

TOOTH GERM-DERIVED STEM CELLS IN BONE TISSUE ENGINEERING:
ANALYSIS OF OSTEO/ODONTOGENIC POTENTIAL



by
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TOOTH GERM-DERIVED STEM CELLS IN BONE TISSUE ENGINEERING:
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*Dedicated to my beloved baby boy
who gave me the best feelings in life*

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ABSTRACT

TOOTH GERM-DERIVED STEM CELLS IN BONE TISSUE ENGINEERING: ANALYSIS OF OSTEO/ODONTOGENIC POTENTIAL

Bone tissue engineering (BTE) needs a reliable stem cell source, appropriate 3D scaffolds and growth factors. Mesenchymal stem cells (MSCs) are attractive stem cell sources for BTE since their differentiation can be controlled and they have high clonogenic capacity. In particular, dental tissue derived MSCs are favorable candidates due to their high osteogenic capacity, easy access, no donor site morbidity and availability of human source without any ethical problems. Aim of this study was to determine most appropriate and applicable dental stem cell source for BTE by comparing clonogenic characteristics and osteogenic differentiation capability of dental germ stem cell (TGSC), dental follicle stem cell (DFSC) and stem cell from apical papilla (SCAP) tissues and to obtain bone-like tissue by seeding the cells with higher osteogenic capacity on fibrous PBS:PLGA composite scaffold. For these purposes, dental germ, follicle and apical papilla tissues of third molars were extracted from 6-month-old pigs due to their similarities to humans and stem cells from these tissues were isolated by explant culture. Mesenchymal characteristics of cells were proved and differentiation studies were performed. Their osteogenic differentiation potential in cell culture plates and on the fibrous composite PBS:PLGA scaffolds which were fabricated by wet spinning were detected in terms of mineral deposition, ALP activity and gene expressions. The best scaffolds were chosen as a result of degradation analysis, mechanical analysis and cell viability test. These studies showed that stem cells from three different sources had great osteogenic differentiation capacity in tissue culture plates but SCAP achieved higher and faster osteogenic differentiation. However, all cells had similar mineralization and bone marker expression on scaffold at the end of 21 days of osteogenic induction. Also, wet spun 20 percent PBS:PLGA (1:1) scaffold was shown to be an excellent candidate for BTE due to its good mechanical properties and physiological features.

ÖZET

KEMİK DOKUSU MÜHENDİSLİĞİNDE DİŞ GERM TÜREVLİ KÖK HÜCRELERİ: OSTEO / ODONTOJENİK POTANSİYELİ

Kemik Doku mühendisliği (KDM), güvenilir bir kök hücre kaynağına, uygun 3B iskelelere ve büyüme faktörlerine ihtiyaç duyar. Mezenkimal kök hücreler, farklılaşmaları kontrol edilebildiği ve yüksek klonojenik kapasiteye sahip oldukları için KDM için çekici kök hücre kaynaklarıdır. Özellikle, diş dokusundan alınan mezenkimal kök hücreler, yüksek osteojenik kapasiteleri, kolay erişimleri, donör saha morbiditelerinin olmaması ve insan kök hücreleri kullanıldığında da herhangi bir etik problem sahip olmamaları nedeniyle uygun adaylardır. Bu çalışmanın amacı, diş germ kök hücrelerinin (DGKH), diş folikül kök hücrelerinin (DFKH) ve apikal papilla kök hücrelerinin (APKH), klonojenik özelliklerini ve osteojenik farklılaşma kabiliyetini karşılaştırarak KDM için en uygun ve uygulanabilir dental kök hücre kaynağını belirlemek ve bu hücreleri kullanarak PBS:PLGA kompozit hücre iskelesi üzerinde kemik benzeri doku elde etmektir. Bu amaçlar için, 6 aylık domuzlardan insanlara benzerliklerinden dolayı diş germ, folikül ve apikal papilla dokuları çıkarıldı ve bu dokulardan alınan kök hücreler eksplant kültür yöntemi ile izole edildi. Hücrelerin mezenkimal özellikleri akış sitometrisi ve farklılaşma çalışmaları ile kanıtlanmıştır. Hücre kültürü plakalarında osteojenik farklılaşma potansiyelleri mineral birikimi, kalsiyum birikimi, ALP aktivitesi ve osteojenik gen ekspresyonları açısından tespit edildi. Daha sonra fibröz kompozit PBS: PLGA iskeleler farklı konsantrasyon ve oranlarda ıslak eğirme yöntemi ile üretildi. Bozulma analizi, mekanik analiz ve hücre canlılığı testi sonucunda en iyi iskeleler seçildi. Daha sonra, üç farklı kaynaktan gelen hücreler, iskelelere ekildi ve çoğalma davranışları ve osteojenik farklılaşma potansiyelleri, MTS, ALP aktivitesi, von Kossa boyama, RT-PCR ve immünofloresan boyama ile değerlendirildi. Bu çalışmalar, üç farklı kaynaktan alınan kök hücrelerin doku kültürü plakasında yüksek osteojenik farklılaşma kapasitesine sahip olduğunu, ancak APKH'nin daha yüksek ve daha hızlı osteojenik farklılaşma sağladığını gösterdi. Bununla birlikte, tüm hücreler, 21 günlük osteojenik indüksiyonun sonunda iskelede benzer mineralizasyon ve kemik belirteci ekspresyonuna sahipti. Ayrıca, ıslak eğirme ile üretilen yüzde 20'lik ıslak eğirme PBS:PLGA (1:1) iskelenin, iyi mekanik özellikleri ve fizyolojik özellikleri nedeniyle KDM için mükemmel bir aday olduğu gösterilmiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

ALP	Alkaline phosphatase
BDO	1,4-butanediol
BMPs	Bone morphogenetic proteins
BMU	Bone basic multicellular unit
Col1	Collagen type 1
DFSC	Dental follicle stem cell
DMEM	Dulbecco's Minimum Essential Medium
DOPC	Determined osteoprogenitor cells
DPBS	Dulbecco's Phosphate Buffer Saline
DSC	Dental stem cell
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
GFs	Growth factors
GM	Growth media
IGF-I	Insulin like growth factor
μl	Microliters
mM	Millimolar
MEM α	Minimum Essential Medium α
MSC	Mesenchymal stem cell
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)- 2-(4-sulfophenyl)-2H-tetrazolium)
OCN	Osteocalcin
OM	Osteogenic Media
OPG	Osteoprotegerin
PBS	Polybutylene succinate
PGA	Poly-glycolic acid
pH	Negative log of hydrogen ion concentration
PLA	Poly-lactic acid
PLGA	Copolymer of PLA and PGA

RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
Runx2	Runt-related transcription factor 2
SCAP	Stem cell from apical papilla
TGF- β	Transforming growth factor- β
TGSC	Tooth germ stem cell
VEGF	Vascular endothelial growth factor



1. INTRODUCTION

The human body in adulthood has 206 bones, 80 of which are axial and 26 of which are perpendicular [1]. That much bones have spread throughout the human body so they are exposed to a lot of external and internal impacts that cause the bone fractures due to high force, stress and some medical conditions like osteoporosis, some cancers, or osteogenesis imperfecta [2]. Bone health is the major concern in the world wide. Only in U.S, approximately 6.3 million fractures arise each year [3,4]. To treat bone fractures, surgical ways are preferred in most cases while nonsurgical ways such as external immobilization of broken bone are used in least severe cases. Surgical procedures include bone grafting (autologous, allogenic, xenogeneic or completely synthetic). Autologous bone grafts are routinely used to fix small defects in the bone, but there are many challenges of this method such as donor site morbidity and source limitation. Main problem of allogeneic and xenogeneic grafting is the immune reaction of patients [5]. On the other hand, usage of metallic implants and screws is a standard relatively successful technique, however, they can delay bone healing process due to poor degradation. Internal fixation with metallic plates and screws cause additional trauma, block the bone blood supply and disrupt tissue integrity. All of them increases the risk of delayed osseointegration and infection [6].

One approach to cope with these troubles may be the application of tissue engineering method to construct tissue-engineered bone that might offer a readily available and probably superior alternative to conventionally used bone graft. From bone tissue engineering point of view, synthetic or biological scaffolds are used as templates to guide and support cell attachment, growth and differentiation before the reformation of bone tissue. Therefore, the combination of biomaterials, cell and biological factors is of great interest. As a cell source, osteoblasts or cells that can become osteoblasts can be preferred for seeding onto scaffolds. Indeed, the potential of MSCs in the bone tissue regeneration has been highlighted over the 30 years. Dental stem cells are the most easily accessible, natural, convenient, noninvasive source of stem cells so they hold great promise for a broad range of potential therapeutic applications [7].

This study mainly focused on the three types of dental stem cells which are dental germ stem cell (TGSC), dental follicle stem cell (DFSC) and stem cell from apical papilla (SCAP) and

their potential of osteogenic and odontogenic differentiation. Also, their differentiation potential towards adipogenic lineage and chondrogenic lineage were examined comparatively. Tissue engineering approach needs a scaffold which is an artificial extracellular environment to give the cells space for attachment, proliferation, differentiation, and new 3D-tissue formation. There are wide variety of biodegradable polymers and scaffolding production methods for bone tissue engineering with their advantages and disadvantages [8]. In this study, combination of two biodegradable and biocompatible synthetic polymers which are poly(butylene succinate) (PBS) and poly(Lactide-co-Glycolide) (PLGA) were used as a scaffold thanks to their mechanical and physiological properties.

1.1. BONE

Bone has mainly three physiological functions in the organism. First function of bones in the body is a mechanical support. It supports the body for motion and gives the shape for posture. Also, it offers protection to sensitive internal organs. Second function of bones is to store calcium and phosphate in the body so it protects homeostasis of these elements in the body. Final function of bones is to provide space for bone marrow and produce haematopoietic cells [9].

There are two main constituents of bone which are cortical bone and trabecular bone. Although trabecular and cortical bones are different from each other with respect to function and mechanical properties, their cellular and molecular compositions and structures are quite similar. Trabecular-also known as cancellous- bone consists of a honeycomb-like network of cancellous plates and rods interspersed in the bone marrow area. Trabecular bones are found in vertebrae at the ends of the long bones and close to the joint surfaces. On the other hand, cortical bones are much more solid and denser than the trabecular bones and they are found in the shafts of long bones (Figure 1.1.).

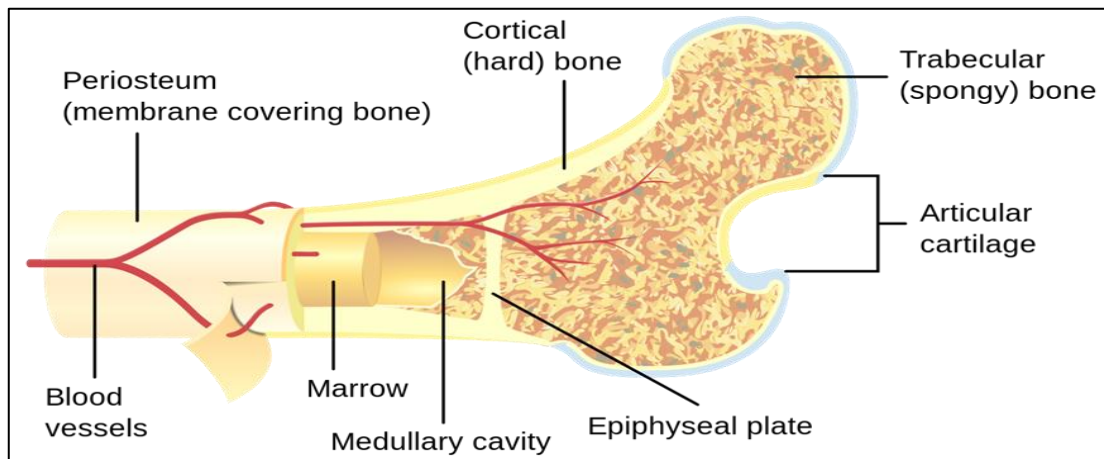


Figure 1.1. Cross-section of a bone and its structures [10]

Cortical bone has two components - outer periosteal surface (periosteum) which is composed of fibrous connective tissue sheath and inner endosteal surface that surrounds the inner surfaces of cortical and cancellous bones and the blood vessels. Exceptionally, the periosteum is absent at the joint surface where bone is surrounded by articular cartilage. Periosteum consists of nerve fibers, blood vessels, osteoblasts, and osteoclasts. Although the cortical bones are compact, solid and hard, they penetrate the blood vessels through haversian canals and Walkman's canal. In the bone organization, osteocytes and their connecting cellular processes stretch along the blood canals and these organizations are called osteons or Haversian systems. Osteons are cylinders of lamellae that are located parallel to the longitudinal axis of the bone and contain centrally oriented vessels and nerves [11]. Bone formation, modeling, remodeling, and fracture healing occur at this site. This structure is responsible for nutrition, and protection of bone [9,12]. Approximately eighty percent of the skeletal mass is formed from cortical bone and remaining twenty percent is formed from trabecular bone (Figure 1.2.) [13].

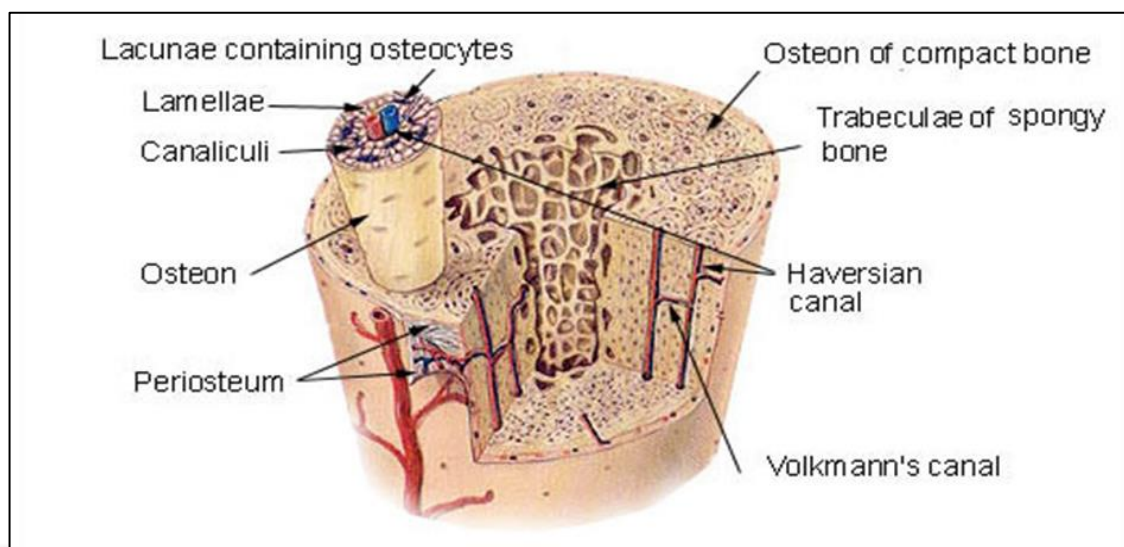


Figure 1.2. Structures of cortical (compact) and trabecular (cancellous) bones [14]

Bones are classified as woven bone and lamellar bone according to the pattern of collagen forming osteoid that is a non-mineralized organic tissue which eventually undergoes calcification [15]. Woven bone has loosely and randomly arranged collagen fibers with various osteocytes which lie in different lacunae. In this bone type, the lacunae's walls aren't defined well. The woven bone is also called primary bone because it is commonly found in the embryonic, cortical and trabecular bones in states of rapid growth, bone replacement, or high bone turnover. The woven bone turns into lamellar bone in normal adult skeleton by remodeling [16]. The lamellar bone has an orderly arrangement of collagen fibers and their cells. The lamellar bone lacunae is uniform and regularly distributed and contains cells with similar morphology. The collagen deposition of lamellar bone shows an arranged lamellar pattern with circular layers of collagen alternating with longitudinal ones [16,17]. The lamellar bone is mechanically stronger due to the lamellar collagen pattern compared to the woven bone. The woven bone is formed when the osteoblast synthesizes osteoid quickly and hence it occurs initially in all fetal bones and in fracture healing. In the adult skeleton, the presence of this bone is seen as a pathology such as Paget's disease of bone, fracture healing sites and osteitis fibrosa cystica [16,17].

Bone consists of organic and inorganic materials in a certain ratio. Generally, organic components form 20 percent of bone and the remaining is inorganic components. This ratio varies according to types and age of bones. Inorganic part of bone is made up of insoluble

salt crystals called hydroxyapatite. Approximately 60 percent of the bones are made up of hydroxyapatite (HAp), which is a mineral form of calcium phosphate. It contains a large volume of calcium phosphate as well as other minerals such as sodium, magnesium and bicarbonate. Mechanical support of bone, its strength and rigidity are provided by these inorganic elements together with some organic elements such as collagen fibers. Another important inorganic element of bone is water which forms roughly 20 percent of bone. Thanks to water, bones gain some flexibility with rigid backbone [18,19].

1.1.1. Cell Types in Bone

Bone has 4 types of cells: osteoblasts, progenitor cells, osteocytes, and osteoclasts that are responsible for growing, shaping, dissolving and maintenance of bone tissue (Figure 1.3.). Bone cells reside in the complex environment with a calcified matrix and mechanical load. Additionally, cross talking between osteoblastic and osteoclastic cells exists in this area as well as numerous local and systemic factors [20].

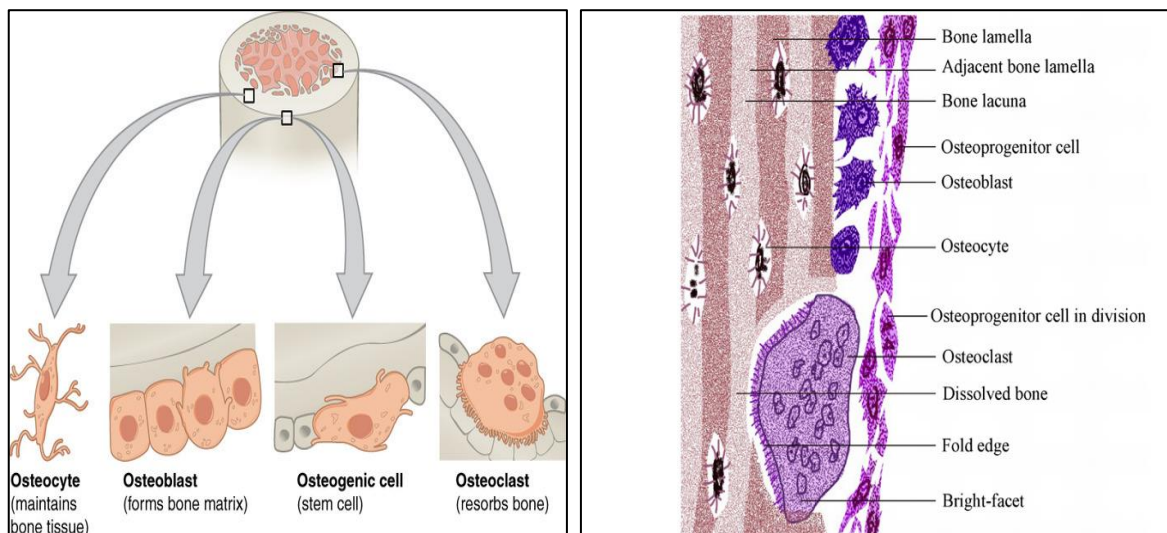


Figure 1.3. Cell types in bone tissue [21]

Osteoblasts are fibroblast-like cuboidal cells originated from bone marrow MSCs and take place in bone formation. Their main role in bone tissue is to synthesize extracellular matrix components like collagen type I (Col1), non-collagenous, proteoglycans, and cell attachment

proteins. In addition, they participate in the bone mineralization by regulating calcium and phosphate ion balance. Having high protein synthesizing activity, these cells have plenty rough endoplasmic reticulum and remarkable Golgi apparatus, including variable secretory vesicles. The Golgi of these cells enlarges and constructs vacuoles that are found at the center of the cell. Osteoblasts have round mitochondria as well as lysosomes, vacuoles, and glycogen granules. Their shapes are variable, usually cubic, flat or round, cylindrical, with a diameter of 20-50 μm . Osteoblasts are functional cells so they rarely show division or proliferation in tissue. Their numbers usually increase with the transformation of osteoprogenitor cells into osteoblasts. Lots of factors are effective in the conversion of osteoprogenitor cells, such as hormone changes and local bioactive matter release after break. In the presence of certain factors, osteoprogenitor cells undergo rapid proliferation and start to differentiate into osteoblasts to achieve osteogenic roles. They take place on the surface of newly synthesized bone by arranging as a monolayer. Osteoblasts have many hairy short protrusions on the surface to connect adjacent cells and form a network. After synthesizing an organic matrix of bone, osteoblast is entrapped in the middle of the osteoid and transforms into osteocytes gradually [22]. In the active form, matrix vesicles of osteoblasts contain alkaline phosphatase, adenosine triphosphatase (ATPase) and inorganic pyrophosphatase and proteinases. Osteoblasts act as starting sites for hydroxyapatite crystal formation through localized enzymatic accumulation of calcium and phosphate. Col1 provides an additional mechanism for mineralization by binding and orientating proteins, like Osteonectin, which also nucleate hydroxyapatite.

There are quiescent flat-shaped osteoblasts that are called bone lining cells in the inactive bone surface in terms of remodeling. These cells communicate with other cell types in bone to maintain bone physiological status. They are responsible for prevention of healthy bone matrix from direct interaction of osteoclasts. Additionally, they produce the receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG) so they take the role in osteoclast differentiation. Bone lining cells together with all cell types in bone form bone basic multicellular unit (BMU) that is an important anatomical part of bone remodeling cycle [23].

Osteoprogenitors have the ability to split propagation and differentiate into osteoblasts. Osteoprogenitor cells are found in the periosteum and bone marrow. According to location and age, periosteum cell layer thickness shows differences. Osteoprogenitors in the

periosteum are derived from the multipotent MSCs in the periosteum. Likewise, osteoprogenitor cells in bone marrow are originated from multipotent stromal stem cells in bone marrow. These two osteoprogenitors are committed to differentiate directly into osteoblast without other inducers. Progenitor cells that have this feature are known as determined osteoprogenitor cells (DOPC). On the contrary, some osteoprogenitor cells are derived from undifferentiated MSCs in specific situations such as fracture repair and heterotopic ossification under osteo-inductive conditions. For example, bone morphogenetic proteins (BMP) can induce MSCs to differentiate into osteoblasts. These cells are called as inducible osteoprogenitor cells and their osteogenic patterns are different from DOPC [22].

The undifferentiated mesenchymal cells that are committed into osteoprogenitor cells need specific genes expression such as members of the Wingless (Wnt) pathways and bone morphogenetic proteins (BMPs). For the osteoblast differentiation, these cells can express Osterix (Osx), Distal-less homeobox 5 (Dlx5), and Runt-related transcription factors 2 (Runx2). Especially, Runx2 is a crucial gene in osteoblast differentiation. Studies have shown that Runx2-null mice do not have osteoblasts. Also, Runx2 plays a critical role in the upregulation of osteoblast-related genes like ColIA1, BGLAP, ALP, OCN, and BSP [23]. Recent findings show that some osteoprogenitor cells can differentiate into osteoclast.

Osteoclasts are large cells which take role in the bone resorption. They are multinucleated cells formed by the fusion of more than one cell. Terminal differentiation of cells to osteoclasts requires RANKL or osteoclast differentiation factors such as macrophage colony stimulating factor (M-CSF) [24]. Their location is the surface of bone mineral. Osteoclasts are derived from macrophage-monocyte cell lineage (Figure 1.4.). Bone resorption is an important mechanism for bone remodeling in response to different factors such as growth, disease, mechanical stress of the skeleton. They form depression which is called Howship's lacunae on the bone surface when the bone resorption occurs.

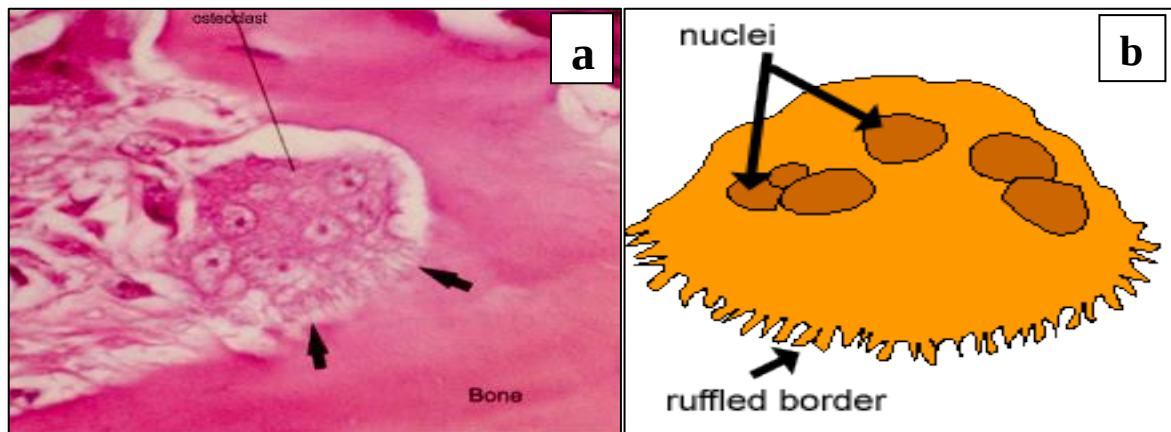


Figure 1.4. Osteoclast (a) microscopic view of osteoclast, (b) drawing of osteoclast [25]

Osteoclasts in the active bone resorption show specialized morphology called a ruffled border that facilitates dissolution of bone matrix. When the osteoclasts attach the bone matrix, they form a sealing zone that is an actin rich area surrounding the ruffled border. In that zone, the ruffled border and bone matrix have close interaction where hydrogen ions are released by osteoclasts. Therefore, the bone matrix environment becomes acidic which causes mineralized bone matrix break down into Ca^{2+} , H_3PO_4 , H_2CO_3 , and H_2O [26]. Osteoclasts are also involved in blood calcium homeostasis. Osteoclasts can be histologically distinguished by staining tartrate-resistant acid phosphatase [27].

Osteocytes are bone resident cells which are found within a fully mineralized bone matrix in the lacuna. They are derived from osteoblasts and formed when the osteoblasts are entrapped in a calcified matrix by surrounding with the products that they secrete. Osteocytes meet their needs for their viability such as gas exchange by extending their cytoplasm along with small channels known as canaliculi. They are the most abundant cells in the mature bone and are long-lived cells [28]. Osteocytes are sensitive to mechanical stress and show response to this stress by communicating with osteoblasts and osteoclasts through the canaliculi. Some studies on mice aged 4 and 22 months have shown that osteocytes produce low levels of collagen type 1 and high levels of alkaline phosphatase [29]. Osteocytes mainly control and regulate bone mineralization. In addition, they have the ability to recruit osteoclasts to the sites of remodeling in healthy or pre-apoptotic conditions [30].

1.1.2. Bone Remodeling

Bone remodeling is an essentially continuous process to maintain and renew bone throughout life. This process allows the shifting of primary (infantile) bone with the secondary bone that is mechanically stronger than the primary bone. Ischemic or micro fractured bone is removed by this process. Also, bone remodeling secures calcium homeostasis between blood and bone. Bone remodeling is the complex process between osteoclasts by breaking down bone and osteoblasts by forming bone [31]. This process requires the synchronized actions of molecular mechanisms and multiple cell types like osteoclasts, osteoblasts, osteocytes, and bone lining cells to guarantee that bone formation and resorption occur sequentially. Imbalances in bone remodeling can result in bone disorders and abnormalities such as rheumatoid arthritis and osteopetrosis. The cells in BMUs have spatially and temporally different arrangements that provide coordination between bone remodeling phases. In this process, osteoclasts destroy bone matrix and osteoblasts synthesize bone matrix. Besides, osteocytes participate in this process with their mechano-sensorial function. Bone remodeling can be divided into six subphases that are activation, resorption, reversal, formation, mineralization, and resting phases (Figure 1.5.) [32,33].

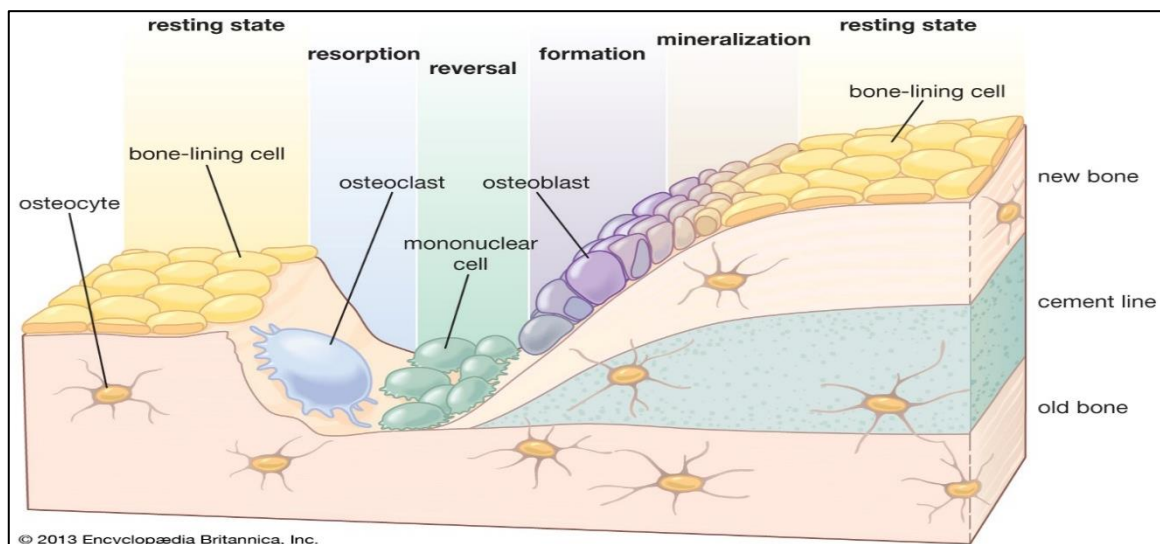


Figure 1.5. Phases of bone remodeling [33]

Activation phase: is initiated when different inputs such as microfracture, mechanical signals, electrical signals or chemical signals come in the bone environment. Chemical signals can be insulin growth factor-I (IGFI), parathyroid hormone (PTH), tumour necrosis factor- α (TNF- α) interleukin-6 (IL-6), and interleukin-1 (IL-1). Osteoclasts also have receptors for calcitonin, androgens, platelet derived growth factor (PDGF), thyroid hormone, and colony stimulating factor-1 (CSF-1). These signals shift quiescent bone surface with bone remodeling surface by activating bone lining cells. As a result of these signals, bone lining cells increase their RANKL expression that communicate with the receptor activator of nuclear κ B (RANK) that is expressed by pre-osteoclasts. This receptor ligand interaction (RANKL/RANK) induces osteoclast differentiation and pre-osteoclast fusion in order to become multinucleated mature osteoclast cells [32,35].

Resorption phase: is divided into different stages within itself. First stage contains proliferation of osteoclast precursors and their terminal differentiation to mature osteoclasts. After osteoclast differentiation, polarized osteoclasts stick to the bone surface and initiate bone dissolution. To degrade inorganic bone matrix, acidification of microenvironment is necessary so osteoclasts secrete hydrochloric acid and then they resorb hydroxyapatite. Acidic dissolution of an inorganic matrix increases mobility to help organic matrix degradation. At the same time, they secrete lysosomal enzymes or proteases such as Matrix metalloproteinase 9 (MMP-9) and cathepsin K in order to degrade organic bone matrix. Dissolution of bone at the specific sites forms resorption lacuna. In the resorption lacuna, degraded bone matrix products are endocytosed by osteoclasts [32,33]. Then, they transport like earth-moving trucks and release them through the extracellular space. If the bone resorption continues in response to signals, osteoclasts detach from the surface and move to the new site of bone degradation. In contrast, if they complete their function, they undergo an apoptosis in order to avoid excessive bone resorption. Molecular mechanism of osteoclast apoptosis is related to osteoprotegerin (OPG), a secretory glycoprotein belonging to the TNF receptor family. OPG is mainly secreted from osteoblast lineages but also secreted from the other cells in bone marrow. OPG blocks RANKL effects by acting as a receptor antagonist. Therefore, it regulates bone dissolution by inhibiting the final differentiation, activating osteoclasts and inducing osteoclast apoptosis. OPG effect is reversible because it is not incorporated into the bone matrix [35].

Reversal phase: contains osteoclast activity that turns into osteoblast activity and it is performed by the reverse cells, whose role has not been yet completely clarified. A cylindrical space is formed between the dissolving area and the refilling area. In this space a cell from the osteoblast lineage removes collagen residue from the bone surface and prepares the surface for subsequent osteoblastic bone synthesis. The length of this area varies depending on the lag between the resorption and formation [32,33].

Formation phase: has osteoblast and osteoblastic activity and bone organic matrix is secreted. Therefore, resorbed bone is synthesized again and gaps are filled. Osteoblasts are affected by various stimuli in an autocrine and paracrine manner. Therefore, osteoblasts contain receptors for parathyroid hormone, parathyroid hormone-related protein, growth hormone, thyroid hormone, insulin, prolactin and progesterone. As well as classical hormones, osteoblasts have nuclear steroid hormone receptors which are receptors for estrogens, retinoids, androgens, and vitamin D3. When the bone matrix is resorbed, some growth factors stored in bone matrix such as BMPs, platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs) and transforming growth factor β (TGF β) are released [34]. These factors are likely responsible for the recruitment of the osteoblasts in the dissolved region of bone. Once recruited, osteoblasts form initially osteoid and then they facilitate mineralization. Imbalances between the resorption and formation phases cause incorrect bone remodeling that leads to a pathological condition. For example, osteoporosis, generally occurring with age, is caused by the inability to produce enough bone from osteoblasts to replace the dissolved bone in the resorption areas [32,33].

Mineralization phase: is that phase in which once osteoid synthesis is accomplished, minerals are deposited within and between the collagen fibers. This process normally lasts about 15 days, which is known as lag time of mineralization. Since the initial rate of mineralization is higher, approximately 60 percent of the mineralization events are accomplished during the first few days, which is called primary mineralization. Then, the mineralization rate is decreased and the remaining mineralization can last around 6 months during the secondary mineralization [32,33].

Quiescence or resting phase: occurs after resorption and formation events are accomplished. Then, the osteoclasts disappear, and the osteoblasts are transformed into osteocytes or Haversian canal lining cells, or they disappear [31].

1.1.3. Bone Defects

The definition of bone defect is the absence of bone where it should normally be. Bone defects can result from accidents, infection, tumors, and bone related diseases such as osteoporosis [36]. Bone fractures commonly occur in the human population but the healing process of bone is complex. Normally, bone has a great regenerative potential, which is able to repair itself without forming a fibrous scar [37]. However, bone healing depends on various different factors such as adequate nutrient intake, bone type, age, smoking, drug therapy, metabolic and endocrine diseases and pre-existing bone pathology [38]. The bone healing process involves three overlapping steps which are inflammation, bone production and bone remodeling [39]. Fracture healing can be classified in the two groups: indirect (secondary) and direct healing (primary). In direct healing, fractured bony fragments come together and fix with compression. After fixation, osteoclasts and osteoblasts undergo a remodeling process with the help of haversian systems and blood vessels to form new bone tissue without callus formation. Therefore, healing can last months to years. Reduction of fracture ends and stable fixation are required for direct healing. These requirements aren't provided naturally so open reduction and internal fixation surgery are necessary. Direct healing is observed through contact healing and gap healing. Both of which contain regeneration of lamellar bone structure. Gap healing occurs on the gap that must be less than 800 μm to 1 mm. However, contact healing occurs on the gap that must be less than 0.01 mm and interfragmentary strain should be less than 2 percent. In many cases, indirect healing that contains endochondral and intramembranous bone healing occurs instead of direct bone healing. Indirect bone healing does not require stable conditions, anatomical reduction and fixation. In this healing, soft callus formation occurs. In the fracture site, small motion or load bearing results in callus formation and following indirect healing. That type of healing is observed in the following cases; non operative fracture treatment, intramedullary nailing, internal fixation of comminuted fractures, and external fixation [37]. This healing process is divided into four different steps: hematoma formation, fibrocartilaginous callus formation, bony callus formation, and bone remodeling, respectively (Figure 1.6.).

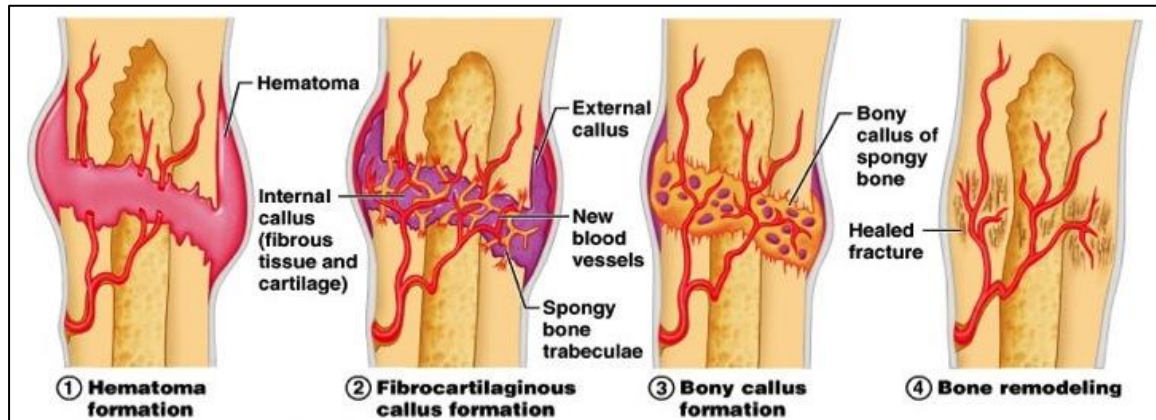


Figure 1.6. Indirect bone healing stages [40]

When the bone is fractured, firstly bleeding occurs in that region, which initiates inflammatory response and clotting. The pro-inflammatory cytokines like BMPs, $\text{TNF-}\alpha$, and certain interleukins like IL-1, IL-6, IL-11, and IL-23 are secreted to the fracture site. These cytokines trigger the vital cellular biology at the fracture site by attracting macrophages, lymphocytes, and monocytes. These acute inflammatory responses are crucial for the healing processes, providing initial structural stability and framework for re-creation of bone. It reaches a peak within one day and ends after 7 days. All cells in this site attempt together to discard damaged, necrotic tissue and synthesize cytokines such as vascular endothelial growth factor (VEGF) to stimulate healing at the defect site. This clot with bone marrow cells and cells from the peripheral and intramedullary blood is called a haematoma that is formed immediately after trauma. The haematoma formation acts like a model for callus formation. After the haematoma formation, release of VEGF results in an angiogenesis at the region, leading to development of fibrin-rich granulation tissue. Further MSCs are recruited to the defect site and differentiate into fibroblasts, osteoblasts, and chondroblasts. As a result, chondrogenesis is initiated and fracture ends are united with a collagen-rich fibrocartilaginous organization that covers the hyaline cartilage sleeve. That cartilaginous tissue provides a soft callus formation to make the break more stable. Simultaneously, a layer of woven bone is produced by the osteoprogenitor cells near the periosteal layers, which creates a hard callus [41]. Besides, adequate blood supply is necessary for the healing. Thus, angiogenic pathways, chondrocyte apoptosis and cartilaginous degradation are essential to bone repair. For mineralization of bone, primary soft cartilaginous callus must be degraded. Woven bone lays down subperiosteally and blood vessels maintain their own proliferation, allowing further transportation of MSCs. As a result

of these steps, callus is calcified and immature bone is formed. An important point is the difference between endochondral ossification described above and intramembranous ossification. Intramembranous ossification is a series of actions by which primitive connective tissue, also known as mesenchymal tissue, is replaced directly with the bone tissue. This process has not cartilage intermediate. The last step of bone repair is the remodeling of bone. Repeated bone remodeling cycles that contain bone resorption and production are initiated within the hard callus as the osteoblast and osteoclast migration continue. The center of this structure becomes a compact bone and its' edges become lamellar bone. Bone remodeling takes months, finally resulting in re-creation of the healthy bone [40,42].

Although the bone has a high self-healing capacity, large defects and significant bone loss caused from serious trauma, developmental abnormalities, revision surgery, infection and tumor resection have poor self-healing. Those defects are called critical size defects and described as the smallest size intraosseous wound in a certain bone which will not repair spontaneously. Additionally, if a break exhibits less than 10 percent bone regeneration during the lifetime of the organism, or a break's length deficiency surpasses two to three times its diameter, it can be defined as critical size defects [42]. They need intervention to heal [43]. There are various surgical methods such as shortening the bone, distraction of osteogenesis, and bone grafting.

Traditionally, large bone defects have been treated by using bone grafting in which defective bone is replaced with the healthy bone that can be taken from different sources to repair bone. This technique has been used for almost 500 years with minor changes. For the first time, in 1505 AD, surgeon Ibrahim Bin Abdullah mentioned bone grafting in his book and described the procedure that is now called cranioplasty. In this technique, dog and goat skull bones have been used to repair human cranial defects. Nowadays, bone grafting, not too much different from his technique, is used as a gold standard to heal large bone defects especially under 2 cm. Autologous (from the patient's own bone), allogeneic (from another human bone), and xenogeneic (animal derived bone) bone graft transplantations are three approaches that are used today [44]. Autologous bone grafting with or without the feeding artery of grafted bone is more preferred than the other grafting types. First choice of surgeons is generally vascularized bone grafts that involve an internal vascular network according to the situation. Unlike non-vascularized bone grafts, re-union with the host bone resembles

the fracture healing that has short union times (3–5 months). However, obtaining vascularized bone is more difficult than non-vascularized bone, therefore, non-vascularized bone is usually used. The most prevalent source for non-vascularized bone graft is the pelvis or the canal of the thigh bone [45]. Obviously, the bone grafting method has many advantages but also major disadvantages such as donor site morbidity, limited availability, risk of disease transmission and prolonged treatment. Additionally, the painful and costly harvesting of patient bone does not represent the ideal method for surgeon and patient. The treatment strategy and process can be long and overwhelming for both patients and clinicians [47,48]. Distraction osteogenesis is also an old technique and its modern form was developed by Ilizarov [48], which is used in defects whose size is between 2 cm and 6 cm. In this method, the bone is cut in a healthy region. After that, this site is distracted by using an external fixator frame or an internal lengthening nail. Bone production in the gap occurs to restore the overall length of the bone. The region of the defective bone can either be shortened simultaneously with distraction osteogenesis surgery, or it can be gradually closed as the new bone is being made. This treatment needs a large amount of effort and complications and setbacks are relatively common [42].

Currently there is no universally accepted procedure that describes how to treat bone defects. Treatment strategies vary case-by-case. Although autologous bone grafts continue to be a successful method for bone regeneration, this may not be applicable in large bone loss. Besides, patients with donor site morbidity and older patients have poor outcomes with this method. As an alternative, allogeneic bone grafts can be applied, however, risks of immune rejection and pathogen transmission always stand in the table. Also, the limited number of donors does not meet the clinical demand. Consequently, demand for alternative approaches to provide efficacious and reliable bone grafts is increasing. At that point, there is a huge focus on the field of tissue engineering to provide new and alternative approaches to old fashioned methods.

1.2. BONE TISSUE ENGINEERING

In today's world, a large human population suffers from bone related diseases. This situation is mainly caused by inadequate or imbalanced nutrition and accidents in heavily populated cities as well as developmental diseases. In the United States alone, roughly 15 million

fractures occur annually, including 1.6 million hospital admissions for traumatic fractures and 2 million admissions for osteoporotic fractures and 1.6 million bone grafts are applied each year. Their costs exceed over 60 billion dollars. Similarly, there is an increasing amount of bone defects in the globe. Worldwide, approximately 2 million people undergo bone-grafting procedures. As a result, the global market value of tissue engineering (TE) is about US \$27.3 billion in 2015. This number is expected to reach US \$94.2 billion in 2022. As can be seen from the statistics, there is an increasing demand for tissue engineering [50,51].

TE is ‘an interdisciplinary field that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain or improve tissue function’ according to Langer and Vacanti who described firstly the TE concept [51]. The main purpose of TE is to produce fully functional tissues which could replace defected tissue. To form fully functional tissue, TE construction should provide the environment for cells, extracellular matrix (ECM) production, blood vessels, nerves, intercellular communication and cell–matrix interaction. Similarly, the aim of bone tissue engineering (BTE) is to induce new bone tissue repairing and regeneration with the help of its construct. BTE has three main components that are cells, biomaterials, and signals as shown in Figure 1.7.

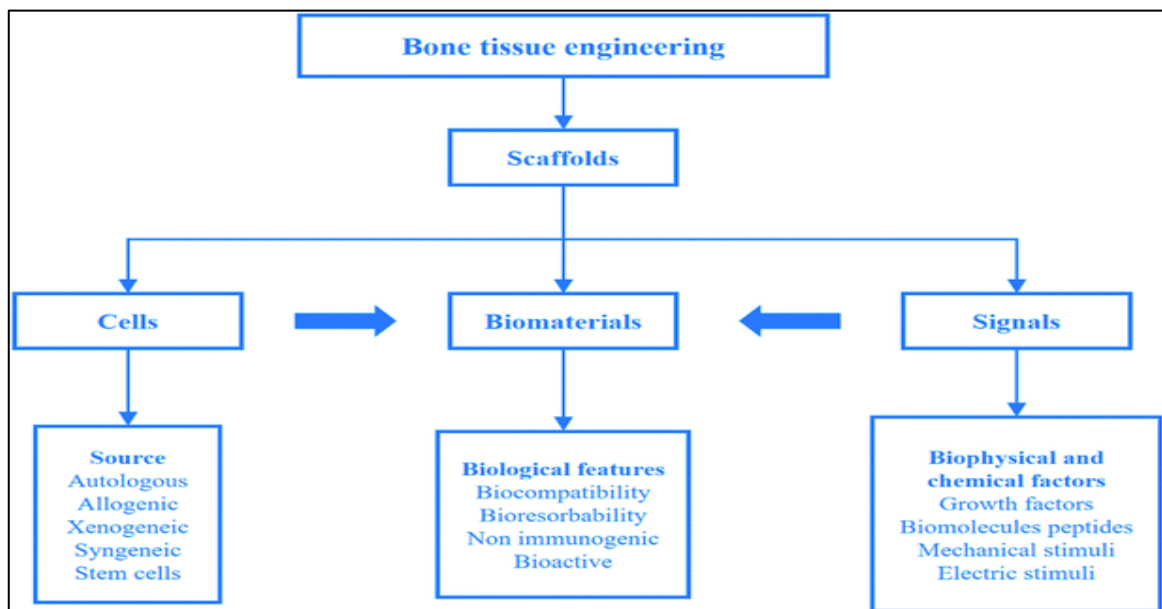


Figure 1.7. Components of bone tissue engineering [52]

An ideal engineered bone tissue should have three fundamental properties: osteogenicity, osteoconduction and osteoinduction. Osteogenicity is the capacity of an artificial tissue to form new bone tissue. Osteoinduction refers to the ability of a material to stimulate differentiation of a host's cell to osteoblastic cells for the formation of bone [53]. Final term is osteoconductivity, which is the ability to promote attachment of osteoblastic cells on the surface and throughout the interior of biomaterial [54]. To sum up, an ideal engineered bone tissue should contain osteogenic cells, osteoinductive signals and osteoconductive scaffolds. Additionally, it should have some properties such as mechanical stability and ability to provide vascularization.

1.2.1. Scaffolds

Scaffolds take an essential role in BTE and their aim is to imitate the structure and function of the extracellular matrix of natural bone. They create a three-dimensional (3D) environment to enhance the proliferation, adhesion, and differentiation of osteoblastic cells to regenerate bone tissue. Scaffolds should have adequate physical features for regeneration of healthy bone tissue. An ideal scaffold should have following properties: biodegradability, biocompatibility, bioactivity, osteoconductivity and osteoinductivity. Also, it should have easy sterilization without loss of its bioactivity.

First of all, scaffold material is one of the most important issues in application and fabrication of scaffold. Historically, metallic scaffolds have predated usage than the other materials. Particularly, usages of iron (Fe) and magnesium (Mg) based metals such as Mg-Ca, Mg-Fe alloys, Fe-Mn alloys, pure Fe, and Fe foam are common. These scaffolds have superiority in terms of mechanical strength. For example, Fe possesses a 211 GPa elastic modulus, Mg possesses 41 GPa and its alloys have 44 GPa [55]. However, the study on Fe aorta stent of rabbits showed that Fe scaffold caused systemic toxicity and inflammatory response [56]. Magnesium has bioresorbability, biodegradability, non-inflammatory responses, and suitable mechanical properties. Although superior properties of Mg and its alloys, their usage in medicine is limited because of their high corrosion rate and toxins [58,59]. Another commonly used metal as a scaffold in BTE is titanium and its alloys. Porous titanium (Ti) scaffolds also have great mechanical strength but they aren't biodegradable and they don't unite with biomolecules. They inhibit bone-cell proliferation and reduce bone formation

markers [59]. Due to their great mechanical properties, biocompatibility and osteoconductivity, metals are preferable biomaterials. However, they have generally shown poor osseointegration and inhibition of bone formation markers. Most of the time, they stimulate bone loss and resorption because of corrosion byproducts which may cause inflammatory response. Additionally, biological factors usually cannot recognize metallic scaffolds. As a result, metals are commonly used as permanent implants rather than scaffold [60].

Another extensively used biomaterial in BTE is ceramics that contain bioglasses, glass ceramics, calcium phosphates (CaPs), and hydroxyapatite (HAp). Ceramics have the ability of osseointegration unlike the metallic implants because they resemble the bone mineral structures [61]. The mineral phase of bone is already composed of ceramics such as hydroxyapatite and tricalcium phosphate (TCP) [62]. Therefore, they provide a higher osteoblasts adherence and proliferation in comparison to other materials. The most extensively used ceramics as a bone tissue scaffold are hydroxyapatite, β -tricalcium phosphate, their combinations and their derivatives [63]. They have good mechanical strength to require mechanical needs at the defect area. For example, HA possess a great compressive (500-1000 MPa) and bending strength (115-200 MPa), which fulfill the mechanical needs of human cortical bone. However, ceramics are innately brittle and may perform poorly in response to impact. Again, looking at the HA example, its fracture toughness (1 MPa) is much less than that of cortical bone (2-12 MPa) [64]. While designing ceramic scaffolds, appropriate physical and chemical properties, and osteoinductive potential are important. Therefore, they are usually combined with polymer materials with higher fracture toughness to adjust optimized scaffold performance.

Another important biomaterial used in BTE is polymers. They are favorable biomaterial sources due to controllable physicochemical characteristics such as biocompatibility, pore size, porosity, solubility and enzymatic reactions. Additionally, polymers have higher workability and lower price compared to other materials. Polymeric scaffolds, thanks to their versatility, are quickly replacing ceramic and metal scaffolds by overcoming the limitations of these materials. To use as a scaffold, biocompatible polymers, which do not cause immunological or foreign body reaction, are required. Also, polymer degradation rate should be compatible with tissue regeneration rate and their degradation product shouldn't be toxic and they should be easily excreted from the body. Many types of polymer meet these

requirements. The polymers formed by covalent bonds like elastomers, textiles, thermoplastics, and thermosets are in this category. They can be classified as natural polymers such as collagen and synthetic polymers such as poly(lactic acid) (PLA) mainly based on their source [44,51,66].

Natural polymers are originated from natural sources like human or animal tissues and can be proteins (collagen, gelatin, fibrinogen, elastin, keratin, silk fibroin), or polysaccharides (chitosan, alginate, glycosaminoglycans, hyaluronic acid, cellulose, amylose, dextran, chitin), or polynucleotides (DNA, RNA) [60]. They have superior properties like bioactivity, providing natural remodeling and biomimetic surface. They are recognized by osteogenic cells in the body so they support cell adhesion and function. The most commonly used and studied natural polymer in BTE is a collagen since it originally occurs in the bone matrix [66]. Firstly, Mizuno and colleagues showed collagen I ability in osteogenesis by using BMSCs cells in 1997 [67]. After that collagen has been used *in vitro* and *in vivo* studies extensively. Natural polymers, besides their advantages, have many disadvantages such as antigenicity, and microbial contamination. Also, they have reduced adjustability, inadequate mechanical strength, uncontrollable degradation rate, and variability in properties. Many natural polymers have limitations in supply so that their price can be high [68].

Synthetic polymers are obtained by synthetic pathways. For example, PLA, which is an extensively used polymer in BTE, is commonly produced via catalytic ring opening polymerization of lactide. Therefore, their production is easy and can be produced on a large scale [50]. Also, physicochemical and mechanical properties of synthetic polymers can be controlled easily according to the purpose of usage. For example, degradation rate of them can be adjusted by altering their chemical composition, molecular weight, and crystallinity. Generally, they have reproducible and predictable mechanical features. Additionally, their size, shape, and microstructure are tunable according to ease of fabrication. Synthetic polymers are biocompatible, biodegradable, and bioresorbable just like natural ones but natural polymers have poor mechanical features and relatively higher degradation rate. Other good features of synthetic polymers are relatively long shelf life and easy sterilization [69]. The major drawback of synthetic polymers is poor cell recognition sites and osteoconductivity but these problems can be eliminated by coating them with bioactive molecules.

Synthetic polymers cover acrylic polymers, poloxamers, polyurethanes, cellulose derivatives, polyesters, polyamides polyanhydrides, phosphorus based polymers and poloxamines. Among the synthetic polymers, aliphatic polyesters are the most widely used ones in BTE like Poly(ϵ -caprolactone) (PCL) and Poly(lactide-co-glycolide) (PLGA) [70].

PLA, polyglycolic acid (PGA) and their co-polymer PLGA are the most extensively used polymers for segmental bone defects. The Food and Drug Administration (FDA) approved usage of PLGA in the human body [71].

PLGA can be obtained from various synthetic pathways and each pathway's parameters affect the physico-chemical properties of the end product. One of the synthetic pathways to obtain PLGA is the solution poly-condensation of lactide (LA) and glycolide (GA) under water-removal conditions at temperatures above 120°C. This process produces low molecular weight PLGA ($M_w < 10$ kDa). Another pathway to obtain PLGA is a direct poly-condensation from LA and GA in the azeotropic solvent diphenyl ether. This technique increases the cost due to the solvent that makes the end product difficult to purify and control the process. Then, a new melt and melt/solid poly-condensation processes were developed and decreased the cost by excluding any azeotropic solvents. However, the optimum pathway to obtain high molecular weight PLGA is the ring opening polymerization of lactide and glycolide at high temperatures (130–220°C) by using metal catalysts such as the stannous octoate ($\text{Sn}(\text{Oct})_2$) as shown in the Figure 1.8. [72].

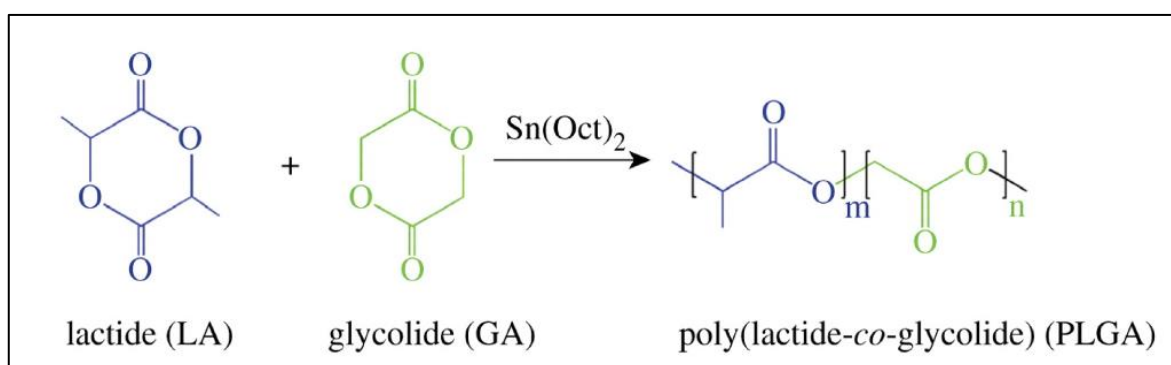


Figure 1.8. Synthesis of PLGA [73]

Apart from these pathways, an enzymatic polymerization and different randomly distributed atactic or syndiotactic PLGA obtaining processes are available.

PLGA has different properties from pure polylactic and polyglycolic acid. PLGA is soluble in a wide range of solvents such as tetrahydrofuran, chlorinated solvents, acetone and ethyl acetate unlike polylactic and polyglycolic acid. LA has two enantiomeric forms that are D and L form based on the position of pendant methyl group on the alpha carbon of PLA but the methyl side group does not exist in GA that makes PGA highly crystalline. However, PLGA copolymers are amorphous.

Different factors such as initial molecular weight of the monomers, molecular weight of end product, the LA:GA ratio, polymerization process' parameters and storage temperature affect the physical and chemical features of PLGA. For example, random PLGA degrades faster than analog-sequenced PLGAs that are prepared by ring-opening polymerization. Additionally, mechanical strength of the PLGA is influenced by physical properties like polydispersity index, the degree of crystallinity, and molecular weight [74]. According to the application field of PLGA, different PLGA forms can be chosen by considering its physico-chemical features, especially LA:GA ratio.

PLGA degradation occurs through the hydrolysis of its ester linkages in water. Their degradation is initiated with the cleavage of covalent bonds that causes a decrease in the molecular weight of PLGA. Then, carboxylic end groups catalyze the degradation process automatically and backbone covalent bonds are cleaved. Thus, mass loss begins and polymer loses its integrity. The fragments after backbone cleavage undergo further disruption to their molecules that are soluble in the water. PLGA hydrolysis is a good example of bulk or heterogeneous erosion [75]. PLA is more hydrophobic than the PGA because of presence of methyl side groups so PLGA copolymers with higher LA content are more hydrophobic than the PLGA with higher GA content. As a result of this, degradation of lactide rich PLGAs are slower than the glycolide rich ones, excluding the 50:50 ratio of PLA/PGA, which shows the fastest degradation. Hydrolysis of PLGA leads to changing descriptions of a solid formulation with time. Thus, moisture content, molecular weight, and the glass transition temperature (T_g) can be changed in shelf. The melting point of PLGA and its' crystallinity degree directly depend on its' molecular weight. The glass transition temperature of the PLGA copolymers are higher than 37°C and hence occur in glassy form naturally. The T_g of PLGAs increases with an increase in molecular weight and in glycolide content in the copolymer [76].

The degradation of PLGA commonly result from enzymatic and hydrolysis disintegration but its biodegradation is mainly performed by non-enzymatic hydrolysis *in vivo*. As a result of PLGA biodegradation, non-toxic lactide and glycolide residues that participate in the metabolic pathway of the tricarboxylic acid cycle are released in the mitochondria. Finally, they are converted into ATP, CO₂ and H₂O and excreted by the kidneys and lungs [43,77].

Poly(butylene succinate) (PBS) is an aliphatic biodegradable polyester with great biocompatibility. PBS is one of the thermoplastic polymer characterized by high rigidity [77]. Moreover, it has good processability for copolymerization or blending with other polymers and allows to tailor its properties. Hence, it can fulfill the requirements for a wide variety of applications. It has been used for cartilage, bone regeneration, and soft tissue engineering.

PBS is produced from succinic acid or dimethyl succinate and 1,4-butanediol (BDO) by condensation polymerization in the presence of a catalyst. The monomers participating in the PBS synthesis (succinic acid and 1,4-butanediol) can be obtained from both fossil resources and renewable resources such as bacterial fermentation of glucose, starch, and xylose [78]. Both monomers of PBS are commonly derived from maleic anhydrides. Production mechanism of PBS from various microorganisms is performed via biotechnological processes. In this way, succinic acid is produced and then converted into 1,4-butanediol through hydrogenation by hydrogenation process, which is called bio-based PBS. Indeed, PBS synthesis via poly-condensation is a two-step reaction. First one is esterification reactions including water or methanol removal. The second one is the removal reaction of BDO at higher temperature and under reduced pressure. Hence, PBS with high molecular weight is produced. Different catalysts play a role in the synthesis of PBS such as titanium (IV) butoxide (TBT), organometallic or metal-oxide compounds. Additionally, the way of obtaining PBS by using enzymatic catalysis has been reported in recent years [79].

PBS has good tensile strength and moderate hardness and stiffness like those of conventional polyolefins. PBS can be classified as tough and resistant material compared to PLA. Thermal stability and melting point of PBS are lower than those of other commonly used biodegradable polymers due to its semi-crystalline nature. Its ductility is relatively lower than that of commercially available polymers [80]. Therefore, studies on PBS got successful results after modifying its properties by blending with other polymers. Apart from these, hydrophilicity of PBS offers good surfaces for cell attachment [81]. In this perspective,

blending PBS with other biodegradable polyesters is suggested as the most promising way to get sustainable materials with enhanced and tailored features.

The degradation products of PBS are finally converted into small molecules that can be excreted with the circulatory system without causing immune rejection and inflammatory response [82]. Biocompatibility properties of PBS have been shown by many previous studies so it provides surfaces for cell attachment, viability, and proliferation both *in vivo* and *in vitro*. Various studies on pure PBS, PBS- β -tricalcium phosphate and PBS-chitosan composites have reported encouraging results with rat fibroblastic, osteoblastic cells and human MSCs. Moreover, a few studies have shown osteoconductivity of PBS scaffold. For example, Li et al. have shown higher alkaline phosphatase (ALP) activity in murine osteoblasts on PBS compared to osteoblast on polystyrene [83]. Costa-Pinto and colleagues showed that 3D porous compression-moulded PBS-chitosan composites provide *in vivo* bone formation in a mouse critical-sized calvarial defect model [84]. Meanwhile, Wang and colleagues have reported osteogenesis of murine osteoblasts on PBS surfaces modified by plasma immersion ion implantation [85]. These articles suggest that PBS might have potential for BTE scaffold, especially blending with other biodegradable polyesters like PLGA.

After scaffold material, scaffold fabrication and design is another important issue for scaffold applications. There are various scaffold fabrication techniques according to material type and application requirements. These techniques can be mainly divided into two groups that are conventional techniques and rapid prototyping. The conventional fabrication techniques contain melt molding, phase separation, gas foaming, wet spinning, electrospinning, freeze-drying, and solvent-casting particulate leaching. On the other hand, the rapid prototyping methods include sheet lamination, laser stereolithography, and fused deposition molding. Each of these methods fabricates scaffolds with different properties in terms of internal structure (including pore size, pore structure and interconnectivity) and mechanical properties. The most convenient scaffolds are selected according to a holistic review and comparison of all suitable methods [87,88].

Rapid prototyping technique (sometimes called additive manufacturing or three dimensional (3D) printing) created a revolution in material science and related fields. The most commonly used types of this method are fused deposition modeling (FDM), selective laser sintering (SLS), inkjet printing (IJP), and dynamic light processing (DLP). There is a specific

branch for biomedical application among 3D printing methods, which is called 3D bioprinting. According to usage area, 3D bio-printing can produce non-biocompatible, non-biodegradable and biocompatible, degradable and biocompatible, and biomimetic cell-laden scaffolds. These methods have advantages such as efficiency on-demand manufacturing, high reproducibility, compatibility with computer aided design (CAD) systems and possibility of both small- and large-scale production [87]. Additionally, these methods offer precise control on the pore size, interconnectivity, and shape of scaffold and biological substances can be integrated in the scaffolds during printing. On the other hand, micrometers or submicron scale scaffold architecture production still remains challenging. Also, bio-printing with biological additives such as cells have many limitations like temperature limit, toxic chemical or solvent usage. Besides, establishment of the printer devices and creation the infrastructure are expensive compared to conventional techniques [88].

Solvent casting particulate leaching is a basic and the most extensively used technique for tissue engineering construct. In this method, polymer or ceramic particles are dissolved in a solvent and then mixed with water soluble salts. Subsequently, the mixture is casted into the predefined mold of the desired shape. In the mold, the solvent is evaporated or lyophilized and then the salts are leached out in order to get a porous scaffold. Its simplicity, adequate control on pore size, and easy preparation are the advantages of this method. On the other hand, the pore shape cannot be changed from the cubic crystal shape of the salt. Also, removal of soluble particles from the interior of a polymer matrix becomes difficult as scaffold thickness increases, therefore, scaffold thickness ranges from 0.5 to 2 mm. Likewise, removal of solvent can be difficult and residual chemicals in scaffolds can be harmful for cells. The interporous connectivity of scaffold produced with salt leaching method is limited, hence it does not provide uniform cell seeding and tissue growth [87,88].

Freeze-drying is similar to solvent casting and particulate leaching methods but it does not require particles to form a porous structure. In this method, polymers are dissolved in their solvents and frozen in a mold with liquid nitrogen. Then, the frozen scaffold is lyophilized and a highly interconnected porous scaffold is obtained. One of the advantages of this method is a scaffold production without high temperature. The pore size can be controlled via freezing rate, polymer concentration and temperature. However, the pores of the scaffold produced by this method are irregular and small [89].

Gas foaming method also creates porous scaffolds by using gas as a porogen. This technique eliminates the drawback of residual solvent of other methods. In this method, carbon dioxide (CO₂) is commonly used as a gas for the construction of polymer foam. Firstly, solid polymer chambers are saturated with high pressure CO₂. Then, CO₂ gas is quickly released from polymer systems, which creates thermodynamic instability. Finally, the nucleation and growth of gas bubbles occur in the material. The major drawback of this method is that it produces mostly a nonporous surface and closed-pore structure, with very low interconnected pores. This feature can be developed by combining particulate leaching method with the gas-foaming process. However, complete elimination of closed pores structure is still challenging [87,91].

The controlled thermally induced phase separation (TIPS) is another conventional method that produces scaffolds with relatively good mechanical strength. In this method, the polymer solution is prepared at high temperature. Then, liquid–liquid or solid–liquid phase separation is triggered by decreasing the solution temperature. The following removal of the solidified solvent-rich phase via sublimation creates a porous scaffold. The main benefit of this method is the fabrication of scaffold with good mechanical strength compared to salt leaching method. On the other hand, this method produces scaffolds with pore size ranging from 10 µm to 100 µm, which is not appropriate for osteoblastic cell proliferation and bone tissue growth [87,92].

Electrospinning is a conventional method to produce fibrous scaffold by using electrical charge. In this approach, a polymer solution is injected with an electrical potential to form a charge imbalance. At a critical voltage, the charge imbalance exceeds the polymer solution's surface tension, which creates an electrically charged jet. The jet in an electric field is directed toward the ground target. The solvent evaporates as it moves in the electric field and then fibers emerge. Fiber diameter and morphology depend on solution viscosity, electric field strength, polymer's molecular weight, and charge density. This method produces scaffolds with higher porosity and interconnectivity so it is useful for bone tissue constructs. Also, fiber diameters can be adjusted from a few microns to the nanometer range according to purpose of application. Removal of residual solvent is one of the disadvantages of this method. Also, the shapes of electro-spun scaffolds, other than cylinders or sheets, have not been produced via this method [93,94]. Schematic drawings of some conventional methods are shown in Figure 1.9. [87].

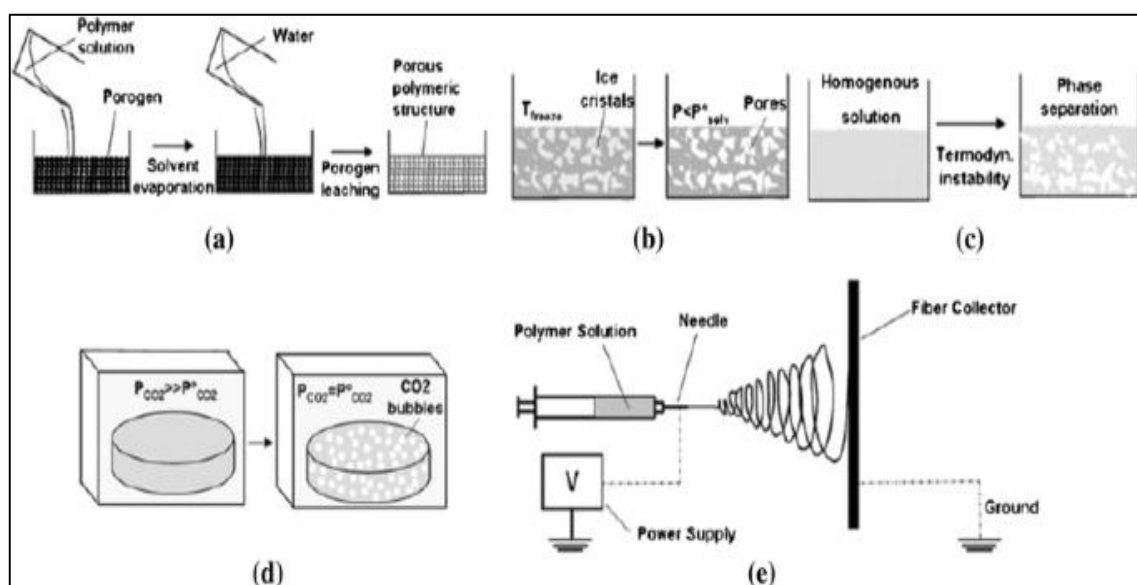


Figure 1.9. Conventional methods for scaffold production (a) solvent casting particulate leaching, (b) freeze drying, (c) Thermally induced phase separation, (d) gas foaming, (e) electrospinning

Wet spinning is the oldest of these processes, which was discovered in the textile industry in the early 1900s to fabricate synthetic fibers like viscose rayon. In this method, the polymers are dissolved in an appropriate solvent and filled in a syringe. Then, polymer solution is injected into a spinning non-solvent chemical bath. The function of a chemical bath is to precipitate and then solidify polymer solution (Figure 1.10.). In this way, polymer fibers form in the ground of the chemical bath. The most commonly produced fibers are spandex, acrylic, aramid, rayon and modacrylic by using this method [94]. This technique is useful not only in the textile industry but also in biomedical applications. The collagen fibers from collagen triple helices suspension can be produced with non-toxic solvents such as acetic acid by using this method [95]. This method has various advantages such as simple processing and easy incorporation with additives. Another advantage is the usage of very low amounts of polymers for massive fiber production [96]. The wet spinning method is advantageous to fabricate a wide range of fiber shapes and sizes. In addition, this method produces scaffolds with higher porosity compared to electrospinning. Therefore, cell attachment, viability, and proliferation on to scaffold are improved and bone tissue growth through the interior part of scaffold can be favorable. The strong fiber structure, higher

porosity and easier cell seeding and penetration make the wet spinning method convenient for fabricating scaffolds for bone tissue regeneration [97].

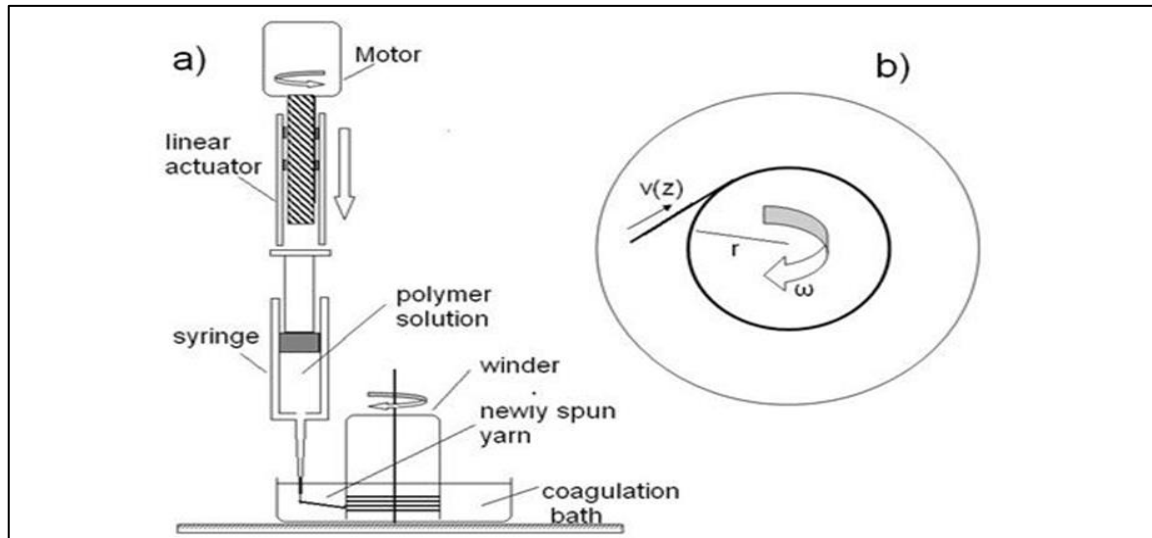


Figure 1.10. (a) The drawing of the wet spinning mechanism and (b) factors affecting fiber diameter in coagulation bath (r : radius of the circle formed by the coiled fibers, ω : angular velocity of the rotating coagulation bath, v : velocity of polymers leaving the syringe)

There are different factors affecting the wet spinning process such as concentration of polymer solution, temperature and composition of coagulation bath, polymer solution pumping rate of extruder, and spinning rate of bath. The polymer concentration in solution depends on polymer solubility and extrusion pressure limitation. The most important factor that affects microstructure and ultimate properties is the mass transfer rate [98].

Design of 3D scaffolds should meet several requirements to get used in BTE. First requirement is sufficient porosity and high interconnectivity. The scaffolds should have high porosity (≥ 90 percent) in order to provide osteoblastic cell adhesion, proliferation and differentiation. Also, pore distribution and size are essential for the appropriate function of the scaffold for bone regeneration. The optimal pore size has been ranged between 100 and 350 μm [99]. Interconnectivity is important for uniform cell seeding, distribution, gas exchange, metabolite and nutrient transport inside the scaffold. Second requirement is adequate mechanical properties to support a suitable environment for healthy bone formation. The degradation rate of scaffold should be synchronized to the rate of bone

regeneration to preserve the structural integrity. The final requirement is that scaffolds should have proper surface chemistry to adhere to bone cells [100].

1.2.2. Cells

If we consider tissue engineering as a tripod, one of the most important components of it is the cell. Although the most intensively investigated area in BTE is the standards of appropriate scaffolds, the cells gain attention for bone formation on scaffold *in vitro* and *in vivo* because they have acceleratory role in bone healing by secreting various cytokines and ECM that is crucial for bone reunion. To use the cells in BTE, some criteria such as a lack of the induced host immune response, ease of availability, potential for osteogenic lineage differentiation, and cell abundance are important [102,103]. Up to date, both differentiated such as osteoblasts and undifferentiated cells such as embryonic, fetal, and adult stem cells are being used for bone regeneration. The usage of differentiated cells has limitations including the restricted proliferation *in vitro*. Thus, there is not much research about the direct use of pre-differentiated or differentiated osteoblastic cells for *in vivo* studies in comparison to stem cells. Many studies have focused on undifferentiated cells including embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), adipose derived stem cells (ADSCs), induced pluripotent stem cells (iPSCs), and dental stem cells (DSCs) due to their ability to promote bone repair and formation [103].

Embryonic stem cells (ESCs) are isolated from ‘embryos’ that occurs immediately after the fertilization. The use of ESC is ethically controversial and thus, isolation of ESCs is performed from extra embryos during *in vitro* fertilization to eliminate ethical issues. Their application in regenerative medicine is highly desirable due to extreme proliferative activity and pluripotency. They can be differentiated from a single source into multiple cell lineages found in mature bone like osteoblastic cells, nerve cells, and vascular cells [102,105,106]. The undifferentiated ESCs show specific surface antigens that are stage-specific embryonic antigen-4 (SSEA-4), tumor rejection antigens TRA-1–81, and TRA-1–60. ESCs have a couple of main histo-compatibility complex (MHC) class I substances and thus, they aren’t highly immunogenic. Also, they are characterized by high telomerase and alkaline phosphatase activity. To maintain pluripotency, they express Oct4, Sox2, and Nanog transcription factors [102,107]. Various studies have shown the strength and potency of

ESCs in BTE. They were combined with various 3D constructs such as PLGA scaffold and successful results were obtained with regards to bone regenerations [108,109]. However, there are some important concerns about usage of ESCs in tissue engineering applications. Firstly, stability of donor ESCs is problematic because they can be tumorigenic. Also, extended culture of undifferentiated ESCs may cause spontaneous development of abnormalities such as teratoma formation in *in vivo* implantation. Secondly, the immunological incompatibility may occur between donor ESCs and host cells despite the lower immunogenicity of ESCs. Finally, culture conditions and requirements for ESC may be tough and demanding compared to other stem cell cultures [101].

The induced pluripotent stem cells (iPSCs) are reprogrammed stem cells from non-stem cell sources to gain pluripotency like ESCs. For the first time, Yamanaka et al. succeeded to develop the iPSCs by inserting four specific genes that encode Sox2, Klf4, cMyc, and Oct4 transcription factors into the somatic cells [103]. Many studies have exhibited the effectiveness and potential of iPSCs in bone tissue regeneration. One study among them showed that iPSC-derived MSCs have the ability to regenerate critical size bone defects in mice more effectively than the bone marrow MSCs (BM-MSCs) in the presence of BMP6 overexpression by using a non-viral transfection method [109]. The main advantage of iPSCs is to eliminate ethical issues of ESCs. Also, host immune reaction is bypassed with autologous cell usage to make iPSCs. On the other hand, the concerns about the possibility of *in vivo* tumor formation is still on the table. Also, reprogramming and culture of reprogrammed cells are demanding. Moreover, differentiation potential of iPSCs and *in vivo* function of tissues formed from such iPSCs may be affected from the inherited epigenetic memory of the starting somatic cells. Thus, the extra research is required to choose the best starting adult cell for iPSC generation for the *in vivo* applications. Finally, non-viral induction and virus-free techniques should be developed for clinically safe iPSCs to be programmed for BTE applications [102,104].

Fetal structures such as the amnion and the chorion membrane, and amniotic fluid have stem cells that are different from ESCs and adult stem cells. Fetal stem cells have distinct characteristics from each other according to tissue of origin. These are umbilical cord blood MSC, endothelial progenitor cells, umbilical cord Wharton's jelly MSCs, amniotic fluid-derived stem cells, amniotic membrane stem cells, and chorion mesenchymal stem cells. Also, some stem cells can be isolated from placenta and maternal circulation. Fetal stem

cells have the ability to preserve clonality, high proliferation and plasticity. Fetal stem cells have the low risk of teratoma formation and infection transmission compared to ESCs and adult stem cells [104,111]. In 2007, the differentiation potential of amniotic fluid-originated stem cells into osteoblast was observed in an osteogenic medium *in vitro*. Furthermore, fetal stem cells seeded on 3D printed alginate/collagen scaffolds were implanted subcutaneously into mice and highly mineralized tissue was observed *in vivo* [111].

Adult stem cells are found in mature or immature tissues and their role is to provide homeostasis and regeneration of tissue when any damage occurs in the human body. These cells are in the quiescence, where the cells do not proliferate and their cell cycle is arrested reversibly, in order to protect their stem characteristics by avoiding early differentiation. These tissue-specific cells have been isolated from the bone marrow, muscle, fat, skin, muscle, etc. [112].

Adipose tissue-derived stem cells (ADSCs) are considered as a kind of MSC that can be differentiated into osteoblasts, myocytes, chondroblasts, adipocytes, and cardiomyocytes in proper conditions. Although ADSCs express the common stem cell surface markers, they can express different surface markers from MSCs due to their heterogeneity. ADSCs have some superiority by being widely available, easily accessible, and abundant source of autologous osteogenic cells. For example, adipose tissue contains 100–500 times higher number of stem cells in comparison to bone marrow, which makes ADSCs an attractive stem cell source for clinical usage [114-116]. The osteogenic differentiation potential of ADSCs was reported both *in vitro* and *in vivo* and their differentiation was found similar to bone marrow-derived MSCs (BMMSCs). *In vitro* osteogenic potential of ADSCs on β -tricalcium phosphate (β -TCP) scaffold was demonstrated by Hottori et al. by comparing the BMMSCs osteogenic potential under the same conditions. Moreover, this study has reported *in vivo* ectopic bone formation on β -TCP scaffold with ADSC cells [115]. However, ADSCs weren't effective to repair a canine defect model when transplanted locally even after osteogenic differentiation. Genetic modification of ADSC with BMP-2 overexpression can increase bone formation but their contribution to bone structure still has question marks. Similarly, Muscle-derived stem cells (MDSCs) have the same cell surface markers as BMMSC and have an easy access and the same culture like ADSC [116]. The studies showed that genetically modified MDSC with BMP-4 overexpression had better results in bone regeneration. However, both ADSCs and MDSCs have weak osteogenic potential *in vivo*

but *in vitro* osteogenic differential potential of them is adequate. Generally, they play a role in production of osteogenic factors to recruit endogenous cells in defect sites [118,119].

The most used and studied stem cell source for bone regeneration is BMMSCs, which is isolated from bone marrow as the name implies. The main functions of BMMSCs are to deliver osteoblasts for the bone remodeling process and support bone and bone marrow homeostasis. BMSCs can be isolated from a patient's own bone marrow aspirate but abundance of BMMSCs in the bone marrow is quite a few (less than 0.01 percent) and hence *in vitro* expansion is required for clinical usage [118]. The collection of ADSCs is easier than the BMMSCs and donor site morbidity for BMMSCs is still challenging for autologous transplantation. The differentiation potential of BMMSCs is influenced by culture conditions such as culture media, cell separation procedures, concentration, timing of passaging, type and origin of sera, number of passages, cell seeding density, reagents for induction, and the period of induction. In addition, the shape, size, and material of scaffolds affect the differentiation of BMMSCs into osteoblast [119]. Stemness quality and stem cell number in bone marrow decrease with aging. Additionally, adult human BMMSCs lose their *in vivo* bone forming ability quickly and bone formation may not occur after the third passage. Therefore, osteogenic induction can increase bone formation *in vivo*. Despite the usage of BMMSC is extensively common both experimentally and clinically, success of them fiercely depends on conditions and method of delivery and by themselves BMMSCs are not quite as efficient as exogenous osteoblast progenitors [121,122].

Dental Stem Cells (DSCs)

Dental tissues are rich sources of stem and progenitor cells, which have been recently utilized in the field of BTE. They are identified as multipotent postnatal MSCs derived from neural crest and can differentiate into osteoblasts, adipose, muscle, cartilage, neuron and endothelial cells [121]. The different types of stem cells have been identified according to tooth structure from which they are isolated. They are dental pulp stem cells (DPSCs), gingiva-derived MSCs (GMSCs), periodontal ligament stem cells (PDLSCs), stem cells from human exfoliated deciduous teeth (SHEDs), dental follicle stem cells (DFSCs), tooth germ stem cells (TGSCs), and stem cells from apical papilla (SCAPs) (Figure 1.11.) [104,124,125]. Dental stem cells express common stem cell surface markers, including CD90, CD73, CD105, CD44. Besides, CD34, CD31, CD45 are lack on the surface of them. DSCs have superiority over the BMMSC such as accessibility, abundancy and lack of donor site

morbidity. Despite the fact that all DSCs come from tooth related structures, their expression markers and potentials of differentiations show slight differences as well as other specific properties.

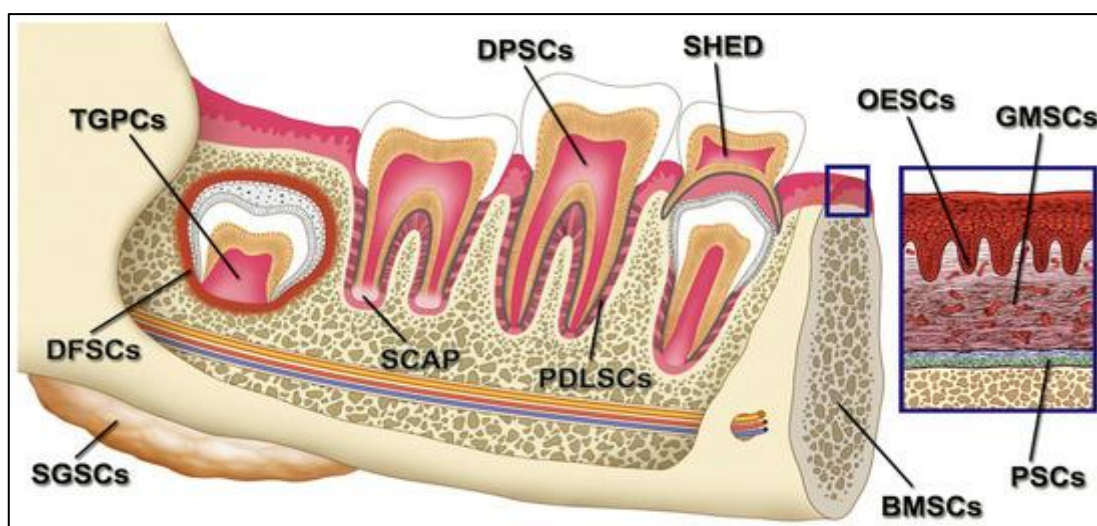


Figure 1.11. Dental stem cells (OESCs: oral epithelial progenitor/stem cells, PSCs: periosteum-derived stem cells, SGSCs: salivary gland-derived stem cells) [124]

In 2000, Gronthos et al. identified DPSCs for the first time as an dental stem cells [125]. They can be isolated with two ways: (1) by selecting single colony from digested dental pulp tissue, (2) by selecting cells derived from digested dental pulp with anti-STRO-1 antibody and magnetically activated cell sorting (MACS). DPSCs have high proliferation and osteogenic differentiation capacity in osteogenic medium. Moreover, the *in vivo* study in rats demonstrated successful fibrous lamellar bone formation after DPSCs transplantation [126]. El-backly and colleagues reported highly mineralized tissues of DPSC on porous 50/50 PLGA scaffold both *in vitro* and *in vivo* cultures [127].

Another MSCs in dental tissue is PDLSCs that are isolated from the periodontal ligament of teeth even when they are extracted. These cells have the ability to regenerate periodontal tissues such as periodontal ligament, cementum, and alveolar bone in animal models [129,130]. SHEDs are other DSCs, isolated from the pulp tissue of exfoliated human deciduous teeth. In 2003, Miura et al. firstly identified and characterized SHEDs with highly proliferative capacity and differentiation ability to various cell lines [130]. SHEDs demonstrated higher osteo-odontogenic differentiation potential and proliferative activity

than the DPSCs [132,133]. Moreover, SHEDs have the differentiation ability to neuronal and glial cells in the presence of a neurogenic inductive media [133]. Up to now, all stem cells from dental tissues were isolated from mature dental structure but dental stem cells from developing dental tissues such as dental follicle, apical papilla and germ have also been identified. Although the morphology of human teeth (i.e., incisor, canine, premolar, and molar) is different, their developmental process has the same stage. Human teeth development and human teeth structures are shown in Figure 1.12. to elucidate stem cell source [134].

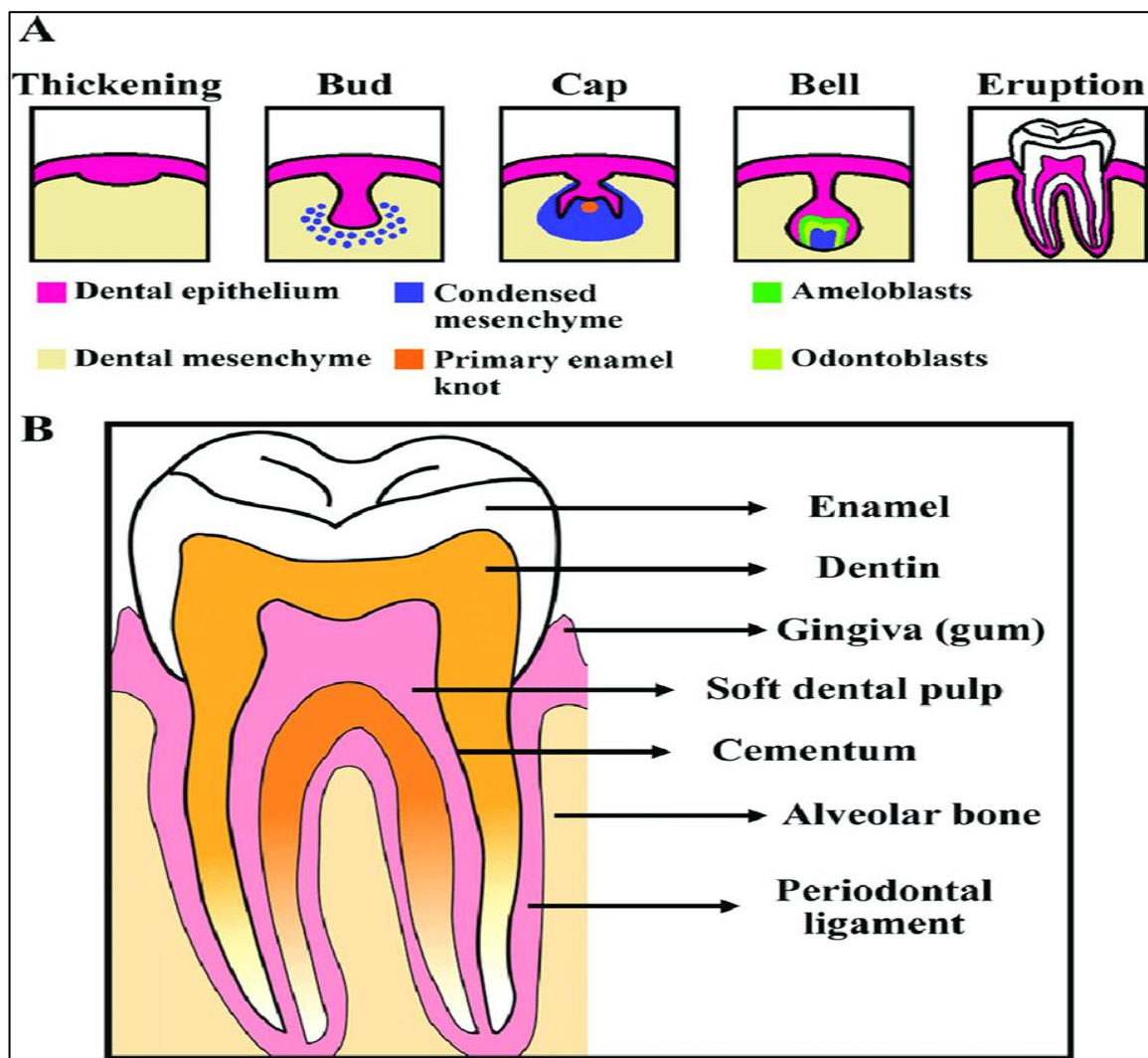


Figure 1.12. Human tooth (a) development and (b) structure [134]

SCAP is one of the MSCs isolated from the upper dental papilla of developing tooth root that turns into dental pulp tissue in mature tooth. The third molars or wisdom teeth and teeth with open apices are extremely important sources of SCAP cells. These stem cells were characterized for the first time by Sonoyama and colleagues in the tooth root apex of developing teeth. In this study, stem cell characteristics (i.e., self-renewal and differentiation ability) of SCAPs were shown via immuno-histochemical, histological, and immunocytofluorescence analyses. SCAPs can be differentiated into osteoblasts, odontoblasts and adipocytes *in vitro*. Also, *in vivo* differentiation capacity to osteo-odontoblast of these cells were proved [136-138]. SCAPs show positive profile for CD90, CD105, CD49d, CD51/61, CD56, CD73, CD106, CD146, CD166, and CD29 and negative profile for CD14, CD45, CD150, CD18, CD34, and CD117. They demonstrate heterogeneous cell population in terms of STRO-1 expression [126,128]. Additionally, SCAPs exhibit some neural markers such as GAD, nestin, GFAP, neurofilament M, NeuN, NSE, and CNPase. SCAPs have a very high proliferation rate among the postnatal stem cells, especially compared to DPSC. Moreover, plasticity and osteogenic differentiation capacity of SCAPs are greater than DPSC, which makes them promising stem cell sources for regenerative medicine [137]. Apart from these, SCAPs can be isolated from inflamed periapical tissue and have high osteogenic differentiation ability both *in vivo* and *in vitro* by maintaining their MSC characteristics. Also, SCAPs can be obtained not only in the developing stage but also in the process of development from the root apical papilla of extracted wisdom teeth. Therefore, typical MSC with embryonic-like properties can be obtained postnatally [140,141].

TGSC is the second type of MSCs derived from developing tooth germ which consists of both premature apical papilla and follicle in the late bell stage of tooth development. Firstly, they were isolated and identified by Ikeda et al. as tooth germ progenitor cells from third molars and later named and characterized as tooth germ mesenchymal stem cells by Yalvac et al. [142,143]. Ikeda and colleagues demonstrated the characterization and differences of the TGSCs and they reported differentiation ability of TGSCs into three germ layers including osteoblasts, neural cells, and hepatocytes *in vitro* as well as their high proliferation rate [140]. Likewise, Yalvac and colleagues have reported differentiation ability of them into osteogenic, neurogenic, and adipogenic lineages as well as showed tube-like structure formation in Matrigel assay under inductive conditions. They also found that human TGSCs exhibited a significant level of sox2, c-myc, and klf4 messenger RNA (mRNA) expression

compared to human ESC [141]. TGSCs show positive profile for CD29, CD44, CD73, CD90, CD105, CD106, CD166 and negative profile for CD14, CD34, CD45 surface markers. They show differential expression for the STRO-1 surface marker similar to SCAP [124]. One of the studies reported the *in vitro* differentiation ability of TGSC loaded onto fibronectin coated PBS scaffold into osteogenic lineages [142].

The last type of stem cells from developing teeth structure is DFSCs, which are isolated from the dental follicle tissue. The dental follicle is a dental sac surrounding the developing tooth and then differentiates into the periodontal ligament and consists of loose vascular connective tissue. A number of studies demonstrated the contribution of DFSCs to the cementum, periodontal ligament, tooth-root, and alveolar bone formation. They differentiate into cementoblast and osteoblast-like cells in the presence of osteogenic induction [145-147]. DFSCs exhibit positive profile for CD9, CD10, CD13, CD29, CD44, CD49d, CD59, CD73, CD90, CD105, CD106, CD166 surface markers and negative profile for CD31, CD34, CD45, CD133 surface markers [124]. One study reported that subcutaneous implantation of DFSCs loaded onto beta TCP scaffold led to ectopic bone formation in immuno-deficient mice [143]. DFSCs have the ability to differentiate into osteogenic lineage by exhibiting a significant level of alkaline phosphatase activity and matrix mineralization. Furthermore, DFSCs have *in vivo* bone formation capacity in a critical size rat calvarial defect model which is as good as the one with BMMSCs and PDLSCs after 4 week of transplantation [145]. When the DFSCs are compared with DPSCs, DFSCs expresses cementoblast marker much more than DPSC. These two cell types show similar proliferation ability and surface markers and both of them have hard tissue formation capacity *in vitro* and *in vivo* [146]. These studies suggest a potential application for DFSC in BTE.

As a mesenchymal stem cell source, dental tissues gain importance and dental stem cells hold great promise for cell therapy approaches in regenerative medicine due to their superior properties including plastic adherent ability with the formation of colonies *in vitro*, immuno-privileged properties, higher proliferation capacity than the ordinary marrow-derived MSCs and easy accessibility. Also, all dental stem cell studies have encouraging results in their ability to regenerate bone and periodontal tissues. Remarkably, findings of the studies suggest that developing dental tissues can be a better source for immature stem cells than the developed dental tissues. Therefore, stem cells from immature teeth tissues (i.e., DFSC, TGSC, and SCAP) may provide promising cell sources for bone tissue engineering.

However, there is no comparative study in the literature about which cells are the most suitable for usage in bone tissue engineering, so the differentiation potential of these cells should be investigated comparatively.

1.2.3. Growth Factors

Biomaterials can combine with various growth factors such as FGFs, PDGF, IGF, TGF- β , BMPs, and epidermal growth factor (EPG) to induce bone tissue growth and vascularization inside the scaffold. BMPs are the most studied and extensively used osteoinductive factors for osteogenesis [147]. They are the members of TGF- β superfamily with 18 known proteins such as BMP-2, BMP-4, BMP-6, BMP-7 and BMP-9. They can be found in homodimeric or heterodimeric forms [148]. BMPs have two type of serine-threonine kinase receptors on the cell surface that are BMPRI and BMPRII. BMPs trigger different cellular signaling pathways such as receptor-regulated Smads and MAPK pathways. These signal transduction pathways induce different transcription factors such as Runx2 and Osx that regulate osteogenic gene expressions [149]. The certain molecules such as gremlin, crypto betaglycan, noggin, chordin, and follistatin inhibit the binding of BMPs to the receptors as their antagonist.

After many studies on animal models with successful results, BMPs have been used in human segmental bone defects. For example, Geesink et al. reported that osteogenic activity of OP-1 bone morphogenetic protein (BMP-7) is effective in a human fibular defect [150]. Additionally, a randomized, controlled trial on the tibial fractures showed that allograft with BMP-2 is safe and as effective as conventional autologous bone-grafting [89].

IGFs are another bioactive molecule, which take role in both bone resorption and formation. Their induction role in bone regeneration has been reported by many articles [154,155]. Likewise, prostaglandins (PGs) that are bioactive substances in bone have both stimulatory and inhibitory effects for bone formation.

Osteogenesis is performed by the orchestrated expression of many substances, covering growth factors, and transcription factors. All of these molecules stimulate cellular actions in order to commit and differentiate the cells for bone modelling. The strategy of incorporation of these molecules with scaffolds is a highly smart idea to improve the healing process.

1.3. AIM OF THE STUDY

The aim of this study was to create 3D scaffolds by blending PBS and PLGA and using wet spinning method for the treatment of bone defects frequently encountered in the mouth and surrounding tissues, and to determine the ideal dental stem cell source that can be used in bone tissue formation with this scaffold.



2. MATERIALS

2.1. KITS

- CellTiter 96® Aqueous One Solution Cell Proliferation Assay Kit (MTS, Promega, USA)
- RNA Isolation Kit (GeneJET RNA Purification Kit, Thermo Scientific, USA)
- Alkaline Phosphatase (ALP) Assay Kit (SIGMAFAST™, Merck, USA)
- Calcium Assay Kit (QuantiChrom™ Calcium Assay Kit, BioAssay Systems, USA)
- Alcian Blue Staining Kit (ALCIAN BLUE pH 2,5, Bio-Optica Milano Spa, Italy)
- Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA)
- iScript c-DNA Synthesis Kit (Bio-Rad, USA)
- von Kossa Stain Kit (Diagnostic BioSystems, CA)

2.2. CHEMICALS

- Bovine Serum Albumin (BSA) (Sigma Aldrich, USA)
- Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco, Germany)
- MEM Alpha Medium (1X) (Gibco, Germany)
- Dulbecco's Modified Eagle Medium high (Gibco, Germany)
- Dulbecco's Modified Eagle Medium low (Gibco, Germany)
- Trypsin-EDTA (Sigma-Aldrich Corporation, Germany)
- Fetal Bovine Serum (FBS) (Gibco, Germany)
- Plasmocin Prophylactic (2.5 mg/ml) (InvivoGen, USA)
- Recombinant Human TGF-beta 1 Protein (R & D Systems, USA)
- Penicillin-Streptomycin-Amphotericin (PSA) Sigma-Aldrich Corporation, Germany)
- Tween® 20 (Sigma Aldrich, USA)
- Triton X-100 (Sigma Aldrich, USA)
- Dichloromethane (Merck Millipore, USA)

- Isopropanol (Merck Millipore, USA)
- Formaldehyde (Fluka, Switzerland)
- Dexamethasone
- Insulin
- IBMX
- Beta glycerophosphaldehyde
- ITS Premix
- L-ascorbic acid-2-phosphate
- Proline
- Na-pyruvate
- Indomethacin

2.3. REAGENTS

- Alizarin Red solution (2 percent, pH=4.1) (Milipore, USA)
- Oil Red O (Sigma Aldrich, Germany)
- Poly (D, L-lactide-co-glycolide) (PLGA)(75:25 LA:GA, Mw=66,000-107,000) (Sigma-Aldrich Corporation, Germany)
- DAPI (4', 6-diamidino-2-phenylindole) (Sigma-Aldrich, USA)

2.4. CD ANTIBODIES

- Anti-CD90 antibody (Abcam, UK)
- Anti-CD45 antibody
- Anti-CD44 antibody
- Anti-CD14 antibody
- Anti-CD105 antibody
- Anti-CD34 antibody
- Anti-STRO-1 antibody (Abcam, UK)

2.5. CONFOCAL ANTIBODIES

- Collagen I (Rabbit polyclonal to Collagen I) antibody (Abcam, UK)
- Osteocalcin (Mouse monoclonal to Osteocalcin) antibody (Abcam, UK)
- Runx2 (Rabbit monoclonal to Runx2) antibody (Abcam, UK)
- Goat antimouse IgG (Alexa Fluor®555) (Abcam, UK)
- Goat antirabbit IgG (Alexa Fluor® 647) (Abcam, UK)

2.6. INSTRUMENTS

- Laminar Flow Cabinet (ESCO Lab. Culture Class II Biohazard Safety Cabinet 2A, Singapore)
- CO2 Incubator (Thermo Scientific, USA)
- Brightfield Microscope (Carl Zeiss, USA)
- Scanning Electron Microscope (Carl Zeiss EVO, Germany)
- Fluorescence Microscope (Carl Zeiss, USA)
- Inverted Microscope (Nikon Eclipse TC 100, USA)
- Confocal Microscope (Leica, Germany)
- Centrifuge (Hettich micro 22R and Sigma 2-5 centrifuge, Germany)
- Nanodrop Spectrophotometer (Thermo Scientific, USA)
- pH Meter (Hanna, Germany)
- ELISA Plate Reader (Bio-Tek EL x 800, USA)
- Varioskan™ LUX Multimode Microplate Reader (Thermo Scientific, USA)
- Vortex (Stuart, UK)
- FACS Calibur (BD Biosciences, USA)
- CFX96 Touch™ Sequence Detection System (Bio-Rad, USA)
- Sputter Coater (Bal-tec SCD 005, Germany)
- Confocal Microscope (Leica, Germany)
- Hemocytometer (Hausser Bright-Line, USA)
- 3 Well Chamber (Ibidi, Germany)
- 24-well plate

- 48-well plate (Surface non-treated)



3. METHODS

3.1. ISOLATION OF SCAP, DFSC AND TGSC FROM THIRD MOLARS OF DOMESTIC PIG

Cells used in this study were isolated with ethics committee approval that was taken from Acibadem University (Appendix A). The six months old male pig was used as a donor for isolation of stem cells. Firstly, the pig was anaesthetized and the jaw bone was carved with a dental drill. Tissues of third molar tooth were brought out into the open and apical papilla, follicle and germ tissues were taken into separate falcon tubes which contain harvest medium (MEM Alpha, 10 percent PSA). After harvesting, stem cells from these tissues were obtained by explant culture. Briefly, tissues were cut into small pieces and placed into tissue culture flask and then MEM alpha medium containing 1 percent (v/v) PSA and 10 percent (v/v) Fetal Bovine Serum, which was named as growth medium (GM), was added into flasks (Figure 3.1. a-f). Flasks were incubated at 37°C and 5 percent CO₂ and medium was changed twice. When the stem cells reached to confluency, they were passed into new flasks.

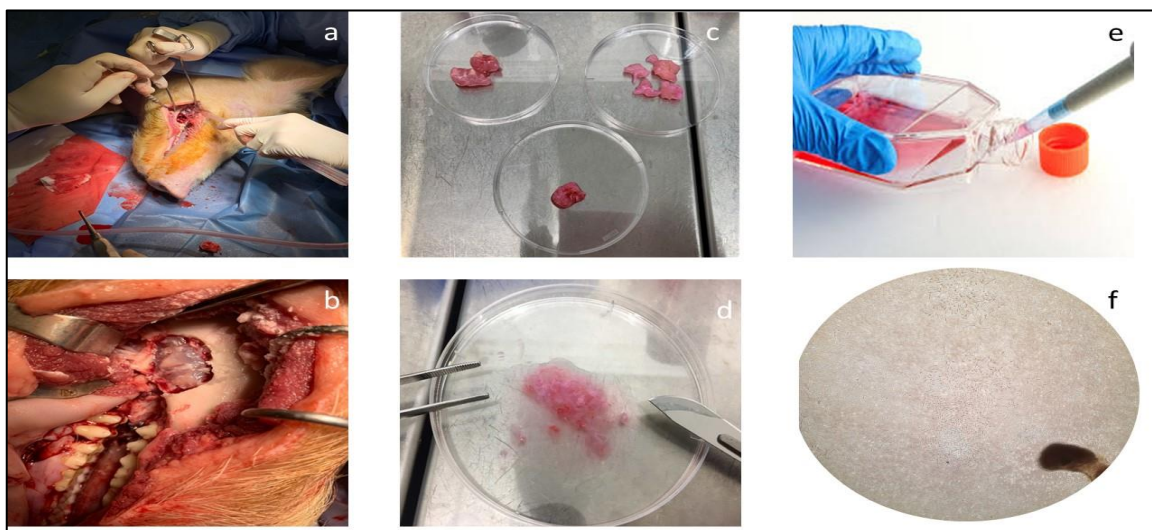


Figure 3.1. (a) Isolation of tissues from six months old pig, (b) immature tooth embedded in the carved jawbone, (c) dental follicle, dental germ, and apical papilla tissues of developing tooth, (d) minced tissues for explant culture, (e) tissue culture flask containing minced tissues and growth medium, (f) adherent cells with a piece of residual tissue

3.2. CHARACTERIZATION OF STEM CELLS

Expression of surface antigens that are CD90, Stro-1, CD45, CD73, CD14, CD105, CD34, CD44 were detected for DFSC, TGSC and SCAP, respectively. For these purposes, cells between passage 2 and 4 were trypsinized and counted. Pelleted cells were divided into 9 tubes having 5.10^5 cells in each tube. Cells were fixed with 3.7 percent formaldehyde. After fixation, monoclonal PE conjugated CD105, FITC conjugated CD90, CD45, and CD44, Stro-1, PE-Cy5 conjugated CD34, CD14 antibodies were added into tubes and incubated for 1 h at 4°C. Tubes were centrifuged and excess antibodies were washed with DPBS. After washing, pellets were resuspended with DPBS and analyzed with a flow cytometry instrument (FACSCalibur Flow Cytometry System). The flow cytometry data was analyzed using Cell Quest software.

3.3. EVALUATION OF OSTEO/ODONTOGENIC CAPACITY OF STEM CELLS

3.3.1. Cell Proliferation (MTS) Assay

CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was performed to determine the cell proliferation after 7, 14, 21 days of incubation in osteogenic differentiation medium (DMEM low glucose supplemented with 10 percent FBS, 100µg/mL PSA, 50 µM ascorbic acid, 10 mM β-glycerophosphate and 100 nM dexamethasone). Briefly, MTS reagent diluted with MEM alpha at 1:6 ratio was added into each well after the medium was discarded from the wells. Then, the wells were incubated at 37°C in a CO₂ incubator for 2 h. At the end of the incubation, their absorbance values were obtained at 490 nm by using Elisa Plate Reader.

3.3.2. Alizarin Red Staining

After 7, 14, and 21 days of osteogenic induction, cells were stained with Alizarin Red solution in order to examine mineralization. For these purposes, cells were fixed with 4 percent formaldehyde for 45 mins. After washing with dH₂O, Alizarin Red solution was

added to samples and they were incubated at RT in dark for 45 mins. After that, samples were washed with distilled water and photographed under the inverted microscope.

3.3.3. von Kossa Staining

After 7, 14, and 21 days of osteogenic induction, von Kossa staining was performed in order to examine calcification according to the manufacturer's protocol. Briefly, 5 percent silver nitrate solution was added to each well after fixation of cells and incubated for 45 mins by exposing UV light. The wells were washed with dH₂O and 5 percent sodium thiosulfate reagent was added and incubated for 3 mins. After washing, nuclear fast red solution was added into the wells and incubated for 5 mins. Then, the wells were washed until redness disappeared. Finally, the wells were analyzed with light microscopy and pictures were taken.

3.3.4. Alkaline Phosphatase (ALP) Assay

After 7, 14, 21 days of osteogenic induction, ALP assay was performed to all samples to detect alkaline phosphatase activity. For these purposes, SIGMAFAST™ p-Nitrophenyl phosphate tablets were used according to the manufacturer's protocol. Briefly, one gold tablet (tris buffer) and one silver tablet (p-Nitrophenyl Phosphate (pNPP)) were dissolved in 20 mL dH₂O. After washing the cells with DPBS, ALP assay solution was added and incubated at RT in the dark for 1 hour. Then, the absorbance values were measured at 405 nm by using Elisa Plate Reader. In this study, absorbance values were converted to the amount of ALP via ALP calibration curve and ALP activity was compared between cell types and days (Appendix B).

3.3.5. Calcium Deposition Assay

Calcium deposition of cells was measured with QuantiChrom Calcium Assay Kit on 7, 14, 21 days of osteogenic induction according to the manufacturer's protocol. Firstly, the samples were washed with DPBS twice. Decalcification of samples was performed with 0.6 M HCl. Cell plates were scraped from the surfaces and collected in tubes for overnight incubation. For measurement, 10 µl of sample and calcium dilutions were transferred to a

96-well plate and 200 µl of working solution from the kit was added. The culture plate was incubated at RT for 60 mins and the absorbance values were read at 612 nm using the Varioskan™ LUX multimode microplate reader. Then, the absorbance values obtained from the samples were converted into concentration of calcium by using calcium calibration curve established with standards (Appendix C).

3.3.6. Real-Time PCR (RT-PCR) Analysis

For RT-PCR experiments, cells were cultured in growth and osteogenic medium for 21 days. Complete RNA isolation was performed with GeneJet RNA Purification Kit from the samples collected on days 7, 14 and 21 during the culture process. Before RT-PCR, cDNA was obtained from the RNA that was isolated from the samples with the iScript cDNA synthesis kit. Primer sequences used in the experiment are given in Table 3.1. and PCR conditions applied are given in Table 3.2. Osteocalcin, Runx2, Osteonectin, Osteopontin, DMP-1 and DSPP gene levels were calculated according to the SYBR® Premix Ex protocol. β -actin was used as a housekeeping gene to normalize RNA expression. Each sample was triplicated and analyzed with CFX96 Touch™ Real-Time PCR Detection System.

Table 3.1. Primers used in RT-PCR

Primers	Primer Sequence (5'→3')	Length of primers	Tm (°C)
B-actin	F: TCGTCCACCGCAAATGCTTC	211	56
	R: TGCTGTCACCTTCACCGTTC		56
ON	F: ACCACTTCCCCTCACTTTTC	316	56
	R: TTTTACAACCTTCCTGCGCC		56
Runx2	F: AGTTTGTTCTCTGACCGCCTCA	230	56
	R: ACCTGCCTGGCTTTTCTTACT		56
OP	F: GAAGAAGACAAACACCTGAAAC	241	56
	R: CCATGAAGCCACACACTAAAC		56
OC	F: ACCAACAGACGCACCATGAG	281	56
	R: AAGCCGATGTGATCAGCCAG		56
DMP-1	F: CAGAAGCGGTGAGGACTCTG	393	56
	R: CTGGCATACCCCACTGATCG		56
DSPP	F: GAAGGCAGTATCACAGAACTCA	298	56
	R: GAATGGCCCATGCTATTGCC		56

Table 3.2. RT-PCR Conditions

95 ° C	95 ° C	56 ° C	72 ° C	Go back second step	72 ° C	4 ° C
10:00	0:15	0:30	0:30	45X	10:00	∞

3.4. EVALUATION OF CHONDROGENIC CAPACITY OF STEM CELLS

Alcian Blue Staining

After 7, 14, 21 days of incubation in chondrogenic differentiation medium (DMEM-high glucose containing 1 percent PSA, 1 percent ITS Premix, 40 µg/mL proline, 100 µg sodium pyruvate, 50 µg/mL L-ascorbic, 100 nM dexamethasone, and 10 ng/mL TGF-β1), cells were fixed with 4 percent formaldehyde for 45 mins. After washing with dH₂O, the samples were stained with Alcian Blue (pH 2.5) kit according to the kit's protocol. After that, samples were analyzed under the inverted microscope.

3.5. EVALUATION OF ADIPOGENIC CAPACITY OF STEM CELLS

Oil Red O Staining

Oil Red O staining was applied to samples in order to observe lipid accumulation of cells after 7, 14 and 21 days in adipogenic medium (DMEM high glucose containing 10 µg/ml insulin, 500 µM IBMX, 100 µM indomethacin, 1 µM dexamethasone, 10 percent FBS, 1 percent PSA). Firstly, samples were fixed with 4 percent formaldehyde for 45 mins. After washing with dH₂O three times, the cells were incubated in 60 percent isopropanol for 5 mins. Then, isopropanol was discarded and the wells were allowed to dry. The Oil Red O dye was dissolved in isopropanol at a concentration of 3 mg/mL. Before using, Oil Red O solution was mixed with water in 3:2 ratio. This working solution was added to wells and incubated at RT for 30 mins. After washing with dH₂O, the stained lipid droplets were observed under the inverted microscope.

3.6. PREPARATION OF PBS:PLGA SCAFFOLDS

Polybutylene succinate (PBS) and Poly Lactic-co-Glycolic Acid (PLGA) were dissolved in dichloromethane at 10 percent, 15 percent, 20 percent, and 25 percent w/v ratio. For each total polymer concentration in dichloromethane, PBS and PLGA were put in a 1:1 and 1:3 ratio. All solutions were stirred in a magnetic stirrer at 150 rpm in RT for 1 day. Wet spinning setup was prepared with a syringe pump fixed in vertical direction (Figure 3.2.). Turntable of wet spinning setup was placed in horizontal direction and a coagulation bath was put on it. Syringe pump had the ability to fix the flow rate of PBS:PLGA/dichloromethane solution and the turntable helped to rotate the coagulation bath. The flow rate of solutions was fixed at 20 $\mu\text{l}/\text{min}$ via syringe pump. Coagulation bath contained isopropanol and distilled water at different ratios which were 100:0, 85:15, 75:25, 50:50, 25:75, 15:85, 0:100. Solutions in different concentrations and different polymer ratios were filled into a syringe with 21 G needle and spun via this setup. After wet-spinning, these scaffolds were incubated in the coagulation bath at 4°C overnight. They were frozen at -80°C and freeze-dried for one day. After drying, scaffolds were cut into equal circular pieces with 0.9 cm diameter in order to be used in *in vitro* studies.

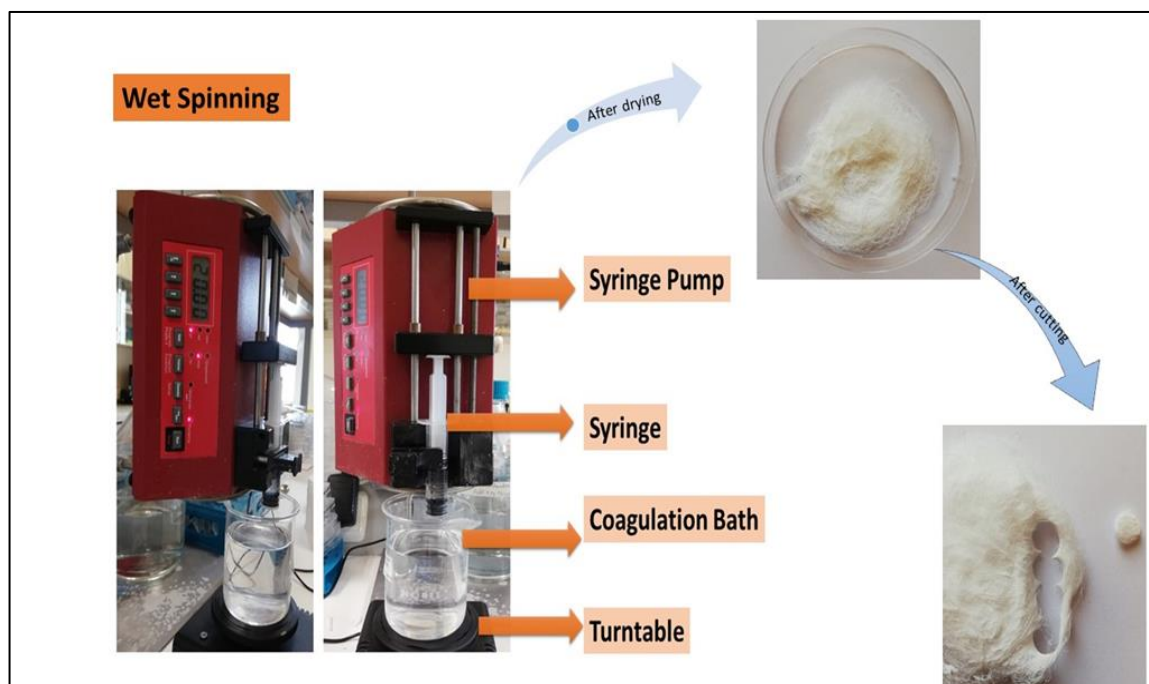


Figure 3.2. Wet spinning setup and fibrous scaffold

3.7. CHARACTERIZATION OF SCAFFOLDS

3.7.1. Scanning Electron Microscopy (SEM)

PBS:PLGA scaffolds with the composition of 10 percent (1:1), 10 percent (1:3), 15 percent (1:1) 15 percent (1:3), 20 percent (1:1), 20 percent (1:3), and 25 percent (1:3) were cut as a circle with 1 cm radius and attached onto adhesive carbon tabs with 12 mm radius which were affixed onto the slide. All samples were coated with gold by Sputter Coater and analyzed with a Scanning Electron Microscope to determine their surface characteristics.

3.7.2. Mechanical Analysis

Compression and tensile tests were performed on 15 percent (1:3), 15 percent (1:1), 20 percent (1:3), 20 percent (1:1) and 25 percent (1:3) scaffolds with Instron Universal Testing Machine. For the compression test, scaffold fibers were filled into cylindrical molds with an area of 0.32 cm^2 . Before compression values were recorded, all molds were pressed with 5N for 5 sec. Then, their heights were measured for young's modulus calculation. Compression tests were performed with a speed of 1 mm/min. Load (N) versus displacement (mm) graphs were obtained after each test and elasticity modulus (E) values were calculated. Mechanical testing setup is shown in Figure 3.3.



Figure 3.3. Compression test setup

Likewise, tensile tests were performed on the same scaffolds with the same universal testing machine. Scaffold fibers were attached from both sides with the help of double-sided tape as seen in Figure 3.4. For each setup, initial width and length were measured to calculate Young's modulus (E). Tensile tests were performed with a speed of 1 mm/min. Load (N) versus displacement (mm) graphs were obtained after each test and modulus of elasticity (E) values were calculated.

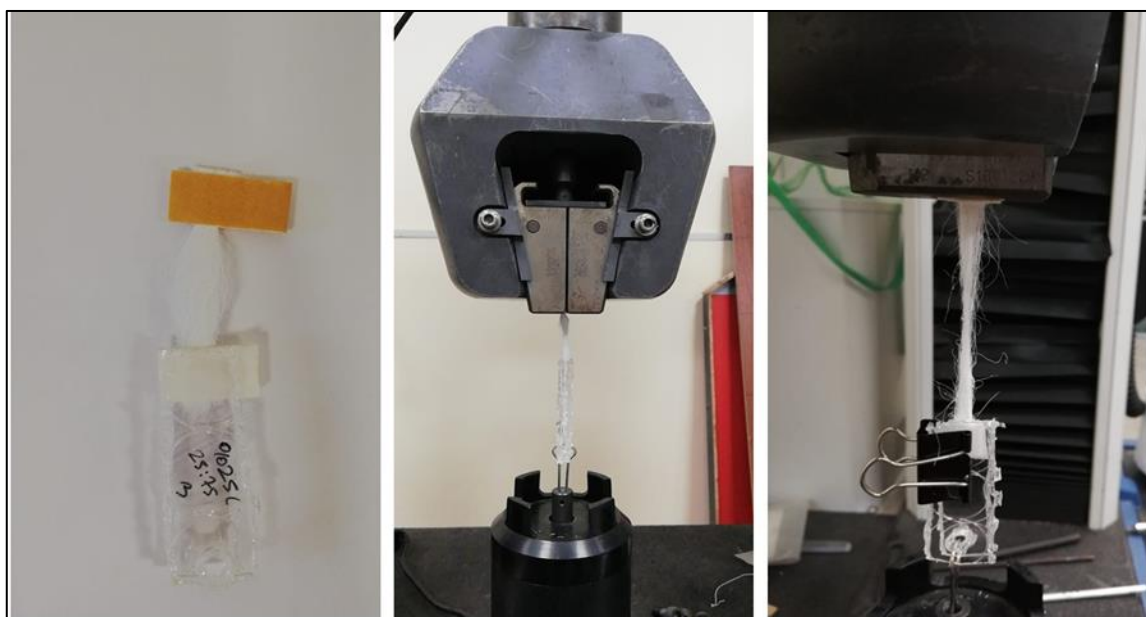


Figure 3.4. Tensile test setup

3.7.3. Degradation Analysis

Degradation analysis of PBS:PLGA scaffolds prepared at concentrations of 15 percent (1:3), 15 percent (1:1), 20 percent (1:3), 20 percent (1:1) and 25 percent (1:3) were evaluated. PBS:PLGA tissue scaffold structures obtained by wet spinning method were prepared as discs with a diameter of 9 mm and placed in falcons filled with the degradation solution that was prepared from DPBS (pH = 7.4) containing 0.09 percent sodium azide. All falcons were kept in the degradation solution for 90 days in a shaking water bath at 37°C. On 30th, 45th, 60th, and 90th days, the pH value of the solution was measured and samples were taken from

the solution. All samples were washed with distilled water and lyophilized after freezing. Next day, weights of the degraded scaffolds were measured and the percent weight loss was calculated according to their initial weight.

3.7.4. Cell Viability

After the results of mechanical testing and degradation analysis, 20 percent (1:1), 20 percent (1:3) and 25 percent (1:3) PBS:PLGA scaffolds with the best performances were selected. Each scaffold was prepared as 9 mm diameter discs. Sterilization process was performed on scaffolds before viability assessment. For that purpose, they were immersed in 70 percent ethyl alcohol for 1 h. After washing with DPBS, they were immersed in DPBS solution containing 100 μ M PSA for 2 h. Again, scaffolds were washed with DPBS and then were kept in alpha-MEM containing 10 percent FBS and 1 percent penicillin, streptomycin and amphotericin at 37°C, 5 percent CO₂ and 90 percent moisture in an incubator overnight. Each of the sterile and scaffolds was placed in non-treated surface 48-well cell culture plates. Then, cells (40,000 cells/scaffold) were seeded on the scaffolds and incubated for 4 h in the incubator at 37°C. TGSCs and human osteoblast cells (hOB) were used to evaluate the effect of scaffolds on cell viability.

3.8. IN VITRO SCAFFOLD AND STEM CELL INTERACTION

3.8.1. Stem Cell Seeding onto the Scaffolds

After evaluation of the effects of the scaffolds onto cell viability, the best scaffold (20 percent (1:1) PBS:PLGA) was selected and used for the rest of the experiments. For *in vitro* studies, each scaffold was prepared as 9 mm diameter discs. They were immersed in 70 percent ethyl alcohol for 1 h, then in DPBS solution containing 100 μ M penicillin/streptomycin/amphotericin (PSA) for 2 h. After washing with DPBS, scaffolds were soaked into 20 μ g/ml fibronectin solution and left for 20 mins. Then, the scaffolds were kept in growth medium at 37°C, 5 percent CO₂ and 90 percent moisture in an incubator. Each of scaffolds was placed in non-treated surface 48-well cell culture plates. Then, cells (150,000 cells/scaffold) were seeded on the scaffolds and incubated for 4 h. Then, the growth

medium was added through the wall of the culture plate slowly. After two days of cell seeding, media of half the cells were changed with osteogenic medium and then cultured throughout 21 days of incubation.

3.8.2. Cell Proliferation (MTS) Assay

On days 7, 14, 21, MTS assay was carried out. For this assay, MTS reagent diluted with MEM alpha at 1:6 ratio was added into the scaffolds after media were discarded from the wells. Then, the samples were incubated at 37°C in the CO₂ incubator for 2 h. After that, their absorbance values were obtained at 490 nm by using Elisa Plate Reader.

3.8.3. Alkaline Phosphatase (ALP) Activity

ALP assay was carried out for samples after days 7, 14, 21. For this purpose, scaffolds were washed with DPBS and put in a new sterile wells. After that, the same protocol in section 3.3.4. was followed.

3.8.4. Real-Time PCR Analysis

RNA isolation was carried out from scaffolds by using GeneJet RNA Purification Kit and the amount of RNA isolated from samples was measured by NanoDrop after days 7, 14, 21. The following steps were the same with section 3.3.6. Similarly, these RNA samples were converted into cDNA by using iScript cDNA synthesis kit. To carry out RT-PCR reaction, these cDNA, primers given in Table 3.1. and Maxima SYBR Green/ROX qPCR Master Mix were used.

3.8.5. Immunocytochemistry

On days 7, 14 and 21, samples were fixed. Fixation of samples was carried out in 3.7 percent formaldehyde for 20 mins at RT and then in absolute methanol for 10 mins at -20°C. After fixation, the samples were washed with DPBS and waited in DPBS at 4°C until staining. Before staining, the samples were incubated in DPBS containing 0.1 percent Triton X-100

for 10 mins at RT to increase permeability of the cells. Following washing with DPBS, samples were incubated for 1 h at RT in the blocking solution containing DPBS, 1 percent BSA, 0.1 percent Tween® 20. Collagen type I, Osteocalcin, and Runx2 antibodies were diluted at a ratio of 1:500 with blocking solution. Then, the diluted antibodies were dropped on the marked areas of the samples and incubation was carried out at 4°C overnight. The next day, after washing with DPBS, the secondary antibodies (goat antimouse IgG and goat antirabbit IgG that were diluted with blocking solution at 1:500 ratio) were added to the samples and incubated at 4°C overnight in the dark. The next day, excess secondary antibodies were washed with DPBS. DAPI was diluted with DPBS solution at 1:1000 ratio and put onto the samples and left for 15 mins at RT in dark. After washing, the samples were dried and Prolong Gold antifade reagent, which is a mounting medium, was put onto them with coverslips. They were incubated overnight at RT in the dark. The next day, nail polish was applied around the coverslip in order to prevent air dry. Finally, the samples were examined and images were recorded via confocal microscope.

3.8.6. von Kossa Staining

von Kossa staining was carried out for samples after days 7, 14, 21. For this purpose, scaffolds were washed with DPBS and put in new sterile wells. After that, the same protocol in section 3.3.3. was followed.

3.9. STATISTICAL ANALYSIS

Statistical analysis was done with GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA). Two-Way ANOVA and Tukey multiple comparison tests were used. Statistical significance was set at $p < 0.05$.

4. RESULTS

4.1. ISOLATION OF SCAP, DFSC, AND TGSC FROM THIRD MOLARS OF DOMESTIC PIG

SCAP, DFSC, and TGSC were isolated from six months old domestic pigs by explant culture technique. The cells migrated from fragmented tissues to tissue culture plates within two days and this process took 1.5 weeks on average. These primary cultures were observed via bright a field microscope (Figure 4.1. a-c).

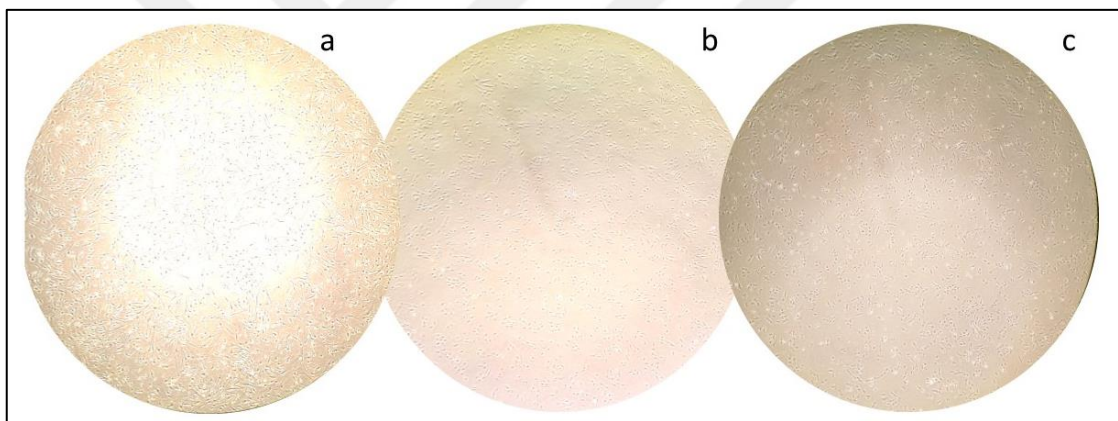
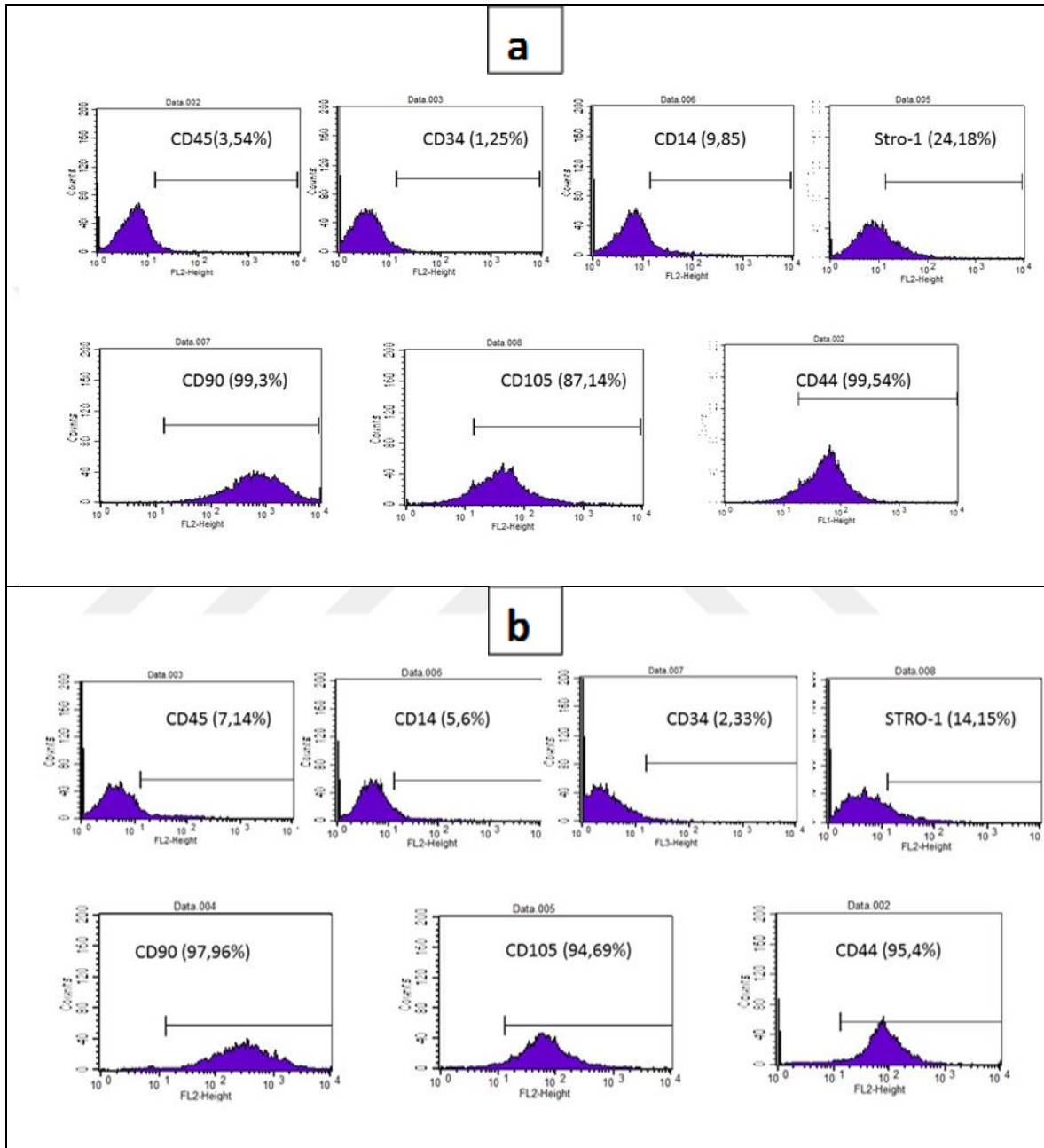


Figure 4.1. Bright field microscopy images of (a) SCAP, (b) DFSC, (c) TGSC under 4x objective

4.2. CHARACTERIZATION OF STEM CELLS

For TGSC and SCAP cells, CD44, CD90 and CD105 were positive, while CD14, CD34, and CD45 were negative among the studied surface markers (Figure 4.2. a, b). Similarly, DFSC had positive profiles for CD90 and CD105 and had negative profiles for CD14, CD34, and CD45 and (Figure 4.2. c). However, DFSC showed lower CD44 expression in comparison

with other cell types while being still positive. The STRO-1 marker, which is important in differentiation into bone, expressed in all three cell types between 14-33 percent.



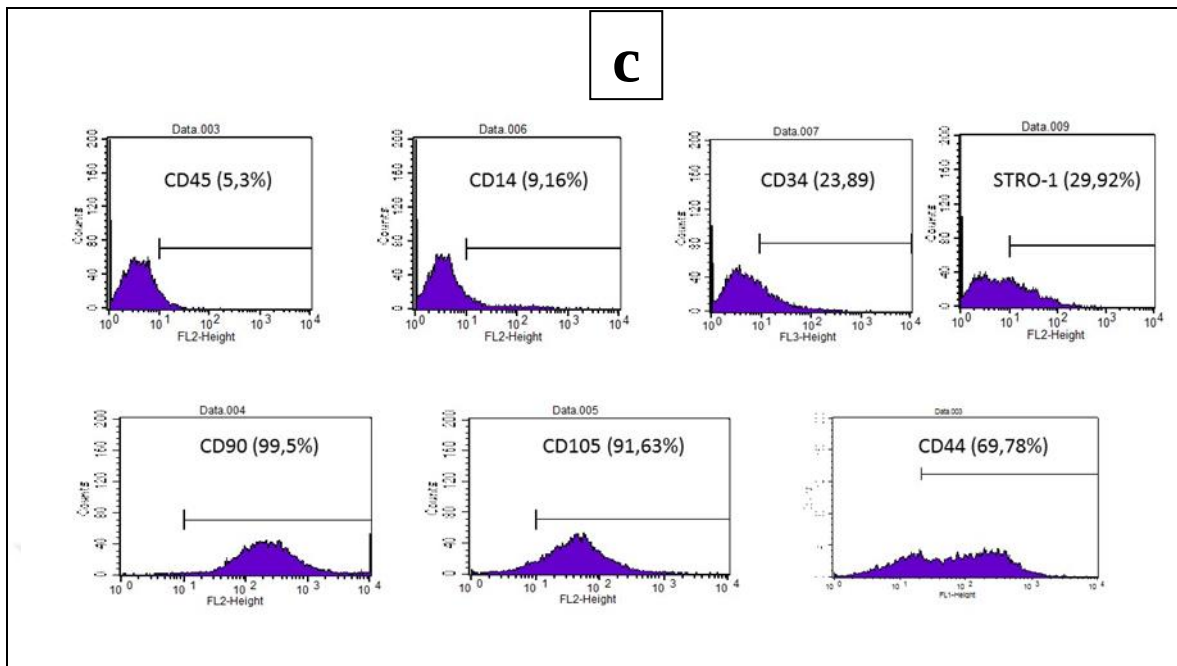


Figure 4.2. Flow cytometry histogram of (a) TGSC, (b) SCAP, (c) DFSC

4.3. EVALUATION OF OSTEO/ODONTOGENIC CAPACITY OF STEM CELLS

4.3.1. Cell Proliferation (MTS) Assay

The results of the MTS cell proliferation assay are shown in Figure 4.3. While an increase in cell number was observed throughout 21 days of incubation in the SCAP and TGSC groups cultured in growth medium, a decrease in cell number was observed in the DFSC group after day 14.

For osteogenically induced groups, cell proliferation in the SCAP group decreased significantly on day 14 and increased again on day 21. On the other hand, the cell numbers in TGSC and DFSC groups were the highest on day 14. Then, a significant decrease in cell number was seen on day 21 after they showed their highest proliferation level on day 14. The addition of osteogenic medium did not suppress cell proliferation in all cell groups at day 7 and the absorbance values between the same cell types groups were found similar. However, a decrease in cell number in the osteogenic medium was observed at day 21 in all cell groups.

In growth media, the highest cell numbers were observed at day 21 in TGSC groups. SCAPs and TGSCs reached their highest cell number on day 21 while DFSCs reached their highest cell number on day 14 in the growth medium. In osteogenically induced media, the highest cell number was recorded on day 14 in TGSC groups. SCAPs on day 21 have higher cell number compared to other days while DFSCs have higher cell number on day 14 compared to other days.

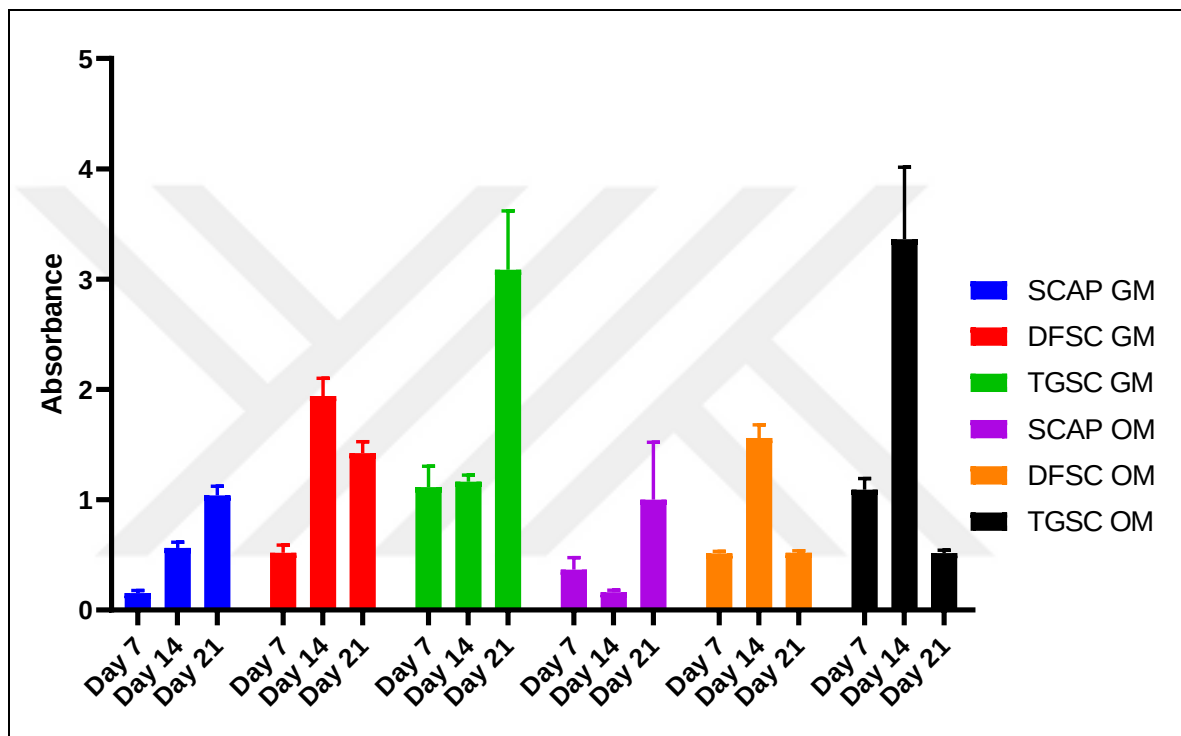


Figure 4.3. SCAP, DFSC, and TGSC proliferation in growth and osteogenic medium throughout 21 days of incubation

4.3.2. Alizarin Red Staining

In the osteogenic media, all three cell types did not show calcified regions on day 7 (Figure 4.4.). On day 14, SCAP had a small number of calcified areas in osteogenic medium while DFSC and TGSC did not form mineral deposition. At the end of day 21, all cell types had mineralized areas. Indeed, SCAP and TGSC had more intense mineralization than DFSC.

In the growth media, calcified and mineralized areas were not observed in all three cell types on days 7, 14 and 21 (Figure 4.5.).

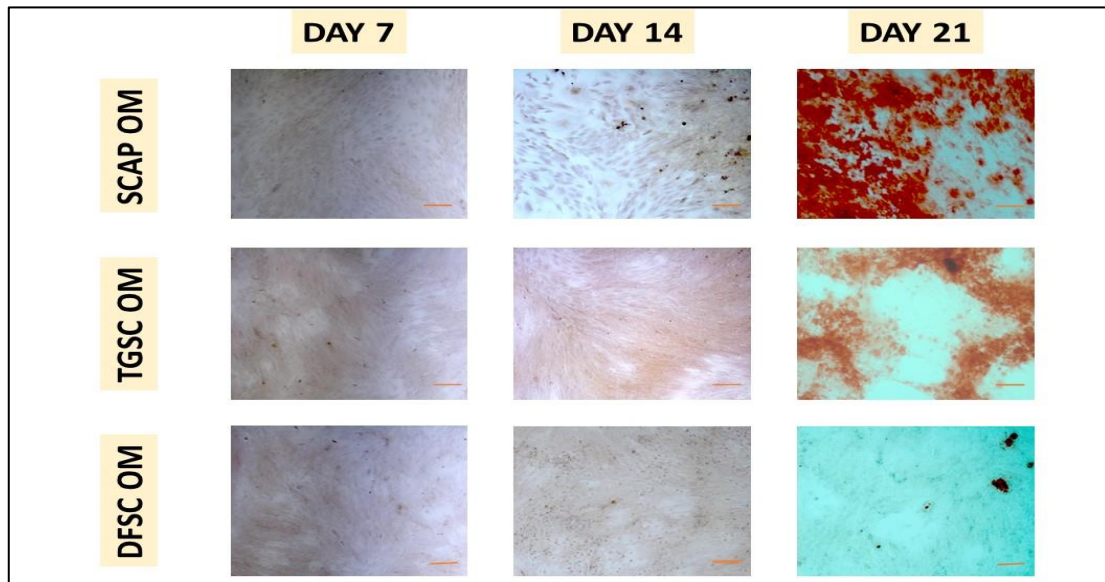


Figure 4.4. Microscopic images of mineralization with alizarin red staining of SCAP, DFSC, and TGSC in osteogenic medium throughout 7, 14, and 21 days of incubation under 10X objectives (Scale: 100 μ m)

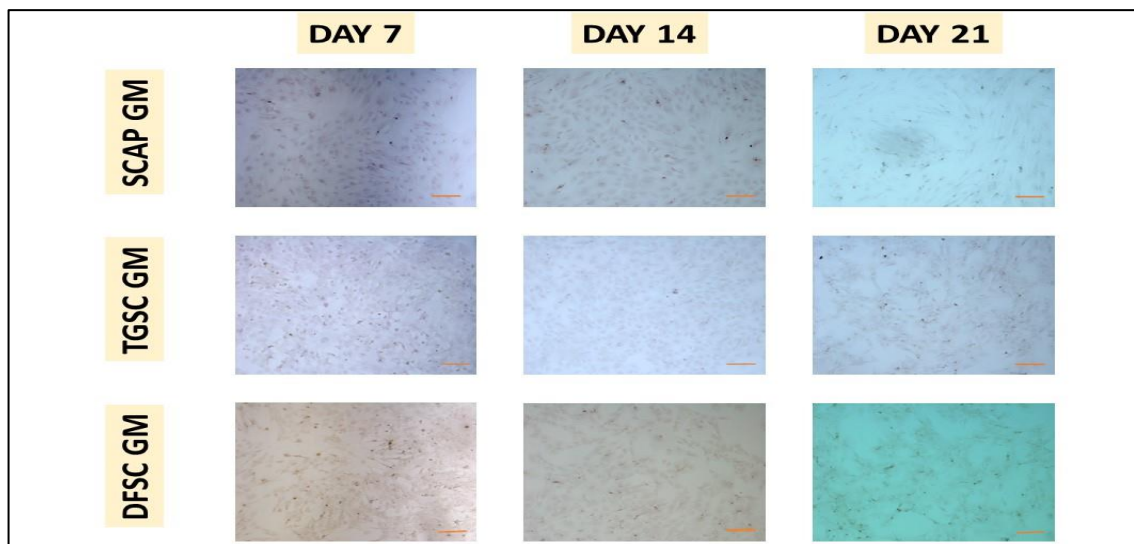


Figure 4.5. Microscopic images of mineralization with alizarin red staining of SCAP, DFSC, and TGSC in growth medium throughout 7, 14, and 21 days of incubation under 10X objectives (Scale: 100 μ m)

4.3.3. von Kossa Staining

In this study, no mineral accumulation was observed in the cells grown in osteogenic medium on day 7 (Figure 4.6.). However, mineralized nodules were noticed only in SCAP on day 14 upon osteogenic induction. On day 21, mineralized areas became common and organized matrix areas were detected in all cell types in the osteogenic media. On the other hand, mineralized areas were not observed throughout 21 days of incubation in all three cell types cultured in growth medium (Figure 4.7.).

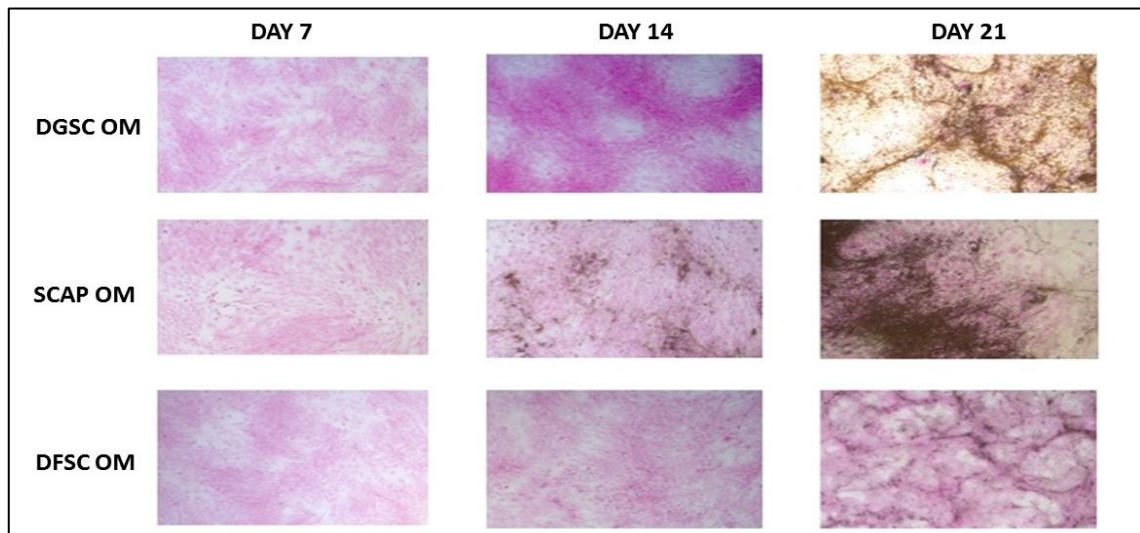


Figure 4.6. Microscopic images of mineralization with von Kossa staining of SCAP, DFSC, and TGSC in osteogenic medium throughout 7, 14, and 21 days of incubation under 10X objectives

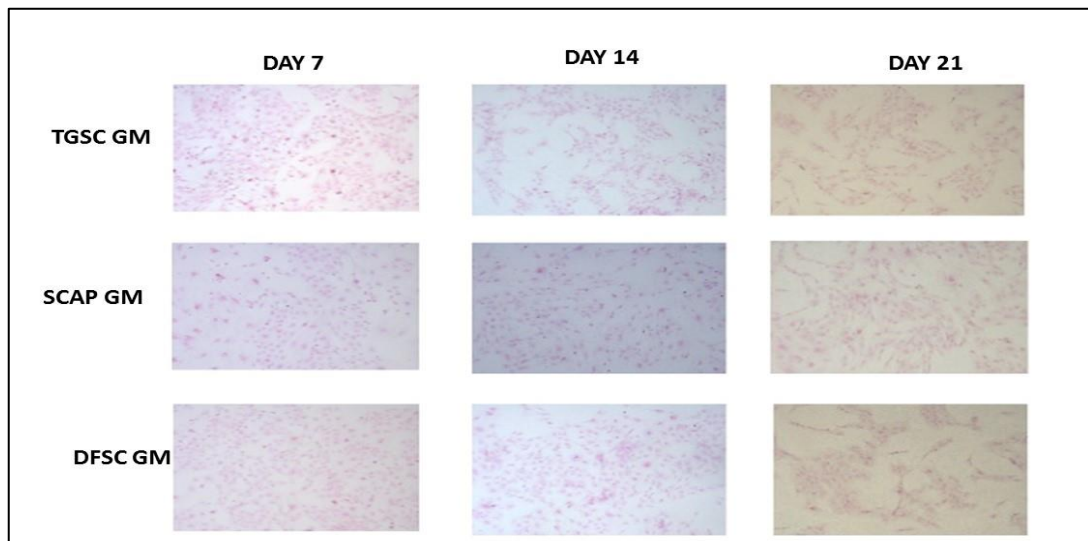


Figure 4.7. Microscopic images of mineralization with von Kossa staining of SCAP, DFSC, and TGSC in growth medium throughout 7, 14, and 21 days of incubation under 10X objectives

4.3.4. Alkaline Phosphatase (ALP) Assay

In the growth medium, only statistically significant differences were seen on the SCAP group compared to other cell types on days 7, 14 and 21 (Figure 4.8.). On the other hand ALP activity increased in osteogenically induced groups from day 7 to day 21 for all cell types. During 21 days of incubation, ALP activity of DFSC and TGSC grown in the osteogenic medium was significantly higher than the ones grown in the growth medium. Although the osteogenic media groups of SCAP showed higher ALP activity compared to growth media groups during culture periods, only statistically significant difference was detected on day 14. On the 14 days of osteogenic induction, TGSC and DFSC had significantly increased ALP activity compared to SCAP. At the end of 21 days of osteogenic induction, there was no significant difference between the cell groups. At this point, all cell types had increased ALP activity by osteogenic induction.

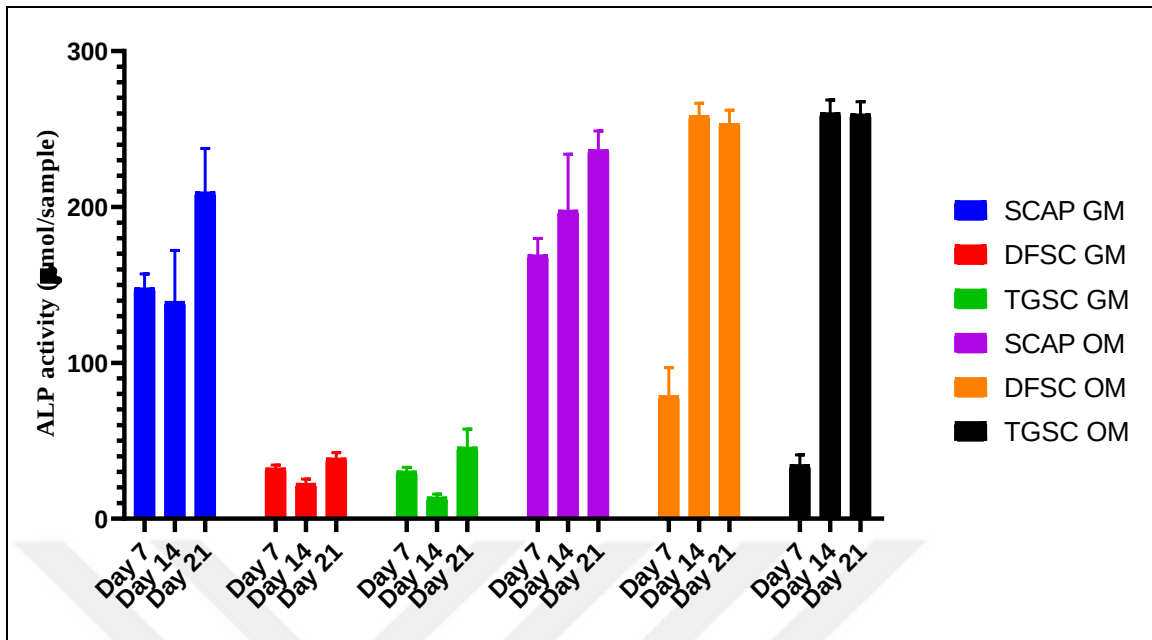


Figure 4.8. ALP activity of SCAP, DFSC, and TGSC throughout 21 days incubation in growth and osteogenic medium

4.3.5. Calcium Deposition Assay

In this study, there was no significant difference in calcium accumulation at any time point among the three osteogenically induced cell types throughout 21 days of incubation (Figure 4.9.). When osteogenically induced cultures were compared to growth medium cultures, there was no difference in calcium accumulation in any cell type on day 7. On day 14, only a statistically significant difference was observed between growth and osteogenic media groups of SCAP ($p = 0.0055$). At the end of 21 days of culture, calcium accumulation in all cell types was found to be significantly higher in osteogenic media groups compared to growth media groups ($p < 0.0001$).

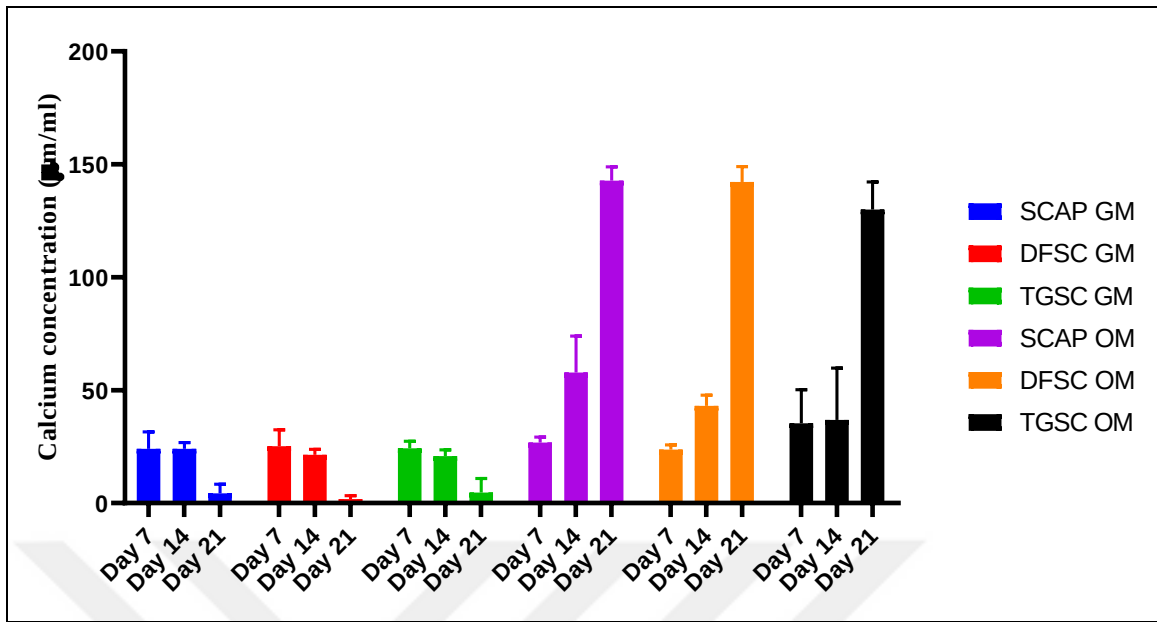


Figure 4.9. The amount of calcium deposition of SCAP, DFSC, and TGSC during 21 days culture in osteogenic and growth media

4.3.6. Real-Time PCR (RT-PCR) Analysis

In this analysis, osteogenic and odontogenic differentiation of cell groups were evaluated according to gene expression at mRNA level. As marker genes, Runx2, Osteocalcin, Osteonectin, osteopontin, DSPP, DMP-1 were used and their levels were normalized by using beta actin as housekeeping gene.

Runx2 had a similar expression pattern for all cell groups in growth media (Figure 4.10. a). Expression level of Runx2 increased from day 7 to day 21 in growth media groups while its levels increased on day 14 and decreased on day 21 in osteogenic media groups. Similar to growth media groups, osteogenic media groups exhibited the same expression trend among themselves. However, only the SCAP group had a statistically significant increase at day 14 ($p = 0.0007$) and a decrease at day 21 compared to day 14 ($p = 0.0019$).

Osteocalcin expression showed statistically significant increase after day 14 in all three cell types in osteogenic medium compared to growth medium groups ($p < 0.005$) (Figure 4.10 b). In osteogenic media groups, there was a significant increase between day 7 and day 21 and

between day 14 and day 21 in all three cell types. However, cells in growth medium did not exhibit statistically significant difference between the other groups at any incubation point.

Osteonectin (ON) expression was high in each group according to other gene expressions in terms of fold change (Figure 4.10. c). There was no statistically significant difference between osteogenic and growth media groups of DFSC and TGSC cell types, which exhibited high Osteonectin expression. SCAP in 14 days of osteogenic induction had the highest Osteonectin level and showed a significantly increased expression among all groups ($p < 0.005$). However, SCAP in 7 and 21 days of osteogenic induction did not show significant difference from the other groups.

Osteopontin gene expression didn't exhibit a statistically significant difference in the growth medium for all cell types at any time point (Figure 4.10. d). In the osteogenic media groups, SCAP and DFSC exhibited a significantly higher osteopontin expression on day 21. SCAP in osteogenic medium on day 21 showed the highest osteopontin expression among all other groups. Although TGSC in osteogenic medium had an increase from day 7 to day 21, this increase in expression was not statistically significant.

Odontogenic differentiation was evaluated by the expression of the DMP-1 and DSPP genes. All three cell types showed statistically significant DMP-1 expression on day 21 in osteogenic media compared to day 7 and day 14 (Figure 4.10. e). In addition, SCAP in osteogenic medium was significantly higher DMP-1 expression level compared to DFSC and TGSC on day 21. However, there was no significant difference in DMP-1 expression between the groups in the osteogenic and growth medium on day 7 and day 14 for all cell types.

Although DSPP gene expression level was low, DSPP expression was detected in all groups on day 7 and day 14 with no significant difference between the groups (Figure 4.10. f). DSPP expression in the TGSC on day 21 was significantly higher compared to day 7 and day 14 in the osteogenic media. Also, all three types of cells had elevated level of DSPP expression in osteogenic medium on day 21.

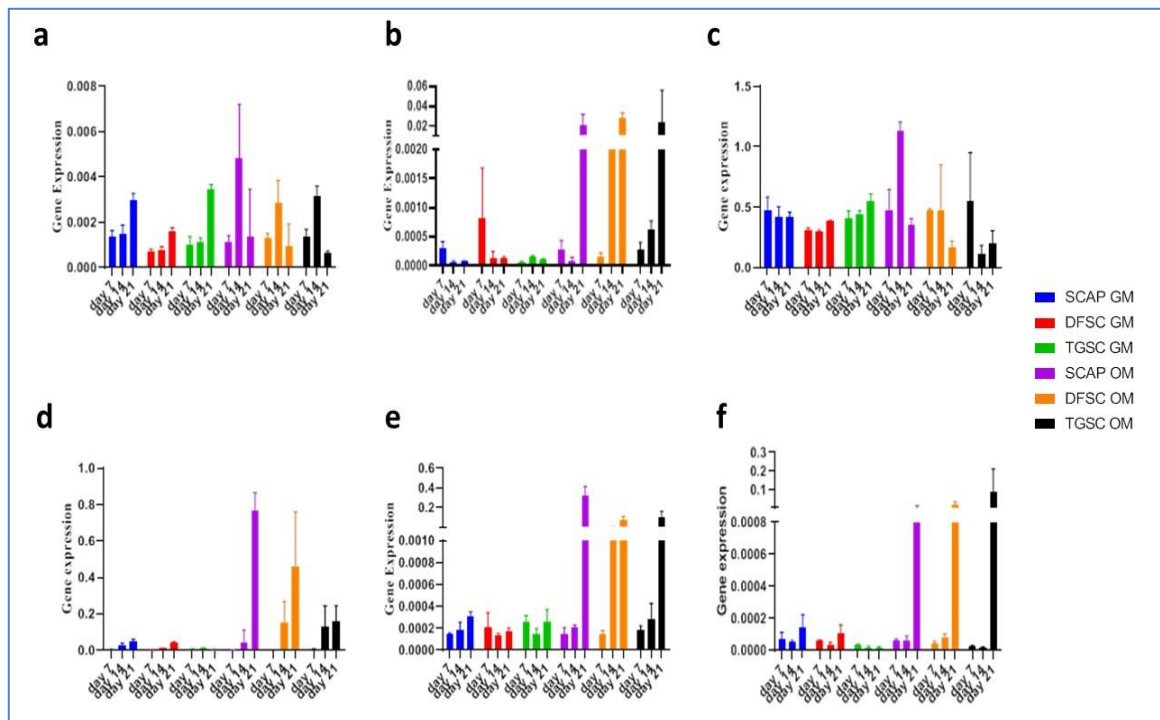


Figure 4.10. Gene expression graphs of (a) Runx2, (b) Osteocalcin, (c) Osteonectin, (d) Osteopontin, (e) DMP-1, (f) DSPP

4.4. EVALUATION OF CHONDROGENIC CAPACITY OF STEM CELLS

Alcian Blue Staining

In this study, Alcian blue staining stains sulfated proteoglycans in cartilage tissue to detect chondrogenesis in cells. In the chondrogenic medium, there were no blue colored regions indicating the presence of glucose aminoglycan in SCAP and TGSC on day 7 while DFSC had some blue colored areas (Figure 4.11. a). On day 14, DFSC and TGSC had more blue colored areas compared to SCAP in chondrogenic medium. After 21 days of chondrogenic induction, positive staining was observed in all cell types compared to the growth media groups. On day 21, the cells switched to a more regular structure and showed dense cartilage-like extracellular matrix formation.

In the growth medium, a very low amount of staining was observed in three cell types at any time point except the 21st day of DFSC that had some blue colored areas indicating chondrogenesis (Figure 4.11. b).

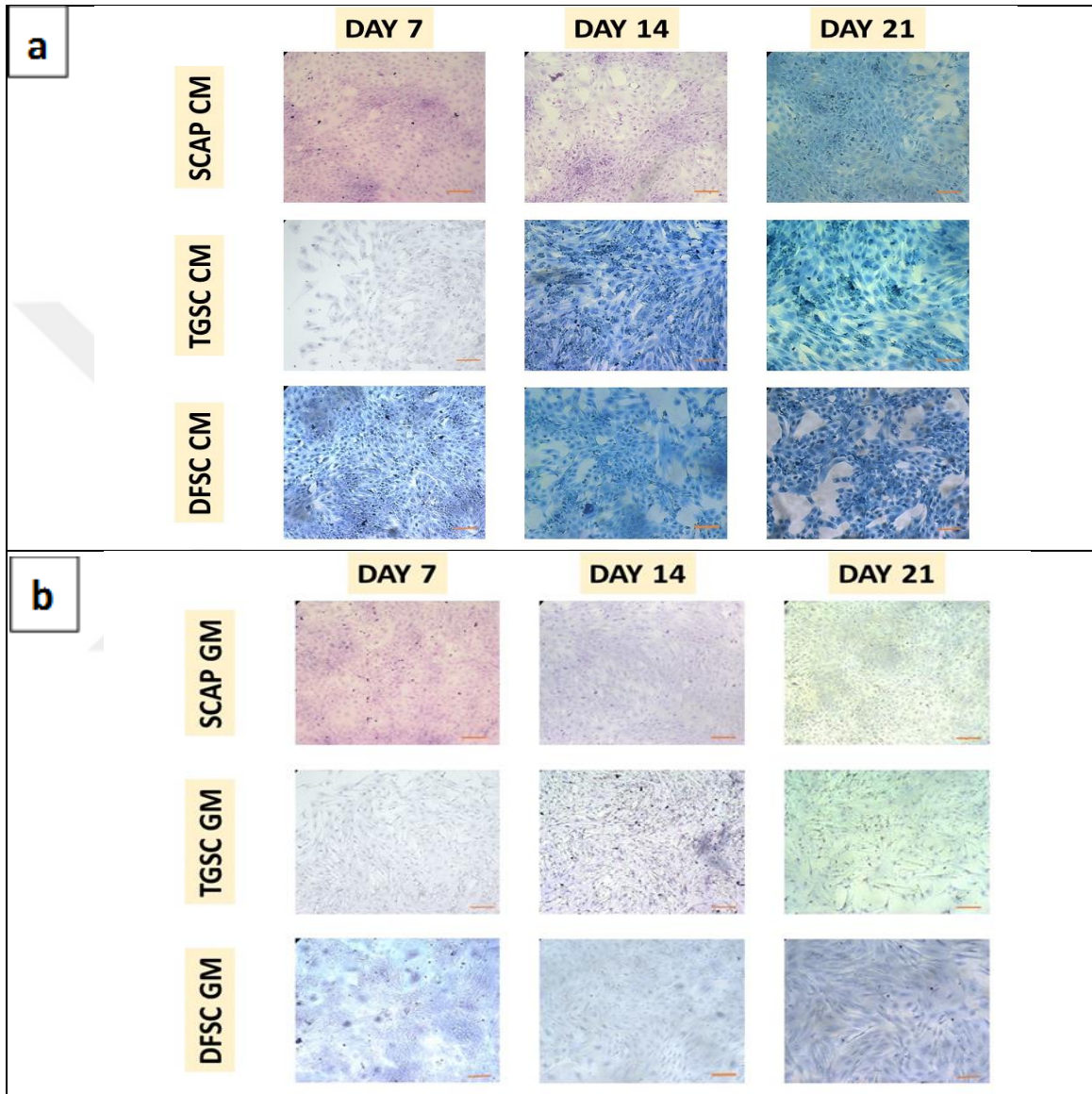


Figure 4.11. Alcian Blue staining of SCAP, DFSC, and TGSC in (a) chondrogenic medium and (b) growth medium throughout 21 days of incubation (Scale: 100 μ m)

4.5. EVALUATION OF ADIPOGENIC CAPACITY OF STEM CELLS

Oil Red O Staining

A small amount of adipogenic differentiation was detected with the presence of oil droplets as bright red stains in all three groups at the end of the 21 days of adipogenic induction (Figure 4.12. a). The amount of oil droplets increased from day 7 to day 21 in all three groups. Oil droplets were the most prominently observed in the TGSC group. There was no oil droplet and adipogenic differentiation in the growth medium groups of all cell types at any time point (Figure 4.12. b)

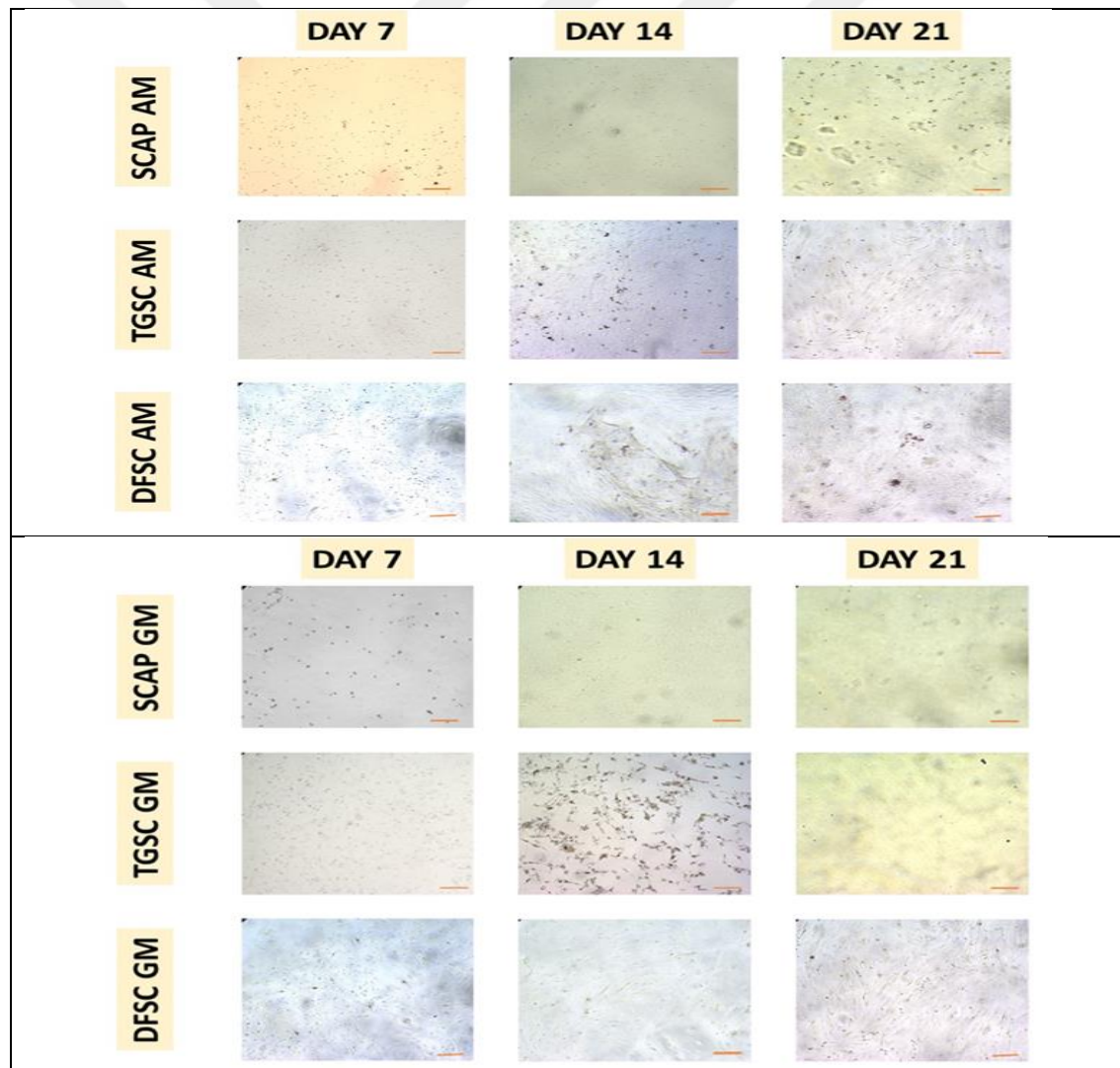


Figure 4.12. Oil Red O staining of SCAP, DFSC, and TGSC in (a) adipogenic media (b) growth media throughout 21 days of incubation (Scale: 100 μ m)

4.6. PREPARATION OF PBS:PLGA SCAFFOLDS

Firstly, 25 percent PBS:PLGA (1:3) solution in DCM were spun in the wet spin setup by using different concentrations of isopropanol-water coagulation baths in order to determine the best coagulation bath. Fiber formation was not observed in coagulation baths where the water ratio in the mixture was over fifty percent. In coagulation baths where the water ratio in the mixture was below 20 percent, the fibers were rapidly coagulated and the tip of the syringe needle was blocked. Therefore, there was difficulty in making continuous fiber, especially in solutions with high polymer concentration. When 75:25 isopropanol:distilled water and 60:40 isopropanol:distilled water coagulation baths were compared, the coagulation bath with a higher alcohol ratio was observed to have a faster and more continuous fiber structure. Since it was thought that the mechanical properties of the scaffold would increase as a result of more continuous fiber structure, it was decided to use 75:25 ratio of coagulation bath for the rest of the study.

Next, 10 percent PBS:PLGA (1:1), 10 percent PBS:PLGA (1:3), 15 percent PBS:PLGA (1:1), 15 percent PBS:PLGA (1:3), 20 percent PBS:PLGA (1:1), 20 percent PBS:PLGA (1:3), 25 percent PBS:PLGA (1:3) were spun successfully by using 75:25 isopropanol:distilled water coagulation bath. The 25 percent PBS:PLGA (1:1) solution was not prepared because polymers did not dissolve at that concentration in DCM.

4.7. CHARACTERIZATION OF SCAFFOLDS

4.7.1. Scanning Electron Microscopy (SEM)

Morphological images of the scaffolds and their fiber structures were evaluated by SEM. After the preparation of scaffolds with the ratio of 1:1 and 1:3 PBS:PLGA prepared in concentrations of 10, 15, 20, and 25 percent in 75:25 isopropanol: distilled water coagulation bath, they were glued to carbon discs and plated with gold by Sputter Coater. Then, the images were taken by SEM. The results showed that 10 percent PBS:PLGA scaffold at both (1:1) and (1:3) ratio did not exhibit continuous fiber structure (Figure 4.13. a-d). Also, these scaffolds had the lowest fiber diameter compared to other concentrations (Figure 4.14.). Thus, 10 percent scaffolds were eliminated from this study based on SEM images.

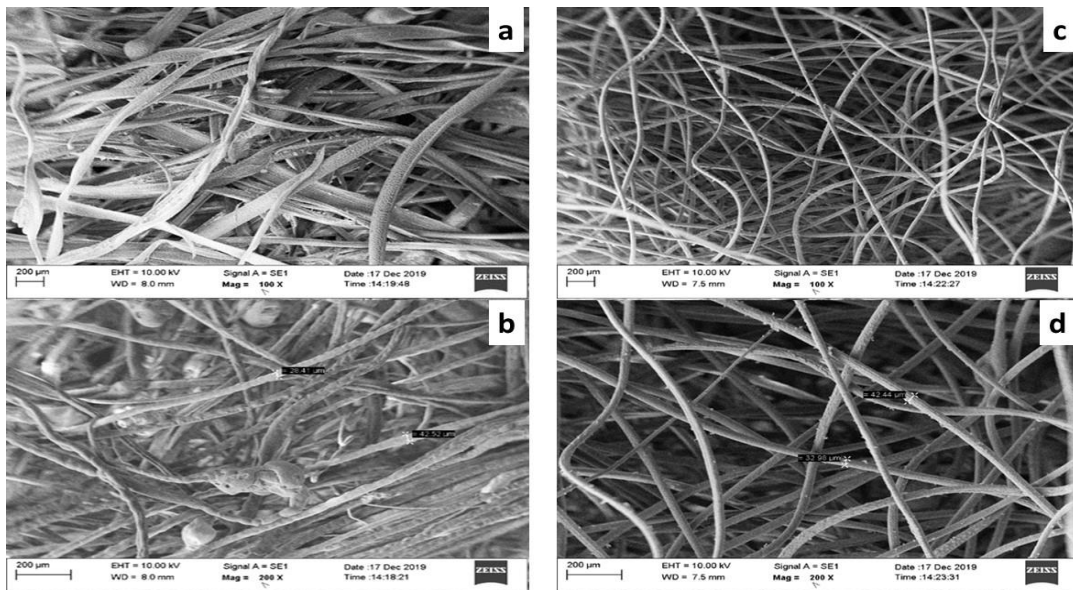


Figure 4.13. Scanning electron micrographs of 10 percent (a, b) 1:1 and (c, d) 1:3 scaffolds at (a, c) 100X magnification, (b, d) 200X magnification

It was observed that the fiber diameters were in the range of 25-100 μm in the scaffolds. Scaffolds with high PLGA ratio (1:3) had the smaller fiber diameter for the same polymer concentration although there was no statistically significant difference among them.

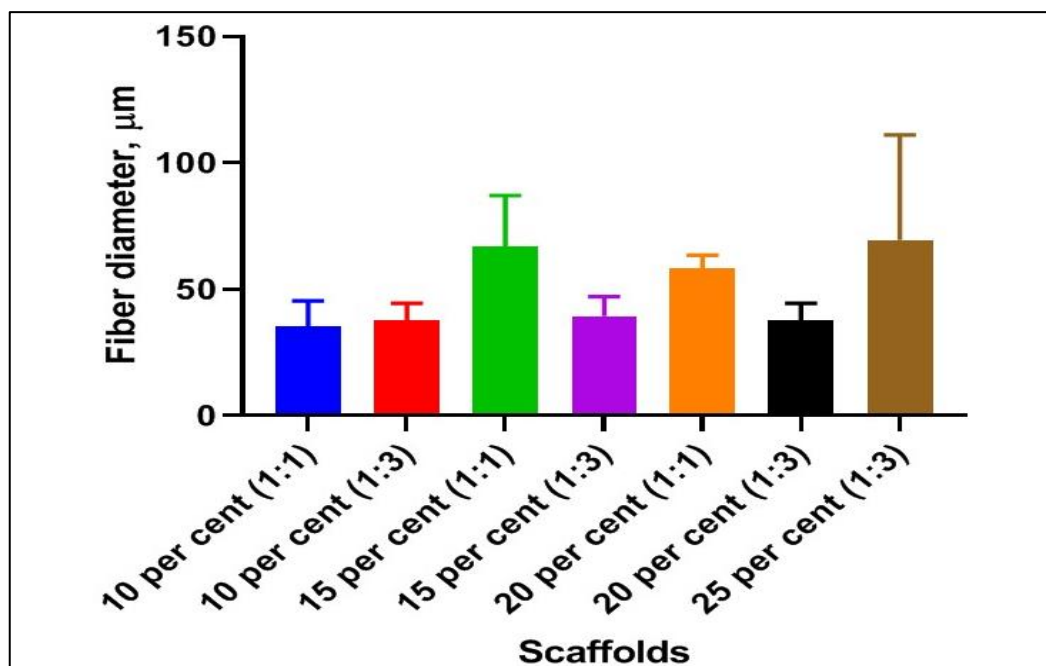


Figure 4.14. Fiber diameter of scaffolds

The 15 percent (1:1), 15 percent (1:3), 20 percent (1:1), 20 percent (1:3), 25 percent (1:3) scaffolds demonstrated continuous fiber structure and suitable fiber diameter for cell attachment (Figure 4.15. a-e). Therefore, these scaffolds were used in the following experiments.

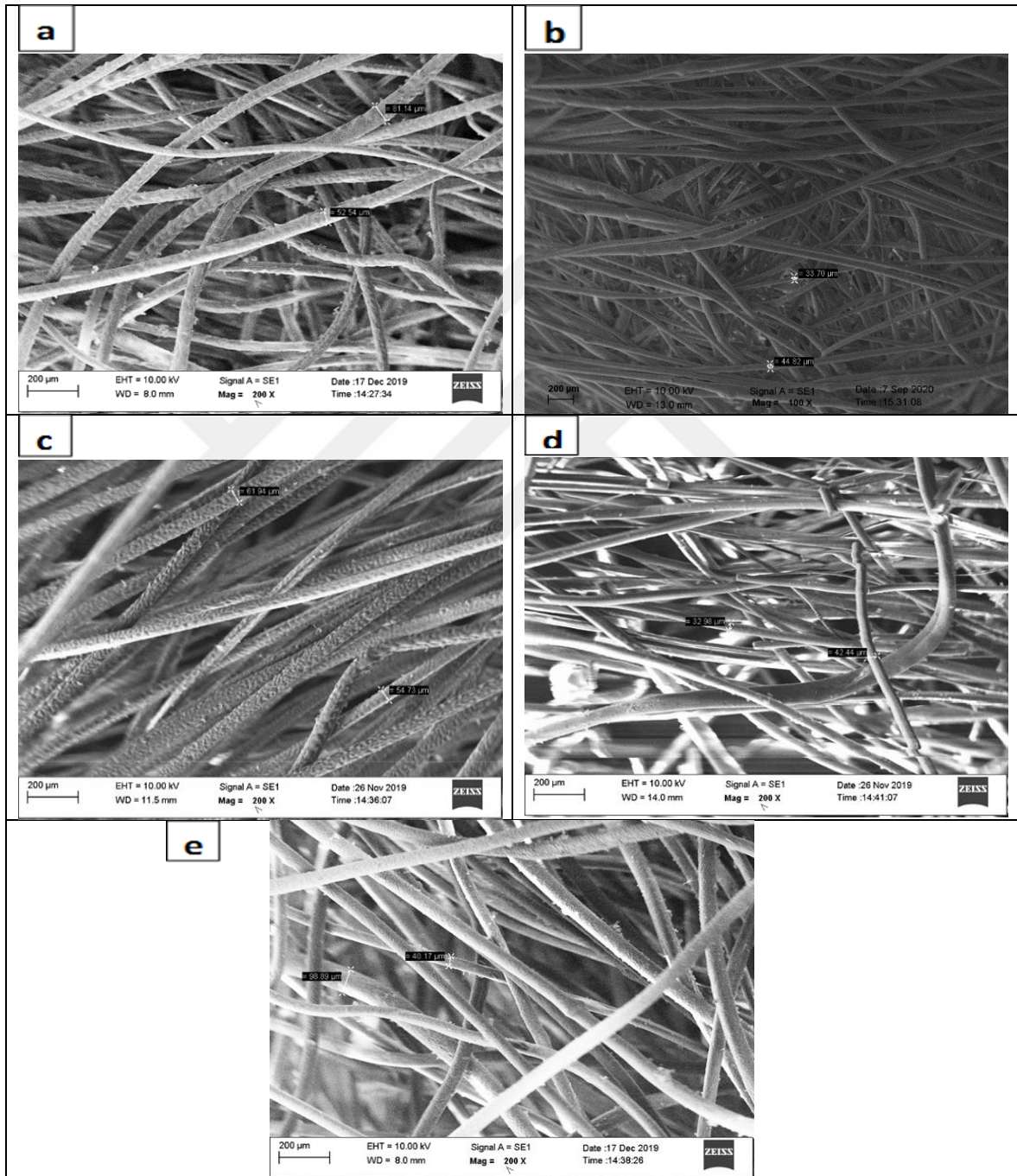


Figure 4.15. Scanning electron micrographs of (a) 15 percent (1:1), (b) 15 percent (1:3), (c) 20 percent (1:1), (d) 20 percent (1:3), (e) 25 percent (1:3) scaffolds at 200 x magnification

4.7.2. Mechanical Analysis

In order to assess the mechanical characteristics of scaffolds, compression and tensile modulus values were measured with an Instron. In mechanical analysis, 15 percent (1:1), 15 percent (1:3), 20 percent (1:1), 20 percent (1:3) and 25 percent (1:3) PBS:PLGA scaffolds were prepared in the suitable form and tested. Compression modulus analysis showed that as the concentration of polymer increased from 15 to 25 percent, the Young's modulus also increased (Figure 4.16. a). Additionally, it was observed that scaffolds with the higher PLGA ratio (1:3) gave the lower compression modulus values compared to the scaffolds with equal polymer ratio for the same concentration. Similar to the compression test, it was observed that the tensile strength increased as the polymer concentration increased from 15 to 25 percent (Figure 4.16. b). Also, scaffolds with the higher PLGA ratio showed higher tensile strength in scaffolds at the same concentrations unlike the compression test.

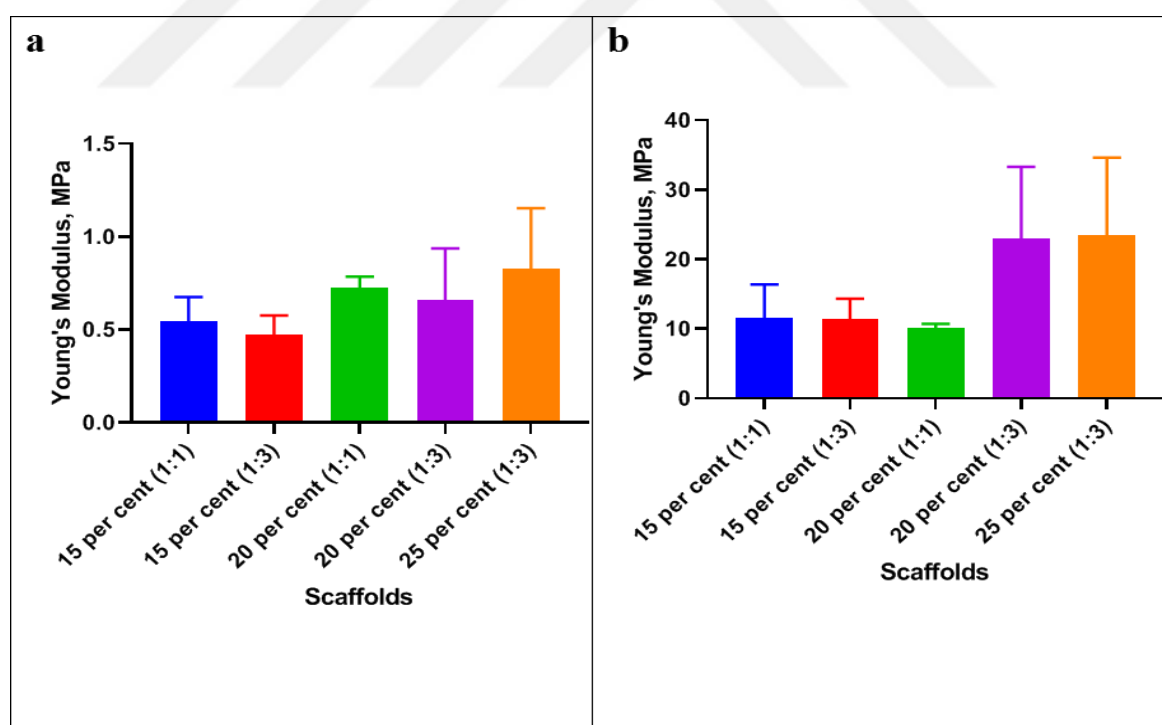


Figure 4.16. Young's Modulus of scaffolds under (a) compressive forces, (b) tensile forces

4.7.3. Degradation Analysis

According to the results of SEM, the wet spun 15 percent (1:1), 15 percent (1:3), 20 percent (1:1), 20 percent (1:3), and 25 percent (1:3) PBS:PLGA scaffolds were selected for degradation studies throughout 90 days of incubation. Initial scaffold weights and pH values were measured on 30, 45, 60 and 90 days. Percent weight loss of scaffolds were calculated based on their initial weight. According to the results, no significant change was observed between the scaffolds until day 60 (Figure 4.17. a). After that point, the 25 percent (1:3) scaffold showed the highest degradation from day 60 to day 90 and lost 57 percent of its initial weight. At the same time, the highest pH change was observed in this scaffold (Figure 4.17. b). The 20 percent (1:1) scaffolds exhibited the least degradation from days 60 to 90 and showed only 14 percent weight loss according to initial weight. As expected during the degradation analysis, the pH rates of all scaffolds decreased from days 30 to 90.

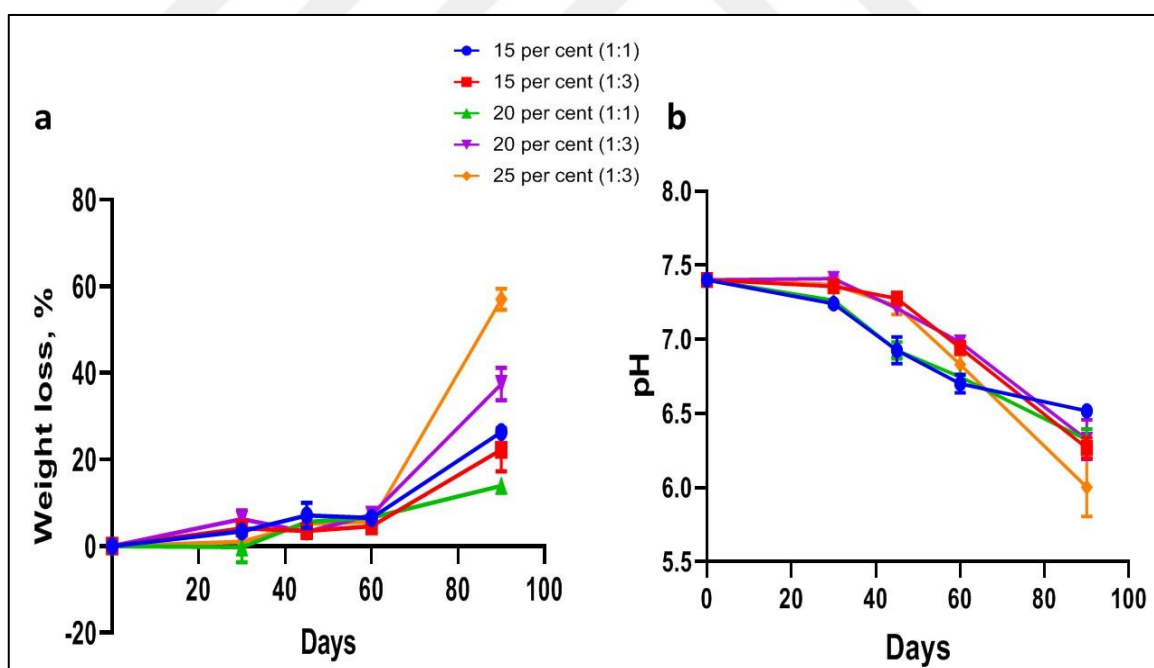


Figure 4.17. Degradation analysis of scaffolds throughout 90 days of incubation with the rate of (a) weight loss and (b) pH decreasing as a function of time (day)

4.7.4. Cell Viability

After the characterization of scaffolds, 15 percent (1:1), 15 percent (1:3) scaffolds were eliminated from this study based on mechanical strength and degradation studies. Then, 20 percent (1:1), 20 percent (1:3) and 25 percent (1:3) scaffolds were selected to evaluate cell viability with CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS).

TGSCs were seeded on these scaffolds and cultured for seven days for the first viability test. As a result of the MTS, it was seen that the cell number in all scaffolds decreased from day 1 to day 4 and then started to increase at day 7 (Figure 4.18. a). According to the results, it was thought that 20 percent (1:1) scaffold would be better. Another cell type, human osteoblast cell, was also used to show cell viability on these scaffolds for 14 days of incubation. According to second viability test result, the cell numbers gradually decreased on 25 percent (1:3) scaffold (Figure 4.18. b). The 20 percent (1:1) scaffold showed the higher cell density than the 20 percent (1:3) scaffold from day 1 to day 14. As a result of both viability tests, 20 percent (1:1) scaffold was chosen and used for the rest of the study.

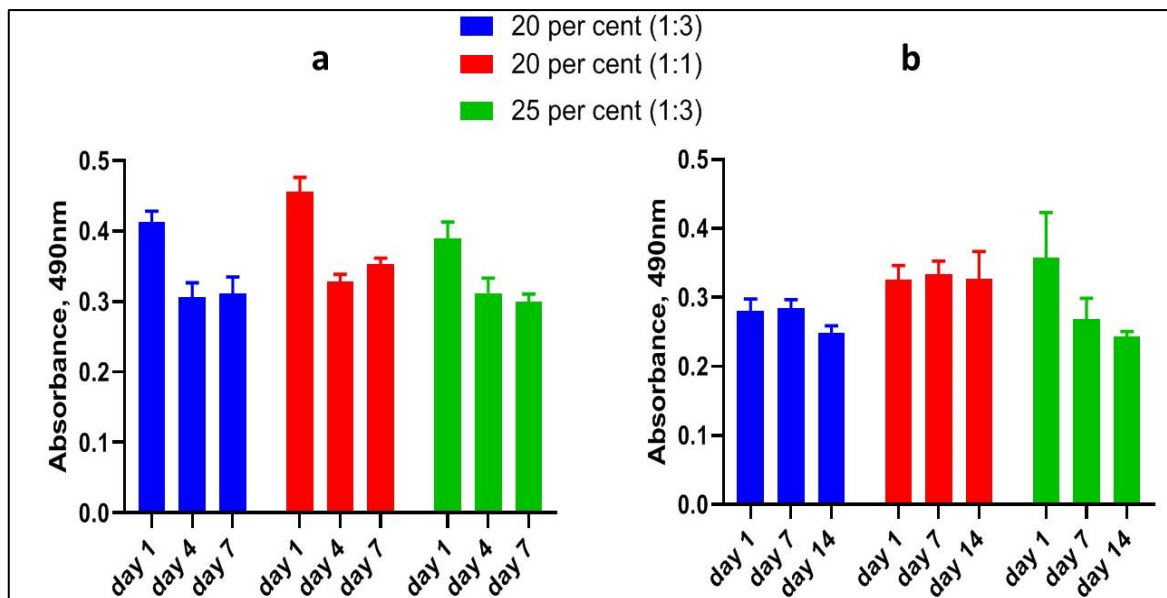


Figure 4.18. MTS assay on the scaffolds with (a) TGSC throughout 7 days and (b) human osteoblasts throughout 14 days of incubation

4.8. IN VITRO SCAFFOLD AND STEM CELLS INTERACTION

4.8.1. Cell Proliferation (MTS) Assay

SCAP, DFSC, and TGSC were seeded on selected 20 percent (1:1) scaffolds. As a control group, the same cells were seeded into the cell culture plates (OC). CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was applied for viability to all cell groups in growth medium and osteogenic medium. According to the MTS, all seeded cells adhered to the PBS:PLGA scaffold but DFSC proliferation on the scaffolds was less than the other cell types (Figure 4.19.). In the growth medium, SCAP and TGSC groups showed a significant increase from day 7 to day 14 ($p < 0.0001$) but there was no significant difference between day 14 and day 21. In the DFSC group, there was no significant increase or decrease between all days both in growth and osteogenic media ($p > 0.05$). In the osteogenic medium, there was a significant increase from day 7 to day 14 in the SCAP group, following a significant decrease on day 21 ($p < 0.0001$; $p = 0.004$, respectively). On the other hand, a significant decrease was observed from day 7 to day 14 in the TGSC group ($p = 0.003$) which was followed by an increase on day 21 ($p = 0.003$).

When the cell groups were compared with each other, there was no difference in cell viability between the groups in growth media on day 7. However, there was a significantly higher cell viability in the TGSC group in osteogenic media compared to TGSC in growth medium on day 7 ($p = 0.046$).

The lowest cell viability in the osteogenic medium was observed in the DFSC group and there was a significant difference compared to the TGSC group ($p = 0.02$). On day 14, SCAP had the highest cell viability in the osteogenic medium and both SCAP and TGSC had the higher viability than DFSC in the growth medium. On day 21, the TGSC group showed significantly higher cell viability in the growth medium compared to the osteogenic medium. In the osteogenic media, SCAP and TGSC had similar cell viability but the DFSC group appeared to have significantly lower viability than the others. While no significant difference was observed between the growth and osteogenic medium groups of the SCAP and TGSC, high cell viability was found in both groups.

In the control groups, an increasing cell proliferation was observed from day 7 day 21 in both osteogenic and growth medium except for TGSCs grown in osteogenic medium.

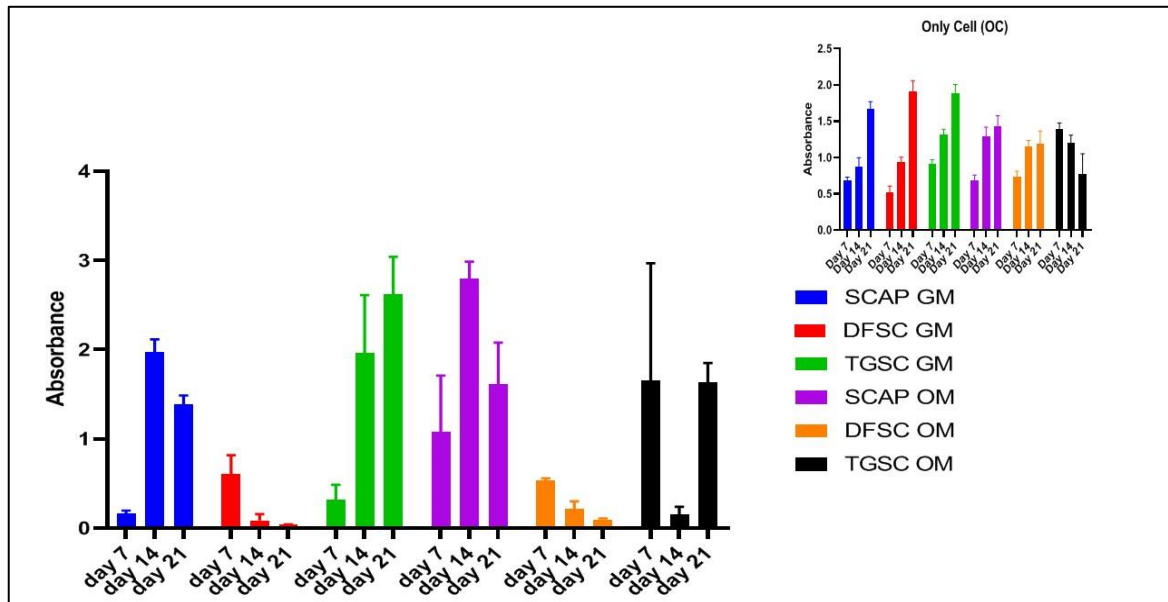


Figure 4.19. Cell proliferation on 20 percent (1:1) PBS:PLGA scaffolds after 7, 14 and 21 days of incubation in osteogenic and growth medium. Initial cell density was 150,000 cells/scaffold. Upper right corner shows the cell proliferation on Tissue Culture Plate (TCP) and named as only cell (OC)

4.8.2. Alkaline Phosphatase (ALP) Activity

Alkaline phosphatase activities of all three cell groups on 20 percent (1:1) PBS:PLGA scaffold and on culture plate as a control were investigated at days 7, 14 and 21. Results show that cells seeded on tissue culture plates (OC) exhibited significantly higher levels of ALP activity than the cells seeded on the scaffolds for all groups and all time periods (Figure 4.20.). Each group showed the highest ALP activity within itself on day 7 compared to the other days. TGSC had the highest ALP activity both in osteogenic and growth media. It was followed by DFSC group both in osteogenic and growth media. DFSC and TGSC groups exhibited a significant decrease in ALP from day 7 to day 14 in both osteogenic and growth

medium but they did not show a statistically significant difference between day 14 and day 21. In the SCAP group, a significant decrease was observed in both growth medium and osteogenic medium from day 7 to day 21.

On day 21, the lowest ALP activity among the groups was observed in the SCAP both in growth and osteogenic media. In general, DFSC and TGSC showed a similar ALP activity pattern by exhibiting a decrease in ALP activity from day 7 to day 21, but SCAP showed a significant decrease from day 7 to day 21, with no difference between day 7 and day 14.

In the control groups, an increase in ALP activity was observed from day 7 to day 21 in the growth medium. In the osteogenic medium, TGSC had the highest ALP activity on day 14. As mentioned before, a higher rate of ALP activity was observed in the cells that were seeded on culture plates (OC) when compared with the cells seeded on scaffolds. When evaluated together with the MTS results, DFSCs and TGSCs showed the higher ALP activity, although the DFSC group exhibited lower viability values.

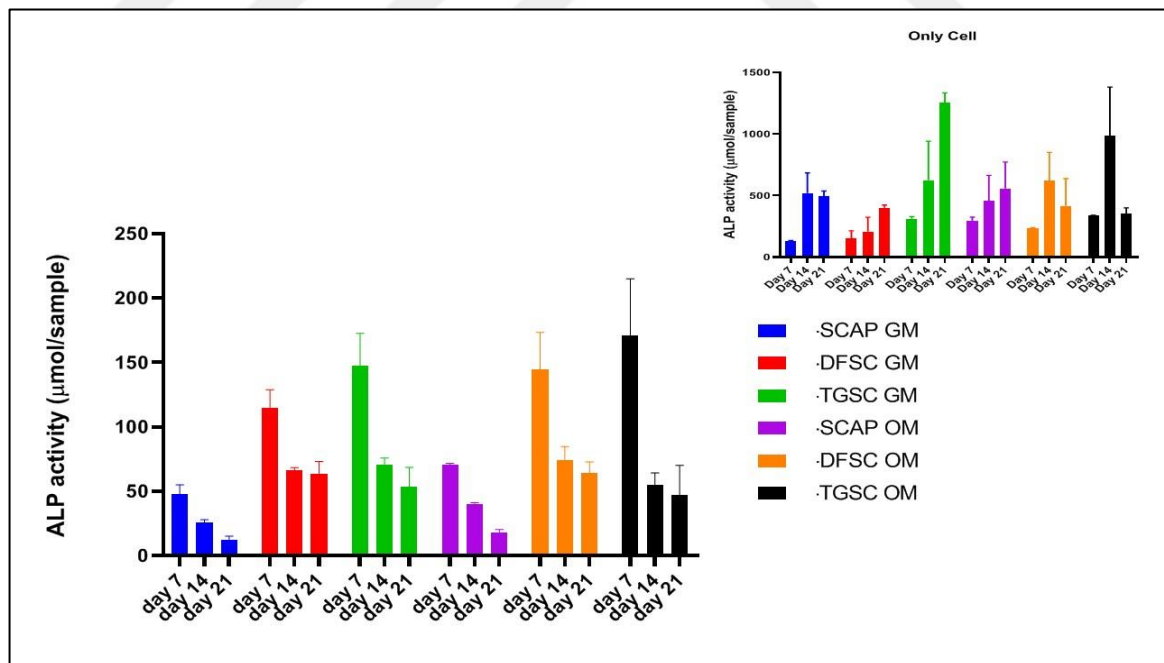


Figure 4.20. ALP activity on 20 percent (1:1) PBS:PLGA scaffolds after 7, 14 and 21 days of incubation in osteogenic and growth medium. Graph of all cells' ALP activity on Tissue Culture Plate (TCP) is shown upper right corner and named as only cell (OC)

4.8.3. Real-Time PCR Analysis

In this analysis, RNA was isolated from the cells on the scaffold in order to evaluate differentiation into osteogenic lineage by examining osteogenic and odontogenic gene expressions such as Runx2, Osteocalcin, Osteonectin, Osteopontin, DSPP, and DMP-1.

According to Runx2 expression results, there was no significant difference among the growth medium groups during 21 days, except for the TGSC group ($p=0.033$) (Figure 4.21. a). In the osteogenic media groups, DFSCs group did not exhibit significant difference at different time point. While SCAP group showed an increase in the Runx2 expression throughout the culture process in osteogenic medium ($p<0.0001$), TGSC group showed a significant increase only from day 7 to day 14. When the expression levels were evaluated, the SCAP group had the highest Runx2 gene expression on day 21 compared to all other groups ($p < 0.0001$).

For Osteocalcin expression, there was no statistically significant difference between all groups on the scaffold (Figure 4.21. b). However, it was observed that its expression significantly increased in the osteogenically induced TGSC group from day 7 to day 21 ($p = 0.02$).

It was found that Osteonectin expression levels were higher than the other markers (Figure 4.21. c). In growth medium, there was no significant change in the Osteonectin expression levels in cells during culture period. In the osteogenic medium, a significant decrease was observed from the beginning to the end of the culture process in the TGSC group ($p = 0.03$). On the other hand, SCAP and DFSC groups demonstrated an increase from day 7 to day 14 following with a decrease on day 21. This change was observed at a significant level especially in the SCAP group ($p < 0.0001$; $p = 0.0004$). When all groups were compared, Osteonectin expression of osteogenically induced SCAP group on day 14 was found to be significantly higher than all other groups ($p < 0.05$).

When osteopontin levels were evaluated, no significant difference in its expression was detected between the groups at day 14 and day 21 in the growth medium (Figure 4.21. d). On day 7, the SCAP in the growth medium showed significantly lower expression than all other groups, while the DFSC in the osteogenic medium showed the highest level of expression by showing a significant increase compared to all groups. While expression levels

of SCAP and TGSC tended to increase as the days passed, expression levels of DFSC tended to decrease in the osteogenic medium ($p < 0.0001$).

DMP-1 and DSPP expressions were examined in order to evaluate the odontogenesis process. Although the expression of these genes could be determined, no significant difference was observed between the groups. DSPP and DMP-1 expression levels of cells were significantly lower than the osteogenic genes (Figure 4.21. e,f). DMP-1 expression was low in all cell groups in the growth medium and the gene expression did not change with the addition of osteogenic medium (Figure 4.21. e). When the graph showing the expression of DSPP regarding the regulation of mineralization of hard tissue was examined, it was found that SCAP and DFSC had high DSPP expression in the growth medium on day 14 (Figure 4.21. f). However, DSPP expression in SCAP decreased on day 21 while the one in DFSC remained at a high level in the growth medium. In the osteogenic medium, DSPP expression was reduced in DFSC on day 14 compared to the one in growth medium while it was maintained at a similar level until day 21 in SCAP.

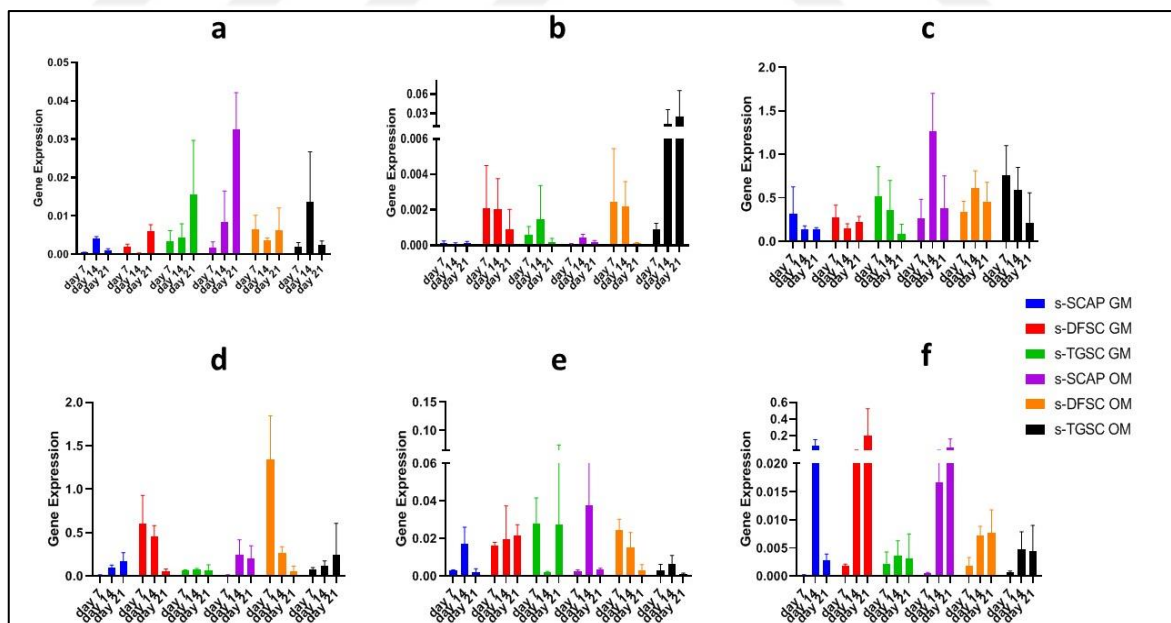


Figure 4.21. Expression levels of (a) Runx2, (b) Osteocalcin, (c) Osteonectin, (d) Osteopontin, (e) DMP-1, and (f) DSPP after 7, 14 and 21 days of incubation in growth medium and osteogenic medium

4.8.4. Immunocytochemistry

All three cell types were seeded on scaffolds and on cell culture plates and stained for confocal microscopic analysis. Runx2, Osteocalcin, Collagen Type I (Col1) and DAPI stainings were performed on all cells in both growth medium and osteogenic medium. Double immunostaining where the two proteins were stained at the same time and on the same sample with different dye was applied.

When DFSCs stained with Osteocalcin, Col1, and DAPI and grown in tissue culture plates were evaluated, positive staining was observed in both osteogenic and growth media, but more intense Osteocalcin staining was observed especially in osteogenic media (Figure 4.22.). While significant and increasing collagen staining was observed in the cells grown in the osteogenic medium from day 7 to day 21, there was no significant collagen staining on the cells grown in the growth medium even on day 21. Additionally, cells in the growth medium tended to spread and expand across the culture plate while cells in osteogenic media tended to clump together.

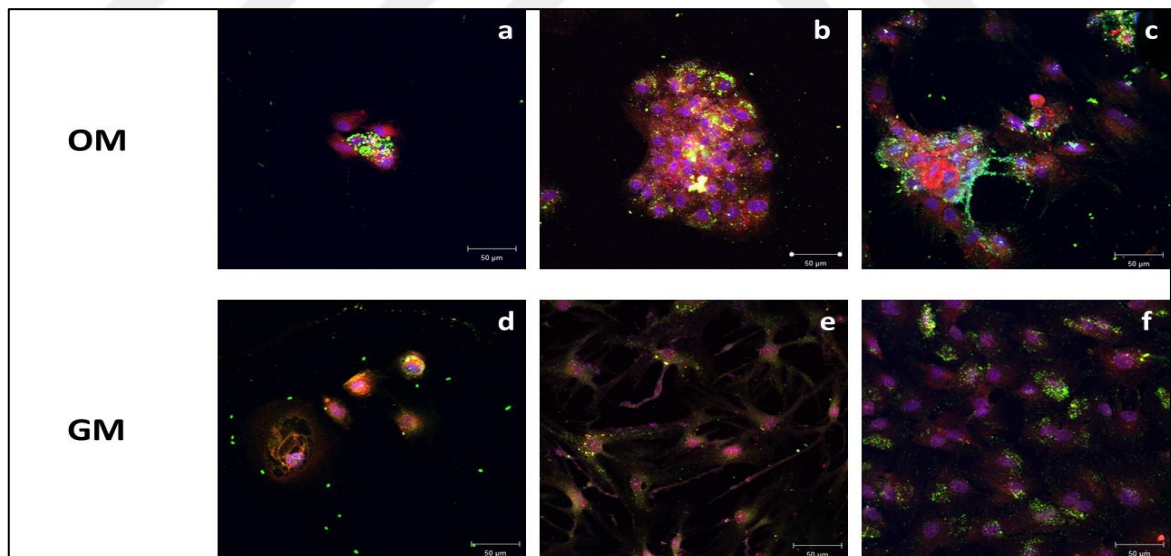


Figure 4.22. Confocal microscopy images of DFSCs stained for Osteocalcin, Col1 and DAPI in (a, b, c) osteogenic medium and (d, e, f) growth medium after (a, d) 7, (b, e) 14, (c, f) 21 days of incubation. Scale: 50 μ m. Green: Osteocalcin, Red: Col1, and Blue: DAPI.

GM: Growth medium, OM: Osteogenic medium

When DFSCs stained with Runx2, Col1 and DAPI and grown in tissue culture plate were investigated, it was observed that while less Runx2 expression was seen in DFSC in growth medium in all time points, a pronounced Runx2 staining was observed in osteogenic medium, especially on day 14 (Figure 4.23.). Col1 was apparently seen in the extracellular matrix of the cells in the osteogenic medium at days 14 and 21, but not in the ones grown in the growth medium. In addition, the cells in the osteogenic medium presented more aggregated form than the cells in the growth medium.

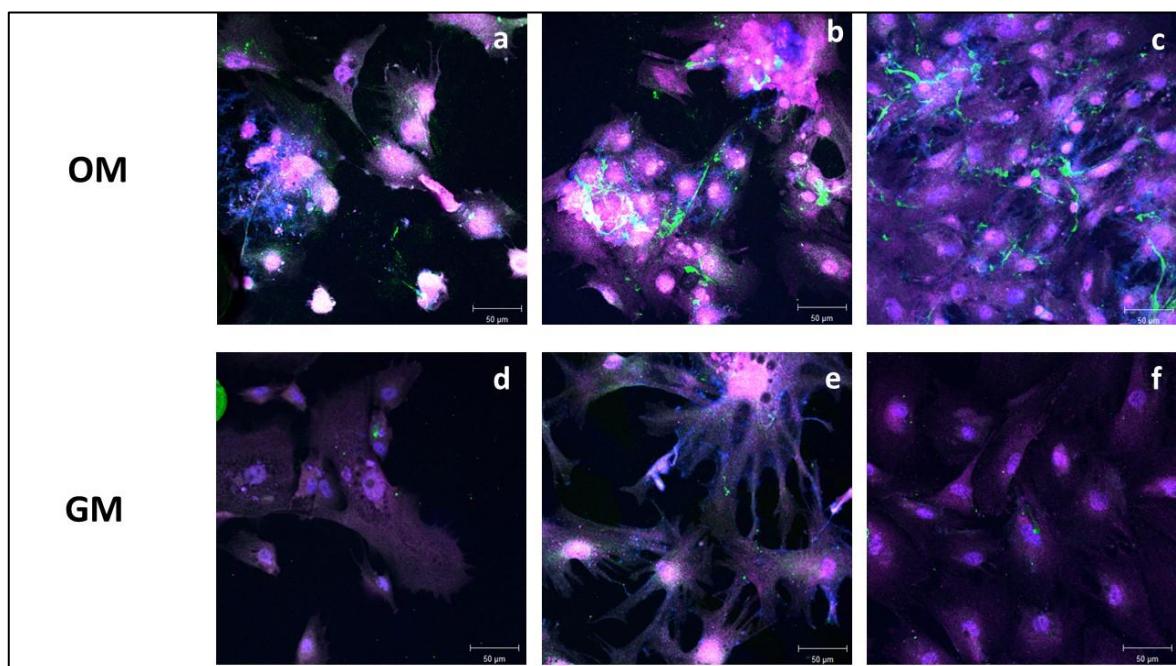


Figure 4.23. Confocal microscopy images of DFSCs stained for Runx2, Col1 and DAPI in (a, b, c) osteogenic medium and in (d, e, f) growth medium after (a, d) 7, (b, e) 14, (c, f) 21 days of incubation. Scale: 50 μm. Magenta: Runx2, Green: Col1, and Blue: DAPI. GM: Growth medium, OM: Osteogenic medium

After seeding DFSCs on scaffolds, it was observed that only cell nuclei were stained with DAPI in the growth medium, while Osteocalcin staining was not observed (Figure 4.24.). Osteocalcin staining was positive only after day 14 in the osteogenic medium. When the Runx2 expression on the scaffolds was evaluated, more intense staining was observed in DFSCs grown in osteogenic medium than the ones grown in growth medium (Figure 4.25.). In the osteogenic medium, there was an increasing staining from day 7 to day 21. However, there was an increase from day 7 to day 14 in growth medium and then, Runx2 staining

stayed at the same level on day 21. When the Col1 expressions were evaluated in both Figure 24 and Figure 25, there was no sign of the Col1 in DFSCs grown in the growth medium throughout the days of incubation. However, very few Col1 expression was observed only at the end of the osteogenic induction which was day 21.

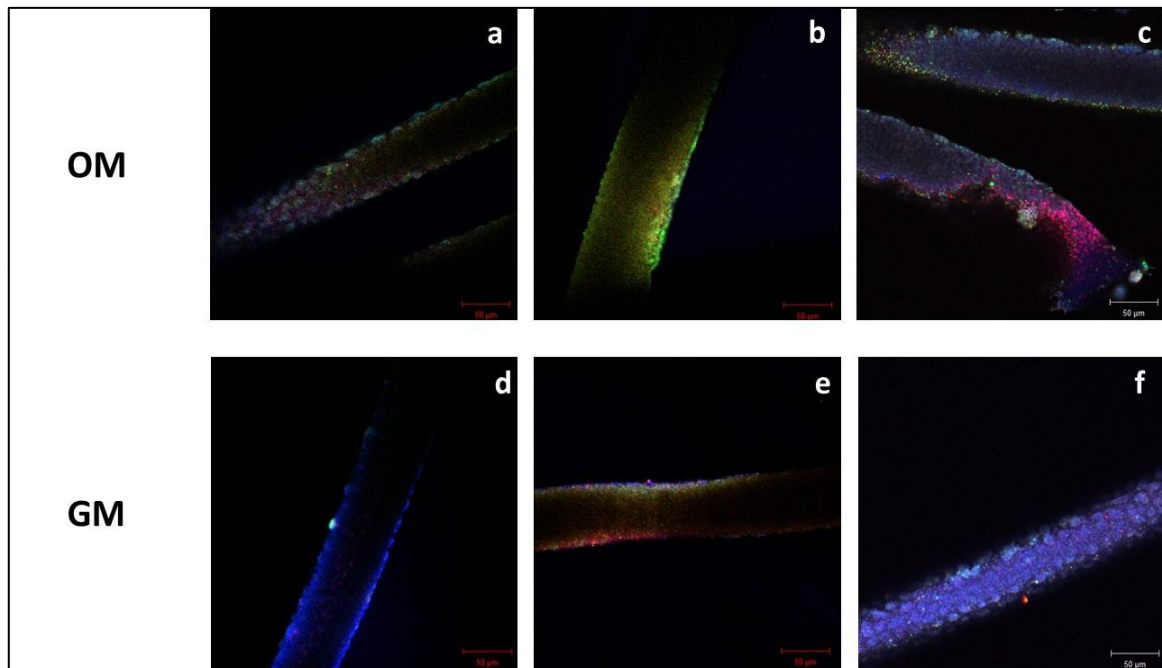


Figure 4.24. Confocal microscopy images of DFSCs seeded on the scaffolds and stained for Osteocalcin, Col1 and DAPI in (a, b, c) osteogenic medium and (d, e, f) growth medium after (a, d) 7, (b, e) 14, (c, f) 21 days of incubation. Scale: 50 μ m. Green: Osteocalcin, Red: Col1, and Blue: DAPI. GM: Growth medium, OM: Osteogenic medium

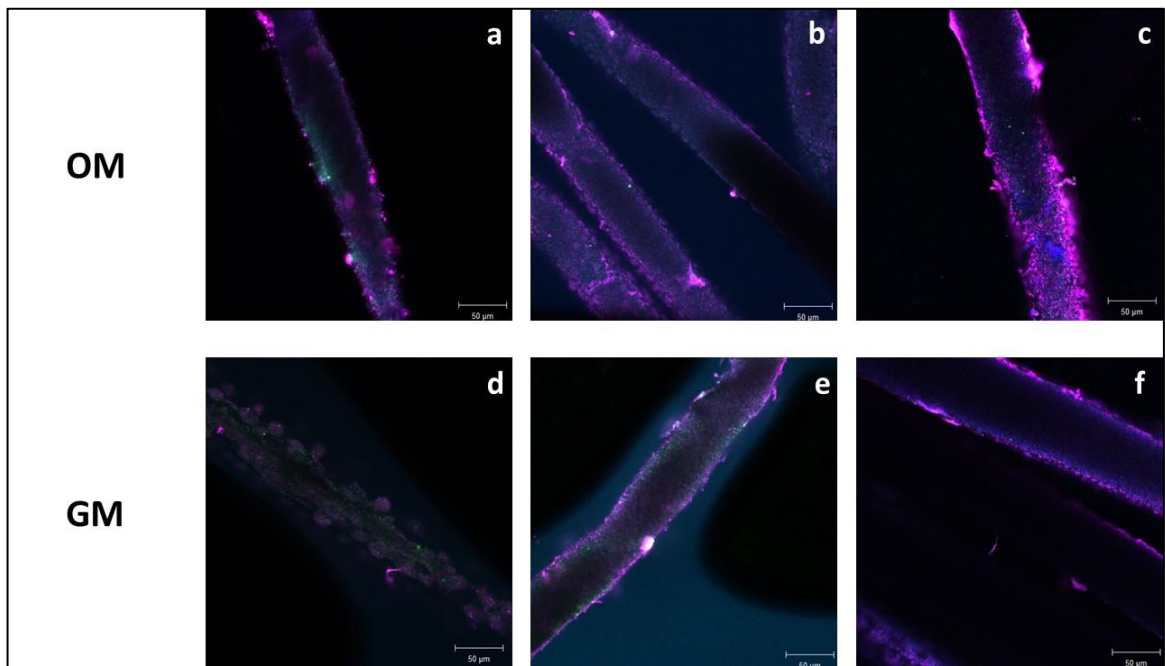


Figure 4.25. Confocal microscopy images of DFSCs seeded on the scaffolds and stained for Runx2, Col1 and DAPI in (a, b, c) osteogenic medium and (d, e, f) growth medium after (a, d) 7, (b, e) 14, (c, f) 21 days of incubation. Scale: 50 μ m. Magenta: Runx2, Green: Col1, and Blue: DAPI. GM: Growth medium, OM: Osteogenic medium

When TGSCs stained with Osteocalcin, Col1, and DAPI and grown in tissue culture plate were evaluated, it was observed that Osteocalcin staining was significantly increased in the osteogenic medium on day 14 and day 21, while it was less positive in the growth medium (Figure 4.26.). Col1 was observed in the cytoplasm of cells grown in the osteogenic medium on day 7 and then extracellular Col1 was observed in the following days between the clustered cells. Col1 expression was the highest in the osteogenic medium on day 21. Also, the cells in the growth medium tended to spread across the TCP while the cells in the osteogenic medium appeared to be aggregated and encapsulated with the ECM containing massive Col1. On the other hand, Runx2 was detected intensively in the cells grown in the osteogenic medium compared to the ones grown in the growth medium (Figure 4.27.). On day 7, the cells in the osteogenic medium exhibited Runx2 expression while the cells in the growth medium did not. Runx2 expression peaked on day 14 in osteogenic medium and its expression decreased on day 21. At the end of the culture period, both cells in the growth medium and osteogenic medium had nearly the same amount of Runx2 expression.

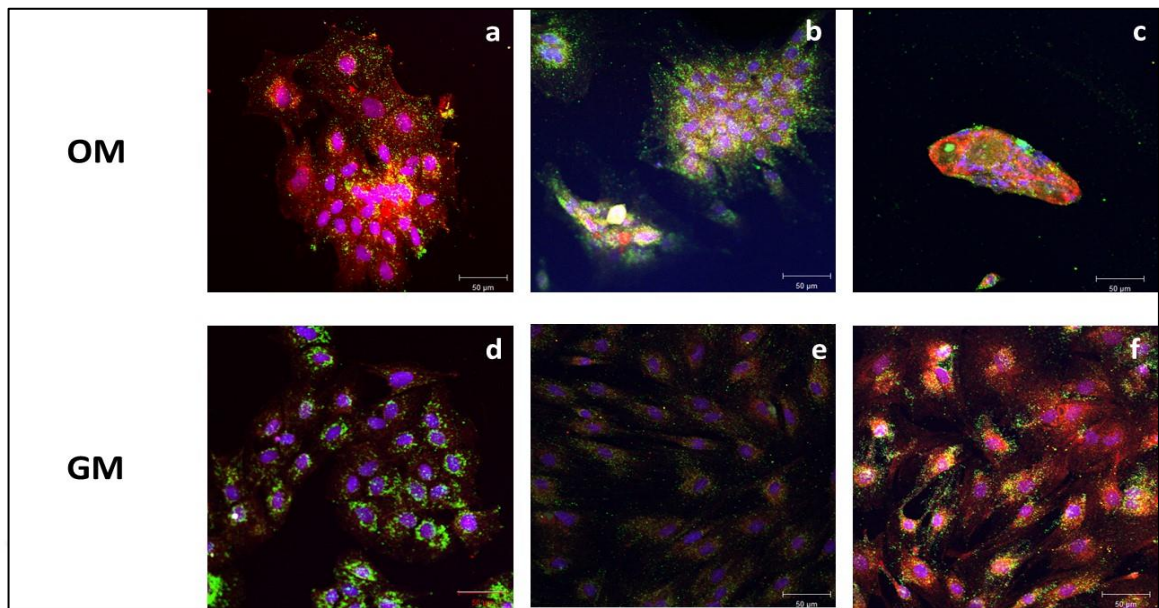


Figure 4.26. Confocal microscopy images of TGSCs stained for Osteocalcin, Col1 and DAPI in (a, b, c) osteogenic medium and (d, e, f) growth medium after (a, d) 7, (b, e) 14, (c, f) 21 days of incubation. Scale: 50 µm. Green: Osteocalcin, Red: Col1, and Blue: DAPI.

GM: Growth medium, OM: Osteogenic medium

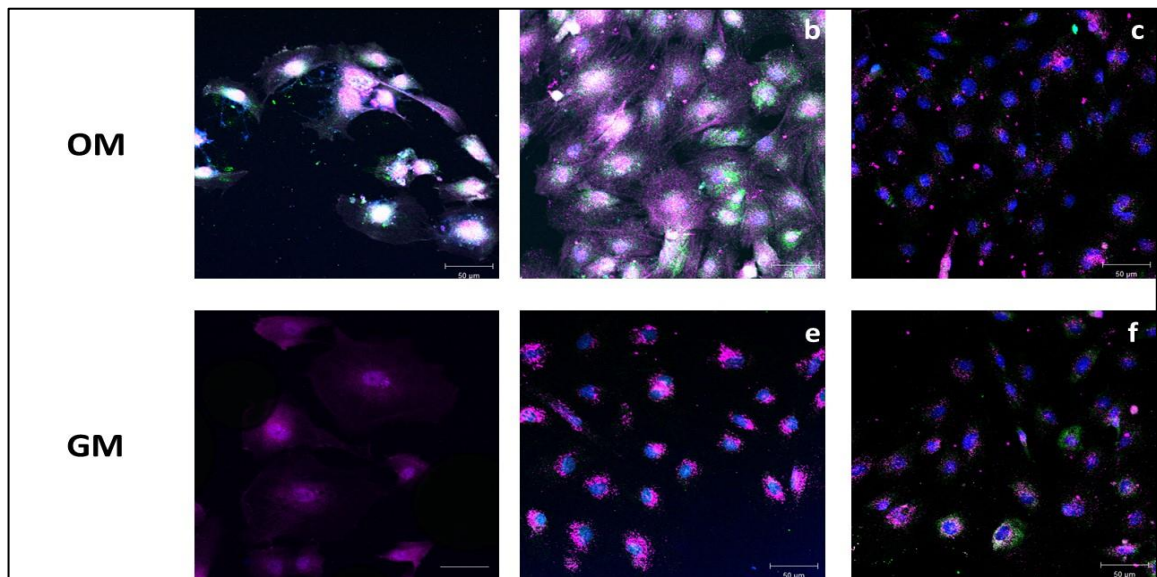


Figure 4.27. Confocal microscopy images of TGSCs stained for Runx2, Col1, and DAPI in (a, b, c) osteogenic medium and (d, e, f) growth medium after (a, d) 7, (b, e) 14, (c, f) 21 days of incubation. Scale: 50 µm. Magenta: Runx2, Green: Col1, and Blue: DAPI. GM:

Growth medium, OM: Osteogenic medium

After seeding TGSCs on scaffolds, no osteogenic marker was detected in the growth medium, while Runx2 and Osteocalcin expressions were observed in the osteogenically induced group, but not intense (Figures 4.28. and 4.29.). In the growth medium, only DAPI staining was observed.

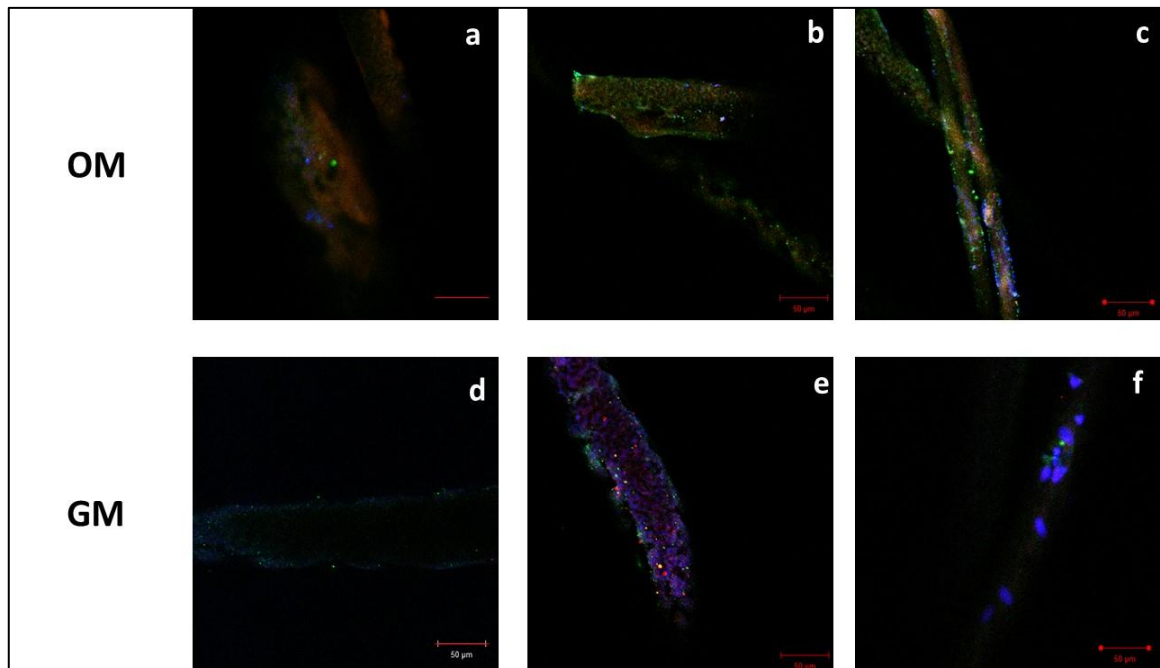


Figure 4.28. Confocal microscopy images of TGSCs seeded on the scaffolds stained for Osteocalcin, Col1 and DAPI in (a, b, c) osteogenic medium and (d, e, f) growth medium after (a, d) 7, (b, e) 14, (c, f) 21 days of incubation. Scale: 50 μm. Green: Osteocalcin, Red: Col1, and Blue: DAPI. GM: Growth medium, OM: Osteogenic medium

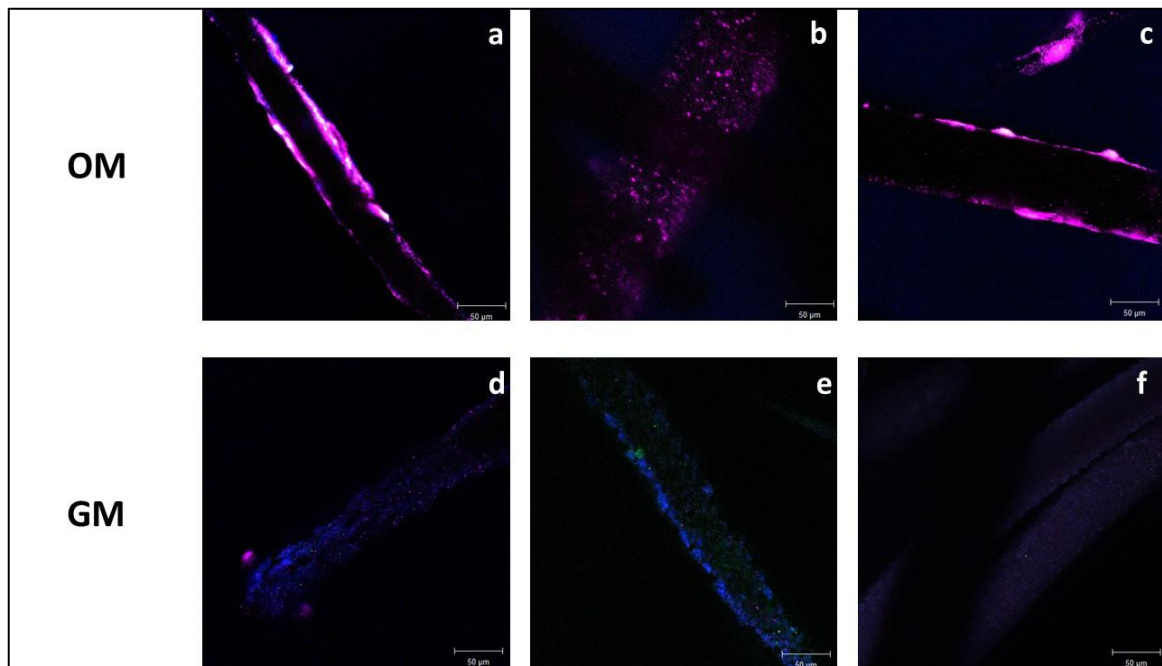


Figure 4.29. Confocal microscopy images of TGSCs seeded on the scaffolds stained for Runx2, Col1 and DAPI in (a, b, c) osteogenic medium and in (d, e, f) growth medium after (a, d) 7, (b, e) 14, (c, f) 21 days of incubation. Scale: 50 μm . Magenta: Runx2, Green: Col1, and Blue: DAPI. GM: Growth medium, OM: Osteogenic medium

When SCAPs stained with Osteocalcin, Col1 and DAPI and grown in tissue culture plate were investigated, more intense Osteocalcin staining was observed in SCAP compared to other cell types (Figure 4.30.). It was observed that the amount of Osteocalcin in the osteogenic medium was concentrated on day 14. Extracellular collagen fibers began to be seen on osteogenic medium from day 7 and their amount increased as time passed. Although significant Osteocalcin production was found in SCAPs grown in the growth medium, extracellular Col1 was not observed. SCAP had significant Runx2 expression from day 7 to day 21 in the osteogenic medium compared to the cells grown in the growth medium (Figure 4.31.). In the osteogenic medium, extensive Col1 and Runx2 expressions were observed on day 21 together with higher ECM.

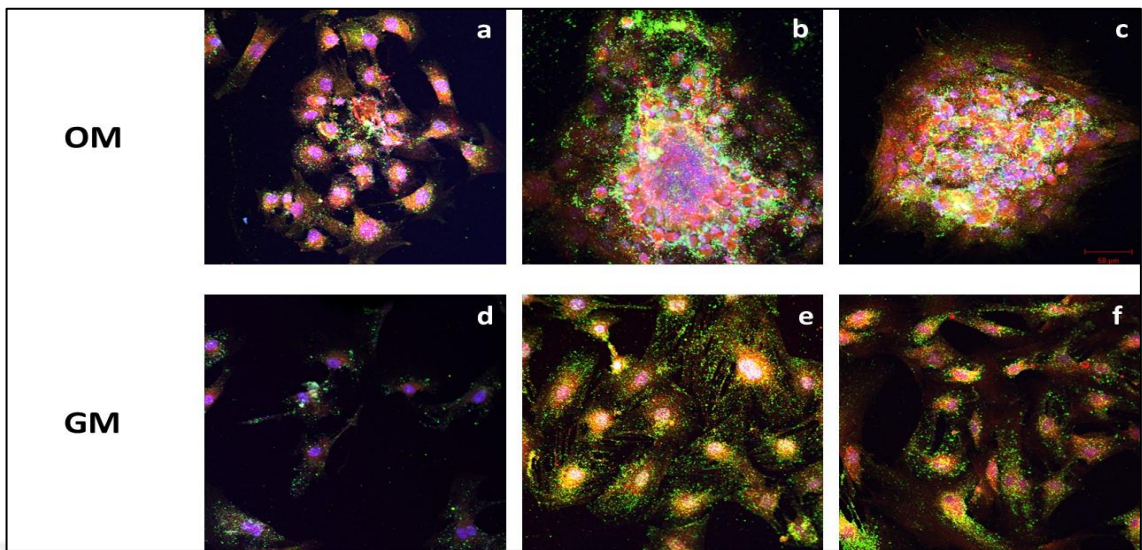


Figure 4.30. Confocal microscopy images of SCAPs stained for Osteocalcin, Col1 and DAPI in (a, b, c) osteogenic medium and (d, e, f) growth medium after (a, d) 7, (b, e) 14, (c, f) 21 days of incubation. Scale: 50 μm. Green: Osteocalcin, Red: Col1, and Blue: DAPI.

GM: Growth medium, OM: Osteogenic medium

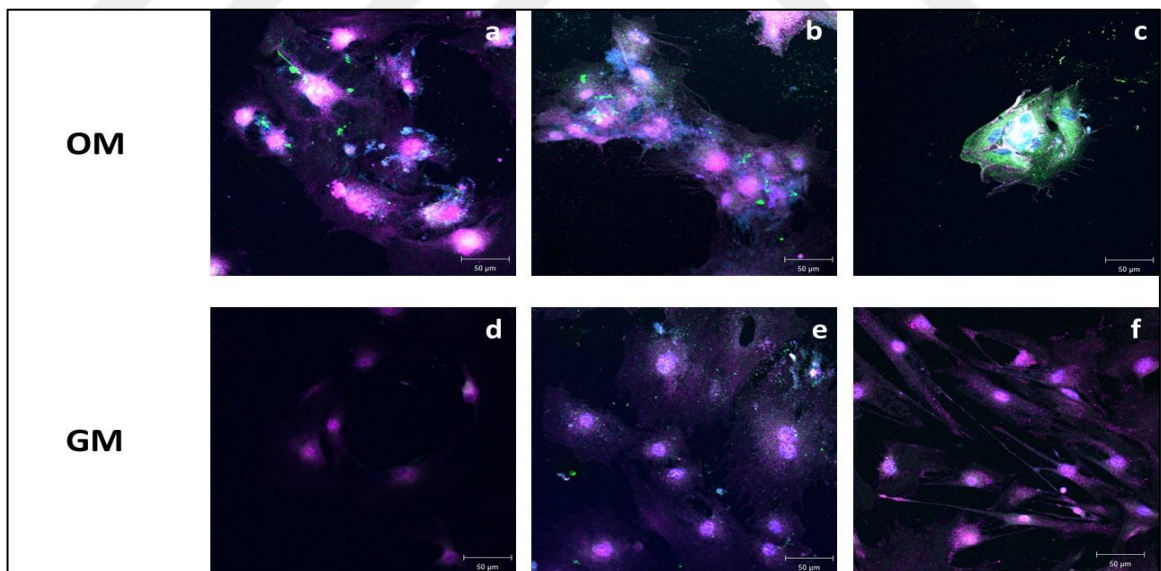


Figure 4.31. Confocal microscopy images of SCAPs stained for Runx2, Col1 and DAPI in (a, b, c) osteogenic medium and (d, e, f) growth medium after (a, d) 7, (b, e) 14, (c, f) 21 days of incubation. Scale: 50 μm. Magenta: Runx2, Green: Col1, and Blue: DAPI. GM:

Growth medium, OM: Osteogenic medium

In the group of SCAPs seeded on the scaffolds, increasing Osteocalcin staining was observed in the osteogenic medium, although significant Osteocalcin staining was observed only on day 21 in the growth medium (Figure 4.32.). On the other hand, Runx2 was observed in small amounts in both growth and osteogenic medium (Figure 4.33.). While more Runx2 expression was observed in the early stages of the culture process, it decreased in both media towards the end of the culture period. In both Figures 4.32 and 4.33, Col1 expression was not observed Col1 expression in growth medium. On the contrary, prominent Col1 expression was observed in the osteogenic medium on day 14 while it was not observed on day 7. However, the amount of Col1 was reduced on day 21 in osteogenic medium.

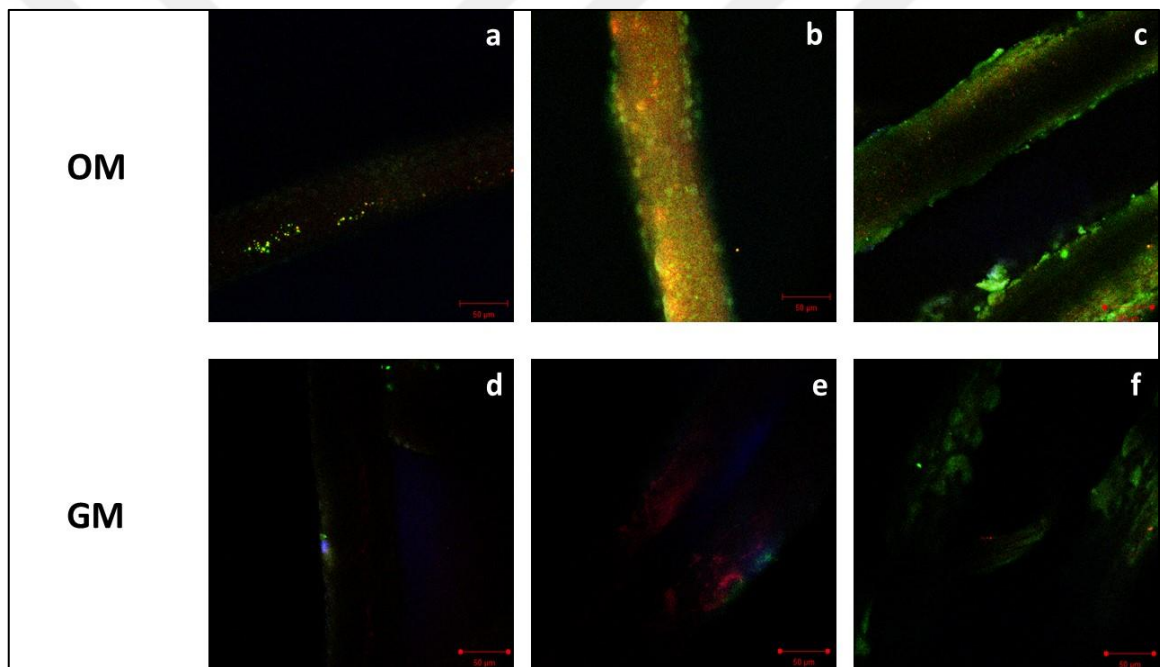


Figure 4.32. Confocal microscopy images of SCAPs seeded on the scaffolds stained for Osteocalcin, Col1 and DAPI in (a, b, c) osteogenic medium and (d, e, f) growth medium after (a, d) 7, (b, e) 14, (c, f) 21 days of incubation. Scale: 50 μ m. Green: Osteocalcin, Red: Col1, and Blue: DAPI. GM: Growth medium, OM: Osteogenic medium

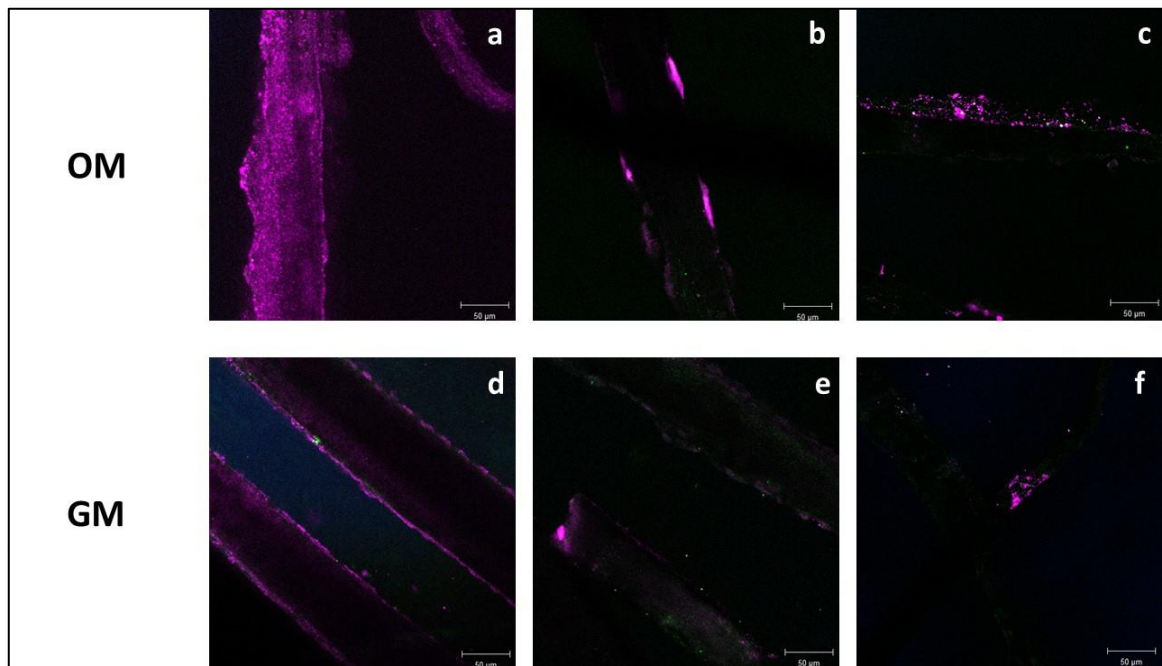


Figure 4.33. Confocal microscopy images of SCAPs seeded on the scaffolds stained for Runx2, Col1 and DAPI in (a, b, c) osteogenic medium and (d, e, f) growth medium after (a, d) 7, (b, e) 14, (c, f) 21 days of incubation. Scale: 50 μ m. Magenta: Runx2, Green: Col1, and Blue: DAPI. GM: Growth medium, OM: Osteogenic medium

4.8.5. von Kossa Staining

von Kossa staining was applied to 20 percent (1:1) PBS:PLGA scaffolds containing three different cell types in growth and osteogenic media in order to get information about calcium deposition of cells. Dark brown color on the samples indicates calcium deposition in this assay.

According to the results, TGSCs had no calcium deposition in the growth medium both in day 7 and day 14 but at the end of the culture process they showed significant calcium deposition (Figure 4.34.). In the osteogenic medium, some degree of calcium accumulation in the TGSC group was observed on day 14 while they did not show calcium accumulation on day 7. Following days calcium accumulation continued until day 21 and they exhibited significantly higher calcium deposition in the osteogenic medium.

According to the result of von Kossa staining performed on SCAP, calcium accumulation was not observed in the growth medium on day 7 but there was some degree of calcium accumulation on day 14 (Figure 4.35.). On day 21, there was a similar level of calcium accumulation as in the osteogenic medium and it was more than the one on day 14 in the growth medium. In the osteogenic medium, there was a constantly increasing calcium accumulation from day 7 to day 21.

The DFSCs, on the other hand, showed no calcium accumulation both in osteogenic and growth media on day 7 while they had some degree of calcium deposition at the same level on day 14 (Figure 4.36.). On day 21, cells in the osteogenic medium showed higher level of calcium deposition than the ones in the growth medium but both of them had significant calcium accumulation compared to other time points.

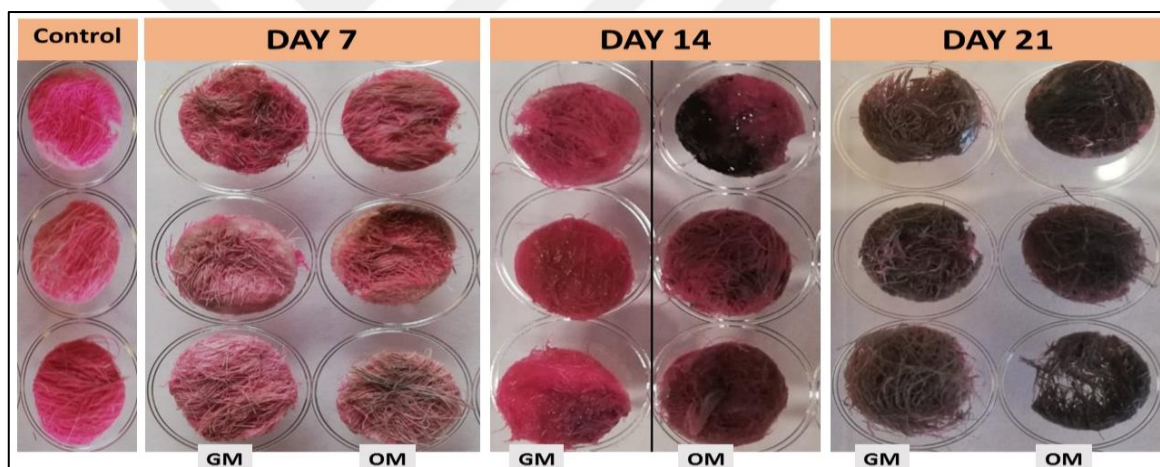


Figure 4.34. von Kossa staining of 20 percent (1:1) PBS:PLGA scaffolds that were seeded with TGSCs and grown in both growth and osteogenic media after 7, 14, and 21 days of incubation. Control represents the scaffold without cells. GM: Growth medium, OM: Osteogenic medium

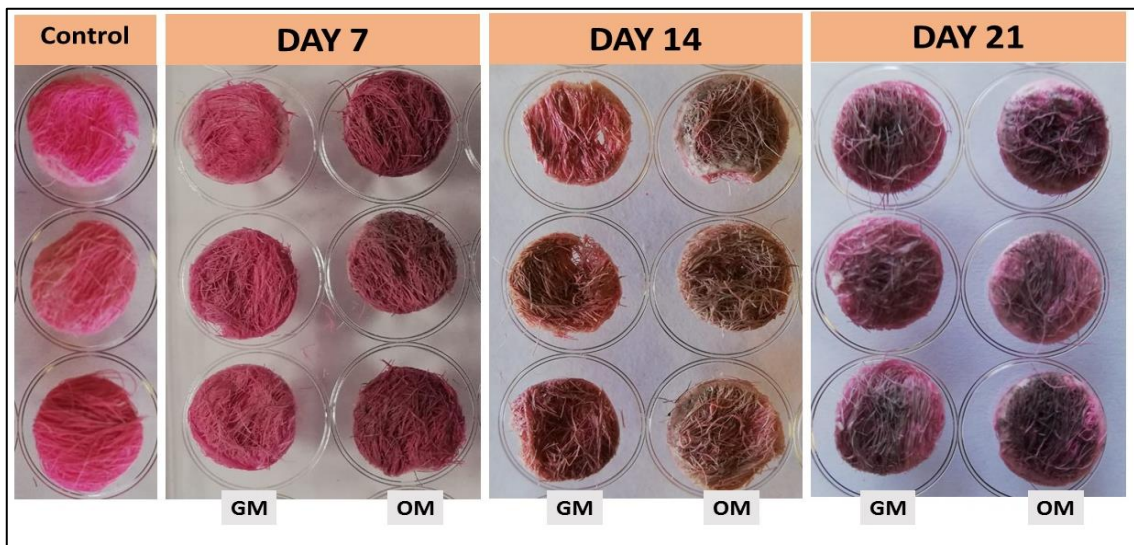


Figure 4.35. von Kossa staining of 20 percent (1:1) PBS:PLGA scaffolds that were seeded with SCAPs and grown in both growth and osteogenic media after 7, 14, and 21 days of incubation. Control represents the scaffold without cells. GM: Growth medium, OM: Osteogenic medium

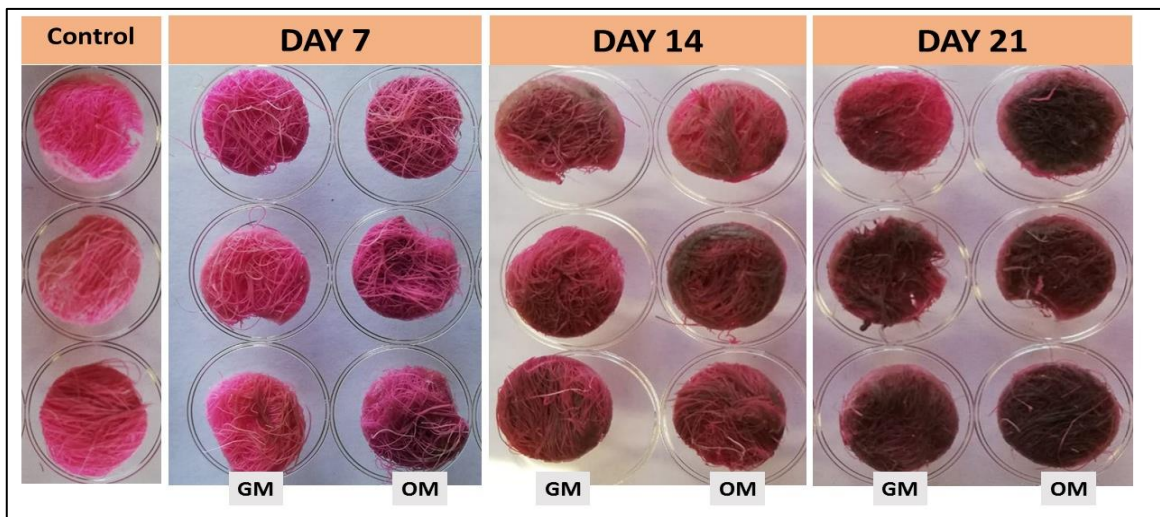


Figure 4.36. von Kossa staining of 20 percent (1:1) PBS:PLGA scaffolds that were seeded with DFSCs and grown in both growth and osteogenic media after 7, 14, and 21 days of incubation. Control represents the scaffold without cells. GM: Growth medium, OM: Osteogenic medium

5. DISCUSSION

The success of bone tissue engineering in combination with bone tissue regeneration depends on the cells and cellular interaction with solid support known as a scaffold as well as bioactive molecules in the environment. It is important to determine the best source of cells to meet the requirements of bone tissue regeneration. Dental stem cells are promising feasible candidates for BTE. The purpose of this study was to determine a multipotent, effective and easily obtainable dental stem cell source to be used in cases where sufficient bone regeneration cannot be achieved and to deliver a suitable carrier serving as a scaffold on which stem cells can remain viable and differentiate.

MSCs can be isolated from different dental tissues such as dental pulp, apical papilla, dental follicle, human exfoliated deciduous teeth, tooth germ, periodontal ligament, and tooth gingiva with ease of accessibility [154]. This study focused on stem cells isolated from immature tissue obtained from the third molar tooth since there are several superiorities of using DSCs derived from developing teeth. First of all, third molars preserve embryonic tissues from dental lamina in quiescent and undifferentiated form until the sixth year of life so cells obtained from third molars stay young developmentally and replicatively [155]. Secondly, third molars usually undergo orthodontic extraction due to prophylactic reasons as part of a routine treatment and therefore cells can be obtained easily. Finally, these cells are adult mesenchymal cells so there is no ethical controversy like in the case of ESC [156]. Various previous reports stated that all DSCs, particularly SCAPs and DFSCs, had greater proliferative potential than BMMSCs [157].

Apart from these, several studies suggested that cells from immature tooth tissues are better than the cells from mature tooth in terms of differentiation capacity, replication rate and isolation efficiency. For example, Chen et al. showed that SCAPs had significantly higher proliferation rate and colony-forming capacity than PDLSCs as well as exhibited higher levels of ALP and Osteocalcin expression [156]. Also, many studies have provided evidence that a higher number of MSCs was obtained from apical papilla than mature dental pulp tissue [156]. Another study conducted with human DFSCs, SHEDs, and DPSCs showed that DFSCs began to proliferate and reached 70 percent confluency earlier than SHEDs and DPSCs after isolation [158]. Since, DFSCs are more undifferentiated comparing with

DPSCs. Thus, dental germ tissue as a whole is more productive mesenchymal stem cell source compared to mature dental pulp [159].

Isolation of cells was performed as previously described in the literature. For the SCAPs, cells were isolated by detaching apical papilla which is the soft tissue that is loosely attached to the apices of immature permanent teeth with a pair of tweezers [160]. The important fact is that SCAPs can be isolated only at a certain stage of tooth development, since apical papilla develops into dental pulp during the crown and root formation. For the DFSCs, cells were isolated from the dental follicle by separating from the surface of the immature tooth using a scalpel and by pulling the papilla off from the apical part of the tooth with a different scalpel [161]. In the study that was firstly identified dental germ progenitor cells, dental mesenchyme of the third molar tooth germs at the late bell stage, one of the four stages of tooth development, were used by separating from dental follicle in order to harvest TGSCs [123]. However, Yalvac et al. performed stem cell isolation from all soft tissues in tooth germ including the dental mesenchyme and follicle tissue inside the crown [141]. One recent study mentioned that the follicle tissue was difficult to separate from the apical papilla although it could be easily peeled from the tooth [162]. In addition, dental mesenchymal tissue and dental follicle tissue in the earlier stage of tooth development cannot be physically separated from each other by certain boundaries [163]. For these reasons, it may be more practical to take dental germ tissue as a whole during the developmental period. Moreover, the stem cell isolation from whole dental germ tissue can be a more effective method due to the interaction of ectoderm and mesoderm originated stem cells with each other, which could affect their differentiation potential. These three cell types were selected for this study due to all their prominent features.

All DSCs used in this study had plastic adherent fibroblast-like morphology under identical culture conditions. DSCs migrated from the minced tissues after 2 days of initial seeding. After that, they formed small globule-like cells that appeared in the flask after 3-4 days of seeding. However, the migration rate of cells was not uniform for all tissues. Some cells migrated after a week and their colony formation was delayed. Also, it was observed that cells had different transitional stages prior to first passage. At the beginning of the primary culture, cells had more round shapes that formed a mesh-like network. Consequently, they achieved spindle-like shape in confluent culture. Although each tissue showed different

migration even within itself, cells in germ tissue migrated faster than the other two tissues on average and became more confluent.

The MSC characteristics of DSCs in this study were confirmed by flow cytometry analysis by using cell surface markers expressed on these three cells. Three cell types were positive for the typical MSC markers (CD44, CD90 and CD105), in agreement with previous studies [165,166], whereas CD14, CD34, and CD45 (blood cells specific surface markers) were negative. These results suggest that our cell isolation and primary culture procedures were rigorously performed. Also, primary cell culture populations of MSCs which were not contaminated with blood cells were established successfully. On one hand, all cells have STRO-1 expression ranging between 15-30 percent positive. According to previous reports, STRO-1 positive cells are more clonogenic, more proliferative and have higher colony-forming abilities in comparison with STRO-1 negative cells or parental unsorted cells [167,168]. The observation of STRO-1 positive cell subpopulations in all three isolated cell types is a supportive finding for the cells to form a population with high osteogenic potential. It was reported that DFSCs and bone BMMSCs, which express approximately 10–20 percent STRO-1 antigen, show similar STRO-1 expression [169,170]. Apart from this, one previous article demonstrated that pig TGSCs expressed almost 40 percent STRO-1 antigen [170] while another article stated that pig TGSCs and human TGSCs expressed 21.6 percent and 11 percent STRO-1 antigen [171], respectively. However, the highest STRO-1 expression was observed in DFSCs with almost 30 percent in our study. These differences may be resulted from the passage number of cells, and tissue origin related factors since various studies stated that STRO-1 expression decreases as the passage number increases [173-175]. The study which found 40 percent STRO-1 expression in pig TGSC used cells in passage number 2 but cells in passage number 4 were used in our study, which obviously led to difference between studies. Nevertheless, all three stem cells in this study had readily higher STRO-1 expression than the most of DSCs such as human DPSCs (0.02–9.56 percent) [172], SHED (9 percent) [174], stem cells from miniature pig deciduous teeth (10.9 percent) [175], and human PDLSCs [176]. In addition to surface antigens, isolated cells achieved osteogenic adipogenic and chondrogenic differentiation. All results collectively proved that isolated cells in this study had mesenchymal characteristics.

International Society for Cellular Therapy (ISCT) proposed some criteria for a cell type to be dental MSCs. These are: (1) cells should be adherent like other MSCs, (2) they should

have ability to give rise to different cell lineages such as osteogenic, neurogenic, adipogenic, and chondrogenic, (3) they should express specific surface antigens of dental MSCs were previously reported and typical antigens of MSCs while should not express typical hematopoietic surface antigen markers [177].

For osteogenic differentiation, three cell types achieved successful mineralization according to von Kossa staining results after 21 day of osteogenic induction. It has been demonstrated that new bone formation is strongly characterized with mineral matrix formation and calcium deposition. Many previous studies showed osteogenic differentiation of these three stem cells by using von Kossa staining. For instance, latest study which was evaluated odonto/osteogenic differentiation capacity of dental stem cells (DFSC, SCAP, DPSC, and PDLSC) was visualized mineral nodules in all cell types after two weeks of induction [178]. Similarly, another study showed that pig TGSC formed mineralized matrix irrespective to STRO-1 expression after 14 days of osteogenic induction [179]. However, only SCAPs showed prominent mineral nodules after two weeks of osteogenic induction in our study. TGSCs and DFSCs required prolonged incubation time according to SCAP and they exhibited successful mineral deposition in their extracellular matrix after 21 days of incubation. These differences between the differentiation times may be resulted from donor related factors and culture conditions.

Chondrogenic differentiation potential of cells was evaluated with alcian blue staining. Alcian Blue stains belong to cuprophthalocyanine that binds to polyanions of acid mucopolysaccharides through saline bounds. In this technique, a sodium tetraborate solution converts the stain into Monastral blue pigment, which is insoluble and cannot spread during further processing. Alcian blue reacts with polyanions whose components are sulphuric and carboxylic radicals and do not react with phosphate radicals of nucleic acids [180]. According to results of alcian blue staining all three cell types can differentiate into chondrogenic lineage in response to chondrogenic induction after 21 days. Moreover, DFSCs showed blue colored areas (positive staining) indicating earlier response to chondrogenic induction. Recent study reported that DFSCs demonstrated a rapid cell aggregation as chondrocytes spheroids, which increased in volume over time and were strongly positive for alcian blue staining while SCAPs exhibited a much slower and less intense staining through 28 days of chondrogenic induction [181].

Adipogenic differentiation potential of cells was evaluated with Oil Red O staining. Oil Red O is a fat-soluble dye used for staining of neutral triglycerides and lipids on the sample as red [182]. In our study, all stem cell types had slightly visible oil drops after 21 days of adipogenic induction. Some previous studies stated that these cells' adipogenic differentiation potential were lower than their osteogenic and chondrogenic differentiation potential. For example, Petrescu et al. could not achieve adipogenic differentiation of SCAPs while they differentiated into chondrocytes and osteocytes in 14 days of culture period. However, DFSCs exhibited some sort of lipid droplets at the end of 14 days as well as osteogenic and chondrogenic differentiation in the same study [181]. However, Sonoyama et al. got successful adipogenesis of human SCAPs by forming Oil Red O positive lipid clusters following 5 weeks of adipogenic induction [135]. Another study achieved adipogenesis of DFSCs and SCAPs after 21 days of adipogenic induction. In addition, they stated that adipocytes from DFSCs had a larger number of stained lipid vacuoles compared to SCAPs and DPSCs [183].

When looking at all the characterization and differentiation results of this study collectively, all three cell types used in this study fulfilled the ISCT criteria. Thus, there is no doubt that the three cell types used in this study were MSCs [177].

In MTS cell viability test, the yellow tetrazolium compounds in the MTS solution turn into purple formazan products, which are reduced in the electron transport chain in the mitochondria of the cells. Since Formazan gives maximum absorbance at 490 nm wavelength, the solution in which the cells are incubated is read at 490 nm wavelength, and gives indirect information about cell viability and number. The number of cells in the well is directly proportional to the amount of formazan in the solution and thus the absorbance at 490 nm [184]. As a result of MTS assay, all cell groups had gradual increase in cell number from day 7 to day 21 in growth medium, excluding DFSC at day 14. This was thought to be due to the lack of space in the culture plate for the cells to grow and the cells began to die since DFSC at day 14 reached the highest cell number among the cell groups in growth medium. When these results are evaluated together with MTS results of control groups of scaffolds on TCP, it can be considered that the DFSC had the higher proliferation capacity than SCAPs in the growth medium while TGSCs had the highest proliferation potential in the growth medium. Patil et al. stated that as a result of *in vitro* culture studies, the DFSCs had a significantly higher proliferation property compared to SCAPs and DPSCs.

Furthermore, they supported this observation by finding a larger number of DSCs in follicle tissue in the proliferative phase of cell cycle than the ones in pulp and papilla tissue [183]. Likewise, Lei and colleagues showed that DFSCs have higher proliferation capacity than SCAPs due to the high molecular expression of CD13 and MARCKS [162]. Moreover, previous studies indicated that dental stem cells proliferate faster than BMMSCs [185]. TGSCs exhibited the highest proliferation rate at the end of 21 days incubation. In many comparative studies with dental stem cells, proliferation rates of DSCs have been shown to be similar. However, a comparative study involving TGSCs is not available in the literature. Son et al. reported that proliferation rates of SCAP, DFSC, DPSC, and PDLSC obtained from same donor could not show statistically significant differences from each other in terms of population doubling time when they investigated proliferation characteristics of these cells in passages 1, 2, 3, 4 in every 72 h [178].

As a result, it was observed that all three cell types had high and similar proliferation and migration rates. The proliferative characteristics of the cell groups were similar, and in some experiments different groups showed higher viability at different time periods, but any of the groups showed a dominantly superior feature. This situation may be attributed to the difference in the host characteristics of the isolated tissues, their age and developmental stages. Although the tooth germ tissue is a more primitive tissue compared to other tissues, proliferation properties were not found to be higher. On the other hand, the proliferation ability of cells showed a different pattern in the osteogenic medium. SCAPs and DFSCs exhibited slight proliferation compared to TGSCs in the osteogenic medium. This situation could indicate that SCAPs and DFSCs suppressed their proliferation and underwent osteogenic differentiation. However, TGSC showed dramatic proliferation from day 7 to day 14 and then a decrease in cell number was observed due to lack of growing space in TCP. When these results are evaluated with alizarin red and von Kossa staining, TGSCs underwent osteogenic differentiation but differentiation did not suppress proliferation of TGSCs. Underlying molecular mechanism of this situation has not been elucidated. However, one previous study stated that STRO-1 positive TGSCs had a higher proliferation rate irrespective to osteogenic induction. They attributed this situation to the fact that osteoprogenitor cells could be found in STRO-1 positive cells [170]. Also, some studies showed that TGSC have primitive stem cell properties due to expressing two transcription factors for pluripotency: Oct-4 and Nanog that are involved in the regulation of cell growth

and differentiation and restricted to pluripotent cells of the developing embryo [186]. The fact that it is a more primitive cell may explain why TGSCs had a high growth rate even when differentiating.

When the osteogenic differentiation potential of the cells was evaluated via ALP assay, calcium deposition assay, alizarin red staining, von Kossa staining, and RT-PCR analysis, it was observed that all cell types successfully formed mineralization and differentiated into osteoblastic lineage at the end of 21 days. Alizarin red staining is a simple, rapid screening method in order to detect microcrystalline or noncrystalline calcium phosphate salts by using light microscopy. This method is helpful in detecting apatite crystal clumps and small calcium pyrophosphate dihydrate crystals. As a result of alizarin red staining, the newly formed bone tissue was stained red due to deposition of the calcium phosphate mineral [187]. Likewise, von Kossa staining method is used to illustrate mineralization such as calcium phosphate. Its principle is the precipitation reaction in which silver ions react with phosphate that is found in calcium phosphate in the presence of acidic material. After that, degradation of silver phosphate to silver happens under light. In this staining, brown color indicates mineralized nodules [188].

Apart from the staining methods, ALP and calcium deposition assays indicate osteogenic differentiation as colorimetric methods. ALP is one of the most credible markers for osteogenic differentiation, since osteogenic cells like osteoblasts produce high amounts of this enzyme [189]. ALP activity was measured by using p-nitrophenylphosphate tablets. This tablets contain the substrate of ALP enzyme. ALP enzyme converts this substrate into p-nitrophenol which is a yellow colored product. This yellow colored p-nitrophenol gives a peak at 405 nm. The absorbance value of the product is directly proportional to the amount of ALP enzyme in cells [190]. On the other hand, calcium deposition is as important as ALP enzyme activity for osteoblastic lineage differentiation. The calcium assay kit contains a phenolsulphonephthalein dye that gives a blue colored complex specifically with free calcium. The intensity of the color measured at 612 nm and absorbance value is directly proportional to the calcium concentration in the sample [191]. Many studies in the literature have previously shown that three cell types can be differentiated into osteoblastic cells [161,165,194]. As a result of alizarin red staining, SCAPs and TGSCs differentiated earlier and had more mineralized areas than DFSCs. It has been demonstrated that SCAPs had a greater differentiation capacity than DFSCs according to a study conducted with a Raman

microspectroscopy. In this study, cellular components of cells were deeply investigated in terms of nucleic acid, lipid, glycogen and protein contents of cells [193]. Another study reported that SCAPs had higher DSPP expression as a marker of osteo/odontogenic differentiation than DFSCs obtained from same donor with SCAPs after osteogenic induction under same culture conditions. When looking at all osteogenic induction results holistically, it was observed that the SCAPs responded faster to osteogenic induction in our study. Also, ALP activity in growth medium was higher in SCAPs and they exhibited high ALP activity in the early period of osteogenic medium. When proliferation analysis was examined simultaneously, the number of SCAPs decreased significantly on day 14 compared to day 7 and increased again on day 21 in the osteogenic medium. This situation supports the fact that cells decrease their proliferation while they are differentiating. In addition, SCAPs in osteogenic medium showed mineral foci with positive staining of von Kossa on day 14 and was the only group that showed a significant increase in calcium concentration compared to day 7.

Besides, Runx2 gene expression, one of the early bone markers, reached the highest value on day 14 in SCAPs in osteogenic medium comparing with all three cell types during incubation period. Moreover in osteogenic medium, in SCAPs, gene expression levels of Osteopontin and Osteonectin, which are the other important bone markers, were found to be higher on day 14 compared to other cell types. All of these RT-PCR results supported early differentiation of SCAPs comparing to other cell types. These results are not surprising considering that SCAPs are the precursors for primary odontoblast *in vivo* [196,197]. On the other hand, one previous study found that the differentiation capacity of SCAPs were higher than DFSCs when interpreting Raman microspectroscopy results [193]. Also, Sowmya et al. showed that DFSCs did not express Runx2 and not have ALP activity in the growth medium although they expressed 20-25 percent PLAP-1, FGF-2 and CEMP-1, fibroblast and cementoblastic markers. They stated that DFSCs could have a higher tendency to differentiate into fibroblastic and cementoblastic cells compared to osteogenic cells without induction [196].

Dentine sialo phosphoprotein (DSPP) is an essential protein in the tooth dentine formation and it is split into two parts (dentin sialoprotein and dentin phosphoprotein) in the process of dentine formation [197]. Although used as odontogenic markers in the many previous reports, DSPP expressions were detected in bone despite the fact that the expression level in

bone and dentin is quite different [198]. For this reason, DSPP is not specific to dental tissues, thereby, it is not considered only an odontogenic marker. The DMP-1 is released by osteocytes and odontoblasts as a non-collagenous extracellular matrix protein and takes the role in the regulation of hard tissue mineralization [199]. DMP1 gene is located within the bone sialoprotein locus and within the locus for Osteopontin on chromosome [200]. Thus, it is found in both dentin and bone. Although DMP-1 and DSPP are frequently used as an odontogenic marker, there is no specific gene used as an odontogenic differentiation marker in the literature. The similarities in tooth and bone structure and the interactions of the genes involved in mineralization processes, as well as the molecular mechanisms have not yet fully elucidated. Besides, it is not possible to make a clear distinction between *in vitro* osteogenic and odontogenic differentiation. In this study, both DMP-1 and DSPP were expressed in significant levels by all three cell types at the end of the osteogenic induction period. However, DMP-1 expressed earlier in SCAPs in osteogenic medium as well as Osteonectin, Osteocalcin, and Runx2. This indicated that all stem cells achieved odonto/osteogenic differentiation within 21 days but SCAPs underwent differentiation faster.

As a result of osteogenic induction, all three cell types were osteogenically differentiated and it has been shown that they had similar mineralization potential with calcium accumulation values and mineral staining that did not differ significantly at the end of the culture process, but gene expressions of osteo-odontogenic differentiation differed from each other.

Current findings and studies show that these dental cells are more prone to osteogenic differentiation due to their role in formation of dentin and explain their predisposition to tooth development and calcification. Thus, these cells are more suitable for osteogenesis.

One of the scaffold materials used in this study was PLGA due to its superiorities among polymers such as biodegradability, high biocompatibility, high mechanical strength and being one of the most widely used synthetic polymers in BTE. Also, its mechanical properties and degradation rate are tunable for the application purposes [93]. Similarly, PBS was another scaffold material in order to make composite scaffolds in this study for basically the same reasons. PBS has interesting thermo-mechanical properties and biodegradability as well as acceptable raw material and production costs. Furthermore, the reactants worked in the synthesis of PBS can be obtained from renewable resources, thereby, PBS becomes a fully bio-based and sustainable polymer [79]. The mechanical properties of brittle polymers

such as PLA can be changed with a ductile material such as PBS in order to make them more durable [201]. Although many studies conducted with PLA and PBS blends exist in literature, there is no study that investigated PLGA and PBS blends and their scaffold, which makes this study distinctive. This polymer blend, which can be prepared as fiber, has sufficient degradation time and exchangeable mechanical properties to allow new tissue formation. The advantage of polymer blends is that they do not have to conform to a chemical structure such as a copolymer and are particularly suitable for nanofiber production.

For BTE, these materials have to meet specific requirements like adequate internal architecture and surface properties as well as mechanical resistance and porosity. These biodegradable scaffolds have to be porous so as to efficiently propagate cells and nutrients, and also for supplying oxygen. Also, scaffolds must have three-dimensionally interlinked pores that allow the generation of bone tissue and facilitate fluid transport through the scaffold [202]. To compensate all these needs, wet spinning methods were chosen to fabricate porous interconnected scaffolds. Although there are various methods to produce porous scaffold such as solvent casting, particulate leaching, 3D printing, and electrospinning, wet spinning is an easy and inexpensive method. Also, this method, which does not require a specialized and expensive device, can be easily preferred to produce tissue engineering scaffolds in many laboratories [203].

Besides, the scaffolds produced by wet spinning method fulfill the requirements mentioned above by creating adequate pore size and fiber diameters. The wet spun scaffold mimics the extracellular structure of the bone consisting of collagen fiber structure with the desired properties. In our study, it was observed that a large number of scaffolds can easily be obtained with a small amount of material for *in vitro* experiments. Although electrospinning is a more common method to fabricate fibrous scaffolds, more porous structure and a larger pore size can be obtained by wet spinning method [97]. Thus, cell attachment and proliferation are increased in the inner regions of the scaffold. Moreover, Jin et al stated that Polysulfonamide (PSA) fibers fabricated by wet spinning had better thermal stability and mechanical features than the PSA fibers produced by electrospinning combined with more regular molecular chain arrangement of the fiber [204]. In the wet spinning method, polymer concentration, content and concentration of the coagulation bath, and speed of both the syringe pump and turntable can affect the structure of the scaffold. The basic process of wet

spinning is the immersion of an injected polymer solution to a coagulation bath containing a nonsolvent for polymer that should be miscible with the polymer solvent. Polymer precipitation and fiber formation are achieved by exchanging between the solvent and nonsolvent [205].

In our study, DCM was used as a solvent for polymers and isopropanol was used as a nonsolvent. One study conducted with the poly4-hydroxybutyrate (P4HB) fibers stated that addition of a chemical that was nonsolvent for polymer and poorly miscible with polymer solution to wet spinning system increased the coagulation rate of the polymer [206]. Thus, water was added to the coagulation bath to obtain more neat and regular fibers. In the current study, different isopropanol: distilled water (IP:DW) ratios (100: 0, 85:15, 75:25, 50:50, 25:75, 15:85, and 0: 100 IP:DW) were tested. In order to determine the best coagulation bath, 25 percent (1:3) PBS:PLGA solution was spun because high polymer concentration have high coagulation speed. Therefore, fiber formation could be observed even in low performance coagulation baths. As the water ratio increased in the coagulation bath, polymer coagulation and fiber formation slowed down. Moreover, polymer coagulation did not occur when the distilled water ratio was over 50 percent in the bath, which is in agreement with the previous studies [97]. Besides that, the polymer coagulation was extremely fast, especially in higher polymer concentrations, causing blockages in the needle tip when the alcohol ratio was over 75 percent in the bath. Therefore, the other coagulation bath ratios were excluded from this study except 75:25 IP: DW ratio and the following experiments were continued with 75:25 IP: DW coagulation bath, which provided the best uniform, neat and smooth fiber formation. Apart from these, fibers after coagulation were incubated for 24 h since some studies showed that elongated coagulation time resulted in more even and smooth fiber formation [207].

The polymer concentration plays an important role in determining the physical properties of the scaffold. Many studies showed that the fiber diameter and linear density increase as the polymer concentration increases, causing an increase in the mechanical strength [97][206]. This can be caused by more swelling of the extruded polymer as it left the needle as a result of increasing polymer concentration. In the current study, PBS and PLGA polymers were dissolved in dichloromethane at 10 percent, 15 percent, 20 percent and 25 percent concentrations. Also, different PBS and PLGA ratios which were 1:1 and 1:3 were tested. The highest polymer concentration could be prepared as 25 percent since the polymer did

not dissolve at room temperature above 25 percent. Also, 25 percent PBS:PLGA (1:1) concentration could not be prepared due to insoluble PBS residues when 25 percent polymer solution contained the same amount of PBS and PLGA. Thus, the polymers with 25 percent concentration could only be prepared as 1:3 PBS:PLGA ratio. According to SEM micrographs, a uniform and fracture-free fibers were not obtained with 10 percent (1:1) and (1:3) scaffolds even if they could be spun. Low polymer concentration might cause a micro-gap formation between continuous fibers. If the enough polymer does not exist at the point where coagulation occurs, beads occur between the fibers and continuous fiber network cannot be achieved at this point. As a result, the fiber structure changes when polymer concentration is too lowered [208]. Additionally, low concentration of polymer solution causes weak mechanical properties. That's why, in our study, low concentration scaffold fibers fractured during handling. After that, 10 percent (1:1) and 10 percent (1:3) scaffolds were eliminated from this study.

In mechanical tests, it was observed that compression modulus increased as the polymer concentration increased and higher values were obtained as the PBS concentration increased in the samples having the same concentration. Similarly, in the tensile modulus, it was observed that increase in the polymer concentration also increased the mechanical properties, but the tensile strength increased as the PLGA concentration increased in the samples having the same concentration contrary to the compression modulus. The mechanical strength of fibers which is directly proportional to polymer concentration tends to incline as the elongation viscosity of the polymer solution increases [209]. Thus, the polymer composition, rheology, and coagulant composition reflect the fiber morphology and mechanical strength.

In addition, fibrous scaffolds were kept in phosphate buffered saline solution throughout 90 days to evaluate degradation behavior of scaffolds. It was observed that all scaffolds maintained at least 90 percent of their weight during first 60 days irrespective to polymer concentration and polymer composition. Nevertheless, an increase in the degradation rate of all scaffolds was observed after that point and all scaffolds, especially 25 percent (1:3) PBS:PLGA scaffold, approximately lost half of their weight. This situation can be resulted from slower surface erosion through 60 days followed by a faster bulk erosion. When the polymers are degraded, acidic monomers are released to the environment such as lactic acid, glycolic acid. All acidic polymers decrease the pH of samples causing autolytic cleavage of

PLGA and accelerating degradation of scaffolds [210]. The highest pH change and degradation of 25 percent (1:3) scaffold with the highest PLGA content suggests that the lactic acid and glycolic acid released during the degradation process of the PLGA reduced the pH and the acidic environment increased the degradation of the material. Consistent with the weight loss results, no excessive pH change was observed in the first 60 days. After that point, pH slumped dramatically, especially in 25 percent (1:3) PBS:PLGA scaffold. A sudden decline in pH can cause the tissue necrosis near the scaffold and that affects the success of scaffold in tissue regeneration. Therefore, 25 percent (1:3) PBS:PLGA scaffold was eliminated from this study considering the highest weight loss and pH decrease. When we evaluate other scaffolds' pH change, they are in the tolerance range of the body since acidic monomers of polymers are balanced with blood which is an efficient buffer system in the body and they are rapidly cleared from the body via the renal system [211]. In addition, extended degradation time is better for scaffolds to be used in bone tissue engineering for bone healing. The 20 percent (1:1) PBS:PLGA scaffold, which maintained its weight around 85 percent on day 90 and had good mechanical properties, was the most suitable scaffold for BTE among the other scaffolds used in this study. Moreover, it was observed that this scaffold was more compatible with the cells in the first cell viability assay. Therefore, 20 percent (1:1) PBS:PLGA scaffold was selected as a main scaffold for determining the *in vitro* scaffold and three different stem cell interaction.

The viability and proliferation characteristics of all cell types on scaffold in both osteogenic and growth medium were evaluated with MTS. The viability of the cells in the cell culture plates increased more in the growth medium than the one in osteogenic medium. It was observed that in the cell culture plates TGSCs had the highest cell viability on day 7 in osteogenic medium. However, cells started to die after day 14. In this group, one hundred percent cell confluency was observed nearly day 14 and then many gaps showing cell detachment were observed under the microscope after that point. This event reflected a decrease in absorbance value. However, the underlying mechanism of higher TGSCs proliferation in osteogenic medium could not be elucidated yet.

On the scaffolds, cells behaved differently from each other in terms of cell viability. TGSCs and SCAPs were successfully attached and proliferated onto the scaffold, but DFSCs did not attach as effectively as them. Nevertheless, DFSCs had higher ALP activity than SCAPs, although they had similar ALP activity with TGSCs in growth medium. This shows that

DFSCs could differentiate in the scaffold although the small number of cells attached to it. The higher ALP activity in DFSCs was supported by a previous study conducted with BMMSCs, DPSCs, SCAPs, and DFSCs. Kumar et al. in this study reported that DFSCs had the highest ALP activity among these cells while they had lowest Runx2 expression. Also, SCAPs had the highest Runx2 expression among these cells while they had the lowest ALP activity in this study [197]. Similarly in our study, the highest Runx2 expression was observed in SCAPs on days 7 and 14 in growth medium and on day 14 in osteogenic medium in our study. These results suggest that differentiation of these dental stem cells to bone tissue might be with the participation of different marker or the participation of different amount of markers in different pathways.

In the ALP activity assay on scaffolds, all cell types showed similar pattern which was a significant increase on day 7 followed by a decrease on days 14 and 21 in both growth and osteogenic media. As expected, cells grown in osteogenic medium had higher ALP activity than the ones grown in growth medium. This situation could result from ALP being early bone marker. In one study conducted with DFSCs, PDLSCs, and DPSCs, ALP activities of cells increased greatly on day 7 followed with a decrease on days 14 and 21 after osteogenic induction while all cell groups grown in growth medium had continuous increase in ALP activity [169]. This article suggested that alterations in the ALP activity definitely do not have deterministic influence for osteogenic differentiation. Another study conducted with pig TGSCs stated that ALP activity in TGSCs reached the highest level on day 7 in osteogenic medium and then decreasing ALP activity was observed on days 14 and 21 [170]. Furthermore, ALP activity of TGSCs reached the highest level on day 14 and diminished ALP activity was observed on day 21 in growth medium in this study.

When the previous studies and our results are holistically evaluated, it can be seen that osteogenic induction resulted in high ALP activity of cells in early days of incubation and ALP activity was diminished in the later stages of osteogenic differentiation. In our study, TGSCs showed the highest ALP activity on scaffold on day 7 in both media and then TGSCs' ALP activity showed similar levels with DFSC's ALP activity on days 14 and 21. When the ALP activity of cells on scaffolds was compared with the ones in the cell culture plate, ALP activity was higher in groups of cells seeded in culture plates as a control. This finding suggests that dental stem cells with high differentiation potential did not show high ALP activity in the scaffold. However, confocal study and gene expression analysis with RT-PCR

revealed that cells attached to scaffolds expressed bone markers. Although an increase for each marker varied depending on cell types, expression of bone markers increased through time periods.

In RT-PCR analysis, induced SCAPs exhibited the highest Runx2 expression on day 21 and the highest Osteonectin expression on day 14. Additionally, they showed the highest DSPP expression among osteogenic induction groups. However, DSPP and DMP-1 levels of cells were very low and there were no significant differences between the cells. This might be due to the isolation of cells from more primitive tissues in the tooth. Aonuma et al. mentioned that DSPP and DMP-1 expression were absent in DFSCs throughout 10 days of incubation in both osteogenic and growth media [212]. Son et al. could detect DMP-1 and DSPP expressions in SCAPs, DFSCs, DPSCs, and PDLSCs after 14 days of incubation in osteogenic induction medium. However, DSPP and DMP-1 expressions in SCAPs and DFSCs were significantly lower than DPSCs [178]. The expression of odontoblastic markers of DFSCs, SCAPs, and TGSCs might be downregulated because they were derived from not fully formed but developing tooth tissues. The highest level of Osteocalcin expression was found in TGSCs on day 21 while the highest level of Osteopontin expression was observed in DFSCs on day 7.

In the confocal study, the cells attached to the scaffold after immunofluorescence staining were positive for Osteocalcin and Runx2, suggesting that all three cell types preserved their bone differentiation potentials on the scaffold. Moreover, positive von Kossa staining on the scaffolds indicates that cell types maintained their differentiation potential on this scaffold. TGSCs on scaffold demonstrated the most intensive color characterized by mineral deposition among the other cells at the end of the culture period.

In an *in vitro* study in which the PBS and chitosan combination (50:50) disc-shaped scaffolds were coated with fibronectin, human osteosarcoma cells (SaOs-2) were seeded on the scaffolds and cell attachment and proliferation were evaluated [213]. This study stated that PBS increases osteoblastic activity while chitosan, a natural polymer, increases cell adhesion. Also, higher proliferation values were observed in pure PBS scaffolds. Likewise, it was observed that viability of cells increased as the PBS ratio increased in our study. However, the problem of this PBS/Chitosan study is that viability and ALP activity were evaluated only within the first 7 days. This period does not give a clear idea that this polymer combination works in the long term. Additionally, this study mentioned that cell

proliferation of the control group in the cell culture plate was significantly higher than the one in the scaffold as in our study. In another study, DFSCs were seeded on the polycaprolactone (PCL), a synthetic polymer, scaffold and osteogenic differentiation was evaluated both *in vivo* and *in vitro* [214]. In the *in vitro* evaluation, the attachment of the cells to the scaffolds was successful on day 1 and day 3. Also, 50 percent recovery was observed in bone defects in the 8th weeks of *in vivo* study in rats. Although results of this study are promising, evaluation of effectiveness of DFSCs on bone regeneration requires a longer *in vitro* period similar to our study. In addition, Morsczeck et al. mentioned that DFSCs in the osteogenic medium could form osteoclasts *in vitro* [215]. However, they lost their ability to form hard tissue with osteogenesis after *in vivo* transplantation. Therefore, our results require to be supported by a follow-up *in vivo* study although all cell types achieved similar and successful mineralization at the end of the culture process in our *in vitro* study.

6. CONCLUSION

A solid understanding of dental stem cells is crucial for tissue engineering and regenerative medicine due to easy accessibility, lacking donor site morbidity, no ethical issues and great differentiation potential. Although many studies have been conducted about DPSCs, PDLSCs, and SHEDs, more primitive dental tissues such as apical papilla dental follicle and especially tooth germ remained out of focus. There is no study to compare potentials of these three stem cells in the literature. Also, wet spun fibrous PBS:PLGA composite scaffolds have not been used before in tissue engineering applications although the PLA and PBS blends exist and PLGA is one of the most common polymers used in tissue engineering. Therefore, the aim of this study was to compare the *in vitro* differentiation potential of stem cells derived from apical papilla, dental follicle and tooth germ and to investigate the potential of wet-spun PBS:PLGA composite scaffold to favor bone tissue engineering due to its biological safety and tuneable degradation properties. In this study, we demonstrated that the culture of these stem cells was fast and easy. These cells could easily proliferate to the desired number of cells to use *in vitro* studies, especially TGSCs was more favorable. In *in vitro* culture, DFSC was more prone to differentiate into chondrogenic lineage than the SCAPs and TGSCs. However, all these stem cells had lower adipogenic potential and their adipogenic differentiations were more prolonged than their osteogenic and chondrogenic differentiations. All cells had great osteogenic differentiation capacity in the cell culture plate in terms of mineralization, calcium deposition and bone marker gene expressions. However, their proliferation and differentiation capacity was diminished in 3D culture although all of them expressed bone markers and achieved mineralization. In the cell culture plate, cells produced prominent Col1 together with Osteocalcin and Runx2 but Col1 expressions were significantly reduced on the scaffold while Osteocalcin and Runx2 expressions were maintained. Apart from these, it was observed that the 20 percent PBS:PLGA (1:1) scaffold had sufficient physical properties for bone tissue engineering and appropriate degradation kinetics in terms of releasing pH values and degradation speed. In conclusion, 20 percent (1:1) scaffold with these stem cell types can be promising alternative for BTE.

7. FUTURE PROSPECTS

In this study, the comparative analysis of three different dental stem cells were performed and an alternative fibrous composite scaffold was fabricated for bone tissue engineering. In the future, first of all, the success of this scaffold and cells should be tested *in vivo* experimental models and tested for longer culture period. There are many factors such as signaling molecules, immune cells, loaded forces that can affect scaffold structure, bone formation on scaffold, and stem cells response on scaffolds in the body.

Secondly, this scaffold can be enhanced with addition of osteoinductive or osteoconductive materials. Also, a layer of drug or bioactive molecule released systems such as hydrogels and micro-particle can be added. In the present study, it was observed that the three cell types in the scaffold could differentiate into osteogenic lineage after osteogenic induction, but their differentiation can be enhanced with various methods. For example, bioactive molecules such as BMP-2 can be used to increase osteogenic differentiation, but the use of these molecules increases the cost [216]. In our study, it was also aimed to produce an economically low cost scaffold, thus, easily accessible synthetic polymers PBS and PLGA were chosen as a scaffold material. Also, osteoconductive materials like hydroxyapatite (HAp) can be added to scaffolds in the fabrication step. In one study conducted with the bilayered PLGA/PLGA-HAp composite scaffold, they got successful osteochondral defect healing via this scaffold both with and without MSCs as seed cells [217]. While seeding of cells led to better results, the PLGA/PLGA-HAp scaffold showed satisfactory *in vivo* efficiency after implanted into the rabbit femur for 16 weeks.

Thirdly, various strategies can be tried to increase cell attachment to scaffolds. The main challenge in our study was to increase cell adhesion rather than increase osteogenic differentiation. Since the surface of the PLGA material does not have a natural structure that enhances the adhesion of cells, extracellular matrix, extracellular matrix-like macromolecules and functional peptide fragments can be used to make the polymer surface more biologically suitable. Therefore, various methods such as fibronectin, vitronectin and collagen coating can be used to create a biomimetic interface between cell-polymer [220,221]. Fibronectin is involved in many processes such as cell migration, cell adhesion and tissue repair. Integrin subunits on osteoblasts recognize the RGD sequence on fibronectin [220]. Therefore, fibronectin may also contribute to osteogenesis in the process

leading to bone differentiation. In another study, laminin or fibronectin modification on PBS scaffold increased the proliferation of tooth germ cells for 10 days culture period [142]. Another method to increase cell attachment in an economical way could be physical surface modification such as oxygen plasma treatment which modifies the chemical properties of the surface and increases its roughness.

Finally, this scaffold can be used in different tissue engineering applications such as cartilage and nerve tissue engineering with different cell types. In this study, stem cells could be obtained from different donors. Thus, comparing stem cells from a single donor at the same time can give more accurate results by eliminating differences caused by different donors.



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APPENDIX A: ETHICAL COMMITTEE PERMISSION

SAYI: HDK-2016/39

25.11.2016

KONU: ACU-HADYEK 2016/38 sayılı başvurunuz

Sn. Yrd. Doç. Dr. Pınar ERÇAL

ACU-HADYEK 2016/38 sayılı “**Kemik Doku Mühendisliğinde Dış Germ Kaynaklı Kök Hücreler: Osteo-Odontojenik Potansiyel**” isimli projeniz Acıbadem Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu’nun **15.11.2016** tarihli **29.** toplantısında incelenmiş ve etik olarak uygun bulunmuştur.

ACU-HADYEK 2016/39 sayılı karar ektedir.

A blue ink signature of Prof. Dr. Güldal SÜYEN.

Prof. Dr. Güldal SÜYEN

Acıbadem Üniversitesi
Hayvan Deneyleri Yerel Etik Kurulu Başkanı



**ACIBADEM ÜNİVERSİTESİ HAYVAN DENEYLERİ YEREL ETİK KURULU
KARAR FORMU**

BAŞVURU TARİHİ: 27.10.2016	KARAR TARİHİ: 15.11.2016
BAŞVURU SAYISI: 2016/38	KARAR SAYISI: 2016/39

İstanbul Kemerburgaz Üniversitesi Diş Hekimliği Fakültesi Ağız Diş ve Çene Cerrahisi Anabilim Dalı'ndan, Yrd. Doç. Dr. Pınar ERÇAL'ın yürütücüsü olduğu "**Kemik Doku Mühendisliğinde Diş Germ Kaynaklı Kök Hücreler: Osteo-Odontojenik Potansiyel**" isimli proje başvurusu Acıbadem Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu'nun "15.11.2016" tarih ve "29" sayılı toplantısında görüşülmüş ve etik açıdan

☒ Uygun ☐ Düzeltmesi Gerekir ☐ Koşullu Olarak Uygun ☐ Uygun Değil

olarak değerlendirilmiştir.

Kurul Üyesi	İmza	Karara Katılıyorum	Karara Katılmıyorum
Prof. Dr. Güldal Süyen (Başkan)		()	()
Prof. Dr. Serap Arbak (Başkan Vekili)		()	()
Prof. Dr. İsmail Hakkı Ulus		()	()
Prof. Dr. Alp Bayramoğlu		(X)	()
Prof. Dr. Yeşim Işıl Ülman		(X)	()
Yrd. Doç. Dr. Devrim Öz Arslan		(X)	()
Yrd. Doç. Dr. Figen Demir		()	()
Sabiha Turgut Genç		(X)	()
Vet. Hek. Samed Özer (Sekreter)		(X)	()

APPENDIX B: ALP CALIBRATION CURVE

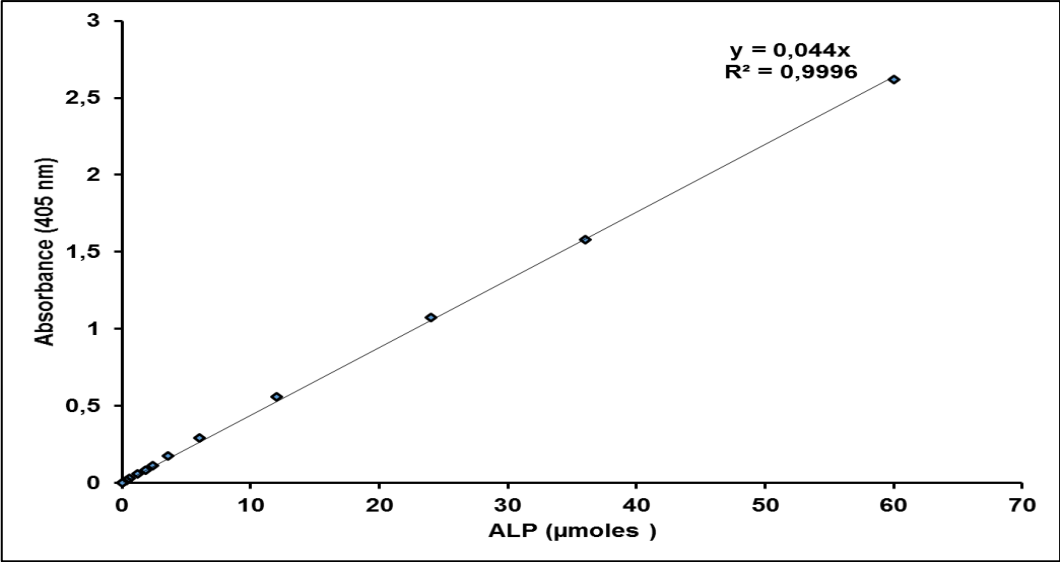


Figure B.1. ALP calibration curve

APPENDIX C: CALCIUM CALIBRATION CURVE

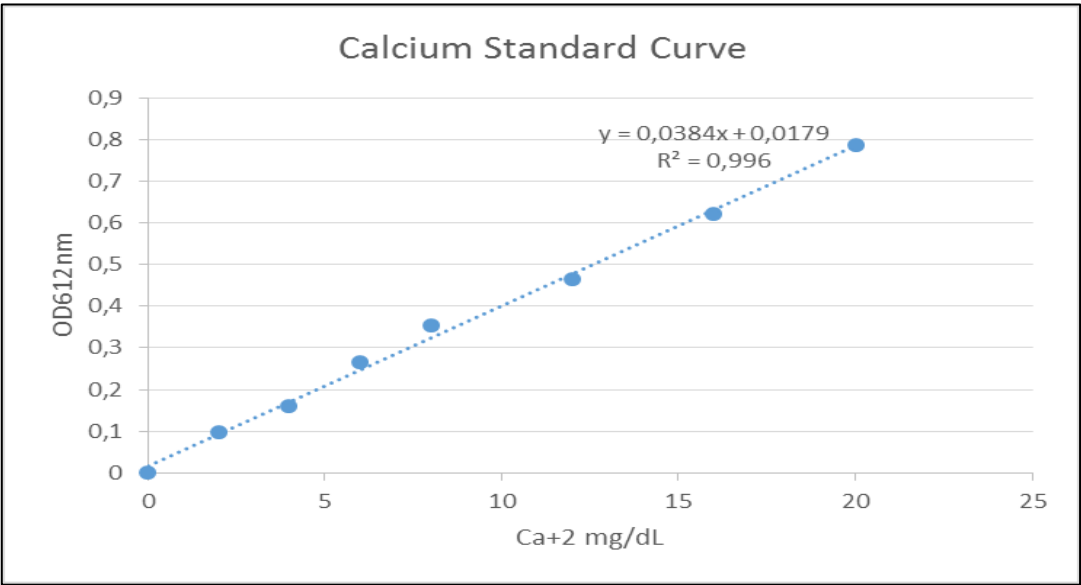


Figure C.1. Calibration curve of calcium standards