SMOOTH MUSCLE CELL DIFFERENTIATION FROM RABBIT AMNIOTIC CELLS

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ABSTRACT

SMOOTH MUSCLE CELL DIFFERENTIATION FROM RABBIT AMNIOTIC CELLS

Congenital anomalies (CA), also known as birth defects are structural and/or functional anomalies that occur during intrauterine life. Treatment strategies like organ transplantation and surgical reconstructions are still being used despite their disadvantages like immune rejection. On the other side tissue engineering studies have promising results. Especially in cases of smooth muscle tissue defects, which are commonly diagnosed prenatal malformation tissue engineering could be alternative treatment strategy. Amniotic fluid is a liquid layer that provides mechanical support and movement of the fetus during embryogenesis. In 2014, researchers show that about 1 per cent of the total amniotic fluid cells contains mesenchymal stem cell surface markers. The main priority of this study is to detect whether amniotic fluid stem cells could be the new source for tissue regeneration in smooth muscle defects and if it is, define new treatment strategy. To achieve this goal, preliminary studies were performed with rabbit amniotic fluid. Amniotic fluid stem cells ($CD90^+$ $CD44^+$ $CD29^+$) were separated from the total amnion fluid cells with flow cytometer. After separation, rabbit amniotic fluid mesenchymal stem cells were differentiated to smooth muscle cells with using differentiation agents (platelet-derived growth factor-BB, PDGF-BB and a multifunctional cytokine TGF-beta1). With parallel to ongoing differentiation study samples were collected for characterization from both experimental and control group in 7^{th}, 14^{th} and 21^{th} days of differentiation. For characterization, morphologic, molecular (Real-time PCR and immunocytochemistry) and functional (contractile assay and Flou-4 calcium signaling assay) properties of induced cells were evaluated. Finally with selected suitable polymer, well-designed PLGA scaffold was fabricated and cell attachment capacity on the scaffold was tested. Obtained data indicate that, fully functional smooth muscle cells could be generated from rabbit amniotic fluid, PLGA polymer is suitable scaffold material for amniotic fluid mesenchymal stem cells and SM cells generated from RAFMS cells can be useful for congenital anomaly treatment.

ÖZET

TAVŞAN AMNİYOTİK HÜCRELERİNDEN DÜZ KAS HÜCRE FARKLILAŞTIRMASI

Doğumsal kusurlar olarak da bilinen doğumsal anomaliler, intrauterin yaşam sırasında ortaya çıkan yapısal ve/veya fonksiyonel anomalilerdir. Organ nakli ve cerrahi rekonstrüksiyon gibi tedavi stratejileri hala kullanılmasına rağmen bağışıklık reddi gibi dezavantajları bulunmaktadır. Öte yandan, doku mühendisliği çalışmaları ile umut verici sonuçlar elde edilmiştir. Özellikle düz kas doku bozuklukları gibi yapısal bozukluklar için doku mühendisliği alternatif bir tedavi yöntemi olabilir. Amniyotik sıvı hamilelik esnasında fetüsü mekanik darbelerden koruyan ve hareketine olanak sağlayan sıvı tabakasıdır. 2014'te araştırmacılar, toplam amniyotik sıvı hücrelerinin yüzde 1'inin mezenkimal kök hücre yüzey markörleri içerdiğini göstermiştirler. Bu çalışmanın ana önceliği, amniyotik sıvı kök hücrelerinin düz kası kusurlarında doku mühendisliği için alternatif bir kaynak olabileceğini göstermek ve yeni tedavi stratejisi tanımlamaktır. Bu amaca ulaşmak için, tavşan amniotik sıvısı ile ön çalışmalar yapılmıştır. Amniyotik sıvı kök hücreleri (CD90 + CD44 + CD29 +) toplam amniyon sıvı hücrelerinden akış sitometresi ile ayrılmıştır. Ayırmadan sonra, tavşan amniotik sıvı mezenkimal kök hücrelerinden düz kas hücresi farklılaştırmasına farklılaşma ajanları (trombosit türevli büyüme faktörü-BB, PDGF-BB ve çok işlevli bir sitokin TGF-beta1) kullanarak başlanmıştır. Farklılaştırma çalışmaları devam ederken daha sonra uygulanacak karakterizasyon çalışmaları için deney ve kontrol gruplarından 7., 14. ve 21. günlerde örnek toplanmıştır. Farklılaştırılan hücrelerin morfolojik, moleküler (Gerçek zamanlı PCR ve immünositokimya) ve fonksiyonel (kasılma ve Flou-4 kalsiyum sinyal analizi) özellikleri incelenmiştir. Son olarak, seçilen uygun polimer ile iyi tasarlanmış PLGA scaffold'u elde edilmiş, amniyon hücrelerinin bağlanma kapasitesi test edilmiştir. Elde edilen veriler tavşan amniyotik sıvısından tam fonksiyonel düz kas hücrelerinin farklılaştırılabildiğini ve PLGA scaffoldunun amniotik sıvı mezenkimal kök hücreler için uygun olduğunu göstermektedir.

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AF	Amniotic Fluid
AFC	Amniotic Fluid Cell
AFMSC	Amniotic Fluid Mesenchymal Stem Cell
AFSC	Amniotic Fluid Stem Cell
ASC	Adult Stem Cell
α-SMA	alpha Smooth Muscle Actin
BMMSC	Bone Marrow Mesenchymal Stem Cell
CA	Congenital Anomalies
CM	Congenital Malformation
CSMC	Contractile Smooth Muscle Cell
CVS	Chorionic Villi Sampling
DAPI	4’,6-diamidino-2-phenylindole
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethylsulfoxide
dNTP	Deoxyribonucleotide triphosphate
DPBS	Dulbecco’s Phosphate Buffered Saline
ESC	Embryonic Stem Cell
EUROCAT	European Surveillance of Congenital Anomalies
EtBr	Ethidium Bromide
EtOH	Ethanol
FACs	Fluorescence-Activated Cell sorting
FBS	Fetal Bovine Serum
HMDS	Hexamethyldisilazane
ICM	Inner Cell Mass
KCl	Potassium Chloride
LFA-1	Lymphocyte Function-Associated Antigen
MHC-1	Major Histocompatibility Complex-1
MHC11	Muscle Myosin Heavy Chain
MRI	Magnetic Resonance Imaging

MTS	3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium
NaOH	Sodium Hydroxide
PDGF-BB	Platelet-Derived Growth Factor BB
PFA	Paraformaldehyde
PLGA	Poly (lactide-co-glycolide)
PS	Penicillin-Streptomycin
SEM	Scanning Electron Microscopy
SMc	Smooth Muscle Cell
SNPs	Single Nucleotide Polymorphisms
SSMC	Synthetic Smooth Muscle Cell
TE	Tissue Engineering
TGF-β1	Transforming Growth Factor beta-1
WHO	World Health Organization

1. INTRODUCTION

1.1. CONGENITAL ANOMALIES

Congenital anomalies (CA), also known as birth defects are structural and/or functional anomalies that occur during intrauterine life. Between 2003 and 2007, 23.9 congenital anomalies were detected over 1.000 of birth by EUROCAT (European Surveillance of Congenital Anomalies) [1]. Since some malformations are incompatible with life, CA is one of the important causes of neonatal death. WHO (World Health Organization) estimate that 303.000 newborn die every year because of congenital anomalies (Figure 1.1) [2].

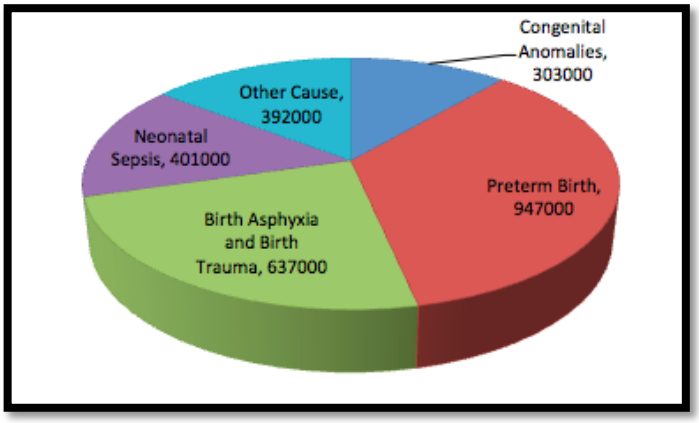

Figure 1.1. Worldwide causes of neonatal death in 2015 [2].

Congenital anomalies classified in five groups; congenital abnormalities (structural malformations), fetal disease, genetic disease, intrauterine growth retardation and disability [3]. Congenital malformation (CM), which developmental delay resulted with structural deformation represents the largest group between these categories. CM divided into two groups, which are major and minor anomalies [4]. In major anomalies, patients have serious medical and cosmetic consequences, that might be lethal other case need medical intervention. On the other hand, in minor anomalies patient do not present medical or

cosmetic symptoms. Incidence of congenital malformation in newborn infants is about 3-5per cent [5].

1.1.1. Causes of Congenital Anomalies

For congenital malformation timing is important impact on the occurrence and the type of malformation produced [6]. Since in embryonic period (before 14th week) numerous mitotic divisions and organogenesis occurs the embryo is very sensitive. In this period malformation ended with spontaneous abortion or major anomalies. On the other side malformations that occur in fetal period generally ended with minor anomalies (Figure 1.2) [7].

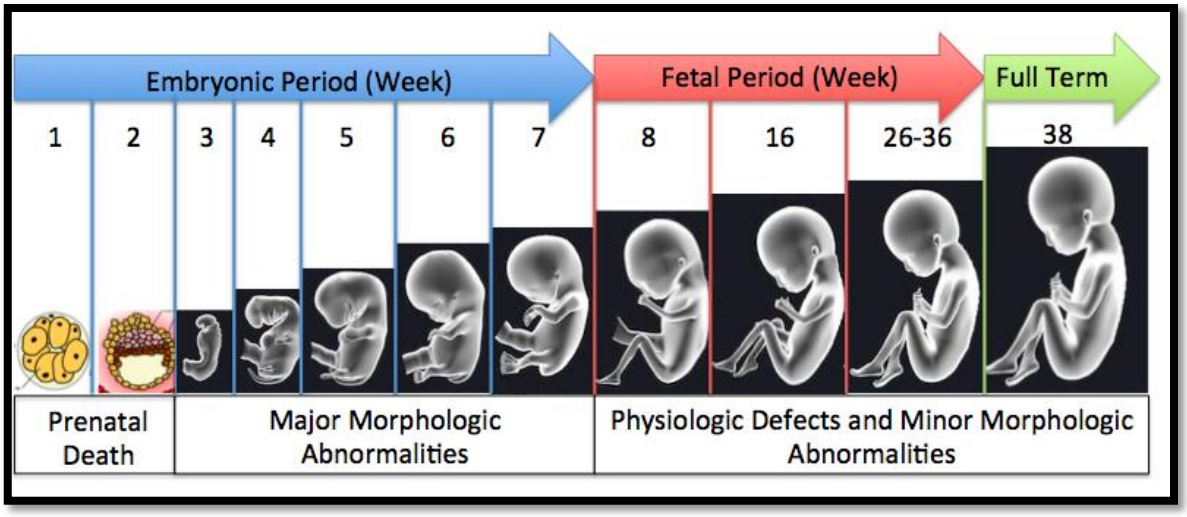

Figure 1.2. The critical period of development for various organ systems and the resultant malformation [8].

Even congenital malformations known and studied for decades, the exact cause remains unknown for many cases. Known causes of malformation can be grouped into three major categories-genetic, environmental and multifactorial (both genetic and environmental) [8,9]. In addition to genetic anomalies such as chromosomal aberration, single gene mutation, microdeletion and genetic imprinting environmental factors like smoking and

drug addiction, radiation exposure, infection and maternal disease can also affect development of major organs (Table 1.1) [10].

Table 1.1. Cause of congenital malformation [6].

Cause		**Malformation (per cent live birth)**
Genetic	Chromosomal Aberration	10-15
	Single Gene Effect	2-10
Environmental	Infection	2-3
	Maternal Disease	6-8
	Drug and Chemicals	1
	Irradiation	1
Multifactorial		20-25
Unknown		40-60

1.1.2. Diagnosis of Congenital Anomalies

From routine prenatal investigation, suspension of congenital anomaly may rise. For diagnosis, both genetic and clinic examination is necessary. Fetal visualization (ultrasound and Magnetic resonance imaging (MRI)) is the key to the detection most congenital malformations [11,12]. In addition to visualization tests, according to CA type and family history (in case of any inherited CA) genetic test might offer to mother. For decades, CA have traditionally been diagnosed by karyotyping following amniotic fluid or chorionic villi (CVS) sampling [13]. With using invasive samples and molecular techniques like linkage analysis and DNA sequencing monogenic disease can also diagnose [10]. Invasive prenatal tests continue to be the gold standard for detection of CA. On the other hand, since the sampling techniques have small albeit potentially significant risk for procedure induced miscarriage, non-invasive techniques like ultrasound examination, biomarkers detection from maternal serum and with recent technological improvement examination of fetal

DNA from maternal blood (Noninvasive prenatal test- NIPT) is tend to eliminate the need for invasive tests [14,15].

1.1.3. Treatment of Congenital Anomalies

Unfortunately for many congenital anomalies like Down syndrome and Fragile X syndrome there is no treatment that ended with full recovery. In these cases, for maximize the child development early intervention programs are offered [16]. On the other hand, for many congenital malformations, structural or functional problems can be treated with pediatric surgery [17].

Congenital malformation might affect every organ system in different level. In case of severe organ failure, organ transplantation might be needed. Although with medical and technological improvement success rate is increased for adult organ transplantation, in pediatric surgery it is still limited. One of the biggest restriction about pediatric organ transplantation is shortage of organ donors, largely because of organ size mismatch between the adult donors and the recipient [18]. Many of children die before organ become available. Even if suitable donors identified, transplantation is challenging and after surgery patient faces life-long problems caused by the risk of donor organ rejection and toxic effect of immunosuppressant [19-21].

In case of tissue losses, with surgical reconstructions and artificial prostheses affected body part can restore anatomically and functionally. Even with these methods problems caused by rejection can be overcome, they also present varying disadvantages [22]. Surgical reconstruction has problems according to donor site. For example, in intestinal tissue autograph might develop metaplasia and malignant transformation and in vein grafts that use in coronary bypass might ended with calcification and stenosis [23,24]. On the other hand, artificial prostheses are responsible for chronic irritation at the implantation site and because of limitation in plasticity especially for pediatric surgery they have limited durability [25-27].

Given the aforementioned limitations tissue engineering (TE) studies have been performed to restore damaged tissue or organ with living cells. From the first studies, tissue

engineering consists of basically three general strategies, which are: determination and isolation of cell source, tissue-inducing substances and placement of cells on matrices [28].

1.2. STEM CELLS

Stem cells refer to cells that have ability to proliferate indefinitely (self-renewal) and can differentiate into different cell types [29]. Depending on their differentiation nature, they divided into three groups; Totipotent stem cells, which can differentiate both embryonic and extra embryonic cell types, Pluripotent stem cells that have the potential to develop all derivatives of three primary germ layers and multipotent stem cells, which is able to differentiate multiple cell types of single germ layer (Figure 1.3) [30].

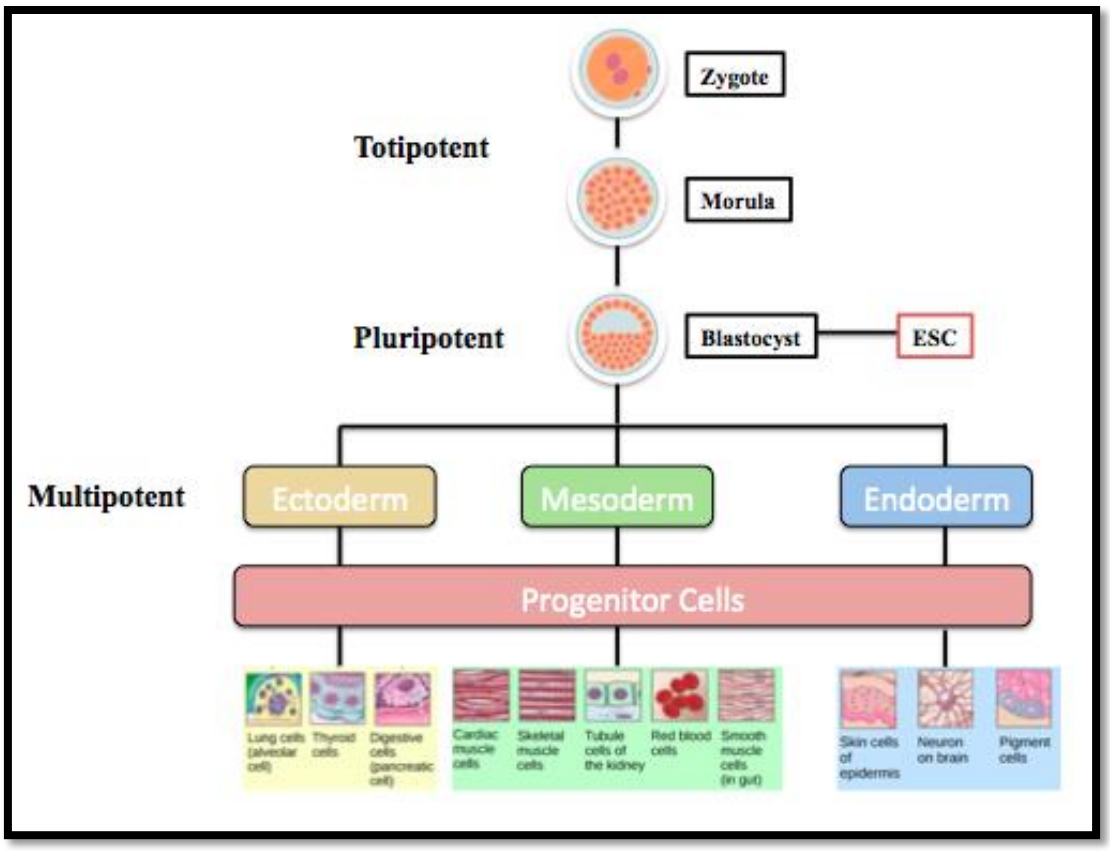

Figure 1.3. Categories of stem cells according to their proliferation potency [31].

According to their origin, stem cells further divided into two main groups. Embryonic stem (ES) cells are obtained from the inner cell mass of the blastocyst (ICM, also called embryoblast) and able to differentiate into all three germ layer cells (endoderm, ectoderm and mesoderm) [32,33]. Adult stem (AS) cells are isolated from almost all organs and tissues (niche) like skin, muscle and hematopoietic system and the ability of proliferation is high but limited when compare to ES cells and they are only multipotent [34].

1.2.1. Stem Cell Based Tissue Engineering

In tissue engineering cell source do not necessarily to be stem cell-based. However because without cellular senescence and dedifferentiation, isolation and culture of mature cells that isolated from adult tissue is difficult, stem cells are one of the main source of tissue engineering [35]. According damaged tissue and application source of stem cells may also vary (Table 1.2). Engineered tissues with stem cells are endless and range from skeletal to neural tissues [22].

Table 1.2. Comparison of Stem Cell sources [36].

Name	Source	Plasticity	Tumor Formation	Life Span (in vitro)	Ethical Issue	Clinical Trial
ES cell	Early Stage of Embryo	Pluripotent	Yes	Long	Yes	No
AS cell	Adult tissue	Multipotent	No	Short	No	Yes
AFS cell	Amniotic Fluid	Broadly Multipotent	No	Long	No	No

Embryonic stem cells have great therapeutic potential for many disorders including congenital malformation since they have unlimited self-renewal capacity and can differentiate almost all types of adult cells [31,37]. Dated from their first isolation,

differentiation of all three embryonic lineages has been performed, which are adipogenic, osteogenic, neurogenic, myogenic, hepatic and epithelial [38-41]. Even tough ES cells nature raised the interest of usage ES cell in tissue engineering studies, there is no approved treatment for clinic because of ethical and safety concerns. First of all, since isolation of ES cells require 'the instrumental use of pre-implanted embryos' [42] a large part of the society do not approve these studies. Furthermore their tendency to form teratoma [43] and possible host immune rejection since they might trigger the expression of MHC-class I antigen [44] cause potential risk for patients.

ES cell and AS cell share many properties like ability to differentiate into all three germ layers and to maintain the telomere length during cell division [45]. In addition to that, AS cells can obtain from all organ and tissue types without destruction of human embryos and do not present formation of tumor after injected *in vivo* [22,45]. Although they have some limitations like limited proliferation capacities, when compared with ES cells, given properties render adult stem cells optimum for TE studies and recent research demonstrates promising results for medical treatment [46].

Additionally amniotic fluid, chorionic villi and umbilical cord blood are another potential source for stem cell [47]. Because these samples are also used in prenatal diagnosis, they can be preferred in the treatment of congenital malformation.

1.3. AMNIOTIC FLUID

1.3.1. Function, Origin and Composition of Amniotic Fluid

Amniotic fluid (AF), which surrounds the developing fetus within the amniotic cavity is clear, aqueous body fluid formed by the amnion. The roles of AF are protect the fetus and provide mechanical support, serve as a shock-absorbing pillow and create an appropriate environment for fetus to grow and move [48,49].

The content and amount of AF changes according to fetal development and gestational age (Figure 1.4). From 2 weeks of gestation, amniotic fluid can observed and after epiblast (future embryo) and amnioblast (future amnion) separate each other, the volume starts to increase [50]. The amount of the fluid increase from 20 mL (measured in week 7) to 1000

mL (measured in week 34) during the normal pregnancy [50,51]. Since AF has important role in development, abnormalities of amniotic fluid volume, increase (Polyhydramnios) or decreased (oligohydramnions) might resulted with poor pregnancy outcome [52,53].

Amniotic fluid mainly comprise of water (98-99per cent). Additionally it contains electrolytes, carbohydrates, proteins, peptides, fats, lactate, enzymes, hormones, pigments and cells [49]. From second half of the gestation, fetal skin keratinization occurs and fetus starts to swallow and urinate the fluid, which results with major alteration in content (Figure 1.4) [54,55].

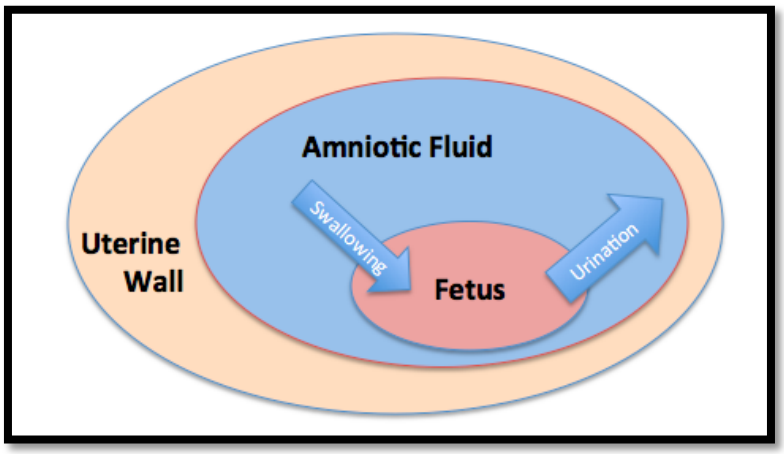

Figure 1.4. Amniotic fluid pathway [49].

Amniotic fluid (AF) cells originated from embryonic and extra-embryonic tissues and express all three germ layer cells markers [56]. Like volume and other components, AF cells are also variable according to fetal development. In 2008 Perin et al. show that level of endoderm and mesoderm markers, which were highly expressed during early gestational age was started to decrease in late gestational age. On the other hand stable ectodermal marker expression was detected in both early and late fetal development [57]. AF cells divided to three main groups according to their morphology and biochemistry, which are epithelioid-like cells (E-type, 33.7 per cent), fibroblast-like or mesenchymal cells (F-like, 5.5 per cent) and amniotic fluid specific cells (AF-like, 60.8 per cent) [58,59]. In case of

developmental abnormality, the number of these cells might affect and other type of cells can be found in amniotic fluid [60].

Amniotic fluid is valuable sample for prenatal diagnosis because cells display fetal genotype. Although amniocentesis has procedure related miscarriage risk (about 1 to 300), amniotic fluid is still gold standard for diagnosis of inherited disease and chromosomal aberrations [14,61].

1.3.2. Amniotic Fluid Stem Cell

Several studies have been performed to understand exact properties and potential of amniotic fluid cells that display stem cell properties [62,63]. According to their plasticity (capacity of self-renewal) and cell-surface antigenic profile amniotic fluid cells were categorized into two groups.

First group is the amniotic fluid mesenchymal stem (AFMS) cells, which are multipotent and can differentiate towards mesoderm-derived cells. AFMS cells firstly detected as hematopoietic progenitor cells by Torricelli and his co-workers [64]. Following studies shows that these cells can successfully differentiate to osteocytes and adipocytes [65,66]. Similarly with other mesenchymal stem (MS) cells, AFMS cells have also spindle-shaped fibroblast like morphology [58]. In addition to that, AFMS cells also share the cell-surface antigenic profile with mesenchymal stem (MS) cells. Whereas mesenchymal markers, which are CD90, CD73 and CD105 was observed in flow cytometry studies, hematopoietic and endothelial markers like, CD45, CD34, CD14, CD133 and CD31 was detected as negative [59,63]. Culture of AFMS cell studies demonstrate that, even they have high proliferation rate (higher than bone marrow-derived MS cells) AFMS cell maintain their normal karyotype [67]. From 2-5 mL of amniotic fluid, isolable amount of AFMS cells is about 0.9-1.5per cent [68].

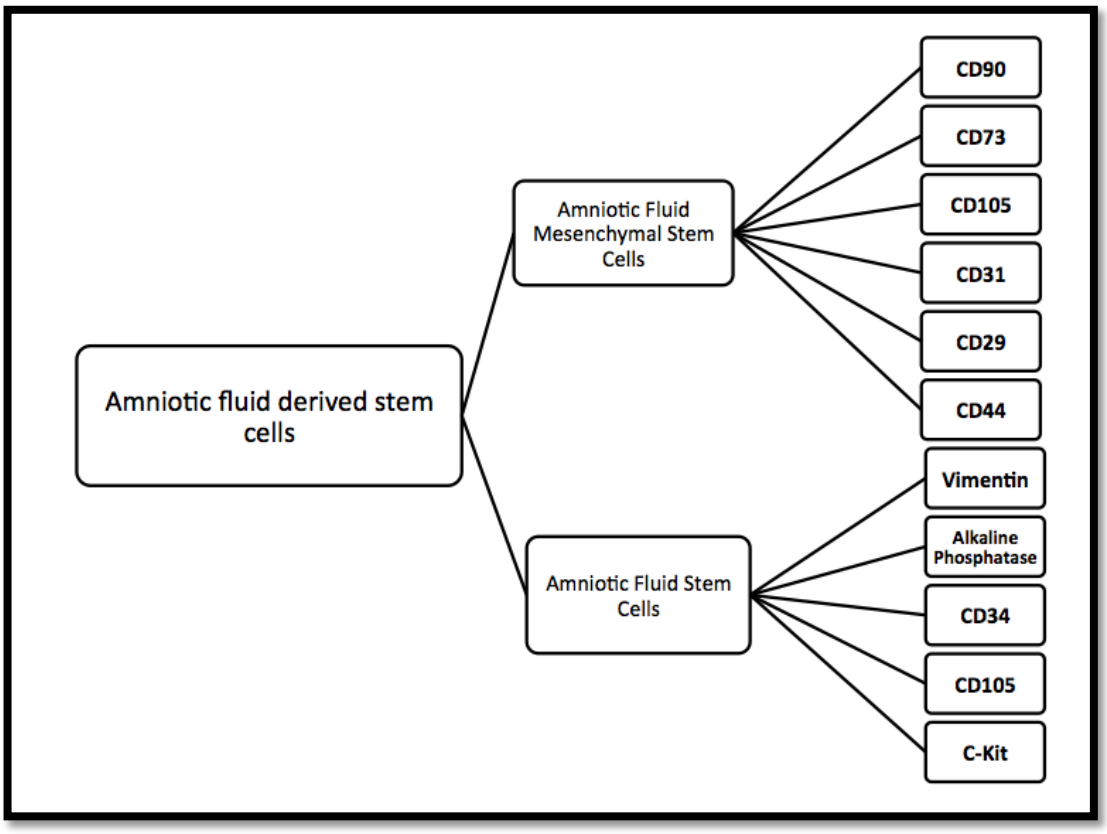

Figure 1.5. AFMS cells and AFS cells specific market proteins.

After AFMS cell detection and characterization, hypothesis of 'existence of ES cell like pluripotent cells (amniotic fluid stem cell-AFS cells) in amniotic fluid' was suggested. In 1999, first supportive data was obtained, which shown some amniotic fluid cells have telomerase activity [69]. Despite their rapid proliferation, they can preserve the telomere length between passages. First evidence for existence of AFS cells was provided by Prusa and his colleagues [70]. They demonstrated the expression of ES cell markers, Oct-4 in 0.1-0.5 per cent of total AF cells at both transcriptional and protein levels. Further studies show that in addition to Oct-4 (which has important role in differentiation and proliferation) other ES cell markers; vimentin, alkaline phosphatase, CD34, CD105 and c-Kit is also express in AFS cells [71]. Finally, AFS cells have similar differentiation potential with ES cells.

1.3.3. Amniotic Stem Cell Based Tissue Engineering

As it mentioned in stem cell based tissue engineering section, even both ES cells and AS cells are potential source for tissue engineering, have some limitations. On the other side of the spectrum amniotic fluid stem cells (both AFMS cell and AFS cells) share similar biological characteristics with ES cells and AS cells (capacity of proliferation and ability of differentiation), moreover have some alternative advantages.

First of all, isolation and culturing of AFS cells are easier and safer. Differently from ES cells, AFS cells do not have ethical concerns related to the destruction of the embryo [72]. Since amniocentesis has been performed for decades, it is well-known and safer compared with other isolation methods. AFS cells do not need feeder in culture and differently from AS cells, lower DNA damage and higher life span have been observed [46,58]. AFS cells can retain long telomeres even in late passages [62]. Secondly unlike ES cells, AFS cells do not form teratomas after transplantation in vivo [73]. Finally studies indicate that AFS cells have unique immunological profile. Flow cytometry analyses with immune-associated antigen show that even after several passages, AFS cells expressed LFA-1 (lymphocyte function-associated antigen) and MHC-1 (major histocompatibility complex-1) significantly lower [48].

Given the aforementioned advantages AFS cells have been frequently studied in TE researches (Table 1.3). When compared to other stem cell types, usage of AFS cells in pediatric cases (especially in congenital malformations) might be optimal choice [74-76]. For prenatally detectable congenital malformation, after diagnosis amniocentesis can be performed and required tissue can be obtained with induced AFS cells. In addition to congenital malformation treatment studies demonstrated that, AFS cells are also useful for treatment other congenital anomalies (severe combined immunodeficiency, congenital blood disorders) in utero [77].

Table 1.3. Recent researches of Differentiation AFS cells.

Title of Publication	Author Name	Year	Differentiation Lineage	References
In Vitro Comparison between Adipogenic Differentiation of Mesenchymal Stem Cells Derived from Human Adipose Tissue and Amniotic Fluid	El-Ghareeb et al	2016	Adipogenic	[78]
In situ vascularization of injectable fibrin/poly(ethylene glycol) hydrogels by human amniotic fluid-derived stem cells	Benavides et al.	2014	Endothelial	[79]
Simvastatin induces osteogenic differentiation in human amniotic fluid mesenchymal stem cells (AFMSC)	Janz et al	2012	Osteogenic	[80]
Human amniotic fluid stem cell injection therapy for urethral sphincter regeneration in an animal model	Kim et al	2012	Myogenic	[81]
Neurogenic differentiation of amniotic fluid stem cells	Rosner et al	2012	Neurogenic	[82]

1.4. SMOOTH MUSCLE CELLS

Smooth muscle (SM) cells play a crucial role in many tissues and organs including gastrointestinal, urinary, respiratory, cardiovascular and reproductive tracts [83]. The primary function of SM cells is contraction [83]. Basically SM cells perform all internal, involuntary movements except breathing (diaphragm) and heartbeat (cardiac muscle cells).

SM cells are originated from a large variety of embryonic tissues like, neural crest, paraxial somatic mesoderm and lateral plate mesoderm [84]. According to their morphology, proliferation and function, they divided into two groups, which are synthetic smooth muscle (SSM) cells and contractile smooth muscle (CSM) cells (Figure 1.6) [85].

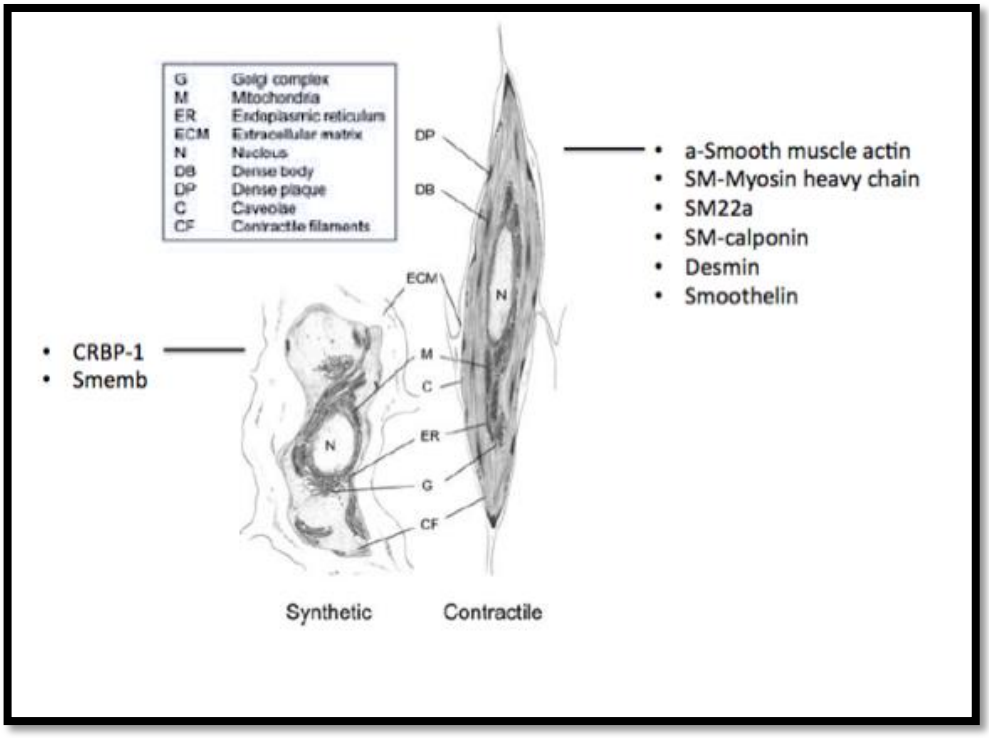

Figure 1.6. Structural characteristics and specific markers of contractile and synthetic SM cells [85].

Because SM cells play an important role in hollow organ function, they are frequently use in tissue engineering studies such as blood vessels, bladder, food borne and intestine.

Smooth muscle cells can be obtained from blood vessels and other internal tissues, but their proliferative ability is limited and may cause some complications [36]. For this reason, the production of smooth muscles by differentiation of stem cells is a promising alternative with many advantages.

1.4.1. Differentiation of Smooth Muscle Cell

From stem cells, SMC differentiation is a complex and poorly defined process. Studies indicate that various signaling pathways and molecules (retinoid receptor, TGF family, extracellular matrix, reactive oxygen species) and regulatory mechanisms (epigenetic modifications, gene transcription and translation, post transcription and posttranslational) play an important role in this process (Table 1.7) [86,87]. Interestingly, even mechanical stress can regulate SM cell differentiation [88].

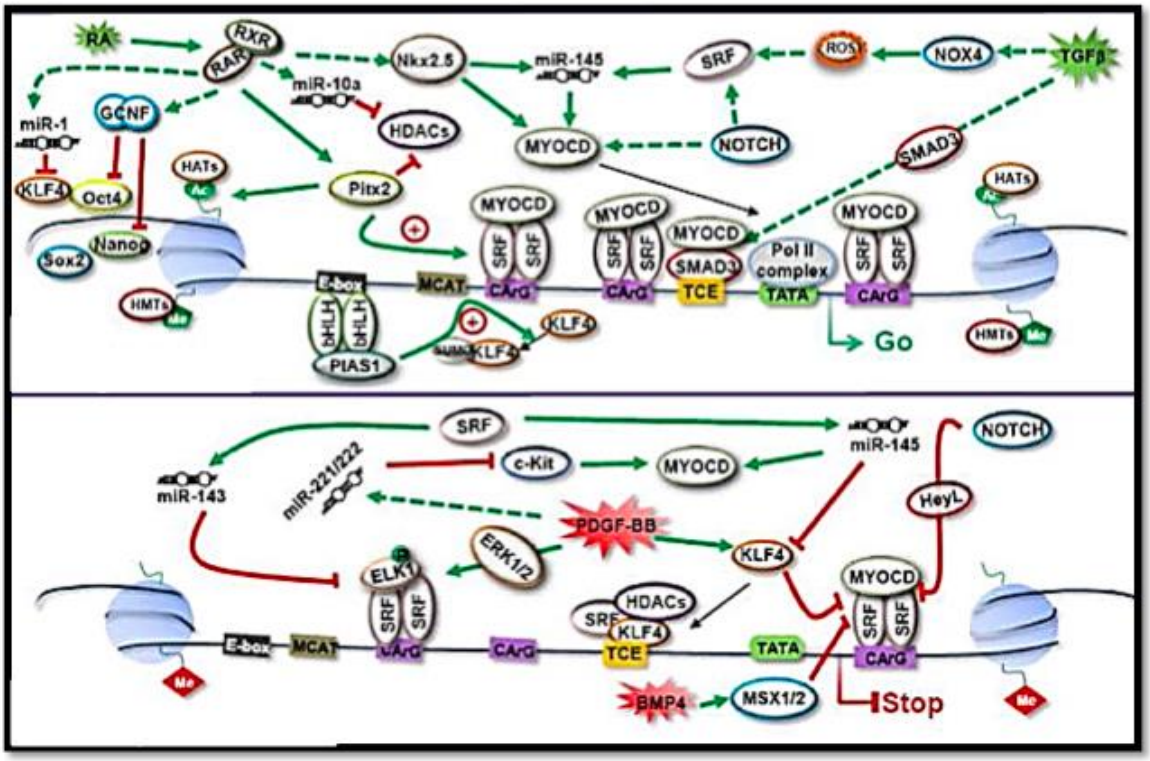

Figure 1.7. Schematic of genes involved in smooth muscle differentiation [86].

Different cell types have been used to study smooth muscle cell differentiation, including embryonic stem cells, adult stem cells and other stem cells [89-92]. Many differentiation studies tested differentiation efficiency with increasing expression of cell specific markers however for SM cells further functional tests are also necessary to show its electrophysiological and metabolic properties. Studies with bone marrow mesenchymal stem (BMS) cells demonstrated that even differentiated SM cells were highly expressed SM cell specific markers, they were failed to display contractile properties [93]. A small number of studies with AFS cells have shown that smooth muscle cell differentiation can be accomplished successfully with these cells [94].

Platelet-derived growth factor BB (PDGF-BB) and transforming growth factor beta 1 (TGF-b1) are considered as key growth factor that stimulate SMC differentiation [86]. Whereas TGF-beta is increase the expression of smooth muscle cell-specific genes by activating Notch signaling, PDGF-BB induce cell division of SM cells [95,96].

1.5. AIM OF THE STUDY

Congenital malformations are one of the important causes of neonatal death. For many cases surgical treatment can result with full recovery however it has some limitations. The biggest limitation of surgical treatment is finding suitable tissue. Recently tissue engineering studies are getting promising results to overcome this problem. Both ES and AS cells has been used in tissue engineering studies however both have some restriction. Amniotic fluid derived stem cells have many similarities with both ES cells and AS cells; in addition to that they also have some advantageous properties.

The main purpose of this study is to identify differentiation capability of AFMS cells into the smooth muscle lineage. Besides, the possible employment of rabbit as a host organism in *in-vitro* studies of amniotic fluid-related tissue engineering clinical trials was aimed to be shown.

2. MATERIALS AND CHEMICALS

2.1. INSTRUMENTS

Laminar Flow Cabinet (Thermo Holten Safe 2010 Class II Biological Safety Cabinet, USA), CO_2 incubator (Thermo Heracell 150, USA), FACs (BD FACSARIA 3, USA), inverted phase contrast light microscope (Leica Microsystems 659 135 001100, Germany), fluorescence microscope (Olympus BX61, Japan), confocal microscope (Zeiss LSM-700), Scanning Electron Microscope (Carl ZEISS, Germany), sputter coater (Baltec, USA), Real-Time PCR Detection System (Roche Light Cycler 480 II, Switzerland), Thermal Cycler (Bio-Rad My Cycler, USA), centrifuge (Hettich Rotofix 32A, Germany), pH meter (InoLab WTW series, Germany), microbalances (Denver Instrument Timberline Balances, USA), vortex (IKA MS3, Germany), mini centrifuge (Labnet C1301B, Canada), Nano photometer (Impleni, Germany), LS 6000 liquid nitrogen storage (Taylor-Wharton, USA), -80^{o}C freezer (Thermo Scientific Forma 906, USA), -20^{o}C freezer (Arçelik 2041D, Turkey), heater (DRI-Block DB.2A), water bath (Memmert WNB14, Germany), autoclave (HICLAVE HG 80, Germany).

2.2. EQUIPMENTS

T-25, T-75 and T175 cell culture flasks (Sarstedt, Germany), 6, 12, 24, 96 multi-well cull culture plates (Corning, USA), serological pipettes (25 ml, 10 ml, 5 ml) (Sarstedt, Germany), micro pipettes 1000 µl, 200 µl, 100 µl, 10 µl, 2.5 µl (Labcon, USA), 50 mL and 15 mL centrifuge tubes (Isolab, Germany), 2 mL and 1.5 mL microcentrifuge tubes (Isolab, Germany), 0.5 mL PCR tube (Axygen, USA), 0.22 um, 0.45 um Syringe Filter (Corning, USA), 10mL, 20mL and 50 mL Sterile Syringe (Hayat, Turkey), cryovials (Corning, USA), BrightLine hemacytometer (Marienfeld, Germany), microscope slides (Thermo Scientific, USA), cover glass (Isolab, Germany), 96-well PCR plate and sealing film (Axygen, USA).

2.3. CHEMICALS

Dulbecco's Modified Eagle's Medium with high glucose (Sigma, USA), Kaighn's Modification of HAM's F-12 with L-Glutamine (F12-K) (ATCC, USA), RPMI Medium 1640 (1X) (Gibco, UK), AmnioMAX™ - II (Gibco, UK), AmcelGrow® Medium (Empire Genetics, USA), Fetal Bovine Serum–cell culture tested (Gibco, UK), Penicillin-Streptomycin (Gibco, UK), Dulbecco's Phosphate Buffered Saline (Gibco, United Kingdom), 0.05per cent Trypsin-EDTA (1X) solution (Gibco, United Kingdom), Dimethyl Sulfoxide (Sigma, USA), Trypan Blue Stain (0.4per cent) (Lonza, USA), Recombinant Human PDGF-BB (Invitrogen, USA), TGF B1 Recombinant Human (Invitrogen, USA), DAPI (BioLegend, US), Rat Monoclonal CD90 antibody (BioLegend, US), Integrin B1 Antibody, FITCH (Millipore, USA), Rat Monoclonal CD44 antibody (BioLegend, US), Collagen (rat tail) (Roche, Switzerland), Carbocol (Millipore, USA), Potassium Chloride (Merck, USA), Agarose (Sigma, USA), Gelatin (Sigma, USA), bovine serum albumin (Santa Cruz, USA), Anti-alpha smooth muscle actin (Abcam,UK), Mouse anti-smooth muscle myosin heavy chain (Millipore,USA), Goat-anti mouse (Santa Cruz, USA), Phalloidin (Thermo Fisher, USA), Triton-X-100 (Merck, Germany), paraformaldehyde (Merck, Germany), Tris Acetate-EDTA buffer 10X (Sigma USA), Acetic Acid (Merck, USA), Flou-4, AM (Thermo Fisher Scientific, USA), Pluronic F-127 powder (Sigma, USA), Poly(D,L-lactide-co-glycolide) (Sigma, USA), Hexamethyldisilazane (Aldrich, USA), n-Hexane (VWR, UK), Cyclohexane (Millipore, USA), 1,4-Dioxane (Merck, USA), Glutardialdehyde (Merck, USA), Cacodylic Acid Sodium Salt Trihydrate (Santa Cruz, USA), D(+)-Sucrose (Carlo Erba, France), Sterile Water Molecular Grade (Wisent Inc., Canada), Ethanol (J. T. Beaker, USA).

2.4. KITS AND SOLUTIONS

TRIzol Reagent (Thermo Fisher Scientific, USA), M-MLV Reverse Transcriptase cDNA Synthesis (Thermo Fisher, USA), 3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS assay) (Promega, UK), TaqMan Universal PCR Master Mix (Applied Biosystems, USA), TaqMan Gene Expression Assay, ACTA2, OC03399251-M1 (Applied Biosystems, USA), TaqMan Gene Expression Assay,

MHY11, OC03397925-M1 (Applied Biosystems, USA), TaqMan Gene Expression Assay, DES, OC04252345-M1 (Applied Biosystems, USA), TaqMan Gene Expression Assay, GAPDH, OC03823402-G1 (Applied Biosystems, USA).

3. METHODS

3.1. SAMPLE COLLECTION AND PREPARATION

In this study, collaboration was done with Gaziosmanpaşa University. The Ethical Committee of the University Gaziosmanpaşa Health Science Center in 05/09/2013 approved this protocol.

Pregnant rabbits on day 14-20 of gestation were used. After breeding female rabbits transferred to separate cage and check up every day. On day 14-20 of gestation, animals were anesthetized and amniocentesis was carried out under continuous ultrasound control. After this procedure, amniotic fluids were transferred to Yeditepe University under appropriate conditions. Each of female rabbit survived after procedure.

3.2. RABBIT AMNIOTIC CELL CULTURE

Centrifuge was used for separation amniotic fluid cells from amniotic fluid. Basically samples were centrifuged at 1000 rpm for 10 minutes. After centrifugation supernatant was discarded and the obtained cell pellet was suspended in a fresh medium.

Five different mediums (Table 3.1) were used for medium optimization. After centrifugation, pellets were re-suspended with each of medium separately, seeded to cell culture flask and carried in humidity incubator at 37 °C and 5 per cent CO_2. Three days later, medium change was performed to discard non-adherent cells and debris. Cell culture flask was routinely control every day. In the end of first week AmnioMAX was detected as optimum medium for rabbit amniotic fluid cell culture.

When the confluency reaches 80 per cent, cell subculture was performed. The spent cell culture medium was removed and discarded. Cells were washed with 0.05 per cent Trypsin-EDTA solution. After washing, pre-warmed Trypsin-EDTA solution was added to cell culture flask and flask was gently rocked to get complete coverage of the cell layer. Culture vessel was incubated for approximately 2 minutes at 37 °C. Cells were observed

under inverted phase contrast light microscope for detachment. Fresh medium was added immediately after detachment to neutralize Trypsin-EDTA solution activity. Cells were transferred to 15 mL centrifuge tube and centrifuged at 1000 rpm for 10 minutes. Supernatant was discarded and cell pellet was re-suspended with pre-warmed fresh medium. If necessary, cell counting can be performed with using hemocytometer in this stage.

Table 3.1. List of medium used in RA cell culture optimization

Name of medium	**Supplemented with**
AmnioMAX™-II	1 per cent PS
AmnioGrowth	1 per cent PS
DMEM	1 per cent PS +10 per cent FBS
RPMI	1 per cent PS +10 per cent FBS
HAM's F-12	1 per cent PS +10 per cent FBS

To prevent loss of stem cell characterization and random differentiation, cells were freeze before the passage number of 7. For freezing, cells were gently detached from tissue culture vessel with procedure followed in subculture. After centrifugation cells were re-suspended with freezing medium that contain 95 per cent medium and 5 per cent dimethylsulfoxide (DMSO) at a concentration of $1*10^6$ cells/ml. Aliquots of the cell suspension were dispensed into cryogenic storage vials. Cryogenic vials were placed to Mr. Frosty and transferred to -80°C freezer. For long-term storage, cells were placed to liquid nitrogen.

After freezing, to understand the efficiency cells were thawed and Trypan Blue viability test was applied in first four month. Basically cells were agitated for 2 minutes in 37 °C water bath. After thawing, pre-warmed growth medium was immediately added to cryogenic vials to dilute DMSO. Cells were centrifuged at 1000 rpm for 10 minutes and cell pellet was re-suspended with pre-warmed fresh medium. Trypan Blue viability test was applied according to manufacturer recommendation. Before usage, Trypan Blue solution was filtered with 0.45 um Syringe Filter to get rid of the particles in solution.

Samples were diluted with filtered Trypan Blue dye in 1:1 ratio and incubated in room temperature for 2 minutes. After incubation, cells were transferred to hemocytometer and viable and non-viable cells were counted.

3.3. FLOW CYTOMETRY ANALYSIS

For characterization and isolation of RAFMS cells from AF cells, which were passage 4-6 flow cytometry analyses was performed with cell surface markers that described in literature [59]. Cells were detached with 0.5 per cent Trypsin-EDTA solution as describe in 4.2 Cell Culture section. Pellets were washed with Ca^{++} and Mg^{++} free PBS, counted and centrifuged at 1200 rpm for 5 minutes. Samples were dissolved in 500 µL Ca^{++} and Mg^{++} free PBS and separated into two one was labeled as 'Stained' and other one was 'Unstained' (control group) to avoid any dye overlap. Proper amount of antibodies, which were detected according to the number of cells, was added to stained group and both groups were incubated for 30 minutes on ice. After incubation, samples were washed with Ca^{++} and Mg^{++} free PBS to remove the free antibodies. Pellets were re-suspended with prepared Ca^{++} and Mg^{++} free PBS, 2 per cent FBS and DAPI mixture, which decrease the harmful effect of procedure.

Table 3.2. List of Antibodies used in Flow Cytometry Analysis

Name	Related Protein	Conjugated Dye
CD90	Thy-1	PE-CY7
CD44	Hyaluronic acid receptor	PE
CD29	Integrin Beta-1	FITC

Samples were analyzed with FACS Aria III. For eliminate non-viable cells DAPI solution was used. After characterization two sub-populations, which are $CD90^{+}CD44^{+}CD29^{+}$ and $CD90^{-}CD44^{-}CD29^{-}$ were separated and sorted.

3.4. DIFFERENTIATION OF RABBIT AMNIOTIC FLUID MESENCHYMAL STEM CELLS

As it mentioned in Theory Section 1.4.2, there are four different mechanisms play important role in smooth muscle cell differentiation. In this present study, extracellular signaling mechanism was used with growth factors; platelet-derived growth factor BB (PDGF-BB) and transforming growth factor β1 (TGF-β1).

Isolated RAFMS cells ($CD90^{+}CD44^{+}CD29^{+}$) were divided into two (control and experimental group), seeded onto gelatin coated cell culture vessels and cultured in growth medium until cells adhered was observed. In order to SMC differentiation, growth medium was discarded and experimental group cells were cultured with differentiation medium (high-glucose DMEM containing 15 per cent FBS, and 1 per cent penicillin/streptomycin supplemented with 5 ng/ml PDGF-BB and 2.5 ng/ml TGF-β1) for about 3 weeks. Cells were controlled daily after differentiation medium was given and every 48 hours medium was changed (Same procedures was also applied to control group).

3.5. CHARACTERIZATION OF DIFFERENTIATED SMOOTH MUSCLE CELL

In this study morphologic, molecular and functional properties of differentiated SMC from RAFMS cell was tested using a combined characterization analysis.

3.5.1. Morphological Examination

During differentiation to show morphological change, images were taken with digital camera integrated to inverted phase contrast light microscope in specific days (7^{th}, 14^{th} and 21^{th} days of differentiation).

3.5.2. Cell Viability Assay

To determine the effect of differentiation on growth, cell viability assay was performed in every day of first week and on 14^{th} day of differentiation to both control and experimental

group. RAFMS cells were seeded to 24-well plate (for first 7 days) and T75 culture flask (for 14th day) and cultured with differentiation medium at 37 °C and 5 per cent CO_2. Before MTS (3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium) treatment, medium was discarded and cells were washed with PBS. MTS reagent was added to each well and samples were incubated for 1 hour at 37 °C. Color change, caused by degraded tetrazolium salt was observed after incubation. To measure the amount of soluble formazan, supernatant was transferred to 96-well plate and absorbance of cells was measured at 490 nm (nanometer).

3.5.3. Gene Expression Analysis

In order to characterize differentiated SM cells, expression level of SM specific genes (Smooth Muscle Actin (ACTA2), Myosin Heavy Chain 11 (MHY11), Desmin (DES)) was analyzed with Real-time PCR.

Table 3.3. Real-Time PCR primers

Gene	Sequence	Amplicon (bp)
ACTA2	OC03399251-M1	86
MHY11	OC03397925-M1	85
DES	OC04252345-M1	96
GAPDH	OC03823402-G1	82

Total RNA isolation was performed to collected samples in day 7, 14 and 21 of differentiation with using Trizol reagent. Briefly, samples were re-suspended in 900 µL of Trizol solution and incubated at room temperature for 5 minutes. 200 µL of chloroform was added to each sample for homogenization, tubes were immediately shaken by hand for 15 second and incubated for 3 minutes in room temperature. Incubated samples were centrifuged at 12000 g for 15 minutes at 4^{o}C. In the end of the centrifuge, the mixture separated into three phases, which were lower red phenol-chloroform phase, intermediate phase and colorless upper aqueous phase. Aqueous phases that contain RNA were

transferred to new tube and 500 µL of chloroform was added. Samples were incubated at room temperature for 10 minutes and centrifuged at 12000 g for 10 minutes at 4^{o}C. Supernatants were removed and pellets were washed with 1 mL of 75 per cent ethanol. Samples were centrifuged at 7500 g for 5 minutes at 4^{o}C. Ethanol was discarded and samples were re-suspended with Nuclease-free water. To solve RNA, samples were incubated at 65^{o}C for 10 minutes and sample quality was measured with nanodrop.

Table 3.4. Reaction mixture-1 for M-MLV transcription

Component	**Amount**
10 mM dNTP mix	1 µL
Oligo $(dT)_{23}$	1 µL
RNA template	Variable (500-1000 µg)
Nuclease-Free Water	Variable
Total volume	10 µL

Complementary DNA (cDNA) synthesis was performed with M-MLV Reverse Transcriptase Kit according to protocol described. RNA samples were diluted and concentration was adjusted. For each RNA sample, master mixes (described in Table 3.4) were prepared.

Table 3.5. Reaction mixture-2 for M-MLV transcription

Component	**Amount**
10X M-MLV Reverse Transcriptase Buffer	2 µL
M-MLV Reverse Transcriptase	1 µL
Nuclease-Free Water	7 µL
Total volume	10 µL

Samples were mixed gently and incubated at 70^{o}C for 10 minutes. After incubation, following mixture was prepared and added to each tube (Table 3.5) and samples were incubated at room temperature for 10 minutes to ensure elongation of Oligo (dT) $_{23}$ primers before the higher reverse transcriptase temperature. Samples were placed in the Thermal Cycler and the thermal cycling protocol given in Table 3.6 was set and run.

Table 3.6. Thermal cycling protocol of the cDNA synthesis with M-MLV transcription kit

Temperature	**Duration**
37^{o}C	50 min
87^{o}C	10 min
4^{o}C	2 min
4^{o}C	∞

Real-time PCR was performed with TaqMan Universal PCR kit following the manufacturer's instruction. For each sample master mix was prepared containing 5 µL TaqMan Universal PCR master mix, 2,5 µL dH_2O and 0,5 µL primer and dispensed to 96-well plate. Later on, 4 µL template was mixed with master mix and 96-well plate was centrifuged in 1500 g for 1 minute to collect the reaction mixture in the well bottom. Plate was loaded to the instrument rack and Thermal cycling program (Table 3.7) was started. Each experiment was repeated in duplicate and GAPDH was used for normalization.

Table 3.7. Thermal cycling program for Real-Time PCR

Step		**Temp.**	**Duration**
UNG Stabilization		60 ^{o}C	1 min
Initial Denaturation		95^{o}C	10 min
Denaturation	**x45 cycles**	95^{o}C	20 sec
Annealing		62^{o}C	20 sec
Extension		72^{o}C	78 sec

Final Extension	72^{o}C	3 min

3.5.4. Immunofluorescence

Immunocytochemistry analysis was performed to show the changing amount and localization of SM cell specific proteins (Smooth Muscle Actin and Myosin Heavy Chain 11) after differentiation as described previously . For this purpose, two groups of cells were analyzed, which were cells in 7th day of differentiation and cells in 21th day of differentiation (with their own control).

Cells were detached as describe in section 4.2 and counted with hemocytometer. Cells were seeded to the gelatin coated cover slip at a density of 75x10^{3} and incubated at 37 ° C for 2 days. After incubation the media were discarded and coverslips were washed with cold PBS. For fixation, 2 per cent freshly prepared paraformaldehyde (PFA) in PBS solution was added to the each well and incubated in room temperature for 30 minutes on shaker. PFA was used instead of methanol or acetone because it results with better preservation of cellular morphology. The PFA solution was discarded and the fixed cells were washed with PBS for three times. Cells were permeabilized with non-ionic detergent solution (Triton X). 1 mL of 0.2 per cent Triton X solution was added to the wells and incubated for 30 minutes in room temperature. For blocking, cells were incubated with PHEM, which was prepared with 5 per cent goat serum, 10 mM Pipes, 25 mM Hepes, 10 mM EGTA and 2 mM $MgCl_2$ for 1 hour in room temperature. Diluted primary antibodies (Table 3.8) were dropped onto coverslips. Coverslips were covered with Paraffin that was cut in coverslip size and incubated at 4^{o}C for overnight on shaker.

Table 3.8. List of Antibodies used in Immunocytochemistry and their dilution rate

Name	Dilution Rate
Anti-alpha smooth muscle actin	1:100
Mouse anti-smooth muscle myosin heavy chain	1:100
Goat-anti mouse	1:200
Phalloidin	1:1000

After incubation, paraffin was discarded and coverslips were washed with PBS 3 times to remove non-binding primary antibodies. Diluted secondary antibody and diluted Phalloidin dye (Table 3.8) were dropped onto coverslips. Phalloidin, which stains the F-actin was used to visualize cell cytoskeleton. Coverslips were covered with Paraffin and incubated at room temperature for 1 hour in dark. Samples were washed with PBS 3 times and placed face down onto a slide. Samples were analyzed and captured with confocal microscopy.

3.5.5. Collagen Gel Contraction Assay

The main function of SM cell is contraction and relaxation. For measure contractile force in differentiated SM cells, collagen gel contractile assay was used as described previously [97, 98]. For this study, cells in 21^{th} day of differentiation and their control were used.

0.2 per cent acetic acid was prepared, filtered and cooled at 4 °C. For preparation 3mg/mL of Collagen type I solution, 0.2 per cent acetic acid was added to the tubes and mixture was shaken at 4 °C for overnight. Proper amount of 1 M sodium hydroxide (NaOH) that use for solidify the collagen mixture was determined according to NaOH titration.

Cells were detached from the culture vessels as it described in section 3.2. After centrifugation, cells were washed with PBS to remove remaining trypsin. Cells were suspended with complete culture medium and counted with hemocytometer. Cell suspension, in which concentration of cells was adjusted $1.5*10^5$ cells /mL was prepared. 0.4 mL of cell suspension was mixed with 0.2 mL of collagen solution on ice. Appropriate amount of 1 M NaOH was added to the solution and mixed well with pipet. 500 µL of solution was immediately transfer to 12 well-plate and incubate at room temperature for 20 minutes to polymerize the collagen gel. After incubation gel was disconnected from the well corner with 200 µL pipet tip to allow the contraction of gel. Before start the initial diameter was noted. To trigger the contraction and relaxation, complete medium with 1 mM carbocol, with 60 mM potassium chloride (KCl) and complete medium without any agonist was given to well separately. The 12 well-plate was placed into incubator and diameter changes were recorded in several time points (0, 2h, 4h, 8h and 16h).

3.5.6. Calcium Signaling Measurement with Flou-4 Staining

Calcium (Ca^{+2}) stimulate muscle contraction and has important role in regulation of metabolism and gene expression in smooth muscle cells [99]. Because of this reason, after contractile assay, amount of free Ca^{+2} was also measured with Flou-4 (labeled calcium indicator) in differentiated SM cells. For this study, cells in 21^{th} day of differentiation and their control were used.

Flou-4 solution was prepared according to manufacturer recommendation. Flou-4 powder was dissolved with 5 mM DMSO solution and mixed with 20 per cent Pluronic F-127 in DMSO in 1:1 ratio. Pluronic F-127, which is a non-ionic detergent allows the nonpolar Flou-4 to dissolve homogeneously in the media. To decrease the toxic effect of DMSO, mixture was diluted to a final concentration of 5 μM of DMSO with medium.

Cells were detached with heated 0.5 per cent trypsin, counted and seeded to 24 well-plate, which was coated with gelatin. After attachment was observed, medium was discarded and cells were washed with PBS to remove remaining medium. 500 μL of diluted Flou-4 mixture was added to each well and cells were incubated for 1 hours at 37°C. Before fluorescence measurement, cells were washed with indicator free medium to remove the dye that nonspecifically associated with cell surface and further incubated for 30 minutes with complete medium supplemented with 1 mM carbocol and with 60 mM potassium chloride. The results were examined by fluorescence microscopy.

3.6. TISSUE ENGINEERING

3.6.1. Fabrication of PLGA Scaffolds

3.6.1.1. Preparation of Paraffin Spheres

PLGA scaffold was prepared using solvent casting/particulate leaching method. In order to create interconnected pore structure, paraffin spheres was prepared as it described previously [100]. Basically, 20 g of paraffin wax was added in 100 mL of 8 per cent gelatin solution at 80 °C. Emulsion was stirred until homogenous solution was obtained and in that point it was immediately poured into ice-cold water to solidify paraffin spheres.

Spheres were washed twice with distilled water and dried under vacuum. Pore diameters were determined for AFMS cells according to literature and to obtained suitable paraffin spheres, dried paraffin were passed through 212-425 μm sieves.

3.6.1.2. Fabrication and Characterization of Porous PLGA Scaffold

Poly (lactide-co-glycolide) (PLGA) polymer was chosen for this study because even it's hydrophobic property, it is approved by FDA and also suitable for SM cell tissue engineering studies.

Selected paraffin spheres were placed into Teflon mold and to obtain pore interconnectivity, it was heated at 45 °C for 30 minutes. Spheres were cooled in room temperature and 1 ml of 12 per cent PLGA/1,4-dioxane solution was added onto paraffin spheres by drop-wise. The system was frozen at – 25 °C and followed by freeze drying to remove 1,4-dioxane. To leach the paraffin assembly, the PLGA porous scaffold was incubated with 400 mL of hexane for two days. The hexane was refreshed every 6 hours. Since hexane is highly toxic for cells, to remove hexane completely in the end of leaching step hexane was exchanged with cyclohexane and further incubated at room temperature for 6 hours. After incubation, cyclohexane was discarded and scaffold was frozen. Samples were freeze-dired to remove remaining cyclohexane. Until the experiment day, scaffolds were stored in liquid nitrogen.

Scanning Electron Microscopy (SEM) was used to characterize the pore morphology and perform microstructure evaluation of the scaffold. Samples were sputter-coated with gold about 15 nm and the cross-sectional surface of scaffold was examined with Arl ZEISS scanning electron microscope. To measure and examine varying pore size of the PLGA scaffold, images were taken from different section of the sample.

3.6.2. Cell Planting and Post-Plant Control

PLGA scaffolds needs to sterilize before cell seeding because in fabrication procedure freeze dry part could not performed under sterile conditions. As it mentioned before PLGA is hydrophobic. Sterilization strategy was changed because of this property. In the beginning, samples was sterilized with UV. However even scaffolds were treated with

medium for 20 minutes to make them hydrophilic, after cell seeding scaffolds did not immerse. After this observation, alcohol sterilization was performed for PLGA scaffolds to increase the time of exposure to liquid. Basically, 70 per cent ethanol was added to the 6 wells, in which scaffolds were placed before and incubated in room temperature for 45 minutes. Ethanol was discarded, evaporated and washed with PBS 3 times in order to remove remaining alcohol (in each washing, samples were incubated for 30 minutes). After washing, scaffolds were treated with medium for 2 hours in room temperature (during this time the polymer was checked for 15 minutes).

Cells were detached from the culture vessels as it described in section 3.2. Cells were suspended with complete culture medium and counted with hemocytometer. $2*10^4$ RAFMS cells in 30 μL medium were added onto the PLGA scaffolds by drop-wise, which was placed agarose coated 6-well to inhibit cell attachment to any surface rather than scaffold. Samples were incubated for 3 hours in 37°C to allow cell attachment. After initial incubation, 500 μL of medium was added to each well and samples were incubated at 37 °C and 5 per cent CO_2 in a humidified incubator for 5 days.

Morphology of RAFMS cells seeded on the scaffolds were examined with SEM after 5 days. Briefly, after medium was discarded, samples were washed with PBS 3 times. Scaffolds were incubated in room temperature for 45 minutes in dark with fixation solution, which contains 3 per cent glutaraldehyde, 0.1 M sodium cacodylate and 0.1 M sucrose. After incubation, samples were dehydrated using an ethanol-graded series (30, 50, 70, 80, 90, 95, and 100 per cent). To remove liquid completely, samples were treated with Hexamethyldisilazane (HMDS) for 20 minutes twice. Finally samples were dried in vacuum dryer and prepared to SEM examination as it described previous section. SEM images were obtained by using SEM Zeiss EVO 40.

4. RESULTS AND DISCUSSION

Congenital malformations are one of the important causes of neonatal death and frequency of CM is about 3-5 per cent in newborn infants [5]. Treatment strategies like organ transplantation and surgical reconstructions are still being used despite their disadvantages like immune rejection. On the other side tissue engineering studies have promising results [76, 101]. Especially in cases of smooth muscle tissue defects, which are commonly diagnosed prenatal malformations tissue engineering could be alternative treatment strategy.

Large number of smooth muscle cell is necessary for generation fully functional smooth muscle tissue. Up to now, differentiation studies along SM cell have been performed with both ES and AS cells. However even ES cell studies have promising results, because of ethical and safety concerns, there is no approved clinical treatment [43,42]. AFS cells do not form teratomas and since amniocentesis performed in prenatal diagnosis for decades with low risk there is no ethical regulation [56]. Especially for pediatric malformation AFS cells, have more advantageous than other stem cell types [36].

As it mentioned in Section 1.3.2, two different types of stem cell have been identified in amniotic fluid. Differently form AFMS cells, AFS cells represent a rare population in the total amniotic fluid cells and number of cells decrease to undetectable level in the third trimester [71, 102]. Generation of fully functional SM cells from AFS cells have been recently reported by Ghionzoli et al. [94]. On the other hand, even expression of galectin-1, which regulate smooth muscle cell differentiation was detected in AFMS cells [68], so far SMC differentiation from AFSM related study is not available.

As a model, in differentiated smooth muscle related tissue engineering studies, sheep and rat have been used [81,103]. However using sheep is unfavorable because of financial and logistical burden and rat is not suitable for transplantation of smooth muscle tissue defects like bladder exstrophy. In this present study we aimed to perform SM cell differentiation via rabbit AFMS cells and create model animal option that can be used for clinical trials.

4.1. ISOLATION AND CULTURE OF RABBIT AMNIOTIC FLUID CELLS

Since amniotic fluid stem cells are recently identified and have heterogeneous cell population, there is no standard method for isolation and culture [56]. So far 4 different approaches have been reported, which can be distinguished into (a) single-step cultivation, in which primary cell culture is left undisturbed for 7 days [104,105]; (b) two-step cultivation; in this protocol after 5 days in primary cell culture, non-adherent cells are collected and cultured separately [106]; (c) selective isolation, in which with stem cell specific marker AFS cells are sorted before culture [107,108] and (d) starter cell cultivation; in this protocol after 3 days in primary cell culture, non-adherent cells and debris are discarded [109]. Tsai and his colleagues shown that stem cells isolated with two-step cell culture method shares many properties with AFMS cells like morphology and cell surface markers (CD29, CD44, CD90 and CD105 (+)) rather than AFS cells (CD117(-)) [106]. Additionally with this method isolation of stem cells can perform without interfering diagnostic studies.

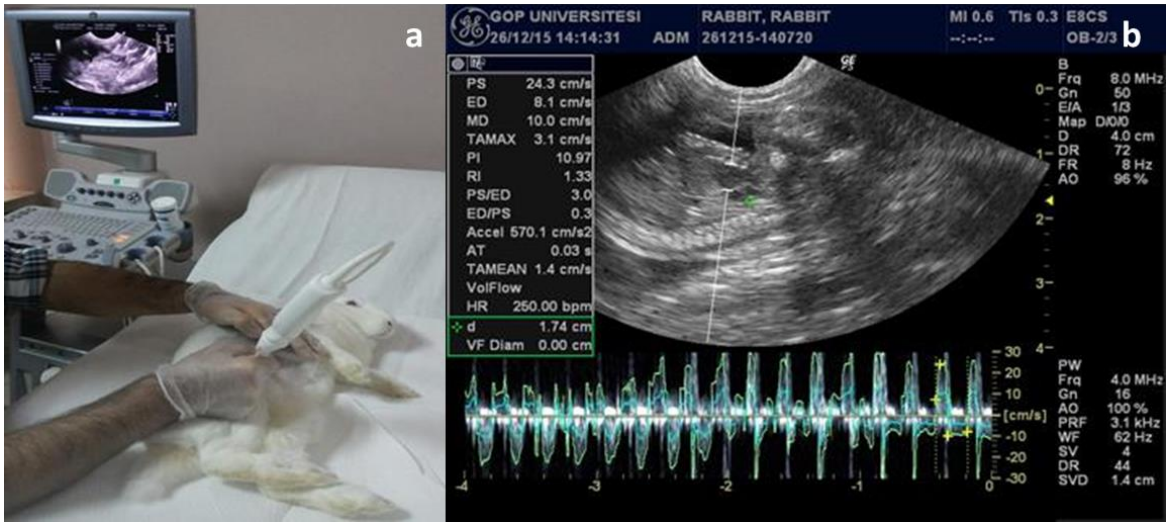

Figure 4.1 Amniocentesis under ultrasound control.

In this present study, two-step cultivation method was used because of mentioned advantages and sample limitation. To not waste our limited samples, non-adherent cells were collected and re-cultured after 5 days of primary cell culture (Figure 4.1). Since for

this study cells did not used in diagnostic test and preliminary data shown no distinguish differences between sorted primary and non-adherent stem cells, stem cells were mixed.

4.2. CHARACTERIZATION AND ISOLATION OF RABBIT AMNIOTIC FLUID MESENCYMAL STEM CELLS

Amniotic fluid mesenchymal stem cells characterization was carried out with CD90 (THY-1), CD44 (hyaluronate receptor) and CD29 (integrin beta-1) antibodies, which are classified as mesenchymal stem cell markers [59,63]. Using DAPI staining, dead cells (DAPI +) and living cells (DAPI-) were separated from each other. With P1 harvesting, alive cells were selected and characterization studies were continued with these cells. Mesenchymal stem cell specific markers expression was detected by FACSAria III device in sub-population of RAF cells as 8.7 per cent CD90, 2.4 per cent CD44 and 1,2 per cent CD29 (Figure 4.2).

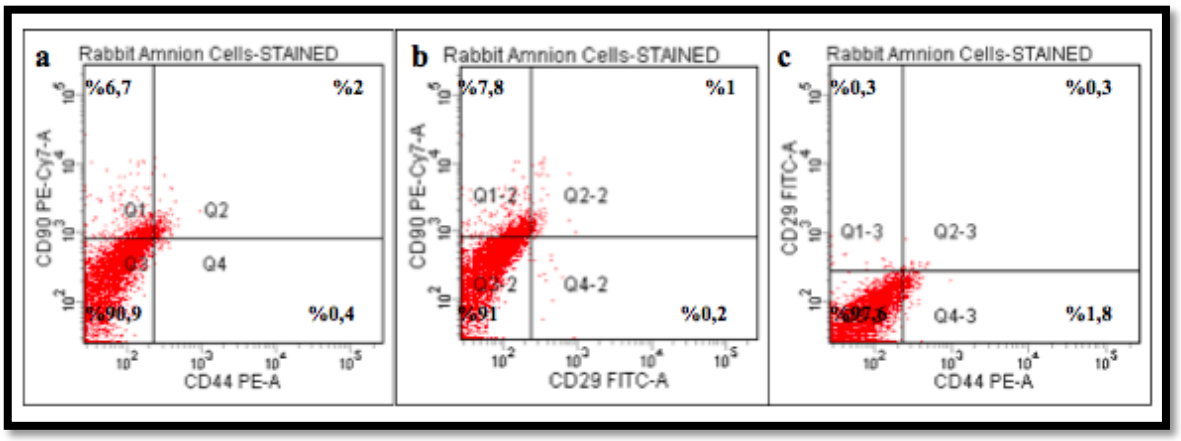

Figure 4.2. Stem cell characterization by mesenchymal stromal markers. (a) CD90 and CD44; (b) CD90 and CD29; (c) CD29 and CD44.

Flow cytometry analysis using PE-CY7 conjugated CD90, PI conjugated CD44 and FITC and conjugated CD29 antibodies revealed 0.3 per cent of the sub-population carrying CD90, CD44 and CD29 surface receptors in amniotic fluid cells (Fig. 4.3).

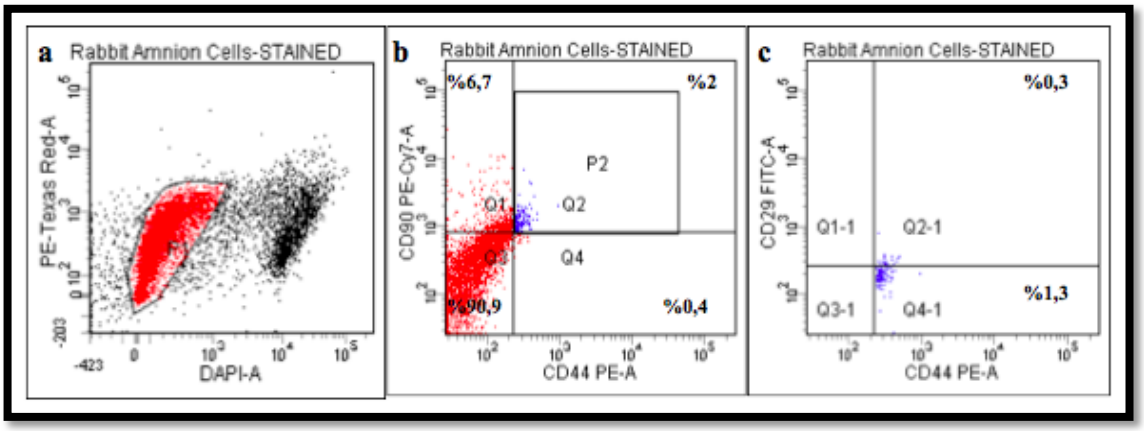

Figure 4.3. Live cells was selected with P1 gate and 0.3 per cent of the population was identified as AFMS cells. (a) DAPI staining for selection dead cells (b) selection of CD90 + CD44 + subpopulation with P2 gate (c) CD29 + CD44 + CD29 + cells total population were identified by specifying CD29 + cells in the subset of this cell.

In the second flow study, not significant but higher expression of MS cell markers was detected (Figure 4.4). RAFMS cells express MS cell markers like CD90 (13.6 per cent), CD44 (4.7 per cent) and CD29 (2.6 per cent).

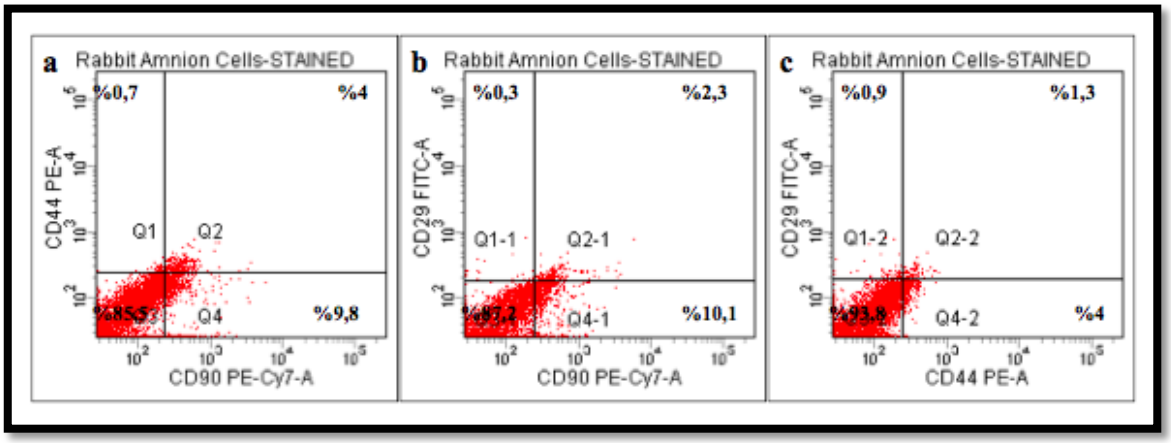

Figure 4.4.Expression level of mesenchymal stem cell markers. (a) CD44 and CD90; (b) CD29 and CD90; (c) CD29 and CD44.

Analysis using PE-CY7 conjugated CD90, PI conjugated CD44 and FITC conjugated CD29 antibodies revealed 1,2 per cent of the population carrying CD90, CD44 and CD29 surface receptors in total amniotic fluid cells (Figure 4.5).

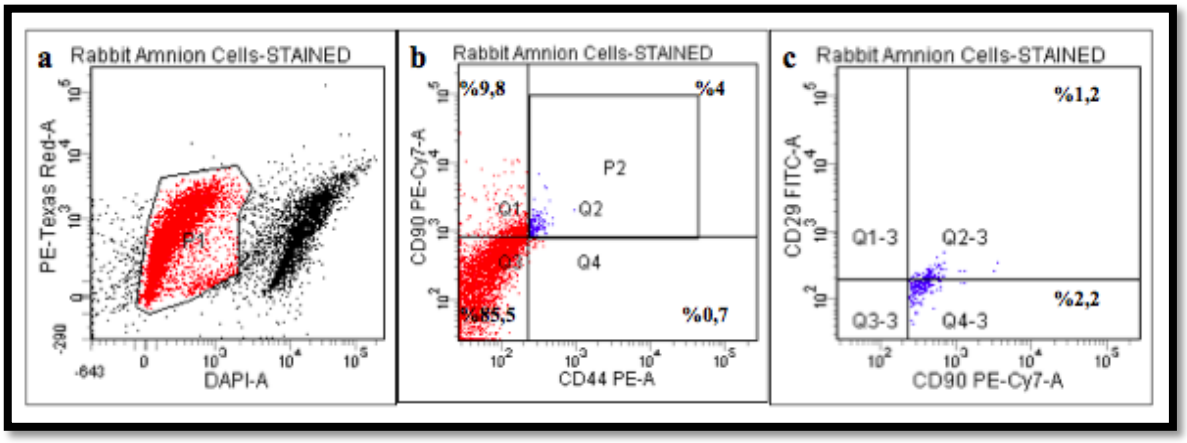

Figure 4.5. Live cells was selected with P1 gate and 1.2 per cent of the population was identified as AFMS cells. (a) DAPI staining for selection dead cells (b) selection of CD90 + CD44 + subpopulation with P2 gate (c) CD29 + CD44 + CD29 + cells total population were identified by specifying CD29 + cells in the subset of this cell.

At the end of characterization and isolation, 40,493 cells that belong to the $CD90^{+}$, $CD44^{+}$ and $CD29^{+}$ sub-populations were separated by 86 per cent efficiency with FACS and the cells were prepared for the next step, the differentiation step.

Roubelakis and coworkers show that AFMS cells represent 0.9 to 1.5 per cent of total AF cell population using flow cytometry analysis of CD45- gated cells with CD90, CD73, CD105, and CD44 markers [68]. In addition to that, highly expressed mesenchymal stem cell markers; CD90, CD44 and CD29 shown in cultured AFMS cells. In this present study with anti-rat antibodies, even in second trial 1.2 per cent of total AF cells were identified as AFMS cells, 0.3 per cent of AFMS cells were detected in first trial and stem cell markers expression (8.7-13.6 per cent CD90, 2.4-4.7 per cent CD44, 1.2-2.6 per cent CD29) was lower than previously detected. Since rabbit is not frequently used as a model, rabbit specific mesenchymal stem markers are not commercially available. Slamecka et al. reported that in RAFS cells characterization with anti-human antibodies only CD 44 (47.8

per cent) expression was detected [110]. For further studies, anti-rabbit antibodies reactive against to mesenchymal stem cell markers need to be designed.

4.3. DIFFERENTIATION OF RAFMS CELLS AND CHARACTERIZATION OF DIFFERENTIATED SMOOTH MUSCLE CELLS

Growth factors and cytokines are crucial for SM cell differentiation and so far various combination of them have been tested [111,112]. Especially, recently reported studies indicate that activation of intracellular signaling like Notch signaling enhanced the transcription of specific smooth muscle proteins [112]. In this present study, combination of PDGF-BB that enhance SM cell proliferation and down-regulate SM cell specific gene expression and TGF-beta, which initiate Notch signaling were used [94,113].

In the end of differentiation studies, using samples that was collected during and the end of growth factor treatment, morphologic, molecular and functional properties of differentiated SMC was tested using a combined characterization analysis.

4.3.1. Morphological Examination

Amniotic fluid stem cells are described as phenotypically and genetically stable cells and differently from bone marrow stem (BMS) cells, proliferation rate and differentiation capacity do not affected by passage number [114]. Here in Figure 4.5, sorted AFMS cells in passage 4 (a) and sub-culture in passage 10 (b) demonstrate that, in morphological level there is no distinguishable change.

Smooth muscle cells display distinct morphological changes during differentiation, as a result of the synthesis of contraction and cytoskeletal proteins [115]. From the first day of growth factor treatment, cell culture plates were observed carefully and photographed under phase contrast microscopy in every day of first week, day 14 and day 21 (Figure 4.6).

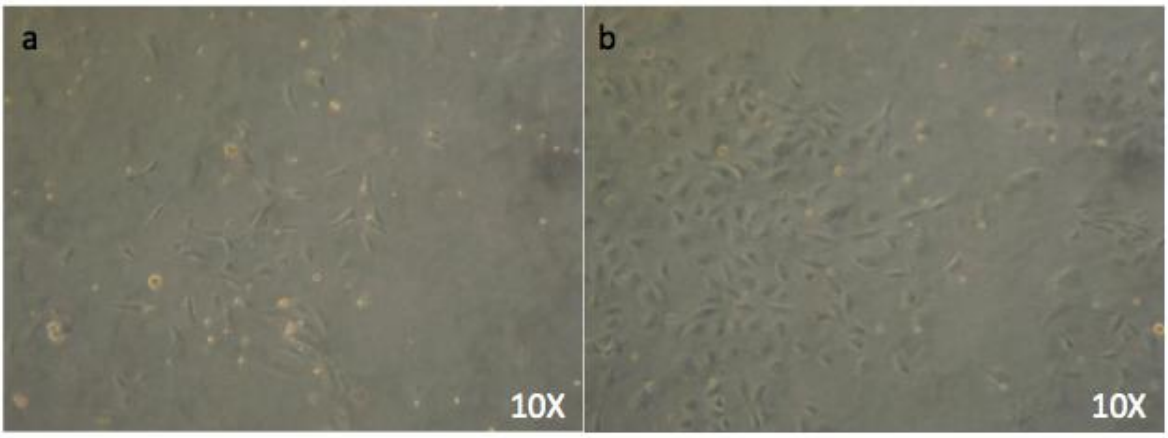

Figure 4.6. Morphology of Rabbit amniotic fluid mesenchymal stem cells before differentiation (a) RAFMS cells from sorting (Passage 4) (b) sub-culture in passage 10.

Microscopy images represents dramatic morphological changes in experimental group after treatment of differentiation agents compared with control group cells. Morphological change started in day 3 of growth factor treatment and reach the maximal level in day 14.

In 7^{th} day of differentiation apparent level of morphological change can be observed (Figure 4.7). When reach the day 14, three different morphology was detected, which were AFMS cells like, contractile SM cells like (elongated and spindle shaped) and synthetic SM cells like (less elongated compared to contractile SM cell and cobblestone shaped). Finally in 21^{th} day of differentiation, remarkable morphological change was observed compared to control group. Cells become larger and display spindle shape morphology. On the other hand, the control group was maintain their fibroblast-like morphology, did not underwent spontaneous differentiation. Same morphological changes have been reported in generation of SM cell from ES cell or AS cell studies [89, 116,117].

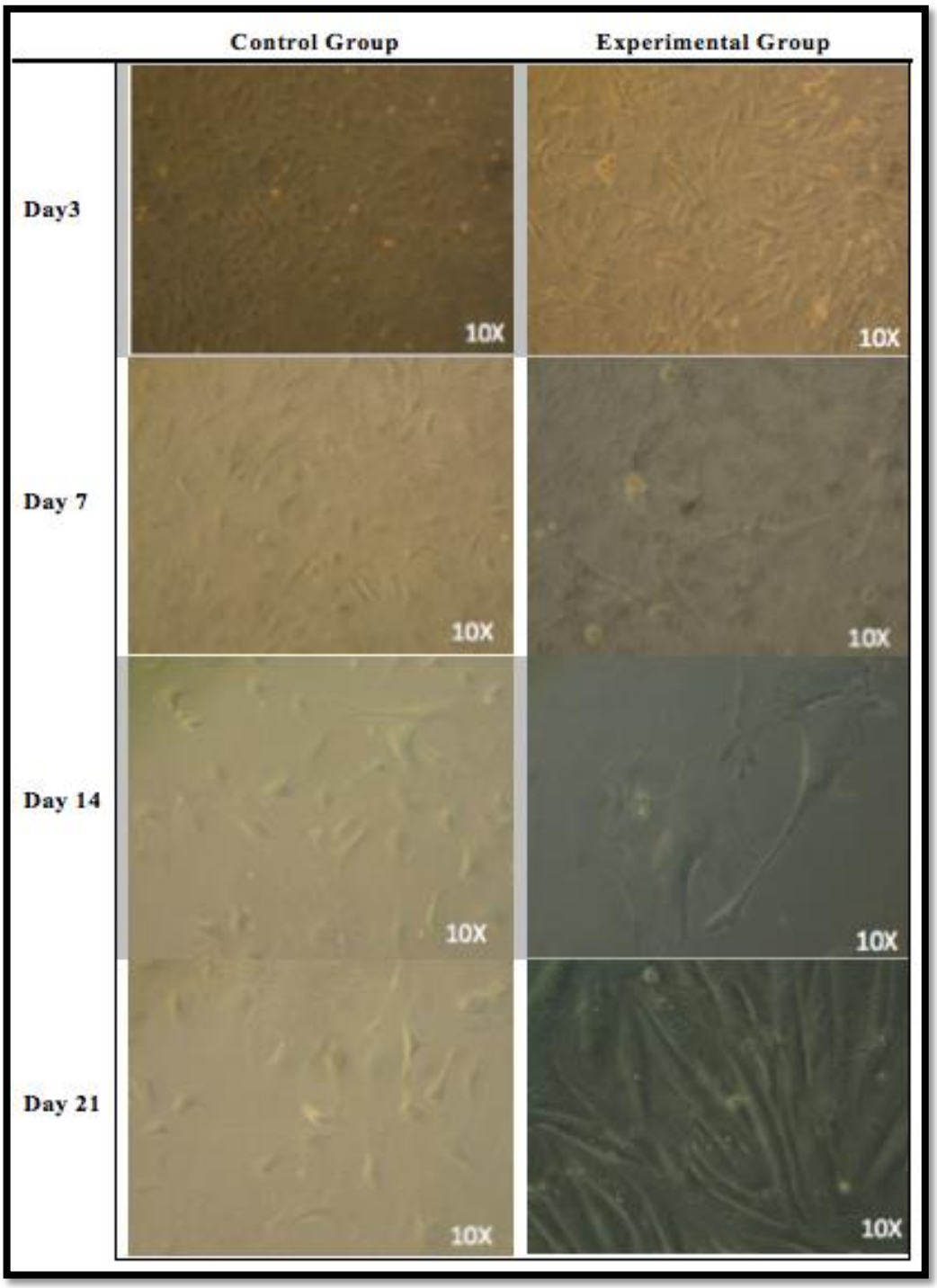

Figure 4.7. Morphological characteristics of RAFMS cells after differentiation. (Day 3: Control group- passage 4 and Experimental group- passage 4; Day7: Control group- passage 5 and Experimental group- passage 5; Day 14 Control group- passage 7 and Experimental group- passage 6; Day 21 Control group- passage 10 and Experimental group- passage 7)

4.3.2. Cell Viability Assay

Cell viability assay was performed to detect the effect of differentiation on proliferation [118]. For this purpose, control and experimental group cells were seeded to 24-well plate (for first 7 days) and T75 culture flask (for 14th day) and cultured with differentiation medium at 37 °C and 5 per cent CO_2.

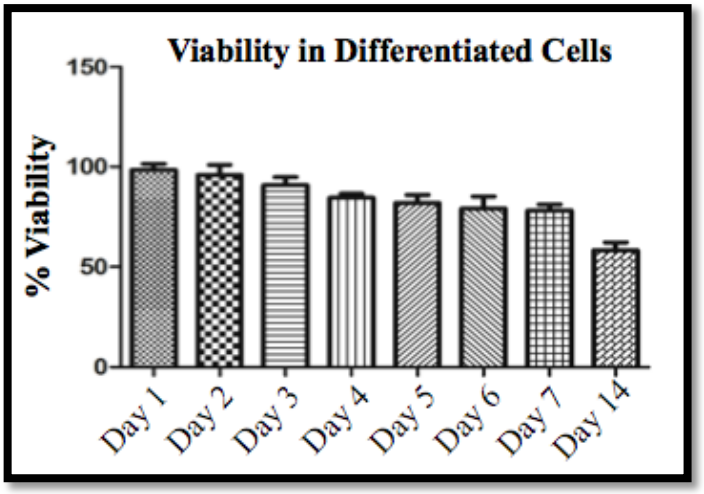

Figure 4.8. Growth rate of RAFMS cells cultured in the presence of growth factors.

Reduced proliferation rate of AFS cells have already been reported in parallel to ongoing differentiation by several groups [94, 119]. Similarly in this study, MTS assay demonstrated that after first week the proliferation capacity decreased (Table 4.8).

4.3.3. Gene Expression Analysis

For characterization of differentiated smooth muscle cells, the expression of several smooth muscle isoform of contractile apparatus use as markers, which have been extensively reviewed [120-122]. In this present study, expression of α-SMA (Smooth Muscle Actin (ACTA2)), Desmin (Des), and MHC11 (Smooth Muscle Myosin Heavy Chain) was examined with isolated RNA samples, which collected in day 7th, 14th and 21th

of differentiation. Each sample was compared with its own internal control and normalization was performed according to GAPDH.

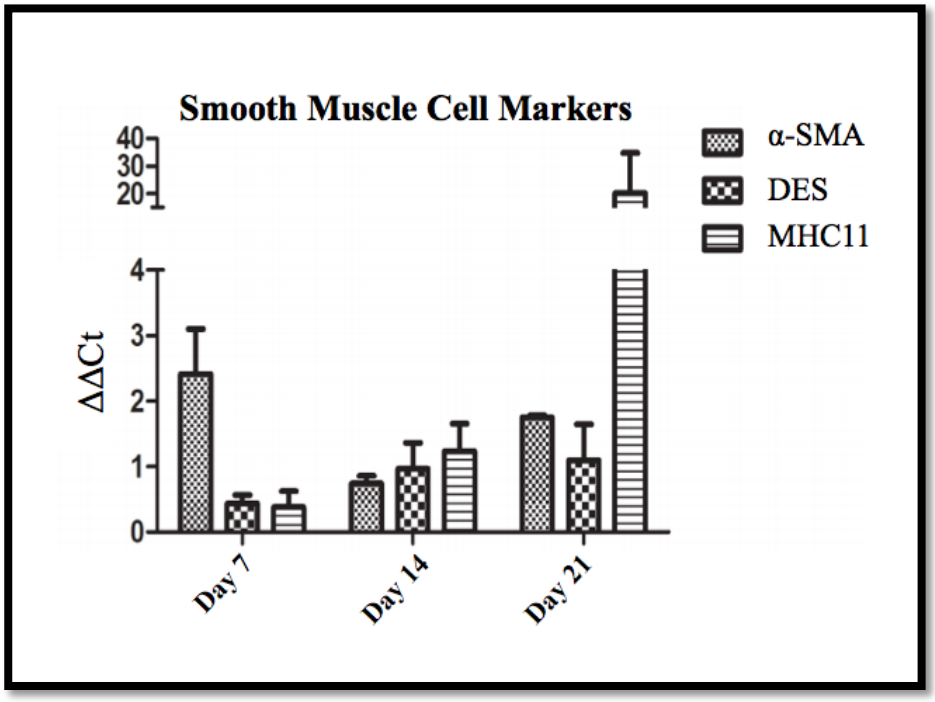

Figure 4.9. Expression levels of SM cells marker in differentiated RAFMS cells. Expression of α-SMA increased compared to the control group on day 7 whereas no change was observed for MHC11 and Desmin. In day 14 and 21, lower expression level was detected for α-SMA compared to day 7 and increased levels were detected for both MHC11 and Desmin.

It was hard to choose the SM cell markers among the markers readily reported because expression of most of these markers demonstrated in other cell types [123]. For instance, expression of α-SMA and SM-calponin was detected in Mesenchymal stem cells [124,125]. In addition to that heterogenic population of somatic SM cells display gradually changing type and expression level of SM cell specific markers. Rensen and his coworkers categorized SM cell specific markers according to SM cell types [85]. Since the main purpose of this experiment was generate fully functional SM cell from AFMS cells, contractile SM cell specific markers was used for characterization.

α-SMA, which is the earliest characterized marker for SM cell, is an early marker of developing smooth muscle cells [129]. Even though some of research group claim that expression of α-SMA is not a supportive data since spontaneously expression of this gene have been detected in pluripotent stem cells [98], α-SMA still widely uses in characterization of SM cells. In this experiment, despite a rapidly increased expression in day 7, decreased α-SMA level compared to day 7 were observed in 14^{th} and 21^{th} day of differentiation (Figure 4.9). Although continuously enhanced expression of α-SMA in SM cell differentiation was reported by Huang et al. [126], similar fluctuations in expression level was observed by other groups [94,106].

Since α-SMA expression is not restricted to differentiated SM cells, expression markers, which are highly specific to SM cells was also evaluated. Desmin is an intermediate filament protein that plays an important role in cardiac and skeletal muscle contractile function [127]. The expression of Desmin is increase during the maturation of smooth muscle cells [128], supportively in present study, low level of Desmin expression was detected in early stage of differentiation but it was increased after day 14 (Figure 4.9).

Myosin Heavy Chain, which is a hexametric motor-enzymic protein, has a crucial role in the contractile force [129]. MHC11 is highly specific marker that only express in mature SM cells [120,130]. Although Xie and his coworkers reported significant upregulation of MHC11 after 5 days of differentiation [117], in many studies MHY11 expression have been reported after 11 to 14 day [94,116,126]. In this study, similarly with Desmin, low level of MHC11 expression was observed in early differentiation and expression was increased abundantly after 14^{th} day (Table 4.9). In day 21, in which according to our hypothesis maturation was complete, expression of MHC11 was increased about 20-fold.

4.3.4. Immunofluorescence

After gene expression analysis, expression and localization of smooth muscle specific cytoskeletal proteins was evaluated with immunocytochemistry staining. For this purpose α-SMA and MHC11 was used, since rabbit-specific Desmin antibody was not commercially available. Phalloidin, which selectively binds to F-actin was provided the

information about structural and volumetric context of the cell [131] and DAPI was used to visualize nuclei.

α-SMA expression and localization was monitored in early (day 7) and late (day21) differentiation. In 7th day of differentiation, cytoskeletal arrangement of α-SMA, which localized in the intermediate filaments was observed. As it mentioned previous section, α-SMA is an early marker for SM cell differentiation [123]. Supportively in this study, α-SMA localization was highly organized in day 7 while Ghionzoli et al reported short bundles of short intermediate filaments in early stage of differentiation [94].

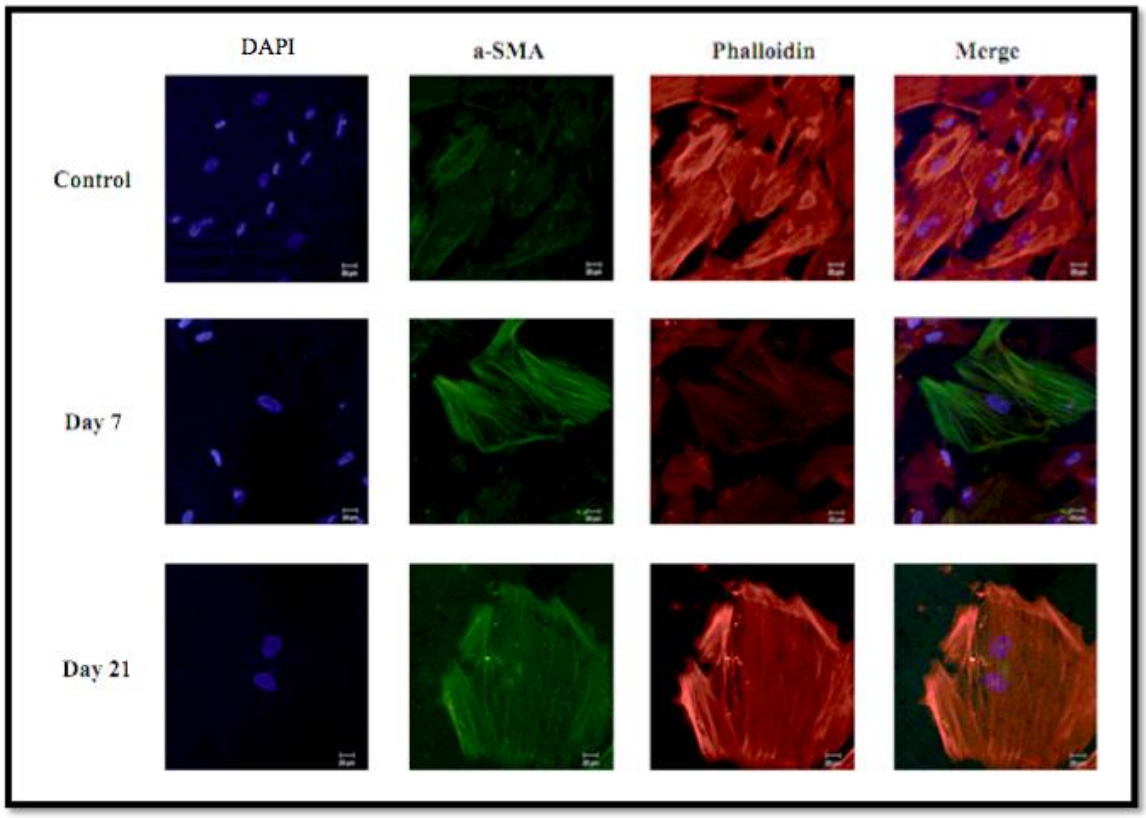

Figure 4.10. Intracellular localization of α-SMA. Immunostaining shows the expression of α-SMA (Green) in day 7 (passage 5) and 21 (passage 7) while expression was not observed in control group. Nuclei was counterstained with DAPI (Blue) and Phalloidin (Red) was used to visualize cytoskeletal structure.

Interestingly in day 21 reduced expression of α-SMA was observed. As it mentioned before α-SMA is not highly specific to SM cells since expression of this marker protein was also detected in MS cells [98]. One possible explanation for this decrease might be disappeared α-SMA, which localized in AFMS cells. However this statement is failed because of low level expression α-SMA was detected in control group (Figure 4.10).

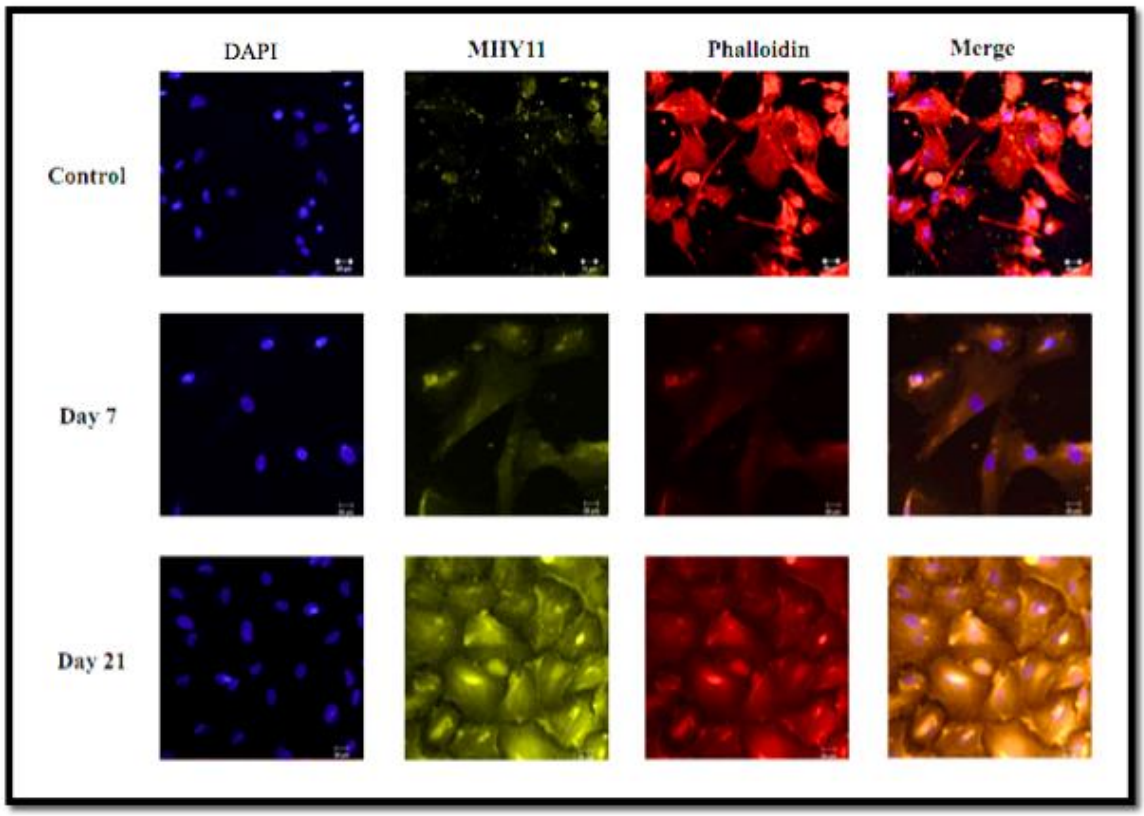

Figure 4.11. Intracellular localization of MHC11. Immunostaining shows the low expression of MHC11 (Yellow) in day 7 (passage 5) and highly expressed MHC11 in day 21 (passage 7) while expression was not observed in control group. Nuclei was counterstained with DAPI (Blue) and Phalloidin (Red) was used to visualize cytoskeletal structure.

De-differentiation of vascular smooth muscle cells have been reported in case of immediate needs for growth [132]. In direction of this information, two different state of SM cells have been identified after maturation; contractile state in which high level of

contractile proteins express and secretory state, that proliferation occurs and only basement membrane proteins express [132,133]. One other possible reason of the low expressed α-SMA in day 21 might be state of differentiated cells. On the other hand, MTS data displayed reduced proliferation in late differentiation and here in next result highly expressed MHC11 was shown in day 21. Further studies are necessary to understand the reason of this decrease.

The expression of α-SMA is not enough to confirm mature SM cells since its early and nonspecific marker. For this reason, MHC11 which is highly specific and late marker of SM cell was also used in this study [120]. Consistent with the Real-time data, ICC analyses shown that MHY11 expression was low in early stage of differentiation whereas, highly expressed and organized MHC11 was detected in day 21 and MHC11 was not express in control group (Figure 4.11). These results are correlated with previous findings [89, 94].

4.3.5. Collagen Gel Contractile Assay

Molecular studies, which explained in previous sections, indicate that differentiated cells seem to have contractile SM cells phenotype (spindle-shaped) and molecular characteristics (express α-SMA, Desmin and MHC11, which are crucial proteins for contraction) rather than synthetic SM cells. To confirm the data obtained via molecular characterization, contraction ability of differentiated cells (in day 21-according to our hypothesis ‘‘mature SM cells’’) compared to RAFMS cells were evaluated with Collagen lattice gel contractile assay.

There are several factors that have crucial effect on collagen gel assay. In addition to cell type and concentration, which were not considered in this study because of short-term incubation after agonist treatment, the alteration of gel surface is highly depend on contraction agents (agonists and inhibitors) [134]. There are several reported contraction agonist, which trigger contraction via precisely different mechanism of action such as membrane depolarization and G protein-coupled receptor activation [135,136]. In present study, Carbachol, which increase the concentration of intracellular Ca^{+} through coupling the muscarinic receptor and G-protein [91] and potassium chloride (KCl) that activates voltage-operated Ca^{+} channels as a result of surface depolarization [98,137] were used to

induce contraction. Serum and agonist free medium was used to determine basal contraction tone of the cells.

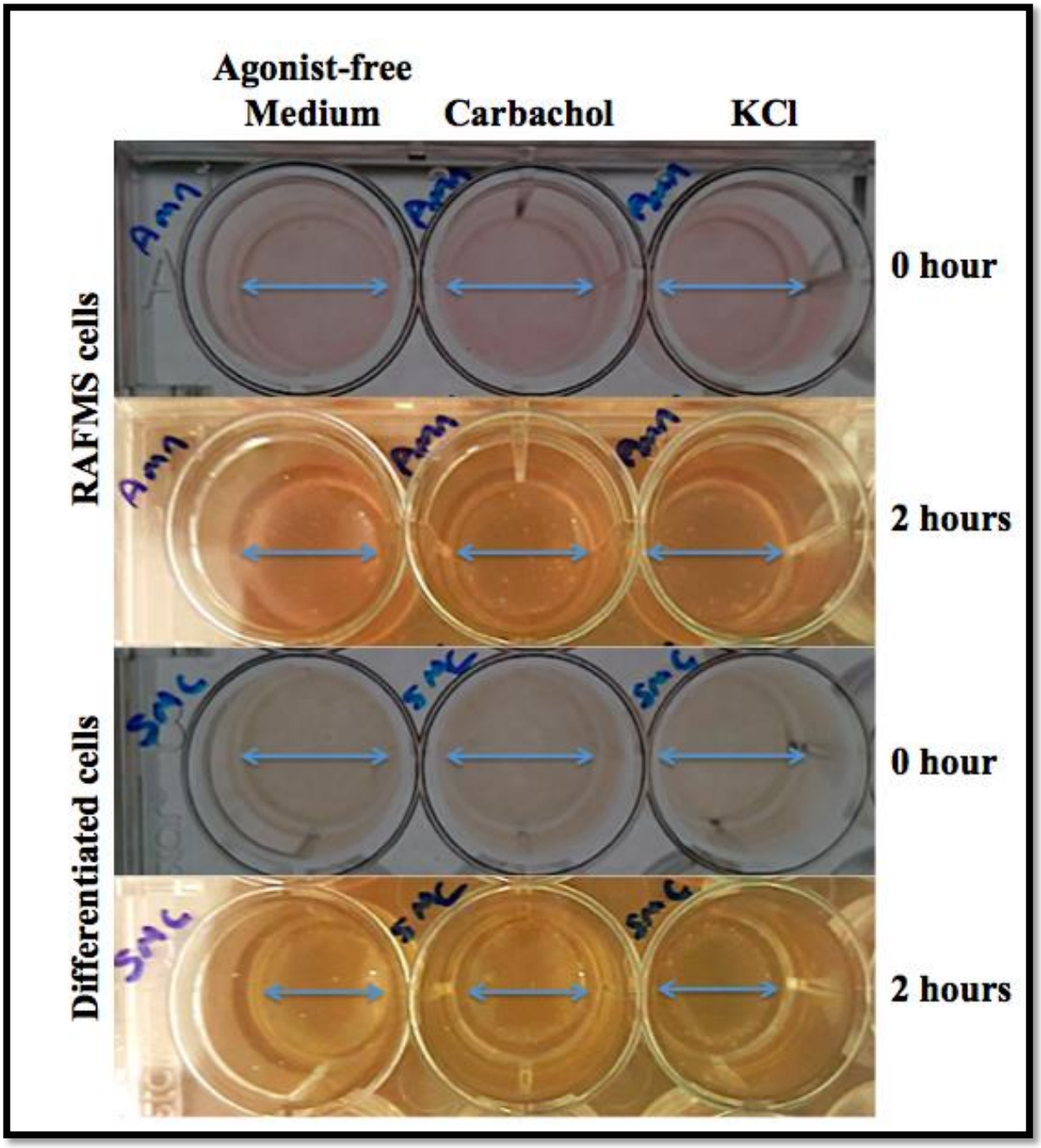

Figure 4.12. Collagen diameters before and after contractile-agonist treatment. After 2 hours, decreased diameters in differentiated cell gels were easily observed, whereas in RAFMS cells collagen gel diameter was stable.

Since the principle function of SM cells is contraction and relaxation, the ability of contraction has been accepted as the main proof of fully functional SM cell differentiation [94]. Bonnet et al. reported that differentiated SM cells through the BMMS cells failed to contract in response to physical stimulus despite highly expressed SM cell specific proteins [93]. In this present study, after 2 hours of agonist treatment differentiated cells gel displayed apparently reduced diameter (Figure 4.12). On the other hand there was no significant change in AFMS cells gel. Interestingly, higher contractile response was observed in KCl treated groups.

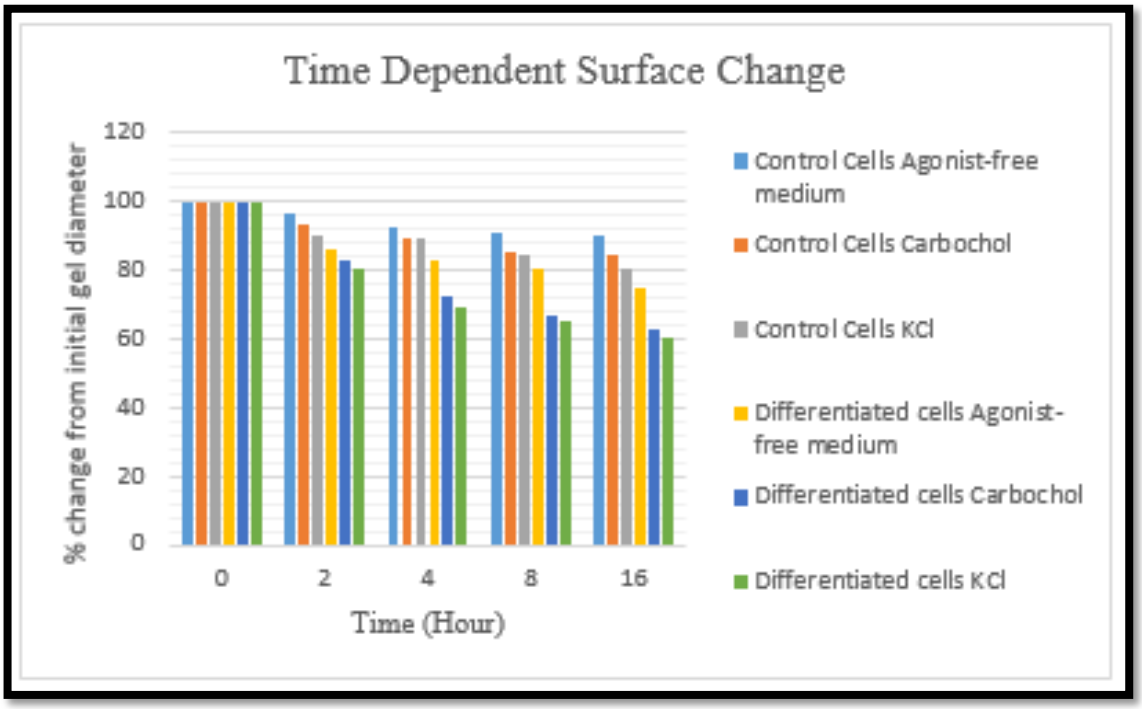

Figure 4.13. Variation in gel diameter with response to contraction agonist treatment

The time-dependent surface area change is shown in Figure 4.13. As expected, remarkable surface area reduction was observed in gel containing differentiated cells while RAFMS cell gels displayed modest contraction. Contracting RAFMS cells exhibited between 10-20 per cent changes of cell surface area whereas differentiated cells displayed 25-40 per cent change. Similar cellular response have been reported by several other independent laboratories [94, 126 ,138].

4.3.6. Calcium Signaling Measurement with Flou-4 Staining

It has been demonstrated that Calcium (Ca^{2+}) signaling play an important role in physiological function of smooth muscle cells, including contraction by mediating through the increase concentration of free calcium in the cell cytoplasm [136]. In addition to that, Ca^{2+} signaling is also responsible to phenotypic variety of SM cells (contractile to synthetic) [139,140]. Therefore, intracellular Ca^{2+} concentration change in response to Carbachol and KCl (contractile agonist used in Collagen Gel assay) were quantified in day 21 differentiated cells and RAFMS cells.

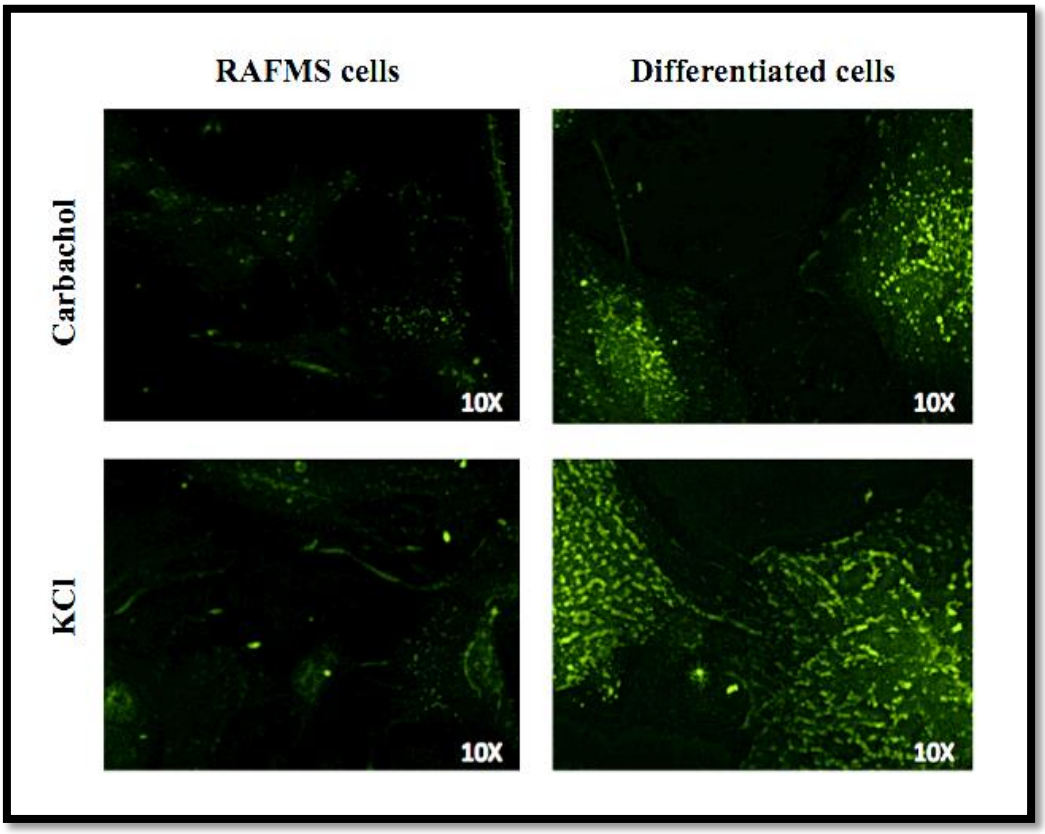

Figure 4.14. Measurement of intracellular Ca^{2+} concentration. Fluo-4 staining followed by 1 mM Carbachol and 75 mM KCl treatment display the Ca^{2+} concentration differences between RAFMS and Differentiated cells.

Egan and his coworkers highlighted that concentration of Ca^{2+} only increase in the fully differentiated cells since intracellular Ca^{2+} directly influences the downstream signaling pathway, which regulate SM cell specific gene expression [141]. In parallel with contractile assay findings and previous studies [142,143], significantly higher accumulation of intracellular Ca^{2+} after both Carbachol and KCl treatment was detected in differentiated cells compared to RAFMS cells (Figure 4.14).

Measured concentration of the Ca^{2} was higher in KCl-stimulated differentiated cells, than Carbachol treated cells. Supportively in collagen gel assay this group displayed higher contraction (Figure 4.13). Both Carbachol and KCl are responsible to the accumulation of Ca^{2+} in the cell cytoplasm through different mechanisms [129] and studies indicate that influences of these agents might differ according to cell types [143,144].

4.4. TISSUE ENGINEERING

AFS cells have already been defined as an alternative source for tissue engineering [58,76]. Even though, generation of fully functional SM cells via ES and AS cells reported by several groups [91,92,95], there are limited data about AFS cells [94]. Up to this section, differentiation capacity of RAFMS cells along SM cells was evaluated and characterization studies display the typical SM cell morphology and molecular properties.

AFS cells are capable to colonize and maintain their functional properties in 3D scaffold [145]. The differentiation capacities of AFS cells in scaffold have already been investigated for bone and vascular tissue engineering [79,120, 146]. For congenital smooth muscle defect treatment engineered smooth muscle grafts derived from amniotic fluid can be use. In second part of the study, stability and colonization ability of RAFMS cells was tested with suitable biodegradable polymer for prospective clinical trials.

4.4.1. Fabrication and Characterization of PLGA Scaffold

The pertinent literature indicate that scaffold materials, which are mostly synthetic or natural polymers have crucial role in tissue engineering since their biophysical and biochemical properties directly affect cells behavior and functions [145,147]. From many

other material U.S. Food and Drug Administration (FDA) approved synthetic polymer, Poly(D,L-lactide-co-glycolide) was preferred for this experiment because of its biodegradable and biocompatibility properties [148]. Even though it's hydrophobic nature causes cell interaction problems, it has been employed extensively in tissue engineering [106, 149 , 150], including muscle engineering [151].

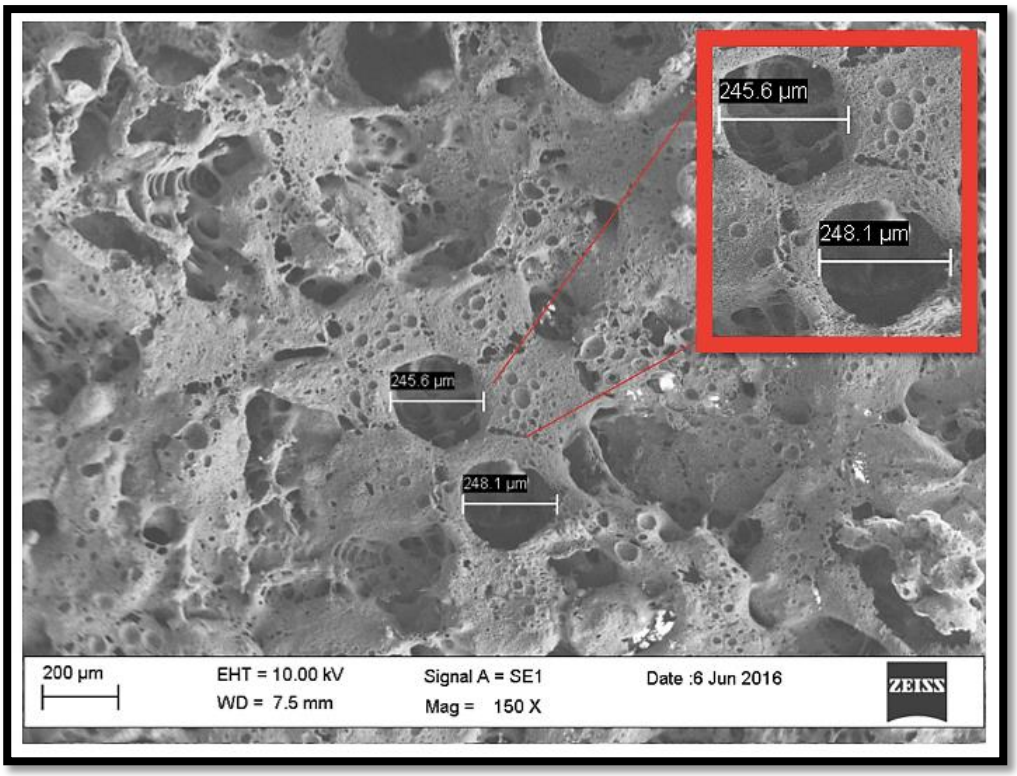

Figure 4.15. Characterization of PLGA scaffold (Sample 1-3). SEM images display the structure of pores and diameters (245.6-248.1 µm). Scale bar is 200 µm and image was taken at 150X.

To provide favorable cell attachment, proliferation and differentiation, interconnected pore structure has crucial importance [152]. There are several strategies to fabricate porous structures, such as porogen leaching, phase separation and electrospinning [148]. In parallel to reported studies, in this present study porogen leaching was preferred since porosity and pore size control is relatively easier than others.

Characterization of fabricated PLGA scaffold was evaluated with SEM. Both Figure 4.15 and 4.16 displayed randomly oriented and open interconnected pore structure of PLGA. Kim and his coworkers stated that this structure could provide suitable environment for cell attachment and proliferation [153]. Additionally it should highlight that, according to cell type and study pore size can be variable. With respect to AFS cells related scaffold studies, pore size was determined as 250-420 μm [114].

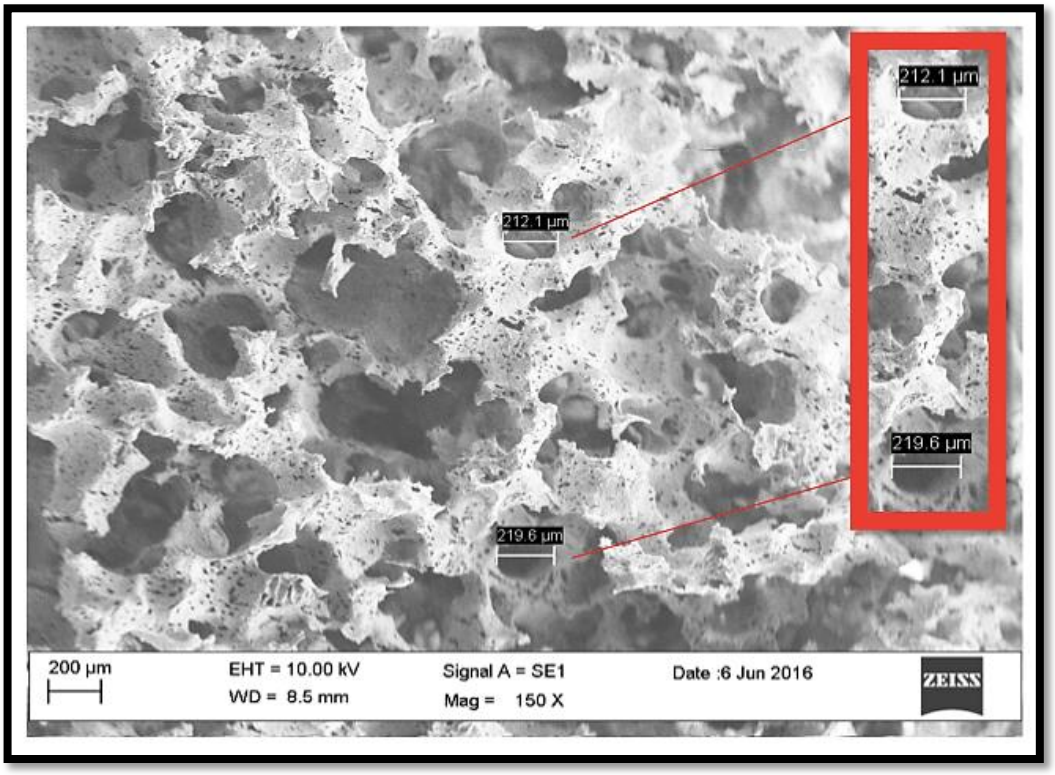

Figure 4.16. Characterization of PLGA scaffold (Sample 2-2). SEM images display the structure of pores and diameters (212.1-219.6 μm). Scale bar is 200 μm and image was taken at 150X.

4.4.2. Cell Planting and Post-Plant Control

As mentioned previous section, according to scaffold material attachment ability and proliferation properties of cells might change. There are limited characterization studies related to AFS cells. Kaviani and his coworkers was detected attached and viable AFS

cells both on polyglycolic acid (synthetic) and acellular human dermis (natural) scaffolds after 48 hours [154]. Additionally, even after long-term culture AFS cells could maintain their proliferation and morphological properties on 3-D PET scaffold [155].

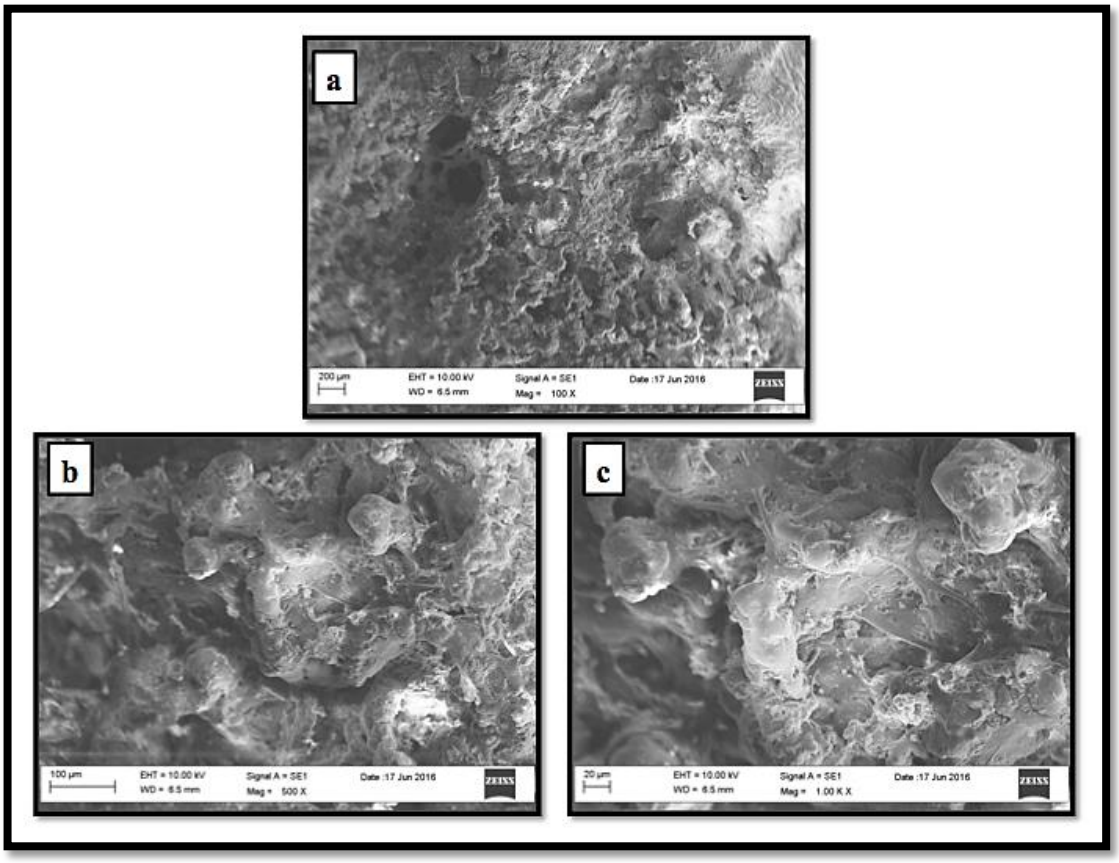

Figure 4.17. RAFMS cells in PLGA scaffold (Sample 3). (a) Distribution of RAFMS cells in low magnification (magnification: 100 X-scale bar: 200 μm (c) 3-D scaffold and cells (magnification: 500 X-scale bar: 100 μm) (d) RAFMS cells on PLGA scaffold at high magnification (magnification: 1.00 K X-scale bar: 20 μm)

In this study, variable morphology in attached RAFMS cells was observed after 3-day culture. Both Figure 4.16 and 4.17 display multi-dimensional cell attachment on 3-D PLGA scaffold. Most cells formed cluster (Figure 4.17), while complex attachment (extensive extra cellular matrix (ECM) network and bridged between fibers) was detected in some other RAFMS cells (Figure 4.17). Similar cell morphology was observed on 3-D PET scaffold Liu et al. [155].

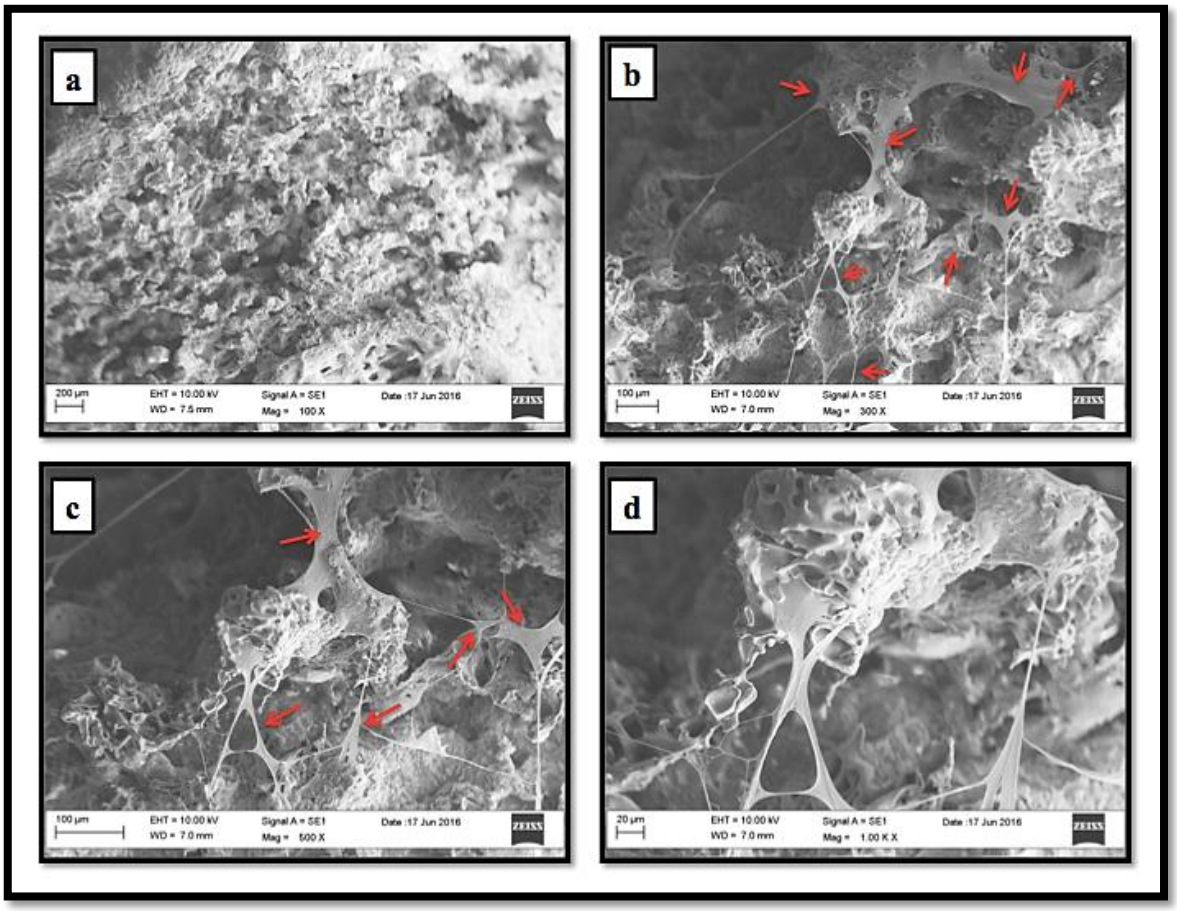

Figure 4.18. SEM image of RAFMS cells in PLGA scaffold (Sample 2). (a) Uniformly distributed RAFMS cells (magnification: 100 X-scale bar: 200 μm) (b) RAFMS cells and their extracellular matrix (ECM), arrows pointed to bridged cells and ECM (magnification: 300 X-scale bar: 100 μm) (c) 3-D scaffold and cells, arrows pointed to bridged cells and ECM (magnification: 500 X-scale bar: 100 μm) (d) RAFMS cells on PLGA scaffold at high magnification (magnification: 1.00 K X-scale bar: 20 μm)

5. CONCLUSION AND RECOMMENDATIONS

Congenital malformations, which occur in 3-5 per cent of newborn are one of the important causes of neonatal death. Treatment strategies of CM such as, organ transplantation and surgical reconstructions are still being used despite their disadvantages. On the other side tissue engineering studies have promising results. Especially in cases of smooth muscle tissue defects, which are commonly diagnosed prenatal malformation tissue engineering could be alternative treatment strategy.

The main purpose of this study is to investigate differentiation ability of amniotic fluid mesenchymal stem cells to smooth muscle cells and define alternative stem cell source for treatment strategy for congenital malformation.

Figure 5.1 Tissue engineering strategies with AF-derived stem cells. In parallel to ongoing pregnant, AF cells derived from amniocentesis, culture and seeded onto suitable scaffold. With this strategy autologous bioprosthesis is ready for implantation before birth.

Results indicated that from AFMS cells fully functional SM cells can be generated and PLGA polymer is suitable scaffold material for amniotic fluid mesenchymal stem cells. With further clinical trials, AFMS cells based smooth muscle engineering might become candidate for highly efficient treatment option in congenital malformation cases.

AFS cells have been already defined as new source for tissue engineering. Even though fully functional smooth muscle generation from AFS cells have already reported by Ghionzoli et al., AFS cells represent a rare population in the total amniotic fluid cells and number of cells decrease to undetectable level in the third trimester. On the other hand AFMS cells can isolate in all tree trimester and number of cells is higher than AFS cells. Fully functional SM cells have been generated from AFMS cells. Further *in vivo* and *in vitro* are necessary for clinical application.

With selected suitable polymer, well-designed PLGA scaffold was fabricated and cell attachment capacity on the scaffold was detected. The differentiation capacities of AFS cells in scaffold have already been investigated for bone and vascular tissue engineering however smooth muscle engineering did not reported yet. Additional investigation is necessary for smooth muscle defects.

AFS cells could be used not only for congenital malformation or later injuries but also for cancer and some neurodegenerative diseases treatment. In 2009 first private amniotic stem cell bank, Biocell Center was opened in USA. In addition to aforementioned advantages, this study shown that amnion banks should be opened in Turkey as well.

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