The Effects of Environmental Factors on Smooth Muscle Cells Differentiation from Adipose-Derived Stem Cells and Esophagus Tissues Engineering

Wang, Fang

DOI (link to publication from Publisher):
10.5278/vbn.phd.med.00023

Publication date:
2015

Document Version
Publisher's PDF, also known as Version of record

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BY
FANG WANG

DISSERTATION SUBMITTED 2015

AALBORG UNIVERSITY
DENMARK

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Dissertation submitted

July 2015

Thesis submitted: July 2015

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PhD Series: Faculty of Medicine, Aalborg University

ISSN (online): 2246-1302
ISBN (online): 978-87-7112-325-8

Published by:
Aalborg University Press
Skjernvej 4A, 2nd floor
DK – 9220 Aalborg Ø
Phone: +45 99407140
aauf@forlag.aau.dk
forlag.aau.dk

Printed in Denmark by Rosendahls, 2015

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CV

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ACKNOWLEDGEMENTS

The whole work related to PhD thesis was carried out during my employment as a PhD student at the Laboratory for Stem Cell Research, the Biomedicine Group, the Department of Health Science and Technology, the Faculty of Medicine, Aalborg University from 2011-2014. During my four years PhD study, the challenges of the scientific work and international working environment foster my scientific mode of thinking and independent problem solving capabilities, which is the precious and memorable period of time in my life.

First of all, I would especially like to express the gratefulness to my supervisor, the head of the School of Medicine and Health, Associate Professor Jeppe Emmersen, PhD, who gives me this great opportunity to come to this beautiful and hospitable country starting a brand new life and a challenging work. His high efficient working style, constructive suggestions and great patience are very impressive and indispensable for the completion of this study. His support and guidance during the whole thesis is sincerely appreciated.

Senior Surgical Research Fellow Yasuko Maeda, PhD, Sir Alan Parks Physiology Unit, St. Mark's Hospital, United Kingdom, is acknowledged for the first article revision and valuable comments. Senior Scientist Tahera Ansari, PhD, Northwick Park Institute for Medical Research (NPIMR), United Kingdom, is acknowledged for providing esophageal scaffold and the third article revision. Associate Professor Cristian Pablo Pennisi, PhD, Laboratory for Stem Cell Research, Aalborg University, is acknowledged for the technical support for the second study and article revision. Professor Vladimir Zachar, PhD, Laboratory for Stem Cell Research, Aalborg University, is acknowledged for the first and second article revision.

Fellow PhD student, Jens Isak Andersen, Laboratory for Stem Cell Research, Aalborg University, is acknowledged for technical support of the second study. My former colleague Fellow PhD student, Michael Henriksen, Laboratory of Cancer

Biology, Aalborg University, is acknowledged for help in initial stage of my work. Also, I would like to thank technicians Helle Skjødt Møller and Ole Jensen for help in cell lab. In addition, I would like to express my thanks to all colleagues of Laboratory of Stem Cell Research group for their kindness and support in the past four years.

Finally, I would like to thank my family and my daughter Juntao Mao for their support financially and spiritually.

This study is generously supported by grants from Kræftens Bekæmpelse. S. C. Van Fonden is acknowledged for financial support.

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Aalborg, Denmark

July, 2015

ENGLISH SUMMARY

Adipose-derived stem cells (ASCs) are increasingly being used for regeneration medicine and tissue engineering due to abundance and easy accessibility.

Smooth muscle cells (SMCs) can be obtained from ASCs via various approaches: different growth factors, enhancement by mechanical force stimulation or changes in oxygen environment. Oxygen is a key factor influencing the stem cell proliferation and differentiation.

The smooth muscle layer constitutes the intermediate layer of the esophagus and plays an important role for food transportation from pharynx to the stomach. A number of diseases might lead to esophageal anatomic damage and functional disorders. Utilizing tissue engineering approach to regenerate a smooth muscle layer is a prerequisite for successfully constructing tissue engineering esophagus.

The goal of this thesis was to explore the effects of hypoxia, biochemical factor stimulation as well as mechanical stretching on differentiation of SMCs from human adipose-derived stem cells (hASCs), and investigate the feasibility of reconstructing the esophageal smooth muscle layer using porcine derived esophageal acellular matrix (EAM) scaffolds and SMCs differentiated from hASCs.

In the first study, the effect of hypoxia on differentiation was investigated at oxygen concentrations of 2, 5, 10 and 20%. Contractile human aortic smooth muscle cells (hASMCs) were used as a control. Real time reverse transcription polymerase chain reaction (RT-PCR) and immunofluorescence staining results were used to evaluate the expression of smooth muscle cell (SMC)-specific markers including the early marker smooth muscle alpha actin (α-SMA), the middle markers calponin and caldesmon and the late marker smooth muscle myosin heavy chain (MHC). The specific contractile properties of cells were assessed using both a single cell contraction assay and a gel contraction assay. The combined results of marker

expression and contraction assays showed 5% hypoxia to be the optimal condition for differentiation of hASCs into contractile SMCs.

In the second study, the combined effects of biochemical factor stimulation, mechanical force and oxygen levels on smooth muscle differentiation were studied. Both normal hASCs and hASCs preconditioned at 5% oxygen for 1 week were cultured on 6-well flexible-bottomed culture plates. HASMCs were used as a control. After reaching subconfluence, cells were subjected to either 10% cyclic tensile strain (CTS) alone or in combination with stimulation for 1 week. A combination of the biochemical factors transforming growth factor-β1 (TGF-β1) and bone morphogenetic protein-4 (BMP4) was used as differentiation factors. Cell reorientation, F-actin remodeling as well as SMC-specific markers were detected by immunofluorescence staining and real time RT-PCR assays. Cells were reoriented and F-actin cytoskeleton was realigned perpendicular to the direction of strain after 10% CTS for 1 week. In addition, the cells differentiated with combined treatments for 1 week promoted the MHC expression as compared to the biochemical factors alone.

In the third study, the potential for using differentiated ASCs to replace SMCs to regenerate the smooth muscle layer of EAM was studied. HASCs expanded and differentiated respectively in 5% or 20% oxygen concentrations were seeded onto muscle layer of porcine EAM scaffold. HASMCs were used as a control. The constructs consisting of scaffold and different types of cells were cultured for 24 hours or 7 days. The morphology of EAM scaffold was evaluated by haematoxylin and eosin (H&E) staining, picrosirius red staining for collagen as well as Miller's elastin staining for elastin. Cell proliferation ability, viability and migrate depth were examined via propidium iodide (PI) staining, cell counting as well as double staining assays. Our results showed that both proliferated and differentiated hASCs in 5% or 20% could attach on the porcine EAM scaffold in vitro after 24 hours and survive until 7 days. There is no significant difference between hASMCs and differentiated hASCs in terms of viability and migration depth, thus ASCs might be

a substitute for SMCs in the construction of tissue engineering (TE) esophageal muscle layer.

In conclusion, the findings of this thesis have demonstrated that:

1) HASCs can be differentiated into SMCs with biochemical factors TGF-β1 and BMP4 in combination, and 5% is the optimal oxygen concentration for differentiation process.

2) Combined treatments containing cyclic stretch and biochemical factors promote SMC-specific marker MHC expression for both hASCs and hASCs preconditioned in 5% as compared to the biochemical factors alone.

3) The SMCs differentiated from hASCs can attach, spread and survive on the EAM scaffold in vitro until 7 days, which is similar to hASMCs performance.

Together the three studies indicate the feasibility of using ASCs in future clinical applications involving tissue regeneration of smooth muscle containing tissue.

DANSK RESUMÉ

Adipøst afledte stamceller (ASCs) bliver i stigende grad anvendt til regenerativ medicin og genskabelse af væv, på grund af forholdsvis let tilgængelighed af en stor mængde celler.

Glatte muskelceller (SMCs) kan udledes fra ASCs ved hjælp af forskellige metoder: vækstfaktor påvirkning, mekanisk stimulation eller ændringer i oxygen koncentration. Specielt oxygen er en vigtig faktor, der påvirker stamcellers proliferation og differentiering.

Glatte muskelceller udgør det mellemliggende lag i spiserøret, og spiller en vigtig rolle for fødevaretransport fra svælget til maven. En række sygdomme i spiserøret kan medføre anatomiske skader og funktionslidelser. Brug af vævsgenopbyggelses teknikker til at regenerere et glat muskel lag er derfor en forudsætning for en vellykket rekonstruktion af spiserøret.

Formålet med denne afhandling er at optimere dannelsen af glat muskelcellevæv fra humane adipøse stamceller (hASCs) ved at variere faktorer i differentieringsprocessen som oxygen, cytokiner samt mekanisk påvirkning. Desuden skal muligheden for at translatere resultaterne fra de basale undersøgelser til klinisk brug undersøges ved at undersøge de differentieredes cellers evne til at anlægge glat muskelcelle lag ved i esofagus acellulære matricer (EAM) afledt fra porcin esophagus.

I første studie blev oxygens påvirkning på differentieringen af stamcelle til glat muskelcelle blev undersøgt ved 2, 5, 10 og 20% oxygen. Kontraktile humane aorta glatte muskel celler (hASMCs) blev anvendt som kontrol. Real time reverse transcription polymerase chain reaction (RT-PCR) og immunfluorescensfarvning blev anvendt til at evaluere ekspressionen af glatte muskelcelle (SMC)-specifikke markører, herunder tidlig-stadie markøren smooth muscle alpha actin (α-SMA), midt-stadie markørerne calponin og caldesmon og sen-stadie markøren smooth

muscle myosin heavy chain (MHC). De specifikke kontraktile egenskaber af celler blev bekræftet med både enkelt-celle kontraheringsforsøg og gel kontraktions-forsøg. De kombinerede resultater af markør udtryk og sammentrækning analyser viste, at 5% hypoxi er den optimale betingelse for differentiering af hASCs til kontraktile SMCs.

I studie 2 blev den kombinerede effekt af mekanisk stimulering og ilt niveauer på glat muskel differentiering undersøgt. Både normale hASCs og hASCs, konditioneret ved 5% oxygen i 1 uge, blev dyrket i 6 brønds dyrkningsplader med fleksible bunde. HASMCs blev anvendt som kontrol celler. Efter at have nået sub-konfluens, blev celler udsat for enten 10% uniaksialt cyklisk stræk (CTS) alene eller i kombination med biokemisk faktorer i 1 uge. Cytokin transforming growth factor-β1 (TGF-β1) og bone morphogenetic protein-4 (BMP4) blev brugt til at differentiere stamcellerne. Celle reorientering, F-actin remodellering samt SMC-specifikke markører blev detekteret ved immunfluorescens og RT-PCR assays. Tre typer af celler blev reorienteret og cytoskelet blev observeret som værende orienteret vinkelret på retningen af den mekaniske stimulering efter 10% CTS i 1 uge, vurderet på farvning af F-aktin. Endvidere blev der, hos cellerne differentierede med kombinations behandling i 1 uge (mekanisk og biokemisk induktion) observeret nedsat α-SMA-ekspression men øget MHC-ekspression, i forhold til celler differentieret alene med biokemiske faktorer.

I studie 3 blev mulighederne for at anvende ASCs som erstatning af SMCs til at regenerere glat muskel lag i acellulære esophagus matricer undersøgt. HASCs blev ekspanderet og differentieret i henholdsvis 5% eller 20% oxygen. HASMCs blev anvendt som kontrol. Disse blev efterfølgende udsået på det oprindelige muskellag i EAM matricen. Konstruktionerne bestående af EAM matrice samt forskellige typer af celler, blev dyrket i 24 timer og 7 dage. Morfologien af matricen blev evalueret ved hæmatoxylin og eosin (H&E) farvning, picrosirius red farvning for kollagen samt Miller elastin farvning for elastin. Celleproliferation, levedygtighed og migrationsdybde blev undersøgt via propidium iodid (PI) farvning. Celle tælling samt dobbelt farvning assays. Vores resultater viste, at både hASMCs og hASCs

kan hæfte på porcine EAM matricer in vitro efter 24 timer og efterfølgende overleve i mindst 7 dage. Der var ingen signifikant forskel mellem cellekontrol og differentierede hASCs med hensyn til levedygtighed og migrationsdybde. Derfor kan viser studiet at ASCs er en mulig erstatning for SMCs i opbygningen af det esophageale glatte muskellag.

Sammenfattende har resultaterne af denne afhandling vist, at:

1) Adipøse stamceller kan differentieres til glatte muskelceller med biokemiske faktorer TGF-β1 og BMP4 i kombination, og 5% er den optimale oxygen koncentration i differentieringsprocessen.

2) A-SMA ekspression inhiberes, men MHC ekspression fremmes for hASCs, når disse behandles med en kombination indeholdende cyklisk mekanisk strækning samt biokemiske faktorer, i forhold til biokemiske faktorer alene.

3) SMCs differentieret fra hASCs kunne vedhæfte, spredes og overleve på EAM matricer *in vitro* i mindst 7 dage, tilsvarende kontrolceller.

Samlet peger dette på en vigtig rolle for adipøse stamceller i fremtidig klinisk applikationer af vævsregeneration involverende glatmuskelcelleholdigt væv.

LIST OF MANUSCRIPTS

Study I: Hypoxia Enhances Differentiation of Adipose-Derived Stem Cells to Smooth Muscle Cells

Fang Wang, Vladimir Zachar, Cristian Pablo Pennisi, Trine Fink, Yasuko Maeda, Jeppe Emmersen.

Manuscript in preparation

Study II: Combined Effects of Biochemical Factors and Cyclic Strain on the Smooth Myogenic Differentiation from Adipose-Derived Stem Cells Preconditioned in Hypoxia

Fang Wang, Cristian Pablo Pennisi, Jens Isak Andersen, Vladimir Zachar, Jeppe Emmersen.

Manuscript in preparation

Study III: Regeneration of the Esophageal Muscle Layer from Esophagus Acellular Matrix Scaffold using Adipose-Derived Stem Cells

Fang Wang, Yasuko Maeda, Vladimir Zachar, Tahera Ansari, Jeppe Emmersen.

Manuscript in preparation

Review: Molecular Mechanisms of Smooth Muscle Cells Differentiation from Adipose-Derived Stem Cells

Fang Wang & Jeppe Emmersen.

Manuscript in preparation

ABBREVIATIONS

ASCs	Adipose-derived stem cells
A-SMA	Smooth muscle alpha actin
BMP4	Bone morphogenetic protein-4
BM-MSCs	Bone marrow-derived mesenchymal stem cells
EAM	Esophageal accelular matrix
HASCs	Human adipose-derived stem cells
HASMCs	Human aortic smooth muscle cells
HIF	Hypoxia-inducible factor
HIF-1α	Hypoxia-inducible factor-1 α
LG-DMEM	Low glucose Dulbecco´s modified Eagle's medium
MHC	Smooth muscle myosin heavy chain
MRTF-A	Myocardin-related transcription factor-A
MRTF-B	Myocardin-related transcription factor-B
MSCs	Mesenchymal stem cells
RT-PCR	Reverse transcription polymerase chain reaction
SMCs	Smooth muscle cells
SMDS	Smooth muscle differentiation supplement
SMGS	Smooth muscle growth supplement
SRF	Serum response factor
SVF	Stromal vascular fraction
TE	Tissue engineering
TGF-β1	Transforming growth factor-β1
CTS	Cyclic tensile strain
VSMCs	Vascular smooth muscle cells

1. INTRODUCTION

Smooth muscle cells (SMCs) constitute an important part of the anatomic micro-structure in esophagus. Esophageal congenital defects like atresia, tracheoesophageal fistula and acquired disorders such as esophageal cancer require the removal of the segmental esophagus and replacement with tissue engineered esophagus.[1] Obtaining functional SMCs is indispensable to successful reconstruction of in vitro tissue engineered esophagus. SMCs can be obtained from autologous tissue, but the limited regeneration capability of mature SMCs limits their application. Adipose-derived stem cells (ASCs) are a preferred stem cell source due to the easy and repeatable access to adipose tissue and simple isolation procedures, thus SMCs differentiated from ASCs have been an ideal solution.[2] The differentiation is determined by numerous local environmental cues and extrinsic factors including oxygen tension, biochemical factors as well as mechanical forces.[3] SMCs can be differentiated from ASCs using both biochemical and mechanical stimuli. Oxygen is a key signaling molecule in the stem niche and hypoxia influences stem cell proliferation and differentiation.[4] Stem cells cultured or preconditioned in hypoxia have been extensively investigated to enhance the yield of stem cell or improve stem cell-based tissue engineering (TE) application. Therefore, hypoxic conditions have a profound significance for the optimization of ASCs differentiation, as well as adipose-derived stem cell (ASC)-based treatment and TE application.

2. BACKGROUND

2.1 STEM CELL AND ADIPOSE-DERIVED STEM CELL

Stem cells are characterized by their self-renewal and replication capability. Stem cells have two types of division. Symmetric division can produce two identical daughter cells with stem cell properties. By asymmetric replication, the stem cell produces one cell retaining its self-renewing capacity, the other cell generating one or more specialized cell types.[5] Stem cells are classified into two sources with very different capabilities: embryonic stem cells (ESCs) and adult stem cells. ESCs are pluripotent and derived from the inner cell mass of the blastocyst.[6] Multipotent adult stem cells originate from one of the three germ layers, the endoderm, mesoderm, ectoderm and contribute to the maintenance of tissue homeostasis.[7]

ASC is a kind of adult stem cell originating from the mesoderm layer of the developing embryo. The stromal vascular fraction (SVF) pellet is a heterogenous combination of several stromal cells, adipose tissue stem cells, endothelial cells, erythrocytes, fibroblasts, lymphocytes, monocytes/macrophages and pericytes.[8] Pure ASCs can be isolated from SVF pellet which has been verified in previous studies.[9, 10] Briefly, after obtaining the lipoaspirate, a wash step with PBS and further digestion step with crude collagenase are carried out. The resulting digested adipose tissue is centrifuged to remove mature adipocyte and obtain the SVF pellet. The SVF pellet is further centrifuged and filtered after lysis of erythrocytes, and subsequently seeded onto a plastic surface overnight in a standard incubator, after 24 hours the non-adherent mononuclear cells are removed and mostly pure ASCs are produced (Figure 2-1).

Mesenchymal stem cells (MSCs) are characterized by the expression of cell-specific proteins and CD markers. In 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed three criteria to define the identification of human MSCs.

1) The plastic-adherent feature.

2) The ability to differentiate into adipogenic, chondrogenic and osteogenic lineages.

3) The presence of molecular markers CD73, CD90, CD105, and absence of CD11b or CD14, CD19 or CD79α, CD34, CD45, HLA-DR.[11]

MSCs can be found in different mesenchymal tissues including adipose tissue and bone marrow. A recent review from Bourin P, *et al.* compared the phenotypical differences between ASCs and bone marrow-derived mesenchymal stem cells (BM-MSCs). ASCs can be distinguished from BM-MSCs by their expression of CD36 and lack of CD106.[8] However, due to the lack of a single definitive marker, the identification of ASCs, to satisfy all the criteria, still needs all of stem cell properties such as tissue origin, CD marker profile, self-renewal ability and pluripotency.

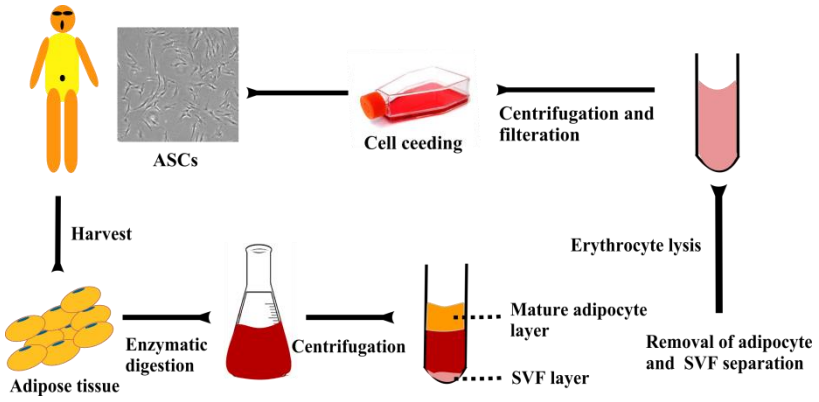


Figure 2-1. Isolation process of ASCs from adipose tissue via enzymatic digestion and centrifugation method. Abbreviations: SVF, stromal vascular fraction; ASCs, adipose-derived stem cells.

In addition, ASCs meet the important criteria in regenerative medicine applications based on the review from Gimble JM, *et al.*[12]

1) Abundant numbers can be obtained from the adipose tissue. Krawiec *et al*. have stated that ASCs yield between 100,000 and 1,000, 000 stem cell per gram of fat,

whereas the yield of MSCs is 100 to 1,000 cells from one milliliter of bone marrow. [13, 14,]

2) Differentiation ability along multiple cell lineages is reproducible.

3) The harvest procedure is minimally invasive and ASCs can be manufactured via current good manufacturing practice (cGMP) and transplanted safely to the host.

Induction of ASCs under defined conditions can result in their differentiation to multiple cell types containing fat,[15] bone,[16] cartilage,[17] muscle,[18] endothelium,[19] cardiomyocyte,[20] neurons[21] and liver lineages.[22] Thus, ASC have been widely used as a promising stem cell source in regenerative medicine applications.

HASCs used in this study were obtained from adipose tissue of two healthy patients in accordance with the above separation procedure. The study from our group has shown its adherence feature to plastic culture plates, proliferation abilities, as well as expression of MSC molecular markers CD29, CD44, CD73, CD90 and CD105.[23, 24, 25] Likewise, our study has suggested that ASCs can be differentiated into adipocytes, chondrocytes and osteocytes.[26, 27] Thus ASCs used in this study have been shown to satisfy all criteria, as stated preciously, and been approved be reliable and multipotent stem cells.

2.2 SMOOTH MUSCLE CELL

2.2.1. SMOOTH MUSCLE CELL CHARACTERISTICS

The SMCs originate from varied progenitors including neural crest, secondary heart field, somites, mesoangioblasts, proepicardium, splanchnic mesoderm, mesothelium and various stem cells and constitute part of major components of human body, including the respiratory, digestive, cardiovascular, urinary, reproductive and excretory systems.[28]

SMCs exhibit two phenotypes with different morphological characteristics, proliferative and migration ability, as well as specific gene marker expression.

Contractile SMCs are elongated spindle cells with rich contractile filaments in the cytoplasm. Synthetic SMCs contain highly developed organelles such as rough endoplasmic reticulum (RER) which is crucial to protein and extracellular matrix (ECM) synthesis. In addition, synthetic SMCs exhibit higher growth rates and higher migratory activity than contractile SMCs. Contractile phenotypical SMCs are characterized by increased expression of specific contractile proteins including smooth muscle alpha actin (α-SMA), calponin, caldesmon, smooth muscle myosin heavy chain (MHC) and specific contractile function. Both visceral and vascular SMCs are not terminally differentiated cells in the adult organism and are capable of switching between phenotypes in response to local environmental changes include mechanical forces, a variety of biochemical factors and growth factors (Figure 2-2).[29, 30, 31]

The contraction of SMC is regulated by calmodulin (CaM), a kind of cellular calcium receptor in the smooth muscle. The contraction can be initiated once the calcium ions binding to CaM. The CaM-calcium complex activates myosin light chain kinase (MLCK), which phosphorylates the regulatory subunits of myosin light chain (MLC20). The phosphorylation of MLC20 activates myosin ATPase, eliciting the cycling of myosin heads (crossbridges) binding to the actin filament causing smooth muscle contraction.[32, 33] Thus the increased expression of myosin protein is essential for contractile phenotypical SMCs and specific contractility.

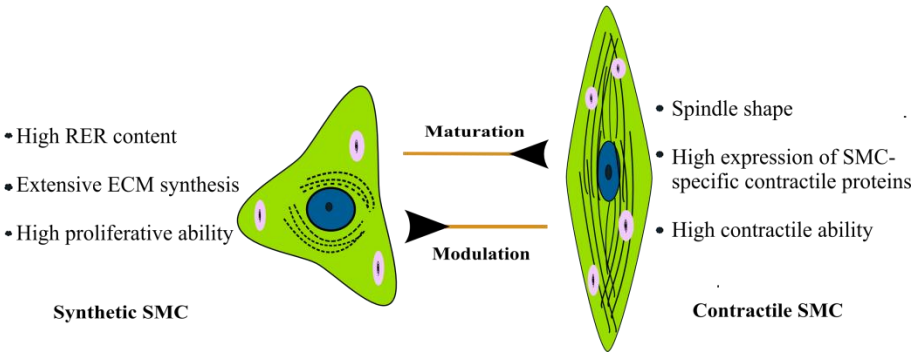


Figure 2-2. Characteristics of synthetic and contractile phenotypes of SMC. Abbreviations: RER, rough endoplasmic reticulum; ECM, extracellular matrix; SMC, smooth muscle cell.

2.2.2. SMOOTH MUSCLE CELL DIFFERENTIATION

Control of cellular differentiation is regulated by the level of gene transcription.[34] Gene transcription and SMC differentiation is controlled by a dynamic array of local environmental cues and extrinsic factors (Figure 2-3).[3, 35] Oxygen is one of the most important environmental components within stem cell specific niche, serving as metabolic substrate and signaling molecule and influencing the self-renewal and differentiation potential.[4] The effects of hypoxia on proliferation and differentiation on stem cells have been extensively explored. It has been shown that hypoxia promotes undifferentiated cell states and enhances cell proliferation in various stem cell populations such as neural stem cells, rat mesencephalic precursor cells, hematopoietic stem cells and ASCs.[36, 37, 38, 39] However, the effect of hypoxia on differentiation varies markedly depending on oxygen concentrations and committed cell lineages. According to the review from Zachar *et al.* 1% or 2% O_2 decreased chondrogenesis of ASCs when cells were seeded as human 3-D cultures. On the contrary, 2% O_2 increased the chondrogeneis of ASCs in human alginate cultures. Both 2% and 5% O_2 decreased osteogenesis of ASCs.[24]

In addition to oxygen regulation, biochemical factors associated with signaling pathways are critical elements. A number of different protocols have been classified to drive the differentiation of ASCs towards a smooth muscle like cell type, exhibiting similar morphology, gene and protein expression profiles as well as contractility.

1) Rodriguez *et al*. used 100 unit/ml heparin in medium MCDB131 for 6 weeks to successfully drive hASCs to differentiate into phenotypic and functional SMCs.[40]

2) Wang *et al.* used combination of TGF-β1 and BMP4 for 1 week to obtain SMC-like cells from ASCs.[41]

3) 5 ng/mL transforming growth factor-β1 (TGF-β1) along with 50 ng/mL platelet-derived growth factor (PDGF)-BB increased SMC-specific marker expression in ASCs.[42]

4) TGF-β1 alone (2 ng/ml for 3 weeks) enhanced SMC-specific marker expression in ASCs.[43]

5) Bradykinin, sphingosine1-phosphate, angiotensin II, sphingosylphosphorylcholine have shown to be able to induce ASCs to express SMC-specific markers.[44, 45, 46, 47, 48]

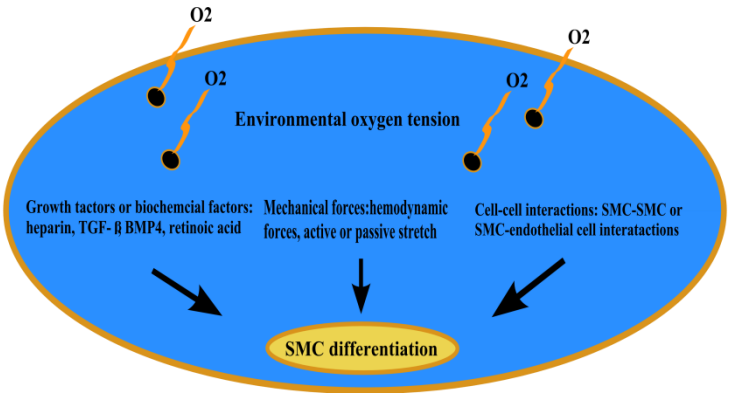


Figure 2-3. The major influencing factors of SMCs differentiation. Abbreviations: TGF-β, transforming growth factor-β; BMP4, bone morphogenetic protein-4; SMC, smooth muscle cell.

Apart from chemical signal modulation, cells and tissues are continuously subjected to diverse mechanical forces. It has been demonstrated that mechanical stimuli affect stem cell morphology, proliferation and differentiation.[49] For example, ASCs stimulated with 10% strain at 1Hz for 7 days inhibited proliferation and caused the cellular realignment perpendicular to the strain direction.[50] Huang et al. suggested that mechanical strain (10% cyclic stretching, 0.5 Hz, 48 hours) enhanced the proliferation of aging ASCs.[51] Another study suggested that cyclic uniaxial strain (10% cyclic strain, 1Hz, 24 hours) caused the myogenic differentiation of rat ASCs.[52]

With respect to underlying mechanism of SMCs differentiation, studies are roughly divided into two types, some studies explore the mechanism of phenotypical switch and SMC-specific markers expression, other studies induce stem cells to differentiate into SMCs using chemical stimulation and investigate the underlying mechanism. However, two kinds of studies have been shown the consistent results. During the processes of both phenotypical switch of SMCs and stem cell differentiation into SMCs, the critical signaling molecules involve serum response factor (SRF), myocardin family containing myocardin, myocardin-related transcription factor-A (MRTF-A) and myocardin-related transcription factor-B (MRTF-B), CArG ($CC(AT)_6GG$) box, a 10 bp cis-element located in the promoter of many genes restricted to adult SMCs. The nuclear localization and recruitment of the transcription factor SRF and coactivator myocardin binding to the CArG sequence initiating the SMC gene transcription.[53, 54, 55] For mechanical stimulation-associated SMCs differentiation mechanism, several key elements include ECM ligands such as collagen and fibronectin, the focal adhesions consisted of clustered integrins and accumulated cytoskeletal proteins, and phosphorylated signaling molecules such as focal adhesion kinase (FAK).[56] In addition, small GTPase RhoA/Rho associated kinase (ROCK) and intact cytoskeleton are essential for the expression of differentiation-related proteins in SMCs.[57]

A set of SMC-specific marker proteins have been used as a measure to detect differentiation of ASCs towards SMCs. SMCs express contractile proteins that are important for the physiologic needs in different stages of maturation and differentiation. Some of the most important SMC markers are α-SMA, caldesmon, calponin and MHC. All of these markers are contractile proteins that contribute to the contractile function.

Actins are highly conserved proteins including α, β, γ actins. The α-actins are found in muscle tissues including α-smooth muscle, α-cardiac, α-skeletal considered as tissue-specific actins. They are major constituents of the contractile apparatus. The β and γ actins co-exist in most cell types as components of the cytoskeleton. A-SMA is a 42-KDα globular protein (G-protein) and forms two-stranded helical filaments

(F-actin) after polymerization. Normally α-SMA can be expressed in vascular smooth muscle, but it can also be expressed in myofibroblasts. It is the first known protein detectable in differentiated SMCs and its level of expression goes up initially as the cell matures. It is also an abundant structure protein accounting for 40% of total cell protein and required for the generation of mechanical forces and contraction of differentiated SMCs.[3, 31, 32,58]

Calponin is known as a family of actin filament-associated proteins. Calponin is expressed in both smooth muscle and non-smooth cells containing three isoforms: h1, h2 and h3 calponin. The h1 isoform of calponin is specific to differentiated smooth muscle cells. The h2 and h3 calponins are found in various tissue including smooth muscle and non-muscle tissue. Calponin is an inhibitor of actin-activated myosin ATPase. Calponin binding to actin leads to inhibition of the actomyosin Mg^{2+}-ATPase and decrease of the sliding of actin filaments over myosin. When calponin is phosphorylated in vitro by protein kinases either Ca^{2+}/CaM-dependent protein kinase II (CAMK II) or protein kinase C (PKC), the inhibitory action is reversed. Thus calponin plays an important role in the regulation of actin-myosin interaction.[59, 60, 61]

Caldesmon is a thin filament-associated, actin and CaM-binding protein with an ample quantity in a variety of smooth muscles. Caldesmon has two kinds of isoforms, the heave caldsmon (h-CaD) found in differentiated SMCs and light isoform (l-CaD) in most types of cells. In SMCs, caldesmon also inhibits the actomyosin ATPase activity and myosin binding to actin. The inhibition is reversed when this protein is phosphorylated by a number of protein kinases including CAMK II, protein kinase A (PKA) or PKC. Therefore, caldesmon modulates the SMCs contraction process. Calponin and caldesmon constitute mid-phase markers of SMCs differentiation.[60, 62, 63]

MHC is a hexamer composed of two heavy chains and four light chains of 20 KDα and 17 KDα (MLC_{20} and MLC_{17}). The heavy chains comprise globular heads and helical tails. Each globular head contains a binding site for actin and actin-activated

magnesium-adenosine triphosphatase (ATPase). The phosphorylation of light chain of myosin is necessary for activating myosin ATPase, leading to the heads of myosin heavy chain repeatedly binding to the actin filament. Therefore, MHC plays a key role in the regulation of smooth muscle contraction. In addition, MHC, as an later marker of mature SMCs, is the most important marker for identification of differentiated SMCs.[31, 60, 63]

2.3. OXYGEN, A KEY MODULATOR

2.3.1. HYPOXIA AND HYPOXIA–INDUCIBLE FACTOR

Stem cells reside in specific niche and oxygen is a critical component of this microenvironment affecting the stem cell proliferation and differentiation.[64, 65] In general, environmental hypoxia refers to oxygen concentrations below the atmospheric 20% O_2 concentration. Physiological hypoxia is an effect of oxygen demand being higher than oxygen supply in the cellular microenvironment.[66, 67] Although most cell cultures and expansion are performed *in vitro* at 20% oxygen concentration, the actual oxygen tensions of many tissues in physiological environment are considerably lower: 1) in arterial blood around 13%, 2) in the veins 5%, 3) 1-6% in the bone marrow, 4) 2-8% in adipose tissue.[24, 64] Therefore, the "normoxic" oxygen concentration (20% O_2) used in cell biology studies does not reflect in vivo real conditions. On the contrary, traditional environmental hypoxia concentrations (below the atmospheric 20% O_2) should more precisely be referred to as in situ normoxia or physiological normoxia depending on the tissues.[67, 68]

The hypoxia-inducible factor (HIF) is a master regulator controlling cellular responses to hypoxia and maintenance of cellular homeostasis. HIF-α has three isoforms, hypoxia-inducible factor-1α (HIF-1α), hypoxia-inducible factor-2α (HIF-2α) and hypoxia-inducible factor-3α (HIF-3α). Hypoxia-inducible factor 1(HIF-1) is a heterodimer composed of HIF-1α and a constitutively expressed hypoxia-inducible factor-1β (HIF-1β), which is also known as the aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-1 is a member of the basic helix-loop-helix-Per-ARNT-Sim (bHLH-PAS) family of environmental sensors and can bind to hypoxia

response element (HRE) DNA sequences. In contrast to the HIF-1β subunit, the protein stability, cellular location, and transcription factor activity of the HIF-1α subunit is affected by oxygen. HIF-1α is rapidly degraded by the ubiquitin proteasome system under normoxia. On the contrary, the HIF-1α subunit is stabilized in hypoxic conditions, allowing the HIF-1 heterodimer to act as a transcription factor facilitating cell specific gene expressions (Figure 2-4).[69, 70, 71] The study from Jiang *et al.* quantitated HIF-1 DNA-binding activity and protein level in Hela cells under different oxygen concentrations indicating that HIF-1 DNA-binding activity and protein expression increased exponentially when cells were exposed decreasing oxygen concentrations. The maximum response was around 0.5% oxygen concentration, and a half maximal response between 1.5 and 2%. 5% was a critical point where HIF-1 DNA-binding activity and protein level were almost equal to the 20% level, which implied that oxygen concentration below 2% is real hypoxia, but from 5% to 20% was not the real hypoxia for Hela cells.[72] However, for distinct cell lines, the hypoxic critical point which can activate HIF-1 binding to DNA varies thus leading to different cellular responses for hypoxia levels.

When the expression of HIF-1α is increased due to hypoxic stimulation, hypoxia responsive genes and cellular physiological activities are regulated or controlled by HIF-1α along the signaling pathway direction.[73] For instance, hypoxia promoted the undifferentiated cell state in various stem and precursor cell populations.[74, 75] Hypoxia environment improves growth kinetics, genetic stability thus increasing in vitro expansion for MSCs by regulating HIF-1α mediated gene expression.[64] Gustafsson *et al*. demonstrated hypoxia (1% O_2) led to recruitment of HIF-1α to a Notch-responsive promoter and increased the Notch downstream genes, blocking myogenic differentiation in a Notch-dependent manner in myogenic cell line C_2C_{12}.[76] In addition, prolonged growth of ASCs in 5% oxygen enhanced vascular endothelia growth factor (VEGF) expression.[39] The hypoxia-induced expression of VEGF is mediated by the transcription factor HIF-1.[77] VEGF is able to activate mitogen-activated protein kinase (MAPK) signaling pathway which controls cell fate and differentiation processes.[78]

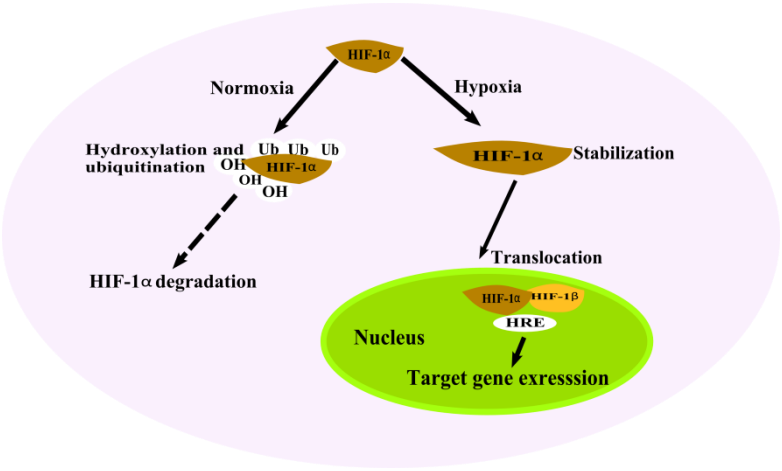


Figure 2-4. The effects of normoxia and hypoxia on HIF-1 protein. Abbreviations: HIF-1α, hypoxia-inducible factor-1α; HIF-1β, hypoxia-inducible factor-1β; OH, hydroxylation; Ub, ubiquitination; HRE, hypoxia response element.

2.3.2. HYPOXIA CONDITONING

Hypoxia has been shown to modify the metabolism and gene expression of MSCs thereby modulating their proliferation and differentiation abilities.[79] As a consequence, different levels of hypoxia have been tried as factors in stem cell differentiation and yield improvement.

In vivo different types of stem cells exist in a hypoxia microenvironment, which is beneficial for the maintenance of these cells and continuous replenishment.[38] Likewise, ASCs in the body are in relatively oxygen-deficient environment (1-5% O_2).[80] Several studies compared the environmental hypoxia concentrations with 20% O_2 in terms of the effects of hypoxia on the stem cell proliferation and maintenance of stemness showing consistent results: Närvä *et al*. suggested that prolonged hypoxia (4% O_2 for 7 days) enhanced the self-renewal ability and maintenance of the pluripotent state in human ESCs.[81] 1% oxygen promotes the maintenance of stemness of ASCs, 2% and 5% oxygen enhance the proliferation ability of ASCs.[39, 82, 83] The results can be explained by proliferating cells have a high metabolic rate

requiring more oxygen supply, cells in real tissues of the body may experience lower oxygen due to oxygen depletion and diffusion limitation, therefore, in vitro hypoxia culture condition might be more similar to in vivo stem cell niche microenvironment in terms of oxygen concentration leading to the better proliferation rate in hypoxia. However, studies regarding differentiation of ASCs under hypoxic conditions focusing on chondrogenesis and osteogenesis of ASCs showed different results: Malladi *et al.* indicated that 2% oxygen strongly inhibited chondrogenesis and osteogenesis in ASCs, but other groups suggested that 5% oxygen enhanced chondrogenesis and osteogenesis in ASCs and rat BM-MSCs.[84, 85, 86] Interestingly, Pilgaard *et al*. demonstrated that 15% oxygen provided the most suitable environment for inducing chondrogenesis in ASCs.[26] The varied results are depending on the committed differentiation lineages, hypoxia level as well as culture conditions.

The effect of hypoxia on SMCs proliferation and phenotypic switch has been investigated. Chronic level of hypoxia at 1% was shown to induce SMC proliferation and prolong cell life.[87] Hypoxic exposure at 3% level for 48 hours leaded to an increased cell number and a significant downregulation of SMC-specific marker.[88] However, according to Berthelemy *et al*., 5% O_2 switched the cellular morphology to SMC-like spindle shape and expressed the SMC contractile phenotypic markers when peripheral blood mononuclear cells were cultured with specific angiogenic growth factors.[89] These results highlight the importance of oxygen as well as differential regulatory role of hypoxia on the physiological proliferation and differentiation processes.

In addition to affecting ASCs proliferation rate and differentiation commitment, hypoxia can modulate the paracrine activity improving the survival and angiogenesis.[90, 91] Cell survival and angiogenesis are key factors to stem cell-based treatment and TE applications.[92] According to Barros *et al.* study, aging-related decrease of hASCs angiogenic potential was improved by hypoxia preconditioning.[93] Similarly, another study indicated that gene expression of pro-angiogenic factors including VEGF, placental growth factor (PIGF) and hepatic

growth factor (HGF) were downregulated with age, but partially restored by hypoxia (1% O_2 for 48 hours). Sun *et al.* study concluded that the conditioned medium from hypoxia-preconditioned ASCs improved wound healing in a rat skin defect model via angiogenesis and recruitment of circulating stem cells.[94] Another study from Hollenbeck *et al*. suggested that conditioned medium from hypoxia-preconditioned ASCs (0.5% O_2) increased VEGF levels and enhanced endothelial cell tubule formation. Additionally, this kind of conditioned medium improved flap viability likely through the effect of VEGF release in the rat model of flap ischemia.[95]

All these results demonstrated that hypoxia preconditioning is a feasible method to ameliorate ASC-based treatment.

2.4. GROWTH FACTORS

The generation of SMCs from ASC progenitors has been extensively investigated, indicating different biochemical factors are able to induce the ASCs differentiation into SMCs. The transcription regulation of SMC markers is related to the complex combination of *cis*-acting elements and *trans*-acting factors. *Cis*-acting elements are located within the promoters of vascular smooth muscle cells (VSMCs). The CArG box found in the promoters of contractile genes is a *cis*-acting element which is vital for the regulation of VSMCs gene expression.[30] Extensive studies have shown TGF-β to be the potent soluble growth factor promoting a number of cell types including ESCs, BM-MSCs, neural crest stem cells differentiate into SMCs.[96, 97, 98, 99] TGF-β1 is a multifunctional protein which plays critical roles in a variety of biological processes including cell growth, differentiation and migration.[100, 101, 102] Bone morphogenetic proteins (BMPs) are multifunctional growth factors representing the largest group in the TGF cytokine superfamily.[103, 104] Lagna *et al.* reported that the BMPs signaling pathway effectively induced contractile phenotype and SMC-specific genes transcription. Nuclear localization and recruitment of the MRTF-A and MRTF-B transcription factors to a smooth muscle α-actin promoter were observed in response to BMP4.[105] In addition, a combination of TGF-β1 (5 ng/ml) and BMP4 (2.5 ng/ml) stimulation for 1 week was shown to drive efficiently the

ASCs into mature contractile SMCs.[41] Similarly, TGF-β1 and BMP4 was shown to reduce VSMCs proliferation and migration and promote expression of VSMCs contractile genes.[43] The study from Lagna *et al.* provided evidence that SMC phenotypic switch induced by BMP4 from synthetic to contractile was dependent on the Smad and RhoA/Rho kinase signaling pathway. The BMP4 pathway activated transcription of SMC genes by inducing nuclear translocation of the transcription factors MRTF-A and MRTF-B, binding the CArG box found within many SMC-specific gene promoters.[105]

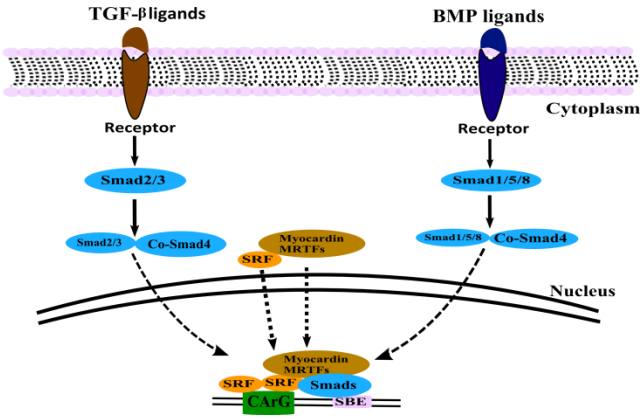
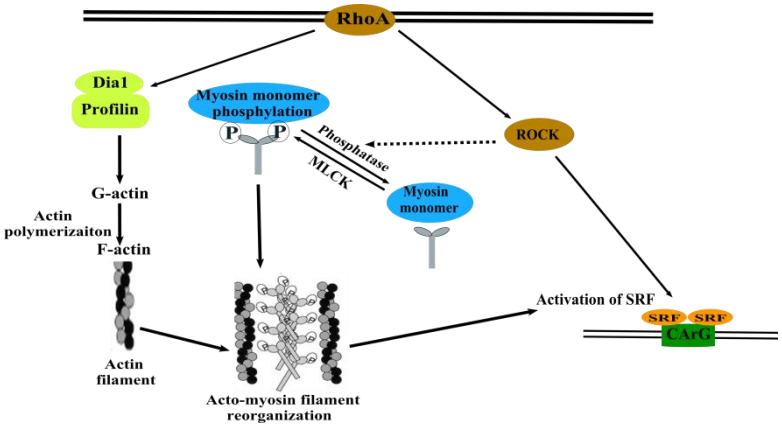


Figure 2-5. TGF-β and BMP signaling pathway of SMCs differentiation. Abbreviations: SRF, serum response factor; MRTFs, myocardin-related transcription factors; SBE, Smad binding element; CArG, $CC(A/T)_6GG$.

Therefore, both TGF-β1 and BMP4 induce SMC contractile genes, but may in combination exert a synergistic influence on differentiation through two independent, but crosstalking signaling pathways (Figure 2-5). Based on the Kretschmer *et al.* study, Smad2, 3 and 4 were shown to contribute to the regulation of TGF-β responses to different extents.[106] Signal transduction studies revealed that Smad1, 5 and 8 are the immediate downstream molecules of BMP receptors and play a central role in BMP signal transduction.[104] In general, signaling is initiated with ligand-induced oligomerization of serine/threonine receptor kinases and phosphorylation of the cytoplasmic signaling molecules Smad2/3 for the TGF-β pathway, or Smad1/5/8

for the BMP pathway. Activated Smads complex with the common signaling transducer Smad4 and then translocate to the nucleus.[107] Activation of TGF-β1or BMP4 related signaling molecules induce the expression of SRF and coactivator myocardin and further translocation to the nucleus.[105] The Smads contain DNA binding motifs and can bind to Smad binding elements (SBE). However, due to the low affinity of Smads and SBE, they regulate transcription in combination with additional transcription factors and cofactors.[35] The complex of transcription factor comprising SRF dimers and its coactivators, such as myocardin, MRTF-A or MRTF-B can bind to the CArG sequence initiating the SMC gene transcription.[108]

RhoA/ROCK is closely related to SMC gene transcriptional regulation. As shown in Figure 2-6, RhoA activates its effector Dia1 and actin-binding factor profilin, which triggers actin polymerization. ROCK also inhibits MLC phosphatase, indirectly leading to increased phosphorylation of myosin monomers and myosin filament polymerization. Therefore, RhoA regulates actin and myosin filament polymerization. In addition, SRF activation and nuclear translocation can be induced both by ROCK and by changes in stress fiber dynamics such as cell stretching. SRF dimers are bound to CArG boxes of the promoter triggering the SMC-specific gene transcription processes.[29]



Modified from plasticity in skeletal, cardiac and smooth muscle invited review: molecular mechanisms of phenotypic plasticity in smooth muscle cells. Journal of Applied Physiology, 2001(90), 358-368.

Figure 2-6. RhoA/ROCK signaling pathway of SMCs differentiation. Abbreviations: ROCK, Rho associated kinase; MLC, myosin light chain; MLCK, myosin light chain kinase; SRF, serum response factor; CArG, CC(A/T)6GG.

2.5. MECHANICAL STRETCH

Mechanical stretch also plays a vital role in controlling cell morphology, proliferation, lineage commitment and differentiation.[109, 110] All cells within the context of a 3D microenvironment are exposed to diverse mechanical forces in the body, for instance, the smooth muscle of media of arterial vessel wall is always exposed to mechanical cyclic strain in the circumferential direction. To mimic the physiological mechanical environment in vivo, various techniques of mechanical stimulation have been applied. Such as shear stress generated by flow fluid, mechanical stretching, compressive load exerted by hydrostatic presure.[111, 112] Cyclic tensile strain (CTS) is a common approach to mimic in vivo cyclic circumferential strain experienced by SMCs, as shown in Figure 2-7. Stretching parameters such as mode (uniaxial, biaxial and equiaxial), magnitude and duration have been found to elicit distinct cellular responses.[109, 113, 114] Hamilton *et al*. utilized a FX-4000T strain unit to upregulate a-SMA and h1-calponin in rat bone marrow-derived progenitor cells (BMPCs) after 10% uniaxial cyclic strain at 1 Hz for 7 days.[115] Another study showed that rat BMPCs were able to differentiate toward a SMC-like lineage which was verified by increased expression of SMC markers a-SMA and h1-calponin after BMPCs were suspended in fibrin gel, pipetted into the trough of Flexcell plates and then stimulated with 10% longitudinal cyclic stretch at 1 Hz for 6 days.[116]

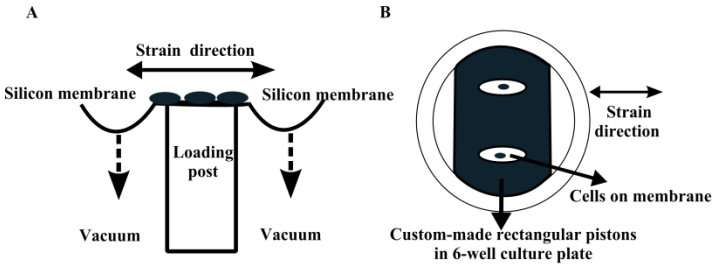


Figure 2-7. (A) Schematic diagram of Flexcell tension system. The system can drop the silicon membrane through the vacuum, therefore producing the horizontal direction stretching for the cells on the membrane. (B) One well of 6-well culture plate showing the custom-made rectangular piston in the middle. Vacuum system can produce the strain for the cell on the membrane.

A number of sensory elements are inherent in cells and can sense external forces such as stretch through mechanosensing process. Mechanotransduction is the conversion process by which the cells sense mechanical stimuli and translate this into biochemical signals.[117] The focal adhesions as a kind of mechanosensory complexes play an important role during mechanosensing and mechanotransduction processes, and the dynamic protein complexes consist of clustered integrins and accumulated cytoskeletal proteins and phosphorylated signaling molecules such as FAK.[56] They link the ECM and actomyosin cytoskeleton serving as a conduit through which signal transduction occurs in response to physical force.[117] The cytoskeleton is the primary mechanical component of cellular structure and it is responsible for maintaining mechanical homeostasis. Also, cytoskeletal reorganization are the main processes during mechanically induced cellular differentiation.[109] The study suggested that Rho and an intact cytoskeleton are essential for mechanotransduction and the expression of differentiation related proteins in SMCs.[118, 119] In addition, RhoA/ROCK is also a key factor for inducing myogenic differentiation through regulating the actin polymerization, reorganization of actomyosin filaments, MLC phosphorylation, as well as activity of SRF and cofactor MRTFs.[29, 57, 120]

Based on the previous studies, the possible SMCs differentiation signaling pathway induced by stretching is summarized in Figure 2-8, including the integrins, integrin-linked kinase (ILK), FAK, RhoA/ROCK, cytoskeletal proteins and cytoskeletal reorganization. Integrins are transmembrane heterodimers receptor composed of non-covalently bound transmembrane α and β subunits. Each subunit of the integrin heterodimers contains a large extracellular domain, a transmembrane domain and a short cytoplasmic tail.[121] Integrins are capable of binding numerous ECM ligands including fibronectin, fibrinogen as well as collagens, transmitting forces from the external environment across the cell membrane.[117, 122] Mechanical stimulation can affect the extracellular matrix, making the ligands in extracellular matrix bind to the integrins. The integrin then transmits extracellular stimuli into intracellular signaling events including phosphorylation of tyrosine kinases such as FAK, Src, as well as adaptor protein p130Cas.[56] FAK phosphorylation can result in tyrosine phosphorylation of cytoskeletal proteins resulting in rapid cytoskeletal reorganization.[122] There are several key focal adhesions involved in establishing and maintaining the integrin cytoskeleton linkage.

1) Talin, α-actinin and filamin, integrin-bound proteins, can directly bind to actin.

2) FAK, ILK and paxillin, also integrin-bound proteins, can indirectly bind the cytoskeleton.

3) Non-integrin bound actin-binding protein such as vinculin.

4) Adaptor and signaling molecules which regulate the interactions of proteins.[121]

Ligand binding and the phosphorylation of signaling molecules lead to the assembly of actin filaments and the activation of downstream small G protein signaling molecule such as RhoA, which can affect actin polymerization and cytoskeleton reorganzization.[121, 123] In addition, ILK is a multidomain adaptor protein which directly binds integrin tails and indirectly associates with actin through its main binding partner parvin. The study showed that ILK and parvin complex can modulate the actin cytoskeleton and the actin polymerization through its interactions

with paxillin, vinculin or other signaling molecules.[124] Thus, a stretch signal can be transduced into internal biochemical signal through a series of cascading proteins resulting in the cytoskeletal reorganization.[122, 124] RhoA/ROCK activation, cytoskeletal organization and stress fibers changes can induce SRF activation and nuclear translocation by binding to the CArG box, inducing SMC-specific gene transcription.

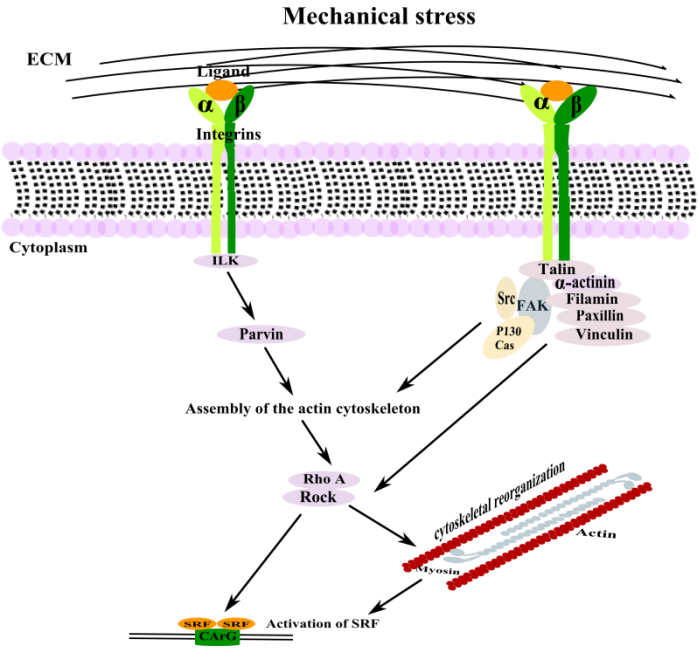


Figure 2-8. Signaling pathway of mechanotransduction of stretching affecting SMCs differentiation. Abbreviations: ILK, integrin-linked kinase; FAK, focal adhesion kinase; ROCK, Rho associated kinase; SRF, serum response factor; CArG, CC(A/T)6GG.

2.6. ESOPHAGEAL MUSCLE LAYER TISSUE ENGINEERING

Esophageal cancer is the leading cause of cancer-related deaths worldwide with high morbidity and mortality. In addition, every year 5000-10000 patients are diagnosed with congenital or acquired diseases such as atresia, Barrett's esophagus and

strictures.[125] Current treatment often requires the removal and reconstruction of segmental esophagus and severe complications limit its clinical application.[126]

A tissue engineered esophagus is an ideal method for the reconstruction of damaged esophagus. The two key elements in tissue engineered esophagus are the suitable scaffold as a support for cell growth and tissue development and the viable specialized cells. Recent decades witnessed the rapid advances in field of the scaffold of esophagus. The use of double layered collagen/silicone tubes, absorbable constructs and decellularized matrices are the most commonly scaffolds in esophageal reconstruction. Earlier artificial conduit replacements made from polytetrafluoroethylene (PTEE) were used to replace damaged esophageal tissue, however, these replacements were unsuccessful due to complications such as leakage, extrusion and stenosis.[127] Some studies used collagen scaffolds and silicon stents for in vivo esophageal tissue regeneration, but post-operation stenosis limits their usage.[128] Synthetic polymers were attempted as a substrate to support epithelial cells or SMCs of tissue engineered esophagus.[129, 130] However, The surface of these synthetic materials are biologically inert impeding the integrity of cells and polymers.[131] As noted in the preceding sections, obtaining mature and contractile SMCs from smooth muscle tissue of human body were rather difficult, thus using ASCs derived SMCs for esophagus TE is an important clinical application.

The esophagus is composed of four distinct layers: mucosa, submucosa, muscularis externa and adventitia. The muscularis externa are divided into two distinct layers: the inner circular muscle cells and an outer longitudinal muscle layer. The esophagus consists of mainly three types of cells: stratified squamous epithelial cells, smooth or skeletal muscle cells and fibroblasts. Skeletal muscle constitutes the upper third, a mixture of skeletal and smooth muscle exists in the middle third, and only smooth muscle in the lower third of the esophageal muscle layer.[127] The esophageal conduit extends from the stomach to the intestine conducting the major function of peristaltic food transport. Esophagus is stretched circumferentially to 50% under 3-5KPa form normal food bolus, which requires the special mechanical properties such as sufficient elasticity.[127] The presence of elastic and collagen fibers is

advantageous for mechanical properties. The SMCs or skeletal muscle cells in the media of esophagus contribute its particular peristalsis and motility. In view of the requirements of structure and function of native esophagus, one review summarized the important factors while designing the scaffold, 1) mechanical properties of the scaffold itself, 2) porosity to meet gas and nutrient exchange, 3) degradation rates and biocompatibility.[132]

All in vivo cells are surrounded by ECM, ECM is not only a simple supporting structure, but provides the appropriate physical and chemical cues guiding cellular survival, proliferation and differentiation.[133, 134, 135] Thus ECM-mimicking scaffolds should be an ideal candidate for constructing tissue engineering esophagus. However, native ECM is composed of many kinds of proteins presenting intricate structures. Some studies have demonstrated that the components of ECM contain collagen, glycosminoglycans (GAGs), fibronectin, laminin, various growth factors as well as a number of unidentified proteins.[136, 137] Therefore, it is difficult to mimic the same composition and microstructure as that of the native esophageal ECM.

However, acellular matrix scaffolds from a variety of tissues such as acellular porcine aorta matrix,[138] gastric acellular matrix,[139] decellularized human skin (AlloDerm),[140] porcine urinary bladder[141] and porcine acellular small intestinal submucosa (SIS)[142] have been widely utilized as a scaffold for cell repopulation in preclinical animal studies for esophagus repair. In comparison with these grafts, EAM scaffold from esophagus has more advantages due to similar microarchitecture, biomechanical properties and biochemical cues to the native esophagus, which is important in directing cells to generate appropriate cellular responses during the structure and functional regeneration.

The production methods of decellularized matrices have been demonstrated to completely remove the cellular components while remaining the ECM intact.[133] Common approaches include physical sonication, cyclic freezing and thawing, chemical alkalis, acids, organic solvents, hypotonic solutions, as well as nuclease treatment.[133] In this study, we used a combination of methods to obtain the EAM

from the porcine esophagus, which has been verified an effective method to remove all cell components while keeping the structure and composition of the native ECM intact, as verified by the integrity of the collagen matrix after cell removal (Figure 2-9).

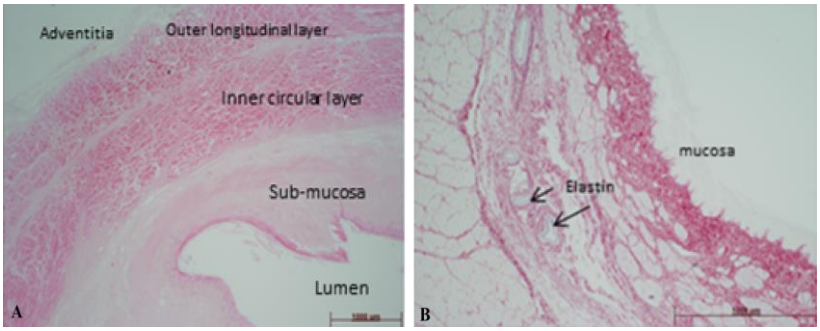


Figure 2-9. Histological evaluation of EAM scaffold. (A) Decellularized porcine esophagus stained with H&E staining. (B) Fine elastin fibers in gray color and collagen in pink color preserved and stained with picrosirius red and Miller's elastin staining.

Several studies compared the performance of combined EAM with stem cells or EAM alone in animal trials to investigate the better methods for clinical application of engineered tissues. Tan *et al*. used engineered esophagus comprised of the porcine acellular SIS and autologous BM-MSCs to repair esophagus excised dogs. Results indicated that the BMSC-SIS construct promoted reepithelialization, revascularization and muscular regeneration as compared with the SIS alone.[142] Similarly, Marzaro *et al* indicated that acellular matrix implant showed SMCs ingrowth and decreased inflammation response compared with acellular matrix alone 3 week after surgery in a pig animal model.[143] Likewise, results from Badylak group showed that ECM bioscaffold plus autologous muscle tissue, but not ECM alone could facilitate in situ reconstitution of esophagus tissue.[144] Based on these data, there are significant positive results when cell-scaffold constructs are implanted in the body of animals. In contrast, scaffold alone as a replacement leads to poor results. Therefore an integrated construct containing SMCs from ASCs and

esophageal EAM scaffold was utilized to reconstruct tissue engineering muscle layer in this study.

3. AIMS AND HYPOTHESES

The goal of this thesis was to explore the effects of hypoxia, mechanical stretching as well as biochemical factor stimulation on differentiation of SMCs from hASCs, and investigate the feasibility of constructing esophageal smooth muscle layer using porcine derived EAM scaffold and SMCs differentiated from hASCs.

There are three hypotheses and corresponding studies in this thesis:

1. Hypoxia can enhance differentiation of hASCs into SMCs. To test this hypothesis, hASCs was differentiated with 5 ng TGF-β1 and 2.5 ng BMP4 in combination for 2 weeks in four different oxygen concentrations. Expression of SMC-specific marker genes and proteins as well as specific contractility were determined.

2. Combined effects of biochemical factors and mechanical stretching can promote the differentiation of SMCs from hASCs or hASCs preconditioned in hypoxia. To test this hypothesis, hASCs or hASCs preconditioned in 5% O_2 were treated with biochemical factors and mechanical stretching in combination. Cell reorientation, F-actin realignment as well as expression of SMC-specific markers were detected.

3. Tissue engineered esophageal muscle layer can be reconstructed using the porcine esophageal EAM scaffold and SMCs differentiated from hASCs. To test this hypothesis, proliferated or differentiated status of hASCs in 5% or 20% O_2 were seeded on the porcine esophageal EAM scaffold. Morphology of EAM scaffold was analyzed and cellular proliferation ability, viability and migration depth were examined.

4. RESULTS

STUDY I

HYPOXIA ENHANCES DIFFERENTIATION OF ADIPOSE-DERIVED STEM CELLS TO SMOOTH MUSCLE CELLS

STUDY II

COMBINED EFFECTS OF BIOCHEMICAL FACTORS AND CYCLIC STRAIN ON THE SMOOTH MYOGENIC DIFFERENTIATION OF ADIPOSE-DERIVED STEM CELLS PRECONDITIONED IN HYPOXIA

STUDY III

REGENERATION OF THE ESOPHAGEAL MUSCLE LAYER FROM ESOPHAGUS ACELLULAR MATRIX SCAFFOLD USING ADIPOSE-DERIVED STEM CELLS

5. SUMMARIZING DISCUSSION AND CONCLUSIONS

ASCs have been a preferred stem cell source in the repair of damaged tissue and reconstruction of diseased organs and have a potential to differentiate into functional SMCs. Thus, SMCs differentiated from ASCs is an ideal source of cells for tissue engineering requiring SMC for normal tissue function such as blood vessels, esophagus, intestines etc. ASCs can be driven into SMCs via different approaches such as biochemical or mechanical stimulation, oxygen being a key factor affecting the differentiation process. This thesis investigated the differentiation process and the effects of the environmental factors oxygen, biochemical and physical stimulation on the differentiation process. In addition, reconstructing the muscle layer of esophagus using the ASCs in different oxygen concentrations was tested to obtain preliminary results anticipating future clinical applications.

1) Hypoxia enhances differentiation of hASCs to SMCs in combination with biochemical factors stimulation

HASCs were differentiated into functional SMCs with TGF-β1 and BMP4 in combination for 2 weeks. Differentiated hASCs expressed the SMC-specific markers α-SMA, calponin, caldesmon and MHC. Differentiated hASCs were able to contract in response to a muscarinic agonist. The dynamic contraction process of differentiated hASCs was clearly shown using different cell contraction assays.

Although there is no previous data on the effect of hypoxia on the SMCs differentiation from ASCs, a study by Lennon *et al.* showed that the markers of osteogenic differentiation were elevated when grown at 5% oxygen in rat BM-MSCs.[86] Khan *et al.* reported that 5% oxygen enhanced chondrogenesis.[145] It was implied that 5% oxygen might be an appropriate oxygen concentration for ASCs differentiation along certain lineages. When comparing the effect of hypoxia on the differentiation of SMCs from hASCs, four oxygen concentrations of 2, 5, 10 and 20%

were performed, the expression of SMC-specific genes and proteins, single cell optimal oxygen environment for the ASC to SMC differentiation process.

Normoxic oxygen concentrations (20% O_2) typically used in cell biology studies does not reflect the in vivo situation since the actual oxygen tensions of most tissues are much lower, for example, the physiological relevant oxygen concentration in normal adipose tissue is approximately 2-8%. Thus, 5% oxygen probably does not reflect the real hypoxia condition for ASCs, instead, it might be closer to normal in situ oxygen levels experienced by the cells providing the optimal oxygen concentration beneficial to ASCs differentiation. Prolonged growth of adipose stem cells in 5% oxygen was previously shown by our group to enhance VEGF expression.[39] In smooth muscle, stimulation with TGF-β was shown to activate VEGF transcription initiated by a Smad3-HIF-1α complex.[146] VEGF was also shown to increase expression of α-SMA and initiate cell contraction measured in a gel contraction assay.[147] Thus we hypothesize that hypoxia enhances the effect of TGF-β, possibly by the autocrine stimulation by VEGF, acting on the VEGF receptors. Both TGF-β and VEGF activates mitogen-activated protein kinase (MAPK) signaling pathway which controls cell fate and differentiation processes.[78]

2) Combined effects of biochemical factors and CTS promote the differentiation of hASCs and preconditioned hASCs into SMCs

We demonstrated in the first study that TGF-β1 and BMP4 in combination with 5% oxygen provides the best conditions for the ASC to SMC differentiation process. Subsequently these were used as reference conditions in the second study on mechanical stimulation. To explore the effects of mechanical stimulation, biochemical molecules as well as oxygen level on the hASCs differentiation into SMCs. HASCs were subjected to differentiation by biochemical factors and CTS for 1 week. In addition, hASCs differentiated in 5% O_2 for 1 week ($hASCs_{pre}$) were used to investigate the effect of hypoxic preconditioning, mechanical stimulation and biochemical factors stimulation on differentiation of ASCs.

MHC, as a later marker of mature SMCs, is the most important gene and protein marker in the differentiation process, therefore we chose MHC to evaluate the differentiation status of SMCs by real time RT-PCR and immunofluorescence staining assays. The results showed the combined treatments promoted expression of MHC for both hASCs and $hASCs_{pre}$. Additionally we showed mechanical stimulation of cells reoriented the cytoskeleton of both hASCs and $hASCs_{pre}$, visualized by F-actin staining. Our study demonstrated that mechanical stimulation, biochemical molecules as well as oxygen level are vital regulators of cell differentiation acting in synergy to promote the ASCs differentiation into SMCs.

Mechanical stretching affects a variety of cellular properties and biological response, such as cell shape, orientation, cell realignment and cytoskeletal remodeling.[148, 149, 150, 151] Cells randomly orient themselves prior to mechanical stimulation, and once exposed to mechanical strain, cells realign the long axis in the direction of minimal strain.[150] Our results showed that after 7 days stretching, cells displayed an elongated morphology compared to biochemical factors stimulation alone. The percentage of cells angles between 80-100 degree reaches 16% compared to 6% prior to stretching. Also our study showed that cellular F-actin realigned perpendicular to the stretching direction. Differentiation can cause changes in cell shape and function, changes in cell shape can alter the differentiation of mesenchymal lineages. In addition, the actin cytoskeletal pattern determines cell shape, which in turn influences cell phenotypic expression.[151] Rho/ROCK signaling pathway is associated with the cytoskeleton and cellular contractility and is responsible for upregulating stress fibers in response to increased force.[152] Rho/ROCK has known to exert a critical effect on the commitment of cells as reduced Rho activity favors adipogenesis and increased Rho activity favors myogenesis.[153] Thus the cell shape switch and F-actin remodeling might be in a certain way by which cells can recognize and activate signal pathway relevant to Rho/ROCK leading to the SMCs differentiation.

3) Differentiated ASCs as an alternative cell source for SMCs in esophageal muscle layer reconstruction

Our study found that the differentiated hASCs were able to attach onto the muscle layer of EAM scaffold when the cells were seeded from the outer muscle layer of a porcine esophagus, independent on oxygen conditions. Differentiated hASCs exhibited similar behavior to hASMCs after 7 days in terms of attachment, proliferation and viability. All these results suggested that the ASCs might be a substitute for SMCs when reconstructing the esophageal muscle layer in tissue engineering applications.

The muscularis externa includes the inner circular muscle cells and the outer longitudinal muscle cells. Using an acellular matrix has the advantage of mimicking the native ECM due to its similar anatomic structure, physical properties and chemical cues. The molecules fibronectin and laminin were demonstrated to be present in the EAM.[154] Ubiquitous fibronectin mediates adhesion of human microvascular endothelial cells to the porcine derived extracellular matrix.[155] Our data showed that after 24 hours, constructs composed of scaffolds and different cells exhibited good integrity between EAM scaffolds and cells, which implied adhesion molecules of surface in EAM were appropriate for the initial cell attachment.

Bhrany *et al.* demonstrated the EAM proteins collagen, elastin, laminin and fibronectin were retained after the decellularization process. However, laminin was either disrupted or disorganized after the decellularization, and this alteration might lead to the decrease of proliferation capability for esophageal muscle cells in the EAM.[154] Consistent with their study, in our study all types of cells attached to the EAM scaffold after 24 hours, but, compared to cells cultured in common culture dishes, proliferation capability of cells on the EAM substrate is limited with thousands of cells per cm^2 after 7 days.

Fibronectin and collagen were located in the intermuscular septae surrounding individual muscle cells.[156] However, the concentration of these two proteins decreased after the decellularization process, moreover, the collagen structure was changed from a characteristic banding pattern to loosely fiber bundles.[154] These alterations might affect the cell migration ability. In respect to cellular viability, the result showed that most cells survived after 7 days in culture on the EAM scaffold, which implied that the oxygen and nutrient was sufficient for most cells on the surface of substrate. In addition, our results showed that the hASCs differentiated in 5% or 20% oxygen concentrations did not show great difference either in the migration depth and cellular viability although we previously confirmed the 5% oxygen increased the expression of SMC-specific markers and contractile ability.

In conclusion, the findings of this thesis have demonstrated that:

1) HASCs could be differentiated into SMCs by in vitro induction with a combination of TGF-β1 and BMP4 for 2 weeks. 5% oxygen was the optimal condition to generate SMCs derived from hASCs. Differentiated ASC exhibited the high expression of SMC-specific genes and proteins as well as SMC-specific contractile ability.

2) Biochemical factors and CTS in combination promoted both hASCs and $hASCs_{pre}$ to express SMC-specific late marker MHC. CTS reoriented hASCs and $hASCs_{pre}$ and realigned the stress fiber perpendicular to the direction of strain. These results make it promising for enhancing ASCs differentiation into SMCs using combined treatments along with the cellular precondition in hypoxia.

3) Differentiated hASCs in hypoxia and normoxia could attach and survive on the porcine EAM scaffold muscle layer in vitro and exhibited abilities similar to hASMCs in terms of attachment, proliferation capability and viability, providing a promising alternative cell source for esophageal muscle layer tissue engineering.

6. LIMITATIONS AND FUTURE PERSPECTIVES

The main theme of this thesis has been describing the role of oxygen in driving the differentiation process of turning adipose stem cells into functional smooth muscle cells, in combination with both biochemical and mechanical stimuli. The results clearly showed an effect of oxygen and added further evidence to the feasibility of using ASCs in future tissue engineering applications requiring smooth muscle tissue and cells. However, the studies in this thesis were not complete due to several limitations.

For example, in the single cell contractile assay, due to experimental constraints, it was only carried out at 20% O_2 which limits the direct comparison of dynamic contractility of SMCs between hypoxia concentrations and 20% O_2. In addition, only two cell lines were used and more samples from different age, sex as well as fat tissue location should be compared to ensure the accuracy of the methodology and clinical application. Although we demonstrated that the SMCs from hASCs were differentiated successfully in gene, protein expression as well as cellular contractile function, the mechanism underlying the differentiation process was not investigated. We also demonstrated that carbachol could induce differentiated hASCs to contract, but the underlying mechanism should be further investigated. 5% oxygen was found to be the optimal concentration both for SMC-specific gene expressions and contractile ability, the expression of proteins HIF1α and relevant signaling molecules VEGF should be investigated to clarify the underlying mechanisms of oxygen signaling in the process of SMCs differentiation from ASCs.

Future studies should link influencing factors to mimic in vivo stem cell microenvironment cues inducing cells to response for the integrated stimulation. Since integrins, FAK and cellular cytoskeletal proteins, Rho/ROCK activation are all related to the mechanical induction of ASC to SMCs differentiation, these

signaling molecules will be relevant target proteins to clarify the converging point of the crosstalk pathway between mechanical and chemical factors.

Mechanical properties and chemical cues of the acellular scaffold obtained from porcine esophagus are similar to original esophagus. The hASMCs were acquired from human aorta. Thus constructs closely resembled in vivo esophageal smooth muscle layer. But constructs were cultured for only one week in vitro, which limits the results in terms of cell migration depth and long term viability of cells. Additionally, this study did not conduct animal trials or clinical trials to verify the results of experiments, which is needed for subsequent clinical application. It has been shown that epithelial cells could be seeded and grown on EAM scaffold. A future study should combine the application of both ASCs and epithelial cells to produce a more complete esophagus structure. Utilizing bioreactor producing dynamic culture environment should be beneficial to oxygen and nutrient supply as well as cell migration.

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SUMMARY

Adipose-derived stem cells (ASCs) are increasingly being used for regenerative medicine and tissue engineering. Smooth muscle cells (SMCs) can be differentiated from ASCs. Oxygen is a key factor influencing the stem cell differentiation. Tissue engineered esophagus has been a preferred solution for diseased esophagus replacement. The first part involved the effect of hypoxia on differentiation. The results showed 5% hypoxia to be the optimal condition for differentiation of ASCs into contractile SMCs. In the second part, the combined effects of mechanical strain (10% cyclic tensile strain) and biochemical factor stimulation on SMCs differentiation were studied. The results showed that combined treatments promoted the late SMC-specific marker smooth muscle myosin heavy chain (MHC) expression. In the third part, the potential for using ASCs to replace SMCs to regenerate the smooth muscle layer of esophagus was studied. Our results showed that both SMCs and ASCs could attach on the porcine esophageal acellular matrix (EAM) scaffold in vitro after 24 hours and survive until 7 days. Thus ASCs might be a substitute for SMCs in the construction of tissue engineered esophageal muscle layer.

ISSN (online): 2246-1302
ISBN (online): 978-87-7112-325-8

AALBORG **UNIVERSITY PRESS**