UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA ANIMAL

The role of microRNA-20a in the TGFβ-ALK5-Smad2/3 signaling pathway and ability to inhibit the Endothelial-Mesenchymal Transition

Ana Cláudia Pacheco Correia

Dissertação

Mestrado em Biologia Humana e Ambiente

2013

UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA ANIMAL

The role of microRNA-20a in the TGFβ-ALK5-Smad2/3 signaling pathway and ability to inhibit the Endothelial-Mesenchymal Transition

Ana Cláudia Pacheco Correia

Dissertacão

Mestrado em Biologia Humana e Ambiente

Orientada por:

Doutor Guido Krenning

(CAVAREM, Department of. Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, The Netherlands)

Professora Doutora Deodália Dias

(Departamento em Biologia Animal, Faculdade de Ciências da Universidade de Lisboa, Portugal)

2013

The study present in this thesis was performed in the Cardiovascular Regenerative Medicine Research Group (CAVAREM), at the Department of Pathology and Medical Biology, University Medical Center of Groningen, University of Groningen, The Netherlands, under the supervision of Guido Krenning, Ph.D.

This work was supported with founding from Groningen University Institute for Drug Exploration (GUIDE) and Netherlands Organization for Scientific Research (NWO)

The study present in this thesis was developed under the frame of the following publication:

Abstract:

Correia AC., Moonen J, Harmsen MC, Krenning G. Role of microRNA-20a in the TGFβ-ALK5-Smad2/3 signalling pathway and ability to inhibit the EndMT process. *20th International Student Congress of (Bio) Medical Sciences*, Groningen, The Netherlands, 2013.

[Selected oral presentation]

| Nota Prévia

A escrita desta tese de mestrado encontra-se na língua Inglesa uma vez que esta é a língua científica universal. Por esta razão, o conhecimento e treino da escrita e gramática revestem-se de uma importância acrescida para quem tenciona seguir uma carreira em investigação científica. A escrita da presente tese nesta língua representa assim um exercício apropriado que poder-se-á revelar proveitoso no futuro.

No decorrer deste mestrado foram reunidas as condições para a escrita de um artigo científico a submeter a revistas internacionais, razão pela qual a presente tese foi escrita sob a forma de uma publicação científica. Desta forma visa-se acelerar o processo de elaboração do manuscrito e a sua subsequente publicação. O manuscrito foi escrito de acordo com as instruções para autores da respectiva revista científica a que se pretende submeter, seguindo as directrizes da revista "Circulation". No entanto, para facilitar a leitura, as figuras e tabelas foram incluidas ao longo dotexto.

Assim como o manuscrito, as referências bibliográficas da Part I foram elaboradas segundo os parâmetros da mesma revista científica internacional, Circulation. Esta é uma das revistas mais relevantes na área em que esta tese foi desenvolvida e possui um sistema de citações cómodo para a leitura de textos de revisão científica. Adicionando o seu elevado factor de impacto na sociedade científica, pareceu apropriada a escolha desta revista como referência para a apresentação da bibliografia.

| Acknowledgments

It has been an intense and challenging project, which would not have been finished without the help of people very dear to me. This work is the result of a very enjoyable period at University Medical Center of Groningen in the CAVAREM group and I would like to express my gratitude to Marco and Guido for accepted me in the group and providing me the opportunity to carry out this work.

I would like to thank Guido, my supervisor, the opportunity he has given me to work in this project. This successful and extraordinary experience wasn't possible without his constant patience, encouragement, support, advices and especially the freedom and trust he has given me. He was an outstanding supervisor and has set an example to follow one day. I would also like to thank for his time and effort in reviewing this work, his corrections and input on scientific matters and English, has improved this thesis. I thank you for everything.

Secondly, I would like to thank Jan-Renier and Marco, for the kind words and guidance, during all year and especially during the preparation for my oral presentation in the conference, without you both have been difficult. Thanks.

I'm equally grateful for all the people I met in CAVAREM and in the U-Lab. Thank you all for the good work enviromment, collaboration an awesome atmosphere in the lab. I loved to be a part of it, it was a great year.

Special thank to Tieme and Veronica for being my good coffee buddies and all the CAVAREM students who were able to handle my distinguish mood in the morning, you are heroes!! Thanks for the friendship, I had a lot of fun with you all.

Thanks to all my friends in Groningen, I already miss you all. Nuria, Alba and Adrian thank you for being my family. To all the people of "Portuguese in Groningen" group, especially Andreia, Lionel and Carlos, thanks for the help and for given me a little bit of home. To Marije and Tieme, thank you for showing your beautiful country.

I would also like to thank Prof. Deodália for the support she gave and the happiness she demonstrate for my success.

To all my friends in Lisbon and in Lagos, even far away always have a place in my heart: Ângela, Catarina, Cláudia, Ana, Janine, Rui and Bruno.

I would like to thank my family: my brother Ricardo, his wife Amanda, my beautiful baby niece Margarida, my sister Laura, my grandmother Anunciada, my uncles Celeste and João and my stepfather Luís. Thank you for all the love and support.

Lastly, I thank to my parents Luisa and Filipe for her love, the unconditional support and freedom to follow my dreams. Thank you for being my parents, I love you too!

| Table of contents

| Resumo

Ao longo das últimas décadas as doenças cardiovasculares têm vindo a aumentar a sua incidência, sendo das doenças que mais mortes provocam a nível mundial. Este grupo de doenças é caracterizado por distúrbios causados ao nível do aparelho cardiovascular, principalmente no endotélio vascular. O endotélio vascular é constituido por uma monocamanda de células endoteliais, formando a face interna de todos os vasos sanguíneos. Devido ao seu contacto directo com a corrente sanguínea, as células endoteliais são extremamente importantes, regulando as trocas que ocorrem entre a corrente sanguínea e os tecidos envolventes. São células extremamente versáteis e multifuncionais, apresentando um conjunto de propriedades sintéticas e metabólicas, entre as quais podemos englobar a regulação da trombose e trombólise, aderência das plaquetas, modulação do tônus muscular e da corrente sanguínea, e regulação de respostas imunitárias e inflamatórias através do controlo da interação dos leucócitos, monócitos e linfócitos, com a parede do vaso sanguíneo.

Sendo as funções das células endoteliais essenciais para a manutenção e bom funcionamento do sistema vascular, disfunções endoteliais promove o desenvolvimento de difunções vasculares levando posteriormente a lesões cardíacas. Muitas destas lesões cardíacas acabam numa via final comum de remodelação do tecido patológico e fibrose cardíaca, conduzindo ao desenvolvimento de insuficiência cardíaca.

A fibrose cardíaca é definida pela deposição de colagénio, elastina, tenascina, entre outras proteínas da matriz, e é induzida pelos fibroblastos cardíacos, que têm um papel importante na remodelação cardíaca após a lesão. Apesar de a fibrose cardíaca apresentar importante papel na cicatrização de lesões, também contribui para o enrijecimento ventricular e para a progressão de falha cardíaca. Actualmente sabe-se que os fibroblastos cardíacos são originados através da transição mesenquimal das células endoteliais que é denominada de transição endotelial-mesenquimal (EndMT; Endothelial-to-Mesenchymal Transition). EndMT é um fenómeno biológico complexo que ocorre quando as células endoteliais perdem os seus marcadores específicos, como é o caso da caderina-endotelial vascular (VE-Cadherin; Vascular Endothelial Cadherin), e adquirem fenótipos mesenquimais ao expressar marcadores mesenquimais específicos, como é o caso de alfa

actina do músculo liso (αSMA; alpha-Smooth Muscle Actin) e colagénio tipo I. EndMT resulta na diminuição do número de células endoteliais e no aumento da produção de colagénio e miofibroblastos, que leva consequentemente a doenças de proliferação vascular. Numa fase embrionária, a EndMT é necessária para uma normal morfogénese valvular e septal a partir da prévia formação do coxim-endocárdico, processo dependente da diferenciação das células endoteliais em células mesenquimais e que tem como principal indutor deste processo o factor de crescimento TGFβ (transforming growth factor-β).

Durante o processo de desenvolvimento celular existem diferentes vias de sinalização que são essenciais. A via de sinalização do TGFβ é especialmente importante uma vez que é igualmente essencial tanto para o desenvolvimento embrionário como na manutenção da homeostase tecidular. O TGFβ pertence a uma superfamília de péptidos multifuncionais que regulam muitos processos no desenvolvimento celular, como a proliferação, diferenciação, adesão, migração e muitas outras funções em diferentes tipos de células. Alteração na sinalização do TGFβ origina malformações congénitas, inflamação e cancro. Os membros da família TGFβ ligam-se a dois tipos de receptores proteicos de serina/treonina quinase, receptores TGFβ do tipo I (TGFβR1 ou ALK5) e receptores TGFβ do tipo II (TGFβR2). Aquando a ligação do ligando TGFβ, TGFβR2 é activado e por sua vez vai fosforalizar ALK5, que leva à activação de cascatas de transdução do sinal, incluindo as vias Smad. As vias Smad regulam a transcrição de genes alvo, através da interacção com vários cofactores nucleares que regulam a transcrição dos mesmos. A fosforilação e activação do Smad2 e Smad3 induz a interação com a Smad4 de modo que entram no núcleo para regularem a expressão genética. O TGFβ pode ser regulado positiva e negativamente por numerosos microRNAs, em que muitos deles têm vindo a ser investigados intensivamente.

A descoberta dos miRNAs e a dos seus mRNAs-alvo permitiu o desenvolvimento de novos mecanismos da expressão genética. Os miRNAs são pequenos fragmentos de RNA não-codificante, aproximadamente 22 nucleótidos, que inibem a produção proteica através da repressão translacional ou da degradação/clivagem do mRNA-alvo. Os miRNAs ligam-se na região não codificante, 3'UTR (untranslated region) de diversos mRNA através do emparelhamento com pares base imperfeitos, regulando um conjunto de cascatas de transdução de sinal, como a sinalização de TGFβ-ALK5-Smad2/3. Os miRNAs são extremamente importantes uma vez que estão envolvidos em diversos processos biológicos

como desenvolvimento, diferenciação, proliferação celular, metabolismo e apoptose. Eles são dos pricipais responsáveis para manter homeostase de diversos tipos de sistemas, como o sistema cardiovascular.

O principal objectivo deste trabalho foi investigar a capacidade do miRNA20a (miR20a) inibir a via de sinalização TGFβ-ALK5-Smad2/3 e deste modo inibir EndMT. Estudos prévios realizados pelo nosso grupo de investigação foi verificou a existência de uma ligação entre TGFβ1 no EndMT, que leva ao aumento da expressão de SM22α (smooth muscle protein 22 alpha). Inicialmente, células endoteliais humanas do cordão umbilical (HUVEC) foram isoladas e estimuladas com TGFβ1 de modo a induzir EndMT. Caracterização da expressão do marcador endotelial, VE-Cadherin, e da expressão do marcador mesenquimal, SM22α, foi feita através de análise por imunofluorescência, assim como os níveis de expressão proteica dos principais alvos desta via de sinalização foram validados por Western Blot.

Não havendo qualquer documentação relativo aos mRNA-alvo do miR20a nesta via de sinalização, foi realizado o ensaio *Luciferase*, para determinar esses mesmos alvos. Células HEK foram transfectadas com 3'-UTR dos genes de interesse na presença ou ausência do miR20a ou controlo (miR608). Foi verificado que miR20a consegue-se ligar a quase todos os mRNAs dos genes de interesse, tendo maior afinidade para os receptores de TGFβ (TGFβR2 e ALK5), SARA e Smad2.

Sabendo que miR20a se consegue ligar a quase todos os genes de interesse da via de sinalização TGFβ-ALK5-Smad2/3, induziu-se a expressão genética do miR20a em HUVECs. Com a expressão genética do miR20a aumentada, foi possivel analisar a capacidade deste miRNA em inibir esta via de sinalização do TGFβ. Verificou-se que as células endoteliais, aquando a sua indução com miR20a, conseguem manter as suas propriedades funcionais apresentado as mesmas caracteristicas que o seu controlo. Este resultado prova-nos que miR20a consegue inibir EndMT e essa inibição ocorre devido à ligação do miR20a ao mRNA de TGFβR2, ALK5, SARA ou Smad2.

De modo a desvendar o processo molecular pelo qual miR20a é expresso nas células endoteliais, estimulamos HUVEC com TGFβ1 e com diferentes factores de crescimento. Através da análise da expressão do miR20a nestas células, foi possível verificar que o factor de crescimento bFGF é o factor de crescimento que leva ao aumento da expressão de

miR20a nas células endoteliais. Deste mode, analisando as diferentes vias de sinalização de bFGF e induzindo HUVEC com TGFβ1, bFGF e com inibidores dessas vias de sinalização, foi possivel verificar que a expressão de miR20a ocorre por via de JNK ou bFGF mediado por JNK ou Erk1/2.

Os resultados adquiridos neste trabalho experimental são de grande interesse para a comuidade cientifica, sendo um base fundamental para futuros trabalhos cientificos mais direccionados para a clínica, de modo a poder contribuir para introdução de terapeutica para o tratamento de doenças cardiovasculares nas quais EndMT está envolvido.

Palavras–chave: MicroRNA-20a; Transição Endotelial-Mesenquimal; TGFβ1; ALK5-Smad2/3

| Abstract

Endothelial-to-mesenchymal transition (EndMT) is a biological process that occurs in advantages stages of endothelial dysfunction and is characterized by decreased expression of endothelial cell markers and functions, together with increased expression of mesenchymal markers and functions. EndMT results in a decreased number of endothelial cells and increase of (myo)fibroblasts, which leads to scar formation in the cardiovascular tissues. EndMT is induced by TGFβ1. Stimulation of endothelial cells with TGFβ1 activates its receptor, ALK5, causing activation of Smad2/3 which results in the induction of mesenchymal genes.

MicroRNAs (miRNAs) are a class of endogenous, small non-coding RNAs (~22 nucleotides) that regulate gene translation. The TGFβ signaling cascade can be regulated by many microRNAs, among them miRNA-20a (miR20a). We investigated if miR20a can inhibit TGFβ-ALK5-Smad2/3 signaling and is thereby able to inhibit the process of EndMT.

Human umbilical vein endothelial cells (HUVEC) were stimulated with TGFβ1 to induce EndMT, leading to an increased expression of SM22α and decreased expression of endothelial marker, VE-Cadherin. Protein expression levels were validated by western blot and had higher levels in the TBFβ receptors and downstream mediators. By expressing miR20a in HUVECs, we could identify miR20a target genes and investigate the influence of miR20a expression on EndMT.

MiR20a is rapidly downregulated when endothelial cells are stimulated with TGFβ1, which coincides with EndMT. VE-Cadherin expression decreased, causing loss of endothelial barrier function, while mesenchymal marker SM22α increased drastically, leading to an aberrant change in cell morphology, e.g. cellular hypertrophy. Ectopic expression of miR20a decreased HUVEC sensitivity to TGFβ1 through targeting TGFβR2, ALK5 and SARA, and abrogated EndMT, as observed by maintenance of VE-Cadherin and inhibition of SM22α expression.

KeyWords: EndMT, miR20a, TGFβ1 and ALK5-Smad2/3

| List of abbreviation

3’UTR Three Prime Untranslated Region

Ago	Argonaute
ALK5	Activin Receptor-Like Kinase 5
ANOVA	Analysis of Variance
αSMA	Alpha Smooth Muscle Actin
β-actin	Beta Actin
BBE	Bovine Brain Extract
bFGF	Basic Fibroblast Growth Factor
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
cDNA	Copy Deoxyribonucleic Acid
Co-Smad	Common Mediator Smad
Ct-value	Cycle Threshold Value
CVD	Cardiovascular Diseases
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DGCR8	DiGeorge Critical Region 8
DMEM	Dulbecco’s Modified Eagle’s medium
ECM	Endothelial Cell Medium
EndMT	Endothelial-to-Mesenchymal Transition
eNOS	Endothelial NO Synthase
Erk1/2	Extracellular Signal-Regulated Kinase 1 and 2
FBS	Fetal Bovine Serum
FTS	Farnesyl Thiosalicylic Acid
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GDP	Growth Differentiation Factors
GFP	Green Fluorescent Protein
GTPases	Guanosine Triphosphate Hydrolase
HEK	Human Embryonic Kidney Cell
HGF	Hepatocyte Growth Factor

HUVEC	Human Umbilical Vein Endothelial Cell
ICAM	Intercellular Adhesion Molecule
IGF	Insulin-like Growth Factor
IgG	Immunoglobulin G
IL-1β	Interleukin-1 beta
IRDye	Infrared dye
JNK	c-Jun N-terminal Kinase
MAPK	Mitogen-Activated Protein Kinase
miRISC	miRNA–induced silencing complex
miRNA	microRNA
miR20a	microRNA-20a
MIS	Müllerian inhibitory substance
mRNA	Messenger RNA
NO	Nitric Oxide
ORF	Open Reading Frame
p38	p38 Mitogen-Activated Protein Kinase
PBS	Phosphate-Buffered Saline
PI3K	Phosphatidylinositide 3-kinases
PIC	Proteinase Inhibitor Cocktail
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
pSmad	phospho–Smad
qRT-PCR	quantitative Real-Time Polymerase Chain Reaction
R204	Endothelial Cell Medium with TGFβ1
RAS	Rat Sarcoma
RIPA	Radioimmunoprecipitation Assay Buffer
RNA	Ribonucleic Acid
RNase III	Ribonuclease III
RNU6B	**S**mall Nuclear Ribosomal RNA 6B
RPMI	Roswell Park Memorial Institute medium
R-Smad	Receptor Activated Smad

SARA	Smad Anchor Receptor Activator
SB-431542	4-(5-benzo[1,3]dioxol-5-yl-4-pyridin-2-yl-1H-imidazol-2-yl)-benzamide
SB-203580	4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole
SCR	**S**cramble Sequence
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
SM22α	Smooth Muscle Protein 22-alpha
SP600125	1,9-Pyrazoloanthrone
TEP	Trypsin-EDTA-PBS
TGFβ	Transforming Growth Factor beta
TGFβ1	Transforming Growth Factor beta type I
TGFβR1	Transforming Growth Factor beta Receptor type I
TGFβR2	Transforming Growth Factor beta Receptor type II
TNFα	Tumor Necrosis Factor alpha
U0126	1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene
VCAM	Vascular Cell Adhesion Molecule
VE-Cadherin	Vascular Endothelial Cadherin
VEGFa	Vascular Endothelial Growth Factor A
WHO	World Health Organization

Part I

| General Introduction

1. Cardiovascular Diseases

According to the World Health Organization (WHO), the incidence of deaths caused by cardiovascular diseases (CVDs) is increasing, being the most prevalent cause of morbidity and mortality.[1] Over the years, the risk of CVD increases due to the exposure of the cardiovascular system to diverse pathophysiological stimuli. Cardiac hypertrophy is a pathological feature common to different CVD such hypertension, ischemic myocardial injury, diabetic cardiomyopathy, vascular dysfunction and aortic stenosis, among others.[2] This condition is a physiological adaptive response to an increase in biomechanical stress such as high blood pressure, metabolic challenges as hyperlipidaemia and hypercholesterolemia, and vascular inflammation.[3, 4] Persistent cardiac hypertrophy is associated with a significant increase in the risk of fibrotic diseases, ventricular dilatation, arrhythmia, heart failure and even sudden death.[2, 3]

1.1. Vascular functions and dysfunctions

The human body has a complex architecture that depends on efficient supply of oxygen and nutrients to all tissue. The vascular system is an important vehicle to achieve with efficiently this task via a highly branched network of blood vessels. The internal surface of all blood vessels is covered by the vascular endothelium (figure 1). This thin monolayer of endothelial cells besides lining the vessel wall, regulate exchanges between the bloodstream and surrounding tissues.[5] Depending on the location, the amount of connective tissue and smooth muscle in the vessels wall can change according to the vessels diameter and function, but the endothelial monolayer is always present.[5]

Endothelial cells interact with different physical and chemical stimuli within the circulation. They regulate the vascular tone, cell adhesion, thromboresistance, vascular permeability, immune and inflammatory responses with the vessel wall and angiogenesis.[6-8] All these functions are essential to maintain blood circulation and therefore tissue homeostasis under physiological conditions.[8] Diabetes, dyslipidemia and oxidative stress are atherogenics stimuli that induce vascular dysfunction, leading to diseases such as atherosclerosis and cardiac fibrosis.[8]

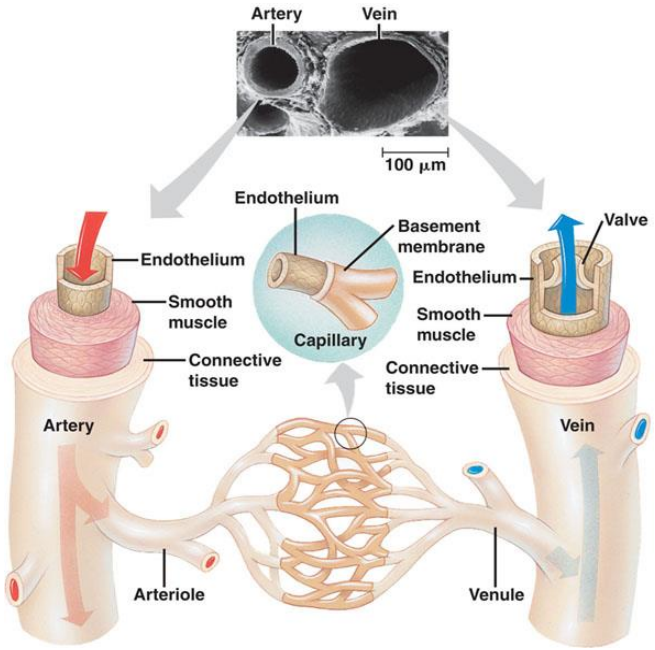

Figure 1| Diagram of different blood vessels of the cardiovascular system in cross section. Structure of different blood vessel were endothelial cells monolayer is always present. Adapted from (http://www.baileybio.com/plogger/?level=picture&id=462)

Endothelial dysfunction refers to a number of dysregulations often observed in CVD. In endothelial dysfunctions an upregulation of adhesion molecules such as intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM) and E-selectin[9], decrease Nitric Oxide (NO) production through endothelial NO synthase (eNOS) uncoupling[10], secretion of pro-inflammatory cytokines, such IL-1b and TNFa[11], and at advanced stages, endothelial-to-mesenchymal transition (EndMT) is observed, often resulting in cardiac fibrosis or atherosclerosis.

1.2. Cardiac Fibrosis

Cardiac fibrosis is defined by the overgrowth, hardening and/or scar formation of diverse cardiac tissues, due to inappropriate proliferation of fibroblast and/or myofibroblasts and excess deposition of extracellular matrix proteins (collagens, elastin and among others).[12, 13] Although, cardiac fibrosis is an essential process in wound healing, the progressive stiffening of the ventricular walls also leads to a loss of contractility, abnormal cardiac conductance, deformed organ architecture and function.[13, 14] Fibroblasts and myofibroblasts are the principal producers of extracellular matrix proteins in the affected tissue, with a significant part of these fibroblasts deriving from the endothelium by a

transforming growth factor β-dependent process, such endothelial-to-mesenchymal transition (EndMT).[14-16]

1.2.1. Endothelial-to-Mesenchymal Transition

EndMT is a complex biological process in which endothelial cells loses their specific endothelial cell markers, such as VE-Cadherin, and acquires a mesenchymal phenotype and expression of mesenchymal cell products such SM22α. Endothelial cells lose their cell-cell contact and disaggregate, change shape and become motile and capable of migrating into surrounding tissues.[17, 18] These morphological changes lead to a decrease number of endothelial cells and increase of myofibroblasts.

EndMT plays a pivotal role in the embryonic development of the heart. With the detachment of endothelial cells from the endothelium, they acquire mesenchymal phenotypes and migrate into cardiac jelly to become mesenchymal cells that form the matrix of the atrioventricular cushion, the valves and septa of the heart.[16] However in adults, EndMT occurs specially during organ pathology, such as cardiac fibrosis, kidney fibrosis, pulmonary fibrosis and even cancer (figure 2).[17]

The transforming growth factor beta (TGFβ) superfamily is key for the activation of EndMT. Although TGFβ is important for other processes in the cell, in case of tissue injury, inflammation and hypoxia, the production of TGFβ is increased by cells surrounding the endothelium leading to EndMT.[18]

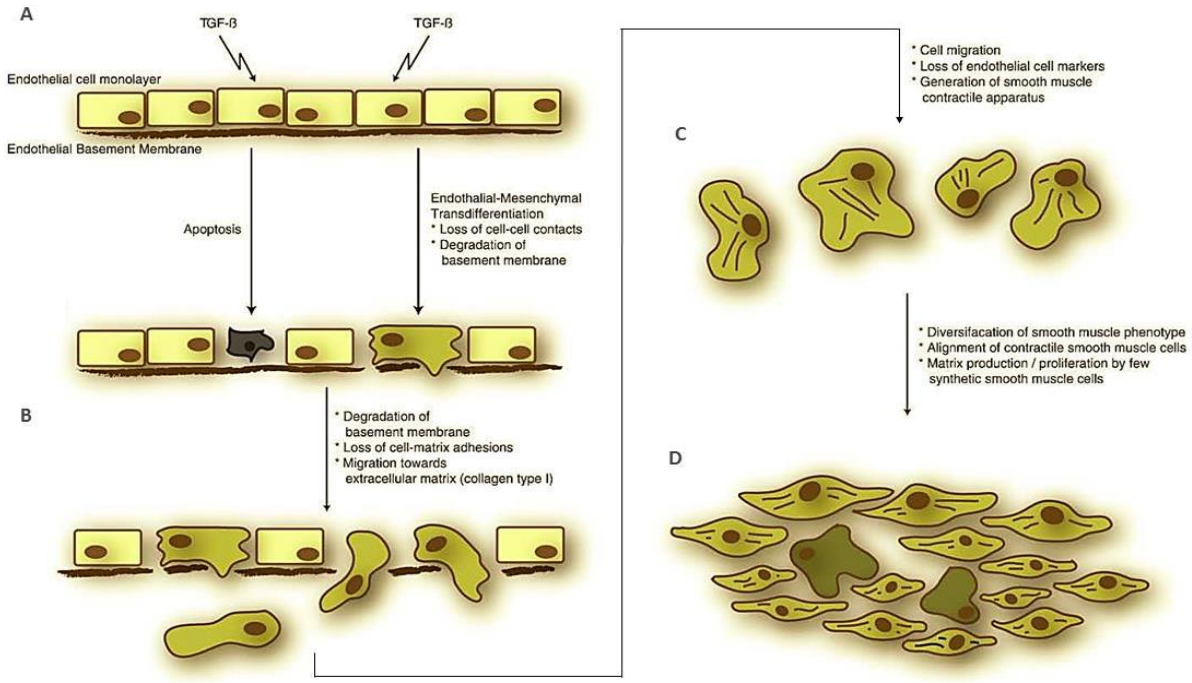

Figure 2| Overview of the EndMT process. **A**, TGFβ levels are increased in endothelial cells initiating EndMT with loss of cell-cell contact. **B**, In EndMT, endothelial cells acquires migratory phenotype and loses cell-matrix adhesion. C, Endothelial cells lose their endothelial cell markers and acquire smooth muscle cell phenotypes. D, Diversification of smooth muscle phenotype and matrix production and proliferation. (Adapted from reference 18)

1.2.2. TGFβ signaling pathway

TGFβ belongs to a large family of related growth factors comprised of at least 30 members. This superfamily, besides TGFβs (1 to 3), also includes bone morphogenetic proteins (BMP), growth and differentiation factors (GDF), activins and inhibins, nodal, leftys, and Müllerian inhibitory substance (MIS).[19] The members of this family are expressed in diverse tissues and have fundamental roles in many processes during embryonic development, but also in maintenance of tissue homeostasis in adult.[19, 20] Abnormal TGFβ signaling is associated with a wide range of human disorders such as cardiovascular diseases and fibrosis in diverse organs, as well as cancer.[20-22]

The effect of TGFβ can be mediated by three TGFβ ligands, TGFβ1, TGFβ2 and TGFβ3. TGFβ transduce their signal, from the membrane to the nucleus, by binding of the dimeric ligand to specific transmembrane receptor at the cell membrane, designated TGFβ type I (TGFβR1) and type II (TGFβR2) serine/threonine kinase receptors (figure 3).[19, 22] Binding of the ligand cause the formation of active heteromeric receptor complex, that results in the phosphorylation and activation of TGFβR1 by TGFβR2. Activation of activin receptor-like kinase 5 (ALK5; also known as TGFβR1) leads to a propagation of signal into the cell with the phosphorylation of the major intracellular mediators of TGFβ signal, the Smad proteins.[19] The non–active receptor-regulated Smads (R–Smads) are capture by SARA (Smad anchor for receptor activation), who bring then to the activated receptor.[23] The TGFβR2–ALK5 complex recruits and phosphorylates Smad2 and Smad3 (R–Smad). Once phosphorylated, the activated R–Smad complexes with the common mediator Smad4 (Co–Smad) and translocate into the nucleus. In the nucleus, the bind of the activated Smad2/3/4 complex to the Smad-binding element (SBE) interacts with other DNA-binding transcription factors, and co–activators and co–repressors, binds to promoter regions of TGFβ target genes and regulates the transcription of specific target genes.[19, 22, 24]

Smads are essential signal transducers in TGFβ signaling, although TGFβ is also known to regulate non-Smad pathways, including Erk1/2, p38 MAPK, JNK, PI3K-Akt and small GTPases (figure 3).

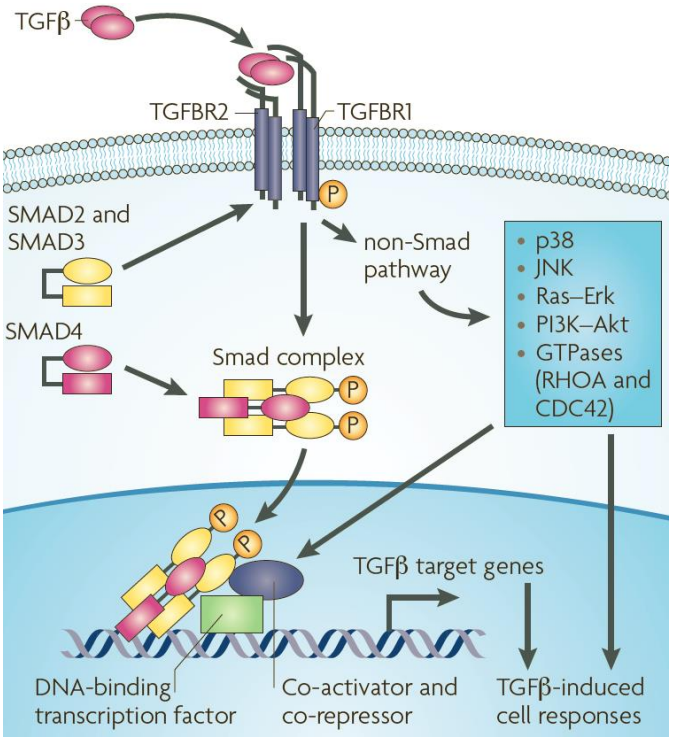

Figure 3| TGFβ signaling pathway. TGFβ signal can be transduced through Smad and non-Smad pathways. TGFβ ligands bind to TGFβR2. TGFβR2 phosphorilates (P) TGFβR1, which subsequently phosphorylates and activates Smad2/3. Activated Smad2/3 complexes with Smad4 and translocate into the nucleus, regulates the transcription of specific target genes. (Adapted from reference 22)

2. MicroRNAs

MiRNAs are a class of endogenous, small non-coding RNAs ~22 nucleotides in length, able to regulate gene expression at the post-transcriptional level by either cleavage/degradation or translational repression of a target mRNA.[25] MiRNAs bind to mRNA targets through Watson-Crick base pairing between the miRNA seed region (i.e. nucleotides 2–8) and sequences usually located in the 3' untranslated region (UTR). [26] The discovery of miRNAs and their target mRNA has uncovered novel mechanisms regulating gene expression.[27] A single miRNA is capable of regulate several distinct mRNA targets, often involved in the same cellular pathway, and are believed to modulate more than one-third of the mRNA encoded in the human genome.[28]

Many signaling cascades, such TGFβ-ALK5-Smad2/3, can be regulated by microRNAs (miRNAs). Utilizing overexpression and knockdown approaches can reveal the distinct roles of miRNAS in several cardiovascular diseases, including cardiac fibrosis.

2.1. MiRNA biogenesis

The mechanism that creates a mature and functional miRNA starts in the nucleus with miRNA gene transcribed by RNA polymerase II or III in pri–miRNA (primary miRNA), a secundary structure containing a 60– to 80–nucleotide hairpin stem–loop (Figure 4).[27] This hairpin structure is cleaved by a protein complex, consisting of Drosha (endoribonuclease; RNase III enzyme) and DGCR8 (DiGeorge critical region 8; binding partner) resulting in the pre–miRNA, which include a 22–bp (base pair) stem, a loop, and a 2–nucleotide 3'overhang.[27, 29] After this first cleavage, the pre–miRNA is exported to the cytoplasm by exportin–5. Once in the cytoplasm, pre–miRNA is cleaved by Dicer (another endoribonuclease; RNase III enzyme), which removes the final loop and releasing ~22–nucleotide mature miRNA duplexes. After unwind, one strand of miRNA is designed the mature miRNA and is the functional guide strand, can bind with the target mRNA. The complementary strand, termed the passenger strand or miRNA*, is rapidly degraded.[25, 27, 29]

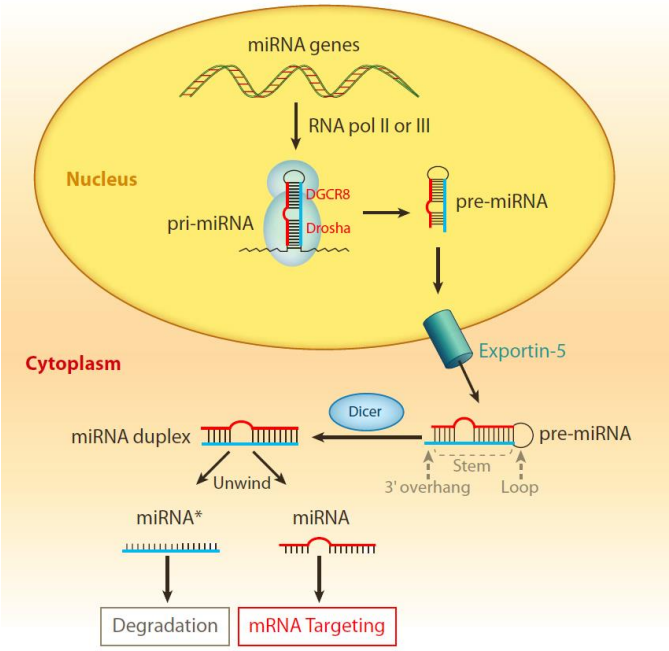

Figure 4| Biogenesis of microRNAs. In the nucleus, miRNA gene is transcribed by RNA polymerase II or III in pri–miRNA and then cleaved by Drosha/DGCR8 complex, resulting in the pre–miRNA. Pre–miRNA is exported to the cytoplasm by exportin–5 and further cleaved by Dicer to remove the final loop. After unwind, mature miRNA duplexes is release. One strand of miRNA is the functional strand and bind to the target mRNA, the complementary strand, miRNA*, is rapidly degraded. (Adapted from reference 27)

Mature miRNA is packed into a ribonucleoprotein complex, miRISC (miRNA–induced silencing complex) and together with Ago (Argonaute) proteins, regulates gene silencing. MiRNA complementary sites can predominantly found at the 3'UTR of the target genes, located at the 3' downstream of the ORF (open reading frame). They bind to their target mRNA in one of two way, through imperfect complementary, which blocks target gene expression at the level of protein translocation, or through perfect (or nearly perfect) complementary leading to cleavage and degradation of mRNA (figure 5).[27, 29, 30]

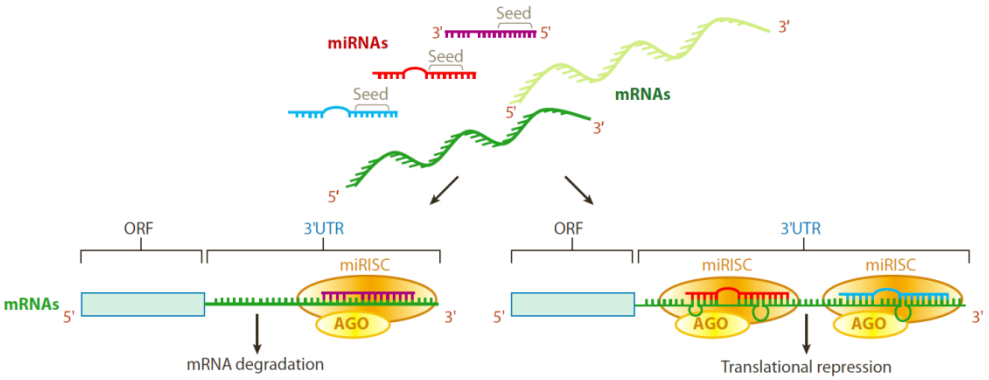

Figure 5| MiRNA targeting. MiRNA seed region targets the 3'UTR of the target mRNA, located at the 3' downstream of the ORF. MiRNA bind the target mRNA through imperfect complementary, which leads to a translational repression of the target gene expression; or through a perfect complementary, which leads cleavage and degradation of mRNA. (Adapted from reference 27)

2.2. MicroRNA in the cardiovascular system

MiRNAs have important roles in many essential biological processes, including development, differentiation, cell proliferation, metabolism and apoptosis, through its fine tuning of gene regulation.[25] They maintain the physiological homeostasis of a wide range of tissues, including the cardiovascular system, being key modulators of both cardiovascular development and disease.

The heart is the first organ to function during vertebrate embryogenesis.[25] Cardiac development derived from diverse cell lineages that differentiate into unique regions.[31] MiRNA-mediated posttranscriptional gene regulation is very important for this development, which is emphasized by loss-of-function mutation studies in genes that encode enzymes essential for miRNA biogenesis, such Dicer, Drosha, Ago2 and DGCR8.[25] Knockout mice lacking these key miRNA-processing genes compromised the normal

development of the vertebrates dying during early gestation with severe developmental defects.[32]

In endothelial cells, genetic knockdown Dicer and/or Drosha expression change gene expression, affecting endothelial cell biology, reducing endothelial cell proliferation and angiogenesis, *in vitro*.[33] Angiogenesis is essential in the development of new blood vessels from existing vascular structures, so the capillary sprouting and tube-forming activity is reduced, which show the importance of miRNAs in the cardiovascular development.[27, 33]

Due to the important role of miRNA in the development is not surprising that miRNA can have a role in cardiovascular diseases. As mentioned before, a single miRNA can target multiple mRNA, and this way regulates a whole signaling cascade, such TGFβ-ALK5-Smad2/3 pathway. Identifying the target genes of specific miRNAs involved in the cardiovascular system can provide more knowledge into the molecular mechanisms behind the disease process. Several miRNA are expressed in endothelial cells and among other, also microRNA-20a (miR20a).[34] This miRNA is a member of the miR-17-92 cluster, which is one of the most extensively studied families and the members of this family play important roles in tissue and organ development.[35] MiR20a is highly investigated as an oncogene in tumors but also plays a role in the regulation of monocyte-macrophage differentiation.[30, 35-37] The members of the miR-17-92 cluster family is involved in some processes of the cardiovascular system playing a role in angiogenesis.[34] However, the role and mechanism of miRNA20a itself in endothelial cells is currently unknown.

3. Aim

Previous results showed the connection between TGFβ1–ALK5–Smad2/3 pathway and EndMT, with the increased expression of the downstream mediator pSmad2, which lead to an increase of SM22α expression.[38]

Recently we identified the miR20a as a possible inhibitor of EndMT, targeting ALK5, Smad2/3 and the co-receptor TGFβR2. Given the importance of miRNA in different biological processes, the main goal of the present study was to investigate the role of miR20a in the inhibition of EndMT, through TGFβ1–ALK5–Smad2/3 signaling pathway, as well as the determination of his target mRNA genes in this signaling cascade. We also focus in the determination of the molecular pathway, in which miR20a expression can be increased in endothelial cells.

Part II

The role of microRNA-20a in the TGFβ-ALK5-Smad2/3 signaling pathway and ability to inhibit the Endothelial-Mesenchymal Transition

Ana C. P. Correia[1, 2], Jan-Renier A.J. Moonen[1], Martin C. Harmsen[1] and Guido Krenning[1]

[1]*Cardiovascular Regenerative Medicine Research Group (CAVAREM), Department of Pathology and Medical Biology, University Medical Center Groningen, The Netherlands*

[2]*Faculty of Sciences, Department of Animal Biology, University of Lisbon, Portugal*

Endothelial-to-mesenchymal transition (EndMT) is a biological process that occurs in advantages stages of endothelial dysfunction and is characterized by decreased expression of endothelial cell markers and functions, together with increased expression of mesenchymal markers and functions. EndMT results in a decreased number of endothelial cells and increase of (myo)fibroblasts, which leads to scar formation in the cardiovascular tissues. EndMT is induced by TGFβ1. Stimulation of endothelial cells with cβ1 activates its receptor, ALK5, causing activation of Smad2/3 which results in the induction of mesenchymal genes. As TGFβ-signalling is regulated by many microRNAs (amongst others miRNA-20a (miR20a)), we investigated if miR20a can inhibit TGFβ-ALK5-Smad2/3 signalling and thereby able to inhibit the process of EndMT.

Human umbilical vein endothelial cells (HUVEC) were stimulated with TGFβ1 to induce EndMT, leading to an increased expression of SM22α and decreased expression of endothelial marker, VE-Cadherin. Protein expression levels were validated by western blot and had higher levels in the TBFβ receptors and downstream mediators. By expressing miR20a in HUVEC, we could identify miR20a target genes and investigate the influence of miR20a expression on EndMT.

MiR20a is rapidly downregulated when endothelial cells are stimulated with TGFβ1, which coincides with EndMT. VE-Cadherin expression decreased, causing loss of endothelial barrier function, while mesenchymal marker SM22α increased drastically, leading to an aberrant change in cell morphology, e.g. cellular hypertrophy. Ectopic expression of miR20a decreased HUVEC sensitivity to TGFβ1 through targeting TGFβR2, ALK5 and SARA, and

abrogated EndMT, as observed by maintenance of VE-Cadherin and inhibition of SM22α expression.

KeyWords: EndMT, miR20a, TGFβ1 and ALK5-Smad2/3

1. Introduction

The development of heart failure is often initiated by cardiac damage, resulting in pathologic tissue remodelling and fibrosis.[1] Cardiac fibrosis contributes to a progressive stiffening of the ventricular walls, resulting in a loss of contractility, abnormal cardiac conductance, deformed organ architecture and function, caused by accumulation of fibroblast and/or myofibroblast.[2, 3] Fibroblasts are cells of mesenchymal origin which cause excessive accumulation of different extracellular matrix proteins in the perivascular and myocardial interstitial compartments during cardiac fibrosis. A significant part of these interstitial fibroblasts are derived from the endothelium by a transforming growth factor β-dependent process, termed endothelial-to-mesenchymal transition (EndMT).[2 ,3, 4] The transforming growth factor-β (TGFβ) superfamily is key in EndMT. This multifactorial process utilizes several membrane-bound receptors and signal transduction pathways, leading to a decrease their specific endothelial markers, such vascular endothelial cadherin (VE-Cadherin), and acquisition of mesenchymal phenotypes, such as smooth muscle protein 22-alpha (SM22α).[5, 6] *In vitro*, EndMT manifest through morphological changes leading to a decrease number of endothelial cells and increase of myofibroblasts.[7, 8]

EndMT occurs when TGFβ binds to the TGFβ type II receptor (TGFβR2) phosphorylating and activating the TGFβ type I receptor (ALK5) within a heterotetrameric complex. The phosphorylated ALK5 activates its catalytic kinase domain, allowing the activation of downstream signal transduction cascades, including the Smads pathway, which is the major intracellular mediator of TGFβ signalling.[1, 2] Non-activated Smad2/3 are capture by SARA (Smad anchor for receptor activation) bring then to the activated ALK5.[7] Smad 2/3 are phosphorylated and there activation are associate with the common mediator Smad 4 (co-Smad). These complexes translocates to the nucleus where regulate transcription of target genes.[1, 2]

The discovery of miRNAs and their target messenger RNAs (mRNAs) has uncovered novel mechanisms regulating gene expression.[9] This small non-coding RNA

fragments, approximately 22 nucleotides in length, play a powerful roles in various biological processes, including cardiac development and functions.[9] MiRNAs are not translated into proteins, but the mature single-stranded miRNA bind to mRNAs to inhibit protein production through translational repression or mRNA cleavage.[10] In general, miRNAs function post-transcriptionally by reducing protein yield from specific target mRNAs.[11] MiRNAs are generated by an RNase III-type enzyme from an endogenous transcript that contains a local hairpin structure.[12] They received great attention, since each miRNA can bind to the 3'UTR of several mRNA targets enabling miRNAs to regulate a whole signalling transduction cascade, such as the TGFβ-ALK5-Smad2/3 signalling.[11, 12]

MiR20a is a member of the miR-17-92 cluster, which is one of the most extensively studied families and the members of this family play important roles in tissue and organ development.[12] However, the role and mechanism of miR20a itself in endothelial cells is not quite know yet. Recently we identified the miR20a as a possible inhibitor of EndMT, targeting ALK5, Smad2/3 and the co-receptor TGFβR2.

Therefore the aim of this study was to investigate the role of miR20a in the TGFβ-ALK5-Smad2/3 signaling pathway and his ability to inhibit EndMT. We also focus in the determination of the molecular pathway in which miR20a inhibit EndMT and how the miR20a expression can be increased in endothelial cells.

2. Material and Methods

2.1. Cell Culture

HUVEC were cultured in gelatin-coated culture flasks in endothelial cell growth medium (ECM). ECM was composed of RPMI 1640 (Lonza) supplemented with 20% fetal bovine serum (FBS; GIBCO/Introgen), 50μg/mL, bovine brain extract (BBE; Lonza), 2mM L-glutamine (Sigma), 1% penicillin/streptomycin (Sigma) and 5 U/ml heparin (Leo Pharma). When HUVEC reached confluence, they were detached using 1% TEP (Trypsin-EDTA-PBS, Sigma) and passaged 1:3. For experiments, HUVEC were cultured in either ECM, or ECM with 5ng/ml TGFβ1 (R204 medium) and appropriate stimuli. Cells were used between passages 3 and 7.

2.2. Western Blot

Cells were lysed with RIPA buffer (Thermo Scientific, MA, USA) supplemented with 0.1% proteinase inhibitor cocktail (PIC; Sigma-Aldrich, MA, USA). Protein concentration were determined using BioRad DC™ Protein Assay (Bio-Rad, VA, USA), following the manufacturers protocol. Samples were loaded with equal amount of protein (30µg/lane) on 10% SDS-polyacrylamide gels and subsequently blotted onto nitrocellulose membranes. The membranes were incubated with appropriate primary antibody solution (table 1) in Odyssey Block Buffer overnight at 4ºC. Subsequently, membranes were incubated with secondary antibodies, anti-rabbit IgG IRDye-680 (Li-COR Biosciences, Germany) or anti-mouse IgG IRDye-800 (Li-COR Biosciences, Germany) diluted 1:10000 in Odyssey Blocking Buffer for 1h at room temperature. Proteins were visualized using the Odyssey® Infrared Imaging System (Li-COR Bioscience). Densitometry analysis was performed using TotalLab TL120 1D (Nonlineal Dynamics ltd.).

Table 1: Description of the primary antibodies and respective dilution factor.

Target		**Dilution**
ALK5	Abcam ab31013	1:500
TGFβ receptor 2	Abcam ab61213	1:500
phospho-Smad2	Cell Signaling #3108	1:500
Phospho-Smad3	Cell Signaling #9520	1:500
Smad2/3	Cell Signaling #3102	1:500
Smad4	Abcam ab40759	1:500
SARA	Abcam ab124875	1:1000
GAPDH	Abcam ab9485	1:500
SM22α	Abcam ab14106	1:500
VE-Cadherin	Cell Signaling #2500	1:500
β-actin	Cell Signaling #4967L	1:1000

2.3. Immunofluorescence Staining

Cells were fixed using 4% paraformaldehyde in PBS at room temperature for 15 minutes. For endothelial marker staining, cells were blocked with 2% BSA () for 10 min

and incubated with primary antibody VE-Cadherin (1:200; Cell Signaling #2500). For mesenchymal marker staining, fixed cells were permeabilized using 1% Triton X-100 in PBS (Sigma) for 10 minutes and incubated with primary antibody SM22α (1:250; Abcam ab14106). Primary antibodies were incubated at 4 ºC, overnight. Samples were washed extensively with 0,05% Tween-20 in PBS and incubated with secondary antibodies Alexa Fluor® 594 Donkey anti-Rabbit IgG (red; AF594αR; Life Technologies A21207) or Alexa Fluor® 647 Donkey anti-Rabbit IgG (green; AF647αR; Life Technologies A31573), both diluted 1:250 in DAPI (blue; 0,001mg/mL in PBS: Sigma) with 2% BSA. Secondary antibodies were incubated for 1h at room temperature. Image analysis was performed on TissueFAXS (TissueGnostics) in the fluorescence mode, in combination Zeiss AxioObserver Z1 microscope. Data analysis was performed using TissueQuest fluorescence (TissueGnostics) software.

2.4. Matrigel Sprouting

10µL of MatriGel™ (BD Biosciences, MA) was solidified in µ-Slide Angiogenesis plates (Ibidi GmbH, Germany). Cells were detached using TEP and a concentration of 2.10^5 cells per mL of ECM was cultured on the solidified MatriGel™, overnight. Sprout formation was analysed by conventional light microscopic analysis.

2.5. Dual Luciferase Reporter Assay

For this assay Human Embryonic Kidney (HEK) cells were used. HEK cells were detached with TEP and placed in 96 wells plate and culture in DMEM (Lonza) supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin, overnight. HEK cells were transfected with UTR-reporters (psiCHECK) in the presence or absence of miR20a or control miR (miR608). Endofectin was used as transfection reagent according to manufacture instructions.. The Dua-Glo® Luciferase Assay System (Promega) was performed according with the manufactures protocol. Firefly and *Renilla* luminescence were recorded with Lunimoskan Ascent Microplate Luminometer (Thermo Scientific) and the relative Luciferase activity analysed.

2.6. *Lentiviral Transfection*

A 2nd-generation lentivurs was used to overexpress miR20a, and in combination with helper-plasmids, were transfected into HEK using endofectin. A lentiviral vector expressing a scramble miR was used as a control. Transformed HEK cells expressed Green fluorescent protein (GFP) and were allowed to secrete viral particles in ECM. The ECM containing the lentiviral particles was collected and supplemented with 4ug/ml Polybrene® (Sigma), and placed on HUVEC for 3 consecutive times. Overexpressed HUVEC with miR20a were selected using puromycin (6µg/mL). Medium was changed for ECM and HUVEC overexpressed with miR20a were detached and divided for different experiments.

2.7. *microRNA Transfection*

MiR20a transfection was made using the Ambion Pre-miR™ miRNA Precursor molecule (# AM-17100), Santa Cruz Transfection Medium (# SC-36868) and Santa Cruz siRNA Transfection Reagent (# SC-29528).

Solutions with pre-miR miR20a and RPMI were mixed with transfection reagent plus transfection medium, and incubated for 45 minutes at room temperature. HUVEC were washed with pre-warmed PBS, added the transfection mix and incubated at 37ºC, 5% CO_2 for 5h before added normal ECM. After incubated overnight, medium was change for ECM or ECM+ TGFβ1.

2.8. *Quantitative Real-Time PCR*

Total RNA isolation was performed using RNA-Bee™ (Bio-Connect) according to manufactures protocol. RNA concentrations and purity were determined using NanoDrop (Thermo Scientific, USA). Subsequently, 20ng/µL of total RNA was reverse transcribed using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) according with the manufactures instructions in a final reaction volume of 10µL (5µL Reverse Transcriptase Mix with miR20a-SL primer or RNU6B-SL primer and 5µL total RNA (20ng/µL)). The qPCR reactions were composed by 10µL of cDNA, 12µL SYBR® Green PCR Master Mix (Applied Biosystems), 0,1µL miR-specific forward primer (miR20a and RNU6B) and 0,1µL miR reverse primer. The qRT–PCR mixes were placed

in 384-Well MicroAmp® Reaction Plate (Applied Biosystems) in a final reaction volume of 20 µL. qRT–PCR was performed in ViiA™ 7 Real-Time PCR System (Life Technologies) according the following steps: activation step (95 ºC, 10min), cycling program (95 ºC, 15seg; 57 ºC, 15seg; 72 ºC; 45seg; for 50 repeats) and melting curve analysis. MiR20a expression was quantified using Ct-values normalized against RNU6B-expression and the ΔCt-method.

Tabel 2: Primer sequence.

primer	Sequence
RNU6B	*Stemloop*: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAAATATGG *Forward:* TGCGGCTGCGCAAGGATGA *Reverse:* CCAGTGCAGGGTCCGAGGTCCG
miR20a	*Stemloop:* GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTACCTGC *Forward:* TGCGGTTAAAGTGCTTATAGT *Reverse:* CCAGTGCAGGGTCCGAGGTCCG

2.9. Statistical Analysis

All experimental data were obtained from at least three independent experiments. Data is expressed as mean ± standard error of the mean (SEM). Data in Gaussian distribution was analyses using one-way ANOVA followed by Bonferroni post-test. Probabilities of p-values < 0.05 were considered to be statically significant. Statistical analysis was performed using GraphPad Prism (Graphpad Software, CA, USA).

3. Results

3.1. TGFβ1 induces EndMT in a ALK5-Smad2/3 dependent manner

TGFβ induces EndMT. Through the stimulation of HUVECs with TGFβ1 is possible to observe a change in morphology, in which has cellular hypertrophy as well a decrease of cell number (figure 1A, D). Theses morphological changes were accompanied by decrease of the expression of endothelial cell marker, VE-Cadherin, and

increase of the expression of mesenchymal marker, SM22α (figure 1B-C and E-F). HUVEC also lose their endothelial function proprieties, not being able to aggregate and sprout to create vessel-like forms in matrigel (figure 1Q; $P < 0.001$; 1S), corroborating the description of EndMT.

SB431542 was previously identified as an ALK5 inhibitor by Callahan et al.[13] Supplementing R204 with SB431542 we can observe that the TGFβ signalling cascade is inhibited (figure 1G). This inhibition allows us to verify that this signaling cascade is pivotal in EndMT and with this inhibition all the endothelial proprieties are maintained. Comparing with the control (figure 1 A-C), they have identical morphology and cell number (figure 1G), as well as the expression of SM22α is lower and VE-Cadherin is equally high (figure 1H-I). They also maintain the function properties, being able to sprout and form the vessel-like forms (figure 1R).

In support of the results above, protein expression levels of all proteins involved in the TGFβ signalling pathway were analysed. The receptor complex, namely ALK5 ($P < 0.01$; figure 1J), TGFβR2 ($P < 0.01$; figure 1K) and SARA ($P < 0.001$; figure 1L), and the downstream mediators, namely pSmad2/Smad2 ($P < 0.05$; figure 1O) and pSmad3/Smad3 ($P < 0.01$; figure 1N), were significantly increased in HUVEC stimulated with TGFβ1. Smad4 expression (figure 1M) was not affected by TGFβ1. The high expression of these proteins means that the TGFβ signalling pathway was activated and as a consequence, the expression of SM22α is higher as well. With the addition of SB431542 we can see that the levels of the receptors complexes and the downstream mediators didn't differ from the control levels, indicating the inhibition of TGFβ signalling pathway with the inhibition of the receptor ALK5.

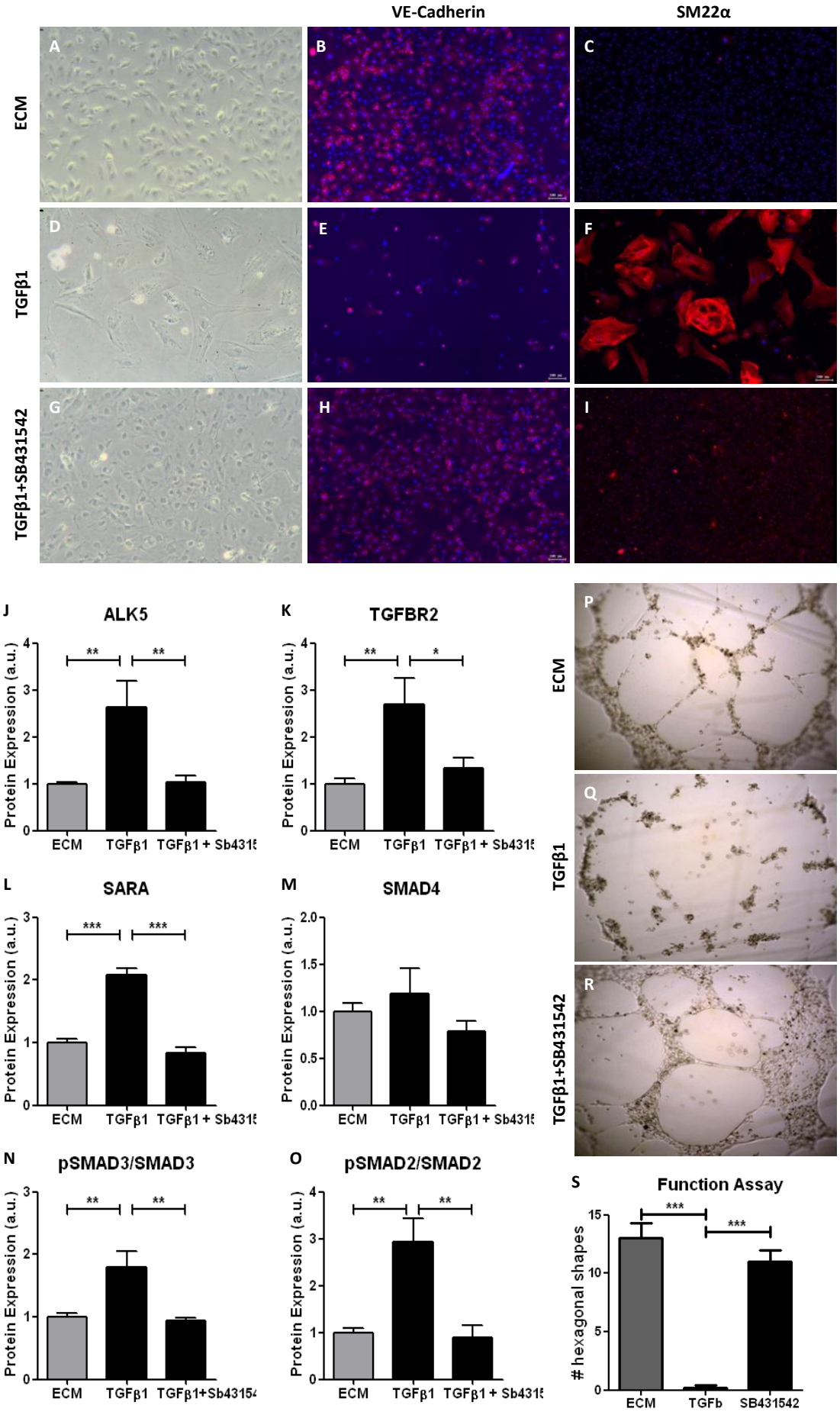
VE-Cadherin
SM22α
ECM
TGFβ1
TGFβ1+SB431542
A
B
C
D
E
F
G
H
I
J
ALK5
K
TGFBR2
L
SARA
M
SMAD4
N
pSMAD3/SMAD3
O
pSMAD2/SMAD2
Protein Expression (a.u.)
ECM
TGFβ1
TGFβ1 + Sb431
TGFβ1+Sb4315
P
Q
R
S
Function Assay
hexagonal shapes
TGFb
SB431542

Figure 1: TGFβ induces EndMT. HUVEC were cultured either in ECM (A-C and P) or R204 (D-F and Q) or R204 with SB431542 (G-I and R). In the light microscopy (A; D and G), at a magnification of 10 times, is possible to see the cell stimulated with TGFβ1 getting hypertrophic compared with control, HUVEC cultured in ECM (A). HUVEC were stained with a endothelial marker, VE-Cadherin (red; B; E; and H), and a mesenchymal marker, SM22α (red; C; F and I), and the expression of both markers was analysed by immunofluorescence. Protein expression was quantified by Western Blot analysis (J-O). Sprouting assay was perform to verified the endothelial function, where the cells were seeded in Matrigel for 1 day and their ability to aggregate and sprout to create a vessels forms was analysed (P-R) and quantified (S), in the light microscopy at a magnification of 5 times. HUVEC = human umbilical vein endothelial cells. ECM = endothelial cell growth medium. TGFβ1 = transforming growth factor β 1.R204 = endothelial cell growth medium with TGFβ1. SM22α= smooth muscle protein 22 alpha. * = $P < 0.05$ vs ECM or SB. ** = $P < 0.01$ vs ECM or SB. *** = $P < 0.001$ vs ECM or SB.

3.2. MiR20a targets TGFβ1-ALK5-Smad2/3 signaling

The knowledge of miR20a target genes in this signaling pathway is unknown. By using luciferase reporter assays we were able to determine the possible target genes. As shown in figure 2, miR20α can target almost all the mRNA of the genes of interest, with significant interaction with the mRNA of both TGFβ receptors, ALK5 ($P < 0.001$; figure 2A) and TGFβR2 ($P < 0.001$; figure 2B), SARA ($P<0.01$; figure 2D) and Smad2 ($P < 0.05$; figure 2E).

Having identify the miR20a target genes, allow us to better understand the mechanism behind the EndMT inhibition by miR20a. Being able to bind to their target mRNA, especially to the TGFβ receptors, TGFβ cannot be capture by his receptors, since the receptors aren't present on the cell membrane. Even when miR20a binds SARA mRNA, this anchor protein for Smad molecules isn't produced and no longer able to bring Smad2/3 in the ALK5 proximities to be phosphorylated. With the binding of miR20a to Smad2, Smad2 cannot associate with Smad4 and the Smad complex cannot translocate into the nucleus and bind with the Smad-binding element (SBE) and the interaction with transcription factors doesn't occur.

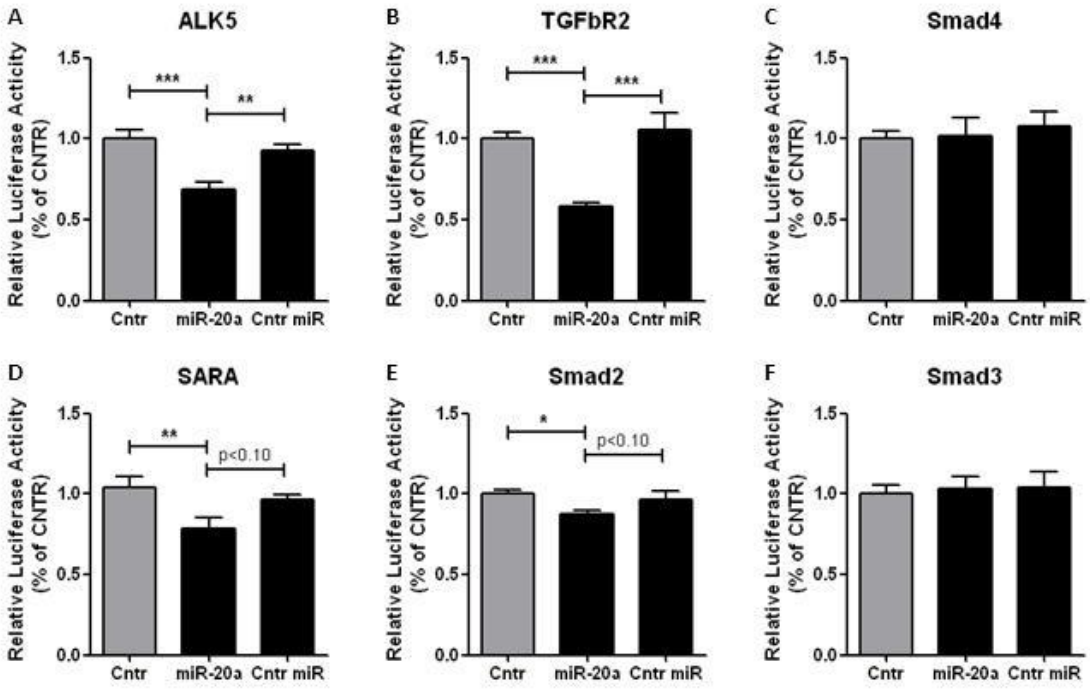

Figure 2: MiR20a target genes. Relative Luciferase activity of miR20a in the target messenger RNA. Cntr = Control; Cntr miR= miR control (miR608); * = $P < 0.05$ vs control. ** = $P < 0.01$ vs control. *** = $P < 0.001$ vs control.

3.3. miR20a gain-of-function inhibits TGFβ1-ALK5-Smad2/3 signaling

The overexpression of HUVEC with miR20a allows to see miR20a inhibiting EndMT. HUVEC transfected with miR20a showed a higher relative expression of miR20a, comparing with the control ($P < 0.001$; figure 3F). Through matrigel sprouting, as controls, SCR and overexpressed HUVECs in ECM, have the same behaviour as the HUVEC in ECM (figure 1P-Q), being capable to maintain their endothelial function proprieties (figure 3A and 3C). Upon TGFβ stimulation, HUVECs that were overexpressed with miR20a show no demise in sprouting behaviour (figure 3D), while in HUVEC treated with a srambled miRNA sequence (SCR), TGFβ inhibits the angiogenic sprouting (figure 3B). This indicates that miR20a can inhibit EndMT by inhibiting the TGFβ -ALK5-Smad2/3 pathway.

Taking into account the previous luciferase results, we can say that TGFβ signaling pathway and this inhibition occur with the binding of miR20a in the mRNA of TGFbR2, ALK5, SARA or Smad2.

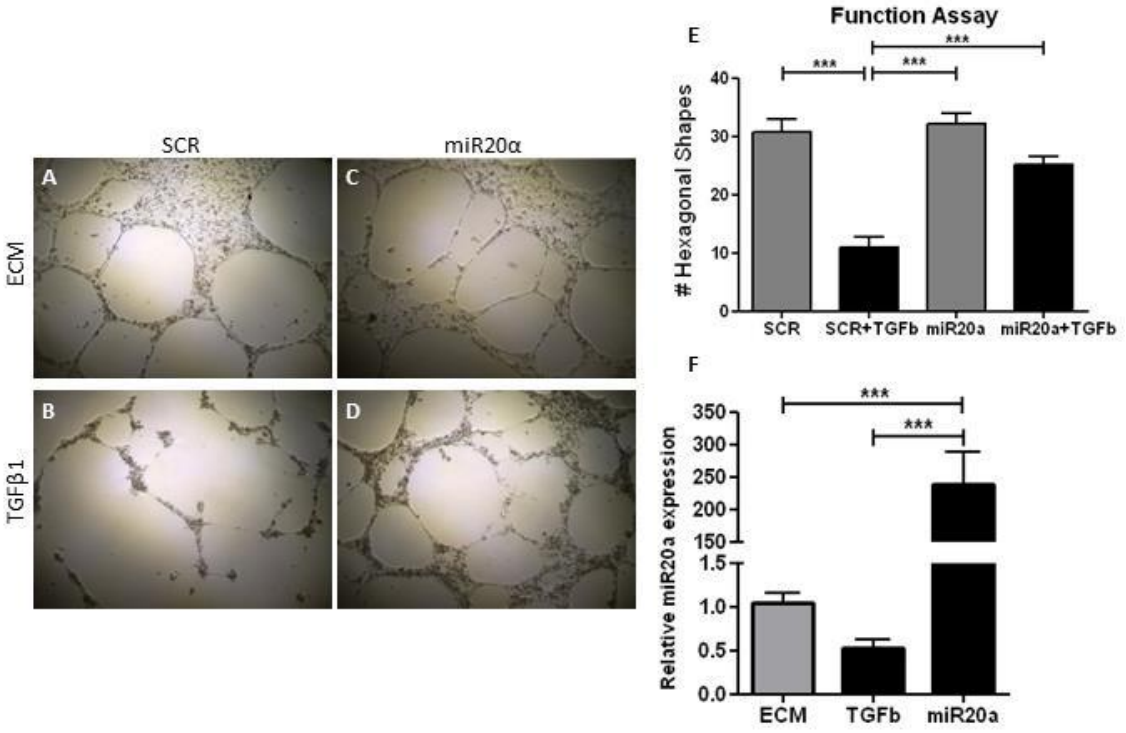

Figure 3: Ectopic expression of miR20a inhibits EndMT. HUVEC were overexpressed with miR20α and SCR (control), using lentiviral transfection and were cultured either in ECM (A and C) or R204 (B and D). Sprouting assay was perform to verified the endothelial function, where the cells were seeded in Matrigel for 1 day and their ability to aggregate and sprout to create a vessels forms was analysed (A–D) and quantified (E), in the light microscopy at a magnification of 5 times. RT-PCR was done to quantify the relative expression of miR20a (F) of HUVEC transfected with miR20a. miR20a = microRNA 20a. SCR = scramble sequence. *** = $P < 0.001$ vs ECM or SCR or SCR plus TGFβ1.

3.4. bFGF induces miR20a expression through JNK and Erk1/2 signaling and inhibit EndMT

MiR20a is expressed in the endothelial cells, but how the expression is regulated is unknown. We speculated that the main pathway of miR20a expression in the endothelial cells occurs via specific growth factors. Indeed, stimulating endothelial cells with various growth factors modulated miR20a levels, notably bFGF indicated the highest miR20a expression ($P < 0.001$; figure 4A). To further identify if bFGF signaling could reduce the expression of TGFβ signaling members, via the upregulation of miR20a, we measured the protein levels of HUVEC stimulated with TGFβ plus bFGF. The proteins levels in the TGFβ receptors ($P < 0.001$; figure 4C-D), Smad4 ($P < 0.01$; figure 4F) and in the downstream mediator pSmad2/Smad2 ($P < 0.01$; figure 4G) are significantly lower than in HUVEC stimulated only with TGFβ.

We further investigated the expression levels of the mesenchymal marker SM22α, which was lower in cells stimulated with TGFβ and bFGF, compared to cells stimulated with bFGF alone ($P < 0.001$; figure 4K). EndMT was inhibited with the addition of bFGF, and that is also seen in Figure 4J, were the expression of SM22α very low. This indicates that miR20a was induced through bFGF pathway.

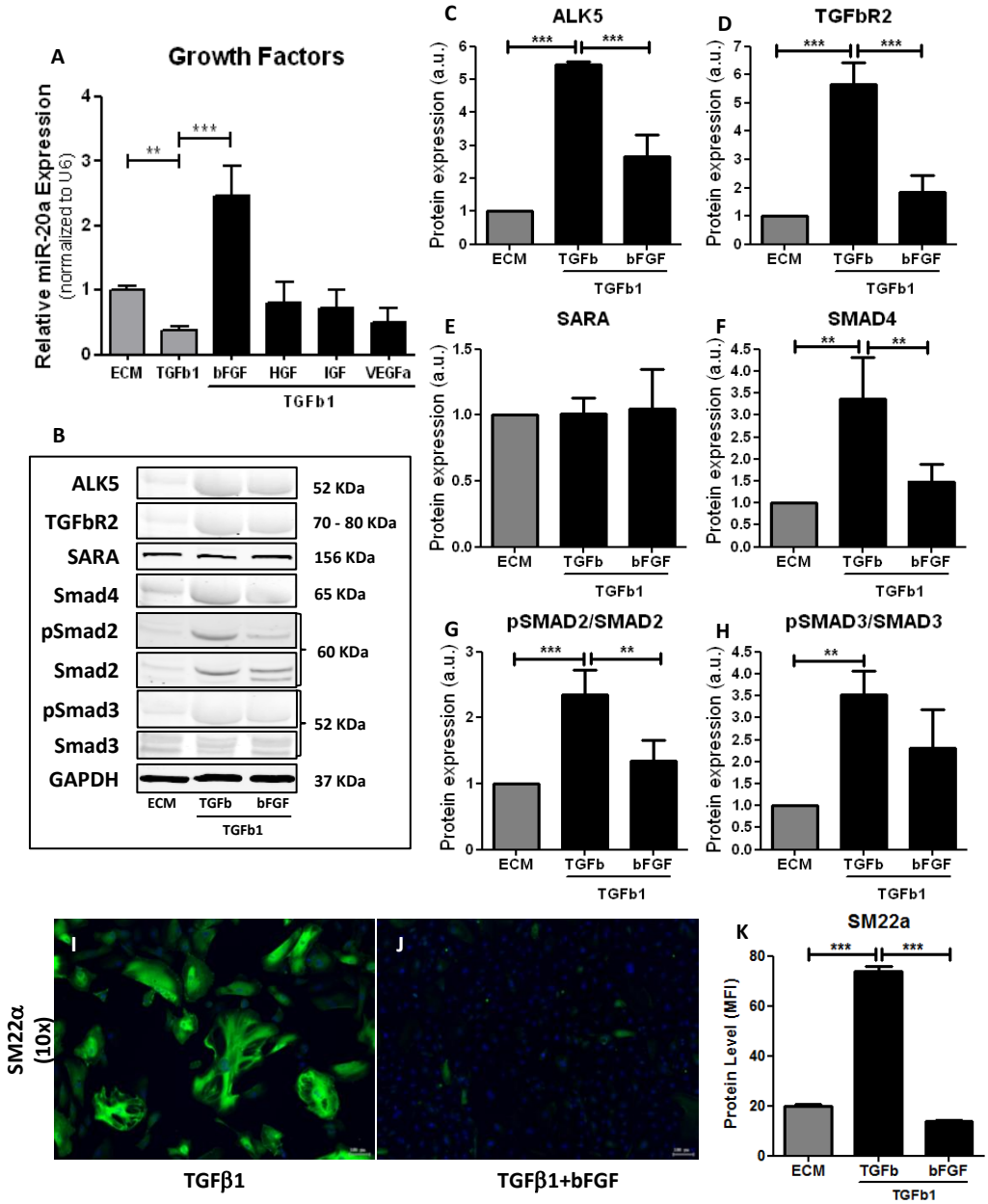

Figure 4: **Immunofluorescence analysis of mesenchymal marker, relative miR20a expression and protein expression analysis of transdifferentiated HUVEC**. HUVEC were cultured either in ECM, R204 or R204 supplemented with bFGF (50ng/μL; Peprotech), HGF (100ng/μL; Peprotech), IGF (50ng/μL; Peprotech) or VEGFa (50ng/μL; Peprotech). RT-PCR was performed to quantify the relative expression of miR20a (A). Protein expression was analysed (B) and quantified (C – H) by Western Blot. Mesenchymal marker, SM22α (green; I – J), was analysed and quantified by immunofluoresce (K). bFGF = Basic

Fibroblast Growth Factor; HGF = Hepatocyte Growth Factor. IGF = Insulin-like Growth Factor. VEGFa = Vascular Endothelial Growth Factor A. * = $P < 0.05$ vs ECM or bFGF. ** = $P < 0.01$ vs ECM or bFGF. *** = $P < 0.001$ vs ECM or bFGF.

Looking into bFGF pathway we could identify different possible ways of how miR20a could be induced in HUVEC. To determine which pathway induces miR20a expression, we additionally treated HUVEC with TGFβ, bFGF and different inhibitors, namely: RAS inhibitor (0,5 mM FTS; Farnesyl Thiosalicylic Acid), PI3K inhibitor (1 mM Wortmannin), Erk1/2 inhibitor (1m mM U0126), JNK inhibitor (1 mM SP600125) or p38 inhibitor (1 mM SB203580).

Measuring the relative expression of miR20a in each sample, we could identify RAS, JNK and Erk1/2 as possible pathways (figure 5A). To further validate these results, we analysed the expression levels of VE-Cadherin and SM22a. In both results, the cells stimulated with TGFβ, bFGF and bFGF inhibitor (RAS inhibitor, JNK inhibitor and Erk1/ inhibitor), had lower expression of VE-Cadherin ($P < 0.001$, figure 5B) and higher expression of and SM22a ($P < 0.001$; figure 5C), compared with the cells stimulated with bFGF alone. The expected level of VE-Cadherin and SM22a are the same as in EndMT (R204; figure 5B-C). However, the levels of VE-Cadherin in JNK inhibitor and Erk1/2 inhibitor are lower than in R204, as well as the levels of SM22a are higher than the values in R204. Being RAS a mediator of JNK and Erk1/2, is expected the identical levels of RAS.

This results show us that the miR20a can be induced via bFGF-RAS-JNK and/or bFGF-RAS-Erk1/2.

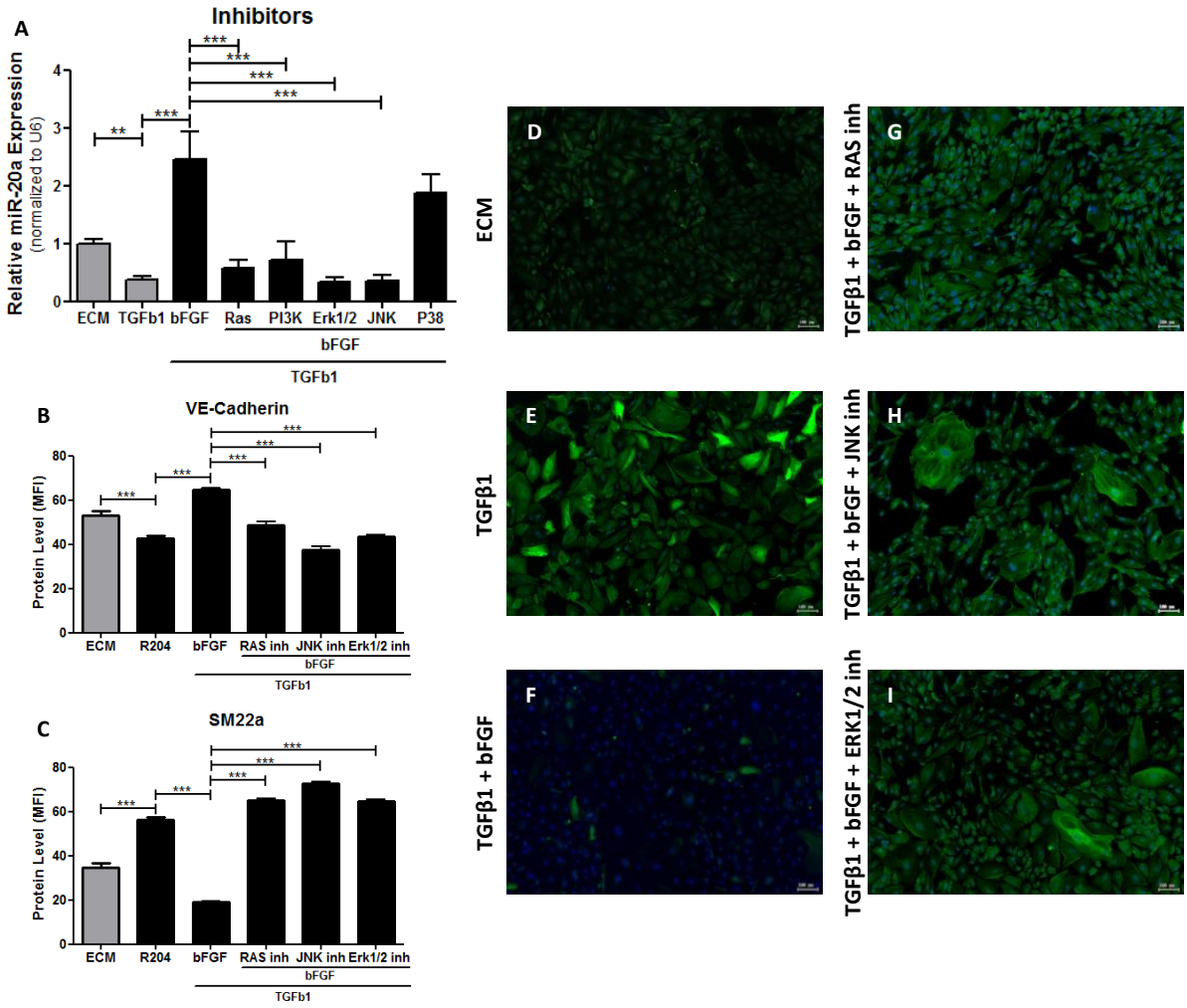

Figure 5: Relative miR20a expression and immunofluorescence analysis of endothelial and mesenchymal marker of transdifferentiated HUVEC. HUVEC were cultured either in ECM, R204, bFGF or R204 supplemented with bFGF and bFGF inhibitor (RAS inhibitor (0,5mM FTS; #10010501, Cayman Chemical), PI3K inhibitor (1mM Wortmannin; #9951, Cell Signaling), Erk1/2 inhibitor (1mM U0126; #V1121, Promega), JNK inhibitor (1 mM SP600125; #420119, EMD Millipore) or p38 inhibitor (1mM SB203580)). RT-PCR was performed to quantify the relative expression of miR20a (A). Endothelial marker, VE-Cadherin (B) and mesenchymal marker, SM22α (green; D – I), was analysed and quantified by immunofluoresce (C). * = $P < 0.05$ vs ECM or bFGF. ** = $P < 0.01$ vs ECM or bFGF. *** = $P < 0.001$ vs ECM or bFGF.

4. Discussion

Inhibition of EndMT is important to reduce the incidence of fibroblast responsible for scar formation in cardiovascular tissues. There are not many studies of miRNAs in cardiovascular diseases, but since miRNAs have a big importance in the development

and function of the cardiovascular system, is important to investigate their role in case of cardiovascular diseases.

When Endothelial-Mesenchymal transition (EndMT) occurs, the most clear phenomenon is the switch in cell markers expression. There is loss in the expression of endothelial marker and increase expression of mesenchymal marker, and this downstream effect of events are initiated by TGFβ. (Figure 1). Being able to induce EndMT in HUVEC using TGFβ1, was possible to study the role of miR20a in the TGFβ-ALK5-Smad2/3 signaling cascade and how this miRNA can inhibit it.

This study shows that miR20a can inhibit EndMT with the inhibition of the TGFβ-ALK5-Smad2/3 signaling cascade. As shown on figure 3, HUVEC previously transfected with miR20a, maintain their endothelial structural properties and function. The inhibition of EndMT by miR20a is due to this miRNA being capable to bind to the 3'UTR mRNA of TGFβR2, ALK5, SARA and Smad2. Knowing the miR20a target mRNA allow us to better understand the mechanism behind the EndMT inhibition by miR20a.

Figure 6 illustrates possible ways by which miR20a can inhibit EndMT. First, miR20a can bind to TGFβ type II receptor not being able to phosphorylate the TGFβ type I receptor (ALK5) and the propagation of TGFβ signal is reduced, as well as the expression of SM22a. Second, miR20a can bind ALK5 and prevent the phosphorylation of Smad2/3, since the receptor is not activated the TGFβ cascade is inhibit. Third, miR20a can also target SARA, not being able to bring the downstream mediators, Smad2 and Smad3, to the activated ALK5 proximities. And last, miR20a can target Smad4, which interfere with the formation of transcriptional complex, Smad2/3/4 formation, not being able to be translocated into the nucleus and the transcriptional activity is compromised.

SM22α is a smooth muscle-specific promoter and is one of the most widely expressed smooth muscle cell markers identified.[14] TGFβ upregulate SM22α gene activation in fibroblast through binding of Smad proteins to two Smad-binding motifs located within the first exon. With the inhibition of TGFβ-ALK5-Smad2/3 signaling cascade, the levels of SM22a gene expression reduced.

When several growth factors were tested for their capacity to induce miR20a expression in HUVECS, we observed that bFGF possess that capacity (figure 4A). HUVECs stimulated with TGFβ and bFGF, leads to a significant decrease of the TGFβ receptors, ALK5 and TGFβR2, Smad4 and Smad2 which means that TGFβ-ALK5-Smad2/3 signaling was inhibit. This results were also shown by Shi, H. X. et al.[15], were

they show bFGF reduce scarring and promotes wound healing by inhibition TGFβ/Smad-dependent pathway. Both finding indicates that miR20a can have anti-scarring effects, being able to minimize the scar formation through the inhibition of EndMT.

Our data also highlight the fact of bFGF–Erk1/2 pathway and/or bFGF–JNK pathways are the main pathways in with miR20a is induced.

In conclusion, our study demonstrates that endothelial cells (HUVECs) can be differentiate into mesenchymal cell by TGFβ-ALK5-Smad2/3-mediated EndMT. This processes can be inhibit by the binding of miR20a to his target mRNA genes, ALK5, TGFβR2, Smad4 and Smad2. MiR20a can be induced in endothelial cells through bFGF–Erk1/2 and bFGF–JNK pathways.

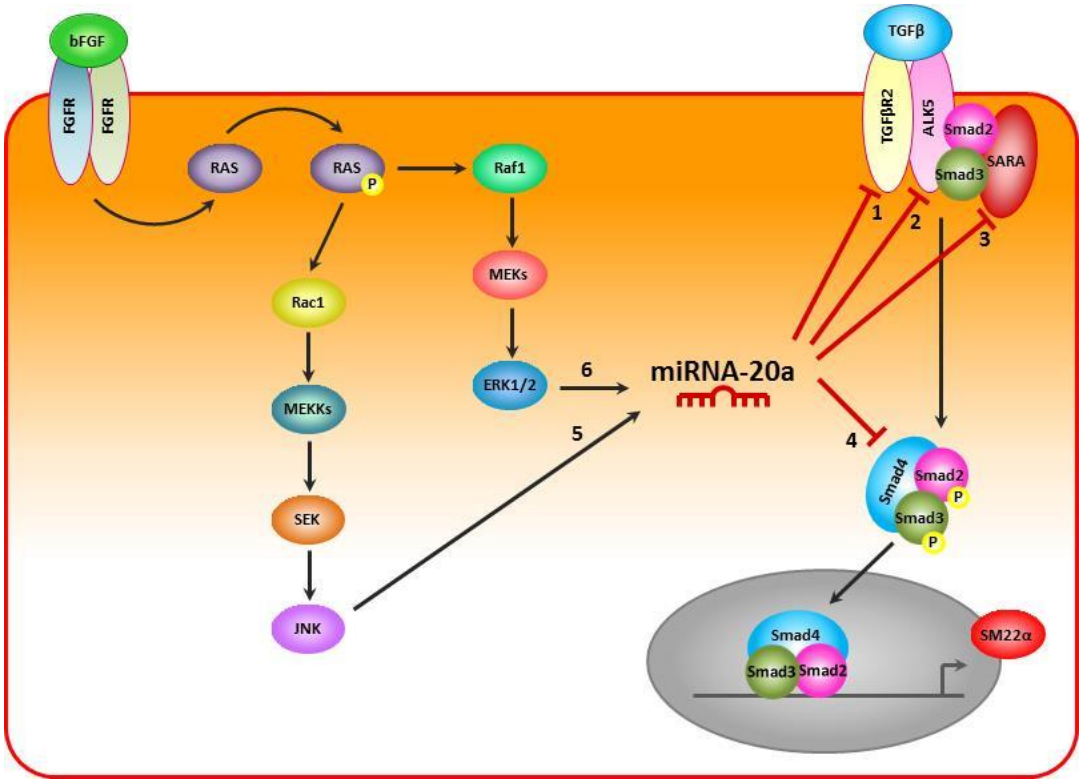

Figure 6: Inhibitory action of miR20a on TGFβ-ALK5-Smad2/3 signaling pathway and possible pathways of miR20a upregulation. Four possible ways of inhibition are illustrated. **1**, miR20a can target TGFβ type II receptor (TGFβR2) and inhibit ALK5-directed phosphorylation. **2**, miR20a can target TGFβ type I receptor (ALK5) and inhibit the activation of the downstream mediators, Smad2/3. **3**, miR20a target SARA not being able to bring the downstream mediators, Smad2 and Smad3, in the activated ALK5 proximities. **4**, miR20a target Smad2, therefore the formation of the complex Smad2/3 with Smad4 is compromised and cannot translocate to the nucleus were can interfering with gene expression. bFGF induces expression of miR20a, as described in the study, through JNK (**5**) or Erk1/2 (**6**) signaling activation

References

1. Yoshimatsu Y, Watabe T. Roles of tgf-beta signals in endothelial-mesenchymal transition during cardiac fibrosis. *International journal of inflammation*. 2011;2011:724080
2. Goumans MJ, van Zonneveld AJ, ten Dijke P. Transforming growth factor beta-induced endothelial-to-mesenchymal transition: A switch to cardiac fibrosis? *Trends in cardiovascular medicine*. 2008;18:293-298
3. Krenning G, Zeisberg EM, Kalluri R. The origin of fibroblasts and mechanism of cardiac fibrosis. *Journal of cellular physiology*. 2010;225:631-637
4. Zeisberg EM, Tarnavski O, Zeisberg M, Dorfman AL, McMullen JR, Gustafsson E, Chandraker A, Yuan X, Pu WT, Roberts AB, Neilson EG, Sayegh MH, Izumo S, Kalluri R. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. *Nature medicine*. 2007;13:952-961
5. Krenning G, Moonen JR, van Luyn MJ, Harmsen MC. Generating new blood flow: Integrating developmental biology and tissue engineering. *Trends in cardiovascular medicine*. 2008;18:312-323
6. Piera-Velazquez S, Li Z, Jimenez SA. Role of endothelial-mesenchymal transition (endomt) in the pathogenesis of fibrotic disorders. *The American journal of pathology*. 2011;179:1074-1080
7. Piera-Velazquez S, Jimenez SA. Molecular mechanisms of endothelial to mesenchymal cell transition (endomt) in experimentally induced fibrotic diseases. *Fibrogenesis & tissue repair*. 2012;5 Suppl 1:S7
8. Maleszewska M, Moonen JR, Huijkman N, van de Sluis B, Krenning G, Harmsen MC. Il-1beta and tgfbeta2 synergistically induce endothelial to mesenchymal transition in an nfkappab-dependent manner. *Immunobiology*. 2013;218:443-454
9. Hata A. Functions of micrornas in cardiovascular biology and disease. *Annual Review of Physiology*. 2013;75:69-93
10. Sun W, Julie Li Y-S, Huang H-D, Shyy JYJ, Chien S. Microrna: A master regulator of cellular processes for bioengineering systems. *Annual Review of Biomedical Engineering*. 2010;12:1-27
11. Nilsen TW. Mechanisms of microrna-mediated gene regulation in animal cells. *Trends in genetics : TIG*. 2007;23:243-249
12. Zhang JF, Fu WM, He ML, Xie WD, Lv Q, Wan G, Li G, Wang H, Lu G, Hu X, Jiang S, Li JN, Lin MC, Zhang YO, Kung HF. Mirna-20a promotes osteogenic differentiation of human mesenchymal stem cells by co-regulating bmp signaling. *RNA biology*. 2011;8:829-838
13. Callahan JF, Burgess JL, Fornwald JA, Gaster LM, Harling JD, Harrington FP, Heer J, Kwon C, Lehr R, Mathur A, Olson BA, Weinstock J, Laping NJ. Identification of novel inhibitors of the transforming growth factor beta1 (tgf-beta1) type 1 receptor (alk5). *Journal of medicinal chemistry*. 2002;45:999-1001
14. Solway J, Forsythe SM, Halayko AJ, Vieira JE, Hershenson MB, Camoretti-Mercado B. Transcriptional regulation of smooth muscle contractile apparatus expression. *American journal of respiratory and critical care medicine*. 1998;158:S100-108
15. Shi HX, Lin C, Lin BB, Wang ZG, Zhang HY, Wu FZ, Cheng Y, Xiang LJ, Guo DJ, Luo X, Zhang GY, Fu XB, Bellusci S, Li XK, Xiao J. The anti-scar effects of basic fibroblast growth factor on the wound repair in vitro and in vivo. *PloS one*. 2013;8:e59966

Part III

| Conclusions

Our results are the first to demonstrate the interaction between miR20a and the TGFβ-ALK5-Smad2/3 signaling cascade in endothelial cells. We show that miR20a has specific target genes in this cascade, namely TGFβR2, ALK5, SARA and Smad2. The binding of miR20a to these target genes allows for the inhibition of the cascade and consequently, inhibition of EndMT. Overexpression of miR20a in HUVEC showed this inhibition, since the endothelial cell can maintain their function and properties.

We also demonstrate that miR20a is induced in endothelial cells through bFGF signaling, specifically through bFGF-JNK and bFGF-Erk1/2 pathway. Knowing that bFGF can reduce scar formation and promotes wound healing by inhibition of TGFβ/Smad-dependent pathway, we hypothesis that miR20a can have anti-scarring effects, being able to minimize the scar formation through the inhibition of EndMT.

Although this results show a major role of miR20a in the inhibition of EndMT, further experiments are required to better understand the molecular mechanism of miR20a in endothelial cells as well as the overall function. Taken together, our results provide new insights into the role of miR20a in EndMT, which serve as starting point for the future research and a novel therapeutic approach wherein miRNAs may function as anti-fibrosis medicines.

| References

1. Alwan A. Global status report on noncommunicable diseases. 2011
2. Shen E, Diao X, Wei C, Wu Z, Zhang L, Hu B. Micrornas target gene and signaling pathway by bioinformatics analysis in the cardiac hypertrophy. *Biochemical and biophysical research communications*. 2010;397:380-385
3. Frey N, Olson EN. Cardiac hypertrophy: The good, the bad, and the ugly. *Annu Rev Physiol*. 2003;65:45-79
4. Moonen JR, Harmsen MC, Krenning G. Cellular plasticity: The good, the bad, and the ugly? Microenvironmental influences on progenitor cell therapy. *Canadian journal of physiology and pharmacology*. 2012;90:275-285
5. Bruce Alberts AJ, Julian Lewis, Martin Rafi, Keith Roberts PW. *Molecular biology of the cell*. Garland Science; 2008:1445-1450.
6. Sumpio BE, Riley JT, Dardik A. Cells in focus: Endothelial cell. *The international journal of biochemistry & cell biology*. 2002;34:1508-1512
7. Deanfield JE, Halcox JP, Rabelink TJ. Endothelial function and dysfunction: Testing and clinical relevance. *Circulation*. 2007;115:1285-1295
8. Hirase T, Node K. Endothelial dysfunction as a cellular mechanism for vascular failure. *American journal of physiology. Heart and circulatory physiology*. 2012;302:H499-505
9. Marino F, Guasti L, Tozzi M, Schembri L, Castiglioni L, Molteni E, Piffaretti G, Castelli P, Cosentino M. Gene expression of adhesion molecules in endothelial cells from patients with peripheral arterial disease is reduced after surgical revascularization and pharmacological treatment. *International journal of vascular medicine*. 2013;2013:412761
10. Yang YM, Huang A, Kaley G, Sun D. Enos uncoupling and endothelial dysfunction in aged vessels. *American journal of physiology. Heart and circulatory physiology*. 2009;297:H1829-1836
11. Kofler S, Nickel T, Weis M. Role of cytokines in cardiovascular diseases: A focus on endothelial responses to inflammation. *Clinical science*. 2005;108:205-213
12. Wynn TA. Cellular and molecular mechanisms of fibrosis. *The Journal of pathology*. 2008;214:199-210
13. Yoshimatsu Y, Watabe T. Roles of tgf-beta signals in endothelial-mesenchymal transition during cardiac fibrosis. *International journal of inflammation*. 2011;2011:724080
14. Krenning G, Zeisberg EM, Kalluri R. The origin of fibroblasts and mechanism of cardiac fibrosis. *Journal of cellular physiology*. 2010;225:631-637

15. Zeisberg EM, Tarnavski O, Zeisberg M, Dorfman AL, McMullen JR, Gustafsson E, Chandraker A, Yuan X, Pu WT, Roberts AB, Neilson EG, Sayegh MH, Izumo S, Kalluri R. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. *Nature medicine*. 2007;13:952-961

16. Goumans MJ, van Zonneveld AJ, ten Dijke P. Transforming growth factor beta-induced endothelial-to-mesenchymal transition: A switch to cardiac fibrosis? *Trends in cardiovascular medicine*. 2008;18:293-298

17. Piera-Velazquez S, Li Z, Jimenez SA. Role of endothelial-mesenchymal transition (endomt) in the pathogenesis of fibrotic disorders. *The American journal of pathology*. 2011;179:1074-1080

18. Krenning G, Moonen JR, van Luyn MJ, Harmsen MC. Generating new blood flow: Integrating developmental biology and tissue engineering. *Trends in cardiovascular medicine*. 2008;18:312-323

19. Weiss A, Attisano L. The tgfbeta superfamily signaling pathway. *Wiley interdisciplinary reviews. Developmental biology*. 2013;2:47-63

20. van Meeteren LA, ten Dijke P. Regulation of endothelial cell plasticity by tgf-beta. *Cell and tissue research*. 2012;347:177-186

21. Gordon KJ, Blobe GC. Role of transforming growth factor-beta superfamily signaling pathways in human disease. *Biochimica et biophysica acta*. 2008;1782:197-228

22. Ikushima H, Miyazono K. Tgfbeta signalling: A complex web in cancer progression. *Nature reviews. Cancer*. 2010;10:415-424

23. Bakkebo M, Huse K, Hilden VI, Forfang L, Myklebust JH, Smeland EB, Oksvold MP. Sara is dispensable for functional tgf-beta signaling. *FEBS letters*. 2012;586:3367-3372

24. Camoretti-Mercado B, Fernandes DJ, Dewundara S, Churchill J, Ma L, Kogut PC, McConville JF, Parmacek MS, Solway J. Inhibition of transforming growth factor beta-enhanced serum response factor-dependent transcription by smad7. *The Journal of biological chemistry*. 2006;281:20383-20392

25. Hata A. Functions of micrornas in cardiovascular biology and disease. *Annual Review of Physiology*. 2013;75:69-93

26. Small EM, Olson EN. Pervasive roles of micrornas in cardiovascular biology. *Nature*. 2011;469:336-342

27. Sun W, Julie Li Y-S, Huang H-D, Shyy JYJ, Chien S. Microrna: A master regulator of cellular processes for bioengineering systems. *Annual Review of Biomedical Engineering*. 2010;12:1-27

28. Shruti K, Shrey K, Vibha R. Micro rnas: Tiny sequences with enormous potential. *Biochemical and biophysical research communications*. 2011;407:445-449

29. Latronico MV, Condorelli G. Micrornas and cardiac pathology. *Nature reviews. Cardiology*. 2009;6:419-429

30. Esquela-Kerscher A, Slack FJ. Oncomirs - micrornas with a role in cancer. *Nature reviews. Cancer*. 2006;6:259-269

31. Kartha RV, Subramanian S. Micrornas in cardiovascular diseases: Biology and potential clinical applications. *Journal of cardiovascular translational research*. 2010;3:256-270

32. Park CY, Choi YS, McManus MT. Analysis of microrna knockouts in mice. *Human molecular genetics*. 2010;19:R169-175

33. Suarez Y, Fernandez-Hernando C, Pober JS, Sessa WC. Dicer dependent micrornas regulate gene expression and functions in human endothelial cells. *Circulation research*. 2007;100:1164-1173

34. Bonauer A, Carmona G, Iwasaki M, Mione M, Koyanagi M, Fischer A, Burchfield J, Fox H, Doebele C, Ohtani K, Chavakis E, Potente M, Tjwa M, Urbich C, Zeiher AM, Dimmeler S. Microrna-92a controls angiogenesis and functional recovery of ischemic tissues in mice. *Science*. 2009;324:1710-1713

35. Zhang JF, Fu WM, He ML, Xie WD, Lv Q, Wan G, Li G, Wang H, Lu G, Hu X, Jiang S, Li JN, Lin MC, Zhang YO, Kung HF. Mirna-20a promotes osteogenic differentiation of human mesenchymal stem cells by co-regulating bmp signaling. *RNA biology*. 2011;8:829-838

36. Poitz DM, Augstein A, Gradehand C, Ende G, Schmeisser A, Strasser RH. Regulation of the hif-system by micro-rna 17 and 20a - role during monocyte-to-macrophage differentiation. *Molecular immunology*. 2013;56:442-451

37. Fontana L, Pelosi E, Greco P, Racanicchi S, Testa U, Liuzzi F, Croce CM, Brunetti E, Grignani F, Peschle C. Micrornas 17-5p-20a-106a control monocytopoiesis through aml1 targeting and m-csf receptor upregulation. *Nature cell biology*. 2007;9:775-787

38. Moonen JR, Krenning G, Brinker MG, Koerts JA, van Luyn MJ, Harmsen MC. Endothelial progenitor cells give rise to pro-angiogenic smooth muscle-like progeny. *Cardiovascular research*. 2010;86:506-515