PhD Thesis:

Functional Roles of microRNA-214 in Modulating Smooth Muscle Cell Functions and Neointima Formation

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Statement of originality

I, Tayyab Adeel Afzal, confirms that all research included within this thesis is my own work. The work carried out by collaborators and/or with the support of other colleagues has been properly indicated and acknowledged below along with my contributions. Previously published research papers are also acknowledged below.

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Collaborations & Contributions

I am extremely thankful for valuable suggestions and practical help from my group members and external collaborators during my PhD work. Dr. Qingzhong Xiao's was very helpful in designing the overall project and performing the experiments. mRNA microarray assay was carried out with the help from Dr. Li Zhang at Zhejiang University, China. I am very thankful to Dr. Le Anh for his valuable suggestions. He also helped me in creating miRNA reporters and generation of NCKAP1 knockdown cell lines. Dr. Feng Yang performed the in vivo experiments pertaining miR-214 agomirs and femoral artery injury models. Dr.Qishan Chen, and Dr.Feng Yang also helped me in performing VSMCs gain and loss of function experiments. I am extremely thankful for continued guidance of Dr. Kenta Yashiro as my 2nd supervisor. Dr.Edmund Wilkes and Dr.Pedro R. Cutillas helped and supervised the proteomics work, generation of volcano plot and relevant data.

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2. Zhao H, Wen G, Huang Y, Yu X, Chen Q, **Afzal TA**, Luong le A, Zhu J, Ye S, Zhang L, Xiao Q. MicroRNA-22 regulates smooth muscle cell differentiation from stem cells by targeting methyl CpG-binding protein 2. Arterioscler Thromb Vasc Biol. 2015 Apr;35(4):918-29.

3. Zhang C, Chen D, Maguire EM, He S, Chen J, An W, Yang M, **Afzal TA,** Luong LA, Zhang L, Lei H, Wu Q, Xiao Q. Cbx3 inhibits vascular smooth muscle cell proliferation, migration, and neointima formation. Cardiovasc Res. 2018 Mar 1;114(3):443-455

4. Yang F, Chen Q, He S, Yang M, Maguire EM, An W, **Afzal TA**, Luong LA, Zhang L, Xiao Q. miR-22 Is a Novel Mediator of Vascular Smooth Muscle Cell Phenotypic Modulation and Neointima Formation. Circulation. 2018 Apr 24;137(17):1824-1841.

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Abstract.

Vascular Smooth Muscle cells (VSMCs) have very important role in pathogenesis of atherosclerosis and neointima formation. VSMCs phenotype switching and resultant increase in migration and proliferation under the influence of certain pro-inflammatory mediators is widely addressed but further research aiming at identifying the molecular mechanisms governing these changes is still required. Since the identification of microRNA (miR) as potent post transcriptional gene regulators, several studies have revealed their role in atherosclerosis and neointima formation. miR-214 was previously considered as oncogene but later studies have pointed at its role in tumourogenesis inhibition. Researchers have postulated a divergent role of miR-214 in cardiovascular diseases ranging from beneficial and cardiomyocyte protector to damaging by causing cardiac hypertrophy but its role in VSMC functional regulation is still unclear. In our study, we analysed its role in regulating VSMC function (migration and proliferation) during neointima formation. By applying loss of function and gain of function experiments, we identified that miR-214 has an inverse relationship to VSMCs functions. Overexpressing miR-214 resulted in reduced VSMC migration and proliferation and vice versa. Proteomics analysis and algorithmic computation models revealed an interesting relationship between miR-214 and Nck associated protein 1 (NCKAP1), a major components of WAVE complex and involved in regulating actin polymerization and lamellipodia formation.

In our study on VSMCs, we have identified NCKAP1 as the functional target gene of miR-214 in regulating VSMC functions in atherosclerosis and neointima formation. By implementing gene and protein biochemical assays including proteomic analysis, we have provided compelling evidence to support a regulatory role for miR-214/NCKAP1 in VSMCs migration and motility. NCKAP1 knockdown in VSMCs recapitulates the inhibitory effects of miR-214 over-expression on actin plymerization, cell migration and proliferation. Furthermore, cotransfection experiments

also revealed that inhibition of NCKAP1 is required for miR-214 medited lamellipodia formation, cell motility and growth. Importantly, locally enforced expression of miR-214 to the injured vessels significantly reduced NCKAP1 expression levels, inhibited VSMC proliferation and prevented neointima hyperplasia after injury.

Our data is in line with the aforementioned role of NCKAP1 as imperative and constitutive component of WAVE complex, leading to lamellipodia formation and actin filament polymerization. We have extended NCKAP1's role in VSMC mobility and proliferation under the effect of pro-inflammatory mediators and under conditions of vascular injury. Our study has uncovered an important relationship between miR-214 and its target gene NCKAP1 in modulating VSMC functions and their effect on neointima hyperplasia. Our findings suggest that miR-214 represents a potentioal therapeutic target for vascular disease.

List of Abbreviations:

2D	2-Dimensional
3D	3-Dimensional
ACE	Angiotensin converting enzyme
ABCA1	ATP Binding Cassette Transporter A1
AGO	Argonaute
Ang-II	Angiotensin-II
APS	Ammonium persulfate
AVSD	Arteriosclerotic vascular disease
AT1R	Angiotensin II type 1 Receptor
Bcl-2	B-cell leukemia/lymphoma 2
BrdU	5-Bromo-2'-Deoxyuridine
BHF	British Heart Foundation
BM derived	Bone Marrow derived
BMP	Bone Morphogenetic Protein
β-ME	β- Mercaptoethanol
BTG3	B cell translocation gene3
CAD	Coronary Artery Disease
CAM	Cell adhesion molecule
CABG	Coronary Artery Bypass Grafting
CArG Box	[CC(A/T)$_6$GG] DNA sequene
CCND1	Cyclin D1
Cdc42	Cell division cycle 42
CHD	Coronary Heart Disease
CHF	Congestive Heart Failure
CO_2	Carbon dioxide
CM	Culture Medium for Vascular Smooth Muscle cells
CREG	Cellular repressor of E1A stimulated gene
CRIB	Cdc42/Rac1 interactive binding domain
CVC	Calcified Vascular Cells
CVD	Cardiovascular disease

CXCL1	Chemokine (C-C-C motif) ligand -1
DAPI	4’6-Diamidino-2-phenylindole
DGCR-8	DiGeorge syndrome critical region gene 8
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNM3	Dynamin-3 gene
DOCK6	Dedicator of cytokinesis 6
DsRBDs	Double-stranded RNA binding domains
EC	Endothelial Cells
ECM	Extra Cellular Matrix
EGFR	Epidermal Growth Factor receptor
Elk-1	ETS-like protein-1
eNOS	Endothelial Nitric Oxide Synthase
eNO	Endothelial Nitric Oxide
ER	Endoplasmic Reticulum
ERK 1/2	Extracellular signal regulated kinase ½
ES Cells	Embryonic Stem Cells
ET-1	Endothelin-1
FBS	Fetal Bovine Serum
FGFR-1	Fibroblast Growth Factor Receptor-1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green Fluorescent Protein
GP	L-Glutamine Penicillin combination
hAoSMC	Human Aortic Smooth Muscle Cells
hr/hrs	Hour/hours
HDL	High Density Lipoproteins
HEK293	Human Embryonic Kidney 293 cells
HIF-1 α	Hypoxia Induced factor-1 alpha
4-HNE	4-Hydroxynonenal
I-1	Protein Phosphatase Inhibitor-1
ICAM-1	Intercellular cell adhesion molecule-1
IFN	Interferons
IGFR-1	Insulin like growth factor receptor-1

IL-1α / b	Interleukin -1 alpha /beta
IL-4/-6	Interleukin-4/-6
IL-8/ -10/ -18	Interleukin-8/ -10/ -18
IL	Interleukins
IR	Ischemia reperfusion injury
7-Keto	7-Ketocholesterol
KLF-2/KLF-4 / KFL-14	Kruppel-like factor -2/-4/ -14
KRAS	K-ras
LDL	Low Density Lipoprotein
LFA-1	Lymphocyte function associated antigen-1
LOX-1	Lectin-type oxidized LDL Receptor 1
LRP-1	Low-density Lipoprotein receptor related protein 1
Ox-LDL	Oxidized LDL
MAC-1	Monocyte adhesion receptor-1
MCP-1	Monocyte Chemotactic Protein-1
M-CSF	Macrophage colony stimulating factor
MECP-2	Methyl CpG-binding protein 2
Mef2c	Myocyte enhancer factor-2c
miR/ miRNA	MicroRNA
MMPs	Matrix Metalloproteinases
MMP-2/ -8/ -9/ -13	Matrix Metalloproteinases-2/-8/-9/-13
mRNA	Messenger RNA
miR-214	MicroRNA-214
Mins	Minutes
MI	Myocardial Infarction
MRTF-A/B	Myocardin related transcription factor A and B
MYOD-1	Myoblast Determination Protein-1
MyoCD	Myocardin
NCKAP-1/ NAP1	Nck Associated protein-1
Nck-1	Non catalytic region of Tyrosine Kinase Adaptor Protein 1
Ncx1	Sodium/Calcium Exchanger 1
NFAT	Nuclear Factor of Activated T-cells

NO	Nitric Oxide
NOR1	Orphan nuclear receptor 1
Nox-1	NADPH Oxidase -1
Nrf2	Nuclear factor (erythroid derived 2)-like 2
Nrf3	Nuclear Factor erythroid 2-related factor 3
OPNG	Ortho-Nitrophenyl-β- D-galactopyranoside
PACT	Protein activator of PKR
PAPP-A	Pregnancy-associated plasma protein-A
PBS	Phosphate buffer saline
PCI	Percutaneous Coronary Interventions
PCNA	Proliferating Cell nuclear Antigen
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived growth factor
PDGFR	Platelet derived growth factor Receptor
PGE_2	Prostaglandin E_2
PGLF	Placental growth factor
PKC-ε	Protein kinase-C-ε
Pim-1	Proviral integration site for Moloney murine leukemia virus 1
PI3K	Phosphatidylinositol 3 kinase
Pre-miRNA	Precursor micro RNA
Pri-miRNA	Primary micro RNA
PDCD4	Programmed cell death 4
PSGL-1	P-selectin glycoprotein ligand 1
PTK2	Protein Tyrosine Kinase 2
PTEN	Phosphate and Tensin Homolog
PTCA	Percutaneous Transluminal Coronary Angioplasty
q-PCR	Real time Polymerase Chain Reaction
RAN-GTP	GTP-binding Nuclear protein
RLC	RISC Loading complex
RNA	Ribose nucleic acid
RNase III	Ribonuclease III
RIIIDs	RNase III Domains

RhoA	Ras Homologue A
ROCK1	Rho associated coiled-coil containing protein kinase 1
RISC	RNA Induced Silencing Complex
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
Sca-1^{+}	Stem Cell Antigen 1 positive cells
SCC	Squamous Cell Carcinoma
SERCA	Sarco/Endoplasmic reticulum Ca^{2+} ATPase
Sec	Seconds
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SHP2	Src homology region 2-containing protein tyrosine phosphatase 2
shRNA	Short hairpin RNA
SirT1	sirtuin 1
SM22 α	Smooth Muscle 22 α
SmαA	Smooth Muscle alpha Actin
SMC	Smooth Muscle Cells
SM-MHC	Smooth Muscle Myosine Heavy Chain
SM Progenitor cells	Smooth Muscle progenitor cells
SMYD5	SET and MYND domain containing 5
Sp-1	Specificity protein-1
SR-A	Scavenger receptor A
SR-I/ SR-II	Scavenger receptors I/ II
Sra-1	Rac1 Associated Protein-1
SRF	Serum Response factor
TIMP	Tissue Inhibitor of MMPs
TGF-β	Transforming Growth Factor β
TNF-α	Tumour necrosis factor α
TRBP	Tar RNA Binding Protein
TLR-2/ -4	Toll like Receptors -2/ -4
Trb3	Tribbles-like protein-3
UTR	Un-translated region

VEGF	Vascular Endothelial Growth Factor
VSMC	Vascular Smooth Muscle Cells
VCAM-1	Vascular cell adhesion molecule-1
WASP	Wiskott-Aldreich Syndrom Protein
WAVE	WASP family Verprolin homology protein
WHO	World Health Organization
YAP	Yes associated protein
XPO5	Exportin-5

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1 Introduction:

Cardiovascular diseases (CVD) are the major cause of morbidity and mortality in the world. About 50% of all cardiovascular deaths are due to ischemic heart disease whereas atherosclerosis is regarded as the major factor in the development of cardiovascular diseases including myocardial infarction (MI), and stroke (Ross 1999). Reduced blood supply due to narrowing of the lumen of artery caused by atherosclerotic plaque leads to hypoxia (a condition characterized by low oxygen supply) in the tissues/organs such as brain and heart, resulting in dysfunctions of these organs. Rupturing of atherosclerotic plaque may cause blood clot and eventually MI, heart attack, stroke and/or other peripheral arterial diseases. Stress, hyperlipidaemia, hypertension, diabetes, smoking, aging and obesity have been regarded as the main risk factors for atherosclerosis.

Most common clinical technique developed and employed successfully to treat atherosclerosis and occluded arteries is Percutaneous transluminal coronary angioplasty (PTCA), which involve insertion of a balloon catheter along with a metallic stent. Inflation of balloon opens up the blocked artery and insertion of stent keeps the artery open for long periods. Despite excellent success rate, there has been some limitation associated with this technique, most prominent of which, is the formation of neointima and thrombosis, resulting in vascular inward remodelling (restenosis) which require repetitive interventions, thus debilitating quality of life.

Vascular smooth muscle cells (VSMCs) have a pronounced role in the pathophysiology of atherosclerosis and neointima formation. Researchers have reported a contributory as well as protective role of VSMC in atheroma development. VSMCs respond to cytokine stimulation produced by inflammatory mediators after vascular injury, by switching to a synthetic phenotype with increased capacity of proliferation, migration, and modulating the synthesis of various extracellular matrix proteins to repair the injured vessel wall. During the early stage of atherosclerotic lesion development, VSMCs migration, proliferation, apoptosis and secretion of

extracellular matrix proteins result in an increase in plaque size, whereas such functions also promotes fibrous cap formation which helps in stabilizing the atherosclerotic plaques and thus protecting against thrombotic eruption. Researchers in the cardiovascular field have work extensively to understand the underlying mechanism involving VSMC phenotype switch and their contributory roles in the pathogenesis of atherosclerosis and neointima formation (Hansson 2005) but still the role of some regulatory factors and their involvement in VSMC behaviour needs to be address.

Among these newly identified regulatory factors are microRNAs (miRNAs or miRs). MicroRNAs are regarded as potent post-transcriptional regulatory agent as a single miRNA can target many mRNAs and hence can regulate multiple downstream signalling pathways. MiRNAs are short, single stranded, non-coding 22 nucleotide RNAs involved in post transcriptional gene silencing. MiRNA along with its associative Argonaute family of proteins binds with the 3' UTR of mRNA, resulting in its cleavage, degradation or de-adenylation and translational repression hence regulating the specific gene and controlling fundamental biological processes like cell proliferation, differentiation, apoptosis and tumour progression or repression. Whereas cardiovascular diseases are concerned, involvement of miRNA has been reported in a number of processes ranging from cardiac development during embryogenesis to the progression of multiple diseases. Role of miR-214 has been described by some researchers in endothelial and myocardial cell survival after ischemia reperfusion injury (IR) but its role in VSMC regulation and its involvement in atherosclerosis and neointima formation remain to be elucidated.

In this study, we will evaluate the possible role of miR-214 in regulating VSMC functions (proliferation, migration, and apoptosis), the potential therapeutic effect of miR-214 invascular injury-induced neointima formation, and investigate the possible candidate genes involved and underlying molecular mechanisms.

1.1 Cardiovascular Diseases (CVD)

Cardiovascular diseases are regarded as disorders of blood circulatory system comprising of heart and blood vessels. According to the world health organisation data, cardiovascular diseases are the number one cause of death globally with an estimated 17.7 million people died in 2015. A study published in 2016 by Nick Townsend estimates CVD related death around 4,002,632 which is 45% of total number of deaths in European region (Townsend, et al. 2016). British heart foundation has estimated that there are around 7 million people living with cardiovascular disease in UK (BHF Stats). Among CVDs ischaemic heart disease and stroke are the two biggest killers accounting for a combined 15 million deaths worldwide in 2015. There are a number of predisposing factors contributing to cardiovascular diseases largely categorised as non-modifiable and modifiable risk factors. Non-modifiable risk factors include gender, age, poverty and low education status, ethnicity family history and genetic makeup. The modifiable risk factors include behavioural risk factors and metabolic risk factors. The behavioural risk factors include smoking/use of tobacco, alcohol, less physical activity, unhealthy dietary habits (excessive use of salt and fats). Metabolic risk factors are hypertension (consistent raised blood pressure), Diabetes (high blood sugar), obesity and hyperlipidemia (high blood cholesterol levels). As aforementioned, coronary artery disease (causing myocardial infarction MI) and cerebrovascular disease (causing stroke) are the two major aliments causing mortality and morbidity, whereas atherosclerosis and neointima formation is regarded as the main processes behind these ailments. A number of factors are involved in triggering the process of atherosclerosis which includes metabolic risk factors, mechanical forces, immunomodulatory cells, inflammatory cytokines, extracellular matrix proteins, and vascular cells.

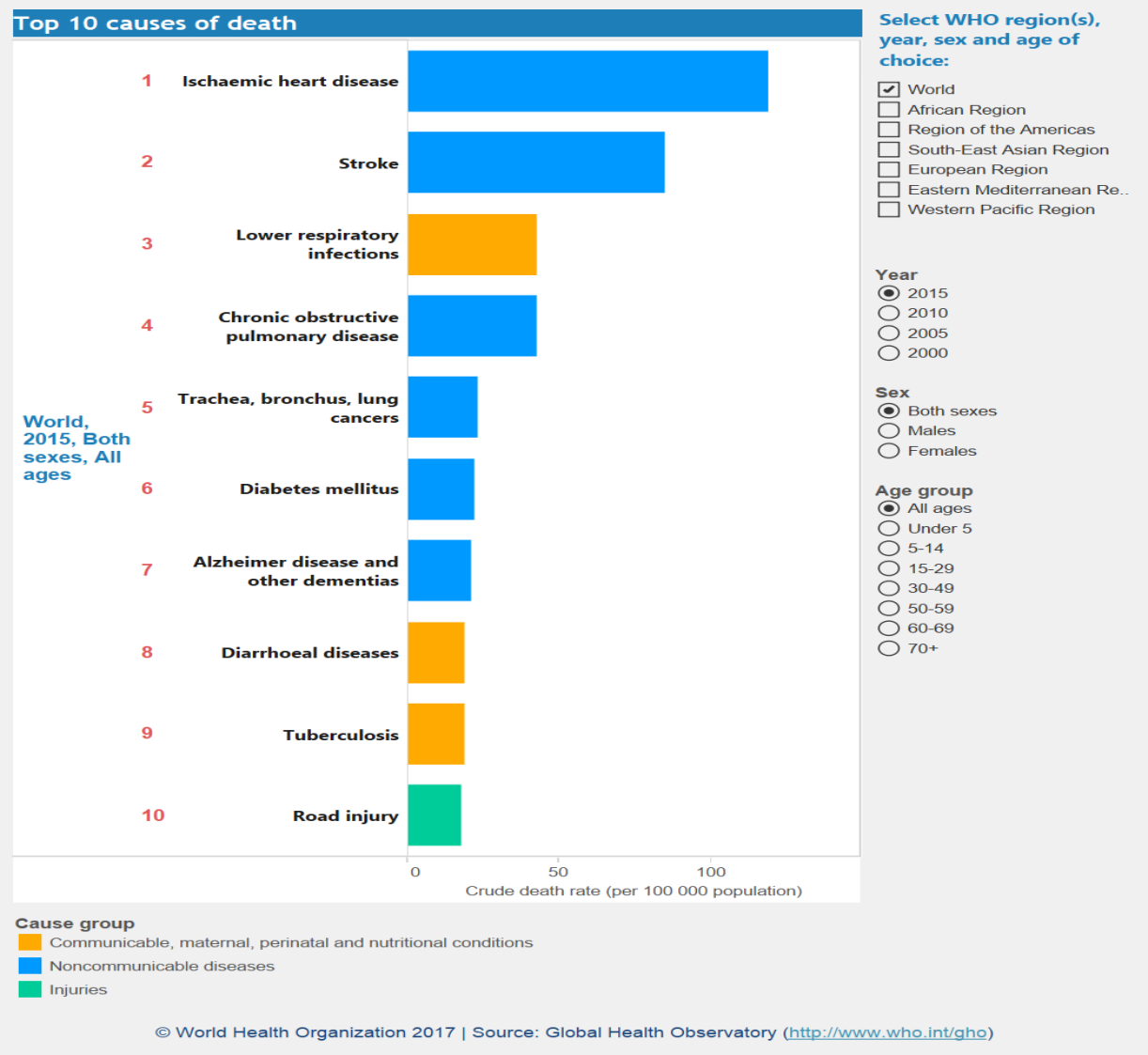


Figure 1. Top 10 causes of deaths worldwide. World Health Organization 2017 http://www.who.int/gho

Figure 2. Prevalence of cardiovascular disease in the UK: bhf.org.uk/heart-health/conditions/cardiovascular-disease

2 Atherosclerosis

Atherosclerosis is an inflammatory disease, characterized by gradual narrowing of the artery lumen due to formation of atherosclerotic plaque. Various metabolic risk factors, mechanical stimuli, immunomodulatory and inflammatory mechanisms trigger the pathogenesis of atherosclerosis (Hansson 2005; Ross 1999). The vasculature is lined by monolayer of cells called endothelium which is selectively permeable, anti-thrombogenic, anti-inflammatory and vasoprotective in nature. Integrity of endothelium plays a critical role in the initiation and development of atherosclerotic plaque. Pathological conditions such as hyperlipidaemia, diabetes, hypertension, smoking, stress, or injury may result in aberrant endothelial cell activation. Meanwhile, endothelial cell programmed cell death (apoptosis) severely damages the integrity and functions of endothelium. Mechanical forces like shear stress and cyclic strain also influence the mechanisms involved in atherosclerosis. A turbulent blood flow at arterial bifurcations may trigger the process of atherosclerosis by effecting endothelial integrity and promoting infiltration of inflammatory mediators, whereas laminar flow exerts anti-atherosclerotic and anti-apoptotic effects.

Currently two major techniques are employed to treat atherosclerosis and to open the occluded vessels, i.e.Percutaneous transluminal coronary angioplasty (PTCA) or Percutaneous coronary interventions (PCI) and coronary artery bypass grafting (CABG). Considering the advantage of rapid procedure with less invasive option, quicker discharge from hospital and earlier return to daily activities, PTCA is considered preferred option by both clinicians and patients. PTCA/PCI involves opening up of stenotic vessel by inserting and inflating a balloon on the catheter tip and implanting a metallic drug eluting stent. This controlled vascular remodelling results in plaque compression and arterial stretching, thereby improving blood supply to relevant tissue/organ.

Despite the development in treatment protocols and introduction of drug eluting stents which results in excellent success rate, treatment with PTCA comes with few limiting factors like vascular remodelling (restenosis) and neointima hyperplasia requiring repeated interventions. Neointima hyperplasia is the development of new thickened layer in arterial intima (comprising largely of vascular smooth muscle cells along with macrophages, leukocytes, lipid/cholesterol particles, and apoptotic cells) formed by the proliferation and migration of cells (VSMCs and fibroblasts) from surrounding tissue or from blood vessel media.

Likewise atherosclerosis, neointima hyperplasia (arising after artery injury) shares same inflammatory pathways, pathological conditions (hyperlipidaemia, hypertension, diabetes, stress) and pre-disposing factors, aggravating the disease/inflammation at much rapid pace than occurs in atherosclerosis (chronic inflammatory process), requiring the need for subsequent intervention in 10-30% of cases in few year time (Serruys, et al. 1988).

2.1 Pathogenesis of Atherosclerosis and Neointima Formation.

2.1.1 Vascular injury/Endothelial integrity.

Blood circulatory system is highly specialized to perform its function i.e. to transport nutrients throughout human body and excrete waste. Blood vessels are uniquely adapted to changes in external and internal environment, for example, inflammation, infection, maintenance of blood pressure and volume, maintenance of vascular tone, etc. Typically, a blood vessel is composed of three distinct layers of cells, named as tunica intima, media and adventitia. The intima is composed of single layer of endothelial cells (ECs) lying on basement membrane composed of collagen type IV, laminin and heparin sulphate proteoglycans (Yurchenco 2011). Tunica media is largely composed of vascular smooth muscle cells (VSMCs) embedded in extracellular matrix containing elastin, collagen type I, fibronectin and other glycoproteins. Tunica media is separated from intima by layer of insoluble contractile elastin fibres called internal elastic lamina

(Wagenseil and Mecham 2009). A similar layer composed of thick elastin fibres called external elastic lamina separates tunica media from adventitia. The adventitia comprises of mainly loosely bounded connective tissue, fibroblast cells, fat cells along with different type of stem/progenitor cells.

As aforementioned, Endothelium being the innermost layer is in direct contact with the flowing blood and hence, constantly exposed to the mechanical force exerted by the blood (cyclic strain and shear stress). Shear stress is the frictional force generated by the flow of blood against the vessel wall and is usually expressed in dynes/cm^2. Unidirectional, laminar flow of blood exerting high shear stress (approx 15 dynes/cm^2) against vessel wall is considered anti-inflammatory, antithrombotic and atheroprotective whereas at vessel bifurcation and bends, the flow of blood is disturbed and results in oscillatory or low shear stress (<5 dynes/cm^2) (Malek, et al. 1999; Warboys, et al. 2011). This disturbed blood flow exposes endothelial cells vulnerable to inflammation by inducing expression of adhesion molecules and contributing to endothelial dysfunction. These areas of arterial tree (bifurcations, bends and branches) are termed as atheroprone areas as they are more vulnerable towards developing atherosclerotic lesions because of low shear stress/blood flow leading to decreased rolling speed and hence, providing better access to leukocytes and low density lipoproteins to transmigrate to the arterial wall as well as increased expression of adhesion factors and chemokines (Kwak, et al. 2014).

2.1.2 Up-regulation of adhesion molecules and recruitment of inflammatory cells.

Modified LDL, free radicals and hemodynamic stress activates ECs resulting in enhanced expression of pro-inflammatory cytokines. Denudation of endothelium as a result of vascular injury (such as stenting) also promotes inflammatory signalling from adjoining intact ECs. The inflammatory signals from ECs, monocytes, leukocytes and platelets intiate an inflammatory

cascade which triggers the pathogenesis of atherosclerosis and neointima formation. Such cytokines in-turn increases leukocyte and monocyte adhesion to endothelium by raising the expression of cell surface leukocyte adhesion molecules e.g. ICAM-1 and VCAM-1 (Butini, et al. 1994; Diacovo, et al. 1994) and by triggering the productionof selectins, particularly E-selectin and P-selectin (Inoue, et al. 1998). Transmigration of monocytes into the site of inflammation is triggered by the interactions between VCAM-1 and its counter receptor, integrin α1β4 on monocytes. Oxidized LDL also stimulates release of MCP-1 from ECs (Cushing, et al. 1990). MCP-1 interacts with its counter receptor integrin on macrophages and T cells leading to their enhanced binding and transmigration across the endothelium (Schober and Zernecke 2007). Monocytes, adhered to the inflamed endothelial layer, starts producing inflammatory cytokines such as IL-1β and tumour necrosis factor-α (TNF-α), thereby further exacerbating inflammation. Reactive oxygen species (ROS) mediate multiple cellular pathways regulating inflammatory responses during atherosclerosis. Among them two major pro-inflammatory signalling pathways: the nuclear factor kappa B (NF-ƙB) and mitogen-activated protein kinase (MAPK) pathway are identified as key regulators because of there effect on multiple downstream pathways to regulate protected and susceptible sites of arterial tree (Passerini, et al. 2004; Yu, et al. 2015; Zhang, et al. 2016a).

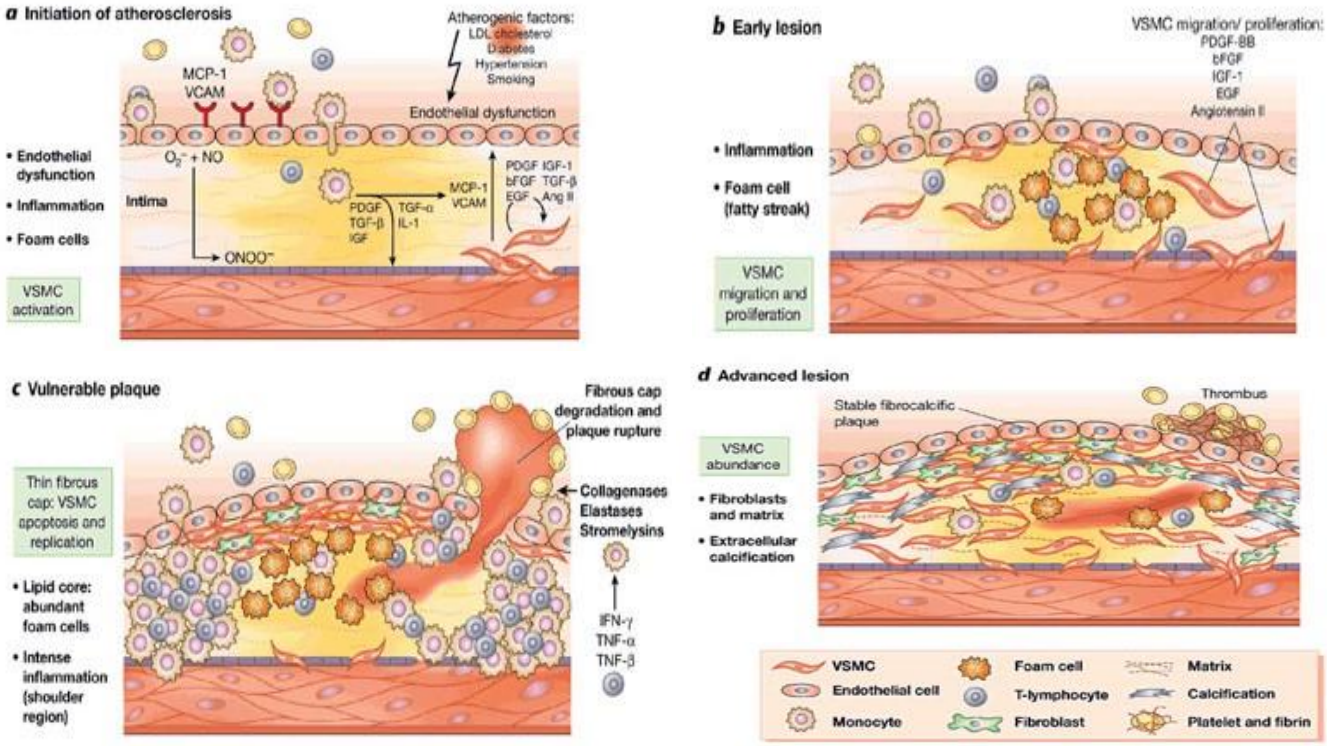


Figure 3. Role of VSMCs at different stages of atheroma development during atherosclerosis. Cardiovascular risk factors or/and endothelial injury initiate a cascade of events involving increased expression of cell adhesion molecules, recruitment of leukocytes, foam cell formation and release of cytokines, chemokines and growth factors by inflammatory cells, EC and VSMCs. VSMCs migration, proliferation and secretion of extra cellular matrix proteins leads to formation of fibrous cap resulting in stable atherosclerotic plaque. At later stage, fibroblast also migrate from adventitia to intima contributing in plaque enlargement, adapted from Dzau VJ,et al, Vasular proliferation and atherosclerosis, Nat Med 2002 (Dzau, et al. 2002).

2.1.3 Formation of Foam Cells.

As aforementioned that chronic inflammation contributes to the development and progression of atherosclerotic plaque, plaque rupture and thrombosis. Oxidative stress due to the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) also induces inflammation (Ooi, et al. 2017; Pashkow 2011). Oxidation of lipoproteins induced by ROS can amplify oxidized low density lipoproteins (oxLDL) formation. Foam cell formation results from the disruption in the normal macrophage cholesterol metabolism which involves uptake, intracellular metabolism and efflux of cholesterol and oxLDL.

Cytokines such as IL-1 up-regulate the expression of scavenger receptors (SR) on macrophages by influencing macrophage colony stimulating factor (M-CSF)(Clinton, et al. 1992; Zoellner, et al. 1992). Upregulated scavenger receptors (SR) on macrophage surface, recognizes and uptake these oxidized lipoproteins (ox-LDL) by the process of phagocytosis and pinocytosis, resulting in formation of foam cells. Scavenger receptor class A (SR-A) and scavenger receptor class B (CD36) are the most important receptors responsible for the uptake of oxLDLs. Studies have documented that about 75% to 90% of oxLDL internalization into macrophages is done by these two receptors (Kunjathoor, et al. 2002). Moreover, lectin-type oxidized LDL receptor 1 (LOX1), toll-like receptor 4 (TLR4) and chemokine (C-X-C) motif ligand are among other receptors responsible for oxLDL uptake (Schaeffer, et al. 2009). CD36 is 88-KDa transmembrane glycoprotein receptor belonging to SR class B family. It is heavily expressed by platelets, monocytes/macrophages, and endothelial cells and exhibits high affinity for ox-LDLs. Studies have demonstrated that genetic deletion of CD36 in ApoE$^{-/-}$ null mice fed with standard rodent chow and western diet were protected against atherosclerotic lesion as compared with wild type mice (Febbraio, et al. 2000). Treatment with moleculaes showing Anti-CD36 activity such as EP80317 (a competitive CD36 ligand) significantly reduce oxLDL internalization and promote

cholesterol efflux by inhibiting CD36 cell surface expression (Marleau, et al. 2005). On the contrary, Moore *et al 2005* reported that ApoE$^{-/-}$ mice lacking CD36 or SR-A displayed increased atherosclerotic area and macrophage foam cell formation in aortic intima (Moore, et al. 2005). Furthermore, some clinical studies showed that patients with CD36 deficiency exhibit severe and enhaced atheroscleritc disease (Yuasa-Kawase, et al. 2012). The pathorgenic role of ox-LDL in atherosclerosis largely depends on CD36 but its actions as proatherogeneic mediator needs to be reassessed.

Another scavenger receptor actively involved in internalization of oxLDLs is SR-A, which is 77-KDa trimeric transmembrane glycoprotein and member of class A SR family. SR-A is highly expressed on monocytes, macrophages and endothelial cells. Inhibition of SR-A in macrophages significantly ameliorates foam cell formation and atherosclerosis in ApoE$^{-/-}$ mice (Dai, et al. 2012). Silencing either CD36 or SR-A reduces atherogenesis in LDL receptor (LDLR) deficient apolipoprotein B100 mice but no beneficial effect was observed when both receptors were blocked suggesting that compensatory activation of both receptors is sufficient for the uptake of modified LDL (Makinen, et al. 2010; Yu, et al. 2013).

In addition to the cholesterol uptake, the balance of free cholesterol (FC) and cholesterol esters (CE) is very crucial for the regulation of intracellular cholesterol content in macrophage foam cells. After internalization, modified LDLs are delivered to the lysosomes where lysosomal acid lipase (LAL) hydrolyse the excess CE to FC. To prevent cells from FC-associated cell toxicity, FC is effluxed from the cell by ABC transporters (ABCA1 and ABCG1) or re-esterified to CE by enzyme acyl-CoA:cholesterol acyltransferase (ACAT1) within endoplasmic reticulum (ER) and stored as cytoplasmic lipid droplets. Disruption in this process results in excessive accumulation of CE in macrophages resulting in the formation of foam cells (Linton, et al. 2000).(Ooi, et al. 2017)

With the progression of atherosclerosis, due to oxidative stress and generation of ROS, cellular antioxidant defence system comes into play to maintain redox homeostasis. One of the major mechanism involved in maintaining cellular redox homeostasis is the Nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 is a transcription factor closely associated with the development of atherosclerosis and act as master redox switch in activating cellular antioxidant defence mechanism. Nrf2 belongs to the cap "n" collar family of basic region-leucine zipper (CNC-bZIP) transcription factors. It regulates genes which contain antioxidant/electrophile response elements (ARE/EpRE), including antioxidant and phase II detoxification enzymes, ABC transporters and other stress response protein expressions (Sykiotis and Bohmann 2010).

Several studies have documented that the transcriptional activation of Nrf2 signalling mechanism protects the cells against oxidative stress which may lead to inflammation, apoptosis, aging and cellular transformation. Activated Nrf2 suppresses endothelial cell activation by inhibiting p38 mitogen activated protein (MAP) kinase activity and suppressing VCAM-1 expression (Zakkar, et al. 2009). Studies have postulated that shear stress and laminar flow also exert their action by activating Nrf2 signalling pathway leading to ARE gene expression and suppression of EC activation (Kim, et al. 2012). Under normal homeostatic and stress free conditions, Nrf2 stays inactive by binding to its inhibitor Keap1, an adaptor protein of a cullin3 (Cul3)-ring-box 1 (Rbx1). Nrf2-Keap1 complex keeps cytosolic Nrf2 protein at low levels by constant polyubiquitination and subsequent proteosomal degradation of Nrf2, hence preventing transcription of downstream target genes (Kobayashi, et al. 2004). But when cells are exposed to oxidative stress, ROS and proinflammatory cytokines, the low affinity interactions between Keap1-Nrf2 breaks down due to a modification in the cysteine residue of Keap1 leading to a conformational change in its structure. This disruption results in release of Nrf2 from Keap1 and its translocation into the nucleus where they form heterodimers with small Maf proteins, followed by transcriptional

activation of cell defence genes, resulting in increased resistance to stress (Bryan, et al. 2013). Apart from Keap1 dependent regulation, Nrf2 activation is also mediated by p38, PKC, PI3K/AKT, MAPK/ERK and JNK via phosphorylation of the serine or threonine residues of Nrf2 (Bryan, et al. 2013; Chen, et al. 2015a). Nrf2 exerts its protective effects against oxidative stress by regulating the expression of multiple genes involved in cholesterol influx and efflux, scavenger receptors, antioxidant enzymes and ABC transport protein such as heme oxygenase 1 (HMX1), glutathione peroxidise 1 (GPX1)], glutamate-cysteine ligase modifier subunit (GCLM) and NADPH quinine oxidoreductase 1 (NQO1) (Ishii, et al. 2004; Jyrkkanen, et al. 2008; Maltese, et al. 2017; Singh and Ramji 2006a).

Macrophage foam cells produce several cytokines and growth factors such as TNF-α, TGF-β, IL-1, and fibroblast growth factors (FGF) which promotes infiltration and proliferation of VSMCs into the intima. VSMCs also play a crucial role in the development of foam cells by their ability to take up oxidized lipids by endocytosis. VSMCs expresses several scavenger receptors like SRA-I, SRA-II, Lectin-like ox-LDL receptor-1 (Lox-1) and CD36, all of which are involved in the process of endocytosis of oxidized or acetylated LDLs and many other heterogeneous compounds (Camino-Lopez, et al. 2007; Moore and Freeman 2006). Cationic proteins are endocytosed through interactions with negatively charged glycocalyx whereas anionic proteins such as LDLs interact with their specific SRs (Mitra, et al. 2011; Sprague, et al. 1985). LDLs can also activate tool like receptors (TLRs) especially TLR-4 which inturn participate in lipid uptake and generation of subsequent inflammatory responses (Choi, et al. 2009b). The endocytosis and subsequent accumulation of LDLs into VSMCs converts them into lipid laden foam cells containing large lipid droplets within their cytoplasm (Mitra, et al. 2011). The accumulation of foam cells leads to the formation of fatty streaks which is considered as the initial lesion-leading step to the development of atherosclerosis (Geng and Hansson 1992; van Lenten and Fogelman 1992).

Activated endothelium, macrophages, leukocytes and T-cells produce inflammatory cytokines and growth factors which regulate the proliferation, migration and/or apoptosis of VSMCs from the media to intima (Libby and Clinton 1992).

2.1.4 Migration and proliferation of VSMCs from media to intima.

It has been well established that VSMCs play an important role at different stages of atheroma development during atherosclerosis (Figure 3). Activated platelets accumulated at the site of injury along with ECs and macrophages, produce a potent growth factor, platelet derived growth factor (PDGF)(Heldin, et al. 1981; Libby 2002) which stimulates the migration, proliferation and accumulation of medial VSMCs into sub-endothelial spaces (Myllärniemi, et al. 1997). PDGF and other pro-inflammatory cytokines also influence VSMCs to change their phenotypic properties from elongated and contractile nature (called differentiated VSMCs as observed in medial layer) to cobblestone, secretary and non-contractile nature often known as 'synthetic VSMCs' (Mack 2011). Enhanced expression of matrix metalloproteinases (MMPs)(especially MMP-2, MMP-8 and -9) also facilitates VSMC migration by degradation of basement membrane and remodelling of extracellular matrix. Furthermore, apoptotic VSMCs are engulfed and degraded by the adjacent VSMCs through phagolysosomes leading to secretion of TGF-β and MCP-1, hence further exacerbating inflammation. Healthy human VSMCs are potent phagocytes of apoptotic VSMCs (Clarke, et al. 2010) but during hyperlipidemic conditions as observed in atherosclerotic plaque, the reduced ability of VSMCs to phagocytose or efferocytose the apoptotic VSMCs results in secondary necrosis and an increase in lipid content of the plaque (Van Vre, et al. 2012). Recent *in vitro* evidence has mentioned the transfer of cholesterol loaded lysosomes from foam cell macrophages to VSMCs hence inducing VSMC foam cell formation (Weinert, et al. 2013). Death of foam VSMCs also promotes proliferation and migration of adjacent VSMCs into intima and subsequent plaque calcification (Clarke, et al. 2008) thus participate in plaque progression.

Migration and accumulation of VSMCs and their excessive secretion of extracellular matrix proteins (e.g. collagen) results in hardening of fibrous cap of atherosclerotic lesion (Owens, et al. 2004). Gradual thickening of arterial wall results in the development of atherosclerotic plaques which represents the core cause of CVDs and a major cause of morbidity and mortality worldwide (Libby 2002; Ross 1999).

2.1.5 Plaque Calcification.

As disease progresses, increased apoptosis and loss of SMC contractile proteins results in calcification of VSMCs and subsequently of advanced atherosclerotic plaque. Calcium phosphate deposition in arterial wall (VSMC and atherosclerotic plaque) results in reduced elasticity leading to vasoconstriction and raised systolic hypertension, and left ventricular hypertrophy, diastolic dysfunction, valve incompetence and increase in oxidative stress (Karwowski, et al. 2012). Several mechanisms including inflammatory factors (e.g., TNF-α, Gas6, and TGF-β), metabolic factors incorporating oxidative stress and oxLDLs, vitamin D, and developmental factors (e.g., bone morphogenetic proteins (BMPs), RUNX and the osteoprotegerin/RANKL axis (Johnson 2014; Magne, et al. 2005; Maziere, et al. 2013; Sage, et al. 2010) are postulated to play a role in this process. Calcium deposition results in formation of so-called calcified vascular cells (CVCs). VSMC/plaque calcification has been broadly divided into two groups, spotty or micro-calcification and dense or macro-calcification. Researchers have observed that the pattern of calcification is more indicative of plaque stability as compared to the total amount of calcification hence spotty or micro-calcification indicates a culprit plaque prone to erupt whereas macro-calcification increases the burden of plaque but associated with stable lesion (Ehara, et al. 2004; Pflederer, et al. 2010; Yang, et al. 2013c).

2.2 Additional mechanisms in atherosclerosis and neointima formation,

Aforementioned 'the response to injury' paradigm proposed by Ross has been really helpful in demonstrating the role of injury, inflammation and subsequent repair process in atherosclerosis and neointima formation. In this model, involvement (migration, proliferation and secretion of extracellular matrix proteins) of medial wall SMCs is regarded as the most likely mechanism of neointimal development. But over the past few years, several studies have also reported various stem/progenitor cells such as circulating smooth muscle progenitor cells and adventitial stem cells/fibroblasts as additional origins of cells within neointima (Figure 4).

2.2.1 Endothelial Progenitor Cells.

In response to vascular damage, vascular repair is initiated by local endothelial cells and bone marrow derived cells called Endothelial Progenitor Cells (EPCs). EPCs were first described as adult vascular progenitor cells in late 1990s (Lu and Li 2018; Marcola and Rodrigues 2015). Since then, there has been extensive research in the field of EPCs but continues to be controversial because of absence of any unique identifying marker. EPCs are circulating; bone-marrow derived stem/progenitor cells, which have the ability to participate in vasculogenesis and vascular homeostasis as they can differentiate into mature endothelial cells (Bonello, et al. 2012). After vascular injury, EPC rapidly move to the site of vascular damage to fill in the empty spaces and initiate the process of re-endothelialization (Ben-Shoshan and George 2007; Werner, et al. 2005). Some researchers have postulated that circulating levels of EPCs correlates with the progression of certain diseases like Takayasu arteritis and peripheral artery disease, hence labelling them as potential biomarker (Bitterli, et al. 2016; Keskek, et al. 2017). The phenotype of EPCs is

characterized by the co-expression of stem cell surface markers (CD34, CD133), endothelial cell markers (CD144, VEGFR2/KDR2) and by their ability to form endothelial colony forming units.

As aforementioned, the precise characterization of EPCs is difficult because many of the cell surface markers used in phenotyping them are common to both hematopoietic stem cells and mature endothelial cells. Surface antigens CD34, VEGFR2 and CD133 are non specific and also expressed by hematopoietic progenitor cells. CD34 is expressed by bone-marrow derived stem cells which also exhibit the ability to differentiate into smooth muscle progenitor cells and inflammatory cells and only a small portion of CD34 positive cells are actually EPCs (Tesfamariam 2016; Urbich and Dimmeler 2004).

Another limitation in phenotypic characterization of EPCs is the presence of other circulating endothelial cells (CECs) in the peripheral circulation. After vascular damage, resident endothelial cells initiate the repair process which results in the release of CECs from vascular intima. Circulating endothelial cells are mature cells that are characterised by the presence of mature endothelail cell surface markers (CD31 and CD144) and absence of hematopoietic progenitor cell markers (CD45, CD133). CECs came in the circulation either by detaching from the vessel wall in response to injury, by sloughing of endothelial cells into the circulation, as part of normal turnover process, or as an effect of damaging factors (e.g. infectious agents and oxidative stress) (Ahmed, et al. 2016; Burger and Touyz 2012; Richardson and Yoder 2011). They have limited proliferative capacity as compared to the EPCs but their levels increases significantly following endothelial damage as occurs during intravascular stent implantation (Blann, et al. 2005; Quilici, et al. 2004).

2.2.2 Circulating Smooth Muscle Progenitor Cells.

Several researchers have reported the involvement of circulating vascular progenitor cells in neointima development from different sources (bone marrow derived as well as non-bone

marrow sources), thus challenging the classical model of medial VSMC based neointima hyperplasia (Iwata, et al. 2010; Saiura, et al. 2001; Sata, et al. 2002). Many investigators have identified and provided evidence for the role of circulating progenitor cells infiltrating into the intima and differentiating (under the influence of PDGF and VEGF) into ECs (Miyata, et al. 2005) and VSMC (Sata, et al. 2002; Shimizu, et al. 2001) during vascular repair and neointoma formation. In cardiac/aortic allograft transplantation studies, researchers have demonstrated the proportional involvement of SMCs from recipient (72%-90%) and donor (10%-20%) within the atherosclerotic/neointimal lesion (Caplice, et al. 2003; Sata, et al. 2002) indicating the transmigration of progenitor cells into intimal layer and differentiation into VSMCs. Although circulating smooth muscle progenitor cells may arise from different potential sources, they are mainly categorized into bone marrow derived and of non-bone marrow origin. Bone marrow serves as major reservoir of hematopoietic and mesenchymal stem cells which differentiate into blood cells as well as cardiovascular cells (cardiomyocytes, ECs and SMCs). Scientists have used bone marrow transplantation models to investigate the potential involvement of stem cells to differentiate into VSMC by infiltrating through the arterial wall. But conflicting data has been reported by different scientist regarding the involvement of BM derived SMCs in neointima formation (ranging from 11% to as high as 56%) (Hillebrands, et al. 2002; Hu, et al. 2002)

In addition to bone marrow derived SMCs, significant amount of data also refers to the non-bone marrow origin of SM progenitor cells. Although the exact source of these progenitor cells is still unknown, several possible sources have been investigated including liver, spleen, intestine and the vascular system. Progenitor cells from these organs also present in the circulation to be trarnsferred to the area of inflammation to initiate their specific action. Different researchers have reported these organs as potential reservoirs harbouring specialized cells which undergo differentiation into ECs and SMCs once arrived at their location (intima and/or media) and after

stimulation with appropriate stimuli (mainly PDGF, VEGF) (Caplan 2009; Crisan, et al. 2008; Groenewegen, et al. 2008).

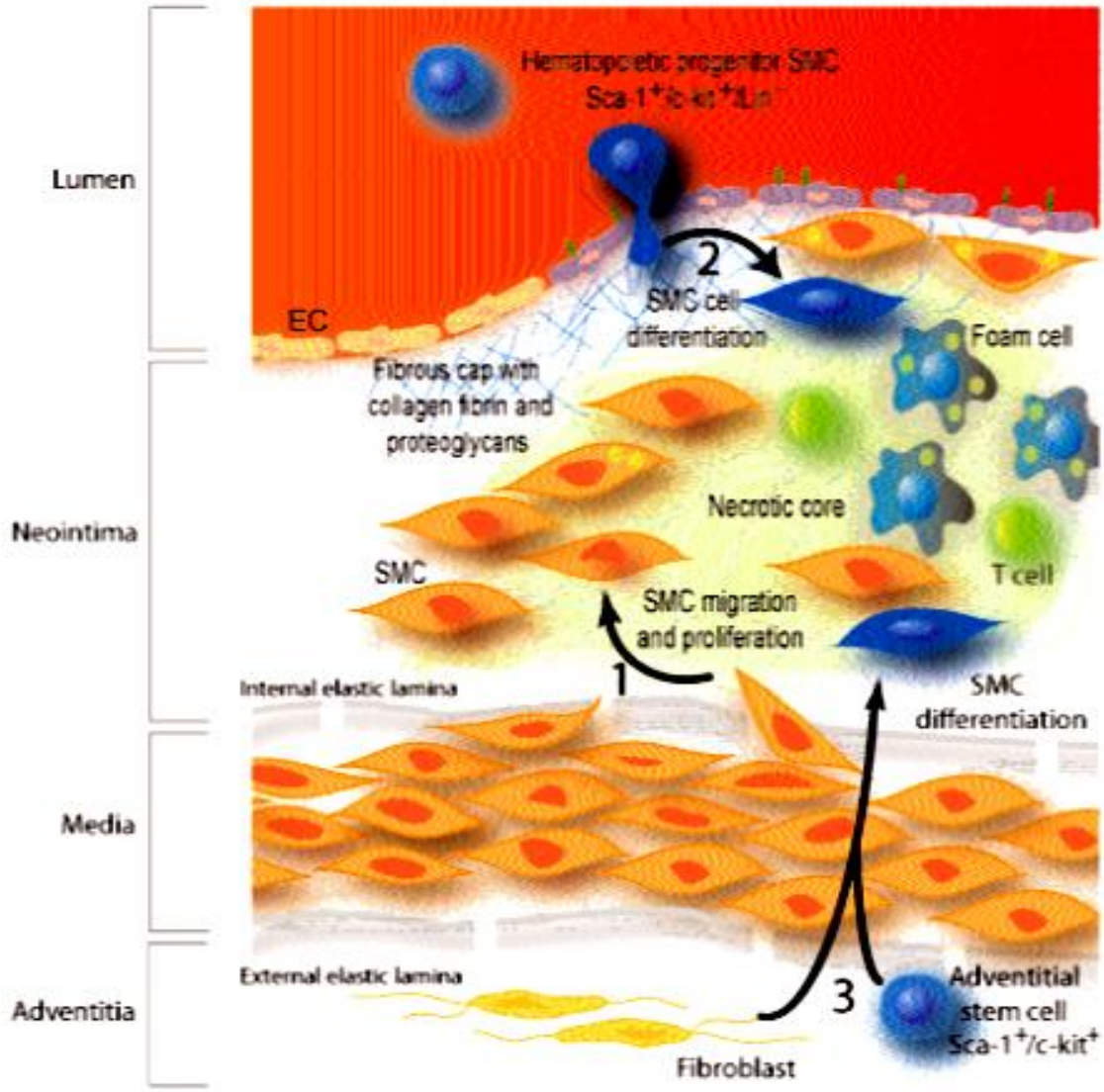


Figure 4. Origins of neointima SMCs.

1-Vascular SMCs from media attain a synthetic phenotype and migrate, proliferate and accumulate in neointima.

2-Circulating smooth muscle progenitor cells, hematopoietic and mesenchymal stem cells can differentiate and contribute to neointimal growth.

3-Adventitial stem cells or fibroblasts can convert into myofibroblast under the effect of certain inflammatory mediators and can migrate into neointima.

Image adapted from diverse origin of vascular smooth muscle by (Zhou, et al. 2005) Zhou X, et al, Anatol J Cardiol 2005

2.2.3 Role of adventitial fibroblasts/ stem cells.

Another hypothesis regarding neointima development focuses on the involvement of vascular adventitial cells i.e. fibroblast and any progenitor population residing within adventitia. Adventitial fibroblasts are heterogeneous population, indicating two possible sources of origin during embryonic development (one distinct progenitor and the other might be same progenitor that gives rise to VSMCs). Similar to VSMCs, fibroblasts are capable of switching structural and functional phenotype according to environmental factors e.g. inflammation, thus referred as myofibroblasts (Hu, et al. 2004). During pathogenesis of atherosclerosis and neointima development, fibroblasts can differentiate toward myofibroblasts under the influence of various growth factors and cytokines (TGF-β, PDGF, TNF-α, GM-CSF) and can migrate to the intima (Li, et al. 2000). Scott *et al* and Shi *et al* have reported significant hyper-proliferative fibroblast response (in models of artery injury) followed by increased migration towards medial layer and to neointima (Scott, et al. 1996; Shi, et al. 1996a). Their trans-migratory capacity were further established by Li*et al* who reported Lac-Z expressing fibroblasts presence and participation in media and neointima, 7 days after the fibroblasts have been transplanted to the adventitia of rat carotid arteries following balloon injury (Li, et al. 2000).

Various other studies have reported existence of Sca-1+/c-Kit+ resident progenitor cells within adventitia. These cells upon activation by PDGF can differentiate into SMC like cells expressing specific surface markers and contributing to neointima formation (Shi, et al. 1996b; Shi, et al. 1996c). Thus adventitial fibroblasts and resident progenitor cells present another cell population that might play a role in neointima development.

2.3 Factors affecting Atherosclerosis and Neointima Formation.

2.3.1 Hypertension.

Hypertension (high blood pressure) is defined as sustained high blood pressure usually more than140/90mm of Hg. Generally high blood pressure is asymptomatic and thus neglected for proper treatment. High blood pressure is a major risk factor for developing coronary artery disease (CAD) and/ or stroke, and is positively associated with the severity of disease. Hypertension also contributes to congestive heart failure, renal impairment, aneurysm, peripheral vascular disease and visual impairment due to damage to retinal blood vessels.

2.3.2 Hyperlipidemia.

Hyperlipidemia (higher levels of blood cholesterol/lipids) is a major risk factor for coronary heart disease (CHD). Cholesterol (lipophilic molecule) is transported in the body as Lipoprotein. Lipoprotein profile is categorised into LDL, high density lipoprotein (HDL) and triglycerides. Total blood cholesterol is a measure of all aforementioned types of cholesterol (LDL, HDL, triglycerides and other lipid molecules). Accumulation of LDL and subsequent oxidation in the blood vessels contributes to atherosclerosis whereas HDL removes the bad cholesterol from walls of arteries and thus protects against atherosclerosis. High levels of triglycerides also increase the likelihood of atherosclerosis.

2.3.3 Diabetes.

Diabetes mellitus (raised blood sugar levels) is a chronic disease caused by inherited or acquired deficiency in insulin production by the pancreas and by resistance to insulin effects. It is defined as having fasting plasma glucose level of ≥ 7.0mmol/l (126mg/dl). Such deficiency results in increased concentrations of glucose (Hyperglycaemia) and other metabolites in the circulation.

Hyperglycaemia induced damage occurs in the cells of certain tissue types as they fail to reduce the transport of glucose inside them when exposed to hyperglycaemia, for example, endothelial cells of retina, mesengail cells, schwann cells, and neurons of peripheral nerves.

One of the major mechanisms involved in hyperglycaemic induced damage is through activation of protein kinase C (PKC) isoforms. Hyperglycaemia increases the synthesis of diacylglycerol DAG which activates PKC isoforms α, β and gamma (Ighodaro 2018; Nishizuka 1995). Activation of PKC results in decrease production of endothelial nitric oxide (eNOS), increased production of endothelin-1, VEGF (increases permeability, angiogenesis), PAI-1 (vascular occlusion), TGFB (increases collagen), NFkB (pro-inflammatory gene expression) and NADPH oxidase (Inoguchi, et al. 2000). Hyperglycaemic induced PKC activation can directly increase permeability of macromolecules across endothelial barriers by phosphorylating cytoskeletal proteins or by regulating activity of various growth factors (Giacco and Brownlee 2010).

Multiple vascular complications have been associated with diabetes, including changes in blood flow, basement membrane thickening, extracellular matrix expansion, vascular permeability, angiogenesis, generation of reactive oxygen species, cell growth and enzymatic activity. The most prominent structural abnormalities of the vasculatures are thickening of capillary basement membrane and matrix expansion. These changes can alter vascular permeability, cell adhesion, proliferation, differentiation and gene expression (Kizub, et al. 2014). Thickening of capillary basement membrane is due to extracellular matrix collagen, fibronectin and laminin. Hyperglycaemic induced PKC activation is responsible for TGF-B1 mediated increased levels of matrix protein. Inhibition of TGF-B1 activity significantly reduces collagen synthesis (Lee 2018; Shi, et al. 2018). Expression of TGF-B1 and Connective Tissue Growth Factor (CTGF) play a key role in the development of basement membrane thickening and ECM in diabetes. Both TGF-B1 and

CTGF regulate ECM accumulation and stimulate production of collagen IV, fibronectin and laminin (Klaassen, et al. 2015).

Diabetic patients are two to three times more at risk of developing CVD as compared to healthy non-diabetic individuals. About 60% of all mortality in diabetic patients is because of CVD events, which makes diabetes as one major risk factor among others.

2.3.4 Obesity.

Obesity (over weight) is a major health problem worldwide and an estimated 2.8 million people die each year due to obesity. Obesity is defined through the body mass index (BMI) which gives the estimation of adipose tissue in the body by calculating the person's total body weight (Kg) divided by square of height in meters. According to the World heath organization (WHO) classification: underweight (BMI <18.5 kg/m2), normal weight (BMI 18.5-24.9 kg/m2), overweight (BMI 25.0-29.9 kg/m2), class I obesity (BMI 30.0-34.9 kg/m2), class II or moderate obesity (BMI 35.0-39.9 kg/m2), and class III or severe obesity (BMI ≥ 40 kg/m2), hence a person is considered obese if their BMI is equal or greater than ≥25kg/m^2 (Alpert, et al. 2014). Obesity has a strong positive relation to major cardiovascular ailments and contributes to the development of heart failure (HF) and ischaemic stroke. Obesity is also regarded as a major contributor to cardiovascular risk factor such as hypertension, dyslipidaemia, diabetes, metabolic syndrome, myocardial infarction and arterial fibrillation (Lavie, et al. 2014; Parto and Lavie 2017).

Obese individuals are observed to have higher circulating blood volume which inturn increases left ventricular (LV) stroke volume that helps in increasing cardiac output. But consistent higher stroke volume and cardiac output pose heavy burden on heart muscles resulting in alteration in ventricular (both left and right) structure leading to ventricular enlargement and hypertrophy, predisposing to heart failure (HF) (Lavie, et al. 2013). According to a big study conducted by Abdullah *et al* involving 5036 participants of Framingham Cohort study who were followed every

two years for ≤ 48 years concluded that every 2 years living with obesity increases the risk of cardiovascular mortality by 7% (Abdullah, et al. 2011).

Vascular endothelial cell activation also occurs in the adipose tissue in obesity. These activated endothelial cells initiate inflammatory process by upregulating the expression of adhesion molecules and chemotactic factors which in turn further recruit inflammatory cells and adipocyte dysfunction (Nakamura, et al. 2014). Hence, the systemic inflammatory state and endocrine abnormalities induced by obesity contribute significantly to elevate risks for CVD (Ortega, et al. 2016; Parto and Lavie 2017).

2.3.5 Hemodynamic forces (shear stress).

Various hemodynamic forces consistently act on blood vessels. Among them, two main mechanical forces exerted by blood on vasculature are cyclic strain and fluid shear stress. Cyclic strain is induced by the pulsatile blood pressure against the vessel wall. The frictional force generated by the flow of blood against the vessel wall and acting parallel to vessel lumen is termed as fluid shear stress. There are specific mechanoreceptors expressed by ECs which sense and respond according to the changes in microenvironment and subsequently guide the process of development during embryogenesis and remodelling during adult life. Studies have revealed the magnitude of fluid shear stress in human ranges from 10-70 dyn/cm^2 in arteries and 1-6 dyn/cm^2 in veins (Girerd, et al. 1996; Malek, et al. 1999). Generally, the larger, straight segments of vessels experience higher fluid shear stress following unidirectional flow acting parallel to lumen surface but at points where arteries split naturally into branches and at curvature of arteries or through surgical interventions such as stent implantation and as in bypass grafting, this normal blood flow gets disturbed and result in turbulent blood flow. Various studies have reported the athero-protective effect of unidirectional, high laminar shear due to the induction

of anti-inflammatory, antithrombotic and antioxidative genes such as flow-dependent transcription factor KLF2 and nuclear factor (erythroid-derived 2) like-2 (Nrf2)(Nigro, et al. 2011). On the contrary, a disturbed blood flow with low shear stress results in generation of ROS, an increase NF-kB and MAPK activity, increased cytokines and receptor expression for leukocyte and monocyte recruitment, reduced NO production (Yurdagul, et al. 2013) and increased endothelium permeability (Chiu and Chien 2011; Gimbrone, et al. 1997; Green, et al. 2014; Zhou, et al. 2014). The oxidative stress and increased production of ROS lead to the cellular injury and apoptosis through direct effects (lipid peroxidation) or indirect effects (activation of redox signalling pathway). MAPK and NF-kB (activated under low shear stress) are labelled as major pathways involved in regulating and contributing to inflammation and lesion development by inducing the expression of genes encoding for E-selectin, IL-8 and VCAM-1 (Passerini, et al. 2004). To counteract the oxidative stress, an endogenous cellular defense mechanism using Vitagenes becomes activated. This mechanism comprised of cytoprotective vitagenes involved in antioxidant defense, being activated by nuclear factor erythroid 2-related factor (Nrf2). Nrf2 is a transcription factor (member of cap-n-collar family of basic leucine zipper proteins) which was first isolated as an activator of β-globin gene expression but later described as major regulater of intercellular redox signalling and a sensor of oxidative stress. Nrf2 is activated by high shear stress and protects the endothelial layer by negatively regulating the MAPK pathway. Keap1 (Keelch-like ECH-associated protein-1) is the main intracellular regulator of Nrf2. Under normal conditions, Nrf2 is suppressed by binding to the cytoplasmic Keap-1 which targets it for ubiquitination and proteasomal degradation (Warboys, et al. 2011). Under conditions of oxidative stress, the Nrf2-Keap1 interaction is disrupted leading to dissociation of Nrf2 from Keap-1. Free and newly synthesized Nrf2 translocates to the nucleaus and heterodimerizes with small Maf proteins. The heterodimers recognize the ARE sequences present in the regulatory

regions of Nrf2 target genes and hence, induces the expression of many antioxidant genes (e.g. ferrintin, heme oxygenase-1, and glutathione S-transferase) responsible for encoding detoxifying enzymes, stress response proteins and redox balancing factors (Bellezza, et al. 2018; Zakkar, et al. 2009). Apart from Nrf2, several studies have postulated an atheroprotective role for Kruppel-like factor-2 (KLF2) in response to high laminar shear stress (SenBanerjee, et al. 2004). KLF-2 inhibits the expression of proinflamatory genes such as VCAM-1 and E-selectin, thus inhibiting endothelial activation and leukocyte transmigration in cultured endothelial cells (Parmar, et al. 2006). KLF2 exerts its anti-inflammatory effects through inhibiting NF-ƙB mediated transcriptional pathways by sequestering critical coactivators of NF-ƙB, cyclic AMP response element binding protein (CBP) and p300 (Wang, et al. 2006). Aforementioned evidence suggests that the expression pattern of Nrf2 and KLF2 in response to different blood hemodynamics has a profound role in pathogenesis of atherosclerosis as well as other clinical disorders like arterial aneurysm, in-stent restenosis, and ischaemia/reperfusion injury.

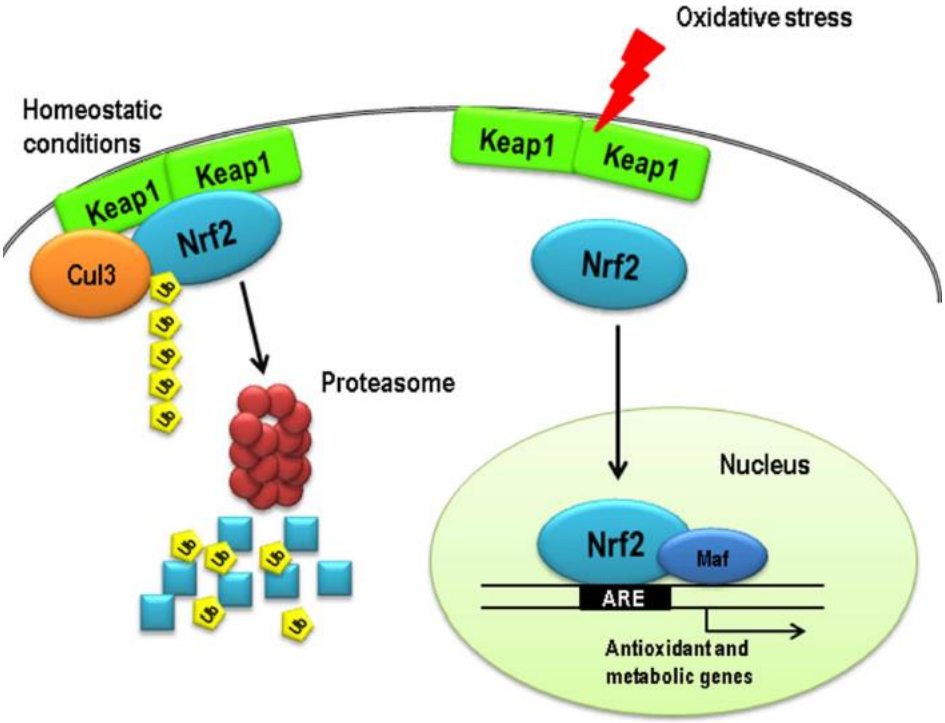


Figure 5: Schematic illustration to present Nrf2/Keap1 interactions in normal and under stress conditions.

Under homeostatic conditions, Nrf2 is kept inactive by binding to its endogenous inhibitor Keap1, associated with F-actin cytoskeleton. Keap1 functions as a substrate adaptor protein for a Cul3-dependent E3 ubiquitin ligase complex, which degrades Nrf2 protein level through proteosome system and maintains Nrf2 protein at a low level under normal homeostatic condition. But under oxidative stress, Nrf2 detaches from Keap-1, translocates to the nucleus and heterodimerizes with Maf. The Nrf2-Maf heterodimer binds to ARE to induce expression of antioxidant genes. Image adapted from Nrf2-Keap1 signalling in oxidative and reductive stress by Bellezza et al 2018: (Bellezza, et al. 2018)

2.3.6 Cytokines.

Cytokines are smaller size, low molecular weight proteins secreted by various body cells and exert their pleiotropic action by regulating immune system and several inflammatory responses. The term cytokines originated from two Greek words, *cytos* means the 'cell' and *kinein* means 'to move' so as to distinguish them as immune-modulator molecules differentiating from growth factors. Cytokines are grouped into several major families depending upon high degree of homology among their primary amino acid sequence orbased on their tertiary structure and spatial organization. **Interleukins** (designated by prefix IL) has originally been thought to responsible for signalling between leukocytes but now known to regulate numerous immunological responses involving multiple cell types. **Tumour necrosis factor (TNF**) is the first monocyte/macrophages derived cytokine, isolated and identified for its specific role as cytotoxic protein for certain tumour cells. **Interferons (IFN)** were first identified as selective antiviral agents due to their role as immunomodulatory agent but because of their effect on cell growth and differentiation, they are categorized among cytokines. Forth group comprises of hematopoietic growth factors (**Colony stimulating factors**) because of their role in cellular growth and differentiation. Another group of cytokines consist of growth factors such as **transforming growth factor-β** (TGF-β)(Young, et al. 2002). **Chemokines** are also considered as a major group among cytokines modulating various cellular functions. Cytokines have two main characteristic features i.e. property of redundancy (structurally dissimilar cytokines can have similar spectrum of action) and the pleiotropy of cytokine action (individual cytokine can exert multiple actions on different cells). Till now about 100 cytokines have been isolated and identified, many of which are actively involved at different stages during the pathogenesis of atherosclerosis (Ramji and Davies 2015).

2.3.7 Cytokine signalling in atherosclerosis.

As atherosclerosis is an inflammatory process, hence many cytokines get involved from the earliest stages of atherosclerosis, contributing to the progression of atherosclerotic plaque by exerting pro- and anti-atherogenic effects (Figure 5). A number of factors mediate cytokine activation such as hypertension, oscillatory hemodynamic/shear stress, hyperlipidaemia and OxLDL accumulation, free radicals and infection. Interleukin such as IL-1α, IL-1β and IL-18 and TNFα (classical pro-inflammatory cytokines) exerts pro-atherogenic effects by increasing leukocyte adhesiveness and promoting their transmigration across endothelium via upregulating expression of ICAM-1 and VCAM-1 (Raines and Ferri 2005). On the contrary, IL-4 and IL-10 is considered as anti-inflammatory cytokine, reducing macrophage infiltration and VSMC proliferation (Charo and Ransohoff 2006). Pro-inflammatory cytokines such as IL-18 and TNFα trigger the release of chemokine MCP-1 from activated platelets, ECs, and VSMCs which further enhances the binding and transmigration of monocytes through endothelium (Gawaz, et al. 1998; Schober, et al. 2004).

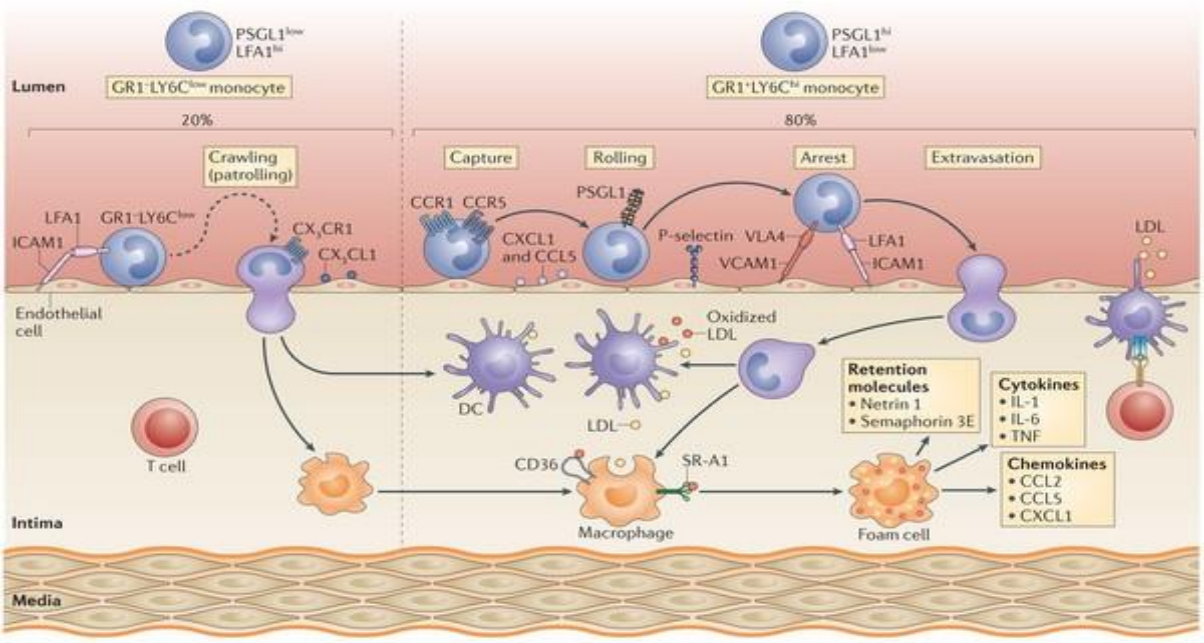


Figure 6. Illustration of the role of different cytokines and chemokines.
Monocyte adhesion and formation of foam cell during pathogenesis of atherosclerosis. Adapted from *Macrophages in atherosclerosis: a dynamic balance by.*Moore KJ et al, *Nat Rev Immunol*. 2013 (Moore, et al. 2013)

Monocyte differentiation into macrophages inside intima is mediate by cytokine macrophage colony stimulating factor (M-CSF)(McLaren, et al. 2011). Inside arterial intima, macrophage exists in 3 phenotypes, M1, M2, and M4. Among these M1, considered as a pro-inflammatory phenotype of macrophage, is responsible for generating IL-6, IL-12 TNFα (pro-inflammatory) cytokines, whereas M2,considered as anti-inflammatory phenotypes, is involved in the production of IL-10 and TGF-β (anti-inflammatory) cytokines (Chinetti-Gbaguidi, et al. 2015; Leitinger and Schulman 2013). Foam cell formation is an important homeostatic step towards progression of inflammation and atherosclerosis. IFNϒ disrupts this process by regulating the genes responsible for the expression of SR and hence promotes uptake of modified LDL (Wuttge, et al. 2004). IFNϒ also decreases cholesterol efflux by inhibiting ATP biding cassette transporter A1 (ABCA1) gene expression, resulting in higher intracellular levels of cholesterol esters (Li, et al.

2010b). Contrary to this, IL-1RA and IL-33 exerts anti-inflammatory actions and inhibit foam cell formation (McLaren, et al. 2011).

2.3.8 Chemokines.

In addition to ILs and TNFα, another family of cytokines, the chemokines along with its receptors play an important role in pathophysiology of neointimal hyperplasia (Schober and Zernecke 2007). Chemokines are small chemotactic cytokines which are divided into 4 subfamilies depending upon their amino acid sequence (according to the configuration of first two N-terminal cysteine residues). The four subfamilies of chemokines and their receptors are designated with the letters CXC, C, CX3C, and CC with a consecutive number (Allen, et al. 2007). The CXC chemokine stromal cell-derived factor (SDF)-1α induces vascular repair by mobilizing bone marrow (BM) derived smooth muscle progenitor cells migration through its receptor CXCR4 (Bleul, et al. 1996). Three distinct chemokines such as RANTES (CCL5), MCP-1 (CCL2), and Fractalkine (CX3CL1) are upregulated during vascular remodelling/repair process and influence leukocyte recruitment and adhesion to the injured area (von Hundelshausen, et al. 2001; Zeiffer, et al. 2004; Zernecke, et al. 2005). CXC chemokine keratinocyte-derived chemokine (KC)/ growth regulated oncogene (GRO)-a (CXCL1) promotes endothelial recovery through its receptor CXCR2 resulting in reduction in neointima formation (Boisvert, et al. 2006; Liehn, et al. 2004). In addition to their role in leukocyte and monocyte infiltration, upregulated MCP-1 and Fractalkine also induces SMC accumulation and proliferation in neointima through their respective receptors CCR2 and CX_3CR1 (Chandrasekar, et al. 2003; Roque, et al. 2002; Schober, et al. 2002). CX_3CL1-CX_3CR1 axis exerts an important homeostatic function by protecting macrophages and monocytes against apoptosis and promotes their survival in atherosclerotic lesion (Landsman, et al. 2009). Inductionof CXCL12-CXCR4 axis promotes SMC migration into the atherosclerotic lesion

and subsequent secretion of extracellular matrix (collagen), hence help in stabilisation of plaque (Akhtar, et al. 2013).

2.3.9 Transforming Growth Factor (TGF).

Members of TGF super family consist of two small (molecular size 12-15kDa) subunits linked together by a disulphide bond. Around 35 members have been identified in different organisms with all of them sharing similar structural homology. Whereas the role of TGF in atherosclerosis is concerned, different studies have proposed contradictory effects comprising both pro and anti-inflammatory roles. TGF-β1, TGF-β2 and TGF-β3 are the 3 isoforms of TGF-β, sharing 70-80% sequence homology and displaying similar properties. All the cells involved in inflammatory cascade (EC, SMC, monocytes/macrophages, platelets, myofibroblasts) expressTGF-β.

TGF-β exerts anti-inflammatory action on macrophages by suppressing the expression of KLF-4 (mediator of proinflammatory signalling in macrophages), inhibiting the expression of MCP-1 as well as reducing the production of NO and superoxide radicals (Feinberg, et al. 2005; Feinberg, et al. 2004; Singh and Ramji 2006a). TGF-β enhances production of anti-inflammatory cytokine IL-10 (Maeda, et al. 1995) as well as play a crucial role in cholesterol efflux by promoting the expression of ABCA1 and apoE (proteins involved in macrophage cholesterol uptake and efflux) (Panousis, et al. 2001; Singh and Ramji 2006b). Contrary to the anti-inflammatory role of TGF-β, several researchers have proposed its pro-inflammatory role. Van Royen has reported that exogenous application of TGF-β enhances monocyte transmigration through endothelial by up-regulation of monocyte adhesion receptor (MAC-1)(van Royen, et al. 2002). TGF-β also up-regulates expression of pro-inflammatory cytokine IL-1 and IL-6, as well as lymphocyte function associated antigen-1(LF-A1) in monocytes, which facilitates adherence of monocytes to extracellular matrix (Turner, et al. 1990; Wahl, et al. 1993; Wahl, et al. 1987).

2.3.10 Platelet Derived Growth Factor (PDGF).

PDGF was first discovered as constituent of platelet α granules influencing cellular growth and development for SMCs and fibroblasts. Gradually the pivotal role of PDGF in regulating cell proliferation and development become clearer and by the fact that it was being released by the cells involved in inflammatory responses, PDGF was considered as one of most potent chemoattractant involved in cell proliferation, chemotaxis, matrix production and tissue repair. Later on, it was evident that in addition to platelets, PDGF is also produced by various cells under pathological/inflammatory conditions. PDGF is a 30kDa dimeric molecule composed of two A and B disulphide-bonded chains. PDGF activates its downstream signal pathways through binding one or both PDGF receptors PDGFR-α and PDGFR-β.

Multiple cells (ECs, VSMCs, platelets, macrophages) involved in the pathogenesis of atherosclerosis produce PDGF. PDGF is actively involved in VSMC phenotype switching by inducing the downregulation of mature SM-markers and promoting VSMC proliferation and migration. Several researchers have elegantly presented studies on the role of PDGF in regulating VSMC proliferation during atherosclerosis (Hu and Huang 2015; Raines 2004; Rubin, et al. 1988; Wilcox, et al. 1988). Sano et al reported a 67% reduction in aortic atherosclerotic lesion size and 80% reduction in VSMC prevalence in neointima in mice injected with rat monoclonal antibody (APB5) against PDGFR–β receptors whereas no such reduction was observed when mice treated with rat monoclonal antibody (APA5) developed against PDGFRα receptor. Furthermore it was observed that PDGF-β receptor is responsible for regulating signal transduction and subsequent reduction of VSMC migration and proliferation (Sano, et al. 2001).

2.3.11 Role of Extracellular Matrix (ECM).

Extracellular matrix (ECM) is the fundamental constitutive component of blood vessels providing structural integrity and support to vessel wall. A normal vessel wall contains several functionally

distinct types of vascular matrices depending upon the specific cellular and structural requirements. These vascular matrices support different types of cells and contain several distinct proteins and thus can be classified into subendothelial basement membrane, internal elastic lamina, medial ECM, external elastic lamina, adventitial and interstitial matrix. Major constitutive proteins found in vascular ECM include elastin, collagens, fibronectin, laminin, amorphous or soluble proteoglycans, microfibrils and leucine-rich glycoproteins.

ECM is a dynamic structure regulating various cellular functions such as adhesion, phenotype switching, proliferation, migration and construction of tissue architecture. The role of ECM is vital in driving vascular function under normal and pathological conditions resulting in crucial changes in vascular wall structure and physiology (Newby and Zaltsman 1999).

An important characteristic of ECM is the storage and mobilization of growth factors and other signalling molecules that induce or restrict VSMC proliferation and migration. Another distinctive feature of ECM is the presence of binding sites for adhesion molecules (N-cadherin) and integrins that mediate leukocyte/monocyte attachment to ECs and transmigration to subendothelial spaces thus playing a crucial role in atherosclerosis and neointima development (Schwartz 2001; Schwartz and Assoian 2001). Obstructing the interactions and binding of integrins with their respective interstitial matrix proteins sufficiently inhibits VSMC proliferation and migration resulting in reduces neointima formation (Dufourcq, et al. 2002; Kappert, et al. 2001). ECM proteins such as collagen-IV, elastin and laminin suppresses VSMC proliferation and migration, reduce inflammatory gene expression and promote a contractile phenotype, whereas interstitial matrix proteins like collagen I, collagen III, and fibronectin reduce contractile gene expression and promote VSMC growth (Schultz and Wysocki 2009).

During atherosclerosis and vascular injury, the composition of basal lamina also changes with down-regulation in the levels of laminin, collagen IV and perlecan and an increase in the

expression of syndecan-4, fibronectin and osteopontin (Moiseeva 2001). Fibronectin expression is also triggered by proinflammatory cytokines such as TNFα and IL-1β. Qwarnstrom *et al* has reported that VSMCs cultured *in vitro* on fibronectin produces 3 times higher expression of NF-kB which induces proinflammatory phenotype in VSMCs (Qwarnstrom, et al. 1994). Uraemia is a strong risk factor for CVD characterised by dedifferentiation of VSMCs. Depletion of osteopontin resulted in less advanced dedifferentiation of VSMC in uraemic mice thus regulating VSMC phenotype modulation (Pedersen, et al. 2013). Osteopontin also induces expression of IL-1β in VSMCs through NF-kB pathway (Yin, et al. 2009).Deletion of syndecan-4 (a trans-membrane heparin sulphate proteoglycan) also resulted in reduced neointima formation and VSMC proliferation (Ikesue, et al. 2011).

2.3.12 Matrix Metalloproteases (MMP)

Matrix metalloproteinases (MMPs) are a large family of zinc-containing endopeptidases with over 25 enzymes isolated and labelled till now. MMPs are mainly involved in the cleavage and metabolism of proteins with particular reference to ECM components. MMPs have been divided into 6 subgroups according to their structure, substrate selection and proteolytic activity. The major groups and their representative members are as follow: collagenases (MMP-1, -8 & -13), gelatinases (MMP2, & -9), stromelysins (MMP-3, -10 & -11), matrilysins (MMP-7 & -26), membrane-bound metalloproteinases (MMP-14, -15, -16 and -25) and zinc and calcium dependent endopeptidases (MMP-20). Majority of MMPs are synthesized by the cells as pro-enzyme, in an inactive form, which can be converted to active their forms through a strict controlled mechanism.

As collagen represent the major component of vascular ECM, the action and subsequent cleavage of collagen by MMPs shifts the plaque phenotype. Hence, MMPs play an important role in development of atherosclerosis and vulnerability of plaque to rupture. During inflammation,

they are not only released by ECs and VSMCs but also from inflammatory mediators like macrophages. This increase in MMP's activity not only modify the matrix but also triggers the VSMC phenotype switching characterised by enhanced proliferation and migration to the intimal plaque. The high quantity of MMPs in the plaque leads to the degradation of collagen (which forms about 60% of total plaque protein (Smith 1965) and important for the formation of the fibrous cap) leading to large lipid core and thin fibrous cap and rupture prone plaque.

MMP-1, MMP-8 and MMP-13 are responsible for degradation of collagen type I,II and III whereas MMP-2 and -9 (gelatinases) are the main enzymes responsible for cleavage of Collagen type IV along with other major extracellular components e.g. elastin, fibronectin and laminin. They also involved in the cleavage and subsequent activation of several bioactive molecules such as cytokines (pro-TGF-β and pro-TNFα), chemokines (IL-8) and vasoconstrictor endothelin-1. Gelatinases also cleave and activate their own and other MMPs such as pro-MMP-9, pro-MMP-1, pro-MMP-2 and pro-MMP-13.

Morphological analysis of plaques have shown higher MMP-1 levels in carotid lesions with large lipid core and thin fibrous cap (Morgan, et al. 2004). Li *et al* has shown enhanced expression of MMP-2 and MMP-9 in the fatty streaks, calcified atheroma and fully occluded lesions (Li, et al. 1996). In addition to that levels of MMP-2 and MMP-9 were upregulated in fatty streaks and atherosclerotic plaques from diseased human vessels as compared to healthy ones (Kieffer, et al. 2001). On the contrary, in fibrotic atherosclerotic plaques, the levels of TIMP-1 and TIMP-2 were upregulated whereas MMP levels were reduced (Choudhary, et al. 2006). Genetic targeting of MMP-3 resulted in larger atherosclerotic lesions with less collagen and SMC content as compared to the control individuals suggesting a more vulnerable plaque phenotype (Johnson, et al. 2005; Silence, et al. 2001).

Tissue inhibitors of MMP (TIMPs) regulate the MMP activity and provide/maintain endogenous mechanism to balance and prevent excessive degradation of ECM. TIMP family has 4 members referred as TIMP1-4, amongst these, TIMP-1 and TIMP-2 are constitutively expressed to inhibit MMPs activity and maintain a balance between matrix production and degradation. Though in healthy individuals, the MMP-2 to TIMP-1 & -2 ratio is maintained but inclined towards MMP during atherosclerosis and lesion development due to the excessive secretion of MMP-3 & -9 leading to plaque destabilization as mention earlier (Galis and Khatri 2002; Owens, et al. 2004).

2.4 Role of Circulating and Resident Cells in Atherosclerosis and Neointima Development:

2.4.1 Macrophages.

Macrophages are regarded as phagocytic cells because of their ability/function to remove apoptotic cells and cellular debris during metabolic processes. Macrophage originate as monocytes from the same myeloid progenitor cells in the bone marrow as neutrophils and dendritic cells (Mosser and Edwards 2008). Low shear stress at areas of disturbed flow and accumulation of lipoproteins renders endothelial layer dysfunctional and thus paving the way for these inflammatory monocytes to transmigrate through the endothelium into the intima. In the intima, monocytes differentiate into macrophages under the influence of specific cytokines with M-CSF (Macrophage colony stimulating factor) as major facilitator (Bobryshev 2006). Under the effect of cytokines, a heterogeneous population of macrophages is produced, which includes inflammation promoting M1 macrophages and inflammation resolving M2 macrophages. Cytokines like INF-ϒ and IL-1β produces M1 macrophages whereas IL-4 and IL-13 are responsible for M2 macrophage production (Shimada 2009). Imbalance between these two sub-populations

of macrophages M1:M2 may contribute to plaque development due to impaired pro-resolving response and the resulting imbalance between lipoprotein uptake and cholesterol efflux result in foam cell formation.

Inside intima, these macrophage proliferate and convert into lipid laden foam cells by internalizing native and modified lipoproteins (oxidised oxLDL and acetylated AcLDL) through multiple pathways. These includes phagocytosis of aggregated lipoproteins, up-regulation of SR-A, CD36 and SR for phosphatidylserine and oxLDL (SR-PSOX), and by fluid-phase pinocytosis of native lipoproteins (Pluddemann, et al. 2007). Following internalization, lipoproteins are delivered to lysosomes and late endosomes where they are hydrolyzed into free cholesterol and fatty acids by lysosomal acid lipase. Excessive cholesterol is transferred to endoplasmic reticulum (ER) for esterification (Chinetti-Gbaguidi and Staels 2009).

Dysfunctional efferocytosis, disruption of cholesterol metabolism and efflux, ER stressincuded Ca^{2+}release, NF-ƙB activation due to mitochondrial oxidative stress, as well as continued influx of lipoproteins and monocytes and secretion of cytokines result in inflammation and subsequent apoptosis of lesional macrophages. Apoptosis of macrophagesis is an important step towards advanced atherosclerotic lesion development leading to post apoptotic necrosis, thus further amplifying inflammation. Stability of plaque is also hampered by macrophages through secretion of proteases such as serine proteases, and MMPs, thus contributing to thinning of fibrous cap. (Libby 2013)

2.4.2 Endothelial cells (EC).

ECs lined the innermost layer of blood vessels thus forming an interface between the blood and the remaining cells of blood vessel. As they are in direct contact with the blood hence are exposed to various chemical and hemodynamic changes. These cells play important role in regulating vascular tone and maintaining haemostatic functions such as angiogenesis and

vascular remodelling, selective permeability for macromolecules, leukocyte trafficking, monocyte/macrophages transmigration and secretion of various procoagulant, anticoagulant and fibrinolytic agents. ECs are also involved in initiating key inflammatory response by triggering the release of major inflammatory mediators, over expression of surface adhesion molecules and release of chemokines and cytokines.

Alteration in endothelial integrity either because of its denudation resulting from PCI or its dysfunction constitutes pathogenic risk factor leading to several vascular diseases including atherosclerosis and neo-intima formation.

In healthy straight vessels, ECs display elongated/ellipsoidal morphology and are aligned parallel to the primary flow direction but different studies indicated that in certain areas (like arterial bifurcation and branches) where blood flow is oscillatory/disturbed with low shear stress instead of laminar flow with high shear stress, the ECs show cuboidal morphology. These areas with disturbed flow pattern are more prone to atherosclerosis, suggesting that laminar and oscillatory flow patterns can induce different molecular responses in ECs. This dysfunctional endothelial phenotype exhibits proinflammatory, prothrombotic and impaired barrier function leading to initiation of atherosclerosis (Berk 2008).

The pathphysiological consequences of endothelial dysfunction includes (Bonetti, et al. 2003):

- Increased cellular senescence and higher rate of cell turnover leading to endothelial dysfunction and apoptosis.
- Impaired endothelial barrier function leading to increased permeability to macromolecules like cholesterol and lipoproteins (Chien 2003).
- Over-expression of cell surface adhesion molecules such as ICAM-1, VCAM-1 and chemotactic markers like MCP-1, E-selectin and P-selectin (Chiu, et al. 2003).

- Over-expression of proatherogenic genes like NF-ƙB and reduced expression of eNOS (endothelial Nitric oxide synthase)(Cooke 2003; de Nigris, et al. 2003).
- Down-regulation of NRF-2 (Warabi, et al. 2007), KLF-2 and KLF-4 (Kruppel-like factor 2 and 4) which are considered as atheroprotective genes (Atkins and Simon 2013; Villarreal, et al. 2010).

Atheroprone endothelium also produce various proinflammatory cytokines, chemokines, ECM-degrading proteins (e.g., MMPs), and growth factors contributing to enhanced recruitment and transmigration of monocytes/macrophages to the intima and formation of foam cells (Galis and Khatri 2002).

2.4.3 Fibroblast.

Fibroblasts are heterogeneous cell population which constitute the outermost layer of vessel wall called adventitia. For many years, their main function was thought to provide nourishment to the VSMCs and support to the blood vessel, but with the advancement in research, their role in atherosclerosis and development of neointima is under discussion. Under the effect of certain growth factors such as TGF-β, PDGF-BB, and TNFα, these cells can proliferate and express contractile and synthetic phenotype like SMCs (Sartore, et al. 2001). The activated fibroblasts exhibiting SMC features (expression of SM-α Actingene and synthesis of collagen type I) are termed as myofibroblasts (Desmouliere, et al. 1993).

Myofibroblasts can migrate toward intima under the effect of MMPs and TIMPs and thus contribute toneointima thickening (Shi, et al. 1999). Shi *et al* has reported such phenomenon (increased level of myofibroblast transmigration towards medial layer and to the intima) in addition to a hyper-proliferative response of myofibroblasts after coronary artery injury (Shi, et al. 1996a). In another study, Siow *et al* also reported that fibroblasts expressing Lac-Z could express SM-αActin and transmigrate through the media, thus actively involved in neointima

formation (Siow, et al. 2003). Scot *et al* has also described an increased cellular proliferation rate in adventitial layer as compared with medial layer after conducting balloon injury to anterior descending artery in swine (Scott, et al. 1996). Myofibroblasts also produce excessive amounts of ECM which may be responsible for adventitial thickening (Shi, et al. 2000). Hence, above studies demonstrates that activation of fibroblasts resulting from vessel injury or under the effect of inflammatory mediators can have pronounced impact on neointima formation.

2.4.4 Vascular smooth muscle cells (VSMCs)

Smooth muscle cells (SMCs) appear as thin layer of sheets and are found throughout our body, lining the walls of passageways and cavities/hollow organs such as blood vessels (VSMCs), lymphatic vessels, airways/bronchial smooth muscle, intestine, bladder and uterus (uterine smooth muscle). Their primary function is to create contractions under the influence of foreign stimulus. The contractions created by SMCs are due to sliding movement of actin and myosin filaments which are also most abundant proteins (about 40%) in SMCs. The movement of actin filaments not only helps the cell in maintaining its cytoskeleton but also regulate cellular movements under the effect of molecular or mechanical stimulus/injury. The energy required for this movement is provided by hydrolysis of ATP.

In cardiovascular system, VSMCs are not only the constitutive component of blood vessels but also play a crucial role in regulating multiple functions under specific conditions. They along with ECM constitute the middle layer (tunica media) of the vessels thus providing structural integrity. Contraction and relaxation of VSMCs controls the vascular tone and diameter thus regulating volume of blood through them and subsequent blood pressure. Excessive vasoconstriction leads to high blood pressure (hypertension) whereas vasodilation results in hypotension. Interestingly, role and phenotype of VSMCs changes dramatically depending upon the specific stage of development. During early stages of vasculogenesis, VSMCs show high rate of proliferation and

migration which results in formation of cardiovascular system. During vascular development, VSMCs also secret excessive amounts of ECM proteins including collagen, elastin, fibronectin, cadherins and integrins which not only constitute major portion of blood vessel wall but also provide structural integrity (Owens 1995). In contrast, in adult blood vessels, mature VSMCs show a quiescent phenotype characterized by an extremely low proliferation rate, non-migratory and decreased synthesis of ECM components. Mature and fully differentiated VMSCs are contractile, express a unique repertoire of contractile proteins, ion channels, calcium regulatory proteins and specific receptors, and signal transduction molecular pathways. The proteins exhibited by contractile VSMCs includes SM-α Actin, SM22a, Smooth Muscle myosin heavy chain (SM-MHC), calponin, and smoothelin all recognized as selective VSMCs markers. (Owens, et al. 2004)

An important characteristic of VSMCs is their phenotypic plasticity, unlike skeletal muscle and cardiomyocytes, VSMCs are not terminally differentiated and can undergo reversible changes in phenotype upon stimulation from environmental cues (Bobryshev and Lord 1996; Gomez and Owens 2012). For example, in case of vascular injury, the VSMCs play a critical role in vascular repair by actively initiating processes of migration and proliferation towards the damaged area and secret large quantities of ECM components to speed up healing process. Generally, the phenotype switching of VSMCs occurs during neovascularization, vascular remodelling, atherosclerosis and repair of vessel injury, and is accompanied by strictly regulated induction of VSMC genes associated with contractility and subsequent downregulation of VSMC markers associated with proliferation and migration (Fischer, et al. 2006; Gomez and Owens 2012).

Growing evidence also suggests ability for VSMCs to trans-differentiate into macrophage like cells in lipid rich regions of plaque during atherosclerosis. Such VSMCs exhibit properties like lipid molecules uptake (phagocytosis), foam cells formation and start expressing CD68 (a macrophage

marker) along with SM-α Actin (a smooth muscle cell marker) (Chistiakov, et al. 2015; Rong, et al. 2003).

2.5 Vascular Smooth Muscle Cell phenotype switch in atherosclerosis.

Phenotype switching or plasticity of VSMCs (any change in the normal structure or function of differentiated VSMC) plays a critical role in development of a number of major diseases in human including hypertension, cancer, atherosclerosis and neointima formation. As mentioned earlier, mature VSMCs express a unique set of contractile proteins such as smooth muscle α actin (α-SMA), smooth muscle 22α (SM22α), smooth muscle myosin heavy chain (SMMHC), smoothelin and calponin which impart them the specific characteristic of contractility, thus maintaining vascular tone and regulating blood volume and pressure in the circulatory system (Owens 1995; Xiao, et al. 2010). Adult VSMC retain and exhibit the distinct quality of plasticity (phenotype switch) throughout their life cycle which render their ability to proliferate, migrate and secret ECM proteins under pathological condition (atherosclerosis) or after mechanical injury to vessel wall. Based on their property of plasticity, evidence has suggested a profound dual role (propagating and protective) for VSMCs in progression of atherosclerosis (Ross 1993). Under normal circumstances, mature VSMCs are elongated, contractile with expression of specific proteins, show very little proliferation and aligned at their long axis directed longitudinally around the vessel (Figure 6). However, during inflammation as in atherosclerosis or upon vascular injury, VSMCs become less contractile, show enhanced proliferation and migration along with increased production of inflammatory cytokines and proteases, becomes cobblestone in shape and are termed as synthetic or proliferative VSMCs (Rzucidlo, et al. 2007; Sobue, et al. 1999) (Figure 6). This synthetic phenotype plays a major pathophysiological role in the

development of atherosclerotic plaque and neointima formation (Kawai-Kowase and Owens 2007; Yoshida, et al. 2008b). At the beginning, in response to the stimulation of inflammatory cytokines released from activated T-cells and macrophages, VSMCs migrate from media to intima, where they proliferate and switch to synthetic phenotype, thus contribute to plaque formation and growth. However, at the later stage of atherosclerosis progression, the accumulated VSMCs and ECM proteins including collagen, elastin and fibronectin produced by VSMCs contribute to stabilization of the plaque by forming a thick fibrous cap on it. Thus they play a beneficial role by protecting plaque rupture and preventing thrombus formation, which is main cause of the clinical complications of atherosclerosis.

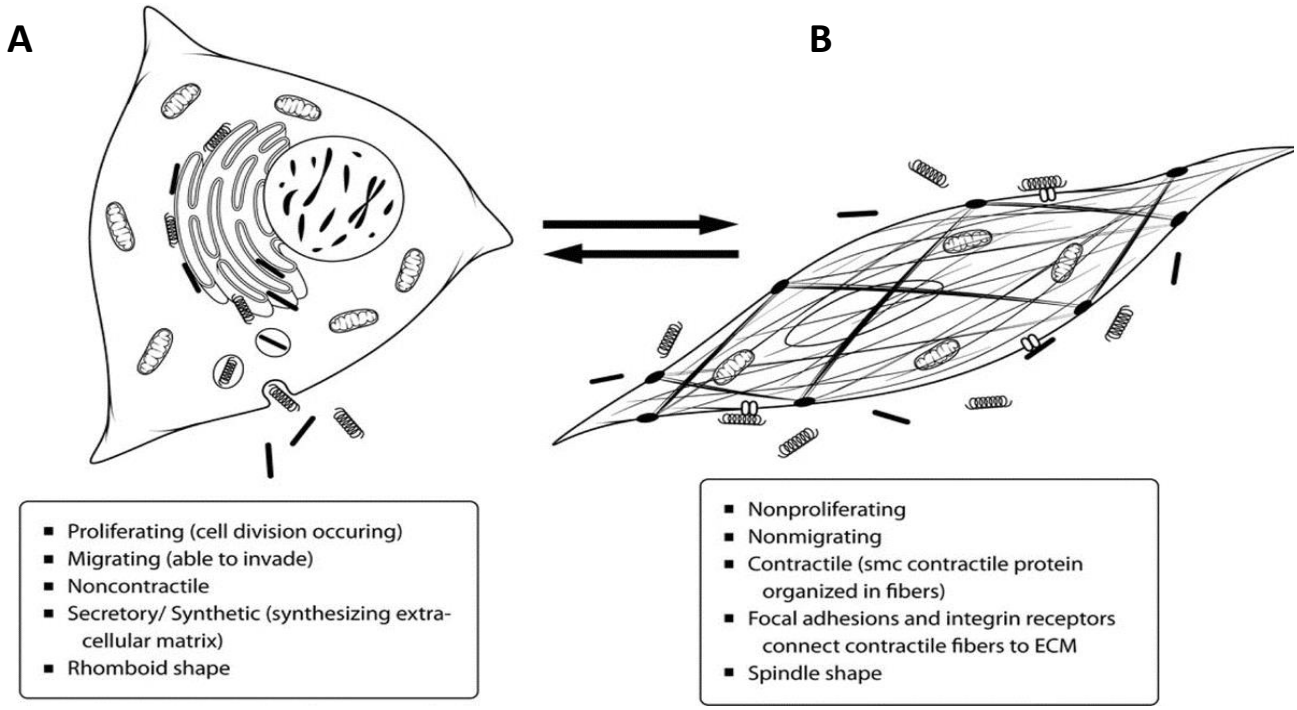


Figure 7. Phenotypic switching by vascular smooth muscle cells.
Mature smooth muscle cells are able to switch between differentiated/contractile and dedifferentiated/synthetic phenotypes. **A: Synthetic/Dedifferentiated Cell**, whose main purpose is proliferation, migration, and modulation of extracellular matrix to repair the vessel wall.**B: Differentiated/ ContractileCells**: Schematic diagram adapted from (Reid, et al. 2010).

2.6 Factors affecting VSMC phenotype switching.

Considering the documented role of VSMC in pathogenesis of cardiovascular diseases, studies focusing on signalling pathways and underlying molecular mechanisms regulating SMC behaviour are crucial for improving our understanding of the pathological processes involve in CVDs and developing novel therapies. As mentioned earlier that mature contractile VSMCs undergo phenotypic switching upon activation by inflammatory stimuli as occurs during atherosclerosis, generating synthetic VSMCs that is pro-atherogenic so it would be interesting to have a brief look at the factors governing this phenotype modulation.

2.6.1 Shear stress.

In healthy individuals, endothelial cells provide a barrier and shield VSMCs from direct exposure to the blood flow and the resulting shear stress. But if EC layer is ruptured due to mechanical injury caused by stent implantation during angioplasty or by rupturing of atherosclerotic plaque, VSMCs comes in direct contact with the flowing blood and results in modulation of several molecular signalling pathways. VSMCs sense and respond to the cyclic strain and shear stress through activation of similar mechanosensory mechanisms as in case of ECs including integrins, receptor tyrosine kinases (RTKs) e.g. PDGFRα and EGFR and ion channels (Ca^{2+} and nonselective ion channels) (Iwasaki, et al. 2000; Mohanty and Li 2002; Wernig, et al. 2003). VSMC mechanosensing leads to the activation of several signalling pathways including focal adhesion proteins FAK and Src, PKC, PI3K/Akt, Ras and Rho family of small GTPases. The interaction of VSMC integrins with the ECM plays a significant role in the initiation of process of mechanotransduction under the influence of cyclic strain. Blocking integrins by using antibodies can significantly effect VSMC proliferation and apoptosis. VSMCs under the influence of stretch, expressed integrin activation as manifested by its association with the adapter protein Shc (Wernig, et al. 2003). Stretch induced phosphorylation of PDGFRα and EGF receptor (EGFR) in

VSMCs in a magnitude dependent manner leads to inhibition of cell proliferation (Hu, et al. 1998; Iwasaki, et al. 2000). Stretch stress induced ion channel activation have been studied extensively in ECs and later in VSMCs. Stretch induced increase in cytosolic Ca^{2+} concentration has been reported through stretch activated Ca^{2+} channels, triggering its release from intracellular Ca^{2+} stores (Mohanty and Li 2002). The active transport of ion via membrane bound ion transport pumps has also been explored. Stretching of VSMCs cause an increase in the mRNA and protein expression of the α-subunit of Na^{+} and K^{+} ATPase resulting in their activation and translocation of α-subunit to the pasma membrane (Sevieux, et al. 2003),leading to VSMC functional changes.

Laminar flow and resultant steady shear stress induces atheroprotective response in VSMCs by triggering prostaglandin E_2 (PGE_2) and eNOS production in ECs leading to increased bioavailability of NO and subsequent vasodilatation of VSMCs (Qiu, et al. 2014). Shear stress also inhibited VSMCs proliferation and migration by attenuating PDGFRβ expression and decrease matrix metalloproteinase 2 (MMP-2) levels (Ueba, et al. 1997). Shear stress induces nitric oxide production and activates AMPK by upregulating its phosphorylation in VSMCs via NOS expression, hence suppressing cell proliferation (Kim, et al. 2017). Laminar flow also modulate VSMC proliferation and migration by downregulating expression levels of Extracellular signal regulated kinase 1/2 (ERK1/2) and phosphatidylinositol 3 kinase (PI3K)/ protein kinase B (Akt) signalling leading to inhibition of MMP-2 production, along with reduction in the levels of PDGFR and TGF-β (Goldman, et al. 2007).

On the contrary, disturbed hemodynamics resulting from turbulent, oscillatory flow results in unstable pattern of mechanical stretch which is recognized by VSMCs through a variety of mechanoreceptors including integrins, PDGFRs, insulin-like growth factor-1 receptor (IGF1R) and Notch receptors (Liu, et al. 2011a; Liu, et al. 2003; Morrow, et al. 2005; Wernig, et al. 2003). Integrins and Notch receptors stimulates VSMC migration by activating RhoA/ROCK1 (Ras

homologue A (RhoA)/rho-associated, coiled-coil containing protein kinase 1 (ROCK1) dependent signalling mechanism through PTK2 (protein tyrosine kinase 2)(Gambillara, et al. 2008). Activation of PDGFR results in upregulation of p53 expression through MEK1/2/p53 MAPK signalling pathway resulting in VSMC proliferation and apoptosis (Mayr, et al. 2002).

2.6.2 Oxidized LDL.

LDL accumulation and subsequent oxidation in the intima holds prime position in initiation of inflammatory response during atherosclerosis. VSMCs express a myriad of specific receptors at their surface which mediate the endocytosis of modified lipoproteins (oxLDLs) resulting in conversion of VSMCs into foam cells thus further exacerbating inflammation. Proatherogenic effects of oxLDL result in up-regulation of these receptors which includes SRA-I, SRA-II, Lectin-type oxidized LDL receptor 1 (LOX-1), CD36, and low-density lipoprotein receptor related protein 1 (LRP1)(Allahverdian, et al. 2012; Lacolley, et al. 2012). Treating VSMCs with ox-LDL components like lysophosphatidylcholine (LPC) resulted in up-regulation of LOX-1 expression and increased ox-LDL uptake (Aoyama, et al. 2000). Ox-LDLs also interfere with differentiation of SM progenitor cells into SM-like cells by regulating PDGF dependent differentiation pathways (Teslovich, et al. 2010). Furthermore, activities of ATP-binding cassette transporter A1 (ABCA1) and apolipoprotein A1 (ApoA1), which are components of cholesterol transport pathway, mediating the efflux of cholesterol from the VSMCs, are downregulated in rat intimal SMCs and human atherosclerotic intimal SMCs when exposed to cholesterol treatment (Choi, et al. 2009a). Increased cholesterol influx alongside decreased efflux further exacerbates VSMC foam cell formation. Continuous accumulation of cholesterol in lipid-laden VSMCs results in cell death and apoptosis, hence promoting migrating and proliferation of adjacent VSMCs to the intima. Hyperlipidemic conditions also reduces phagocytic/efferocytic activities of VSMC which results in accumulation of apoptotic VSMC foam cell in the atheroma, due to impaired clearance leading to

secondary necrosis and enlargement of atherosclerotic plaque (Clarke, et al. 2010; Van Vre, et al. 2012). To sum up, oxLDLs promotes proatherogenic activation and phenotype switching of VSMCs towards synthetic form with a dysregulated cell proliferation, dedifferentiation, migration and apoptosis.

2.6.3 Proinflammatory stimuli.

Proinflammatory cytokines like TNFα and IL-1β play a crucial role in initiating the inflammatory process as well as activation of VSMC. TNFα triggers the release of ICAM-1 and VCAM-1 from VSMCs hence regulating adhesion of monocyte/macrophages (Lee, et al. 2006a). Furthermore, it also induces secretion of MMP-9 from VSMCs which through it proteolytic activity enhances migration of VSMCs into the intima (Lin, et al. 2008). IL-1β also induces the production of ICAM-1, VCAM-1 and MMP-9 through p42/p44 MAPK-p38 MAPK-Jnk-NF-ƙB signalling pathway (Liang, et al. 2007). TLRs are pattern recognising receptors involved in initiation of immune response upon activation by viral and bacterial biomolecules. TLR-2 and TLR-4 on VSMCs are reported to be upregulated during inflammation and atherosclerotic lesion development (Curtiss and Tobias 2009). Both TLR-2 and TLR-4 promote VSMC proliferation, migration and neointima formation by activating various proinflammatory signalling pathways such as Akt, Jnk1/2, p38MAPK and Erk1/2 resulting in the enhanced production of proinflammatory cytokine IL-6 and MMP-2 (Lee, et al. 2012; Liu, et al. 2014). Low concentrations of *Escherichia coli* LPS can activate TLR-4 which results in enhanced production of IL-1α, IL-6, MCP-1 and stimulates ERK1/2 activity (Yang, et al. 2005b). TLR-4 stimulation upregulates NF-ƙB leading to enhanced production of MMP-9, associated with VSMC migration and plaque vulnerability owing to its proteolytic activity (Li, et al. 2012).

Disruption in Calcium signalling/handling and resultant overload through modulation in activity of SERCA (sarco/endoplasmic reticulum Ca^{2+} ATPase) also regulates VSMCs proliferation. VSMCs from athero-susceptible mice showed altered expression of SERCA and impaired calcium

handling (Van Assche, et al. 2007). Lipskaia *et al* demonstrated that SERCA 2a along with protein phosphatase inhibitor-1 (I-1) inhibited VSMC switching to synthetic phenotype by regulating Ca^{2+}handling (Lipskaia, et al. 2005). Furthermore, SERCA also reduced VSMC proliferation and subsequent balloon injury induced neointima hyperplasia by regulation of transcription factor NFAT (Nuclear factor of activated T-cells) activity. Exploring further, Lipskaia *et al* also observed that VSMC phenotype switching and neointimal thickening was reduced by overexpression of I-1 in rat angioplasty models (Lipskaia, et al. 2014).

2.6.4 Transcription factors.

As aforementioned, switching of VSMCs from contractile to synthetic phenotype involves decrease in expression of SM contractile proteins like SM-α Actin and SM-MHC with subsequent increase in expression of genes involved with SM proliferation and migration. These regulations of SM marker genes are controlled by different mechanisms. Transcription factors such as KLF2 and Nrf2 which are associated with regulation of constriction and dilatation of VSMCs are also upregulated under normal blood flow conditions (laminar flow and steady shear stress)(Lee, et al. 2006b; Takabe, et al. 2011). KLF2 is considered as an atheroprotective transcription factor controlling the expression of various genes actively involved in the regulation of VSMCs constriction and dilatation. Transcription factor Nrf2 is responsible for upregulation of many antioxidant enzymes as well as detoxifying agents thus preventing the cells from proatherogenic effects of ROS and oxidative stress (Warabi, et al. 2007). Furthermore, PDGF and oxidized phospholipids regulate Klf-4 expression which results in downregulation of VSMC markers such as SM22α (Dandre and Owens 2004; Salmon, et al. 2012). Myocardin is critical factor for VSMC differentiation and expression of mature SMC marker genes. During atherosclerosis, expression of KLF4 is upregulated in VSMC leading to inhibition of myocardin-mediated expression of VSMC marker genes by downregulating both the myocardin gene and its targets (Liu, et al. 2005).

Transcription factor NF-kB also contributes to inflammation, proliferation and apoptosis. Activation of NF-kB promotes a synthetic phenotype (Cao, et al. 2006) in VSMCs through upregulation of proinflammatory genes such as MCP-1 (Landry, et al. 1997), VCAM-1, ICAM-1, (Cercek, et al. 1997), chemokine (C-X-C motif) ligand-1 (CXCL1) (Kim, et al. 2008) and MMP-1, 2 and 9 (Cui, et al. 2014; Moon, et al. 2004). MCP-1 and CXCL1 initiate the inflammatory response by attracting monocytes and neutrophils to the site of injury whereas MMPs degrade ECM leading to increased migration of VSMCs (Chen, et al. 2013b). NFAT is a family of transcription factors shown to be important in immune response. Upon activation by calcineurin (Ca^{2+} dependent protein kinase) NFAT translocates to nucleus and drives the expression of genes controlling VSMC proliferation and migration (Yellaturu, et al. 2002).

2.6.5 Extracellular matrix proteins.

ECM proteins have a pronounced role in regulating phenotype modulation of VSMCs, the atheroma development and plaque stability. ECM proteins such as collagen, elastin, and fibronectin not only provide the elasticity and integrity to the blood vessel but also suppress phenotype switching by keep the VSMCs in a 'contractile' state. During atherosclerosis, excessive production of proteases like MMPs from macrophages and VSMCs leading to breakdown of ECM (e.g., collagen and elastin) and hence promotes phenotype switching and increases VSMC proliferation and migration. Furthermore appearance of syndecan-4, fibronectin and osteopontin also change the composition of basal lamina and influences VSMC proliferation through Erk-dependent mechanism (Moiseeva 2001).

N-cadherin is a major cell-cell adhesion molecule in VSMCs. Several MMPs (MMP-7,-9,-12) have been reported to influence VSMC activity (proliferation and migration) by cleaving N-cadherin (Dwivedi, et al. 2009; Williams, et al. 2010). Different researchers have proposed the role of oxidative stress in MMP-directed shedding of N-cadherin from VSMCs and resultant phenotype

switching. Lee and Jagadesh have proposed role of Nox1 (NADPH Oxidase-1) in activation of MMP-2 and MMP-9 through two different mechanisms involving TLR-2 ligand interactions with Nox1 and through trans-activation of epidermal growth factor receptor (EGFR) respectively (Jagadeesha, et al. 2012; Lee, et al. 2013). Study showed (Barnes and Farndale 1999) that under inflammatory conditions more MMP-1 and MMP-9 is produced by VSMCs, which degrades collagen-I and collagen IV respectively, leading to the liberation of collagen I in monomeric form. Collagen-I promotes VCAM-1 expression in VSMC via a NFAT dependent mechanism and induces VSMC migration through its interactions with integrin $\alpha_2\beta_1$. (Heino 2000; Minami, et al. 2006; Orr, et al. 2009).

It is important to understand that the MMP levels are strictly controlled in VSMCs of healthy individuals by the synthesis of TIMPs (Fabunmi, et al. 1998). A balance between MMP-2 and TIMP-1 and TIMP-2 is maintained in normal arteries, but such balance shifts towards MMPs favour during atheroma development due to the excessive production of MMP-2 and MMP-9, and thus influence plaque stability (Galis and Khatri 2002). Expectedly, an imbalance between MMP/TIMP is one of major mechanisms underlying the development of atherosclerosis.

2.7 MicroRNAs (miRNA)

Precise regulation of genes and related downstreammolecular mechanisms are extremely important in embryogenesis and development. DNA and chromatin modifiers and transcription regulators are indispensable in proper gene expression patterns. However the gene expression is also regulated at post-transcriptional level. Various post-transcriptional regulatory mechanisms such as alternative splicing, messenger RNA stability and translational control, and RNA silencing have been extensively investigated in the past decades. Most recently, a class of

endogenousnon-coding, small, regulatory RNA molecule called microRNA (miRNA) has emerged as another key regulator of gene expression during development and disease progression.

MiRNAs are endogenous, highly conserved, single strand, short (20-23 nucleotides), non-coding RNAs which were first identified about 30 years ago by Lee and Ambros while working on nematode Caenorhabditis elegans (Ambros and Horvitz 1984). They regulate target gene expression at post-transcription level by interacting with the 3' un-translated regions (UTRs) of the specific mRNAs (Ambros 2004; Wightman, et al. 1993). The fact that single miRNA can suppress many mRNA (thus regulating multiple genes), make them stand out as key gene regulators, controlling fundamental biological processes like cell proliferation, differentiation, apoptosis and tumour progression or repression and the pathways that control developmental processes. Currently more than 2588 mature miRNAs in human genome and around 1915 from mouse musculus have been reported with each regulating dozens of target mRNAs. The miRBASE Registry (http://www.mirbase.org) holds and organised the published data about the miRNA sequence with associated annotations and provide gene naming and nomenclature function.

2.7.1 MicroRNA Nomenclature:

With the advancement in the miRNA research, proper categorisation and classification of all the identified miRNAs is critical for miRNA biology since increasing number of miRNAs have been reported in the literature. Furthermore, as one miRNA could regulate many genes, thus identifying particular target genes for specific miRNA is another area need to be addressed. Sanger institute and welcome trust have jointly developed miRNA sequence database called miRBase (freely available online as http://www.mirbase.org) to help researchers in this field to access a centralise searchable database of all identified miRNA, their sequence and annotations along with specific gene nomenclature and prospective targets.

Briefly, miRNAs are now classified by using a prefix “miR” followed by a specific number such as miR-214. The assignment of these numbers to miRNAs is sequential as earlier identified miRNAs are given lower number whereas newly identified miRNAs are given higher numbers. The genes responsible for their transcription are also assigned same numbers but represented with smaller letter ‘mir’ instead of ‘miR’. To help distinguish between primary and precursor miRNAs, primary miRNA transcript is referred as pri-miR-214 whereas precursor hairpin miRNA is referred as pre-miR-214.

As some miRNAs might be specific to particular organism, hence miRBase also includes three letters abbreviation from organism genus and species to identify particular species, miRNAs are derived from, for example, hsa-mir-214 refers to human miR-214. An additional lower case letter is added after the number if the same miRNA precursor gives rise to multiple miRNAs with difference of 1-2 nucleotide in their sequences (e.g. miR-200a, miR-200b, and miR-200c are closely related miRNAs). Mature miRNA with identical neucleotide sequence can arise from more than one genome locus and different pre-miRNA. In such case, their loci are named with the addition of a number suffix at the end (e.g. mir-125a-1 and mir-125b-2), both generate mature miR-125.Mature miRNAs are double stranded (functional guide strand and passenger strand which generally degraded) but in some case both strand become functional. In such cases mature miRNAs are classified by the addition of suffixes representing their specific arms, e.g. miR-142-5p ‘5’ (from 5’arm) and miR-142-3p ‘3’ (from3’ arm). (Ambros, et al. 2003; Griffiths-Jones 2004; Griffiths-Jones 2006; Lee and Ambros 2001).

2.7.2 MicroRNA Biogenesis.

MiRNAs genes are either distributed throughout the genome as clusters and are transcribed as polycistronic primary transcripts, which are subsequently cleaved into many miRNAs or they may be located within intergenic regions or in intronic sequences of protein-coding or non-coding

exonic sequences. MiRNAs are transcribed as long double-stranded transcripts termed as primary miRNA (pri-miRNAs) byRNA polymerase II in association with certain transcription factors and epigenetic regulators. Although some researchers have proposed RNA polymerase III as transcribing agent for certain pri-miRNAs (Pfeffer, et al. 2005), still majority of pri-miRNAs are transcribed by RNA polymerase II as evident by their polyadenylation and capping. Recent studies have suggested that epigenetic factors like histone modification and DNA methylation may contribute to miRNA gene regulation whereas p53, ZEB1 & ZEB2, MYC and myoblast determination protein 1 (MYOD1) are the reported major transcription factors regulating miRNA transcription (Davis-Dusenbery and Hata 2010; Kim, et al. 2009). pri-miRNA generated after initial transcription is usually quite long (over 1kb) with a characteristic stem loop structure containing mature miRNA sequences and single stranded RNA segments at both 5' and 3' ends. Processing of pri-miRNA into mature miRNA involves catalytic activity of two RNase III family enzymes, Drosha and Dicer along with their associated proteins, in a step wise manner (Figure 7). The first process initiates within nucleus by double stranded ribonuclease III (RNase III) type endonuclease enzyme, Drosha (a 160 kDa protein) and its co-effector DiGeorge syndrome critical region 8 (DGCR-8) protein (also called Pasha)(Wang, et al. 2007). DGCR8 (a 90kDa protein localized within nucleolus and nucleoplasm) works as an essential co-factor with Drosha and together they form a complex called microprocessor. DGCR8 interacts with Drosha through its conserved C terminus whereas its two double-stranded RNA binding domains (dsRBDs) help in recognizing pri-miRNA (Shiohama, et al. 2007). Drosha has two tandem RNase III domains (RIIIDs) and a double stranded RNA-binding domain (dsRBD) that works in conjunction with the two dsRBDs of DGCR8 to provide optimum pri-miRNA binding and cleavage activity. Pri-miRNA has a characteristic hairpin structure consisting of 33-35 base-pairs (encoding portion of the miRNA), a terminal loop, and two single stranded RNA segments upstream and downstream of the hairpin

(5’ and 3’) that is precisely recognised by Drosha and DGCR-8/Pasha complex near the hairpin base. Drosha cuts the 3’ strand of pri-miRNA stem by first RIIID (RIIIDa), whereas second RIID (RIIIDb) cuts the 5’ strand of pri-miRNA (Blaszczyk, et al. 2001; Han, et al. 2004) whereas DGCR8 provides stability to pri-miRNA and function as molecular ruler to support precise cleavage, thus producing the 70-90 nucleotides hairpin shaped stem-loop precursor-miRNA (pre-miRNA) with a staggered 2 nucleotide long 3’ overhang end (Han, et al. 2006; Yeom, et al. 2006). Although majority of pre-miRNAs are generated by Drosha/DGCR8 pathway, however, researchers have proposed that some short intron-derived miRNAs (mitrons) can be processed into pre-miRNA through splicing and lariat debranching enzyme action thus bypassing Drosha/DGCR8 pathway (Okamura, et al. 2007).

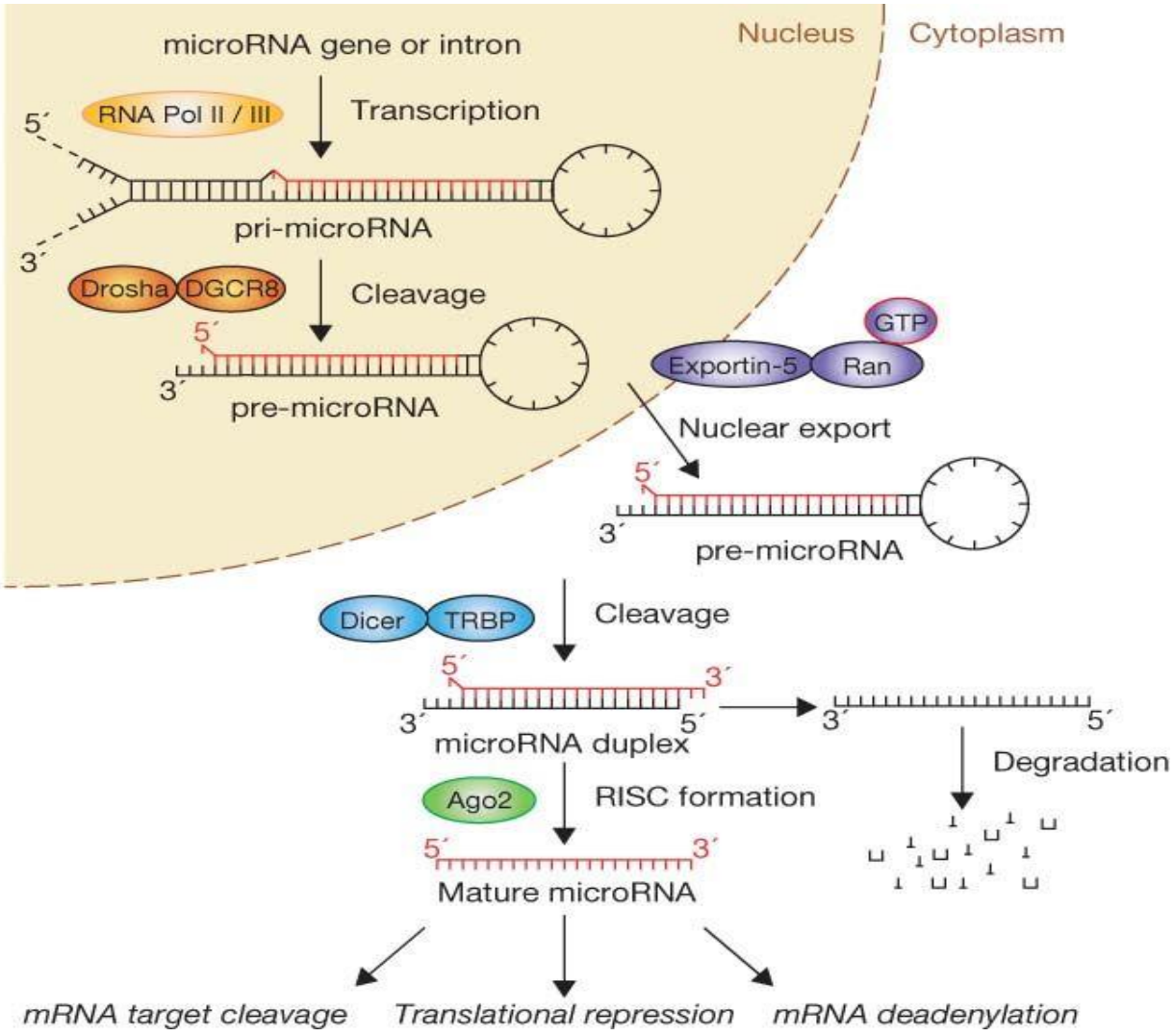


Figure 8. Biogenesis of miRNAs

miRNAs are transcribed in nucleus by RNA Polymerase II/III into pti-miRNAs which subsequently processed into pre-miRNAs. Pre-miRNAs are exported from nucleus by exportin-5 into cytoplasm where they mature into miRNA and incorporate with RISC to perform their action. (Winter, et al. 2009)

Regulation of this microprocessor (Drosha/DGCR8 complex) activity is very crucial in determining miRNA expression level, specificity and abundance. Multiple mechanisms have been identified to control Drosha expression and function. An important one is the autoregulation process between Drosha and DRCG8. At optimum levels of DGCR8, it stabilized Drosha through protein-protein interaction, but under higher concentration of DGCR8, Drosha cleaves the DGCR8 mRNA at a hairpin in the second exon thus stabilizing DGCR8 levels and maintaining homeostasis for microprocessor activity. Other notable mediators of Drosha/DGCR8 activity are post translational modifications (phosphorylation and acetylation) histone deacetylation of DGCR8, MECP2, p68, p72, p68 and SMAD proteins, all of them can promote microprocessor activity.

After nuclear processing, the pre-miRNA is transported from nucleus into cytoplasm by a nuclear pore transport complex consisting of protein, 'Exportin 5' (XPO5) along with GTP-binding nuclear protein RAN-GTP. Once translocated, GTP is hydrolysed, resulting in the disassembly of the complex and release of pre-miRNA in the cytosol (Ha and Kim 2014; Lund, et al. 2004; Yi, et al. 2003). Knockdown of Exportin 5 (XPO5) resulted in reduction in mature miRNA levels without pre-miRNA nuclear accumulation, indicating that Exportin 5 also protects pre-miRNA from nucleolytic activity in nucleus (Yi, et al. 2003).

In cytoplasm, pre-miRNA is further processed by another RNase III family endonuclease enzyme Dicer. About 200kDa in size, Dicer recognizes the pre-miRNA by interactions between its N-terminal helicase domain and the terminal loop of pre-miRNAs and exerts its catalytic activity through its C-terminal tandem RNase III domain (Tsutsumi, et al. 2011; Zhang, et al. 2004). Dicer forms a complex with its double stranded RNA-binding domain proteins called trans-activator RNA binding protein (TRBP) to cleave the pre-miRNA into 22-nt double stranded mature miRNA (Bartel 2004). In some cases the double stranded RNA binding domain cofactor PACT (protein activator of PKR) can also bind with mammalian dicer but its specific role is still unknown.

Although Dicer does not require TRBP and PACT for its pre-miRNA specific cleavage activity, they facilitate and stabilize the cleavage process (Haase, et al. 2005; Lee, et al. 2006c).

After the cleavage of pre-miRNA into a mature miRNA duplex by Dicer, the next step is it's loading into RNA-induced silencing complex (RISC) and subsequent unwinding. RISC assembly is preceded under the control of RISC loading complex (RLC), which consists of Dicer, TRBP, PACT and members of Argonaut proteins (AGO). Although 4 homologues of Ago proteins are expressed in humans (AGO1-4) with all of them capable of inducing translational repression and mRNA degradation, only AGO2 is regarded as most important member in RISC formation and maintenance as well as miRNA directed mRNA cleavage. During RISC formation, the two strands of miRNA duplex subsequently unwound by helicases (p68, p72 RNA helicase A, Gemin ¾ and human Mov10) and AGO-2 into functional guide strand, which is complimentary to the target and the passenger strand, which is subsequently degraded by Argonaute proteins (Okamura, et al. 2004). Although miRNA duplex can give rise to two different mature miRNAs, only one strand incorporates into RISC and performs specific gene regulation. Relative thermodynamic stability/ difference between the 5'end of the two miRNA strands determines the fate by allowing less stable strand to be selected as guide strand and to incorporate into RISC (Khvorova, et al. 2003; Schwarz, et al. 2003). Another determinant of strand choice may be the 1st nucleotide sequence in both strands as AGO proteins select strands with 'U' at nucleotide position 1 as guide strand.

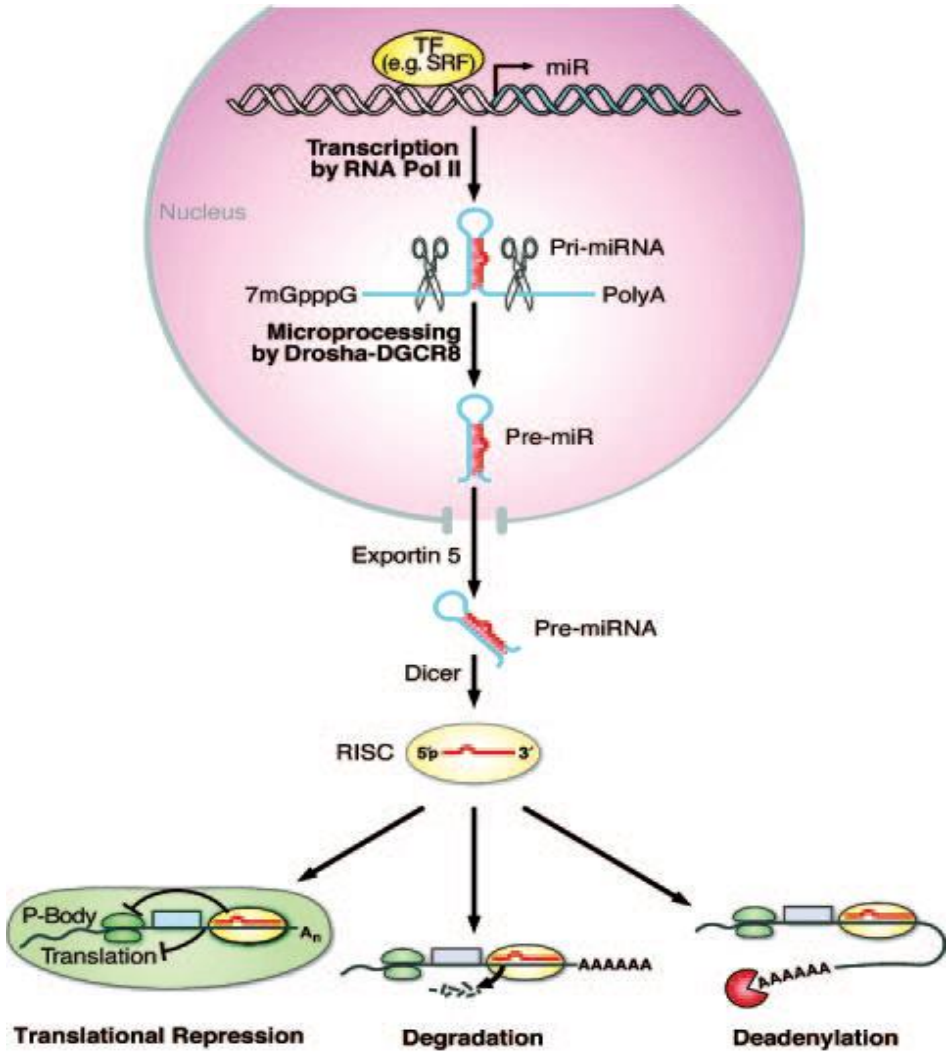


Figure 9. Schematic description of microRNA biogenesis and target prediction.
Adapted from (Zhao and Srivastava 2007)

2.7.3 MircoRNA Target Regulation.

A single mature miRNA can regulate multiple cellular functions by recognizing, binding and inhibiting expression of multiple mRNAs. Interactions between mature miRNA and mRNA are mediated by RISC. As mentioned earlier, RISC is multi-protein complex with Argonaut proteins as essential structural and functional constituents, performing the crucial role of mRNA degradation. 5' UTR end of the miRNA binds to the 3' UTR of their target mRNA in a Watson-Crick base-pairing pattern at the seed region (between base 2 and 8) resulting in repression of mRNA expression by

several mechanisms. A precise complementarity between miRNA seed sequence and 3'UTR of mRNAs results in RNA cleavage by members of Argonaut protein family and subsequent degradation by ribonucleases. A less degree of complementarity results in repression of mRNA translation at ribosomal level, de-adenylation, or accumulation within P bodies (Figure 8)(Filipowicz 2005; Pillai, et al. 2005; Wu, et al. 2006). The ability to repress gene expression by even imperfect base pairing renders the enormous regulatory potential of any given miRNA. Apart from their well established role as translation repression agents, researchers have recently revealed that miRNA can also bind to the 5'UTR of mRNA and therefore can upregulate gene expression (Vasudevan, et al. 2007). Interactions between miRNA 369-3, AGO2, fragile X mental retardation related protein 1 (FXR1) and AU-rich elements (AREs) within the 3'UTR of target gene are imperative for such activation in HEK293 cells. Tili *et al* (2007) has reported an enhanced TNFα translation in macrophages under the influence of miR-155. Other researchers have also reported such upregulatory activities involving multiple miRNAs (miR-373, miR-744) under specific conditions (Huang, et al. 2012; Place, et al. 2008; Tili, et al. 2007). Another characteristic of miRNA is their tissue specificity, which highlights their importance in cell/tissue differentiation and development.

2.7.4 MiRNA and Target Prediction.

As aforementioned, single miRNA can regulate multiple mRNAs and subsequent biological processes, so defining miRNA target genes and their specific regulations is imperative in all miRNA studies. Furthermore, validated targets also help in determining the specificity and efficacy of certain miRNA (mimic or inhibitor) as potential biomarker in clinical therapies. Several techniques are applied in determining/predicting the best suitable targets for specific miRNAs; among those techniques are bioinformatics algorithmic target prediction tools, proteomics analysis, and miRNA/mRNA interactions.

Bioinformatics tools (computational algorithmic models) are commonly used as they give us a lot of information about predicted targets of miRNAs by analysing their seed region (first 2-8 nucleotides) located at their 5' terminus and its complementarity with 3' terminus of possible mRNAs using standard Watson-Crick rules. A number of additional additive approaches are implemented to improve accuracy of target gene prediction and to eliminate possibility of false positive. Most popular target prediction algorithmic tools used in our study were miRanda, TargetScan and PicTar.

Our 2nd approach was to use quantitative proteomics analysis (Mass Spectrometry) to directly observe the influence of miRNA overexpression using whole cell lysate and to predict target proteins by upregulation or downregulation of their expression. Although, as expected, many proteins were downregulated validating miRNA's role as post transcriptional regulator, there are a number of proteins whom expression was upregulated, indicating a role of miRNA's in modulating epigenetic regulators and transcription factors.

Gene specific experiments are commonly employed to validate individual miRNA/mRNA interactions and subsequent targets. These includes series of well-established methods like RT-qPCR, luciferase reporter analysis and western blot to verify both gene and protein expression. RT-qPCR and Western blot analysis reveal up-regulation/down-regulation of target gene (mRNA expression) and protein levels under the influence of given miRNA, whereas luciferase reporter assay provides a direct evidence of miRNA activity whereby the activity of 3'UTR of luciferase reporter is altered under the effect of a particular miRNA. Further validation of miRNA activity can be assessed by introducing binding site mutations in luciferase reporter to better understand and validate the specific binding sites required for miRNA-mediated target gene regulation.

In addition to above mentioned techniques, unbiased biochemical target identification methods are also employed to identify miRNA targets. Microarray analysis or next generation of RNA

sequencing is used to identify target RNAs by immunoprecipitating RISC with anti-Argonaute antibodies, with or without prior RNA crosslinking.

2.8 Role of microRNAs in Atherosclerosis and CVD

MiRNA biogenesis is regulated by Dicer, so researchers have develop dicer deficient mice model to evaluate the role of miRNAs during development and disease progression by conducting loss of function experiments. Dicer knockout in vascular smooth muscle attenuates SMC proliferation and differentiation which resulted in haemorrhage, thinner vessel walls, and impaired contractility (Albinsson, et al. 2010; Yang, et al. 2005a), suggesting a critical role of miRNAs in VSMC functions and CVDs. In this aspect miR-1 and miR-133 are well documented for their role in cardiac myogenesis during embryonic development, where miR-1 cardiac specific over-expression inhibits cardiomyocyte proliferation whereas miR-133 knockdown results in cardiac hypertrophy (Carè, et al. 2007; Zhao, et al. 2005). Additionally, miR-155 was also reported to down-regulate enhanced angiotensin II type 1 receptor activity in VSMCs which is associated with CVDs (Martin, et al. 2007).

2.8.1 Role of MicroRNA in Endothelial Function:

As aforementioned, endothelial integrity plays a pivotal role in pathophysiology of atherosclerosis and neointima development. Endothelial dysfunction can be characterized by many features such as reduced NO bioavailability, increased levels of pro-inflammatory mediators, enhanced expression of adhesion molecules, and oxidative stress. Being regarded as master regulator, role of miRNAs cannot be overlooked in case of endothelial functionality,

MiRNA	Target	Function	Reference
MiR-21	MAPK	VSMCs	(Thum, et al. 2008)
miR-33	ABCA1, ABCG1	Cholesterol efflux, Macrophages,	(Rayner, et al. 2010)
miR-34a	Sirt-1	EC senescence	(Chen, et al. 2015d)
miR-125a	ET-1, ORP9	Regulation of Endothelial function	(Chen, et al. 2009) (Li, et al. 2010a)
miR-126	VCAM-1, Dlk1	Regulation of Monocyte adhesion	(Zernecke, et al. 2009)
miR-133		Cardiomyocytes	(Carè, et al. 2007)
miR-146	TRAF-6, NFkB	EC, Macrophages	(Li, et al. 2015a)
miR-155	Angiotensin type II receptor, eNOS	VSMCs, Macrophages	(Martin, et al. 2007)
miR-92a	KLF4	Angiogemesis, EC	(Fang and Davies 2012)
miR-200	VEGF	Endothelail angiogenesis	(Choi, et al. 2011)

Table 1: Brief list of miRNAs involved in progeression of atherosclerosis

Hence, it is not surprising that investigators have reported a number of miRNAs actively involved in modulating endothelial function. MiR-34a is highly expressed in ECs and its overexpression results in EC senescence and a reduction in cell proliferation mainly due to inhibition of SirT1 protein expression (Chen, et al. 2015c). miR-125a-5p and 125b-5p target and downregulate endothelin-1 (ET-1) gene responsible for deregulation of endothelial function thus protecting against development of atherosclerosis (Li, et al. 2010a). miR-126 is regarded as EC specific miRNA. It exerts anti atherosclerotic properties by regulating monocyte/macrophage adhesion by targeting VCAM-1, thus reducing apoptotic cell content and lesion size (Zernecke, et al. 2009). miR-200 can reduce protein levels of vascular endothelial growth factor (VEGF) resulting in an inhibition of endothelial angiogenesis (Choi, et al. 2011). Finding from our group also revealed that miR-150 and miR-200c play an important role in human endothelial lineage specification and chick embryonic vasculogenesis by targeting ZEB1 (Luo, et al. 2013).

2.8.2 Role of microRNA in macrophage movement:

Macrophages evolved from monocytes under the effect of certain cytokines are indispensable components of innate immunity and provide defence against inflammation, however, formation of lipid laden foam cells from macrophage is a key step in the pathogenesis of atherosclerosis. By using miRNA microarray, Chen et al found that miR-9, miR-146a and miR-125a-5p were upregulated in ox-LDL stimulated monocytes (Chen, et al. 2009). Antagonizing miR-155 in macrophages results in enhanced lipid uptake and increased expression of scavenger receptors indicating a regulatory role of miR-155 towards inflammatory response (Teng and Papavasiliou 2009).

2.9 MicroRNAs regulation of Vascular Smooth Muscle Cells:

VSMCs hold a central role in paradigm of atherosclerosis and neointima formation. The ability of plasticity impart them prime importance in neointima and plaque development. Under normal

conditions, differentiated VSMCs comprising blood vessels are spindle-shaped showing low rate of proliferation, migration and production of ECM, but high level of contractile gene expression. Whereas under pathological conditions evolving due to the loss of ECs resulting from angioplasty (stenting), mechanical forces like hemodynamic sheer stress, cellular apoptosis or/and inflammation, VSMCs undergo phenotype switch and attain a rhomboid shaped phenotype accompanied by high rate of proliferation, migration and excessive production of ECM along with blunted/decreased expression of contractile genes/proteins. Such proliferatory and migratory VSMC are termed as dedifferentiated or synthetic VSMCs. As differentiation and phenotype switching of VSMCs holds prime importance in development of CVDs hence it has been tightly regulated through a complex, multilayered combination of factors involving DNA-binding transcription factors e.g., SRF (serum response factor), Cofactors of DNA binding proteins e.g., Myocardin (MyoCD) and myocardin-related transcription factor A and B (MRTF-A/B), ETS-like protein-1 (Elk-1), KLF4 (Kruppel like factor-4), DNA and histone modifications within promoters chromatin (H4 acetylation at CArG box) and direct interactions of DNA and transcription factors complexes, e.g. SRF/CArG interactions (Kawai-Kowase and Owens 2007; McDonald and Owens 2007). Interestingly, VSMC genes responsible for migration, proliferation, secretion of contractile proteins and production of extracellular matrix have an evolutionary conserved CArG box [$CC(A/T)_6GG$] DNA sequene located within their promoter region (Miano 2003; Sun, et al. 2006). Myocardin is regarded as most potent SRF transcriptional coactivator as it physically associates with SRF and forms a ternary complex with CArG DNA (Spiegelman and Heinrich 2004; Wang and Olson 2004). SRF binding to this CArG box with the help of its cofactors myocardin and myocardin related transcription factor (MRTF-A/B) results in activation of VSMC contractile gene expression and cell differentiation, whereas under the effect of growth mediators/signals, Elk-1

displaces myocardin from its docking site on SRF resulting in suppression of SMC gene expression (Figure 9)(Buchwalter, et al. 2004; McDonald, et al. 2006; Wang, et al. 2004).

Furthermore, PDGF-BB inhibits VSMCs differentiation and promotes cell proliferation and migration by upregulating KLF-4 production. KLF4 represses VSMC differentiation marker genes by inhibition of mycardin expression and suppression of SRF/myocardin induced activation of VSMC contractile gene promoters (Liu, et al. 2003; Liu, et al. 2005). VSMC gene expression is also influenced by histone modifications (acetylation and methylation) as they regulate multiple functions including chromatin remodelling and transcriptional regulation (Cao, et al. 2005; Nie, et al. 2003; Qiu and Li 2002).

During development, SRF binding to the chromatin is also regulated by histone acetylation/deacetylation within the CArG box chromatin of VSMC genes, hence effecting VSMC differentiation process (Bhaumik, et al. 2007). For example, H4 acetylation facilitates SRF access to CArG box DNA by opening up the chromatin structure (McDonald, et al. 2006), whereas, H4 deacetylase dismantle the association of SRF and CArG box DNA by promoting the release of SRF from the methylated histones and CArG box chromatin, thus repressing VSMC gene expression (Yoshida, et al. 2008a; Yoshida, et al. 2008b).

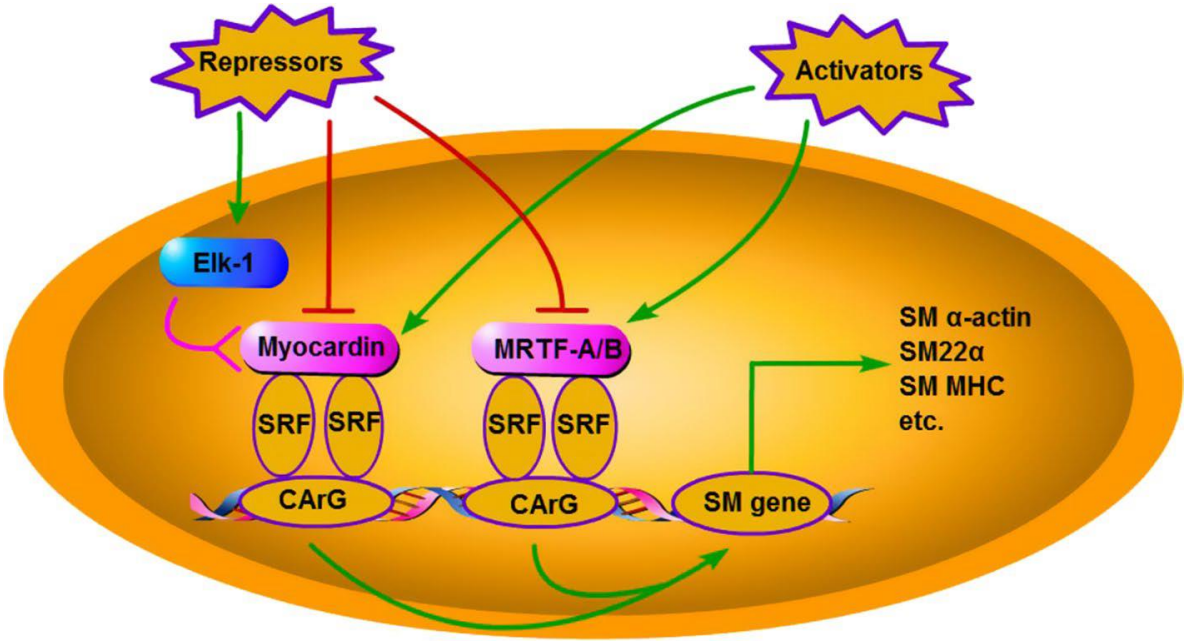


Figure 10. Formation of SRF-CArG-myocardin complex and maintenance of contractile state of VSMCs.

SRF upon activation by MyoCD and MRTF-A/B forms complex with CArG DNA box located within the promoter region of contractile genes. Contrary to this, Elk-1 represses SMC gene expression by competing and displacing the myocardin from the common docking site on SRF in response of growth signals. Activation or suppression of CArG-SRF-myocardin complex regulates many biochemical factor and cytokines associated with VSMC phenotype switching. (Zhang, et al. 2016b)

MiRNA's represents a new regulatory layer providing post transcriptional regulation of gene expression thus controlling numerous physiological and pathological conditions under varying environmental and metabolic stimuli. Several miRNA's are involved in regulation of the VSMC gene expression, differentiation, cell proliferation, migration and apoptosis by fine-tuning protein levels post transcriptionally (Ji, et al. 2007; Kuehbacher, et al. 2007; Lin, et al. 2009).

Albinsson *et al* in 2010, provided a direct evidence of the involvement of miRNA in regulation of VSMC differentiation*in vivo* by using conditional knockout mice for the enzyme Dicer in VSMCs of blood vessels. Deletion of Dicer resulted in dilated, thin-walled blood vessels chracterised by decreased medial thickening mainly due to reduced cellular proliferation and an extensive internal hemorrhage. The contractility of vessels was also reduced due to impaired contractile

ability of mutant VSMCs. Furthermore, Dicer knockdown resulted in late embryonic lethality at E16-17 suggesting that miRNAs are imperative for proper VSMC development and performance.(Albinsson, et al. 2010)

2.9.1 MiRNA and VSMC Differentiation:

Several researchers have reported dynamic changes in the levels of multiple miRNAs during murine Embryonic Stem (ES) cells differentiation to VSMCs (Huang, et al. 2010; Xie, et al. 2011; Yu, et al. 2014; Zhao, et al. 2015), thus postulating an important role of miRNAs during this process. Huang and Xie have reported upregulation in the expression of miR-1 and miR-10a during the differentiation of VSMCs from mouse ES cells, and that their blockade results in reduction in VSMCs differentiation as evident by the percentage of differentiated VSMCs (Huang, et al. 2010; Xie, et al. 2011). Xie had proposed KLF4 repression by miR-1 as possible mechanism determining SMC fate during retinoid acid-induced ESC/SMC differentiation (Xie, et al. 2011). MiR-10a targets histone deacetylase 4 (HDAC4) to mediate its function during retinoid acid-induced SMCs differentiation from ES cells. Interestingly, Huang *et al* have reported a consensus NF-ƙB element in miR-10a gene promoter region and that NF-ƙB binding to its promoter regulates miR-10a expression as they observed that inhibition of NF-ƙB nuclear translocation repressed miR-10a expression and decreased SMC differentiation from ESCs (Huang, et al. 2010).

Importantly, our group has also identified two miRNAs (miR-34a and miR-22) involved in regulation of SMC differentiation from ES cells and adventitial progenitor cells through novel mechanisms. Our data showed that levels of miR-34a and miR-22 were significantly up-regulated during SMC differentiation from ES cells. MiR-22 targets Methyl CpG-binding protein 2 (MECP-2) gene resulting in down-regulation of MECP2 gene expression. MECP2 is known to repress SMC gene expression by modulating multiple SMC transcription factors and thus its repression by miR-

22 is proposed as major mechanism that promotes SMC differentiation from ES cells and adventitial stem/progenitor cells (Zhao, et al. 2015).

In the meantime, Xu has elegantly presented miR-34a as an important mediator for SMC differentiation from pluripotent stem cells by positively regulating its target gene deacetylase sirtuin 1 (SirT1), thus challenging the traditional concept of miRNAs as translational repressor. He observed that miR-34a promoted differentiating stem cells arrest at G0/G1 phase along with significantly reduces incorporation of miR-34a and SirT1 RNA into Ago2-RISC complex upon SMC differentiation. He proposed a positive relationship between miR-34a and SirT1 during SMC differentiation in a cellular context and RNA sequence manner wherein SirT1 acted as transcriptional activator modulating the regulation of SMC genes and cellular differentiation. (Yu, et al. 2014)

2.9.2 MiRNA and VSMC Phenotype Switching:

As aforementioned, that under pathological conditions VSMC undergoes phenotype switching from normal contractile state to synthetic state characterized by increased proliferation, migration, and ECM production. During atherosclerosis and neointimal hyperplasia, synthetic VSMCs constitute the major cellular type in the plaque. Multiple factors exert their effect in dictating this change at the micro-environmental level, including local inflammatory signals, cytokines, intercellular contact and adhesion, ECM interactions, mechanical injury and the effect of PDGF-BB, BMP and TGF-β. PDGF-BB is a potent stimulator of SMCs phenotype switching from differentiated, contractile phenotype to dedifferentiated, synthetic phenotype, thus acting as pro-proliferative agent. Conversely, TGF-β and BMP act as anti-proliferative agent by promoting the contractile, differentiated state of VSMCs through enhanced expression of contractile genes.

Several studies have documented KLF4 as potent repressor of VSMC specific contractile genes by repressing MyoCD levels during VSMC phenotype switch in response to vascular injury, PDGE-BB

or TGF-β. Likewise KLF4, Pim-1 (proviral integration site for Moloney murine leukemia virus 1), an oncogene serine/threonine kinase also induces neointimal hyperplasia by promoting VSMC proliferation. miR-1 targets KLF4 along with Pim-1 to exert its pro-contractile effects on VSMCs, thus regulating the differentiation process. miR-1 levels are significantly higher in contractile and differentiated VSMCs compared to synthetic VSMCs, and downregulated during neointima formation.

TGF-β and BMP play an important role in VSMC phenotype switching by maintaiing VSMCs in contractile state with enhanced expression of contractile genes/proteins. Davis *et al* 2008 have proposed that TGF-β and BMP exerts their effect by enhancing the production of miR-21 which inturn downregulates programmed cell death 4 (PDCD4) which is a negative regulator of SMC contractile genes. TGF-β and BMP promots the processing of pri-miR-21 (primary miRNA) to pre-miR-21 (precursor miRNA) by influencing SMAD signal transducer and hence, regulating Drosha complex (Davis, et al. 2008). Contrary to this, other researchers have proposed a different role of miR-21, by promoting phenotypic modulation and inhibiting differentiation of VSMC, they mark its role as controversial in VSMC phenotype switching. According to those studies, miR-21 work as pro-proliferatory agent as it promotes proliferation, reduced apoptosis and inhibit VSMC differentiation by modulating Sp-1 (Ji, et al. 2007; Yang, et al. 2012).

miR-31 levels are highly expressed in contractile VSMCs, and reduced in proliferative VSMCs induced under the influence of PDGF-BB. miR-31 exerts its effects by suppressing cellular repressor of EIA-stimulated gene (CREG), thus indirectly increasing CREG level and promoting VSMC contractile phenotype (Han, et al. 2008). CREG over-expression promotes VSMC differentiated phenotype whereas shRNA-mediated CREG knockdown results in VSMC dedifferentiation and phenotype switching. miR-133 levels are downregulated in proliferating VSMC but upregulated to normal concentration, once VSMCs are back to contractile state. miR-

133 favours the inhibition of VSMC phenotype switching to synthetic phenotype by inhibiting the transcription factor, specificity protein-1 (Sp-1). Activated Sp-1 is responsible for up-regulation of the KLF4 activity, thus repressing myocardin and subsequently down-regulation of the expression of most of the genes in VSMC (Torella, et al. 2011).

MiR-143/145 are abundantly expressed in VSMCs and several studies have reported a reduction in their levels during VSMCs phenotype switching (Boettger, et al. 2009; Cordes, et al. 2009). Cordes *et al* 2009 has suggested that SRF-MyoCD interactions can directly activate the expression of miR143/145 which was suffiecient to promote VSMC contractile state by enhancing contractile gene expression and subsequently inhibiting the proliferation of cultured VSMCs (Cheng, et al. 2009; Cordes, et al. 2009). miR143/145 knockdown $ApoE^{-/-}$ mice developed spontaneous atherosclerotic lesion in the femoral artery in the absence of hypercholesterolemia. Isolated VSMC from such $miR143/145^{-/-}$ mice showed enhanced migratory activity in response to PDGF-BB compared to wild type VSMCs (Boettger, et al. 2009; Elia, et al. 2009).

miR-145 based therapy to $ApoE^{-/-}$ mice fed on western diet for 12 weeks resulted in significant reduction in plaque size compared to controls, along with an increase in fibrous cap area accompanied by increase in levels of calponin and SMαA. These results mainly attributed to miR-145 mediated reduction in KLF4 levels and elevated MyoCD expression (Lovren, et al. 2012). Studies have revealed multiple targets of miR143/145 including transcription factors like KLF4, KLF5, Elk1, angiotensin converting enzyme (ACE), actin remodelling proteins, and SRF-MyoCD interactions in VSMCs to regulate their contractility and proliferation (Cordes, et al. 2009; Lovren, et al. 2012; Rangrez, et al. 2011).

miR-24 expression levels are upregulated in VSMCs in response to PDGF-BB treatment. Upregulation of miR-24 results in down-regulation of Tribbles-like protein 3 (Trb3) due to its posttranscriptional effect. Trb3 interacts with type-II BMP receptor (BMPRII) and promotes the

degradation of SMAD ubiquitin regulatory factor-1 (Smurf1). Smurf1 is a negative regulator of TGF-β and BMP's SMAD dependent signalling, hence, degradation of Trb3 results in upregulation of Smurf1 and subsequent reduction expression of SMAD proteins leading to inhibition of TGF-β and BMP signalling and enhanced VSMC proliferation and synthetic phenotype (Chan, et al. 2010; Chan, et al. 2007).

miR-26a is also involved in promoting VSMC proliferation, migration and phenotyp switching as its levels are significantly upregulated in synthetic VSMCs. Over-expression of miR-26a targets SMAD-1 and SMAD-4 (two elements of TGF-β and BMP signalling pathways, related to pro-differentiation of VSMCs), resulting in inhibition of differentiation of VSMCs. On the contrary, inhibition of miR-26a promoted VSMC contractile phenotype while inhibiting proliferation and migration (Leeper, et al. 2011).

miR-221 and miR-222 both share a common seed sequence and are significantly upregulated in VSMCs upon PDGF-BB treatment or following balloon injury of the vessel. They regulate multiple functions related to phenotype switching, such as promoting proliferation, migration and inhibiting the expression of contractile genes (Liu, et al. 2009). Knockdown of miR-221 enhanced the expression of VSMC specific markers and consequently inhibiting VSMC migration and proliferation, whereas transfection of exogenous miR-221 results in reduction of VSMC markers and increased VSMC migration and proliferation (Davis, et al. 2009). The effects of miR-221 and miR-222 are attributed to the suppression of multiple target genes including c-Kit, MyoCD, p27 (Kip1), and p57 (Kip2)(Liu, et al. 2012; Tanner, et al. 1998; Yu and Li 2014).

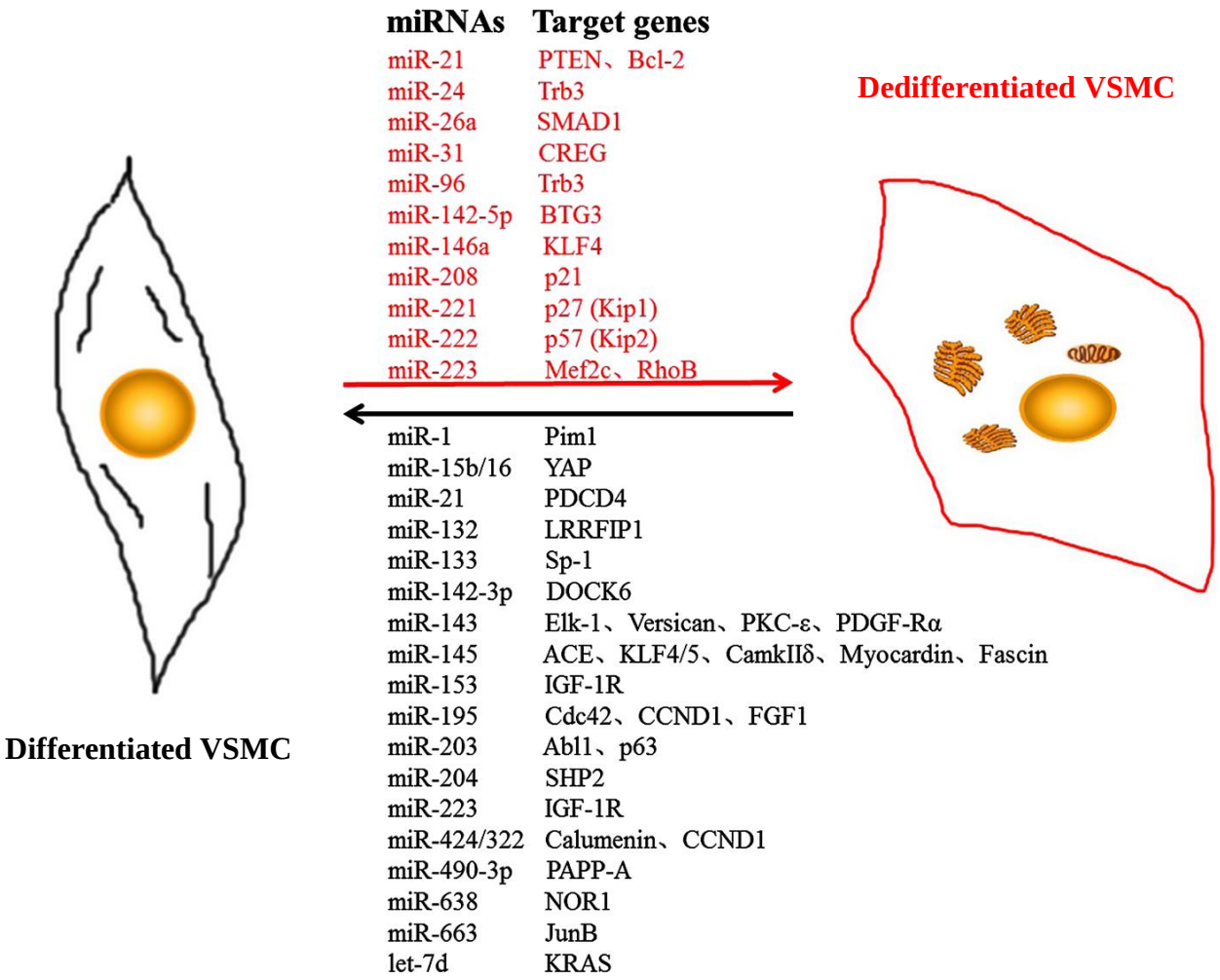


Figure 11. Schematic diagram of VSMC phenotype switching and the role of different miRNAs along with their respective targets.

Schemetic image adapted from (Zhang, et al. 2016b)

2.9.3 MiRNA regulate VSMC Proliferation:

Differentiated VSMCs express higher levels of miR-1 which is significantly down-regulated upon vascular injury and during neointima formation. Myocardin, which is a strong inhibitor of VSMC proliferation, also induce miR-1 expression in VSMCs. miR-1 inhibits the VSMC proliferation by targeting Pim-1, which encodes an oncogenic serine/threonine kinase, necessary for SMC proliferation and neointima formation after vessel injury (Chen, et al. 2011a; Katakami, et al. 2004).

Zheng *et al* 2013 reported that KLF4 significantly upregulated the miR-15a expression in VSMCs, and conversely, KLF4 depletion resulted in reduction in the miR-15a expression leading to subsequent inhibition or activation of VSMC proliferation. Hence, miR-15a serves as a direct transcriptional target of KLF4 that mediates the anti-proliferative and anti-angiogenic actions of KLF4 (Zheng, et al. 2013). miR-133 has pronounced role in VSMC phenotype switching during atherosclerosis and neointima formation following vascular injury. Its over-expression results in reduction in VSMC proliferation and migration, whereas its inhibition results in an increase in VSMC proliferation and migration. Transcription factor specificity protein-1 (Sp-1) is proposed as specific target of miR-133 to perform its inhibitory action (Torella, et al. 2011).

As aforementioned, several studies have mentioned the important role of miR-143/145 in regulating the phenotypic modulation of VSMCs. Their expression is down-regulated in VSMCs following PDGF-BB treatment and after vascular injury. KLF5 and its downstream signaling molecule, myocardin, are suggested to be the possible targets of miR-145 mediated phenotype modulation. Neointima formation in balloon-injured rat carotid artery was blunted after restoration of miR-145 via adeovirus mediated (Ad-miR-145) gene transfer (Cheng, et al. 2009; Cordes, et al. 2009).

Reduced expression of miR-152 was observed when VSMCs were treated with lipopolysaccharide (LPS). VSMC proliferation (following LPS treatment) was inhibited by over-expression of miR-152. DNA methyltransferase 1 (DNMT1) is the most abundant DNA methyltransferase in mammalian cells responsible for maintenance of hemimethylated DNA during DNA replication leading to cell proliferation. MiR-152 exerts its action by down-regulating DNA methyltransferase 1 (DNMT1) and subsequent decrease in the methylation of estrogen receptor α (ERα) gene promoter region leading to reduced cell proliferation (Li, et al. 1992; Wang, et al. 2012a).

VSMCs interactions with collagen are crucial for their migration, proliferation and stability of atherosclerotic plaque. Over-expression of miR-181a inhibits the adhesion of VSMCs with the collagen following Angiotensin-II (Ang-II) treatment. Ang-II mediated osteopontin (OPN) expression (pro inflammatory marker) was also inhibited by over-expression of miR-181a (Remus, et al. 2013).

Cdc42, cyclin D1 (CCND1) and fibroblast growth factor 1 (FGF1) are functionally related genes and are imperative in modulating cell cycle, cell migration and proliferation. Cdc42 is downstream effector of phosphoinositide-3-kinase (PI3K) and can induce CCND1 and FGF1 expression in VSMCs resulting in enhanced migration and proliferation. Over-expression of miR-195 significantly down-regulates the expressions of Cdc42, FGF1 and CCND1 thus inhibiting the VSMC phenotype switching and reducing neointimal hyperplasia (Chotani, et al. 2000; Hanna, et al. 1997; Wang, et al. 2012b). Moreover, miR-195 also inhibits ox-LDL induced VSMC proliferation, migration, and synthesis of inflammatory mediators like IL-1β and IL-6 leading to reduction in neointima hyperplasia (Wang, et al. 2012b). The expression levels of miR-424 (ortholog of rat miR-322) were observed to be upregulated in proliferative VSMCs *in vitro* and following vascular injury. Increased levels of miR-424/322 inhibit VSMC proliferation and reduce neointima

formation after balloon-artery injury by targeting and down-regulating cyclin D1 and calumenin protein levels (Merlet, et al. 2013).

Human atherosclerotic plaques and VSMCs treated with ox-LDL shows significant reduction in the expression levels of miR-490-3p, whereas pregnancy-associated plasma protein A (PAPP-A) and insulin like growth factor (IGF2) expression were up-regulated. VSMCs are enriched with PAPP-A which can act as activator of insulin like growth factor (IGF) and IGF-binding protein-4 (IGFBP-4) leading to VSMC proliferation (Sun, et al. 2013). miR-490-3p mimics inhibited the upregulation of PAPP-A induced by ox-LDL, and subsequently suppressing the matrix metaloprotease effect of PAPP-A on IGFBP-4 leading to inhibition of VSMC proliferation.

Lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) upon activation by ox-LDL can initiate multiple inflammatory signals including superoxided radical formation and upregulation of endothelial pro-inflammatory genes. All these inflammatory stimuli stimulate VSMC proliferation and migration into atherosclerotic plaque and participate in neointima formation. Abnormally low levels of let-7g were observed in serum of human subjects suffering from hypercholesterolemia as well as mice fed on western diet. Over expression of let-7g by transfecting it into VSMCs targets LOX-1 which resulted in significant reduction in VSMC proliferation and migration (Chen, et al. 2011b).

Expression of miR-21 was significantly upregulated in synthetic VSMCs compared to differentiated ones and a similar pattern was observed in the VSMCs from vessel wall after balloon injury. Inhibition of miR-21 resulted in decreased cell proliferation and increased cell apoptosis in cultured VSMCs as well as in balloon-injured rat carotid arteries. PTEN and Bcl-2 are proposed to be the major target of miR-21 to mediate such cellular effects. (Ji, et al. 2007; Yang, et al. 2012).

miR-26a promotes VSMC proliferation and inhibits cellular differentiation by influencing TGF-β signalling. Inhibition of SMAD proteins is being the major underlying mechanism. Over-expression of miR-26a inhibitd gene expression of SMAD-1 whereas expression of SMAD-1 and SMAD-4 were upregulated upon miR-26a inhibition (Leeper, et al. 2011).

In addition to its role in phenotypic modulation of VSMCs, miR-31 is also involved in regulating VSMCs proliferation. The expression of miR-31 is significantly upregulated in proliferative VSMCs. Large tumour suppressor homolog 2 (LATS2) is proposed to be the target of miR-31 to perform its pro-proliferative role. LATS2 is an important regulator of tissue development and plays a major role in cellular proliferation and apoptosis. Inhibition of miR-31 results in downregulation of LATS2 activity leading to reduction in VSMC proliferation ability.(Liu, et al. 2011b)

Arteries of hypertensive individuals show upregulation of miR-130 expression which is thought to be involved in promoting the proliferation of VSMCs through its effect on growth arrest-specific homeobox (GAX). Several factors involved in initiation and propagation of vascular disease including balloon injury, hypertension, Ang-II and PDGF downregulate GAX expression. GAX has potent inhibitory effect on VSMCs proliferation, migration and differentiation and its down regulation by miR-130 significantly enhanced the proliferatory and migratory capacity of VSMCs (Saito, et al. 2005; Wu, et al. 2011; Xia, et al. 2011).

miR-146a is another pro-proliferative miRNA whose expression is upregulated in atherosclerotic vessels compared to normal controlarteries. miR-146a exerts its proliferatory effects by targeting KLF4 in VSMCs. Overexpression of miR-146a decreases KLF4 level, whereas silencing of miR-146a in VSMCs increases KLF4 expression. KLF4 is postulated to express its anti-proliferative effects by upregulating p21, which is a member of cyclin-dependent kinase (CDK) inhibitory protein family. miR-146a mediated inhibition of KLF4 results in reduction in p21 levels leading to blunted inhibitory response and subsequently increased proliferation of VSMCs. Sun *et al* 2011 had

reported inhibition of VSMC proliferation and migration resulting in significant decrease in neointimal hyperplasia with the transfection of antisense miR-146a oligonucleotide into balloon-injured rat carotid arteries (Sun, et al. 2011).

miR-221/222 play an important role in VSMC phenotype switching by regulating multiple functions ranging from promotion of proliferation, and migration to reduction in the expression of contractile genes. Their expression is upregulated in proliferating VSMCs whereas inhibition of miR221/222 results in significant reduction in VSMC proliferation and neointima formation following balloon injury in rat arteries. Down-regulation of p27 and p57 (members of Cyclin dependent kinase inhibitors (CKI) involved in inhibition of cell proliferation) is proposed to be the major mechanism by which miR221/222 regulate and enhance VSMCs proliferation. (Liu, et al. 2012; Liu, et al. 2009)

Function	miRNAs	Targets	Reference
Inhibits proliferation	miR-1	Pim-1	(Katakami, et al. 2004)
	miR-15a	KLF4	(Zheng, et al. 2013)
	miR-133	Sp-1	(Torella, et al. 2011)
	miR-143/145	KLF5, ELK1, MyoCD	(Cheng, et al. 2009; Cordes, et al. 2009)
	miR-152	DNMT1	(Wang, et al. 2012a)
	miR-181a	---	(Remus, et al. 2013)
	miR-195	Cdc42, CCND1, FGF1	(Wang, et al. 2012b)
	miR-424/322	Cyclin D1, Calumenin	(Merlet, et al. 2013)
	miR-490-3p	PAPP-A	(Sun, et al. 2013)
	let-7	LOX-1	(Chen, et al. 2011b)
Promotes prolifaration	miR-21	PTEN, Bcl-2	(Ji, et al. 2007)
	miR-26a	SMAD-1/4	(Leeper, et al. 2011)
	miR-31	LATS2	(Liu, et al. 2011b)
	miR-130a	GAX	(Wu, et al. 2011)
	miR-146a	KLF4	(Sun, et al. 2011)
	miR-221/222	PTEN, Bcl-2, p27, p57	(Liu, et al. 2012; Liu, et al. 2009)

Table 2: miRNAs involved in the regulation of VSMC prliferation

2.9.4 MiRNAs regulate VSMC Migration:

Similar to proliferation, VSMC migration also plays paramount role in the progression of atherosclerotic plaque and neointima formation. Following vascular injury, either because of inflammation or mechanical injury (ballooning with or without stenting), the VSMCs migrate from medial layer to intima (to the site of injury) to perform their rehabilitatory role by proliferation, excretion and deposition of extra cellular matrix. VSMC proliferation and migration are closely associated events which normaly appear simultaneously and are regulated by similar growth and/or inflammatory stimuli.

As mentioned earlier, expression levels of miR-133 also influence VSMC proliferation and migration. A reduction in miR-133 levels after vascular injury results in enhanced VSMC proliferation and migration whereas an increase in miR-133 expression levels decrease VSMC migration. Similar effects were observed by antagonizing miR-133 with anti-miR-133 leading to increase in VSMC proliferarion and migration. Suppression of Transcription factor Sp1 is the proposed underlying mechanism behind miR-133's effects.(Torella, et al. 2011)

Podosomes are highly dynamic, actin rich membrane protrusion involved in the migration of several cell types including VSMCs. PDGF is reported as major promoter of podosome formation in VSMCs through Scr and p53 pathways (Gimona, et al. 2003; Linder and Aepfelbacher 2003). Overexpession of miR-143/145 inhibit podosome formation thus reduces VSMC migration, whereas loss of miR-143/145 in vivo and in vitro results in enhanced podosome formation leading to increased migration, prolifearation and neointima formation. PDGF receptor alpha and protein kinase C epsilon are described as miR-143 targets whereas fascin is postulated as miR-145 target (Quintavalle, et al. 2010; Rangrez, et al. 2011).

miR-181a exerts its anti migratory action by inhibiting the adhesion of VSMCs to collagen in response to Ang-II, which is crucial for migration and proliferation (Remus, et al. 2013). miR-195

inhibits the synthesis of pro-inflammatory cytokines such as IL-1β, IL-6 and IL-8 by ox-LDL, thus reducing the inflammatory conditions and suppressing the proliferation and migration of VMSCs (Wang, et al. 2012b). Synthetic VSMCs exhibit significantly down-regulated expression of miR-638. Overexpression of miR-638 downregulate NOR1/cyclin D1 pathway in order to inhibit VSMC migration and proliferation in response to PDGF (Li, et al. 2013). Let-7g is also a potent inhibitor of VSMC migration and proliferation. It exerts its action by inhibiting LOX-1 and OCT-1 expression induced in response to ox-LDL treatment, thus reducing VSMC proliferation and migration (Chen, et al. 2011b).

Function	**miRNAs**	**Targets**	**Reference**
Inhibits migration	MiR-133	Sp-1	(Torella, et al. 2011)
	miR-143/145	KLF5, ELK1, MyoCD	(Quintavalle, et al. 2010; Rangrez, et al. 2011)
	miR-181a	---	(Remus, et al. 2013)
	miR-195	Cdc42, CCND1, FGF1	(Wang, et al. 2012b)
	miR-638	NDR1	(Li, et al. 2013)
	let-7	LOX-1	(Chen, et al. 2011b)
Promotes migration	miR-26a	SMAD1, SMAD4	(Leeper, et al. 2011)
	miR-29b	DNMT3b	(Chen, et al. 2011c)

Table 3: miRNAs involved in VSMC migration

As miR-26a performs multiple functions during VSMC phenotype switching, hence, it regulates VSMC migration as well along with proliferation. Inhibition of miR-26a results in reduction in cellular migration under the influence of inflammatory mediators and/or growth factors (Leeper, et al. 2011). Expression of miR-29b was upregulated when VSMCs were treated with ox-LDL.

Upregulated miR-29b promotes VSMC migration by targeting DNA methyltransferase 3b (DNMT3b) which results in reduction in DNMT3b expression and leading to enhanced oxLDL-induced DNA demethylation levels of MMP-2/MMP-9. Treating VSMCs with antagomiR-29b knockdown miR-29b expression resulting in attenuation of OxLDL-mediated MMP-2/MMP-9 up-regulation, DNMT3b down-regulation, and DNA demethylation (Chen, et al. 2011c; Urbich, et al. 2008).

2.9.5 MiRNA and VSMC Neointima Hyperplasia:

Neointimal hyperplasia is cellular response to repair injured vessels following any inflammatory (vascular disease) or mechanical demage to the artery such as atherosclerosis or after balloon injury (post angioplasty). The process is tightly regulated involving multiple inflammatory stimuli/factors and cell types such as adhesion molecules, ECM proteins, cytokines, chemokines, EC, macrophages and VSMCs. As described earlier, VSMC phenotype switching after injury plays a key role in regulating their movement towards the damaged area and development of neointimal hyperplasia. A number of miRNAs described above modulate VSMC phenotype switching, and control neointiml hyperplasia formation. Microarray analysis indicated high expression of certain miRNAs in normal murine arteries such as miR-21 (Ji, et al. 2007), miR-221/222 (Liu, et al. 2009) and miR-143/145 (Boettger, et al. 2009; Cordes, et al. 2009). As a result of vascular injury the expression profile of these miRNAs changes significantly with miR-21 and miR-221/222 were upregulated whereas miR-145 expression level was downregulated (Cheng, et al. 2009; Liu, et al. 2009). Upregulation of miR-21 and miR-221/222 act as pro-proliferatory stimuli for VSMC and their inhibition resulted in significant reduction in neointimal hyperplasia in rat carotid artery ballon injury model (Ji, et al. 2007; Liu, et al. 2009). Knockdown of miR-221 and miR-222 expression resulted in decrease proliferation of VSMCs (Dentelli, et al. 2010; Liu, et al. 2009). Sun *et al* 2011 had reported similar phenomenon of blunted neointimal hyperplasia

accompanied by inhibition of VSMC migration with the suppression of miR-146a (Sun, et al. 2011).

On the contrary, Feng *et al* 2018 has elegantly presented miR-22 as a crucial regulator of VSMC phenotype switching and neointimal formation (Yang, et al. 2018). He not only observed variations in miR-22 expression levels in wire injury indueced mice model but also employed normal and diseased human femoral artery injury models to explore the therapeutic potential of miR-22. He identified Virus Integration Site 1 Protin Homologue (EVI1) as a novel target of miR-22 and proposed an inverse relationship between the two. He deduced that miR-22 over expression in the injured vessles significantly reduced the expression of its target gene along with decrease in VSMC proliferation and blunted neointima formation in wire injured femoral arteries model. As expected, similar inverse relationship were observed (reduced miR-22 expression whereas increased EVI1 and MECP2 expression) in diseased arteries copared to healthy femoral human arteries. Thus, he proposed over-expression of miR-22 and/or inhibition of EVI1 as possible therapeutic targets for treating vascular disease and neointimal hyperplasia.

miR-34a also plays an important role in regulating VSMC proliferation, migration and neointima formation by targeting Notch homolog 1 (Notch1). Overexpression of miR-34a inhibited VSMCs proliferation and migration by negatively regulating Notch1. Similar results were observed during *in vivo* experiments by over expressing miR-34a perivascular to wire-injurd mice femoral arteries, which resulted in reduction in Notch1 expression, reduced VSMC proliferation and inhibited neointima formation (Chen, et al. 2015d).

Zhang *et al* (2017) has as reports an interesting role of miR-124 and hnRNAP1 in regulating VSMCs functions. hnRNAP1 (heterogeneous nuclear ribonucleoprotein A1) was reported to regulate VSMC specific gene expression, proliferation and migration by inversely regulating IQGAP1 (IQ motif containing GTPase activating protein 1) (Zhang, et al. 2017). Zhang *et al* has

proposed a relationship between hnRNAP1/miR-124 and IQGAP1 where hnRNP1 is upstream of miR-124, involved in promoting its biogenesis and which inturn, targets and degrades IQGAP1 mRNA. In wire-injured carotid arteries, ectopic overexpression of hnRNPA1 results in higher miR-124 expression levels leading to significantly reduced VSMC proliferation and blunted neointima formation (Zhang, et al. 2017).

Cheng *et al* has presented miR-145 as an important mediator in VSMC phenotype switching and neointima formation. According to him, restoring levels of miR-145 using adenoviral mediated gene transfer to the balloon injured arteries, resulted in inhibition of neointima, along with reduction in VSMCs proliferation and migration but an upregulation of VSMC differentiation markers (Cheng, et al. 2009).

In a different experiment settings, by using 18 months old miR-143/145$^{-/-}$ mutant mice, Boettger *et al* 2009 had reported a significant increase in spontaneous neointimal hyperplasia consisting of VSMCs and macrophages along with high deposits of amorphous collagen I in the femoral arteries (Boettger, et al. 2009). Interestingly, contrary to this, Xin *et* al 2009 reported that miR-143/145 deletion (either single or double) resulted in reduced VSMC migration leading to significant reduction in neointimal development (Xin, et al. 2009). The reported difference in their results may be attributed to difference in vascular injury models (mouse vs rat; young vs old) used as well as difference in genetic modification techniques (virus mediated vs systemically modified). Expression profile of several extracellular proteins e.g. elastin, collagens and fibrillins are downregulated by miR-29 hence regulating the stability of atherosclerotic plaque (Chen, et al. 2011c; Latronico and Condorelli 2010).

miR-195 also inhibits the VSMC phenotype switching and reduces neointima formation by down-regulating the expressions of Cdc42, FGF1 and CCND1 (Chotani, et al. 2000; Hanna, et al. 1997; Wang, et al. 2012b). Furthermore, over expression of miR-195 also inhibits ox-LDL mediated

secretion of inflammatory mediators like IL-1β and IL-6, hence leading to reduction in VSMC proliferation, migration, and neointima hyperplasia (Wang, et al. 2012b)

Concluding above discussion and literature review, miRNA came up as prominent mediators of inflammation resulting from atherosclerosis and vascular injury/repair process by regulating multiple steps involving VSMC phenotype switching (contractile or synthetic), proliferation, migration, excretion of ECM proteins and expression of contractile proteins.

Function	miRNAs	Targets	Reference
Inhibits neointima hyperplasia	miR-22	EVI1, MECP2	(Yang, et al. 2018)
	miR-34a	Notch1	(Chen, et al. 2015c)
	miR124	IQGAP1	(Zhang, et al. 2017)
	miR-143/145	KLF5, ELK1, MyoCD	(Cheng, et al. 2009) (Boettger, et al. 2009)
	miR-195	Cdc42, CCND1, FGF1	(Chotani, et al. 2000; Wang, et al. 2012b)
Promotes neointima hyperplasia	miR-21	PTEN, Bcl-2	(Ji, et al. 2007)
	miR-221/222	PTEN, Bcl-2, p27, p57	(Liu, et al. 2009)
	miR-146a	KLF4	(Sun, et al. 2011)

Table 4. miRNAs involved in regulating neointima formation

2.10 MicroRNA 214

miR-214 is regarded as highly conserved miRNA among species indicating its pivotal role in broad range of physiological functions ranging from cell fate decision, differentiation and morphogenesis of muscles and skeleton from precursor cells, nervous system, tumourigenesis to pancreas and cardiac development. miR-214 is a member of miR-199a-214 cluster (which

contains two members miR-199a and miR-214 approximately 6kb apart)(Watanabe, et al. 2008). (Figure 11)

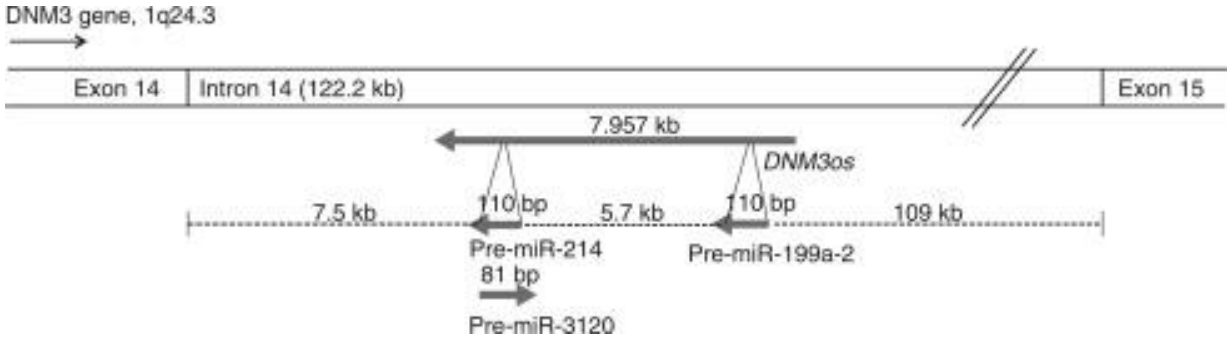


Figure 12. Genomic location of human miR-214.
7.9kb long Non-coding RNA precursor DNM3os carrying miR-214 and miR-199a, 6kb apart, in intron 14 of Dynamin-3 gene. (Adapted from E Penna et al journal of investigative dermatology 2015)(Penna, et al. 2015)

Human gene for miR-214 is located in intron 14 of the Dynamin-3 gene (DNM3) and is encoded into a long (8kb) non-coding RNA named as DNM3os, which is transcribed in the opposite strand of the DNM3 gene (Lee, et al. 2009; Loebel, et al. 2005; Yin, et al. 2010). miR-214 was shown to regulate multiple genes such as Ezh2, P53, Quaking, Sufu, N-RAS, and Ncx1 (Aurora, et al. 2012; Long, et al. 2015; van Mil, et al. 2012; Wang, et al. 2015; Yang, et al. 2013b) as well as various key signalling pathways like β-catenin or tyrosine kinase receptor pathway and PTEN/AKT pathway simultaneously (Gu, et al. 2015; Wang, et al. 2014). Accordingly,many biological process such as regulating mitochondrial morphology and cell cycle, skin and hair follicle development, angiogenesis and differentiation of myoblast cells, dendritic cell activation and regulation of tumour progression growth and suppression are strictly controlled by miR-214 (Ahmed, et al. 2014; Bucha, et al. 2015; Duan, et al. 2015; Gu, et al. 2015; van Mil, et al. 2012).

2.10.1 MicroRNA-214 in Cancer.

As aforementioned, miR-214 has profound role in tumourigenesis and is deregulated in several human tumours including breasts, ovarian, hepatocellular carcinoma and melanoma. miR-214 was first described as an oncogene which regulatestumour growth, stemness, angiogenesis, invasiveness and resistance to chemotherapy, but later studies have also suggesteda divergent role for miR-214 in tumourigenesis since an inhibitory role for miR-214 in cancer cell progression, metastasis, apoptosis and chemoresistancewas reported by various researchers (Gutierrez, et al. 2010; Wang, et al. 2013b; Xia, et al. 2012; Xu, et al. 2012).

Raised expression of miR-214 in pancreatic cancer cells influences ING4 gene (involved in cell cycle arrest, DNA repair and apoptosis) resulting in decrease sensitivity to treatment (Zhang, et al. 2010). miR-214 expression is also up-regulated in lung tumours, nasopharyngeal, stomach and prostate cancer resulting in unfavourable prognosis in overall survival and poor response to therapy (Deng, et al. 2013; Ueda, et al. 2010; Volinia, et al. 2006; Yanaihara, et al. 2006). Contrary to this, down-regulation of miR-214 expression occurs in hepatocellular, bladder and colorectal carcinomas as compared to healthy individuals, resulting in tumour angiogenesis, poor survival, metastasis and malignancy (Chen, et al. 2014; Gutierrez, et al. 2010; Okada, et al. 2015; Ratert, et al. 2013; Wang, et al. 2013b). In hepatocellular carcinoma, overexpressing miR-214 reduces tumour growth and vascularity by targeting hepatoma-derived growth factor (HDGF) (Shih, et al. 2012). Conflicting results have also emerged for miR-214 functionsfrom ovarian cancer studies. Significant deregulations were observed in miR-214 expression level during late-stage and high grade overian cancer tumours. Over expression of miR-214 in ovarian tumours is associated with tumour cell survival and cisplatin resistance through targeting the 3'UTR of PTEN leading to inhibition of PTEN translation and activation of Akt pathway (Yang, et al. 2008). However, miR-214 down-regulation has also been reported in neoplastic ovaries compared to healthy tissue

(Iorio, et al. 2007). Whereas skin cancer is concerned, increased expression of miR-214 in primary malignant cutaneous melanomas and in ocular melanomas is associated with poor prognosis and high metastatic risk (Molnár, et al. 2008; Penna, et al. 2011; Worley, et al. 2008). In squamous cell carcinoma (SCC) of oral cavity and tongue, the levels of miR-214 are up-regulated but is been down-regulated in oesophageal and cutaneous SCC (Yu, et al. 2010).

miR-214 promotes tumour cell proliferation and survival in gastric tumours and nasopharyngeal carcinomas by promoting AKT signalling via PTEN and by targeting lactoferrin and Bim respectively (Deng, et al. 2013; Yang, et al. 2013a; Zhang, et al. 2014). In contrast, miR-214 inhibits hepatic and breast carcinoma proliferation by targeting Ezh2, XBP1 and β-catenin (Derfoul, et al. 2011; Schwarzenbach, et al. 2012; Wang, et al. 2013b). Over-expression of miR-214 down-regulates fibroblast growth factor receptor 1 (FGFR-1) in colorectal and hepatocellular carcinomas and inhibits cancer cell invasion and progression (Chen, et al. 2014; Wang, et al. 2013b).

During tumourigenesis, cell stemness and developmental pathways are re-activated and several studies have highlighted an important role of miR-214 in this scenario. Over-expression of miR-214 results in inhibition of p53 and indirect upregulation of Nanog, leading to increased stemness among ovarian cancer stem cells (Xu, et al. 2012).

Response to treatment (chemoresistance) is another important parameter for cancer treatment. Raised miR-214 levels reduces chemotherapy sensitivity (cisplantin chemoresistance) in tongue squamous cell carcinoma (Yu, et al. 2010) and inhibits cisplatin-induced apoptosis in ovarian cancer by triggering AKT pathway and enhancing cell survival (Yang, et al. 2008). In contrast, another study by Wang et al (2013) found that over-expression of miR-214 inhibits Bcl212 in cervical cancer cells and enhances cisplatin induced cytotoxicity (Wang, et al. 2013a).

To sum up, miR-214 appears as pleiotropic hub that performs contradictory but decisive function during tumorigenesis. Such versatile behaviour of miR-214 depends on a couple of factors. First, as miR-214 modulates several genes by inhibiting specific mRNAs and regulating different transcription factors, so differences in downstream pathways of these players might be the reason of its divergent role. For example, TFAP2 and ALCAM (miR-214 regulated genes) exhibit contrasting behaviour in different cell types. Second, different tumour cells have altered cellular mechanisms and disparities in expression of different molecular and growth factors in distinct tumour cell population may account for these discrepancies in miR-214 action suggesting that the functional implication of miR-214 is cell context dependent.

2.10.2 MicroRNA 214 in CVD

A contradictory but pivotal role of miR-214 (protective and pathological) in cardiovascular disease and in cancer progression has been reported from recent studies. miR-214 is upregulated in response to several factors including cardiac stress, myocardial infarction (MI) and Ca^{2+} overload (van Rooij, et al. 2006; van Rooij, et al. 2008). miR-214 has been reported to protect myocardial cells against excessive Ca^{2+} uptake during ischemia reperfusion (IR) injury by repressing mRNA encoding sodium/calcium exchanger 1 (Ncx1), thus maintaining Ca^{2+} homeostasis and increasing cell survival (Aurora, et al. 2012). In another finding, a decrease in miR-214 levels has been associated with an increase in PLGF (placental growth factor) levels and worsening of atherosclerosis, suggesting its role as a protective agent and promising biomarker for severe CAD (Lu, et al. 2013). It has been proposed that over-expression of miR-214 inhibits left ventricular remodelling and suppress myocardial apoptosis after an acute myocardial infarction via its effect on phosphatase and tensin homolog (PTEN)(Yang, et al. 2016). Study by Lv G et al (2014) has demonstrated its protective role for cardiac myocytes against H_2O_2-induced injury (Lv, et al. 2014). However, in another study by Tao Yang, antagonizing (inhibiting) miRNA

214 is suggested as a new therapeutic approach for treating cardiac hypertrophy. In this study, overexpression of miR-214 resulted in pathological cardiac growth and heart failure, suggesting EZH2 as a proposed target gene for miR-214 mediated cardiac hypertrophy (Yang, et al. 2013b). Aforementioned studies have provided clear evidence that miR-214 plays a role in cardiac hypertrophy, but less is known about its role in regulating VSMC functions and its significance in atherosclerosis or neointima formation.

2.10.3 miR-214 in VSMC differentiation

Data from the microRNA microarray analysis revealed that miR-214 was upregulated during SMC differentiation from Day 0 to Day 8 (Yu, et al. 2014). Our microarray results also identified other miRNAs apart from miR-214 that were upregulated in SMC differentiation from ES cells indicating their specific role at different stages of differentiation and proliferation. miRNAs such as miR-143, miR-145, and miR-133 (which are involved in SMC differentiation), were upregulated in the early stage of SMC differentiation (Day 4) when compared with their expression in undifferentiated ES cells (Day 0). In contrast, miR-21, which has profound role in SMC proliferation, was undetectable at the early stage of differentiation (Day 4) but upregulated at the late stage (Day 8). No significant changes were observed in levels of miRNAs specific for other cell lineages, for example, miR-146a (for T lymphocytes), miR-203 (for epidermal cells), miR-126 (for endothelial cells), miR-206 (for myogenic cells) and miR-124 (for neural cells) as they were increased at the early stage of differentiation and undetectable or downregulated at the late stage. The summary of upregulated and downregulated miRNAs is shown in following table 4.

Name	Fluorescence signal		Reported functions	Reference
	Day4/day0	Day8/day0		
miR-290-5P	0.775761	0.286816	ES cell specific	Houbaviy et al(2003)
miR-291a-5P	0.925415	0.271932	ES cell specific	Houbaviy et al(2003)
miR-292-3P	1.427017	0.468515	ES cell specific	Houbaviy et al(2003)
miR-293	1.18702	0.411204	ES cell specific	Houbaviy et al(2003)
miR-294	1.146818	1.367687	ES cell specific	Houbaviy et al(2003)
miR-295	1.062308	0.265621	ES cell specific	Houbaviy et al(2003)
miR-143	1.470071	1.237568	SMC differentiation	Cordes et al(2009)
miR-145	2.113956	1.460188	SMC differentiation	Cordes et al(2009)
miR-21	UD	9.848351	SMC proliferation	Ji et al(2007)
miR-22	2.20227	11.205712	Tumour suppressor	Xiong et al(2010)
miR-24	0.987122	3.597099	Heart contraction and myoblast differentiation	Li et al (2013b), Sun et al(2008)
miR-214	**3.451106**	**2.024559**	**Muscle cell specification and myogenesis**	**Flynt et al(2007), Liu et al(2010)**
miR-146a	1.225387	UD	T lymphopoiesis	Georgantas et al(2007)
miR-16	0.668986	1.187912	Universal expression	Landgraf et al (2007)
miR-107	0.754878	0.861935	Monocytopoiesis	Georgantas et al(2007)
miR-203	1.103917	UD	Epidermal differentiation	Yi et al(2008)
miR-206	1.563997	1.063436	Myogenic cells	Rao et al(2006), Kim et al(2006)

miR-124	0.707948	0.458512	Brain specific	Landgraf et al (2007)
miR-126-3P	1.455875	UD	Endothelial cells	Fish et al(2008)

Table 5. Selected miRNA expression profile during SMC differentiation from Embryonic stem cells.

In another study, assessing miR-214's role in VSMC differentiation from ESCs, Wu *et al* 2017 postulated suppression of quaking (QKI) expression by miR-214 as major underlying mechanism to regulate VSMCs gene expression during VSMCs differentiation from ESCs. The authors demonstrated that overexpression of QKI down-regulated the expression of SRF, MEF2C and Myocd through transcriptional repression and direct binding to promoters of the SRF, MEF2c and Myocd genes, thus inhibiting VSMC contractile gene expression. Whereas, QKI inhibition by miR-214 resulted in upregulation of SRF, MEF2c and Myocd gene expression followed by VSMC differentiation, thus presenting QKI as a functional modulating target in miR-214 mediated VSMC differentiation from ESCs (Wu, et al. 2017)

2.11 NCKAP1 (Nck associated protein 1).

Cells exhibit astonishing diversity in their shape depending upon their function, for example, neurons forms long branching network to communicate and perform functions; ECs are strictly bound to each other to provide a barrier between blood and neighbouring tissues; Blood and immune cells display ever changing shapes from round to elliptical. At time, cells change their normal shape to adjust and address the dynamic conditions (pathogen, injury or metabolic disorder) in microenvironment. This change in shape enables them to move from one place to another under the influence of certain inflammatory or pathological stimuli (immune cells crawl to catch the pathogen, VSMCs migrate to the intimal layer in atherosclerosis). Cell migration is a

fundamental property of cells and is important in maintaining homeostasis (embryonic development, wound healing) under normal conditions but if it's not tightly controlled, it allows cells to invade nearby tissue and hinder normal functionality as in cancer and neointima formation.

The shape and movement of cells is under the control of their internal framework called cytoskeleton. A highly specialized and dynamic protein called actin is responsible for these changes in cytoskeleton through its reversible polymerization and hence regulate cellular migration. In its globular, monomeric form, actin is soluble but upon polymerization, it forms insoluble filaments with significant mechanical strength to maintain cellular structure. Actin polymerization involves assembly of three actin monomers into a trimmer and is triggered by several factors that stimulate this 'nucleation'. Under certain conditions, when cells need to migrate, these actin filaments spread at the front of the cell pushing its membrane forwards in a sheet-like structure (lamellipodium) or finger like protrusions (filopodia). These cell protrusions forms adhesions at the cellular front and when coordinated with contractions at its tail end, cell can propel itself and move forward at a speed of 30 micrometers per minute (Cory and Ridley 2002). This actin polymerization and subsequent cell migration is beautifully orchestrated by a family of regulatory proteins called Wiskott-Aldreich syndrome protein (WASP) consisting of WASP,N-WASP and SCAR, and WAVEs 1,2 and 3, combined with multiple other proteins including PIR121, Nap125, HSPC300 and Arp2/3 protein complex (Takenawa and Miki 2001). WAVE proteins are accumulated at the extreme edges of these protrusions providing required propulsive force along with additional proteins like signalling adaptors Abi-1 (e3b1), Abi-2 and IRSp53, thus forming a multiprotein complex. WAVEs and WASP are themselves regulated by Rac1 and Cdc42 (members of Rho family of small GTPases). The Rho-GTPases Rac1 and Cdc42, and members of Src homology (SH) domain containing adapter proteins such as NCK1

(Noncatalytic region of tyrosine kinase adaptor protein 1) stimulates actin polymerization by relaying signals to WASP/WAVEs proteins leading to activation of Arp2/3 complex which in turn catalysis the nucleation of actin filaments (Mullins 2000; Rohatgi, et al. 2000). It is well documented that Cdc42 directly interacts with N-WASP through its Cdc42/Rac1 interactive binding (CRIB) domain, which in turn reacts with Arp2/3 complex through its conserved verprolin-homology acidic (VCA) domain and catalysis actin filament nucleation but such direct interactions have not been reported in the case of Rac1-WAVE pathway because of lack of binding sites on WAVE proteins for Rac1 (Kim, et al. 2000; Machesky, et al. 1999; Rohatgi, et al. 2001).

Eden *et al* 2002 have proposed a mechanism by which Rac1 binds to the adapter protein NCK resulting in WAVE1 activation and actin nucleation. According to this model WAVE1 exists in an inactive mode (as heterotetrameric) by forming acomplex comprising proteins such as human PIR121, Nap125 and HSPC300. A combination of Rac1 with the adapter protein NCK causes dissociation of WAVE1 complex thereby releasing active WAVE1 and enhanced actin nucleation (Eden, et al. 2002).

Another appealing study came up 2 years after Eden's work by Anika *et al* 2004, in which the relationship between Rac1 and Nap1/NCKAP1 (NCK associated protein 1) was beautifully elaborated. According to this study Nap1/NCKAP1 and Rac1-associated protein 1 (Sra-1) interact with WAVE2 and signalling adaptors Abi-1 containing complex and thus linking Rac1 to actin nucleation. They presented mammalian Nap1/NCKAP1 and Sra-1 as an integral part of WAVE2/Abi-1 complex, which co-localizes at the tips of lamellipodial protrusion after Rac activation. Furthermore, loss of function of either protein Nap1/NCKAP1 or Sra-1 resulted in severe alteration in actin cytoskeleton owing to complete inhibition of cellular ability to respond to Rac activation and a failure to develop lamellipodia protrusion and membrane ruffles, thus

making them an essential component of Rac dependent actin polymerization complex (Steffen, et al. 2004).

NCK is an adapter protein (serve as link connecting enzymes and phosphorylated proteins) composed of one Src homology (SH2) domain and three Src homology (SH3) domains. NCKAP1 (Nap1) was first identified as 125kDa protein that specifically associates with the first Src homology (SH3) domain of NCK in both in vitro and in vivo (Kitamura, et al. 1996). Further evidence for its role, structure and chromosomal location came from Suzuki *et al* 2000 while working on patients suffering from Alzheimer disease as its been reported as most downregulated gene among 31 other differentially expressed genes. The human NCKAP1/Nap1 gene was mapped to human chromosome 2q32 by using FISH (fluorescence in situ hybridization)(Suzuki, et al. 2000).

In addition to its aforementioned role in lamellipodia formation, a developmental role for NCKAP1/Nap1 has also been discovered as mice lacking this gene showed arrested growth at midgestation and morphogenesis defects of all 3 embryonic germ layers. NCKAP1/Nap1 mutants failed to form single heart tube, showed delayed migration of endoderm and mesoderm along a striking phenotypic condition associated with duplication of the antero-posterior body axis (Rakeman and Anderson 2006).

3 Hypothesis and Aims of the PhD Project:

3.1 Hypothesis

Aforementioned studies have provided clear evidence that miR-214 plays a role in cardiac hypertrophy, but less is known about its role in regulating VSMC functions and its significance in atherosclerosis or neointima formation. Importantly, data from our miRNA microarrays analysis revealed that miR-214 was one of the most up-regulated miRNAs during SMC differentiation from stem cells (Yu, et al. 2014). The functional roles of other most up-regulated miRNAs (miR-22 & miR-34a) in VSMC functions/phenotypic modulation were already under investigation in our group.(Yu, et al. 2014; Zhao, et al. 2015). Furthermore, Studies by Wu *et al* 2017 (our collaborators at Zhejiang University, China) have also proposed crucial role of miR-214 in SMC differentiation (Wu, et al. 2017). Aforementioned evidence prompted us to hypothesize that miR-214 could play an important role in modulating VSMC functions and in vascular remodelling (neointima formation).

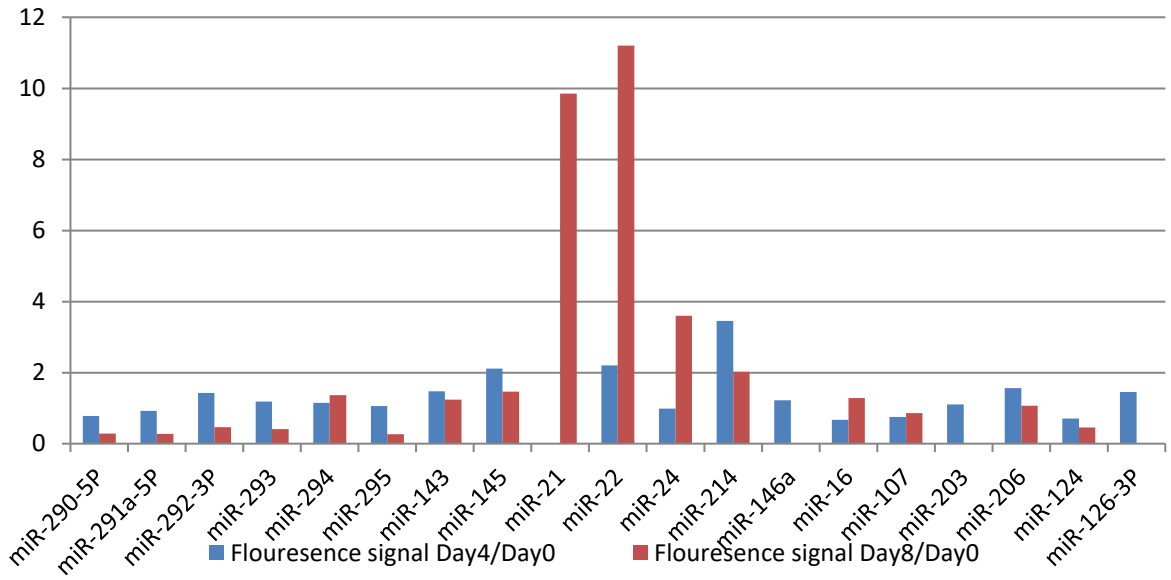


Table 6 Graphcal representation of miRNAs microarray showing miR-214 is consistently expressed and most upregulated miRNA during SMC differentiation (Yu, et al. 2014).

3.2 Aims:

1. To investigate the potential role of miR-214 in regulating SMC behaviour by addressing a possible role for miR-214 in VSMC proliferation and migration.
2. To identify and validate its target gene(s).
3. To delineate the underlying molecular mechanisms through which miR-214/target gene(s) regulate VSMC functions.
4. To explore the functional involvement/significance of miR-214 in vascular remodelling (neointima formation) using femoral artery injury model.

4 Materials and Methods:

4.1 Materials.

Antibody against NCKAP1 (goat, N-12, sc-161124) was purchased from Santa Cruz Biotech, USA. The anti-NCKAP1 antibody used for paraffin-section staining was purchased from Antibodies-online GMBH, Germany. Antibodies against GAPDH (mouse), Ki-67 (rabbit) and PCNA (rabbit) were from Abcam, UK. All secondary antibodies were from Dako, Denmark. Other materials including Phalloidin-FITC (P5282) used in this study were purchased from Sigma unless specifically indicated. Human aortic SMCs were purchased from PromoCell GmbH (C-12533) and cultured in SMC growth medium 2 (C-22062 PromoCell GmbH), according to manufacturer's guidelines.

4.2 VSMC Isolation Solutions

Operation and collection medium: DMEM, FBS 5%, GP 1%

Perfusion medium: HBSS+1% GP,

0.1% Gelatin in PBS,

0.25% Trypsin-EDTA

Digestion Medium: 1mg/ml Collagenase I and 0.744 U/ml Elastase in DMEM

Culture Medium: DMEM, FBS 20%, GP 1%

1% GP: 100U/ml Penicillin, 100 μg/ml Streptomycin, 2mM L-glutamine

DMEM: Dulbecco's modified eagle medium

FBS: Foetal bovine serum

4.3 VSMC isolation protocol.

Primary murine VSMCs were isolated from mouse aorta, and routinely maintained in DMEM supplemented with 10% FBS. The detailed process is as follow:

Preparation

1. Prepare all the buffers and media, sterilize the surgical instruments and culture dishes by UV lights for at least 30min.

2. Coat 12-well plate with 0.1% gelatin for 30-40min.

Retrieval of aorta

1. Asphyxiate mouse with CO_2 for 2 min. Spray the mouse with 70% ethanol thoroughly.

2. Open the chest without cutting any vessels with sterilized forceps and scissors.

3. Perfuse the mouse through a cardiac puncture at the apex with Perfusion medium.

4. Remove the organs to allow a clear view of the whole aorta.

5. Remove the connective tissue around the aorta carefully.

6. Cut down the aorta when it is free of fibrous material and fat tissue. (approximately 1.5cm long)

7. Place the aorta into the collection medium and keep them on ice.

Removal of the adventitia

1. Rinse the aorta in operation medium quickly and place it on the sterile dissection board covering with operation medium.

2. Scrap off as much surrounding tissue of the artery as possible under microscope.

3. Digest the artery in 1 mg/ml Collagenase Ⅰ at 37°C for 15min

4. Strip off adventitial layer and outer portion of the media under the microscope, guaranteeing to gain a clean smooth aorta with as less stretch of the artery as possible.

Enzyme digestion

1. Cut the aorta open longitudinally with scissors and remove the endothelium by gently scrapping the luminal side of the artery with a fine forceps or a scalpel blade.

2. Cut the artery into cubes as small as possible.

3. Digest the tubes in 1 mg/ml Collagenase Ⅰfor 3-3.5hrs.

4. Stop the digestion by adding Culture medium.

5. Spin down the cells at 800rpm*3min and resuspend in culture medium(DMEM with 10% FBS).

6. Plate the cells in 12 well plates pre-coated with 0.04% gelatine and culture at 37℃ in 5% CO_2 incubator. (2-3 aortas in one well of 12-well plate)

4.4 VSMC Culture medium. (CM)

VSMC culture medium contains Dulbecco's Modified Eagle's Medium (DMEM) 90% from Sigma, foetal bovine serum (FBS) 10%, 100 U/ml penicillin and 100 μg/ml streptomycin. To keep culture medium biologically active, it was prepared in small batches of 50ml and kept at 4℃ in refrigerator for frequent use.

4.5 VSMC culture, maintenance, and treatment.

VSMCs between passages 5 to 10 were used in the current study. VSMCs were cultured/ maintained in T25/T75 flasks with 5/15ml completed culture medium subsequently and incubated at 37℃ under constant supply of 5% CO_2. Cells were observed everyday to check cellular growth and morphology. Cell passaging at a ratio of 1:3 were performed once cell reach confluence level of 80-90%.

4.5.1 VSMC Passaging.

When VSMCs reach confluence level of around 80-90%, they were passaged/sub cultured to new T25/ T75 flasks. New flasks were coated with pre-warmed 0.04% gelatine (Sigma-Aldrich) in PBS and kept in incubator at 37℃ for minimum 30 mins. Culture medium was removed from the flask and VSMCs were washed once with pre-warmed PBS and treated with trypsin-EDTA for 1 min, while keeping them in incubator at 37℃. After that, pre-warmed culture medium was added to the cells to neutralize the trypsin and subsequently cells were dissociated into single cells

suspension by pipetting several times. VSMC suspension, then, transferred to new pre-coated T25/T75 flasks at a ratio of 1:3. Fresh CM was added to the flask up to the required level and cells were cultured in a CO_2 (5%) incubator at 37℃ for 2-3 days.

4.5.2 VSMC freezing and thawing

VSMC were preserved by freezing them in -80℃ freezers (for short term) and in liquid nitrogen (for long term preservation) by using specific freezing medium. The medium contains FBS 50%, 40% DMEM (Sigma-Alderich) and 10% of Dimethyl sulfoxide (DMSO, Sigma-Alderich). The procedure is as follow:

After removing old culture medium, the VSMCs were washed once with pre-warmed PBS. After washing, the cells were treated with trypsin-EDTA for about a minute in 37℃ incubator and subsequently added pre-warmed CM to neutralize the trypsin. The resulting cell suspension was transferred to 15ml falcon tube and centrifuged at 1000× g for 3 mins. Discard the supernatant and re-suspend the VSMCs by adding suitable quantity of freezing medium (generally 1 ml for each cryovial). The cells were split into several cryovials at a ratio of 1:4 and transferred to freezing container and placed in -80℃ freezers overnight. For longer preservation, the cryovials were transferred from -80℃ freezers to liquid nitrogen.

For thawing purpose, cryovials containing cells from liquid nitrogen were placed in water bath at 37℃ to defrost the medium containing cells. The cell suspension immediately transferred to 15ml falcon tube and 5ml pre-warmed DMEM were added to it. The cells were subjected to centrifugation at 800× g for 3 mins to collect the cells in the bottom of falcon tube. Supernatant were discarded and cells were re-suspended using CM and finally plated in T25/75 flasks pre-coated with 0.04% gelatine and cultured in 37℃ incubator with 5% CO_2.

4.5.3 VSMC Treatment.

VSMCs were treated with various athenogenic stimuli. Briefly, for PDGF-BB (Biolegend) and serum stimulation, VSMCs were serum starved for 24~48 hours (0.5% FBS), followed by an incubation with 20% FBS and 10ng/ml PDGF-BB for 3, 6, 12, 24 and 48 hours, respectively; For ox-LDL component treatments, VSMCs were serum starved for 24~48 hours, followed by an incubation with 10µM 4-Hydroxynonenal (4-HNE) and 7-ketocholesterol (7-Keto) for 24 hours.

4.6 Transfection

4.6.1 miRNA mimics and inhibitor transfection

Transfection is the process of introducing foreign genetic material (nucleic acid, plasmid) into cells. miRNA mimics or inhibitors and respective miRNA negative controls (25nM, final concentration) were transfected into VSMCs using TransIT-X2 Transfection Reagent (Geneflow Limited, UK) according to the manufacturer's instructions. VSMCs cultured in T75 flasks were washed with 1X PBS once and treated with trypsin-EDTA and subsequently neutralized by adding culture medium to obtain single cell suspension. After counting the cells using hemocytometer, 1.5x105 cell per well were seeded into six-well plate 24 hours prior to transfection. Before transfection, cells were washed with 1x PBS once and replenished with 1.75 ml fresh culture medium containing 5% FBS. TransIT-X2 reagent was warmed to room temperature and 7.5µl of TransIT-X2 reagent was added to 250µl of pre-warmed serum free DMEM and transferred to a sterile Eppendorf tube labelled for each treatment. Five µl of miR-214 mimics/inhibitor or their respective control scramble microRNA mimics/inhibitor (10µM/L in stock) was added and mixed gently using a pipette. This solution was left to incubate at room temperature for 20-30mins to allow the transfection complexes to form. After incubation, the TransIT-X2: miRNA complexes were added on top of the cells in drop-wise manner with circular motions to ensure all the cells being covered by the mixture. The 6-well plate was rocked back-and-forth and side-to-side to

evenly distribute the TransIT-X2: miRNA complexes onto the cultured cells. The transfected cells were cultured for 16-24hrs at 37℃ in 5% CO2 incubator prior to medium change or serum starvation. All miRNAs inhibitors or mimics and respective negative controls were purchased from Sigma- Alderich.

4.7 NCKAP1 stable knockdown cell lines

4.7.1 Generation of NCKAP1 shRNA lentivirus

To generate NCKAP1 knockdown cell lines, NCKAP1 short hairpin (shRNA) lentiviral particles were produced using MISSION shRNA NCKAP1 plasmids DNA (SHCLNG-NM_016965,MISSION® shRNA Bacterial Glycerol Stock, Sigma) according to protocol provided. The shRNA Non Targeting control vector (SHC002) was used as a negative control. Briefly, 293T cells were cultured in T75 flasks for 24 hrs followed by transfection with the lentiviral vector and the packaging plasmids, pCMV-dR8.2 and pCMVVSV-G (both obtained from Addgene) using TransIT-X2 Transfection Reagent (Geneflow Limited, UK) according to the manufacturer's instructions. Transfected the cells overnight, followed by replenishing the culture medium with fresh one on the next day. The supernatant containing the lentivirus was harvested 48h later, filtered, aliquoted in 15ml falcon tubes and stored at –80°C. p24 antigen ELISA (Zeptometrix) was used to determine the viral titre. The Transducing Unit (TU) was calculated using the conversion factor recommended by the manufacturer (10^4 physical particles per pg of p24 and 1 transducing unit per 10^3 physical particles for a VSV-G pseudotyped lentiviral vector), with 1pg of p24 antigen converted to 10 Transducing Units (TU).

4.7.2 Lentiviral infection.

For shRNA lentiviral infection and NCKAP1 stable knockdown cell line generation, VSMCs were plated 24 hours prior to infection in 6 well-plates at 37°C. One transducing Unit per cell (or 2-3x10^5/well) of shRNA or control virus were added into the culture medium with 10µg/ml polybrene (hexadimethrine bromide H9268; Sigma) and incubated at 37℃. Viral constructs were incubated 24 hours with the cells before the media was replaced with fresh complete culture media. For cellular selection puromysin (4µg/ml) (P9620, Sigma) was added to the fresh medium. For selection of transductants, fresh media containing puromycin was replenished every 2-3 day intervals for 10 days. Stably infected cells were split and frozen for future experiments.

4.7.3 Co-transduction of NCKAP1 shRNA lentivirus and miR-214 inhibitor

To evaluate the effect of miR-214 inhibition on NCKAP1 knockdown cell lines to help us better understand the mechanism of action, miR-214 inhibitor and NCKAP1 shRNA lentivirus co-transfection were performed. Vascular smooth muscle NCKAP1 knockdown cell lines generated by infecting with non-target control or NCKAP1 shRNA lentivirus were cultured in 6 well plates for 24 hrs prior to be transfected with miR-214 inhibitor or control miRNA inhibitor (25nM final concentration) as indicated in the figures, using TransIT-X2 Transfection Reagent (Geneflow Limited, UK) according to the manufacturer's instructions. All procedures were same as the single microRNA transfection explained earlier, except that the VSMCs were prior infected with non-target or NCKAP1 shRNA virus as described in the above section.

4.8 Gene cloning and Mutation.

4.8.1 NCKAP1 3'UTR clone and miR-214 binding sites mutation.

Reporter vector harbouring sequences of the murine NCKAP1 was created using cDNA from VSMCs. The 3'-flanking untranslation region (3'UTR, 3605nt ~4403nt) of murine NCKAP1 gene (NM_016965.3) (**Figure 20**) was amplified by PCR with primers shown in **Table 6.** The insert DNA fragment and the pmiR-reporter-basic vector (ThermoFisher Scientific Inc. UK) were first digested with MIuI (Promega) at 37℃ overnight. After purification, the purified inserts and vectors were digested with Hind III and Sac I (Promega) at 37℃ for 12hr. Next day, 1 µl bacterial alkaline phosphatase (BAP; Invitrogen) was added to the digested vector, mixed and incubated at 65℃ for 1-2 hr for vector dephosphorylation. The DNA inserts and vectors were purified with agarose gel electrophoresis and their concentration was measured using Nanodrop spectrophotometer. The purified DNA inserts were ligated into pmiR-Luc vectors in a solution containing T4 DNA ligase and 10 × Ligase Buffer (New England Biolabs) at room temperature overnight. The self ligation vector was used as control. After overnight incubation, the ligation mixture was transformed into JM109 competent cells. The resultant clones were picked and identified by PCR. The correct clones were further amplified and the plasmid DNA was extracted using GenElute Plasmid Miniprep Kit (Sigma). The DNA was sequenced for final verification and the resultant vector was designated as pmiR-Luc-NCKAP1-WT.

Computational algorithmic tools reported three binding sites for miR-214 on NCKAP1 gene as shown in **figure 20**. miR-214 binding site 1, 2, 3 mutation alone or combination were introduced into pmiR-Luc-NCKAP1-WT by using QuikChange™ site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer's instructions. The pmiR-Luc-NCKAP1-WT reporter from -20℃ freezer was thawed in ice. The site directed mutagenesis reaction contains 10 × QuickChange Multi Reaction Buffer, QuickSolution, dsDNA template, dNTP mix, Mutagenic

primers, double distilled water, and QuickChange multi enzyme blend were prepared for thermal cycling under following programme; 95℃ for 2min, 30 cycles at 95℃ for 30sec, 55℃ for 30sec, and 65℃ for 5 min (1 min/ 1kb), 10 min at 65℃, and 4℃ for 2 mins. The amplification product was digested with DpnI (1.5 μl) for 5 min at 37℃.

For transformation, JM109 competent cells were thawed from -80℃ freezer on ice and 30μl were aliquoted for each pre-cold tube and mixed gently with 2 μl β-ME while keeping them on ice. 2μl of Dpnl treated DNA from each mutagenesis reaction was added to each tube of competent cells. The mixture was incubated on ice for 30 mins before giving heat shock treatment at 42℃ for 30-45sec and again putting them on ice for 5mins bringing the temperature down to 2-8℃. 250μl of pre-heated and cooled Luria Broth (LB) medium without ampicillin was added to this treated mixture and incubated for 1 hr on a floor shaker at 37℃ with 225rpm. Afterwards, the bacteria were spread on a culture dish containing LB (Luria Broth) medium with 1000 × ampicillin (50μg/ml) and incubated at 37℃ for 16-20 hrs or overnight. Next morning, the plasmid was extracted using GenElute Plasmid Miniprep Kit (Sigma) and verified by both PCR and DNA sequencing.

The resultant plasmids were designated as pmiR-Luc-NCKAP1-BS1^{mut}, pmiR-Luc-NCKAP1-BS2^{mut}, pmiR-Luc-NCKAP1-BS3^{mut}, and pmiR-Luc-Notch1-BS1/2/3^{mut} mutants, respectively.

4.8.2 Generation of KLF14 and SMYD5 3' UTR Reporters

Reporter vectors harbouring sequences of the murine Kruppel-Like factor 14 (KLF-14) and SET and MYND domain containing 5 (SMYD5) were created using cDNA from VSMCs. The flanking 3' UTR of the murine KLF14 gene (NM_001135093; 3'UTR: 1272nt-2970nt) or SMYD5 (NM_144918;3' UTR:1281nt-2491nt) gene was amplified by PCR with primers shown in table and cloned into the SacI and Hind III sites of the pmiR-reporter-basic vector (Thermo Fisher Scientific

Inc), designated as pmiR-Luc-KLF12 and pmiR-Luc-SMYD5, respectively. The process is exactly similar as described above. All vectors were verified by DNA sequencing.

4.8.3 Generation of miR-214 Gene promotor.

By using similar protocol as described above, two miR-214 gene promoter plasmids were generated. Briefly, the functional full length of miR-214 gene promoter (-640:0) and its truncated form (-640:-357) were recovered from mouse genomic DNA by PCR using respective primers shown in table 5. Amplified DNA fragments were cloned into the Sac I and Hind III sites of the pGL3-basic vector (Promega), designated as pGL3-miR-214_FL and pGL-miR-214-short, respectively. All vectors were verified by DNA sequencing.

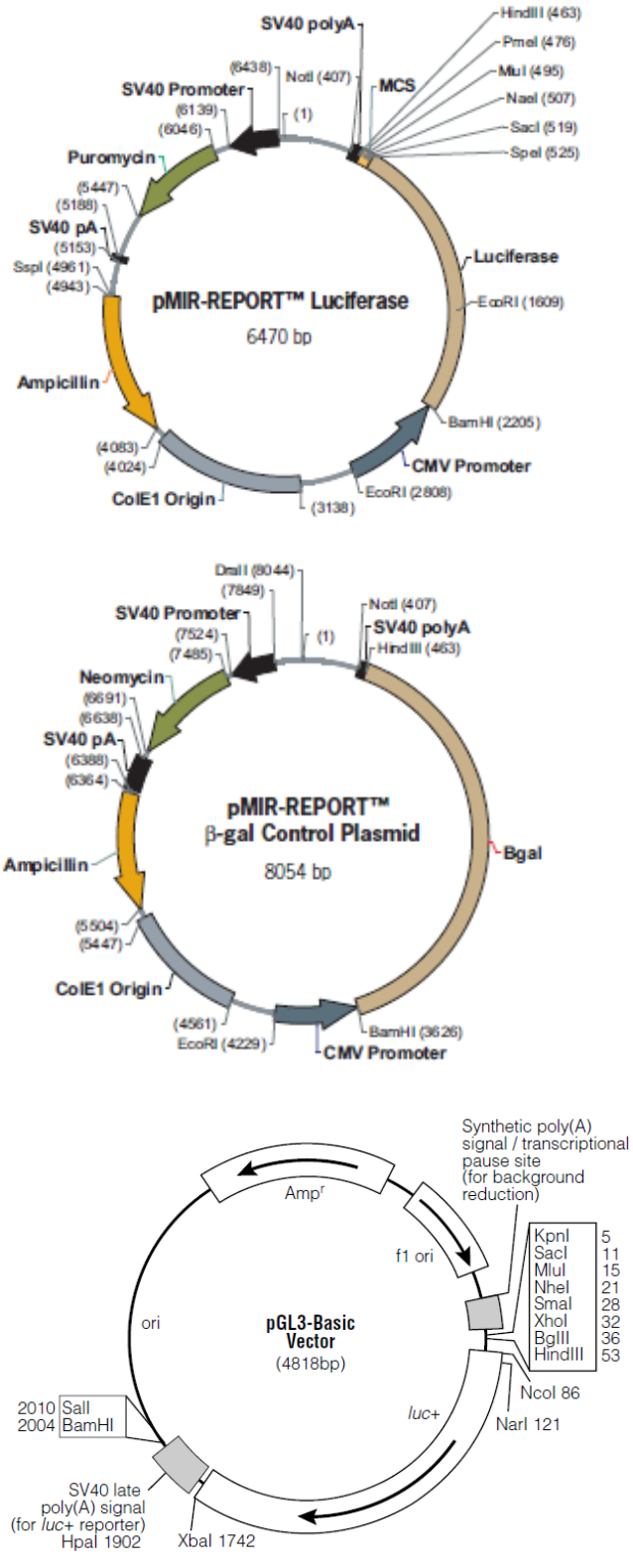


Figure 13: Maps of pmiR-Luciferase Reporter, β-gal Control Plasmid (ThermoFisher) and pGL3 Basic vector (Promega)

Gene names	Forward (5'-3')	Reverse (5'-3')	Application
U6 snoRNA (mu/hu)	GATGACACGCAAATTCGTG	miRNA universal reverse primer (Invitrogen, A11193-051)	Real-time RT-PCR
Mus/hu miR-214 (mature)	CAGGCACAGACAGGCAGT	miRNA universal reverse primer (Invitrogen, A11193-051)	Real-time RT-PCR
Mus Pri-miR-214	GCAAGGCTATGGCACTTACCTA	CCTGTTGTTACTGGCCCTCA	Real-time RT-PCR
Mus NCKAP1	GAGAAGCTCACCATCCTCAAC	CAAGAAGCAAGGACAAGTTTGG	Real-time RT-PCR
Mus PCNA	TTGCACGTATATGCCGAGACCT	ATTGCCAAGCTCTCCACTTGC	Real-time RT-PCR
Mus Twist-1	GAGGTCTTGCCAATCAGCCA	CCAGTTTGATCCCAGCGTTT	Real-time RT-PCR
pmiR-Luc-NCKAP1-WT	GTGGTGGAGCTCAGAC AAGCACGAGTTTCTGTTG	CTGCTGAAGCTTCAAGGTGTGAT ACAGTCTAGTG	NCKAP1 3'UTR reporter clone (Sac I/Hind III)
pmiR-Luc-NCKAP1-BS1^{mu}	gtggatgaaattttcttAaCgcGTGtgt attttctgatcattggc	gccaatgatcagaaaataca CACgcGtT aagaaaatttcatccac	miR-214 binding site 1 mutation
pmiR-Luc-NCKAP1-BS2^{mu}	ggcatctAcactgcctttacAACgcGT Gataataaacaaccagacac	gtgtctggttgtttattatCACgcGTTgta aaggcagtgTagatgcc	miR-214 binding site 2 mutation
pmiR-Luc-NCKAP1-BS3^{mu}	tgctgccgccgcACgtagaaCgcGtac ACtagaaacatctcatcc	ggatgagatgtttctaGTgtaCgcgttcta cGTgcggcggcagca	miR-214 binding site 3 mutation
pmiR-Luc-KLF14	GTCGTC GAGCTC GACTGTCTTGCTGTCTATCT	GTCGTC AAGCTT CTCAGCATTTAAAGATTTATAG	KLF14 3'UTR reporter Clone (Sac I/Hind III)
pmiR-Luc-SMYD5	CACGAC GAGCTC TGTTATCTCACCTGGAAGGC	CACGAC AAGCTT TCACCACTCACATTTTATTGAGAC	SMYD5 3'UTR reporter clone (SacI/Hind III)
pGL3-miR-214-FL	gaggag GAGCTC agggggggagccccaacttatctga	gaggag AAGCTT TTCCTGCACCAGGGGCTTGT	Mouse miR-214 gene promoter (-640:0)clone
pGL3-miR-214-short	gaggag GAGCTC agggggggagccccaacttatctga	ctggtc AAGCTT TGGGGCCCCAGTATGGAAAA	Mouse miR-214 gene promoter (-640:-357) clone

Table 7. Set of Primers used in this study

4.9 Plasmid Amplification and Extraction.

4.9.1 Plasmid Amplification.

Transformation was applied to amplify plasmid DNA using JM109 bacteria. Briefly, Bacteria and Plasmid/DNA was thawed on ice and 1µl of plasmid DNA was mixed with 30-50 µl of JM109 bacteria aliquoted in pre-cold tubes for each amplification reaction. Gently mixed and incubated on ice for 30 mins before giving heat treatment at 42℃ for 1 min and again chilling the mixture for 3-5 mins by keeping in ice 2-8℃. 500µl of LB medium without antibiotics (ampicillin) was added to the plasmid/bacteria mixture and incubated at 37℃ for 1 hr on a floor shaker at 225rpm. After incubation, the plasmid/bacteria mixture was transferred and evenly spread onto the surface of culture dish containing LB medium with antibiotics (ampicillin 50µg/ml) and incubated at 37℃ for overnight. Next morning, distinct single clones were picked and transferred to 14ml polystyrene round bottom tube containing 5ml LB each mixed with ampicillin. These tubes were incubated at 37℃ to help bacterial growth for 14-18 hrs in a shaker at 225rpm. After incubation, the samples were centrifuged at 4,000 × g for 10 mins, discarded the supernatant and bacteria was collected to obtain the plasmids.

4.9.2 Plasmid Extraction.

GenElute Plasmid Miniprep Kit (Sigam) was used to isolate and extract plasmid from bacteria as per manufacturer's instructions.

The collected bacterial pellet from previously described procedure was resuspended in 200µl of resuspension solution containing RNase A solution in 14ml polystyrene round bottom tube and mixed to obtain homogenised solution. This bacterial solution was transferred to 1.5ml microcentrifuge tube and mixed with 200µl lysis solution to lyse the cells. The lysis reaction should be terminated within 5 min by adding 350µl of Neutralization solution to stop the

reaction and help precipitate cell debris. Prolonged lysis permanently denatures supercoiled plasmid DNA and renders it unsuitable for most downstream applications. After mixing neutralization solution, the tube was centrifuged at 12,000×g for 10min. The supernatant clear lysate containing DNA was transferred to DNA binding column (pre-treated with 500µl column preparation solution) with a 2ml collection tube. Keep the apparatus (DNA binding column and the collection tube) at room temperature for 3-5 min to facilitate DNA binding. Centrifuge the column with the tube at 13,000 rpm for 30sec and discarded the flow through. Optional wash solution (500µl) was added to the column to avoid nuclease contamination of the final plasmid product (when the bacterial strain used contain the wild type $EndA^+$ gene. 750µl of wash solution diluted with ethanol prior to use) was added to tube and centrifuged at 12,000 ×g for 1 min and discarded the flow through. The column was placed into new collection tube and 50µl elution buffer was added, incubated for 1-3 min and centrifuged at 12,000× g to collect plasmid in collection tube. Nanodrop spectrophotometer was used to measure plasmid DNA concentration before storing it in -20℃ freezer for future use.

4.10 Luciferase Assay.

4.10.1 Transient transfection and luciferase assay.

Luciferase assay for NCKAP1 3'UTR reporters were conducted by co-transfecting VSMCs (cultured on 24 well, 0.04% gelatin coated plates) with individual reporter gene (pmiR-Luc-NCKAP1-WT, pmiR-Luc-NCKAP1-$BS1^{mut}$, pmiR-Luc-NCKAP1-$BS2^{mut}$, pmiR-Luc-NCKAP1-$BS3^{mut}$, or pmiR-Luc-NCKAP1-$BS1/2/3^{mut}$, 0.15-0.20µg/2.5 x 10^4 cells) and control or miR-214 mimics (25nM) using TransIT-X2 Transfection Reagent (Geneflow Limited, UK), according to the manufacturer's instructions. pmiR-Luc-β-gal (0.20µg/2.5 x 10^4 cells) or Renilla plasmid (15ng/well) was included in all transfection assays as internal control. Luciferase, Renilla, and/or β-galactosidase activities

were detected 48 hours after transfection using a standard protocol. Briefly, VSMCs were refreshed with new culture medium after overnight transfectionand the luciferase assay continued for 48-72hr post transfection. For luciferase assay where Renilla gene was used as control, the culture medium was removed and cells were washed twice with 1× PBS (pre-warm). After washing, 100µl reporter lysis buffer (Promega) was added to each well and placed on 2D rocker for 30 min at room temperature. The cell culture plate with lysis buffer was then incubated in -80℃ freezer for 2h followed by thawing for 30 min on shaker at room temperature to facilitate cell lysis. The cell lysate was then transferred to 1.5ml eppendorf tube and centrifuged at 13,200 rpm for 5 min at 4℃. 15µl of supernatant from each sample were mixed with luciferase or Renilla substrate (Promega) to detect the activity using a Single-Tube Luminometer (Turner BioSystem Sunnyvale, CA, USA). Relative luciferase unit was defined as the ratio of Luciferase activity versus Renilla activity with that of the control set as 1.0.

The activity of LacZ was detected using β-Gal Kit (Invitrogen) when the pShuttle2-LacZ was used as internal control. Similar procedure was adapted as described earlier for measuring luciferase activity, except for detecting β-galactosidase activity, where 10-15µl from cell lysate was diluted to a final volume of 30µl with distilled water and transferred to a fresh 1.5ml eppendorf tube. 70µl of OPNG (Ortho-nitrophenyl-β-D-galactopyranoside) and 200µl of 1 × Cleavage buffer with β-mercaptoehanol (β-ME) were added to the diluted cell lysate. After vortex and brief centrifuge, the mixture was incubated at 37℃ for 30min. Hydrolysis of OPNG to the ONP anion by β-galactosidase produces a bright yellow colour, a distinct feature of β-galactosidase presence and activity. Stop buffer (500µl) was added to mixture to stop the reaction and the mixture was observed at 420nm using a luminometer against a blank reaction mixture containing lysis buffer, OPNG and cleavage buffer. Relative luciferase unit (RLU) was defined as the ratio of Luciferase versus β-galactosidase or Renilla activity with that of the control (set as 1.0).

4.11 RNA extraction and Analysis.

4.11.1 Total RNA Extraction.

Before harvesting cell, all work area was cleaned with 75% ethanol to minimize contamination. Cell scrappers were sterilized in 75% ethanol for 10 mins and rinsed with autoclaved 1X cold PBS. Cell culture medium from flasks or plates was removed and washed once with 1X cold PBS. Cells were scrapped in cold 1X PBS to minimize enzymatic activity and resulting cell suspension was collected in 1.5ml sterilized eppendorf tubes. The cells were centrifuged at 8,000rpm, 4℃ for 2 mins to obtain cell pellet which were either preserved at -80℃ or processed further for RNA extraction.

All RNA related work was performed in RNase free environment using 75% ethanol to clean work surface and by using sterilized/autoclaved tubes and pipette tips to minimise risk of RNA contamination and/or degradation. Total RNA was isolated from the cells using GenElute Mammalian Total RNA Miniperp Kit (Sigma-Alderich) according to manufecturer's instructions.

To fully inactivate RNase activity during RNA extraction, fresh Lysis solution was prepared each time by adding 2-mercaptoethanol at a ratio of 1:100 to lysis buffer (provided in the kit). Appropriate quantity of lysis solution/2-ME mixture was added to each cell pellet (250 or 500 μl for 5×10^6cells or 1×10^7 cells respectively. The cell solution was vortexed to dissolve all cell clumps before transferring it to the GenElute Filtration Column placed in a 2ml collection tube. The Cell solution was centrifuged at 13,200 rpm for 2 mins at room temperature. Filtrate column was discarded from collection tube and equal volume (250-500μl) of 70% ethanol was added to the filtrate and mixed well to precipitate RNA. The lysate/ethanol mixture (500$\sim$700μl) was transferred into a clear GenElute binding column placed in 2ml collection tube and centrifuged at 13,200rpm for 30sec. The flow through was discarded and GenElute binding column was again placed back in the same collection tube. If the volume of lysate/ethanol mixture was greater

than 700μl, then repeat the previous step to allow all RNA to bind to the column. 500μl of wash solution 1 was added to each column and spun at 13,200rpm for 15-30 sec. Discarded the flow through and added 500μl of wash solution 2 to each column and spun again at 13,200 rpm for 15-30 sec. After the final wash and removal of flow through from collection tube, the sample column was centrifuged at maximum speed (13,200 rpm) for 2 mins to remove the traces of ethanol from the column. Binding columns were then transferred to new RNase free collection tubes and 50μl of elution buffer was added to each column. After incubation for 1 min at rom temperature, the column was centrifuged at maximum speed (13,200 rpm) for a minute to elute RNA. All RNA samples were kept on ice after centrifugation and for concentration measurement. NanoDrop spectrophotometer was used to measure concentration of RNA samples. RNA samples were either processed further (reverse transcribed) to obtain complimentary DNA (cDNA) or stored at -80°C for future use.

4.11.2 MicroRNA Extraction.

Total RNA including microRNA was extracted from the cells using mirVana Protein and RNA isolation System kit (Thermo Fisher Scientific Inc) or TRI-reagent (Sigma-Alderich) according to the manufacturer's instructions and subjected to DNase I digestion (Sigma-Alderich) to remove potential DNA contamination.

Briefly, Cell pellet obtained from flasks using cold PBS and cell scrapper (previously described method) were mixed with 625µl ice-cold cell disruption buffer. Samples were vortexed to completely lyse the cells and to obtain a homogeneous lysate. Immediately, an equal volume of 2× denaturing solution was added to the lysate to prevent RNA degradation. Next, 1250 μl of acid-phenol:chloroform was added to the mixture and vortex for a minute. After mixing, the samples were centrifuged at 13,200 rpm for 5 mins at room temperature, which resulted in

separation of sample into upper colourless aqueous phase containing RNA and lower organic phase. The upper aqueous phase was carefully collected into new RNase free tube. 100% ethanol was added to the aqueous phase (collected). The volume of ethanol was 1.25 times to the volume of the recovered aqueous phase. The ethanol/lysate complex was mixed thoroughly and aliquoted onto a filter cartridge placed on the collection tubes. The mixture was centrifuged at 13,200 rpm for 30sec and the flow through was discarded. The process of centrifugation was repeated till the entire ethanol/lysate sample had filtered through the cartridge. MicroRNA wash solution 1 (700μl) was added to the filter cartridge and centrifuged for 30 sec with the flow through discarded. Wash solution 2/3 (500μl) was added to the filter cartridge, centrifuged for 30 sec and discarded the flow through. A repeat wash procedure was performed with wash solution 2/3 to completely wash the microRNA. The filter cartridge was centrifuged empty for a minute to remove any residual fluid. The filter cartridge was transferred into a fresh collection tube and 100μl elution solution (pre-heated to 95℃) was added to the centre of the filter cartridge. The miRNA elute was recovered by centrifugation for 30sec. NanoDrop spectrophotometer was used to measure RNA concentration before further processing or storing the samples at -80℃.

Total RNA including microRNA was also extracted from the cells using TRI-reagent (Sigma-Alderich) according to manufacturer's instructions. The cells (in flasks) were scrapped using sterilized scrapper in cold PBS and transferred to sterilized RNase free 1.5ml eppendorf tubes. Centrifugation of cells at 8,000rpm, 4℃ for 2 mins resulted in cell pellet. Supernatant was discarded and 500μl of TRI-reagent (Sigma-Alderich) was added to each sample. Whereas, if cells were cultured in 6 well plates, 500 μl of TRI-reagent was directly added on top of cells, after being washed with cold PBS. Pipetting several times resulted in homogenised cell lysate, which was transferred to RNase free 1.5ml tubes. The homogenised samples were left at room

temperature for 5 minutes to permit complete dissociation of nucleoprotein complexes. The lysate were centrifuged at 13,200rpm for 1 min to remove cell debris and the resulting suspensions were transferred to new RNase free tube. 200μl chloroform (Sigma-Alderich) was added to each sample and shacked vigorously for 15 sec. The resultant mixture was left at room temperature for 5 minutes before centrifuging the samples at 12,000 ×g for 15 minutes at 4℃. After centrifugation, the sample separated into upper colourless part and lower pink coloured (organic) part containing cellular debris. Upper colourless supernatant containing RNA was carefully collected in new RNase free tubes. 500μl of isopropanol (equal volume to that of TRIzol Reagent) was added to each sample and mixed gently. Samples were incubated at room temperature for 10 minutes before centrifuging them at 12,000 × g for 10 mins at 2-8℃. Centrifugation results in RNA precipitation as small pellet at the bottom of the tube. Supernatant was discarded and RNA pellet was washed with 75% ethanol (500μl, equal volume as that of TRIzole reagent used). The samples were centrifuged at 7,500 × g for 5 mins at 2-8℃ and the resulting supernatant discarded. The resulting RNA pellet was air dried for 10 mins and dissolved in 30μl of RNase free water by gentle mixing. RNA concentrations were measured using NanoDrop spectrophotometer and were preserved in -80℃ freezer.

4.11.3 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

For reverse-transcription polymerase chain reaction (RT-PCR), RevertAid Reverse Transcriptase by Thermo Scientific was used. RNA samples from -80℃ freezer were thawed on ice and gently mixed. 1-2μg of RNA was used as template for each reaction by diluting with RNase-free water and mixed with 1 μl of random primer (50ng/μl, Promega). The final volume of mixture was 13µl. After mixing and briefly centrifuging, this reaction mix was placed in a thermal cycler and run a programme at 65℃ for 5mins followed by cooling down to 4℃. The above treatment destabilizes RNA secondary structure (because of G-C rich sequence) and enhances primer

bonding. Subsequently, master mix for RT-PCR was prepared which includes following components:

5× Reaction Buffer 4µl, dNTPs (2'-deoxynucleoside 5'-triphosphate) mixture (10mM) 2µl, Thermo Scientific RiboLock RNase Inhibitor 0.5µl, RevertAid Reverse Transcriptase 0.5µl. The final volume for each reaction was 20µl. This master mix was combined with the RNA samples and start the thermal cycler with the following programme; 25℃ for 10 mins for annealing followed by an extension phase at 42℃ for 60 mins and finally to stop reaction by enzyme denaturation, the temperature raises to 70℃ for 10 min. The reaction was then cooled down and holds at 4℃. The resultant complimentary Deoxyribonucleic acid (cDNA) product was diluted to working concentration of 5ng/µl and stored at -20℃ for future use.

4.11.4 microRNA Reverse transcription.

The microRNA cDNA was prepared by using NCode VILO miRNA cDNA synthesis Kit (Invitrogen), which synthesize poly(A) tails of all the microRNA and then synthesizing the cDNA from the tailed population in a single reaction. Briefly, 1 µg of template RNA was mixed with 4µl of 5× Reaction Mix and 2µl of 10× SuperScript Enzyme Mix and topped up to 20µl by adding DEPC-treated water. The mixture was mixed and briefly spun down before incubating in a PCR thermal cycler running following programme; 37℃ for 60mins followed by 95℃ for 5 min to terminate the reaction and finally cooled down and hold at 4℃. The final cDNA product was diluted to 5ng/µl and stored in freezer at -20℃ or used immediately.

4.11.5 Polymerase Chain Reaction. (PCR)

The working area was cleaned with 70% ethanol and sterilize pipette tips were used to minimise contamination while preparing Real Time Polymerase Chain Reaction samples. To minimise pipetting error, the PCR reaction mix was prepared by mixing 13µl nuclease free water, 2.5µl 10 × DreamTaq Buffer, 2.5µl dNTP mix (final concentration 2mM), 2µl primers (1µl forward and

reverse primer each) and 0.14 µl DreamTaq DNA Polymerase for each PCR reaction. The mixture was mixed and aliquoted to individual PCR tubes containing 2µl of cDNA. The reaction mix was incubated in PCR thermal cycler (DNA Engine Tetrad 2: MJ Research) and run the programme as described in table 6.

Step	**Temperature**	**Time**	**Number of Cycles**
Initial Denaturation	95	5 mins	1
Denaturation	95	1 min	35
Annealing	55	1 min	
Extension	72	1 min/kb	
Final Extension	72	5-10 mins	1

Table 8. Normal PCR conditions

UltraPure Agarose (Invitrogen) get electrophoresis was performed to detect and identify the final PCR product. Agarose gel is used to separate and purify the DNA segments [nucleic acid fragments] ranging in size from 0.2kb to 20kb [kilo base pairs]. Concentrations of agarose gel preparations vary from 0.3% to 3% depending upon the nature and size of DNA segment to be analysed. Smaller fragments resolve better in gel with higher percentage of agarose and vice versa. For agarose gel preparation, calculated amount of agarose gel powder was mixed with TAE buffer [usually 1.5g in 100ml of TAE buffer] and heated in a microwave. The mixture was stirred after every 30 seconds to make it homogenise. When the solution became clear, transferred it to water bath at 50˚c for 10 mins or cool down under cold running water to bring the temperature down to around 50-60℃ while assessing by touch. 5µl of GelRed Nucleic Acid Stain (10,000 ×in DMSO; Biotium) was added to the solution. Poured the solution in a closed edged casting glass plate to avoid solution spillage. Fixed a comb at one end of the plate and left it for 2 hours to

settle down and solidify. After 2 hours, transfer the gel to a reservoir containing TAE buffer. Filled the wells with 20-30 µl of DNA samples along with 10-15 µl of DNA ladder and run the gel by applying the electrical current of 160V for 25 mins or till the dye front reached the end of the gel. Captured the florescent image depicting expression of cDNA using AlphaImager HP System (Alpha Innotech) with dual wavelength ultra violet illumination (365 nm or 302 nm) and automatic image processing software by Alpha Innotech.

4.11.6 Real-Time Polymerase Chain Reaction (q-PCR)

The working area was cleaned with 70% ethanol and sterilized pipette tips were used to avoid any contamination during qPCR samples/mixtures preparation. cDNA samples were thawed from -20℃ freezer and kept on ice.

For qPCR, KAPA SYBR FAST qPCR Kit Master Mix (KAPA Biosystems) was used. The qPCR master mix contains following components shown in table 7.

Reagents	**Volume for 1 reaction**
KAPA SYBR FAST qPCR Master Mix	5µl
Forward primer	1µl
Reverse Primer	1µl
RNase free water	1µl

Table 9. Components and quantity for qPCR reaction, using KAPA SYBR FAST qPCR Kit

For miRNA samples, the master mix consists of NCode EXPRESS SYBR Green ER qPCR SuperMix Universal (Thermo Fisher Scientific Inc). miRNA specific forward primer was mixed with miRNA universal reverse primer (Invitrogen) in equal quantity and topped up with RNase free water to a final volume of 8µl which then added to each well of 384 well plate. The specific quantities used were as follow:

Reagents	Volume for 1 reaction
NCode EXPRESS SYBR GreenER qPCR Master Mix	5µl
miRNA specific Forward Primer	1µl
Universal Reverse Primer	1µl
RNase free water	1µl

Table 10. Components and quantity of qPCR for mircoRNA

The above master mix was gently mixed and 8µl was aliquoted in each well of 384 wells plate (MicroAmp Optical) along with 2µl of cDNA sample (5ng/µl). The plate was sealed with MicroAmp PCR film vortexed briefly and centrifuged at 2000 rpm for 2 min. An Applied Biosystems 7900 HT TaqMan Real-Time PCR System was used to run the qPCR reaction with the following programme: 95℃ for 10 min, 40 cycles of 95℃ for 15 sec and 60℃ for 1min. Once the reaction completed, the result was analysed using SDS 2.3 software (Applied Biosystems). The raw Ct values and the relative expression abundance of the mRNA of interest were analysed and calculated using relative quantification methods ($2^{-\delta\delta Ct}$). Relative mRNA or miRNA expression level was defined as the ratio of target gene expression level or miRNA expression level to 18S or U6 small nuclear RNA exoression level, respectively, with that of the control sample set as 1.0. The respective endogenous control was set as 18S rRNA for mRNA detection and U6 snRNA was used as the endogenous control to normalise the expression levels of small/micro RNAs.

4.11.7 PCR Quality control

A number of steps were employed to ensure a good quality for real time PCR measurements and the resultant product, which ranges from primer design, RNA and cDNA quality and quantity to experiment protocol which involves conducting the experiment in triplicate, running the RT and qPCR with negative control and general cleanliness to avoid cross-contamination.

Primers were designed with following criteria;

Intron spanning primers were designed, between 18–25 bases in length, and have a Guanine-Cytosine content between 40–60% which helps to prevent mismatch stabilization.

Designed primers to have comparable melting temperatures (Tms) between 58 and 65°C. The Tm difference between the forward and reverse primers should be ≤ 4°C, and the last five nucleotides at the 3' end of each primer should not contain more than two guanines (G) or cytosines (C).

Avoid sequence complementarity within each primer and between all primers used in an assay.

Standard operating and cleaning procedures were employed. Work area was cleaned with ethanol along with sterilised equipment was used to minimize cross-contamination. Quality and quantity of RNA and cDNA was ensured to lie between the optimum values with running negative controls for RT reaction. Running positive and negative control for qPCR. Running the experiment in triplicate and detecting the cDNA for specific product band size by running agarose gel electrophoresis and finally checking for cDNA sequence to valide the PCR product is indeed our intended gene product.

4.12 Protein Extraction and Analysis.

4.12.1 Chemicals and Buffers

Solution name	Components
RIPA Buffer	Tris-HCL: 50 mM, pH 7.4 NP-40: 1% Na-deoxycholate:0.25% NaCl: 150mM
5 × SDS Loading Buffer	Tris-Cl (0.25M, pH 6.8) SDS (Sodium dodecyl sulphate: 10%) Glycerol: (50%) Bromophenol Blue: (0.25%) DTT (Dithiothreitol): 0.5 M)
10 × Tris-Glycine Buffer, **pH 8.4,**	Tris Base: 30.3 g Glycine: 144.1 g Distilled water up to 1 litre
1 × Running Buffer	10 × Tris-Glycine Buffer: 100ml 10% SDS: 10ml Distilled water up to 1 litre
1 × Transfer Buffer	10 × Tris-Glycine Buffer: 100 ml Methanol: 200 ml Distilled water up to 1 litre
10 × TBS Buffer	Tris-HCL: 24 g Tris Base: 5.6 g NaCl: 88 g Distilled water up to 1 litre
TBST (0.1%)	10 × TBS: 100 ml Tweet 20: 1 ml Distilled water up to 1 litre

Table 11. List of Chemicals and buffers used for western blot experiment

4.12.2 Protein Extraction.

The work surface was cleaned using 70% ethanol and autoclaved pipette tips and tubes were used to minimise contamination.

Similar to the process mentioned above for RNA extraction, the cells were harvested from T27/T75 flasks using cell scrapper (cleaned with 70% ethanol and rinsed with PBS) in cold 1 × PBS to minimise proteolytic activity. Centrifugation at 8,000 rpm for 2 min at 4℃ gives cell palette, which after removing the supernatant was preserved at -80℃ or processed immediately for protein extraction. Each cell pellet was mixed with 100µl of lysis buffer (RIPA Buffer containing Protease Inhibitor Cocktail by Sigma- Alderich) and 0/5% Triton on ice. The lysate was vortexed and then sonicated at 4℃ for 20sec to disrupt cell membrane and facilitate release of protein content. The lysate were left on ice for 1 hour while vortexed after every 15 min to completely lyse the cells. After incubation on ice, the cell lysate was centrifuges at 13,200 rpm for 10 minutes at 4℃ to remove cellular debris. Up to 100 µl of supernatant from each sample was transferred to new pre-cooled eppendorf tube and placed on ice. The concentration of protein was measured by using BIO-RAD Protein Assay Reagent (Bio-Rad Herts UK). The Bio-Rad solution was first diluted in a 1:5 ratio by using distilled water. 998µl of diluted Bio-Rad assay solution was added to 2µl of each protein sample, mixed and incubated at room temperature for 10 minutes. A negative control was prepared by adding 2µl of lysis buffer with 998µl of Bio-Rad Protein Assay solution to normalize the final protein concentration values. Protein concentration was measured by using spectrophotometer (Smart Spec 3000: Bio-Rad) at 595nm wavelength. Lysis buffer and pre-warmed 5 × SDS Protein loading buffer was added to each sample to adjust and normalise the concentration. The protein samples were heated at 95℃ for 10 min to denature any secondary structures followed by brief vortex and centrifugation. After cooling down to

room temperature, the samples were used for western blot experiments or stored in -80℃ freezers.

4.12.3 Western Blot.

Western blot electrophoresis was performed using a Mini-PROTEAN Tetra Cell system (Bio-Rad, UK) and proteins were separated by using sodium dodecyl sulphate-polyacrylamidegel electrophoresis (SDS-PAGE) with 6-10% Tris-glycine gels. The 10% separating and 6% stacking gels were prepared fresh on the day of experiment. The constituents and their relative quantity for making 10% separating gel and 6% stacking gel is descried in table 10 and 11.

The 10% separating gel (5ml) was prepared first and poured in glass casting chamber. 1ml Butanol (Sigma-Alderich) was added on top of the gel to level its surface. The mixture was left at room temperature for 20 minutes to let gel solidify, after which the butanol was removed and solidified gel was washed twice with distilled water to remove traces of butanol. Now, 2.5ml of 6% stacking gel was added on top of separating gel and immediately a plastic comb was inserted into stacking gel creating 12 wells per gel. Again, the solution was left at room temperature for 20 min to let stacking gel solidify after which the gel glass cassette was transferred to a chamber for running electrophoresis. 1 × running buffer was added to the chamber to the extent that it covers the gel and the inserted comb. 10-12µl of ColorPlus Pre-stained Protein Ladder (New England BioLabs Inc) was added to first well as marker labelled for specific protein sizes. Calculated amount of protein (usually 20-30 µg) were loaded into each well and the gel was run at 90 Volts for 20-25 min to help stacking the proteins followed by 160 Volts for 70-90 min at room temperature until the protein marker ladder reached the bottom of gel.

Reagents	Quantity for 1 gel (10%)
Double distilled water	1.9 ml
Acrylamide (National Diagnostics, USA) 30% Stock	1.7 ml
Tris-HCL Buffer 1.5M (pH 8.8)	1.3 ml
SDS (10%)	50 µl
APS (10%)	50 µl
TEMED	4µl

Table 12. Reagents for 10% separating gel (final vol. 5ml)

Reagents	Quantity for 1 gel (5%)
Double distilled water	2.1 ml
Acrylamide (National Diagnostics, USA) 30% Stock	0.5 ml
Tris-HCL Buffer 0.5M (pH 6.8)	0.4 ml
SDS (10%)	30µl
APS (10%)	30µl
TEMED	3µl

Table 13. Reagents for 5% stacking gel (final vol. 3ml)

Polyvinylidene difuoride (PVDF) membranes (GE Healthcare) are used to transfer protein form gel to membrane. Before transferring proteins, PVDF membrane is activated by submerging it in methanol for about 5 min. After this, the membrane is soaked in pre-cold 1× transfer buffer along with pre-washed blotting sponges and filter papers. After completion of electrophoresis, the gel was assembled with PVDF membrane along with blotting sponges (transfer buffer soaked) and filter papers in a western blot cassette in correct order/ direction to facilitate protein transfer. The assembly was performed in 1× transfer buffer and air bubbles were carefully

removed by rolling over the surface with a roller. This apparatus is placed into Mini Trans Blot Central Core (Bio-Rad) filled with pre-cold 1× transfer buffer. The proteins were electro-transferred to membrane for 2 hours 30 min at 60Volts. After transfer, the membrane was removed from blotting cassette and blocked with milk (5%) diluted in 1× TBST buffer on a 2D rocker, with the protein facing upwards, for 1 hour at room temperature. The membrane was then incubated with specific antibody prepared in 5% skimmed milk in TBST, overnight on a slow roller at 4℃.

Next morning, membrane were washed with 1× TBST 3 times for 10 min each wash on 2D shaker. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibody in 5% milk TBST for 1 hr while gently shaking at room temperature. After secondary antibody incubation, the membrane is again washed 3 times 10 min each with 1 × TBST buffer. Then the membrane was incubated with ECL-Plus Reagent (Amersham Biosciences, Stockholm, Sweden) for 2-3 min at room temperature. After removing excess ECL solution, the membrane was placed in an autoradiographic cassette and transferred to dark room, where membranes are exposed against a piece of X-ray film (Hyperfilm ECL, GE Healthcare) for normally between 10 sec to 5mins depending upon strength of signals from protein under observation. The film was developed with an X-Ray Film Processor (SRX-101A, Konica Minolta, USA) using Fix buffer and Develop Buffer.In some experiments, the blots were subjected to densitometric analysis with Image J software. Relative protein expression level was defined as the ratio of target protein expression level to GAPDH expression level with that of the control sample set as 1.0.

4.13 VSMC proliferation assays.

4.13.1 Cell counting.

For cell counting, VSMCs were plated ($1x10^5$ per well) and cultured in 6 well plates pre-coated with 0.04% gelatine and supplemented with complete culture medium containing DMEM, 10% FBS and 1% Penicillin/streptomycin-glutamine. The plates were placed in humified incubators at 37˚c and 5% CO_2. After culturing for 24hrs, the cells were transfected with single miR-214 mimics/inhibitor, or co-transduced with miR-214 inhibitor/NCKAP1 shRNA, or respective negative control as indicated in the figures. After 12-16 hrs of transfection, the cells were starved by culturing them in the DMEM supplemented with 1% Penicillin/streptomycin-glutamine and 0.5% serum for further 24 hrs. After starvation process, the cells were treated with 20% FBS or PDGF-BB (10ng/ml) for 48hrs before trypsinizing and manually counting the cells under hematocytometer.

4.13.2 BrdU incorporation assay.

VSMCs were transfected as described above, and were re-cultured ($0.75 x10^4$ per well) in 96 well plates overnight, followed by serum starvation for 24 hours. Starved VSMCs were re-stimulated with 20% FBS or 10ng/ml PDGF-BB, respectively, for 48 hours. Cell proliferations were evaluated using 5-Bromo-2'-deoxy-uridine (BrdU) Labelling and Detection Kit II (Roche) according to the manufacturer's instructions. Briefly, cells were incubated with BrdU at a final concentration of 10μM for 8~12h before measurement. After fixation, cellular DNA was digested by nuclease and labelled with a peroxidase-conjugated BrdU antibody, followed by incubation with the peroxidase substrate. The absorbance of the samples was measured by a microplate reader at 405nm (OD405) with reference measurement at 490nm (OD490). Absorbance (A_{405nm}-A_{490nm}) values representing cell proliferation ability were compared between treatments.

4.14 VSMC migration assays.

4.14.1 Wound healing (Scratch model).

For scratch wound healing assays, VSMCs were cultured on 12-well plates 24h prior to transfection, and then transfected with miR-214 mimics, miR-214 inhibitor, or respective miRNA negative control as described earlier. After 12-16 hrs of transfection, the confluent cells were starved by culturing them in the DMEM supplemented with 1% Penicillin/streptomycin-glutamine and 0.5% serum for further 24 hrs. After starvation process, the cells were treated with hydroxyurea (2mM) to inhibit cell proliferation for 2hrs before subjecting them to 20%FBS or PDGF-BB (10ng/ml) treatment. The cells were scratched using a sterilized 200µl pipette tip in criss-cross manner and rinsed with PBS or DMEM three times to remove cell debris. 2 crosses were made in each well to get duplicate readings for each treatment as well as to better identify the scratched area. The cells were cultured in DMEM supplemented with 20% FBS or PDGF-BB (10ng/ml) in the presence of 2mM hydroxyurea. The observations were made and photomicrographic images were taken at 0hr and 24 hrs, respectively. ImageJ software was used to measure the denuded cell surface of each wound (criss-cross) by two experienced investigators blinded to the treatments, and the percentages of cell closures (migrated area) were calculated as the denuded area difference between hour 0 (A0) and hour 24 (A24) over the denuded area at hour 0, then times 100 [or (A0-A24)/A0*100].

4.14.2 Trans-well migration assay.

Cell migration plays a fundamental role in regulating several pathological and physiological processes such as embryonic development, inflammation, immune response, wound healing, and tumorigenesis. Transwell migration assay is an excellent tool to observe cellular movement under different immunomodulatory and inflammatory stimuli and provides us ability to better

understand underlying chemokinetic and chemotactic signals regulating biological mechanisms. The transwell migration assay involves two compartment system whereby cells are stimulated to migrate from an upper compartment into a lower compartment through a porous membrane under the influence of certain chemoattractant molecule. The relative migratory profile of cells gives us information about cellular responses towards different molecules and is a gradient of cellular function.

VSMCs, cultured in flasks, were transfected with miR-214 (mimics or inhibitor), NCKAP1 shRNA and relevant controls for overnight. Next morning, refreshed the medium and continued culturing the transfected cells for 24 hrs in DMEM containing 0.5% serum for 24 hours. After that, the cells were harvested by using trypsin, washed with PBS and resuspended in serum free medium. Transwell inserts precoated with 0.5% gelatin were placed in 24 well culture plate.

An aliquot (250,000 cells/200µl) of the cells in serum-free DMEM was dispensed into the trans-well inserts (8µm pore size, Greiner Bio-One Ltd, UK. Item number: 662638). Added 500-550µl of culture medium with 20%FBS or 30ng/ml of PDGF-BB in the lower chamber and incubated the trans-well apparatus at 37°C in a 5% CO_2 incubator for 18~24 hours. The cells will migrate across the porous membrane under the influence of chemoattractant signals from the treatment/culture medium placed in the lower chamber. After certain time, removed the culture media from cell culture inserts and lower chamber, and fixed the cells with 4% PFA for 20 mins at room temperature and subsequently washed them twice with PBS. Transfer the cell inserts into new 24 well cell culture plate and incubate them with 0.1% crystal violet added in lower chamber for 15-20 mins at room temp. Removed the stain and washed with PBS twice to remove excessive crystal violet. Carefully removed the non-migrated cells from the inner surface of top culture inserts with cotton swab and observed the migrated cells under the microscope.Images

were captured at five fixed locations (right, bottom, left, up and centre), and migrated cells were counted by two experienced investigators blinded to the treatments.

4.15 Indirect immunofluorescent staining for cells.

For double immunofluorescent staining of NCKAP1 and Phalloidin-FITC, VSMCs transfected with miR-214 and NCKAP1 shRNA and/or respective controls were cultured in chamber slides and subject to different treatments (20%FBS and PDGF-BB). Upon reaching 60%-70% confluence, the treatment medium was discarded and cells were washed with PBS twice before fixing with 4% paraformaldehyde (PFA) at room temperature for 5-10 mins. Fixation immobilizes antigens while retaining cellular and sub cellular structures. After fixation, the cells were washed with PBS 3 times for 10 mins each before proceeding to permeablization process. Permeablization is required when antibody needs access to the inside of the cells to detect proteins. Permeablization was performed by treating the cells with Triton x100 (0.1% Triton x100 in PBS) for 10 mins at room temperature. The cells were then blocked by using 5% BSA in PBST (1x PBS + 0.2% Tween 20) for 1 hr at room temperature before incubating them with primary antibody NCKAP1 and rabbit isotype IgG control at a dilution of 1:500 in blocking buffer overnight at 4°C. Next morning, cells were washed with PBS three times, 10 mins each before incubating them with the appropriate secondary antibody (donkey anti-rabbit IgG) conjugated with CF 568 fluorescence (Sigma, in 1:400 dilution) and Phalloidin-FITC (P5282) according to manufacturer's instructions. After incubating cells with secondary antibody, in dark, for an hour, they were washes with PBS 3 times, 10 mins each and counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma). Finally the cells were mounted with fluoromount-G (Cytomation; Dako, Glostrup, Denmark).

Images were examined using SP5 confocal microscope with Plan-NEOFLUAR 63x objective lenses and Leica TCS Sp5 software (Leica, Germany) at room temperature, and were processed with Photoshop software (Adobe).

For detecting Ki-67-positive cells, VSM cells subjected to various treatments were fixed and blocked as described earlier and labelled with antibody against Ki-67 (Abcam), or rabbit isotype IgG control, and visualized using a donkey anti-rabbit IgG antibody conjugated with CF™ 568 fluorescence (Sigma, in 1:400 dilution).

For Phalloidin-FITC staining alone, similar procedure was applied involving separate setting of cells with different treatments as indicated in the figures. The VSMCs were fixed, blocked and labelled with Phalloidin-FITC (P5282) according to the manufacturer's instructions.

4.15.1 Immunofluorescent Staining for Sections.

Paraffin embedded sections of mouse aorta were numbered and subjected to immunohistological analysis with appropriate antibody. Before proceeding to staining protocol, the slides were deparaffinized and rehydrated by subjecting them through a process which includes treating the slides/tissue with Xylene to remove paraffin and then through ethanol concentration gradient (ranging from 100% ethanol to 50% ethanol) to rehydrate them and finally immersing the slides in cold water. Placed all section slides in a staining chamber to facilitate deparaffinization and rehydration step by ensuring equal treatment to all the sections. The slides holding tissues were kept in xylene for 2 times 5 mins each to get paraffin wax dissolve completely, followed by their transfer to ethanol concentration gradient chambers containing 100%, 90%, 70% and 50% ethanol. The slides were immersed in each chamber for 3-5 mins before finally immersing them in water for complete hydration. After the tissue is rehydrated, it should not be allowed to dry as drying out will cause non-specific antibody binding and hence high background staining. The tissue sections were then subjected to antigen retrieval process.

The antigen retrieval method serves to break the methylene bridges (may formed during fixation process) and expose the antigenic sites in order to allow antibodies to bind. Sodium citrate buffer (Sodium Citrate (10mM) 1.5gm, ddH2O 500ml, pH 6.0) were applied to help with antigen retrieval. The slide chamber was filled with sodium citrate solution and covered with covering film with holes in it to allow evaporation and to refill antigen retrieval solution. The slide chamber filled with sodium citrate solution was placed in microwave and set to full/maximum power until solution starts boiling. Boiled the slide chamber for 20 mins while constantly checking for evaporation and subsequently filling up with more antigen retrieval solution to avoid slides from drying. After 20 mins of heat treatment, took out the slide box and let it cooled at room temperature for 30 mins. The slides were washed then with 1x PBS 3 times, 5 mins each. All tissue sections were then blocked with 5% BSA in PBS (Sigma) for 1 hr at room temperature. After blocking, wash the slides again with PBS, 3 times 5 mins each and then encircle the tissue sections using PARA Pen. Primary antibodies (NCKAP1 or rabbit isotype IgG control) prepared in blocking solution were added in a drop wise manner on the tissue sections and incubated at 4℃ overnight.

Next morning, cells were washed with PBS three times, 10 mins each before incubating them with the appropriate secondary antibody (donkey anti-rabbit IgG) conjugated with CF 568 fluorescence (Sigma, in 1:400 dilution) and Phalloidin-FITC (P5282) according to manufacturer's instructions. After incubating cells with secondary antibody, in dark, for an hour, they were washes with PBS 3 times, 10 mins each. Sections were counterstained with DAPI (4',6-diamidino-2-phenylindole, Sigma) at a ratio of 1.1000 for 5mins and mounted with fluoromount-G (Cytomation;Dako, Glostrup, Denmark).

Images were examined using SP5 confocal microscope with Plan-NEOFLUAR 63x objective lenses and Leica TCS Sp5 software (Leica, Germany) at room temperature, and were processed with Photoshop software (Adobe).

For double immunofluorescent staining of NCKAP1 and Phalloidin-FITC,a separate setting of cells with different treatments as indicated in the figures were fixed and labelled with anti-NCKAP1, or rabbit isotype IgG control. After then, cells were visualized using a donkey anti-rabbit IgG antibody conjugated with CF™ 568 fluorescence (Sigma, in 1:400 dilution) and Phalloidin-FITC (P5282) according to the manufacturer's instructions. Cells were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; Sigma) and mounted in Fluoromount-G (Cytomation; DAKO, Glostrup, Denmark). Images were examined using SP5 confocal microscope with Plan-NEOFLUAR 63x objective lenses and Leica TCS Sp5 software (Leica, Germany) at room temperature, and were processed with Photoshop software (Adobe). For detecting Ki-67-positive cells, cells subjected to various treatments were fixed and labelled with antibody against Ki-67 (Abcam), or rabbit isotype IgG control, and visualized using a donkey anti-rabbit IgG antibody conjugated with CF™ 568 fluorescence (Sigma, in 1:400 dilution). For Phalloidin-FITC staining alone, another setting of cells with different treatments as indicated in the figures were fixed and labelled with Phalloidin-FITC (P5282) according to the manufacturer's instructions.

4.16 Proteomics studies.

4.16.1 Sample preparation for Proteomic analysis.

VSMCs transfected with control or miR-214 mimics were directly lysed in a urea-based lysis buffer [10ml buffer containing 9770 µL of 8M urea in 20mM HEPES, pH 8.0, 100 µL of 100 mM Na3VO4 (sodium orthovanadate), 20 µL of 500mM NaF (sodium fluoride), 10 µL of 1M β-glycerol phosphate, and 100 µL of 250mM Na2H2P2O7 (disodium pyrophosphate) and proteins were digested using trypsin. The enriched peptides were subjected to mass spectrometry.

4.16.2 Mass Spectrometry.

Protein-derived peptides were analysed by LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK) coupled to EASY-nLC (Proxeon, ThermoScientific). Peptide separation was performed in a C18 Pepmap reverse phase column (75 µm I.D, 3µm particle size; proxeon, Thermo-Fisher) using solution A (0.1% formic acid in liquid chromatography (LC)-MS grade water) and solution B (0.1% formic acid in LC-MS ACN) as mobile phases. Gradient runs from 2% to 30% in solution B for 100 min and from 30% to 60% for 10 min followed by a final 10 min wash at 85% in solution B. Full MS scans were acquired in the Orbitrap mass analyser over the range m/z 375–1500 with a mass resolution of 30,000. Tandem MS (MS/MS) was acquired using top seven data-dependent acquisition using high energy collision dissociation (40%). Gas phase fractionation method was applied to acquire MS/MS scans.

4.16.3 Peptide Identification by Database Search.

MS/MS data were converted to mgf files using Mascot Distiller (version 2.2) and searched against the 2012_03 databases of UniProt-TrEMBL (104,945 and 60,427 entries for *Homo sapien* and *Mus musculus* sequences,respectively) and UniProt SwissProt (20,249 *Homo sapiens* and 16,521 *Mus musculus* entries) and a decoy database using the Mascot search engine (version 2.2). The data was searched twice, restricting searches against human or mouse-specific sequences in each separate search. Significance of peptide identification was assessed by comparing results returned by searches against random and forward databases. Fold discovery rates at several cut-off values of Mascot scores and mass tolerances were used to calculate an empirical value of probability of random identification.

4.16.4 Data Analysis and Volcano Plot Analysis.

Relative quantification of peptides across experimental conditions was achieved by comparing peak heights of extracted ion chromatograms (automated by Pescal). The data were normalized

to the sum of all intensities derived from a sample (columns). When comparing peptide signals across different treatments, these were also divided by the average signals of such peptide across all the samples (rows). When comparing the effects of miR-214 over-expression on protein regulation, peptide signals were divided by those of the untreated control samples (control miRNA mimics). The *p* values of differences across treatments were obtained by means of a *t* test of log2 transformed fold changes and these were adjusted for multiple testing through the Benjamini-Hochberg procedure. To identify peptide sequences specific for mouse, peptides returned as positive identifications by Mascot were searched against the UniProt-TrEmbl database restricted to human sequences using a script written in Visual Basic to automate these searches. Failure to detect peptides from human searches in mouse database, indicated that these peptides were specific for mouse. The fold change was transformed using the log2 function, so that the data is cantered around zero, while the Benjamini–Hochberg corrected P value was −log10 transformed for volcano plot analysis.

4.17 Mouse Femoral Artery Denudation Injury and miR-214 Agomir Perivascular Delivery.

C57BL/6 mice were anesthetized and the surgical procedure performed was similar to that as described previously (Xiao, et al. 2006; Xiao, et al. 2014; Zeng, et al. 2006). Removal of the endothelium of the femoral arteries was achieved by 3 to 5 passages of a 0.25mm angioplasty spring wire (tips of cross-IT 200 × guide wire, Abbott laboratories). After the vascular injury, the injured femoral arteries were randomly received miR-214 or Cel-miR-67 agomir treatments, as described in our previous study (Chen, et al. 2015d). Briefly, after injury, 100µl of 30% pluronic gel containing chemically modified and cholesterol conjugated 2.5nmol miR-214 or scramble (Cel-miR-67) agomirs was applied perivascularly to the injured femoral arteries. The miRNA

agomirs were purchased from RiboBio (Guangzhou RiboBio Co, Ltd). The in vivo expression efficiency and stability of such agomirs have been documented extensively by many research groups worldwide. Additional femoral arteries were harvested at 3 days (for gene expression) or 14 days (for protein expression) after injury (3-5 femoral arteries from each group were pooled for each independent experiment, and triplicate experiment were conducted). Total RNAs including small RNAs and protein were extracted for RT-qPCR or western blotting analysis of miR-214, NCKAP1, or PCNA gene/protein expression in injured vessels. Our previous study (Chen, et al. 2015d) showed that perivascular delivery of 2.5nmol agomirs into each injured vessel generally resulted in 5-10 times higher expression level compared with control mice (received Cel-miR-67) or normalized target miRNA expression level in injured arteries to level similar to those in normal uninjured vessels. All animal experiments were performed according to animal (Scientific procedures) act of 1986 (United Kingdom) and all protocols were approved by the institutional committee for use and care of laboratory animals. In addition, the principles governing the care and treatment of animals, as stated in the guide for the care and use of laboratory animals published by the national academy of science (8^{th} edition,2011) were followed at all times during the study. All mice were euthanized by placing them under deep anaesthesia with 100% O_2 and 5% isoflurane, followed by decapitation.

4.17.1 Morphometric Analysis and Quantification of Lesion Formation.

All the procedures used in this study were similar to that described in our previous study (Chen, et al. 2015d). Briefly, the femoral arteries (~1.0μm from injury site) were harvested 4 weeks after the operation. The specimens were fixed in 4% formaldehyde for H&E staining. Sections (5μm) were collected at 100μm intervals (10sections per segment/interval), mounted on slides,

andnumbered. Six digitised sections with same identification number from three segments/intervals (~0.4μm, 0.5μm and 0.6μm from injury site) of each animal (e.g. IV-1/2, V-1/2, VI-1/2 represent the 1st and 2nd section of the 4th, 5th and 6th segment/interval, respectively) were stained with H&E for morphometric analysis. The procedure used for lesion quantification was similar to that described previously (Chen, et al. 2015d; Xiao, et al. 2006; Xiao, et al. 2014; Zeng, et al. 2006). Briefly, EEL (external elastic membrane), IEL (internal elastic membrane), lumen, media and neointimal areas were automatically measured on H&E stained cross sectional femoral artery segments using acomputerized image analysis system (pixel^2, Axiovision software) by two experienced investigators blinded to the treatments. Six sections were analyzed per vessel sample and averaged.

For immunofluorescence staining of NCKAP1 expression in the injured vessels, three digitised paraffin sections with same identification number from same segment/interval (e.g. IV-3, V-3, VI-3) of each animal were deparaffined with xylene and rehydrated with ethanol, and then incubated with 10mM sodium citrate at 100˚C for 10 minutes to retrieve antigens, followed by incubation with 1% bovine serum albumin (BSA) for 30 minutes.Thereafter, the sections were incubated with rabbit anti-NCKAP1 antibody (1:100 dilutions, Antibodies-online GMBH) or rabbit IgG control diluted in blocking buffer in a cold room (4°C) overnight. The tissue sections were then washed and subsequently incubated with a donkey anti-rabbit IgG antibody conjugated with CF™ 568 fluorescence (Sigma, in 1:400 dilution), followed by nuclei staining with 4,6-diamidino-2-phenylindole (DAPI) (1ug/ml). After mounting, the slides were examined using a laser scanning confocal microscope (Zeiss LSM 510 Mark 4) and Zen2009 image software. The mean fluorescence intensity (MFI) for red fluorescence signal from each section was measured with Image J pro software. Three sections were analyzed per vessel sample and averaged.

For immunohistochemistry analysis of PCNA-positive cells in the injured vessels, another set of paraffin sections (e.g. IV-5, V-5, VI-5) were deparaffined and prepared as described above, followed byan incubation with 3% H_2O_2, and then 10% goat serum (Dako). The sections were incubated with rabbit anti-PCNA antibody (1:500 dilutions) or rabbit IgG control diluted in blocking buffer in a cold room (4°C) overnight. The tissue sections were then washed and subsequently incubated with a goat anti-rabbit IgG antibody conjugated with HRP (1:400 dilution). Thereafter, sections were incubated with 3,3´-diaminodbenzidine (Dako), and followed by an incubation with hematoxylin solution. After mounting, the sections were examined using an All-in-One Fluorescence Microscope (BZ-X700, Keyence) and images taken using attached camera. Images were processed with Photoshop software (Adobe). The percentage of PCNA-positive cells over total cells within vessel wall were examined and calculated by two well-trained independent investigators blinded to the treatments. Three sections were analyzed per vessel sample and averaged.

4.18 Statistical Analysis.

Three to five independent experiments were performed for each experiment category (in vitro and in vivo). Results are presented as Mean $\pm$ SEM. Statistical analysis was performed using GraphPad Prism5 (GraphPad Software). The Shapiro-Wilk normality test was used for checking the normality of the data. Data with a Shapiro-Wilk test $P>0.05$ was considered to fit a normal distribution. A 2-talied unpaired Student *t* test was used for comparison between 2 groups, and a 1-way ANOVA test with a Bonferroni post hoc test was applied when >2 groups were compared if the data displayed a normal distribution. An $\alpha=0.05$ was chosen as the significance level, and *P* values of <0.001, <0.01, and <0.05 were considered statistically significant.

5 Results:

Mouse primary VSMCs were used for most of in vitro experiments, apart from three experiments in which human aortic smooth muscle cells (HAoSMC) were used to analyse the effect of miR-214 inhibitor on their proliferation and migration profile, mentioned in result section. **(Result 5.5).** Human aortic smooth muscle cells were purchased from Promocell GmbH (C-12533) and cultured in smooth muscle growth medium 2 (C-22062 PromoCell GmbH) according to the manufacturer instructions. Passage 5-10 VSMCs were used in this study.

5.1 miR-214regulation in VSMCs.

Various pathological stimuli including high concentration of serum (20%), PDGF-BB, oxidized Low density lipoproteins (LDL) or its components such as 4-Hydroxynonenal (4-HNE) and 7-ketocholesterol (7-Keto) have been extensively used to induce differentiated/contractile VSMC phenotype switching to a dedifferentiated/synthetic phenotypeafter serum starvation-induced cell growth arrest (Auge, et al. 2002; Chahine, et al. 2009; Salmon, et al. 2012; Torella, et al. 2011; Wang, et al. 2010). To examine the miR-214 expression during VSMC phenotype switching, under the effect of above mentioned stimuli, serum starved VSMCs were treated with various stimuli and harvested at different time intervals. As expected, both PDGF-BB and 20%FBS significantly down-regulated miR-214 levels in murine VSMCs (**Figure 14A**) after 3-24hr of treatment compared with untreated control cells at 0hr. 4-Hydroxynonenal (4-HNE) is a major active product formed following the oxidation of n-6 polyunsaturated fatty acids. 4-HNE is highly lipophilic and can interfere with the functions of many proteins by forming adducts. 4-HNE is associated with the generation of reactive oxygen species and hence its levels increases significantly in many diseases such as atherosclerosis and diabetes (Chapple, et al. 2013; Selley

1997). Physiological levels of 4-HNE in human plasma range between 0.3-0.7 µM (Selley, et al. 1989) which under pathological conditions increases significantly to 20µM or even higher concentrations (100µM) have been reported in areas suh as plasma membrane (Chapple, et al. 2013; Esterbauer, et al. 1991). 4-HNE levels in plasma also serve as markers of oxidative stress (Chapple, et al. 2013). To analize effect of 4-HNE and 7-Keto on miR-214 expression in VSMCs, different group of cells were cultured in 1%DMSO (vehicle control), 4-HNE (10µM) and 7-Keto (10µM each) as described in our previous study (Chen, et al. 2013a). The dosage of 4-HNE was carefully selected to avoid the undesirable effects of cellular apoptosis whereas creating suitable inflammatory conditions to stimulate cellular responses based on our own experience and previous publications (Pidkovka, et al. 2007). As expected, decreased miR-214 expression was observed with 4-HNE and 7-Keto stimulation compared to vehicle control (cells in 1%DMSO) (**Figure 14C**). Above data suggest a role for miR-214 in VSMC functions, such as proliferation and migration.

To evaluate the expression of miR-214 in Human VSMCs under these stimuli (20% serum and PDGF-BB), human aortic SMCs (hAoSMCs) were subject to above mentioned stimuli treatment. RT-qPCR data (**Figure 14B)** showed a similar response i.e. miR-214 expression level was significantly reduces by both serum and PDGF-BB, confirming a similar regulatory role of serum and PDGF-BB on miR-214 expression in human VSMCs. Cellular viability after above treatments were observed by physically monitoring the cell morphology and by using Annexin V kit followed by FACS analysis.

Furthermore, as miRNA activity depends upon 2 major factors; its synthesis within nucleus (transcription) and maturation in cytosol (biogenesis), so we wondered at which step does the serum and PDGF-BB effects and downregulated miR-214 activity. To differentiate between these two mechanisms, we initially examined miR-214 primary transcript activity by conducting RT-

qPCR analysis with specific primers. Interestingly, the expression level of primary miR-214 was significantly downregulated by serum and PDGF-BB treatment as shown in RT-qPCR analysis **(Figure 14D)** indicating that these pathological stimuli regulate miR-214 activity at transcriptional level.

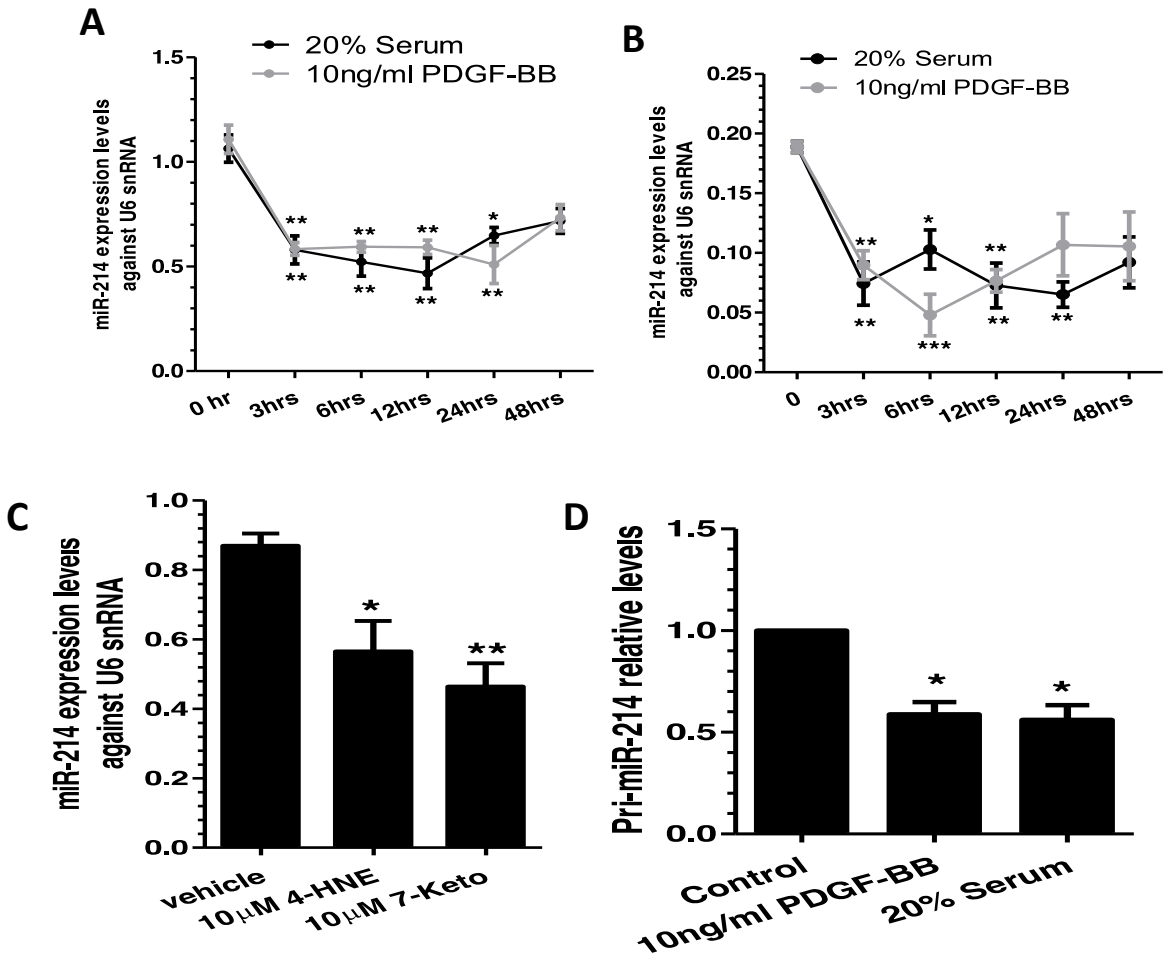


Figure 14. miR-214 was significantly down-regulated in response to various pathological stimuli in Murine VSMCs.

A-C miR-214 is regulated by different pathological stimuli. Murine VSMCs **(A and C)** and human aorta smooth muscle cells (hAoSMC)**(B)** were subjected to serum starvation for 24 hours, followed by four different stimulations. Cells treated with20% serum, 10ng/ml PDGF-BB **(A,B)** were harvested at indicated times, whereas 4-hydroxynonenal (4-HNE) and 7-Ketocholesterol (7-keto) treatments were applied for 24 hrs before harvesting cells and readings were compared with the cells cultured in 1% DMSO (Vehicle). Total RNAs including small RNAs were extracted, and subjected to RT-qPCR (reverse transcriptase quantitative PCR) analyses with specific miR-214 forward primer and universal miRNA reverse primer. Data presented as mean ±SEM (circles and error bars respectively **(A and B)** and as mean ±SEM (bars plus error bars) in **(C)** of 5 independent experiments (n=5).*P<0.05, **<0.01 and ***<0.001; (different treatment groups were compared versus 0hr or vehicle). **(D)** Transcriptional inhibition of miR-214 by PDGF-BB and serum as evident from down-regulation of miR-214 primary transcript. Serum starved VSMCs were treated with 20% serum and PDGF-BB for 3 hrs followed by collection of cells and extraction of total RNA and subsequent RT-qPCR analysis. Data presented as mean ± SEM (bars plus error bars) of 3 independent experiments n=3 *P<0.05,

5.2 PDGF-BB and Serum Down-regulate miR-214 via inhibition of transcription factor Twist-1.

Previous research has documented that miRNA synthesis and maturation is controlled by 2 molecular events; transcription and biogenesis. To differentiate between these two mechanisms, we conducted RT-qPCR analysis with specific primers to examine the miR-214 primary transcript. RT-qPCR data showed that the expression level of primary miR-214 was significantly reduced by serum and PDGF-BB **(Figure 14D)** indicating that miR-214 is regulated by these inflammatory mediators at the transcriptional level. Lee *et al (Lee, et al. 2009)* had reported that miR-214 expression is regulated by transcription factor Twist-1 via an E-box promoter element during development. Therefore, we wonder if similar mechanism was responsible for miR-214 inhibition by PDGF-BB and serum. To address this issue, we generated 2 mouse miR-214 gene promoter plasmids, one containing E-box promoter element termed as pGL3-miR-214-FL and the other devoid of this promoter region was termed as pGL3-miR-214-short, by using similar strategy to that reported by Lee *et al.* **(Figure 15A)**

It has been mentioned in the literature that miR-214 is hypoxia inducible miRNA which can be regulated in a HIF1α dependent or independent pathway but considering our experimental design, it seem unlikely that HIF1α might have been involved in miR-214 regulations as we cultured VSMCs in normoxic conditions.

The murine VSMCs were transfected with these plasmids and cultured in normoxic conditions in incubator at 37℃ for 48h under the PDGF-BB and serum treatment before performing luciferase activity assay as described above. Data showed that the luciferase activity was significantly decreased in VSMCs transfected with pGL3-miR-214-FL under the effect of serum and PDGF-BB (**Figure 15C, 15D)**, whereas no such inhibitory effect was observed in VSMCs transfected with

pGL3-miR-214-Short reporter (lacking E-box), suggesting E-box is critical to perform such inhibitory action. The data demonstrates that miR-214 expression in VSMCs is regulated by various stimuli through a transcriptional mechanism and that E-box element is required for such action. As expected, the expression level of Twist-1 was also dramatically reduced by serum and PDGF-BB (**Figure 15B)** suggesting that these 2 pathogenic stimuli down-regulate miR-214 expression in VSMCs by inhibiting the transcription factor Twist-1.

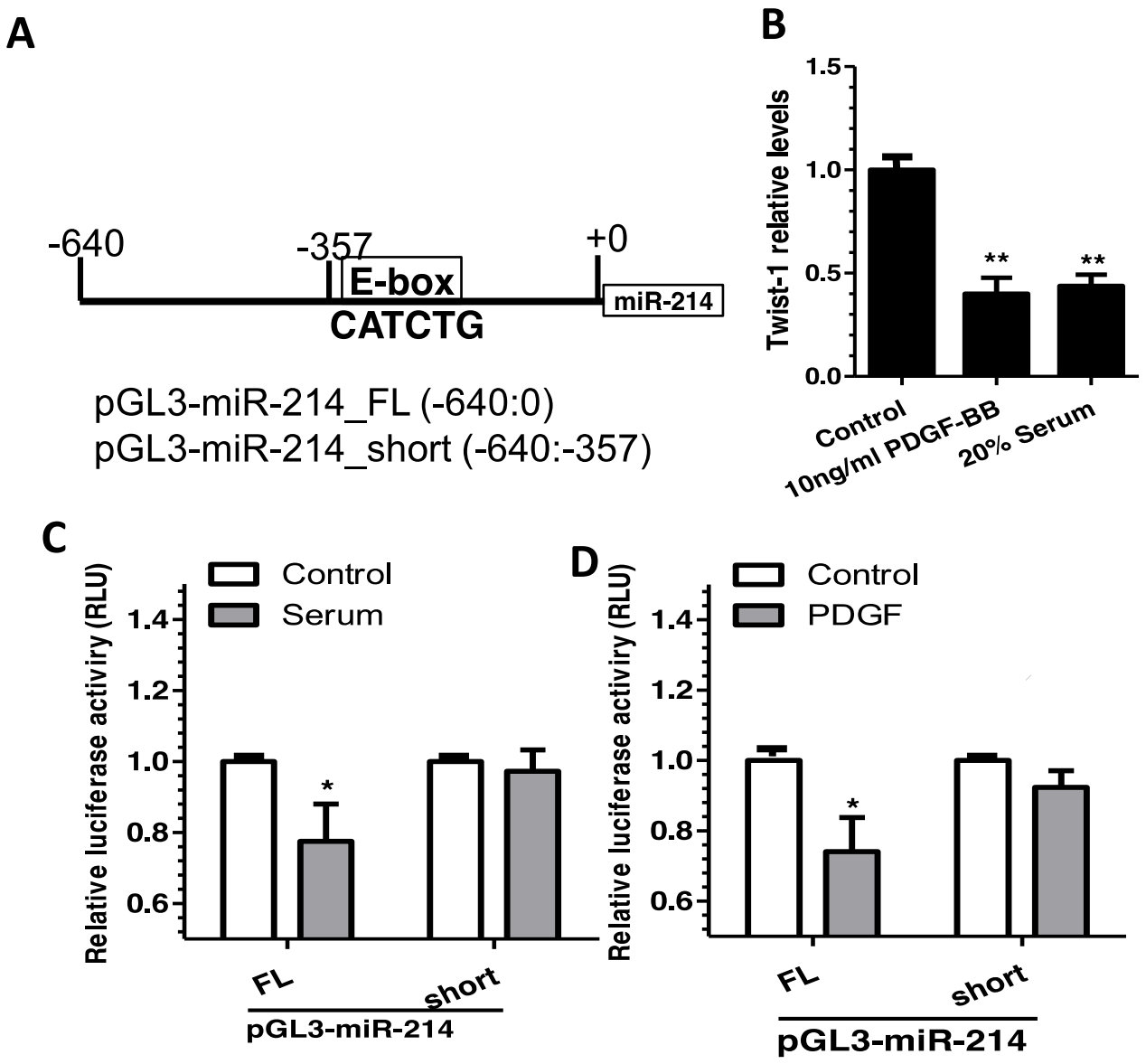


Figure 15. Transcription factor Twist-1 is crucial for miR-214 modulation by PDGF-bb and Serum.

(A) Schematic diagram of mouse miR-214 gene promoter reporter. (**C** and **D)** The E-box element is required for miR-214 gene inhibition. Murine VSMCs transfected with respective reporters harbouring the full length (pGL3-miR-214-FL; -640:0) or truncated form (pGL3-miR-214-short; -640:-357, lacking E-box element) of the miR-214 gene promoter were subjected to serum starvation for 24hrs followed by treatment with 10ng/ml PDGF-BB and 20% serum for 3 hrs. Cell lysate was harvested and subjected to luciferase activity assay. Data presented as mean ±SEM (bars plus error bars) of 6 independent experiments *<0.05 compared with control (n=6). (**B**) Twist-1 expression is inhibited by serum and PDGF-BB in VSMCs. Twist-1 expression levels were examined by RT-qPCR. Data presented as mean ±SEM of 3 independent experiments.**<0.01 compared with control (n=3).

5.3 miR-214 modulates VSMC proliferation.

As mentioned previously, VSMC proliferation/growth and accumulation within intima has been recognised as a major early stage event contributing to development and progression of atherosclerotic lesion and postangioplasty restenosis. We wondered if miR-214 could play a role in VSMC proliferation. To address this question, gain of function and loss of function experiments were performed by transfecting VSMCs with miR-214 mimics, inhibitor and respective controls. miRNA mimics or inhibitors are small, synthetic double stranded RNA molecules designed to mimic endogenous mature miRNA or inhibit its activity by binding to its target miRNA molecule. A complete complimentarity between miRNA inhibitor and its target results in inhibition of miRNA's activity as well as cleavage and degradation of miRNA in a manner similar to the function of siRNA. miRNA mimics and inhibitors can be introduced into the cells using transfection or electroporation. To evaluate VSMCs proliferative profile, actual cell number counting and BrdU incorporation assay was performed as described earlier. Specifically, VSMCs cultured in complete culture medium were transfected with miR-214 mimics or negative control overnight to evaluate the potential effects of miR-214 overexpression on VSMC proliferation. The final concentrations of miRNA (mimics and inhibitor) for VSMC transfection was optimised in our previous study, in which we observed a final concentration of 25nM miRNAs could result in significant miRNA over-expression and inhibition in VSMCs but cause a minimal unwanted side effects such as cell apoptosis (Yu, et al. 2014; Zhao, et al. 2015). Expectedly, the expression levels of miR-214 in VSMCs were significantly up-regulated after transfection with miR-214 mimics (**Figure 16A).** Compared with control treatment, an increased BrdU incorporation (**Figure 16B**) and much higher total cell number (**Figure 16C**) were observed in both PDGF-BB and 20%FBS treatments. Importantly, miR-214 over-expression significantly inhibited both serum and PDGF-BB induced VSMC proliferation. To further confirm the role of miR-214 in VSMC proliferation,

loss-of-function experiments were conducted using miR-214 inhibitor in VSMCs cultured under similar conditions. As shown in **Figure 16D**, over 50% miR-214 inhibition was achieved in VSMCs by using miR-214 inhibitor. Consistent with miR-214 over-expression (gain of function) experiment, inhibition of miR-214 significantly increased PDGF-BB and serum induced VSMC proliferation, as observed by manually counting cell number and applying BrdU assay, respectively (**Figure 16E and 16F**), supporting a role for miR-214 in VSMC proliferation.

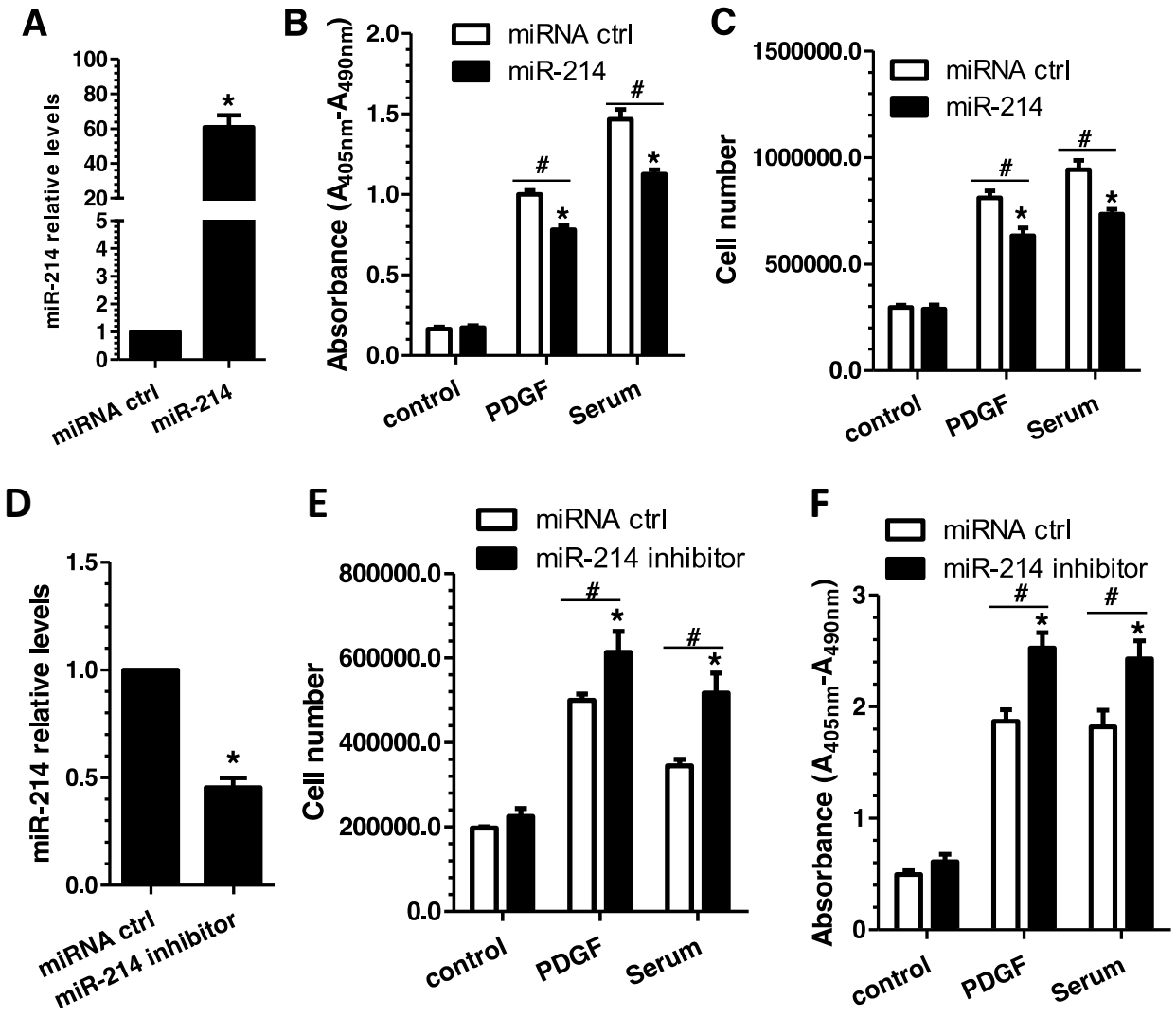


Figure 16. miR-214 modulates VSMC proliferation.

Murine VSMCs were transfected with miR-214 mimics **(A-C),** miR-214 inhibitor **(D-F),** or respective negative control miRNAs, followed by 24 hours serum starvation. After then, cells were induced to growth by PDGF-BB or serum for further 48 hours, followed by BrdU (bromodeoxyuridine incorporation) assays **(B and F)** and cell counting **(C and E),** respectively. Meanwhile, cells were harvested to examine the miR-214 expression levels by RT-qPCR analyses. Data present here as mean +SEM, are average of three to four independent experiments (n=3-4). *P<0.05 (miR-214 mimics or inhibitor versus negative control miRNA); #P<0.05 (treatments versus vehicle).

5.4 miR-214 down-regulates VSMC migration.

Increased VSMC migration into the atheroma is another major contributor to the development and progression of atherosclerotic lesion and post-angioplasty restenosis. To investigate if miR-214 also plays a role in VSMC migration, murine VSMCs were transfected with miR-214 mimics, inhibitor and their respective negative controls overnight, followed by cell migration assays (wound healing and trans-well experiments as described earlier) under similar stimulatory conditions (20% serum and PDGF-BB). The cell migration data showed a similar pattern as of cell proliferation towards miR-214 over-expression and inhibition **(Figure 17).** Percentage of denuded area compared to covered (in wound healing experiment) gives us information about cellular migratory response and is calculated by measuring the denuded area at 0hr (A0) and at specific time point (AT), get the difference between two time points and then divided it by the denuded area measurement at 0hr(A0) followed by percentage. The migratory tendency of VSMCs transfected with miR-214 mimics were significantly lower than their counterparts in control group in response to both PDGF-BB and serum stimulations as observed in wound healing **(Figure 17A)** and trans-well migration **(Figure 17B)** assays**.** On the contrary, the migraotry capacity of cells transfected with miR-214 inhibitor was clearly higher than that of control samples as evident from wound healing **(Figure 17C)** and trans-well migration **(Figure 17D)** experiment data, suggesting that miR-214 inhibits VSMC migration. Cellular viability after above treatments (miR-214 inhibitor transfection and inflammatory mediators) was observed by physically monitoring the changes in cell morphology.

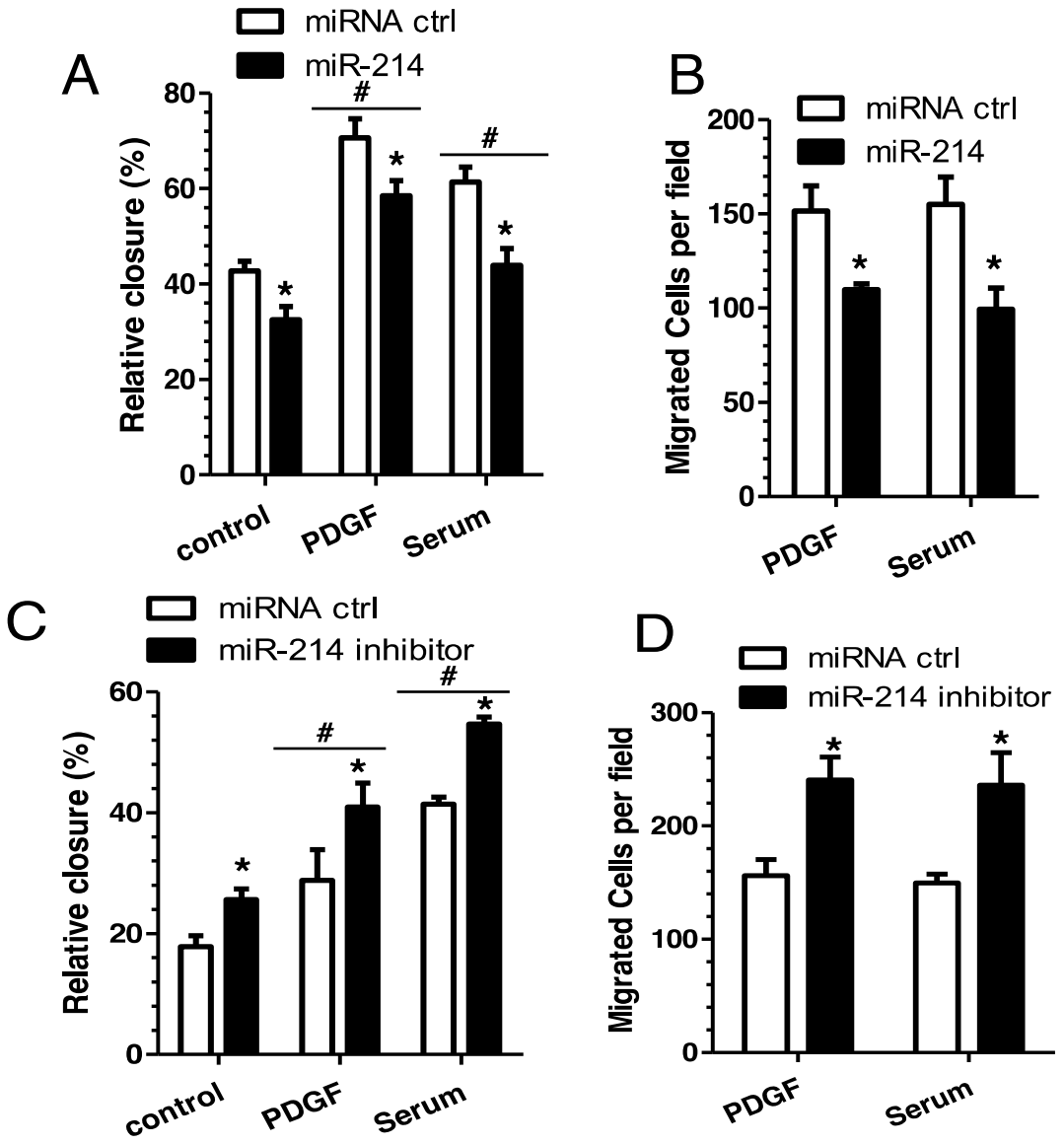


Figure 17. miR-214 regulates SMC migration.

Murine VSMCs were transfected with miR-214 mimics **(A-B),** miR-214 inhibitor **(C-D),** or respective negative control miRNAs, followed by 24 hours serum starvation. After then, cells were subjected to wound-healing **(A and C)** and transwell migration **(B and D)** assays in the presence of PDGF-BB or serum stimulation for another 24hrs, respectively. The percentage of cell closure (%) in panels **A** and **C** was calculated as the percentage of denuded area difference between 0 hour (A0) and indicated time points (AT) [or (A0-AT)/A0*100]. Data presented here as mean+SEM, are average of three to four independent experiments (n=3-4). *P<0.05 (miR-214 mimics or inhibitor versus negative control miRNA); #P<0.05 (treatments PDGF-BB and serum versus control).

5.5 miR-214 modulates Human VSMC Proliferation and migration.

To investigate whether miR-214 also plays a pathological role in human VSMCs, similar (miR-214 loss of function) experiments were performed using human Aortic SMCs and analysing their proliferation by BrdU assay and migration capacity by transwell experiment. Three independent experiments were performed using Human aortic smooth muscle cells (HAoSMC) at different passages to analyse the effect of miR-214 inhibitor on their proliferation and migration profile. Human aortic smooth muscle cells isolated from were purchased from Promocell GmbH (C-12533) and cultured in smooth muscle growth medium 2 (C-22062 PromoCell GmbH) according to the manufacturer instructions. The human aortic SMCs were cultured under normal conditions and transfected by miR-214 inhibitor and respective control overnight, followed by serum strvation and treatment with PDGF-BB and high concentration serum (20%) stimulation. Serum starvation helped in minimizing the interference of many unknown stimulators which may present in serum as well as keeping cells in G0 phase of cell cycle, so that the cellular response would be an indicative of the specific treatment. RT-qPCR data showed an over 50% miR-214 inhibition was achived using miR-214 inhibitor in human VSMCs **(Figure 18A)**. Expectedly, as evident from the results that (BrdU incorporation assay) VSMC proliferation was significantly enhanced by miR-214 inhibition under PDGF-BB and serum stimulation **(Figure 18B)**. A similar pattern was observed interms of VSMC migration **(Figure 18C).**

Untill now it was clear that miR-214 has important role in modulating VSMC functions during inflammation and atherosclerosis.

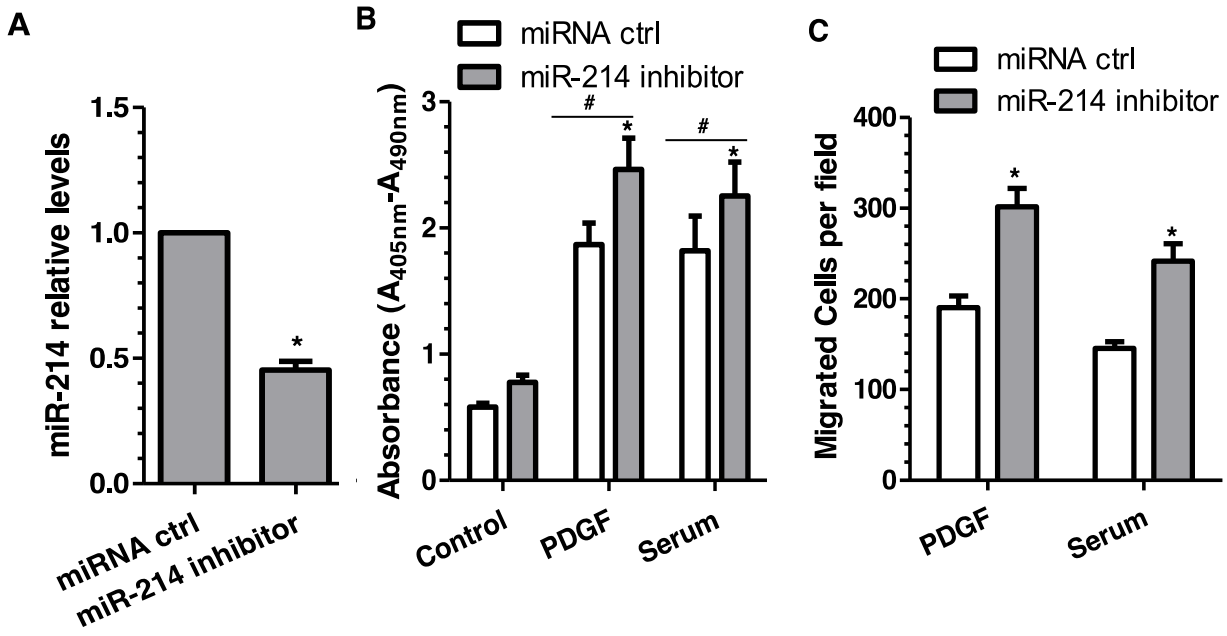


Figure 18. Effect of miR-214 on Human VSMC's Proliferation and Migration.
miR-214 inhibitor and respective negative control were transfected into human aortic smooth muscle cells, followed by 24hrs of starvation. Serum starved cells were treated with PDGF-BB (30ng/ml) and Serum (20%) for another 48hrs, followed by RT-qPCR to examine miR-214 expression level **(A),** bromodeoxyuridine incorporation assay **(B)** and transwell migration analysis **(C)** in 2 different settings. Data presented here is mean+ SEM of 3 independent experiments (n=3) *P<0.05 (miR-214 inhibitor versus negative control miRNA) #P<0.05 (treatments PDGF-BB and serum versus control).

5.6 Proteomics analysis reveals miR-214 modulates NCKAP-1 level.

Till this stage, it was quite clear that miR-214 influences VSMCs proliferation and migration, so our next objective was to investigate the underlying genes/ proteins and the molecular mechanism through which miR-214 performs its inhibitory role in VSMCs. To this end, we applied two methods. First, we conducted protomics analysis of whole cell lysate of murine VSMCs transfected with miR-214 mimics and subjected to mass spectrometer for quantitative proteomics analysis. Second, we applied computational algorithmic prediction tools to predict possible target genes for miR-214.

To conduct proteomics analysis, Murine VSMCs were first transfected with miR-214 mimics or negative control and then lysed to obtain total protein contents, which were then subjected to label-free quantitative proteomics analysis. miR-214 over-expression in transfected VSMCs was confirmed using RT-qPCR **(Figure 19A).** By applying 25% of change as cut-off value, 219 proteins were observed to be modulated by miR-214 over-expression. Among them 59 proteins were down regulated and 160 proteins were up-regulated (**Table 16**). As its widely established that miRNAs perform their function (post transcriptional regulation) by binding to 3'UTRs of target RNAs which results in RNA cleavage, degradation and/or silencing, so we consider only those proteins which were down-regulated by over-expression of miR-214 as potential targets.

Interestingly, Gene Ontology (GO) term enrichment analysis of the down-regulated proteins showed that actin filament polymerization was the highest enriched functional/biological process and was inhibited by miR-214 over expression in VSMCs (**Table 15**). We also observed that most of these down-regulated proteins (28 of 59) are under the GO terms of 'regulation of cell migration'(e.g.,NCKAP1, EMAL1, LIMS2, SPI2), 'proliferation' (e.g.,EMAL1, LIMS2, LTOR3),

'adhesion' (e.g., SNX5, SNX12, LIMS2), 'actin filament reorganisation and actin polymerization' regulating proteins (e.g.,NCKAP1, ARPC2, CAPZB), 'Cell cycle'(e.g.,APC7, ARL3, ULA1), and 'gene expression regulator' proteins (e.g.,KLF14, SMYD5, and LONM), respectively, thus further confirming a role of miR-214 in VSMC proliferation and migration.

Our next step was to further examine the gene sequence to identify possible binding sites for miR-214 among these down-regulated proteins/genes by utilizing several computational algorithmic databases, such as RNA22 (https://cm.jefferson.edu/rna22/), DIANA-microT-CDS(http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=MicroT_CDS/index), TargetScan7.1(http://www.targetscan.org/mmu_71/), miRanda-rel2010(http://www.microrna.org/microrna/getGeneForm.do), and miRDB4.0 (http://mirdb.org/cgi-bin/search.cgi). We identified one or more miR-214 binding sites within 3'UTR of 39 (out of 59) genes that its protein expression levels have been decreased by miR-214 over-expression, presenting potential target genes for miR-214. As such, these proteins likely represent good candidates as the functional direct target genes of miR-214 in VSMCs.

As it is unrealistic to assess whether all identified 39 downregulated genes with predicted binding sites are direct target of miR-214 in current project so to further concise the data, volcano plot was applied to identify the most-meaningful change in our large proteomic dataset. As shown in the volcano graph, with stringent criteria and P <0.05, NCKAP1, also known as NAP1 or NAP125 was identified as the most important target gene for miR-214 in VSMCs **(Figure 19B)(Table 14).** NCKAP1 is 129kDa, type II transmembrane protein transcribed by NCKAP1 gene. NCKAP1 is an important constitutive component of the WAVE2 complex that regulates actin filament polymerisation hence plays crucial role in lamelliopodia (cell membrane protrusions) formation

and regulating cellular migration. Interestingly, computational algorithmic models DIANA-microT/RNA22 showed three binding sites for miR-214 (binding site2 is predicted to be highly conserved across > 10 species including human, whereas binding site 1 and 3 are conserved in mouse and rat) within 3'UTR of NCKAP1 **(Figure 20A).** Moreover, a favourable minimum loop-free energy (dH: -136.5, -143.1 and -150.2 kcal/mol for binding site 1, 2 and 3, respectively) in the formation of the miR-214:NCKAP1 3'UTR duplex stem-loop for all three binding sites was observed by using mFold software (http://mfold.rna.albany.edu/?q=DINAMelt/Two-state-melting) **(Figure 20B).**

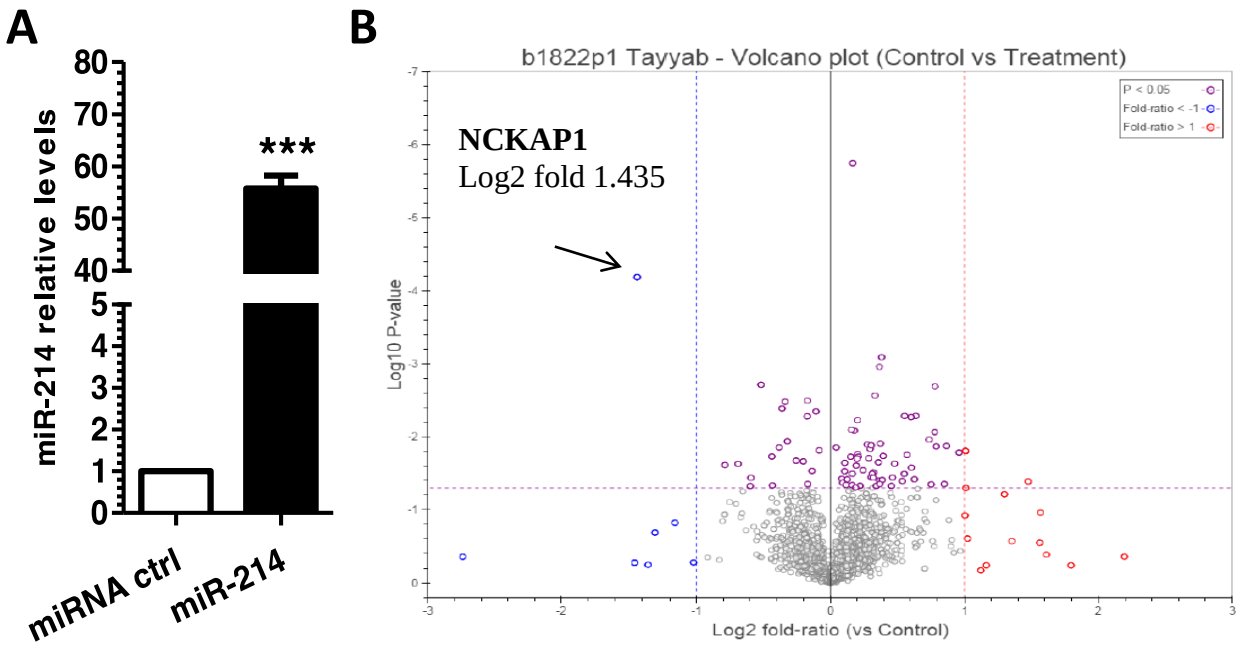


Figure 19. Proteomics analysis: Identification of NCKAP1.

Murine VSMC were transfected with miR-214 mimics. After 48 hours of transfection, cells were lysed directly in urea based lysis buffer containing Na_3VO_4 (sodium orthovanadate),NaF (sodium fluoride),β-glycerol phosphate, and $Na_2H_2P_2O_7$ (disodium pyrophosphate)] and proteins were digested using trypsin. Transfected cells were also examined for miR-214 expression levels by RT-qPCR analyses **(A). (B)** The enriched peptides were subjected to mass spectrometry**.** The fold change was transformed using the log2 function, so that the data is centred around zero, while the Benjamini–Hochberg corrected P value was −log10 transformed for volcano plot analysis versus protein ratio of miR-214 mimics/control (log_2) of all 1594 proteins fulfilling strict quantitation criteria (Red, 14 upregulated proteins with 2-fold changes; Blue, 7 downregulated proteins with 2-fold changes; Purple, significantly changed but such change is <2-fold on miR-214 overexpression ANOVA with P<0.05). NCKAP1 emerged as the most down-regulated protein along with greatest significance value.

Downregulated Proteins	LogFR (Treatment Vs Control)	Fold change (Treatment vs Control)	Upregulated Protein	LogFR (Treatment Vs Control)	Fold change (Treatment vs Control)
PEPD	-2.7379	0.1498	PSB7	0.3232	1.2511
ABL2	-1.4546	0.3648	DPOE1	0.3235	1.2513
LSM8	-1.4546	0.3648	CALX	0.325	1.2527
NCKAP1	-1.4349	0.3698	COPZ1	0.3261	1.2537
HYPK	-1.3531	0.3914	VPS29	0.3275	1.2548
COX2	-1.3027	0.4053	NASP	0.3283	1.2555
EMAL1	-1.1601	0.4474	HNRPK	0.3286	1.2558
SYAP1	-1.0218	0.4924	RTCB	0.3289	1.256
SPTB1	-0.9188	0.5289	UBL4A	0.3299	1.2569
ILEUC	-0.8274	0.5635	H12	0.3303	1.2573
LYPA2	-0.806	0.5719	PHB2	0.3327	1.2593
KLF14	-0.793	0.5771	SC61B	0.3353	1.2616
ARPC2	-0.7868	0.5796	CNPY2	0.3391	1.265
PGM1	-0.7861	0.5798	TRXR1	0.3406	1.2663
PPM1F	-0.7534	0.5931	QCR1	0.3414	1.267
ABHEB	-0.7046	0.6135	RFA2	0.3418	1.2673
TPP1	-0.6898	0.6199	UBE2K	0.3436	1.2689
SNX12	-0.6707	0.6281	NUCB2	0.3483	1.2732
CIRBP	-0.6599	0.6329	ALR	0.3494	1.274
IDHC	-0.6531	0.6358	TIA1	0.3523	1.2766
INT3	-0.632	0.6452	TBC15	0.3538	1.2779
LIMS2	-0.5997	0.6598	LSM6	0.3567	1.2805
SNX5	-0.5951	0.6619	IMPA2	0.357	1.2807
SMYD5	-0.5722	0.6725	VATA	0.3607	1.2841

Table 14. Proteomics data. List of 25 upregulated and downregulated Proteins by miR-214 in VSMCs.

Note: Proteins/genes with Red/yellow-highlighted have been most down regulated by miR-214 mimics whereas Proteins in Green were among most upregulated ones.

Analysis Type:	**PANTHER Overrepresentation Test (release20160321)**						
Annotation Version and ReleaseDate:	**GO Ontology database Released 2016-04-23**						
Analyzed List:	Down-regulated(Musmusculus)						
ReferenceList:	Musmusculus(all genesindatabase)						
Bonferroni correction:	TRUE						
GO Biological process Complete	**Mus musculus-REFLIST (22320)**	**Proteins (56)**	**Proteins (expected)**	**Proteins (over/under)**	**Proteins (fold Enrichment)**	**Proteins (P-value)***	**Protein name**
Regulationof actin filament Polymerization (GO:0030833)	137	6	0.34	+	17.46	1.05E-02	NCKPA1,RHOA,ARPC2, SPTB1,PROF1,CAPZB
Regulationof Protein Polymerization (GO:0032271)	174	7	0.44	+	16.03	2.29E-03	NCKPA1,RHOA,ARPC2, SPTB1,PROF1,CAPZB,
Regulationof Actin Polymerization Or depolymerization (GO:0008064)	156	6	0.39	+	15.33	2.21E-02	NCKPA1,RHOA,ARPC2,
Regulationof actin filament Length (GO:0030832)	157	6	0.39	+	15.23	2.30E-02	NCKPA1,RHOA,ARPC2,
Regulationof Actin Cytoskeleton Organization (GO:0032956)	278	7	0.7	+	10.04	4.99E-02	NCKPA1,RHOA,ARPC2, SPTB1,PROF1,CAPZB,
Organelle Organization (GO:0006996)	2779	21	6.97	+	3.01	1.30E-02	NCKAP1,RHOA,STK25, SNX12,PLSI,THIM,TPP1, CATD,EMAL1,ARPC2, PPM1F,PROF1,ABL2, SMC3,ARL3,SNX5,

Table 15. GO term enrichment analysis of down-regulated proteins by miR-214 in VSMCs.

A

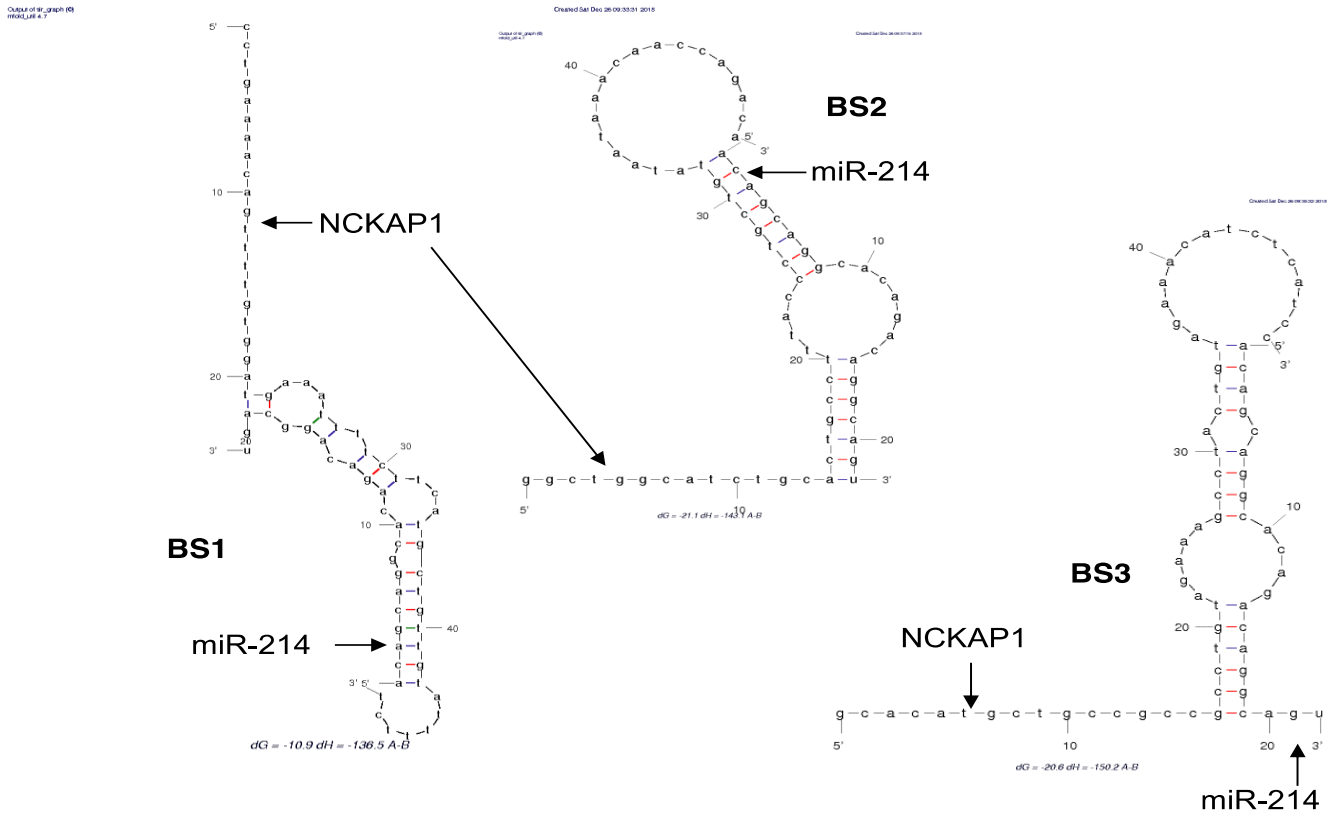
Mouse NCKAP1 (NM_016965.3), 3UTR: 3605~4403

Binding site-1 (BS1) - minimum loop-free energy (RNAhybrid):-51.803
3661 ggaactattt taccttaaag cctgaaaaca gttttgtgga tgaaattttc ttcatgctgt
miR-214: 3'-tgacggacagacac----------ggacgaca-5'
BS1^{mut}: taccttaaag cctgaaaaca gttttgtgga tgaaattttc ttAaCgcGTG
Mlu I (ACGGCT)

Binding site-3 (BS3) - minimum loop-free energy (RNAhybrid): -51.80
4141 tccgaggttc tcagacagca gcacatgctg ccgccgcctg tagaaagcct actgtagaaa
miR-214: 3'-tgacggaca-gacacgga cgaca-5'
BS3^{mut}: tgctgccgccgcACgtagaaaCgcGtacACtagaaa
Mlu I (ACGCGT)

Binding site-2 (BS2) - minimum loop-free energy (RNAhybrid): -51.801
4201 catctcatcc cgtgctgccc ggctggcatc tgcactgcct ttac cctgctgtataataaa
miR-214: 3'-tgacg-gacagacac-ggacgaca-5'
BS2^{mut}: ggcatc tAcactgcct ttac AACgcGTGataataaa
Mlu I (ACGCGT)

B



mFold: http://mfold.rna.albany.edu/?q=DINAMelt/Two-state-melting

Figure 20. Three potential wild type binding sites (BS1-BS3) of miR-214 within NCKAP1 3'UTR. **(A)**As predicted by RNAhybrid and their mutants (BS1/2/3^{mut}). **(B)**The formation of the miR-214:NCKAP1 3'UTR (spanning through miR-214 BS1-3) duplex stem-loop and the minimum loop-free energy for individual loop (binding site) (*http://mfold.rna.albany.edu/?q=DINAMelt/Two-state-melting*)were calculated by and extracted from mFold software.

5.7 Identification of NCKAP1 as a functional target of miR-214.

As NCKAP1 appears as a prominent target for miR-214, we wonder how does it respond to the effect of inflammatory mediators (PDGF-BB and serum). At first, we studied NCKAP1 gene expression in murine VSMCs treated with the atherogenic stimuli like PDGF-BB and serum (20%). As expected, RT-qPCR results showed up-regulated expression of NCKAP1 gene under the effect of these pathological stimuli **(Figure 21A**). Our next step was to check NCKAP1 gene expression levels under the effect of miR-214 over expression and inhibition. miRNA mimics or inhibitors are small, synthetic double stranded RNA molecules designed to mimic endogenous mature miRNA or inhibit its activity by binding to its target miRNA molecule. A complete complimentarity between miRNA inhibitor and its target results in inhibition of miRNA's activity as well as cleavage and degradation of miRNA in a manner similar to the function of siRNA. For this purpose, VSMCs were transfected with 25nM of miR-214 mimics/ inhibitors or respective controls overnight as described earlier. After transfection, murine VSMCs were cultured for 48hr before harvesting them for RT-qPCR and protein expression (western blot). Data from Western blot analyses further confirmed the proteomics data that NCKAP1 protein level was significantly down-regulated by miR-214 over-expression **(Figure 21B).** As expected, NCKAP1 gene expression levels were significantly down-regulated when the cells were transfected with miR-214 mimics (miR-214 over expression) and were up-regulated under the inhibition of miR-214 in VSMCs **(Figure 21C and 21D).** Data also showed a reduction in the miR-214 levels under the effect of miR-214 inhibitor which not only inhibited miRNA-214 funtions by binding to miR-214 but also lead to reduction in its levels by showing a precisely complimentarity and subsequent degradation of miR-214 in a siRNA manner. These data clearly imply that NCKAP1 is the specific target of miR-214 which is inversely regulated in VSMCs under miR-214 over-expression.

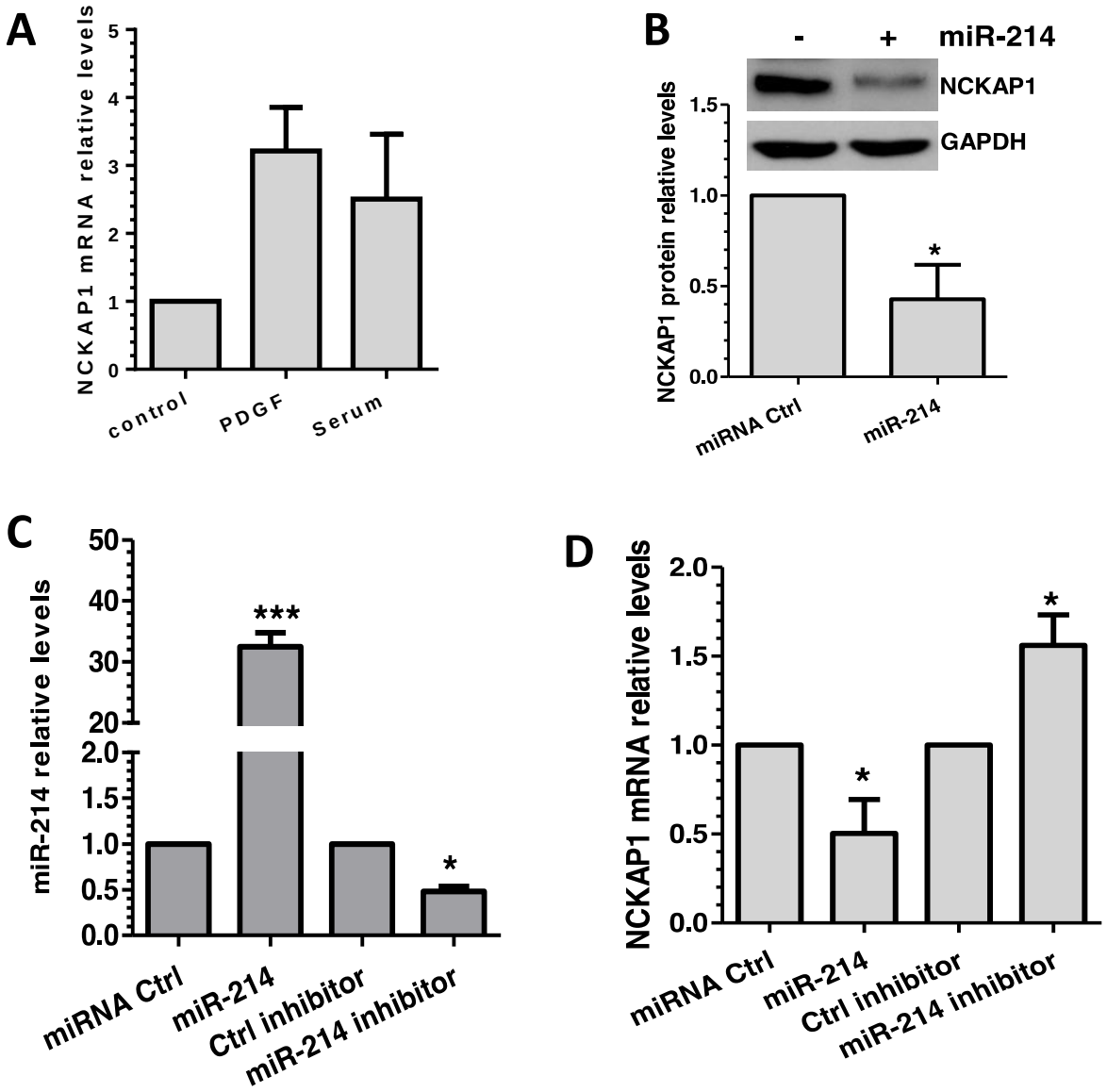


Figure 21. miR-214 negatively regulates NCKAP1 gene expressions in Murine VSMCs.

(A) Levels of NCKAP1 gene expression were upregulated under the influence of inflammatory mediators 20% Serum and PDGF-BB. **(B)** NCKAP1 protein levels were downregulated in VSMCs by miR-214 over expression as shown in western blot. **(C and D)** Effects of modulation of miR-214 expression levels in VSMCs on NCKAP1 gene expression.VSMCs were transfected with miR-214 mimics (miR-214) or inhibitor (miR-214 inhibitor) (final conc. 25nM), or respective negative controls. Total RNAs were harvested and subjected to RT-qPCR analyses with indicated primers. Data presented is representative **(B)** or show mean ±SEM of 3-4 independent experiments (n=3-4), ***<0.001, *<0.05, compared with control miRNA.

5.8 MiR-214 directly regulates NCKAP1 through its binding sites.

As mentioned previously, three binding sites for miR-214 were identified in the 3'UTR of NCKAP1 gene with favourable minimum loop free energy **(Figure 20)**. To determine if miR-214 can directly regulate NCKAP1 and to identify the main binding site among three binding sites (identified using computational data) responsible for NCKAP1 gene repression by miR-214, the 3'UTR of NCKAP1 containing all the three binding sites was cloned into a luciferase reporter. In addition to this, four luciferase reporters each carrying mutation at one binding site (single mutation) named as $BS1^{mut}$ (Binding site mutation 1), $BS2^{mut}$ (Binding site mutation2), or $BS3^{mut}$ (Binding site mutation3) as well as for a combination of three binding sites (combinational $BS1/2/3^{mut}$ mutant reporters), were also generated (according to the guidelines of QuikChange Site-Directed Mutagenesis Kit, Agilent Technologies) to identify the best binding site for miR-214 to perform its function. Luciferase analysis data from our miRNA reporter assay showed that the activity of luciferase from construct harbouring the wild-type NCKAP1 3'UTR, but not the mutants, was significantly inhibited by miR-214 over-expression indicating that all three binding sites are important for NCKAP1 3'UTR reporter activity repression mediated by miR-214 **(Figure 22).** Taken together, these data have clearly demonstrated that NCKAP1 is a true mRNA target of miR-214, which is negatively regulated by miR-214 in VSMCs.

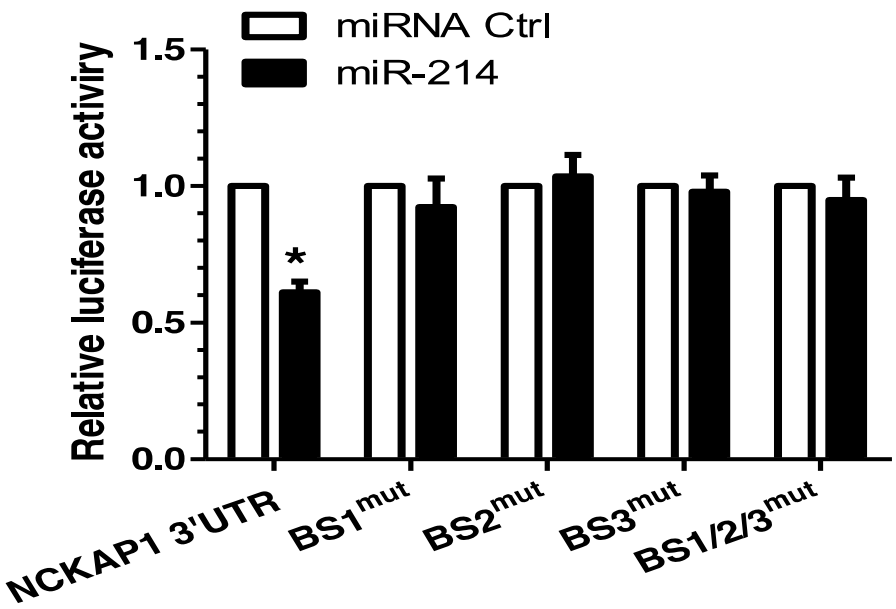


Figure 22. All Binding sites within the 3'-UTR are required for NCKAP1 gene repression by miR-214. Murine VSMCs were seeded in flasks coated with 0.04% gelatin and cultured for 24hrs in Culture Medium before transfection. According to the co-transfection protocol, miR-214 mimics or negative control were co-transfected into VSMCs with wild type NCKAP1 3'UTR reporter, or the indicated single/ combined binding site mutants [bindings site 1 (BS1mut), 2 (BS2mut), 3 (BS3mut), or the combinational mutations (BS1/2/3mut) respectively, for 14-16hrs, followed by medium change to normal CM and the luciferase activities were measured at 48 hrs post transfection. The luciferase activities of the four indicated mutants [pmiR-Luc-NCKAP1-BS1, pmiR-Luc-NCKAP1-BS2, pmiR-Luc-NCKAP1-BS3 and pmiR-Luc-NCKAP1-BS1/2/3] were analysed with over expression of miR-214. All three binding sites are important for miR-214 mediated NCKAP1 gene repression. The data presented here are the mean ± SEM of three to four independent experiments. (n=3-4) *P < 0.05 (treatment vs. control). The ratio of luciferase activity to β-galactosidase (β −gal) activity in the control samples was set at 1.0.

5.9 Effect of miR-214 on SMYD5 and KLF14.

miRNAs are regarded as master regulator as single miRNA can regulate multiple function by targeting many genes and so was observed in our proteomic data, that 59 proteins/genes have been downregulated by miR-214 overexpression. Out of these 59 genes, 39 genes are predicted to have miR-214 binding site in their 3'UTRs as per the algorithmic computational models. To further assess other candidate genes among these 39 downregulated genes with predicted miR-214 binding sites, we selected KLF14 and SMYD5 as possible direct target genes of miR-214 in the context of VSMCs. KLF14 is a member of Kruppel-like factors family of transcription factors and regulate the transcription of various genes including type II receptor of TGFβ (TGFβRII) (Truty, et al. 2009). In addition to that, KLF14 also plays major role in regulating multiple inflammatory diseases like coronary artery disease, Diabetes type II, and hypercholesterolemia (Grarup, et al. 2010; Voight, et al. 2010). The reason for selecting SMYD5 since it is a powerful epigenetic regulator with methyltransferase activity. Moreover, we observed multiple miR-214 binding sites within 3'UTR of KLF14 and SMYD5, suggesting a regulatory role for miR-214 in these two gene expression.

The 3'UTRs of KLF-14 and SMYD5 (containing 2 and 4 miR-214 binding sites respectively) were cloned into luciferase reporter (pmiR-Luc) and transfected into VSMCs along with miR-214 mimics or respective negative control for 14-16hrs. Cells were harvested after 48hrs and subjected to luciferase activity assay. No significant reduction was observed in the luciferase activity with the construct harbouring KLF14 3'UTR, suggesting that miR-214 does not directly represses KLF-14 but rather does its activity in an indirect manner (probably by interacting with another epigenetic factor whose inhibition or over-expression results in KLF-14 repression) **(Figure 23A)**. Importantly, the luciferase activity of the SMYD5 3'UTR construct was significantly

inhibited by miR-214 over expression, confirming it as another target gene for miR-214 (**Figure 23B).**

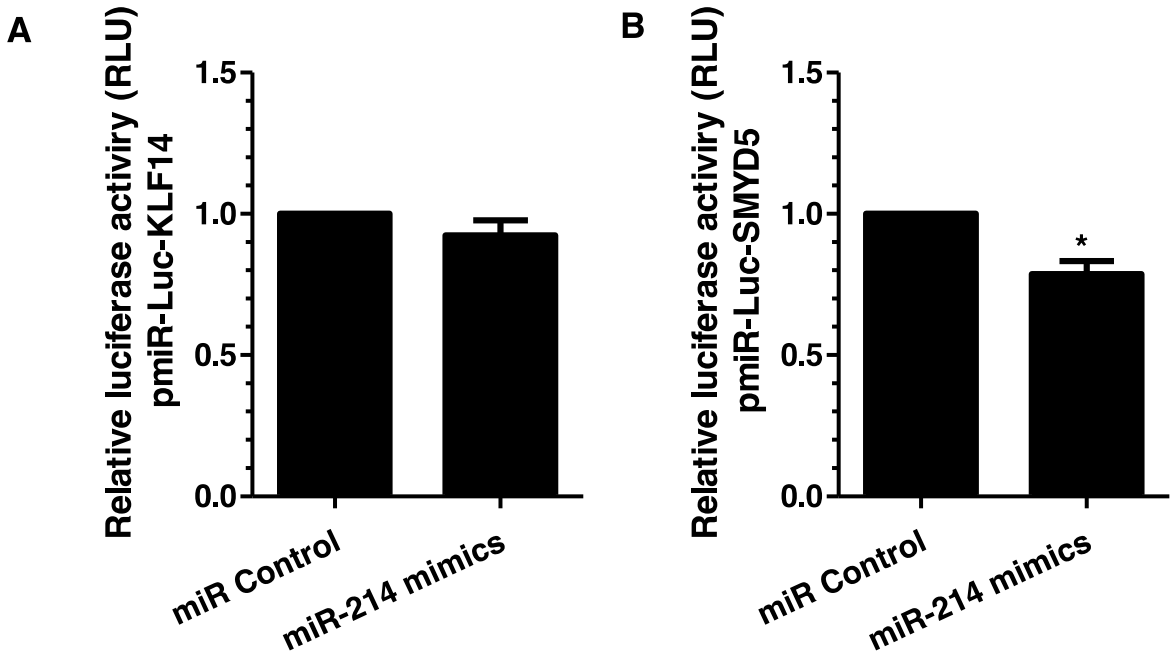


Figure 23. KLF-14 and SMYD5 regulation in Murine VSMCs by miR-214.
Murine VSMCs were seeded in flasks coated with 0.04% gelatin and cultured for 24hrs in Culture Medium before transfection.miR-214 mimics or negative control were co-transfected into VSMCs with KLF14 3'UTR reporter **(A),** or with SMYD5 3'UTR reporter **(B)** (pmiR-Luc-KLF14, and pmiR-Luc-SMYD5) respectively, for 14-16hrs. The luciferase activities were measured at 48hrs post transfection. The data presented here are the mean ± SEM of three to four independent experiments. *P < 0.05 (treatment vs. control). The ratio of luciferase activity to β-galactosidase (β −gal) activity in the control samples was set at 1.0.

5.10 Crosstalk between miR-214 and miR-34a.

Our group have reported that another miRNA, miR-34a also plays an important role in modulating VSMC functions and neointima formation through targeting the Notch1 gene (Chen, et al. 2015c). We wondered if there was any overlap or crosstalk between miR-214 and miR-34a in terms of target gene regulation. To address this issue, we designed an experiment to evaluate the regulatory effect of miR-214 on Notch1 (reported target gene for miR-34a) and vice versa. We performed co-transfection experiments involving miR-214 mimics with Notch1 3'UTR reporter and miR-34a mimics along with NCKAP1 3'UTR reporter in VSMCs. The cells were harvested 48hrs post transfection and subjected to luciferase activity analysis. The data revealed that the Notch1 3'UTR reporter activity was unaffected by miR-214 over-expression **(Figure 24)**. Similar phenomenon was observed in terms of NCKAP1 3'UTR reporter as miR-34a over expression didn't have any pronounced effect on its activity.

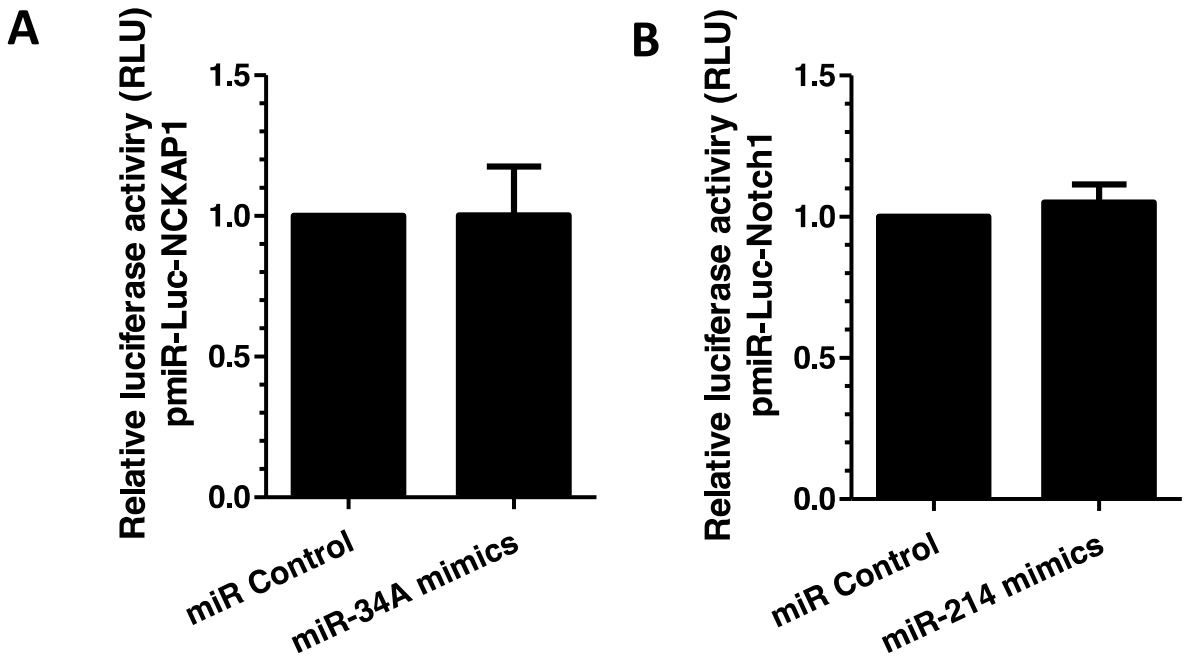


Figure 24. No cross-talk between miR-34a and miR-22 in Murine VSMCs.

Serum starved Murine VSMCs were co-transfected with miR-34a and miR-214 mimics/negative control along with NCKAP1 (**A,** pmiR-Luc-NCKAP1) and NOTCH1 reporter (**B**, pmiR-Luc-Notch1) respectively. Cells were lysed 48hrs post transfection and luciferase activity was measured. No significant effect was observed on reporter activity in either group under the influence of miR-34a **(A)** or miR-214 **(B)** suggesting an independent mechanism of action for both microRNAs in VSMCs. Data presented here are mean ±SEM of four to six independent experiments (n=4-6) P *<0.05 versus miR control.

5.11 Effect of NCAKAP1 knockdown on Murine VSMC functions.

Till now, these results have confirmed NCKAP1 mRNA as a legitimate target of miR-214 in murine VSMCs. NCKAP1 is an important constitutive component of the WAVE2 complex that regulates actin filament polymerisation. NCKAP1 plays crucial role in lamelliopodia (cell membrane protrusions) formation, thus regulating cellular migration.To further analyse/evaluate the role of NCKAP1 in VSMC migration and proliferation process, NCKAP1 knockdown stable cell lines were generated using NCKAP1 shRNA lentivirus. RT-qPCR **(Figure 25A)** showed over 60% NCKAP1 inhibition efficiency was achieved in NCKAP1 knockdown stable VSMCs which was comparable to NCKAP1 levels under miR-214 mimics overexpression. Compared to control, NCKAP1 knockdown stable cell lines exhibited a significant reduction in cell proliferation as demonstrated by cell counting **(Figure 25B)** and cell proliferation marker (Ki-67) staining **(Figure 25C)**. Similar effect was observed with miR-214 over-expression in VSMCs **(Figure 25B and 25C)**. As expected, similar pattern was demonstrated in terms of cell migration profile (low migratory profile in NCKAP1 knockdown stable cell line) as analysed by trans-well migration assay **(Figure 25D).** Furthermore, no significant difference was observed between two groups (miR-214 mimics and NCKAP1 knockdown cells) in terms of inhibition/reduction of VSMCs proliferation and migration confirming comparable effects of miR-214 over expression to NCKAP1 silencing on VSMC functions.

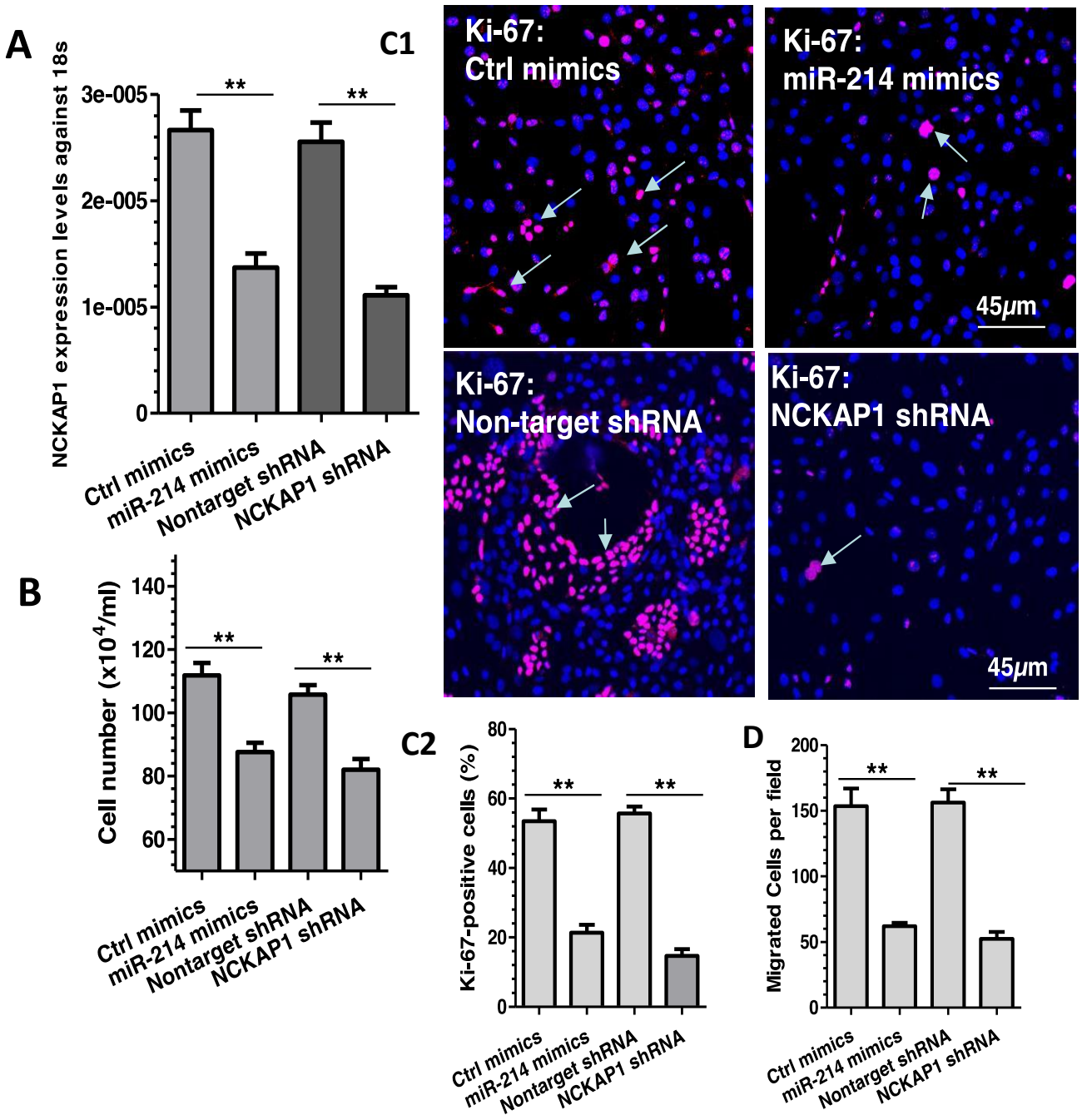


Figure 25. NCKAP1 knockdown in Murine VSMCs recapitulates the effects of miR-214 over-expression on cell migration and proliferation.

Murine VSMCs were infected with non-target or NCKAP1 short hairpin RNA (shRNA) virus, followed by serum starvation (0.5%) and restimulation with 20% serum for 24hrs. Cells were subjected to RT-qPCR **(A),** cell counting**(B)** and immunofluorescence staining with antibody against Ki-67 **(C).**miR-214 overexpression or NCKAP1 knockdown inhibited VSMC proliferation **(B & C)** and Migration **(D). C1**, representative images; **C2**, quantitative data from three independent experiments (n=3). In another set of experiments, (n=3) mVSMCs were transfected with control or miR-214 mimics or infected with nontarget or NCKAP1 shRNA virus, serum-starved cells were subjected to trans-well migration assays in the presence of 30ng/ml PDGF-BB **(D).** Bar graphs are quantitative data (mean±S.E.M.) from three independent experiments (n=3). **P<0.01 (compared with control mimics or non target shRNA, respectively). In C1, white arrows indicate Ki-67 positive cells, and the cell clusters positive for Ki-67 staining in Non-target shRNA group indicate actively proliferating VSMCs.

5.12 miR-214 and NCKAP1 modulate VSMC migration and proliferation through modulating Actin polymerization.

As aforementioned, NCKAP1 is reversely regulated by miR-214 in VSMCs, and NCKAP1 plays a crucial role in cell migration and proliferation through its role in actin cytoskeleton rearrangements and lamelliopodia formation. It is plausible that miR-214 and/or NCKAP1 modulate VSMC migration and proliferation by regulating actin polymerization and/or lamelliopodia formation. Such effect can be examined by observing changes in actin polymerization and resultant lamellipodia formation under the influence of miR-214 over-expression. To address this, VSMCs were transfected with miR-214 mimics and respective control, and subjected to immunofluorescence staining with NCKAP1 antibody and phalloidin-FITC (a high affinity F-actin probe conjugated to FITC) as described in previous chapter at page 158 (**Materials and methods 4.15**). Confocal microscopy showed that typical lamellipodia was formed in VSMCs transfected with control miRNA mimics whereas such characteristic was inconspicuous in VSMCs transfected with miR-214 mimics **(Figure 26A).** Furthermore data from immunofluorescence staining with NCKAP1 antibody showed very little expression for NCKAP1 protein in VSMCs transfected with miR-214 mimics as compared to control, which supports our notion that miR-214 exerts its action by inhibiting NCKAP1 expression. When NCKAP1 knockdown stable VSMC lines were subjected to immunofluorescence staining with NCKAP1 antibody and Phalloidin-FITC, a similar phenomenon in terms of actin polymerization and/or lamellipodia formation was observed in VSMCs with NCKAP1 knockdown that showed a much lower level of actin polymerization as displayed by a weaker F-actin staining intensity in these cells **(Figure 26B).** Interestingly, NCKAP1 was observed to co-localize with F-actin within lamellipodia and membrane ruffles that faces the direction of cell movement in the control cells whereas such

assembly was disrupted in the cells transfected with miR-214 mimics or in NCKAP1 knockdown VSMC lines, indicating that NCKAP1 inhibition can recapitulate the effects of miR-214 over expression on actin polymerization and cell migration. Above data beautifully demonstrated that miR-214 regulates VSMC functions (proliferation and migration) by inhibiting/repressing NCKAP1 expression, thus leading to less lamelliopodia formation and lower actin polymerization, resulting in reduced cell migration and proliferation.

An interesting finding is the higher proportion of NCKAP1 localized in nucleus of control VSMCs infected with shRNA lentivirus (marked as red staining in B) as compared to VSMCs transfected with control miRNA mimics (A), which will be discussed later in chapter 6 (Discussion).

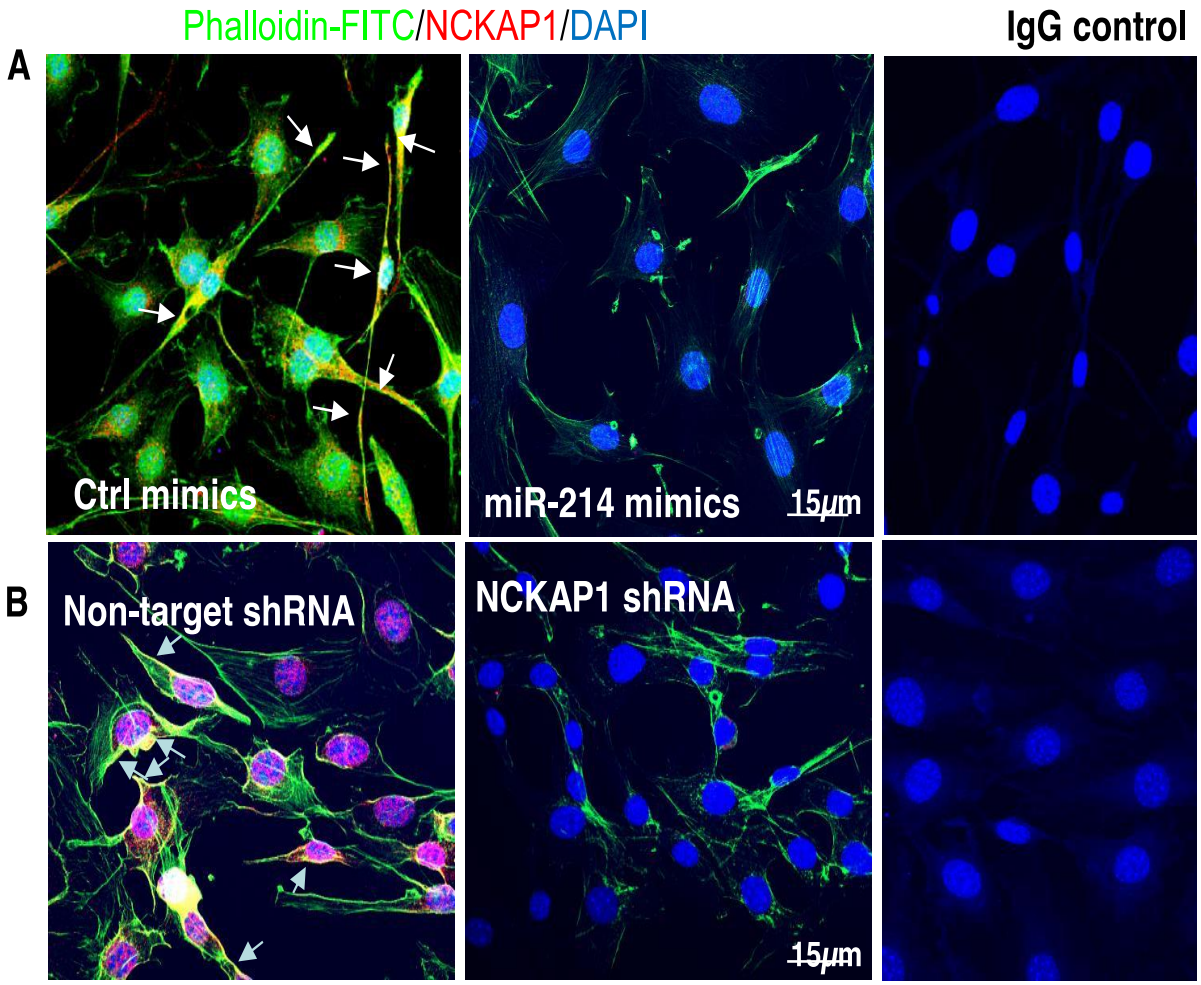


Figure 26. NCKAP1 knockdown in Murine VSMCs recapitulates the effects of miR-214 over-expression on actin polymerization.

(A) Actin polymerization in VSMCs was inhibited by miR-214 over-expression. Murine VSMC were transfected with miR-214 mimics/control, followed by serum starvation for 24 hrs.Serum-starved cells were treated with 20% serum for another 24 hours and subjected to immunofluorescence staining with antibody against NCKAP1 and Phalloidin-FITC (F-actin staining). **(B)** NCKAP1 knockdown decreased actin polymerization in VSMCs. VSMCs were infected with non-target or NCKAP1 shRNA virus, followed by similar treatment and analyses as described in **(A).** Representative images from three independent experiments (n=3) were presented here. Note: white arrows **(A and B)** indicate that NCKAP1 co-localizes with F-actin within lamellipodia and membrane ruffles, which are facing the direction of cell movement.

5.13 NCKAP1 inhibition is required for miR-214 mediated VSMC growth and motility.

We further reasoned that inhibiting miR-214 promotes cell migration and proliferation via interacting with NCKAP1, so we investigated if such interactionis required for VSMC functions regulated by miR-214. To address this aspect, co-transduction experiments were conducted in VSMC using miR-214 inhibitor, NCKAP1 short hairpin RNA (shRNA) lentivirus and respective controls. Following transfection, the cells were serum starved for 24hrs before treating them with serum and PDGF-BB (30ng/ml) and subjected to immunofluorescence staining with antibody against Ki-67, F-Actin staining by using phalloidin-FITC, VSMCs proliferation (cell counting) and transwell migration assays. Levels of both miR-214 and NCKAP1 were down-regulated after treatment with miR-214 inhibitor orNCKAP1 shRNA lentivirus, respectively, as shown by RT-qPCR data **(Figure 27A).** In control VSMCs, treatment with miR-214 inhibitor resulted in an increase in NCKAP1 levels, which were abolished in NCKAP1 knockdown cell lines even under the effect of miR-214 inhibition.Furthermore, expression level of cell proliferation gene PCNA (proliferating cell nuclear antigen) was upregulated in VSMCs treated with miR-214 inhibitor but such expression was down-regulated in NCKAP1 knockdown cell lines **(Figure 27A).** Interestingly, PCNA gene activation by miR-214 inhibition was also blunted by NCKAP1 shRNA. Consistently, cell proliferation was up-regulated as evident from cell number **(Figure 27B)** and Ki-67 staining assay **(Figure 27 C1 and C2)** under the effect of miR214 inhibition but down-regulatedin NCKAP1 knockdown cell lines. Importantly, suppressing NCKAP1 expression in VSMCs transfected with miR-214 inhibitor almost abolished the promoting effect of miR-214 inhibition on VSMC proliferation, suggesting that miR-214 inhibits VSMC proliferation through repression of NCKAP1. Similar results were observed for VSMC migration as demonstrated by trans-well experiments

(Figure 27 D1 and D2). Moreover, phalloidin-FITC staining showed lower levels of actin polymerization as evidenced by a weak staining in NCKAP1 shRNA group as compared to the control and miR-214 inhibitor treated group, and the inhibitory effectof NCKAP1 knockdown on actin polymerization was almost restored by miR-214 inhibition **(Figure 27E),** supporting the notion that miR-214 exerts its inhibitory effects on VSMC proliferation and migration through NCKAP1 repression.

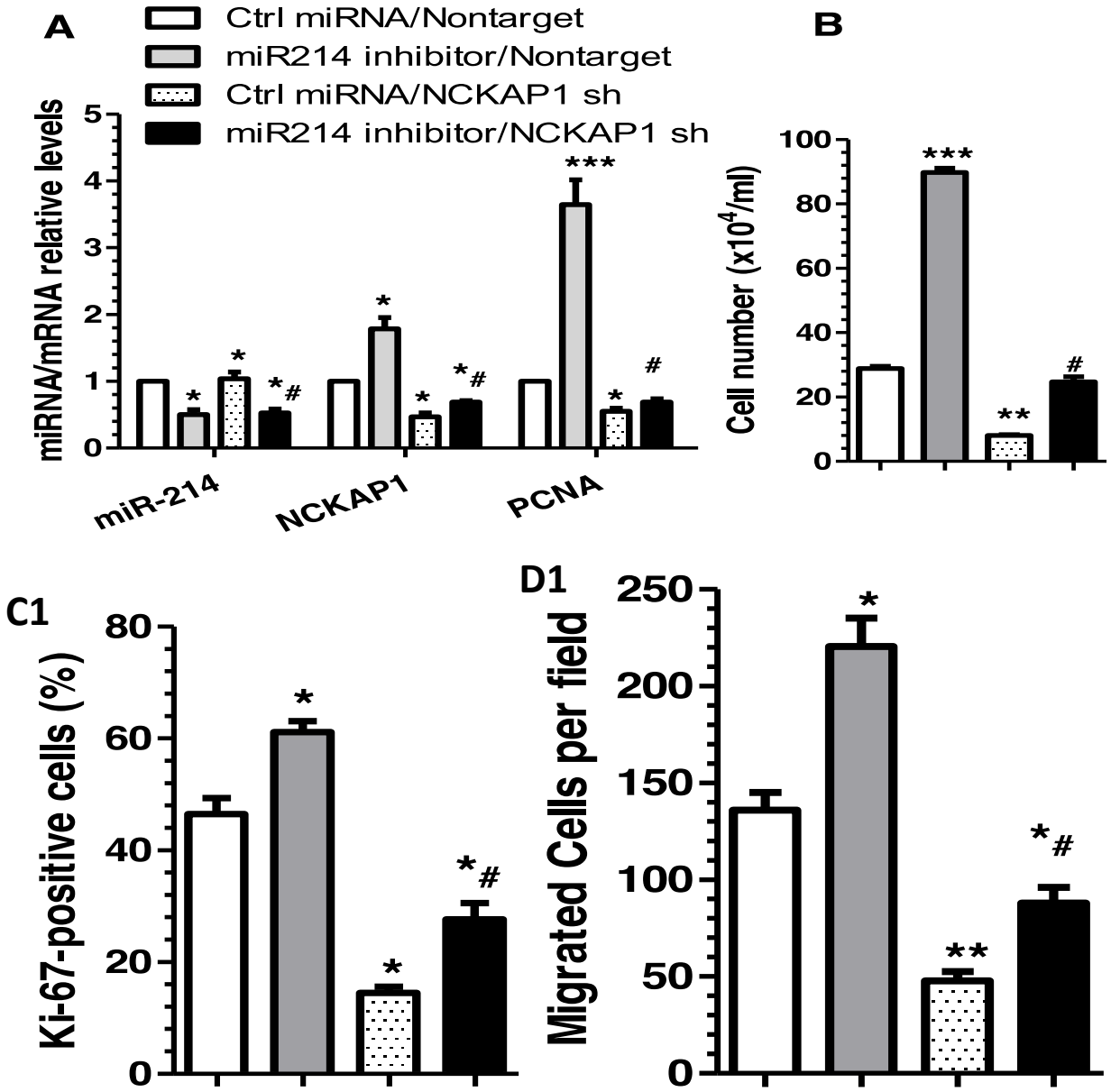


Figure 27. miR-214 mediates VSMC (Murine) growth, motility and actin polymerization through modulating NCKAP1.

Murine VSMCs were co-transduced with miR-214 inhibitor, NCKAP1 shRNA lentivirus (NCKAP1 sh), and/or respective controls (Control miRNA and/or non-target shRNA) as indicated. Transduced cells were serum-starved for 24 hours, followed by treatment with 20% serum **(A-C)** and 30ng/ml PDGF-BBfor trans-well migration **(D),** respectively.Cells were subjected to RT-qPCRanalysis for examining miR-214, NCKAP1 and PCNA (proliferating cell nuclear antigen) gene expression level **(A),** Cell counting **(B),** Immunofluorescence staining for Ki-67 **(C1 and C2)**,trans-well migration **(D1 and D2)** and F-actin staining using Phalloidin-FITC **(E).**Data presented are representative images **(C2, D2, and E**) or mean+SEM **(A,B, C1 and D1)** from three independent experiments (n=3). *<0.05, **<0.01, ***<0.001 (compared various treatments with double control, #P<0.05 (compared NCKAP1 knockdown with control in VSMCs with miR-214 inhibition).

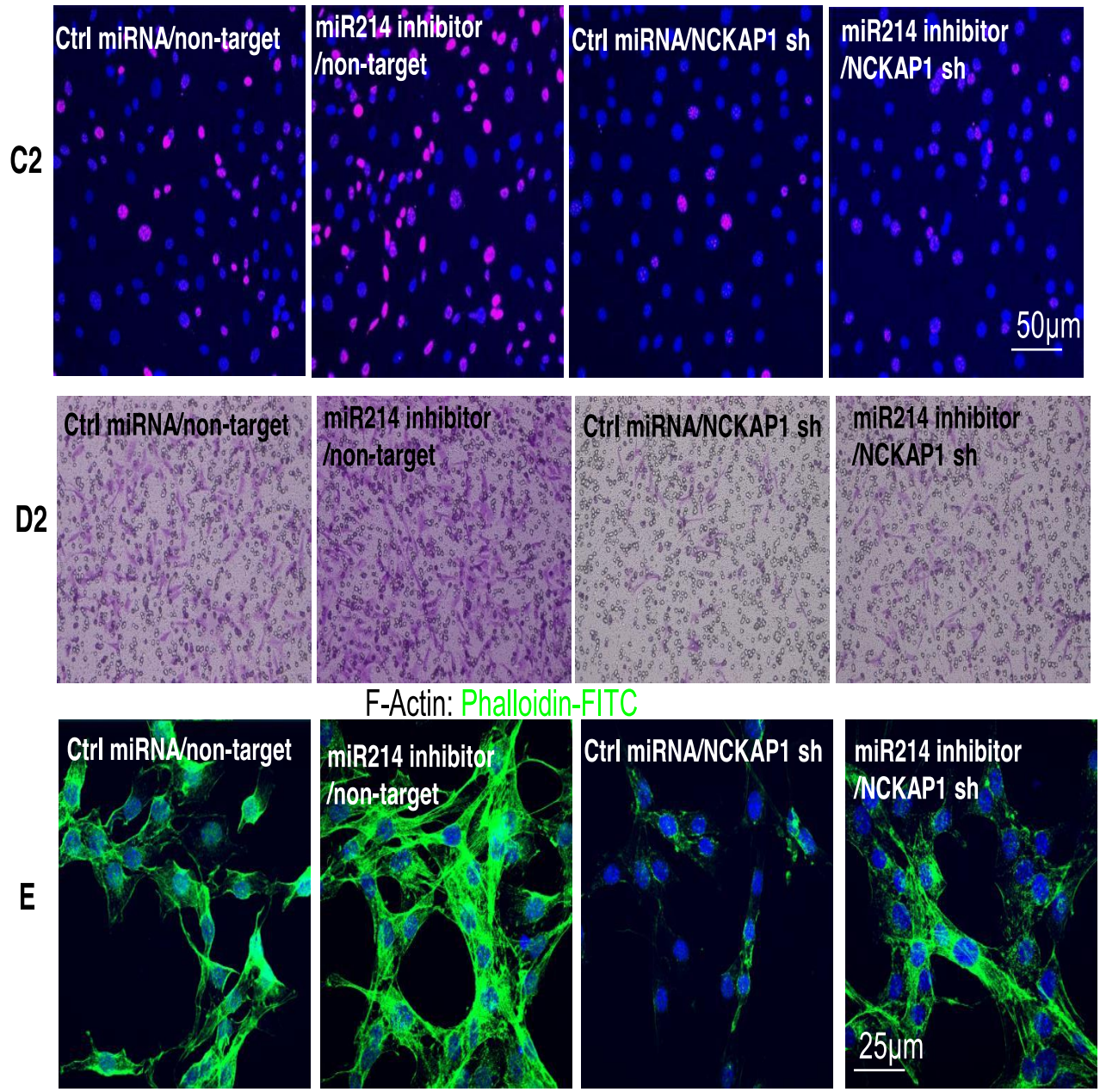


Figure 27. ***Continued***

5.14 In Vivo Experiment: Locally Enforced expression of miR-214 to Injured Artery Reduced NCKAP1 expression, Decreased VSMC Proliferation and Blunted Neointima SMC Hyperplasia.

To explore the functional implication of miR-214 in vascular remodelling after angioplasty, femoral artery wire injuries were conducted in C57BL/6 mice, as described in our previous studies (Xiao, et al. 2006; Xiao, et al. 2014; Zeng, et al. 2006) and elaborated in the **section 4.7** (**materials and methods**), the expression levels of miR-214 was examined by RT-qPCR assay. Consistent with our in vitro findings **(figure 14),** upon vascular injury, the expression levels of miR-214 were substantially downregulated in femoral arteries from day 1 to day 14, whereas their expression levels were almost back to normal at 28 days after angioplasty **(figure 28A),** suggesting a regulatory role of miR-214 in neointima formation. To further determine the effect of miR-214 on VSMCs proliferation and neointimal growth in vivo, 100ul of 30% pluronic gel containing chemically modified and cholesterol conjugated 2.5 nmol miR-214 or Cel-miR-67agomiRs (negative control) was applied perivascularly to femoral arteries immediately after injury, as described in our previous study (Xiao, et al. 2006; Xiao, et al. 2014; Zeng, et al. 2006). Local transferring miR-214 agomiRs increased vascular miR-214 levels 72 hours following wire injury to a level comparable to that of uninjured femoral artery, which was significant higher than that of injured vessel treated with Cel-miR-67 agomiRs **(Figure 28B).** Compared with the control group, enforced expression of miR-214 in the injured vessels dramatically decrease NCKAP1 expression levels, as demonstrated in RT-qPCR **(Figure 28B)** and western blot analysis **(Figure 28C** and **28D)**. These data demonstrate an inverse relationship between miR-214 and NCKAP1 expression in the injured vessels. As expected, a deceased level of PCNA gene **(Figure 28B)** and protein **(Figure 28C** and **28D)** expression was observed in the injured vessel treated

with miR-214 agomiRs, demonstrating that the perivascular enforced expression of miR-214 in the injured vessel inhibits VSMCs proliferation. Consequently, local transfection of miR-214 in the injured vessel resulted in 42% decrease in neointima formation after angioplasty **(Figure 28E** and **28F).** As expected, a thick intima was induced by wire injury of the femoral artery after 28 days in the mice treated with Cel-miR-67 agomiRs (n=11), which significantly reduced the lumen of the vessel. However such remodelling response was significantly inhibited by treatment with miR-214 agomiRs (n=11). Our data suggests that locally restoring the expression levels of miR-214 inhibits neointima SMC hyperplasia induced by vascular injury.

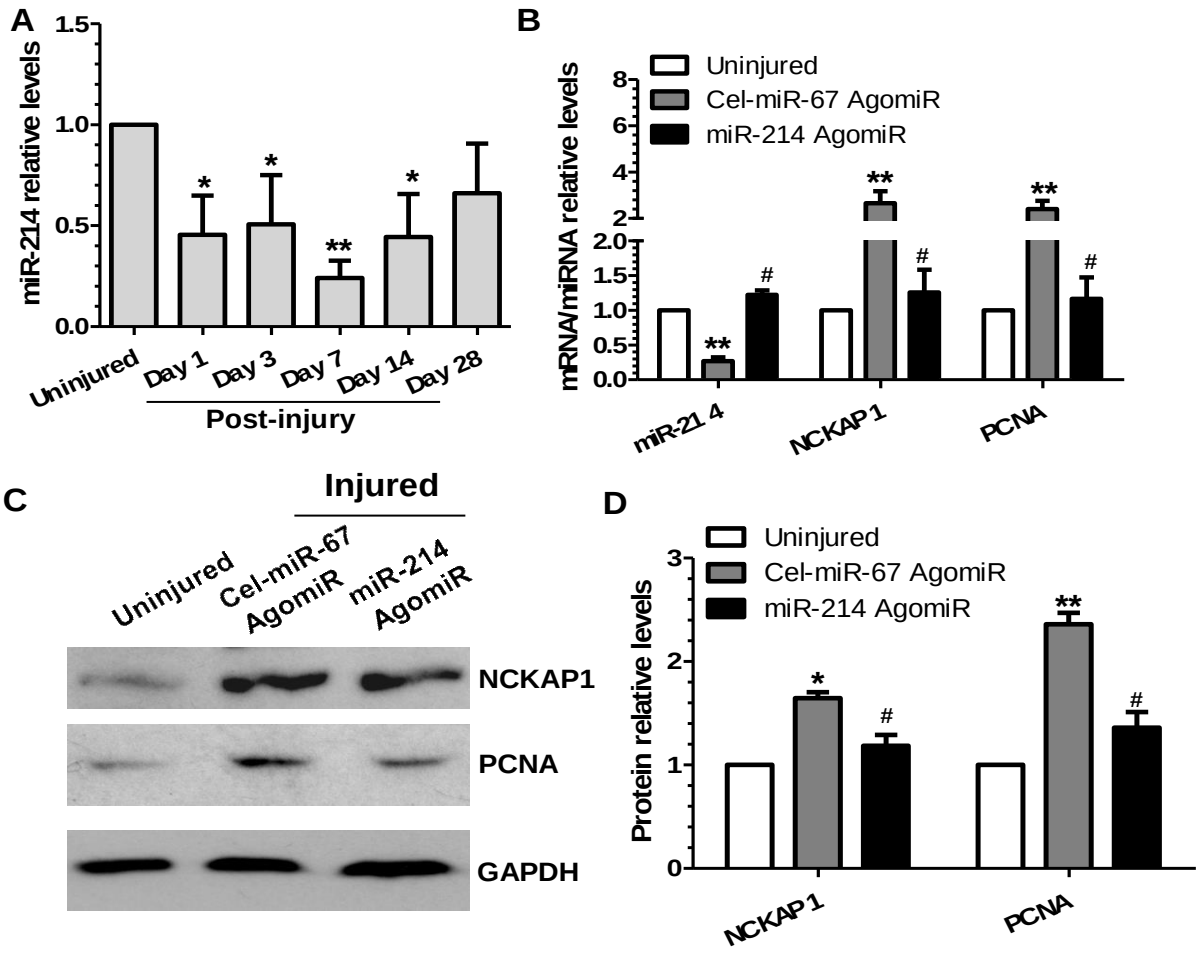


Figure 28. Enforced expression of miR-214 to the injured C57BL/6 mouse femoral artery reduces NCKAP1 expression, decreases VSMC proliferation and blunts neointima hyperplasia.

(A) miR-214 was downregulated after injury. *<0.05, **<0.01 (compared with uninjured control). **(B-D)** Gene/ Protein expression levels in the injured vessel after perivascular delivery of miR-214 agomiRs. After injury, 100ul of 30% pluronic gel containing 2.5 nmol agomiRs per vessel per mice was immediately applied and packed around the injured vessel. At 3 days **(B),** 14 days **(C and D)**, or 28 days **(E and F)** later, injured segments of femoral arteries were harvested and subjected to various studies. Total RNA and proteins were extracted from the uninjured and injured femoral arteries (3-5 mice for each experiment, n=3 experiments) and subjected to RT-qPCR **(B)** and western blot **(C and D)** analysis. Representative images **(C)** and quantitative data as mean+SEM **(B and D**) of 3 independent experiments are presented.$^{\#}$P<0.05 (compared miR-214 agomiRs with Cel-miR-67 agomiRs **(B,D, and F)**. *<0.05, **<0.01 (compared with uninjured vessel **B** and **D).**

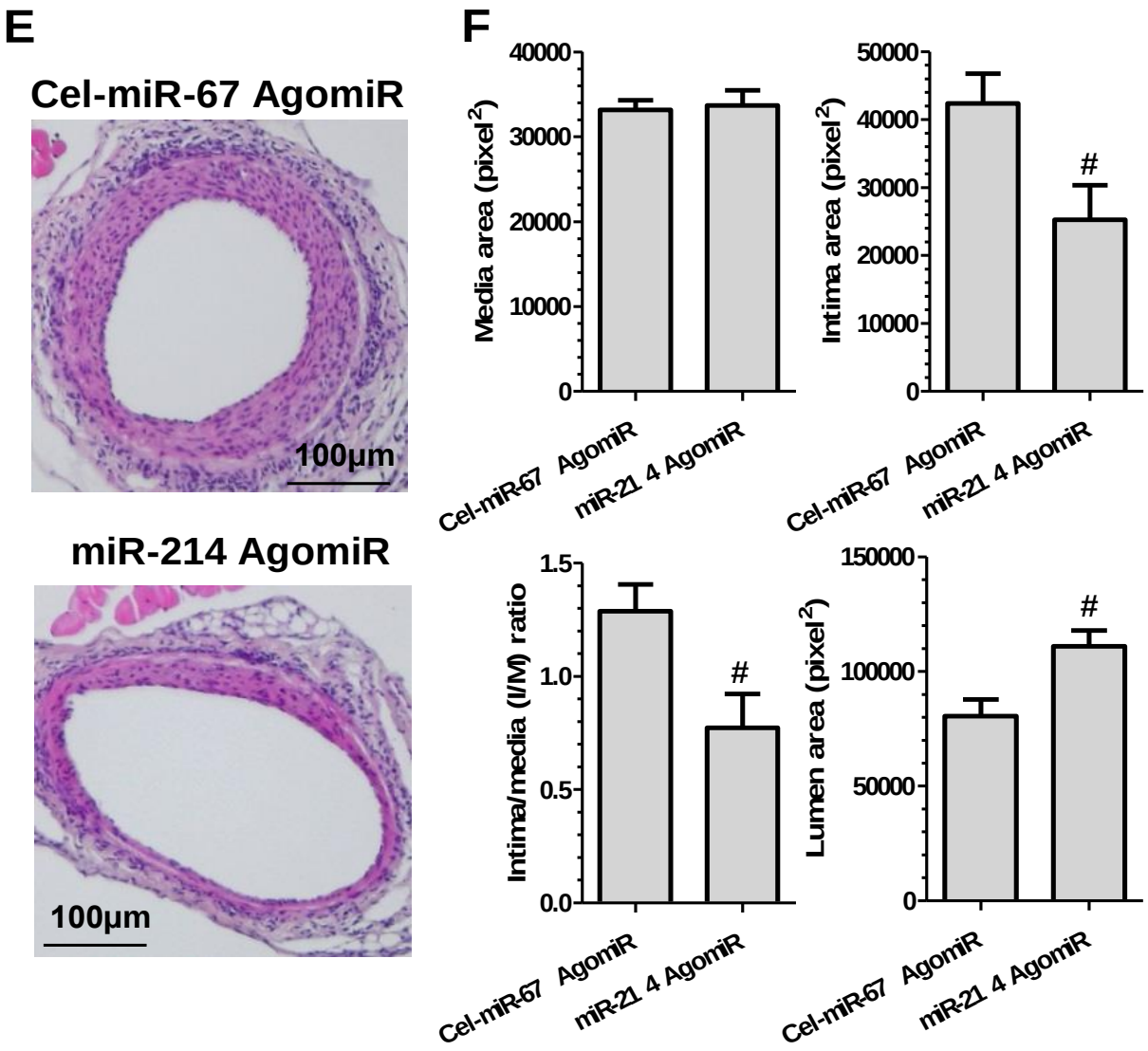


Figure 28 *Continued*. Wire injury induced neointima formation was blunted by miR-214 overexpression (E and F). Paraffin sections from both groups were prepared (n=11 mice for each group) and subjected to hematoxylin and eosin staining analysis. Representative images **(E)** and quantitative morphological characteristics (mean+SEM) including media area, neointima area, neointima/media ratio and lumen area **(F)** are presented. #P<0.05 (compared miR-214 agomiRs with Cel-miR-67 agomiRs **(B, D, and F).** *<0.05, **<0.01 (compared with uninjured vessel **B and D**). For measurements of external elastic lamina (EEL), internal elastic lamina (IEL), artery lumen and Neintimal area on H&E stained cross sections of femoral artery, following calculations were made using computerized image analysis system (pixel^2 Axiovision software).
External Elastic Lamina (EEL) - Internal Elastic Lamina (IEL) = Media area,
IEL - Artery Lumen = Neointima area (NI)
Neointima area (NI)/ Media area= NI/Media ratio

6 Discussion:

Unlike other somatic cells that are terminally differentiated, VSMCs within adult animals retain incredible ability of plasticity and can undergo phenotype switching in response to changes in local environmental cues or stimulations that normally regulate VSMC phenotype. Among the various stimuli, high concentration of serum (20%), PDGF-BB, oxidized LDL or its components such as 4-Hydroxynonenal (4-HNE) and 7-ketocholesterol (7-Keto) have been extensively used to study the molecular mechanisms underlying VSMC phenotype switching. This phenomenon of plasticity plays a crucial role in the development of atheroma as well as in neointimal hyperplasia post angioplasty. MicroRNAs have beens recently identified as a class of non-coding small RNAs involved in post transcriptional regulation of genes through influencing their target gene messenger RNA stability, translation and cleavage/degradation. In this study, we have expanded our knowledge about interactions between VSMCs and miRNAs with particular emphasis regarding the role of miR-214 and explored a new mechanism through which miR-214 regulates VSMC function (proliferation and migration) by influencing NCKAP1 gene function.

6.1 Atherogenic stimuli downregulate miR-214 levels in mVSMCs

Interestingly, we observed that proatherogenic stimuli such as PDGF-BB, high concentration of serum, and ox-LDL components (4-HNE, 7Keto) influence miR-214 expression in VSMCs. Although we didn't examine if other miRNA expression is regulated by PDGF-bb and 20% Serum in this study, our group have previously reported the expression levels of other miRNAs were controlled by these two stimuli in VSMCs. Specifically, a similar response was observed regarding the cellular expression levels of miR-22 and miR-34a when VSMC were treated with PDGF-bb and high conc of serum (Yang, et al. 2018). miR-22 and miR-34a expressions in cultured VSMCs were

reduced in response to PDGF-BB and serum stimulation, while the opposite effect were seen in the serum starved VSMCs (Yang, et al. 2018; Yu, et al. 2014; Zhao, et al. 2015).

4-Hydroxynonenal (4-HNE) is a major active product formed following the oxidation of n-6 polyunsaturated fatty acids. 4-HNE is highly lipophilic and can interfere with the functions of many proteins by forming adducts. 4-HNE is associated with the generation of reactive oxygen species and hence its levels increases significantly during diseases involving oxidative stress and results in generation of ROS as happens in atherosclerosis and diabetes (Chapple, et al. 2013; Selley 1997). Physiological levels of 4-HNE in human plasma range between 0.3-0.7 µM which under pathological conditions increases significantly to 20µM or to even higher concentrations (100µM) have been reported in areas such as plasma membrane (Chapple, et al. 2013; Esterbauer, et al. 1991; Selley, et al. 1989). 4-HNE levels in plasma also serve as markers of oxidative stress (Chapple, et al. 2013). To analize effect of 4-HNE and 7-Keto, different group of cells were cultured in 1% DMSO (vehicle control), 4-HNE (10µM) and 7-Keto (10µM each) (Chen, et al. 2013a). The dosage of 4-HNE was carefully selected to avoid the undesirable effects of cellular apoptosis whereas creating suitable inflammatory conditions to stimulate cellular responses by considering our lab experience and previous publications (Pidkovka, et al. 2007). 4-HNE and 7-Keto stimulation resulted in reduction in the levels of miR-214 (**Figure 14C**) compared to vehicle control (cells in 1% DMSO), suggesting that miR-214 plays a role in VSMC proliferation and migration.Furthermore, decrease in levels of miR-214 was observed during vascular remodelling after injury. Our in vitro data indicates that miR-214 reduces VSMC proliferation and migration, and NCKAP1 has emerged as the functional target of miR-214 in context of VSMC functions. Considering the fact that NCKAP1 is the constitutive component of WAVE complex and plays an important role in actin cytoskeleton and lamelliopodia formation, it is very reasonable to suggest that miR-214 regulates VSMC functions by regulating actin polymerization and

lamelliopodia formation via inhibiting NCKAP1. Translationally, documented evidence suggests that locally enforced expression of miR-214 in the injured vessels (femoral artery wire injury models) resultes in reduced neointimal hyperplasia compared to control group. This beneficial outcome attributes to decreased NCKAP1 expression levels resulting in inhibition of VSMCs migration and proliferation into neointima. Although we didn't examine the direct effect of 4-HNE and 7-Keto on the expression levels of NCKAP1, by considering the role and effect of 4-HNE during oxidative stress and it's involvement in generation of ROS which further deteriorates the micro-environment within atheroma leading to exacerbation of atherosclerosis, one can speculate its role in pathrogenesis of atherosclerosis. Furthermore, as we observed that 4-HNE reduces the levels of miR-214 which is responsible for reduction in VSMCs proliferation and migration by negatively regulating NCKAP1 levels, hence, one can speculate that 4-HNE may be positively influencing the expression levels of NCKAP1 to induce VSMCs migration and proliferation. As mentioned above that NCKAP1 is the constituative component of Wave complex and is required for actin filament polymerization, so it is plausible that a relationship may occur between components generating oxidative stress and ROS (e.g. 4-HNE) and NCKAP1 to regulate cellular response during stress and injury. However, further studies are required to fully elaborate the role of 4-HNE and its effects on NCKAP1 levels and if there is any direct mechanism exists between 4-HNE and NCKAP1.

6.2 MiR-214 reduces mVSMCs proliferation and migration *in vitro* and *in vivo*.

miR-214 is the member of miR-199a-214 cluster and is encoded from DNM3os (opposite strand of DNM gene) as a long non-coding RNA and has emerged as indispensable for normal skeletal development and body growth in mammals (Watanabe, et al. 2008). Increasing amount of data is now supporting a role for miR-214 in many cellular functions including tumorigenesis, cardiac development, cardiac protection, mitochondrial morphology, mitochondrial fatty acid oxidation and cell cycle, gluconeogenesis suppression, angiogenesis, skin and hair follicle development and dendritic cell switching from tolerance to immunity. Initially it has been labelled as oncogene contributing to tumour growth, progression and cell cycle control, enhancing motility, promoting angiogenesis and invasiveness, metastasis, stemness and resistance to chemotherapy (Deng, et al. 2013; Molnár, et al. 2008; Penna, et al. 2015; Ueda, et al. 2010; Yang, et al. 2008; Yin, et al. 2010; Zhang, et al. 2010; Zhang, et al. 2014), but now various studies have suggested aninhibitory role for miR-214 in tumorigenesis, cencer cell survival and metastasis as well (Derfoul, et al. 2011; Gutierrez, et al. 2010; Schwarzenbach, et al. 2012; Shih, et al. 2012; Wang, et al. 2013a; Wang, et al. 2013b; Xia, et al. 2012; Xu, et al. 2012). The reported divergent (or even opposite) roles for miR-214 in different types of cancer may attrebute to the fact that miR-214 can target respective target genes and modulate various downstream signalling networks in different cancers. Another major underlying factor of such discrepancy is the using of different cancer models involving different cancer cell lines, suggesting that cell context is decisive for miR-214's function.

A similar phenomenon is observed when studying role of miR-214 in cardiovascular system. Genetic deletion of miR-214 resulted in heart defects, increased apoptosis and loss of cardiac contractility and excessive fibrosis after ischemia-reperfusion injury. miR-214 protects cardiomyocytes from Ca^{2+} overload by repressing Ncx1 and maintaining Ca^{2+} homeostasis (Aurora, et al. 2012). *Lu et al., 2013* has suggested miR-214 as a cardioprotective agent and promising

biomarker for severe coronary artery disease (Lu, et al. 2013). miR-214 has also been suggested to protect cardiac myocytes against H_2O_2 induced injury, and inhibits left ventricular remodelling after acute myocardial infarction (Lv, et al. 2014). In a recent study by Natsume *et al* have identified miR-214 along with 3 other miRNAs as a novel biomarker for atrial fibrillation (predictive accuracy of 80%) in mice and men. (Natsume, et al. 2018).

Whereas the above studies have provided evidences to support a role of miR-214 in protecting cardiomyocytes from various insults and maintaining cardiac function, in another study up-regulation of miR-214 is associated with cardiac hypertrophy and its antagonization is proposed as therapeutic approach to treat this ailment (Yang, et al. 2013b).

In our study, we have found that miR-214 reduces VSMC proliferation and migration as evident from gain of function and loss of funtion studies under the effect of two proatherogenic stimuli (high concentration of serum and PDGF-BB). It is worth noting that though miR-214 mimics resulted in 60 fold increase in miR-214 levels but we were unable to see such big response (reduction) in VSMCs proliferation. Such observation indicates that multiple signal pathways are responsible for VSMC proliferation and that miR-214 is only one of them.

To better evaluate the effects of any treatment on VSMC functions, we starved the cells in serum starvation culture medium to minimize the possible interference of many unknown stimulators which may present in serum as well as keep cells in G0 phase of cell cycle, so that the cellular response would be an indicative of the specific treatment.

Different concentration of miRNAs (20 to 100nM) has been used in various studies. We titred the concentrations of miRNA transfection (mimics and inhibitor) to 25nM depending upon our previous lab experience which has resulted in significant over-expression and inhibition during our experiments (Zhao, et al. 2015). In this study, miR-214 expression levels in VSMCs were successfully up-regulated and down-regulated by 25nM miR-214 mimics and inhibitor,

respectively, without apparent cell apoptosis and cytotoxicity. There is no significant change in terms of cellular viability after treatment with miR-214 mimics or inhibitor transfection and inflammatory mediators, as observed by physically monitoring the changes in cell morphology.

Furthermore, we elegantly demostrated that miR-214 regulate VSMC migration and proliferation and inhibit neointima SMC hyperplasia by using our well established wire injury-induced neointima formation model and applying enforced expression of miR-214 by perivascular delivery of miR-214 agomiRs into injured vessels. Because these VSMC functions hold prime importance in the pathophysiology of neointimal hyperplasia, so miR-214 could represent a new therapeutic target for the treatment of post-angioplast restenosis. Importantly, findings from our study and previous studies have indicated a divergent role of miR-214 with respect to different organs and diseases. As impact of miR-214 on various cellular functions are conflicting concerning a wide range of human diseases (particularly cancer, cardiovascular diseases, diabetes, and rheumatology), caution should have been taken when considering the therapeutic benefits of miR-214 overexpression and/or inhibition on distinct human disease.

6.3 MiR-214 levels are regulated by Transcription factor Twist-1.

Different studies have proposed changes in miR-214 levels (normally upregulation) under the effect of different stimuli including hypoxia, cardiac stress, H_2O_2, and Ca^{2+} overload, but we observed downregulation in miR-214 levels under the influence of various atherogenic stimuli (PDGF-BB, serum, 4HNE, and 7Keto) when cultured using normoxic conditions. Different mechanism have been proposed for regulation of miR-214 expression. miR-214 has been shown as a hypoxia-inducible miRNA which can be regulated in HIF1-α dependent/independent manner (el Azzouzi, et al. 2013), whereas other study has identified an E-box element within miR-214 promotor, through which the basic helix-loop-helix transcription factor, Twist 1, can regulate miR-214 expression in hepatic stellate cells (Chen, et al. 2015b).

Hypoxia is a physiological and/or pathological state characterized by imbalance between oxygen need and supply leading to insufficient concentration of oxygen to meet cellular or tissue demands. It may arise due to reduced oxygen supply (due to defects in blood vessels leading to insufficient blood supply) or due to an icreased consumption of oxygen relative to the supply (as in areas of rapid cell proliferation). Oxygen consumption rate also varies significantly depending upon specific cell/tissue type, for example the brain consumes 3ml O_2/min 100g tissue whereas heart consumes 8-15ml O_2/min 100g (Braunwald 2001). These differences between O_2 consumption arise from difference in metabolic activity and mitochondrial density. Under hypoxic conditions, cellular transcriptional, translational and post-translational response changes and results in upregulation of expression of certain genes known as hypoxia inducible genes. Genes involved in angiogenesis (VEGFR), erythropoiesis (erythropoietin EPO), cellular metabolism (Pyruvate dehydrogenase kinase 1,Pdk-1) and inflammation (inducible nitric oxide synthase iNOS) are some examples of hypoxia inducible gene. Hypoxia inducible factor 1α (HIF-1α) and HIF-2α act as trascriptional mediators in the induction of above mentioned hypoxic genes.

Atmospheric oxygen is transported to the cell in two forms, bound to Hb and dissolved in blood. Total amount of oxygen which fully O_2 saturated blood can carry is 20.4 ml/100ml blood. From the atmospheric air, O_2 passes through many gradients to finally reach at cellular/tissue level and at each levels its partial pressure (PO_2) decreases. At 1st step, (during breathing) the PO_2 of O_2 which is normally around 19.9kPa reduces to 13.3kPa due to introduction of CO_2 and water vapour partial pressure (Keeley and Mann 2019) . Moving further (from blood to cytosol), the O_2 levels decrease significantly between the systemic arterial circulation and arteriolar vessels which is directly proportional to lumen diameter and inversely proportional to blood flow (Bohlen 1983; Duling, et al. 1979; Kerger, et al. 1995). Significant O_2 gradient is observed in the

vascular wall of arterioles which is proportional to the thickness of artriolar wall (Duling, et al. 1979). In addition to the longitudinal O_2 concentration gradient, radial or transluminal O_2 concentration gradient across vascular wall also significantly depend on the concentration of available O_2. Researchers have demonstrated significant reduction in O_2 levels between the lumen and adjacent thin tissue layer by penetrating the vascular wall using electrodes (Niinikoski, et al. 1973; Santilli, et al. 2000). Different researchers have proposed different and contradictory ideas about this significant difference between the intimal and luminal PO_2. Recent evidence suggests that the major barrier to transfer of O_2 from the lumen to the tissue may be the plasma phase of blood as it creates significant barrier between red blood cells and endothelium. The 3rd gradient for O_2 transfer is from vessels to cytosol. The pressence of longitudinal and radial O_2 concentration gradient in the vasculature, diffusional transfer of O_2 between neighbouring arterioles, capillries and venules results in significant narrow Guassian tissue PO_2 distribution (Tsai, et al. 2007).

Various types of cells including EC, monocytes/macrophages, T lymphocytes, fibroblasts and VSMCs are involved in initiation and development of atheroma plaque. Several studies have docuented the presence of hypoxic regions within plaque. In developed atherosclerosis, oxygen consumption rate increases than supply, leading to the development of tissue hypoxia at the plaque lesion. As aforementioned, the partial pressure PO_2 differes significantly across different gradients while travelling from lungs to the tissue/cells and varies greatly within artery wall even in helathy individuals. During atherosclerosis, due to impared O_2 diffusion capacity and excessive oxygen consumption by marcophages, hypoxic zones develop within atherosclerotic plaques. Hypoxia has been observed in atherosclerotic plaques from human and animal models including ApoE$^{-/-}$ and LDLr$^{-/-}$ mouse models (Bjornheden, et al. 1999; Parathath, et al. 2013). Hypoxia/reoxygenation cycles leads to the formation of ROS which may promote angiogenesis by

regulating HIF-1α expression and activation of NOS and NADPH oxidase activity. HIF-1α plays critical role in proression of atherosclerosis by promoting the formation of foam cells, EC dysfunction, apoptosis and by increasing inflammation. Considering our in vivo model, we can speculate (though its currently unknown) the involvement of HIF-1α for regulating miR-214 expression and resulting NCKAP-1 expression along with the dominant effect of inflammatory stimulators (PDGF-bb and serum) on the transcription factor Twist-1.

Whereas our in vitro model is concerned, we assume that changes in miR-214 levels under the influence of inflammatory stimuli (PDGF-bb and serum) are mainly due to their influence on transcription factor Twist-1 rather than the involvement of HIF-1α dependent pathway because the cells were cultured under normoxic conditions although recent studies do present that cells cultured under in vitro condition experience different PO_2 than the PO_2 of air in the room (Keeley and Mann 2019).

The PO_2 in room (20.9 kPa) is strictly refers to the dry atmospheric air whereas the conditions inside laboratory incubators are somewhat different with 95% of air and 5% CO_2. Moreover incubators run at 75% humidity to reduce growth medium evaporation, compared to around 50% at room air, hence the actual PO_2 in room reduces to 18.5 kPa (Keeley and Mann 2019). In the absence of Hb as happens during in vitro studies, O_2 delivery to the cell monolayer is defined largely by the PO_2 of the culture medium directly adjacent and hence the total availale O_2 is considerably lower. To address any hypoxic condition arising from such situation, gas permeable culture substrate can be effective in minimizing unintentional hypoxic condition. Furthermore, temperature also adversely effects the solubility of gas in water/solution. Higher temperature increases evaporation and thus increase PH_2O at the expense of O_2. Solubility of O_2 in pure water at 37°C is 77% than at 20°C, resulting in about 30% less O_2 in the solution (Keeley and Mann 2019). Another factor influencing O_2 solubility is the salinity of the solution. Equilibrium between

atmospheric and medium O_2 levels is another varient effecting O_2 levels in culture. Normally O_2 levels equilibrates in the solution through the gas/liquid interface and hence addition of poorly equilibrated, overly oxygenated medium to cells adapted to low O_2 may create condition similar to ischaemia/ reoxygenation as observed in stroke and MI.

One limitation in this study was the lack of technology to accurately monitor intracellular O_2 levels in living cells. Requirement for such tool arises because even if the cells are cultured under normoxic condition, they can experience culture-induced hypoxia by consuming O_2 faster than it can diffuse specially when they were actively proliferating as it was in our case. Under such conditions, it may become difficult to differentiate the cellular response to physiological and hypoxic O_2 levels, resulting in incorrect interpretation.

Althought, it is currently unknown whether the above mentioned atherogenic stilmuli downregulate miR-214 expression in VSMCs through HIF1-α dependent and/or HIF1-α independent mechanism; however, it is unlikely that the HIF1-α signalling pathway will be involved in miR-214 modulation by atherogenic stimuli because the VSMCs were cultured under normoxic conditions rather than hypoxic one in this study. Instead our data demonstrate that both serum and PDGF-BB regulate miR-214 expression through a transcriptional mechanism, and the E-box element within the miR-214 promoter region is required for such regulations. So in this regard, our data is consistent with the previous finding that miR-214 expression in hepatic stellate cells and during development is dependent on Twist-1.(Chen, et al. 2015b). Interestingly, we also observed incomplete inhibition of miR-214 when VSMCs were transfected with full length miR-214 gene promoter containing E-box **(figure 15C)** indicating that miR-214 and Twist-1 gene regulation is under controlled by multiple factors (Serum, PDGF-BB, other growth factors or cytokine/chemokines, etc) and/or many other yet unknown signalling pathways.

6.4 NCKAP-1 is the downstream target of miR-214.

As aforementioned, major finding in our study was the identification of NCKAP1 as the functional target gene of miR-214 in the context of VSMC functions. Although multiple miR-214 targets have been reported invarious cellular context and diseases (e.g. ITCH, FGFR1, Quaking, Osterix, Ncx1, β-catenin, Mitofusin2 and ATF4)(Ahmed, et al. 2014; Aurora, et al. 2012; Bucha, et al. 2015; Chen, et al. 2014; Chen, et al. 2015e; Li, et al. 2015b; Shi, et al. 2013; van Mil, et al. 2012), we provided evidence to suggest that NCKAP1 is the novel target gene for miR-214 in VSMCs. Our proteomics data showed NCKAP1 is the most down-regulated protein with the highest significance. Using computational algorithmic data, we identified three binding sites for miR-214 within 3'UTR of NCKAP1. Furthermore, miR-214 over-expression and inhibition experiments showed an inverse relationship between miR-214 and NCKAP1 in VSMCs as evidenced by upregulation of miR-214 results in decrease in NCKAP1 levels and vice versa. NCKAP1 is 129kda protein primarily shown to be associated with NCK (non-catalytic region of tyrosine kinase adaptor protein 1) through binding to the Src homology 3 (SH3) domains of NCK (Kitamura, et al. 1996; Suzuki, et al. 2000). But later studies have suggested a critical role for NCKAP1 in regulating cell motility and adhesion through regulation of actin polymerization (Nakao, et al. 2008). Mice lacking NCKAP1 gene were arrested at midgestation and have defects in morphogenesis of all 3 embryonic germ layers (Rakeman and Anderson 2006). Cellular movement/migration process is triggered with the formation of lamelliopodia (membrane protrusions) and/or filopodia (finger like protrusions), followed by changes in action cytoskeleton which enables cell movement. It has been well established that Rac signalling to actin mediated by WAVE complex holds key role in cell movement. NCKAP1 being integral constructive part of WAVE complex has prime importance in regulating VSMC functions (Ibarra, et al. 2006; Steffen, et al. 2004). NCKAP1 is described as locallized at the protruding ends of lamelliopodia thus playing a key role in regulation of cell motility, contact dependent cell migration and adhesion by driving actin assembly. Though role

of NCKAP1 is well postulated but by now the underlying mechanism for NCKAP1 regulation in VSMCs was not clearly establised.

6.5 NCKAP1 inhibition leads to reduced Actin polymerization, lamelliopodia formtion and cell migration.

By using proteomics analysis, miR-214 over-expression and inhibition experiments, and by generating NCKAP1 stable knockdown VSMC lines, we have, for the first time, provided compelling evidence to show that NCKAP1 is regulated by miR-214 in VSMCs. Futhermore, immunoflourescense staining has provided additional evidence about its localization (lamelliopodial ends) and showed an inverse relationship between miR-214 and NCKAP1 (low levels of F-actin and NCKAP1 in lamelliopodia in miR-214 over-expressing VSMCs). Another technique which could have been employed to investigate co-localization of NCKAP1 and F-actin would be proximity ligation assay (PLA). PLA allows *in situ* detection of endogenous proteins, protein interactions and modifications with high sensitivity and specificity. The detection and cellular localization of proteins can be made with single molecule resolution in unmodified cells and tissues. Two primary antibodies raised in different species are used to detect two unique protein targets. Then two secondary antibodies containing a short unique DNA strand oligonucleotide probe (PLA Probe) binds to the specific primary antibodies. If the two PLA probes (occurs only if the target proteins are very close) are in close proximity of each other, the hybridizing connector oligos joins the PLA probes into a closed circular DNA template required for rolling-circle amplification (RCA). The PLA probe then acts as primer for DNA polymerase, which generates concatemeric sequences during RCA resulting several hundreds fold amplification of DNA circle. Next, flourescent labelled complementary oligonucleotide probes are added to amplified DNA, which being tethered to DNA probe allows localization of the signals.

The resultant flouresence is visualized and quantified as discrete spots by microscopy image analysis. In our case, as it was widely accepted that both NCKAP1 and F-actin are involved in actin cytoskeleton and cellular movement so it was quite logical to speculate that both are present at same location and involved in cell migration which was evidant in our immunoflouresence assay. Luciferase reporter and mutagenesis assay finally confirmed that NCKAP1 is the direct and functional target of miR-214.

Functionally and mechanistically, we have demonstrated that controlling actin assembly and polymerization and lamellipodia formation in VSMCs through modulation of NCKAP1 expression is the primary mechanism through which miR-214 mediates VSMC functions and behaviours including growth and movement. We observed a much lower level of F-actin and lamellipodia formation and a significantly reduces levels of NCKAP1 in miR-214 overexpressing VSMCs **(Figure 26),** suggesting that miR-214 impairs actin assembly and polymerization and lamellipodia formation through inhibition of NCKAP1. Additional data from NCKAP1 knockdown expeiments **(Figure 25C** and **25D)** showed that NCKAP1 inhibition can recapitulate the inhibitory effects of miR-214 overexpression on VSMC functions and behaviours. Finally, evidences from cotransduction experiments (miR-214 inhibition and NCKAP1 knockdown)**(Figure 25)** indicated that NCKAP1 repression is required for miR-214 mediated inhibition of VSMC proliferation, migration and actin polymerization.

One of the surprising findings which warrants further investigations was an unexpected observation about the localization of NCKAP1 within the nuclei of VSMCs particularly after the cells were restimulated with serum after serum starvation **(Figure 26A** and **26B).** The functional implication of such behaviour requires further investigation in a separate study. Furthermore, we observed a higher level of nuclear localization of NCKAP1 in control VSMCs infected with shRNA lentivirus than in VSMCs transfected withcontrol miRNA mimics. Because these two control

VSMCs were subjected to different treatments (cells with control miRNA mimics received transient transfection for 24 hours, whereas the control VSMCs with nontarget shRNA were generated from lentivirus infection and a 10 days puromycin selection) prior to serum starvation, we speculate that such discrepencies is due to the additive or synergistic effects of serum restimulation and lentivirus infection (and/or puromycin selection during the cell line generation), which require further investigation in separate study.

6.6 MiR-214 Crosstalk and effet on other Genes/proteins.

The fact that multiple genes have been identified as functional targets of miR-214 in different studies involving different organs suggests that miR-214 plays a diverse or even contradictory role under different physiological and pathological conditions. So considering the findings of other researchers and from our proteomics data, we can postulate that miR-214 regulates specific genes in a organ/cellular context dependent manner. Furthermore, despite the limitation of our study, majority of the proteins downregulated by miR-214, as per data from our proteomics analysis, showed that they are involved in actin filament reorganization and polymerization, regulation of cell migration, proliferation, cell cycle and gene expression, further supporting a role for miR-214 in modulating VSMC proliferation and migration.

Interestingly, as per our proteomics data, overexpression of miR-214 in VSMCs resulted in upregulation of 160 proteins out of 219 total observed proteins (**Table 14**), whereas just 59 proteins were downregulated by miR-214 in VSMCs, which suggestes that miR-214 might suppress one or more transcription or epigenetic factors which resulted in a positive impact/upregulation of these proteins. As expected, SMYD5, a major epigenetic regulator, was observed to be significantly downregulated by miR-214 in VSMCs. Moreover, data from luciferase assay showed that SMYD5 3'UTR reporter activity is negatively regulated by miR-214 **(Figure 23B),** thus providing a direct evidence to support that SMYD5 is a target gene of miR-214

in VSMCs. SMYD5 belongs to the class V-like SAM-binding methyltransferase superfamily, which has been suggested to play an important role in regulation of gene silencing by modulating the methylation of a variety of histone and nonhistone targets. It would be interesting to further investigate the implication of SMYD5 modulation and the regulation of associated genes under the influence of miR-214 over expression and inhibition, and how such regulation affect VSMC functions and neointima hyperplasia.

In addition to SMYD5, we also selected KLF14 to evaluate miR-214 activity as algorithmic computational models showed 2 binding sites for miR-214 in its 3'UTR. KLF14 is a member of Kruppel-like factor family of transcription factors and plays an important role in regulating expression of many genes involved in various biological processes. As aforementioned many predisposing factors (inflammation, hyperlipidemia, obesity, diabetes) are involved in the progression of atherosclerosis and KLF14 gene expression is closely associated with regulation of these factors. KLF14 regulates the expression of genes involved in cellular proferation, differentiation, apoptosis, inflammation and cardiovascular disease (McConnell and Yang 2010). TGFβ is antiatherosclerotic cytokine which is involved in inhibition of cell proliferation at the initial stage of atherosclerosis and later in the stabilization of atherosclerotic plaque by help in maintaining a fibrous cap. TGFβ treatment results in upregulation of KLF14 expression levels which leads to silencing of TGFβRII promoter, resulting in suppression of TGFβRII activity, thus playing a role in maintaining cellular homeostasis (Truty, et al. 2009). Recent genome-wide association studies (GWAS) presented involvment of KLF14 variant in etiology of atherosclerosis, ischaemic strock and MI (Chen, et al. 2012; Grarup, et al. 2010; Voight, et al. 2010). KLF14 hepatic-specific deletion in apoE$^{-/-}$ mice resulted in accelerated development of atherosclerotic lesion due to decrease cholesterol efflux and reduced HDL-C levels (Huang, et al. 2013). On the contrary, activation of KLF14 resulted in reduction in atherosclerotic lesion suggesting KLF14 as

potential therapeutic target. Study also reported that KLF14 regulates lipid metabolism through HDL biogenesis and by maintaining triglycerides and cholesterol homeostasis (Teslovich, et al. 2010).

Activation, migration and infiltration of macrophages and T-cells is an important step in progression of atherosclerosis and KLF14 plays a profound role in driving T-cells differentiation (Sarmento, et al. 2015). Interestingly KLF14 knockdown significantly decrease the circulating levels of inflammatory cytokines such as TNF-α, IL-6 and MCP-1 in apoE$^{-/-}$ mice (Wei, et al. 2017). Abovementioned roles for KLF14 in multiple processes involved in atherosclerosis prompted us to investigate if KLF14 expression is regulated by miR-214 in VSMCs. The 3'UTRs of KLF-14 were cloned into luciferase reporter (pmiR-Luc) and transfected into VSMCs along with miR-214 mimics or respective negative control for 14-16hrs. Cells were harvested after 48hrs and subjected to luciferase activity assay. No significant reduction was observed in the luciferase activity with the construct harbouring KLF14 3'UTR, suggesting that miR-214 does not directly represses KLF-14 but rather does its activity in an indirect mannar (probably by interacting with another epigenetic factor whose inhibition or over-expression results in KLF-14 repression).

Furthermore, as a major characteristic of miRNAs, it has been widely known that single miRNA can effect multiple target genes and downstream cellular/molecular processes so we can pressume that there might be more miRNAs whose specific roles and involvement in the cardiovascular system still needs to be explore. They might be working in harmony, synchronising cellular response or they may be counteracting each other's influence or might be influencing cellular and biological functions completely independent of each other, working though separate molecular mechanism to produce their effect as we observed in our case of miR-214 and miR-34a. Both miR-214 and miR-34a influence VSMCs functions and neintima formation but through their own respective pathways independent of each other i.e. through NCKAP1 and Notch1

respectively and that there is no cross talk or link between the effect of these two miRNAs with respect to murine VSMCs. But there are possibilities that some other miRNAs (whose role hasn't been explored yet) might be working through similar mechanisms and pathways resulting in similar response on target tissue/organ. Hence, we can speculate that there is possibility that some other miRNA could be working on NCKAP1 or influencing the transcription of miR-214 and thus regulating the cellular responses and functions.

Another area requiring further investigations is other down-regulated proteins/genes by miR-214 in VSMCs. Multiple miR-214 target genes have been reported in different cell population, but among them we only found two genes (β-catenin and PCBP2) were slightly decreased by miR-214 over expression in VSMCs in our proteomics anslysis. All other reported miR-214 target genes failed to pass the strict threshold setting for our proteomic analysis. Possible reason for this discripency might include sensitivity of mass spectrometer in identifying such regulatory proteins from our sample of whole cell lysate. Another reason might be the VSMCs which may contain less amount or lack of expression of these proteins. B-catenin is involved in coordinating intercellular interactions and regulation of gene transcription. Several studies have documented its role in endothelial cells as it regulates cellular growth and adhesion by interacting with VE-cadherin and intracellular actin cytoskeleton (Rho, et al. 2017; Tian, et al. 2011). In our proteomic data, β-catenin protein level was not significantly downregulated by miR-214 overexpression in VSMCs. One possible reason for such observation would be specific cellular context as we were examining VSMCs but not ECs. Furthermore, it is widely accepted that miRNAs behave differently in different cells and can exhibit totally divergent interactions regarding post translational mRNA inhibition, hence, we can presume that miR-214 might have regulated different genes/proteins in VSMCs compared to ECs. Another reason for such discripency might be the inflammatory stimulus employed in our experiment. Finally the limitation regarding the low sensitivity of mass

spectrometer in identifiying β-catenin from our sample could be another reason for such observation.

In the current study, we have mainly focused on the regulatory role of miR-214 in modulating VSMC functions and their involvement towards neointima formation, however its noteworthy to mention another major contributing factor to neointima formation after vessel injury, namely the lack of re-endothelialization and impaired response of ECs towards injury and subsequent initiation of inflammatory response. Several studies (Chan, et al. 2009; Duan, et al. 2015; van Mil, et al. 2012) have reported a role of miR-214 in modulating endothelial response and resulting angiogenesis; therefore, the biological effects of miR-214 on ECs and re-endothelialization after injury may also contribute to the miR-214 mediated effect on decreased neointima formation and resulting vascular remodelling which need to be further investigated in separate study.

Nonetheless, in the current study, we have successfully uncovered the functional involvement of miR-214 in VSMC biology and in vascular remodelling after injury and we can conclude that, miR-214 exerts its function by regulating the lamelliopodia formation and controlling actin polymerization through modulating NCKAP1 expression and hence affects VSMC migration and proliferation during atherosclerois and neointima formation. Although miR-214 exerts a diverse array of functions (as with the other miRNAs) in different organs and cellular context, findings from our study present miR-214 as potential therapeutic target for VSMC related diseases such as atherosclerosis and postangioplasty restenosis.

7 Conclusion, Limitations and Future Plan

7.1 Conclusion

VSMCs phenotype switching and the resultant migration, proliferation and ECM secretion holds fundamental importance in pathogenesis of atherosclerosis and neointima hyperplasia. Determination of underlying molecular mechanism and their regulation can help to modify the disease progression as well as reduce the fatal/disabling outcome. miRNAs are relatively recent class of small non-coding RNAs playing crucial role in biological processes. They modulate cellular processes at post-transcriptional level, inhibiting the translation of mRNA and leading to their degradation. They are regarded as master regulator as single miRNA can regulate multiple genes and the related proteins. Several researchers are investigating their role in multiple biological processes including VSMCs phenotype switching, atherosclerosis and neointimal formation.

In this study, we expanded our knowledge by uncovering a novel role of miR-214 in modulating VSMC phenotype switching by regulating two major VSMC function (proliferation and migration) *in vitro* and *in vivo*. Interestingly, we observed that differet inflammatory stimuli (e.g. PDGF-BB, high concentration of serum, 7-ketocholesterol and 4-hydroxynonenal) can modify miR-214 levels which results in down-regulation of miR-214 levels. A similar pattern of reduced miR-214 expression was observed during vascular remodelling after injury. Consistent with the previous studies, (Lee, et al. 2009) our data indicate that both PDGF-BB and high concentration of serum regulate miR-214 expression through a transcriptional mechanism.miR-214 has an E-box element within its promoter region, which upon binding with basic helix-loop-helix transcription factor Twist-1, regulates its activities. Both atherogenic stimuli, PDGF-BB and high concentration of

serum, downregulate Twist-1 gene expression in VSMCs, thus leading to reduced expression/activity of miR-214.

Regarding cellular function, by using miRNA gain-and loss-of-function analysis, we documented that, 2 critical cellular events in vascular neointima formation, VSMC proliferation and migration were inhibited by miR-214 over-expression, when treated with PDGF-BB and high concentration of serum.

Major finding in our study was identification of NCKAP1 as functional target gene of miR-214 in regulating VSMC functions. miR-214 inversely regulate NCKAP1 expression as overexpression of miR-214 significantly downregulates NCKAP1 and vice versa. NCKAP1 was most downregulated protein in our proteomics analysis as well as we identified 3 binding sites for miR-214 in 3'UTR of NCKAP1. Actin polymerization and lamellipodia formation in response to extracellular stimuli and resultant WAVE signalling is fundamental to cell migration. As a constitutive component of WAVE complex, NCKAP1 plays key role in regulting cell motility and adhesion. Thus inhibition of Actin polymerization and/or lamellipodia formation by downregulating NCKAP1 expression is one of the underlying molecular mechanisms through which miR-214 regulates VSMC functions (growth and motility) and prevents neointimal SMC hyperplasia in response to vascular injury.

Importantly, by using our well-established wire injury-induced neointima formation model and perivascular delivery of miR-214 agomirs into injured vessels, we further demonstrated that miR-214 reduced NCKAP1 expression levels, modulated VSMC proliferation and inhibited neointima SMC hyperplasia after injury, suggesting that miR-214 can be a potential therapeutic target in postangioplasty restenosis.

In conclusion, findings from this study significantly increased our understanding about the interaction between atherosclerotic/inflammatory stimuli and miRNAs, and the subsequent molecular mechanisms behind VSMC phenotype switching leading to neointimal hyperplasia.

This study also highlighted the importance of miRNAs (particularly miR-214) as prospective new targets to address CVD with particular reference to SMC biology, atherosclerosis, and neointimal hyperplasia.

7.2 Study Limitations.

Altough we beautifully elaborated role of miR-214 in modulating neointima formation by regulatig NCKAP1 levels and resultant inhibition of VSMC migration and proliferation, we did come across some obstacles involved in our in vivo works. One of the major study limitations is the difference between our in vivo model (mice) and human disease. Neointima formation is the inflammatory process following angioplasty and stenting leading to restenosis of vessels. Although our mouse femoral artery injury model is well established in generating neointima and to analyse the contributing factors but it does not completely mimics the conditions prevailing at the micro-environmental level after stent implantation in patients, hence it cannot provide us a best alternative to study the underlying factors at such micro level.

Additionally, atherosclerosis (being chronic inflammatory disease) and post-angioplasty neointima formation normaly occurs at later stages in life (generally in middle to old age population) and hence the cellular and molecular response to different inflammatory stimuli is somewat different than in normal young individual and this was the 2nd limitation of our study as we used adult C57BL/6 mice where the cellular response to injury might slightly different from middle to old age human population.

Moreover, hyperlipidemia is considered as a major predisposing factor for atherosclerosis and neointima formation by effecting endothelial integrity leading to increased accumulation, transmigration and oxidation of LDL, generation of ROS and activation of inflammatory mediators. The difference of lipid content represents another limitation of our study model as it has normal levels of cholesterol and phospholipid content as compared to the actual patients which normally presents high levels of cholesterol and triglycerides.

Another potential limitation affecting our in vivo work is the use of GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) as an internal control gene. GAPDH is commonly used as reference

gene while investigating for gene expression and protein studies. GAPDH has long been recognized as an important enzyme for energy metabolism and the production of ATP with highest levels in cytoplasm. Although increased GAPDH gene expression and enzymatic function is associated with cell proliferation, recent studies have highlighted it as multi-functional protein and that its levels are affected by conditions such as oxidative stress which impair GAPDH catalytic activity (Nicholls, et al. 2012). Specifically, in response to oxidative stress, GAPDH translocates to the nucleus under the effect of NO from inducible nitric oxide synthase (iNOS) or neuronal nitric oxide synthase (nNOS) and participate in regulating cell survival and apoptosis (Hara, et al. 2006; Rodacka, et al. 2014). Despite of such a pitfall, I believed that GAPDH is still a valid reference gene in our study since we didn't observe any significant change of GAPDH gene expression in response to wire-induced vascular injury in our study.

7.3 Future plan

Although we did provide compelling evidence about the role of miR-214 and its interactions with NCKAP1 and subsequent effects on VSMC functions and neointima formation, during the course of study we came across some new findings which warrant further investigation.

1. Investigating the other parameters involved in SMC biology (e.g. apoptosis and ECM production/secretion) under the influence of miR-214 would be another area for future work. VSMC apoptosis is another determinant for vascular wall remodelling and atherosclerotic lesion progression. The functional involvement of miR-214 will be explored in the future by using flow cytometry analysis (FITC Annexin V/Dead Cell Apoptosis Kit, Life Technologies, V13242) and/or terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (DeadEnd™ Fluorometric TUNEL System, Promega, G3250).

   For ECM secretion profile, VSMCs will be transfected with miR-214 precursor or inhibitor, and the gene/protein expression levels of various ECM proteins will be examined using RT-qPCR, Western blot and/or ELISA analyses, respectively.
2. Data from the present study demonstrate that miR-214 downregulates NCKAP1 in murine VSMCs thus contributing to inhibition of neointima formation but how we can incorporate these results into human requires experiments on human tissue using human VSMCs.
3. Transfering the results of our finding into clinical benefits requires investigating miR214 and NCKAP1 involvement in neointima hyperplasia using human tissues. (Gene expression and immunohistochemistry).
4. Interestingly, we unexpectedly observed that NCKAP1 can be localized inside the nuclei of VSMCs particularly after the cells were treated with serum starvation (Figure

24B). To investigate the functional implication/importance and mechanisms involved in NCKAP1 re-localization to nucleus in VSMCs upon serum starvation and re-stimulation would be another interesting area to explore in a different study.

5. Proteomics analysis gave us a vast array of data (total 219 proteins regulated by miR-214, out of which 160 upregulated and 59 downregulated) **(Table 13)**, which suggests that miR-214 influences ≥1 major transcription/epigenetic factors to exert such changes. Indeed in this study, we observed SMYD5 (epigenetic regulator) downregulated by miR-214 overexpression. Furthermore, data from luciferase reporter assay showed repressed SMYD5 3'UTR activity by miR-214 overexpression, providing evidence that SMYD5 is also another major target gene of miR-214. SMYD5 plays an important role in regulating gene silencing and hence, SMYD5 inhibition may result in upregulation of several proteins as observed in our proteomics data. Further studies are reqired to fully elucidate the interactions between miR-214 and SMYD5 and their resultant implications on VSMC biology.
6. A major area of our research is converting our findings into treatment perspective: It would be interesting to develop such mechanisms which help enable our findings in lab, transfer into human to treat specific disease. Using nano-particles is emerging as new delivery method to deliver and apply the drug at the microenvironment level. Hence, Site specific delivery of miR214 using nano-particles to reduce neointima hyperplasia would be an interesting and challenging way forward to translate laboratory findings into clinical outcome.

8 Appendix

Proteins	LogFR (Treatment vs Control	P-Value	Fold changes (Treatment vsControl)	Binding sites*	ProteinName
Downregulatedproteinsbymi-214					
PEPD_MOUSE	-2.737973	0.4	0.149895	No	Xaa-ProdipeptidaseOS=Mus musculusGN=PepdPE=2SV=3
ABL2_MOUSE	-1.454600	0.5	0.364856	No	Abelsontyrosine-proteinkinase2 OS=MusmusculusGN=Abl2PE=1 SV=1
LSM8_MOUSE	-1.454600	0.5	0.364856	No	U6snRNA-associatedSm-like proteinLSm8OS=Musmusculus GN=Lsm8PE=3SV=3
NCKP1_MOUSE	-1.434939	0	0.369863	Yes(3)	Nck-associatedprotein1OS=Mus musculusGN=Nckap1PE=1SV=2
HYPK_MOUSE	-1.353127	0	0.391443	yes(1)	Huntingtin-interactingproteinK OS=MusmusculusGN=HypkPE=2 SV=2
COX2_MOUSE	-1.302748	0	0.405353	No	Cytochromec oxidasesubunit2 OS=MusmusculusGN=Mtco2PE=1 SV=1
EMAL1_MOUSE	-1.160103	0	0.447481	yes(1)	Echinodermmicrotubule-associated protein-like1OS=Musmusculus GN=Eml1PE=1SV=1
SYAP1_MOUSE	-1.021893	0.5	0.49247	No	Synapse-associatedprotein1 OS=MusmusculusGN=Syap1PE=1 SV=1
SPTB1_MOUSE	-0.918821	0	0.528941	Yes(2)	Spectrinbetachain,erythrocytic OS=MusmusculusGN=SptbPE=1 SV=4
ILEUC_MOUSE	-0.827446	0.5	0.563526	No	LeukocyteelastaseinhibitorC OS=MusmusculusGN=Serpinb1c PE=2SV=1
LYPA2_MOUSE	-0.806064	0	0.57194	Yes(1)	Acyl-proteinthioesterase2 OS=Mus musculusGN=Lypla2PE=1SV=1
KLF14_MOUSE	-0.793026	0	0.577132	Yes(2)	Krueppel-likefactor14OS=Mus musculusGN=Klf14PE=2SV=1
ARPC2_MOUSE	-0.786859	0	0.579604	no	Actin-relatedprotein2/3complex subunit2OS=Musmusculus GN=Arpc2PE=1SV=3
PGM1_MOUSE	-0.786171	0.1	0.579881	No	Phosphoglucomutase-1OS=Mus musculusGN=Pgm1PE=1SV=4
PPM1F_MOUSE	-0.753456	0	0.593181	Yes(3)	Proteinphosphatase1FOS=Mus musculusGN=Ppm1fPE=2SV=1
ABHEB_MOUSE	-0.704636	0.1	0.613597	No	Alpha/betahydrolasedomain-containingprotein14BOS=Mus musculusGN=Abhd14bPE=2SV=1
TPP1_MOUSE	-0.689847	0	0.61992	Yes(5)	Tripeptidyl-peptidase1OS=Mus

					musculusGN=Tpp1PE=1SV=2
SNX12_MOUSE	-0.670723	0.1	0.628192		Sortingnexin-12OS=Musmusculus GN=Snx12PE=1SV=1
CIRBP_MOUSE	-0.659948	0.1	0.632901	No	Cold-inducibleRNA-bindingprotein OS=MusmusculusGN=CirbpPE=1 SV=1
IDHC_MOUSE	-0.653167	0	0.635883	Yes(2)	Isocitratedehydrogenase[NADP] cytoplasmicOS=Musmusculus GN=Idh1PE=1SV=2
INT3_MOUSE	-0.632013	0	0.645275	Yes(2)	Integratorcomplexsubunit3 OS=MusmusculusGN=Ints3PE=2 SV=2
LIMS2_MOUSE	-0.599727	0	0.659879	No	LIMandsenescentcellantigen-like-containingdomainprotein2 OS=MusmusculusGN=Lims2PE=1 SV=1
SNX5_MOUSE	-0.595149	0	0.661976	Yes(1)	Sortingnexin-5OS=Musmusculus GN=Snx5PE=1SV=1
SMYD5_MOUSE	-0.572227	0	0.672578	Yes(4)	SETandMYNDdomain-containing protein5OS=Musmusculus GN=Smyd5PE=2SV=2
APC7_MOUSE	-0.553523	0	0.681354	Yes(2)	Anaphase-promotingcomplex subunit7OS=Musmusculus GN=Anapc7PE=1SV=3
ACOT9_MOUSE	-0.552868	0	0.681664	Yes(2)	Acyl-coenzymeAthioesterase9, mitochondrialOS=Musmusculus GN=Acot9PE=1SV=1
LONM_MOUSE	-0.550077	0	0.682983	Yes(1)	Lonproteasehomolog,mitochondrial OS=MusmusculusGN=Lonp1PE=1 SV=2
SNP29_MOUSE	-0.543593	0	0.68606	yes(3)	Synaptosomal-associatedprotein29 OS=MusmusculusGN=Snap29 PE=2SV=1
FKB10_MOUSE	-0.528053	0.4	0.69349	No	Peptidyl-prolylcis-transisomerase FKBP10OS=Musmusculus GN=Fkbp10PE=1SV=2
GSTM1_MOUSE	-0.518952	0	0.697878	Yes(2)	GlutathioneS-transferaseMu1 OS=MusmusculusGN=Gstm1PE=1 SV=2
KIME_MOUSE	-0.515848	0	0.699382	yes(1)	MevalonatekinaseOS=Mus musculusGN=MvkPE=2SV=1
ARL3_MOUSE	-0.512953	0	0.700787	Yes(1)	ADP-ribosylationfactor-likeprotein3 OS=MusmusculusGN=Arl3PE=1 SV=1
SAMH1_MOUSE	-0.484792	0	0.7146	Yes(2)	Deoxynucleosidetriphosphate triphosphohydrolaseSAMHD1 OS=MusmusculusGN=Samhd1 PE=1SV=2
SRRT_MOUSE	-0.483568	0	0.715207	Yes(1)	SerrateRNAeffectormolecule homologOS=Musmusculus GN=SrrtPE=1SV=1
AP2B1_MOUSE	-0.474446	0	0.719743	Yes(6)	AP-2complexsubunitbetaOS=Mus musculusGN=Ap2b1PE=1SV=1
PROF1_MOUSE	-0.472765	0	0.720582	Yes(2)	Profilin-1OS=Musmusculus GN=Pfn1PE=1SV=2
GSTM2_MOUSE	-0.470258	0	0.721836	Yes(1)	GlutathioneS-transferaseMu2 OS=MusmusculusGN=Gstm2PE=1 SV=2

CAPZB_MOUSE	-0.463590	0.3	0.725179	No	F-actin-cappingproteinsubunitbeta OS=MusmusculusGN=CapzbPE=1 SV=3
TGM2_MOUSE	-0.461355	0	0.726304	Yes(4)	Protein-glutaminegamma-glutamyltransferase2OS=Mus musculusGN=Tgm2PE=1SV=4
RCN3_MOUSE	-0.457328	0	0.728334	No	Reticulocalbin-3OS=Musmusculus GN=Rcn3PE=2SV=1
SMC3_MOUSE	-0.451019	0	0.731526	Yes(5)	Structuralmaintenanceof chromosomesprotein3OS=Mus musculusGN=Smc3PE=1SV=2
RHOA_MOUSE	-0.447904	0	0.733107	Yes(1)	TransformingproteinRhoAOS=Mus musculusGN=RhoaPE=1 SV=1
G3BP1_MOUSE	-0.444125	0	0.73503	Yes(7)	RasGTPase-activatingprotein-bindingprotein1OS=Musmusculus GN=G3bp1PE=1SV=1
LAMP1_MOUSE	-0.443885	0	0.735152	Yes(1)	Lysosome-associatedmembrane glycoprotein1OS=Musmusculus GN=Lamp1PE=1SV=2
SMC1A_MOUSE	-0.443237	0	0.735483	Yes(1)	Structuralmaintenanceof chromosomesprotein1AOS=Mus musculusGN=Smc1aPE=1SV=4
SPI2_MOUSE	-0.441805	0.5	0.736213	No	SerpinI2OS=Musmusculus GN=Serpini2PE=2SV=1
PLSI_MOUSE	-0.440962	0	0.736643	Yes(2)	Plastin-1OS=Musmusculus GN=Pls1PE=2SV=1
LTOR3_MOUSE	-0.439528	0	0.737376	Yes(3)	Ragulatorcomplexprotein LAMTOR3OS=Musmusculus GN=Lamtor3PE=1SV=1
PSB4_MOUSE	-0.438911	0	0.737691	No	Proteasomesubunitbetatype-4 OS=MusmusculusGN=Psmb4 PE=1SV=1
THIM_MOUSE	-0.437870	0	0.738224	Yes(2)	3-ketoacyl-CoAthiolase, mitochondrialOS=Musmusculus GN=Acaa2PE=1SV=3
STK25_MOUSE	-0.434782	0	0.739805	Yes(1)	Serine/threonine-protein kinase25 OS=MusmusculusGN=Stk25PE=1 SV=2
CATD_MOUSE	-0.434733	0	0.739831	Yes(4)	CathepsinDOS=Musmusculus GN=CtsdPE=1SV=1
ULA1_MOUSE	-0.433753	0	0.740333	No	NEDD8-activatingenzymeE1 regulatorysubunitOS=Mus musculusGN=Nae1PE=1 SV=1
TBAL3_MOUSE	-0.433621	0	0.740401	Yes(1)	Tubulinalphachain-like3OS=Mus musculusGN=Tubal3PE=2SV=2
CSN5_MOUSE	-0.431786	0.1	0.741343	No	COP9signalosomecomplexsubunit 5OS=MusmusculusGN=Cops5 PE=1SV=3
PPP6_MOUSE	-0.421643	0	0.746574	Yes(2)	Serine/threonine-protein phosphatase6catalyticsubunit OS=MusmusculusGN=Ppp6cPE=2 SV=1
RS27A_MOUSE	-0.421124	0	0.746842	No	Ubiquitin-40Sribosomalprotein S27aOS=Musmusculus GN=Rps27aPE=1SV=2
					Chromoboxproteinhomolog1 OS=MusmusculusGN=Cbx1PE=1

CBX1_MOUSE	-0.419343	0	0.747765	Yes(2)	SV=1
MK10_MOUSE	-0.418101	0	0.748409	Yes(3)	Mitogen-activatedproteinkinase10 OS=MusmusculusGN=Mapk10 PE=1SV=2
UpregulatedproteinsbymiR-214					
PSB7_MOUSE	0.323204	0.2	1.251106		Proteasomesubunitbetatype-7 OS=MusmusculusGN=Psmb7 PE=1SV=1
DPOE1_MOUSE	0.323513	0.1	1.251374		DNApolymeraseepsiloncatalytic subunitA OS=Musmusculus GN=PolePE=2SV=3
CALX_MOUSE	0.325062	0.3	1.252718		CalnexinOS=Musmusculus GN=CanxPE=1SV=1
COPZ1_MOUSE	0.326198	0.3	1.253705		Coatomersubunitzeta-1OS=Mus musculusGN=Copz1PE=2SV=1
VPS29_MOUSE	0.327551	0.1	1.254881		Vacuolarproteinsorting-associated protein29OS=Musmusculus GN=Vps29PE=1SV=1
NASP_MOUSE	0.328329	0.1	1.255558		Nuclearautoantigenicspermprotein OS=MusmusculusGN=NaspPE=1 SV=2
HNRPK_MOUSE	0.328623	0.2	1.255814		Heterogeneousnuclear ribonucleoproteinKOS=Mus musculusGN=HnrnpkPE=1SV=1
RTCB_MOUSE	0.328922	0.5	1.256075		tRNA-splicingligaseRtcBhomolog OS=MusmusculusGN=RtcbPE=2 SV=1
UBL4A_MOUSE	0.329965	0.2	1.256983		Ubiquitin-likeprotein4AOS=Mus musculusGN=Ubl4aPE=2SV=1
H12_MOUSE	0.330383	0.3	1.257347		HistoneH1.2OS=Musmusculus GN=Hist1h1cPE=1SV=2
PHB2_MOUSE	0.332705	0	1.259372		Prohibitin-2OS=Musmusculus GN=Phb2PE=1SV=1
SC61B_MOUSE	0.335326	0.2	1.261662		ProteintransportproteinSec61 subunitbetaOS=Musmusculus GN=Sec61bPE=1SV=3
CNPY2_MOUSE	0.339157	0.3	1.265017		Proteincanopyhomolog2 OS=Mus musculusGN=Cnpy2PE=1SV=1
TRXR1_MOUSE	0.340636	0.1	1.266315		Thioredoxinreductase1, cytoplasmicOS=Musmusculus GN=Txnrd1PE=1SV=3
QCR1_MOUSE	0.341453	0.2	1.267032		Cytochromeb-c1complexsubunit1, mitochondrialOS=Musmusculus GN=Uqcrc1PE=1SV=2
RFA2_MOUSE	0.341865	0	1.267394		ReplicationproteinA 32kDasubunit OS=MusmusculusGN=Rpa2PE=1 SV=1
UBE2K_MOUSE	0.343689	0.2	1.268997		Ubiquitin-conjugatingenzymeE2K OS=MusmusculusGN=Ube2kPE=1 SV=3
NUCB2_MOUSE	0.348476	0.3	1.273215		Nucleobindin-2OS=Musmusculus GN=Nucb2PE=1SV=2
ALR_MOUSE	0.349409	0.5	1.274038		FAD-linkedsulfhydryloxidaseALR OS=MusmusculusGN=GferPE=2 SV=2
TIA1_MOUSE	0.352377	0.1	1.276662		NucleolysinTIA-1OS=Mus musculusGN=Tia1PE=1SV=1
TBC15_MOUS	0.353875	0.4	1.277988		TBC1domainfamilymember15

E					OS=MusmusculusGN=Tbc1d15 PE=1SV=1
LSM6_MOUSE	0.356710	0	1.280502		U6snRNA-associatedSm-like proteinLSm6OS=Musmusculus GN=Lsm6PE=3SV=1
IMPA2_MOUSE	0.357044	0.6	1.280799		Inositolmonophosphatase2 OS=MusmusculusGN=Impa2PE=1 SV=1
VATA_MOUSE	0.360771	0.2	1.284112		V-typeprotonATPasecatalytic subunitA OS=Musmusculus GN=Atp6v1aPE=1SV=2
CTBP1_MOUSE	0.364133	0	1.287108		C-terminal-bindingprotein1 OS=MusmusculusGN=Ctbp1PE=1 SV=2
SZT2_MOUSE	0.365471	0.2	1.288303		ProteinSZT2OS=Musmusculus GN=Szt2PE=1SV=1
RBM25_MOUSE	0.365489	0	1.288318		RNA-bindingprotein25OS=Mus musculusGN=Rbm25PE=1SV=2
PTMS_MOUSE	0.366225	0.7	1.288976		ParathymosinOS=Musmusculus GN=PtmsPE=1SV=3
INF2_MOUSE	0.367510	0.1	1.290124		Invertedformin-2OS=Musmusculus GN=Inf2PE=1SV=1
ILF3_MOUSE	0.368909	0	1.291376		Interleukinenhancer-bindingfactor3 OS=MusmusculusGN=Ilf3PE=1 SV=2
PABP2_MOUSE	0.371446	0.1	1.293649		Polyadenylate-bindingprotein2 OS=MusmusculusGN=Pabpn1 PE=2SV=3
ALD2_MOUSE	0.372046	0.2	1.294187		Aldosereductase-relatedprotein2 OS=MusmusculusGN=Akr1b8 PE=1SV=2
TCOF_MOUSE	0.373927	0	1.295875		TreacleproteinOS=Musmusculus GN=Tcof1PE=1SV=1
I2BP2_MOUSE	0.374380	0.3	1.296283		Interferonregulatoryfactor2-binding protein2OS=Musmusculus GN=Irf2bp2PE=1SV=1
H32_MOUSE	0.375301	0.2	1.29711		HistoneH3.2OS=Musmusculus GN=Hist1h3bPE=1SV=2
XRN2_MOUSE	0.376271	0.3	1.297983		5'-3'exoribonuclease2OS=Mus musculusGN=Xrn2PE=1SV=1
CK5P2_MOUSE	0.376365	0.2	1.298067		CDK5regulatorysubunit-associated protein2OS=Musmusculus GN=Cdk5rap2PE=1SV=3
RPN1_MOUSE	0.377760	0.1	1.299323		Dolichyl-diphosphooligosaccharide--proteinglycosyltransferasesubunit1 OS=MusmusculusGN=Rpn1PE=1 SV=1
RS28_MOUSE	0.378317	0.3	1.299825		40SribosomalproteinS28OS=Mus musculusGN=Rps28PE=2SV=1
API5_MOUSE	0.378529	0.3	1.300016		Apoptosisinhibitor5OS=Mus musculusGN=Api5PE=1SV=2
IF2B3_MOUSE	0.379407	0.2	1.300807		Insulin-likegrowthfactor2mRNA-bindingprotein3OS=Musmusculus GN=Igf2bp3PE=1SV=1
CUL4B_MOUSE	0.379845	0.2	1.301202		Cullin-4BOS=Musmusculus GN=Cul4bPE=1SV=1
NXT2_MOUSE	0.380032	0.1	1.30137		NTF2-relatedexportprotein2 OS=MusmusculusGN=Nxt2PE=2
					SV=1

MYL6B_MOUSE	0.382474	0	1.303576		Myosinlightchain6BOS=Mus musculusGN=Myl6bPE=2SV=1
PYR1_MOUSE	0.384037	0.2	1.304988		CADproteinOS=Musmusculus GN=CadPE=2SV=1
RTN4_MOUSE	0.384912	0.1	1.30578		Reticulon-4OS=Musmusculus GN=Rtn4PE=1SV=2
KTN1_MOUSE	0.385735	0.1	1.306525		KinectinOS=Musmusculus GN=Ktn1PE=2SV=1
E2F8_MOUSE	0.386205	0	1.306951		
MALT1_MOUSE	0.386487	0.4	1.307206		Mucosa-associatedlymphoidtissue lymphomatranslocationprotein1 homologOS=Musmusculus GN=Malt1PE=1SV=2
KLC2_MOUSE	0.388877	0.4	1.309374		Kinesinlightchain2OS=Mus musculusGN=Klc2PE=1SV=1
GFPT2_MOUSE	0.393627	0	1.313692		Glutamine--fructose-6-phosphate aminotransferase[isomerizing]2 OS=MusmusculusGN=Gfpt2PE=2 SV=3
BAP31_MOUSE	0.396808	0.1	1.316592		B-cellreceptor-associatedprotein31 OS=MusmusculusGN=Bcap31 PE=1SV=4
RAB10_MOUSE	0.405410	0.4	1.324465		Ras-relatedproteinRab-10OS=Mus musculusGN=Rab10PE=1SV=1
H1T_MOUSE	0.406704	0.2	1.325653		HistoneH1tOS=Musmusculus GN=Hist1h1tPE=1SV=4
NDUA4_MOUSE	0.411036	0.6	1.32964		NADHdehydrogenase[ubiquinone] 1alphasubcomplexsubunit4 OS=MusmusculusGN=Ndufa4 PE=1SV=2
RS27L_MOUSE	0.411764	0.2	1.330311		40SribosomalproteinS27-like OS=MusmusculusGN=Rps27l PE=2SV=3
RRBP1_MOUSE	0.414189	0.2	1.33255		Ribosome-bindingprotein1 OS=Mus musculusGN=Rrbp1PE=1SV=2
D19L1_MOUSE	0.414387	0.2	1.332733		ProbableC-mannosyltransferase DPY19L1OS=Musmusculus GN=Dpy19l1PE=2SV=1
PRUNE_MOUSE	0.414520	0.5	1.332855		ProteinprunehomologOS=Mus musculusGN=PrunePE=2SV=1
UCHL5_MOUSE	0.415921	0.2	1.33415		Ubiquitincarboxyl-terminalhydrolase isozymeL5OS=Musmusculus GN=Uchl5PE=1SV=2
SH3L3_MOUSE	0.420241	0.2	1.338151		SH3domain-bindingglutamicacid-rich-likeprotein3OS=Musmusculus GN=Sh3bgrl3PE=1SV=1
AN32B_MOUSE	0.422776	0.4	1.340504		Acidicleucine-richnuclear phosphoprotein32 familymemberB OS=MusmusculusGN=Anp32b PE=1SV=1
TYB10_MOUSE	0.423131	0.4	1.340834		Thymosinbeta-10OS=Mus musculusGN=Tmsb10PE=2SV=3
VASP_MOUSE	0.425311	0.3	1.342862		Vasodilator-stimulated phosphoproteinOS=Musmusculus GN=VaspPE=1SV=4
IDHP_MOUSE	0.427113	0.2	1.344541		Isocitratedehydrogenase[NADP], mitochondrialOS=Musmusculus
					GN=Idh2PE=1SV=3

SFR1_MOUSE	0.428830	0.3	1.346141		Swi5-dependentrecombinationDNA repairprotein1homologOS=Mus musculusGN=Sfr1PE=1SV=2
CO6A1_MOUSE	0.435420	0.2	1.352304		Collagenalpha-1(VI)chainOS=Mus musculusGN=Col6a1PE=2SV=1
CCD38_MOUSE	0.438081	0.4	1.354801		
PPP5_MOUSE	0.439429	0.5	1.356067		Serine/threonine-protein phosphatase5OS=Musmusculus GN=Ppp5cPE=1SV=3
ILF2_MOUSE	0.445389	0.3	1.361681		Interleukinenhancer-bindingfactor2 OS=MusmusculusGN=Ilf2PE=1 SV=1
ERLN2_MOUSE	0.449599	0.1	1.36566		Erlin-2OS=Musmusculus GN=Erlin2PE=1SV=1
TTC9C_MOUSE	0.456300	0	1.372019		Tetratricopeptiderepeatprotein9C OS=MusmusculusGN=Ttc9cPE=2 SV=1
SRA1_MOUSE	0.458491	0.1	1.374104		SteroidreceptorRNAactivator1 OS=MusmusculusGN=Sra1PE=1 SV=3
RAB1A_MOUSE	0.461907	0.5	1.377361		Ras-relatedproteinRab-1AOS=Mus musculusGN=Rab1APE=1SV=3
QCR6_MOUSE	0.462570	0.4	1.377995		Cytochromeb-c1complexsubunit6, mitochondrialOS=Musmusculus GN=UqcrhPE=1SV=2
SAE2_MOUSE	0.463759	0	1.379131		SUMO-activatingenzymesubunit2 OS=MusmusculusGN=Uba2PE=1 SV=1
H2AZ_MOUSE	0.467525	0.2	1.382735		HistoneH2A.ZOS=Musmusculus GN=H2afzPE=1SV=2
SMAD3_MOUSE	0.476613	0.2	1.391473		Mothersagainstdecapentaplegic homolog3OS=Musmusculus GN=Smad3PE=1SV=2
IKIP_MOUSE	0.477947	0	1.39276		Inhibitorof nuclearfactorkappa-B kinase-interactingproteinOS=Mus musculusGN=IkbipPE=2SV=2
HBA_MOUSE	0.478404	0.4	1.393201		HemoglobinsubunitalphaOS=Mus musculusGN=HbaPE=1SV=2
CALL3_MOUSE	0.479895	0.5	1.394642		Calmodulin-likeprotein3OS=Mus musculusGN=Calml3PE=2SV=1
FBRL_MOUSE	0.481143	0.1	1.395849		rRNA2'-O-methyltransferase fibrillarinOS=MusmusculusGN=Fbl PE=2SV=2
ARF4_MOUSE	0.486244	0.2	1.400793		ADP-ribosylationfactor4OS=Mus musculusGN=Arf4PE=1SV=2
GCR_MOUSE	0.488620	0.1	1.403102		GlucocorticoidreceptorOS=Mus musculusGN=Nr3c1PE=1SV=1
TBCA_MOUSE	0.491108	0.4	1.405524		Tubulin-specificchaperoneA OS=MusmusculusGN=TbcaPE=2 SV=3
OFUT2_MOUSE	0.495153	0.4	1.40947		GDP-fucoseproteinO-fucosyltransferase2OS=Mus musculusGN=Pofut2PE=1SV=1
LTOR2_MOUS					Ragulatorcomplexprotein LAMTOR2OS=Musmusculus
ATPD_MOUSE	0.500446	0.3	1.41465		ATPsynthasesubunitdelta, mitochondrialOS=Musmusculus GN=Atp5dPE=1SV=1
MKLN1_MOUS					

E	0.500446	0.3	1.41465		
MP2K1_MOUSE	0.501973	0.1	1.416149		Dualspecificitymitogen-activated proteinkinasekinase1 OS=Mus musculusGN=Map2k1PE=1SV=2
REPS1_MOUSE	0.508337	0.1	1.42241		RalBP1-associatedEpsdomain-containingprotein1 OS=Mus musculusGN=Reps1PE=1SV=2
DHRS4_MOUSE	0.509393	0.2	1.423451		Dehydrogenase/reductaseSDR familymember4OS=Musmusculus GN=Dhrs4PE=1SV=3
PTMA_MOUSE	0.525322	0.4	1.439254		ProthymosinalphaOS=Mus musculusGN=PtmaPE=1SV=2
NC2B_MOUSE	0.527111	0.1	1.441041		ProteinDr1OS=Musmusculus GN=Dr1PE=2SV=1
FETUA_MOUSE	0.530992	0.3	1.444922		Alpha-2-HS-glycoprotein OS=Mus musculusGN=AhsgPE=1SV=1
LAP2B_MOUSE	0.531212	0.1	1.445143		Lamina-associatedpolypeptide2, isoformsbeta/delta/epsilon/gamma OS=MusmusculusGN=TmpoPE=1 SV=4
PRS10_MOUSE	0.532698	0.4	1.446632		26Sproteaseregulatorysubunit10B OS=MusmusculusGN=Psmc6 PE=1SV=1
NEST_MOUSE	0.533669	0.2	1.447606		NestinOS=MusmusculusGN=Nes PE=1SV=1
NPTN_MOUSE	0.533795	0.1	1.447732		NeuroplastinOS=Musmusculus GN=NptnPE=1SV=3
E41L3_MOUSE	0.537226	0.5	1.451179		Band4.1-likeprotein3OS=Mus musculusGN=Epb41l3PE=1SV=1
MEPCE_MOUSE	0.537434	0.2	1.451389		7SKsnRNAmethylphosphate cappingenzymeOS=Musmusculus GN=MepcePE=1SV=2
LAP2A_MOUSE	0.537485	0	1.45144		Lamina-associatedpolypeptide2, isoformsalpha/zetaOS=Mus musculusGN=TmpoPE=1SV=4
SRSF4_MOUSE	0.550978	0	1.465078		Serine/arginine-richsplicingfactor4 OS=MusmusculusGN=Srsf4PE=2 SV=1
PR40A_MOUSE	0.552884	0	1.467015		Pre-mRNA-processingfactor40 homologA OS=Musmusculus GN=Prpf40aPE=1SV=1
H33_MOUSE	0.562486	0.2	1.476811		HistoneH3.3OS=Musmusculus GN=H3f3aPE=1SV=2
DHRS1_MOUSE	0.567058	0.1	1.481499		Dehydrogenase/reductaseSDR familymember1OS=Musmusculus GN=Dhrs1PE=2SV=1
IF1A_MOUSE	0.571096	0	1.485652		Eukaryotictranslationinitiationfactor 1AOS=MusmusculusGN=Eif1a PE=2SV=3
PP14B_MOUSE	0.595869	0.2	1.511382		Proteinphosphatase1regulatory subunit14BOS=Musmusculus GN=Ppp1r14bPE=1SV=2
IMMT_MOUSE	0.599209	0.1	1.514886		Mitochondrialinnermembrane proteinOS=MusmusculusGN=Immt
					PE=1SV=1
MBB1A_MOUSE	0.601030	0	1.5168		Myb-bindingprotein1AOS=Mus musculusGN=Mybbp1aPE=1SV=2
					Ribonucleoside-diphosphate

RIR1_MOUSE	0.603171	0	1.519051		reductaselargesubunitOS=Mus musculusGN=Rrm1PE=1SV=2
IF2B2_MOUSE	0.607204	0.2	1.523304		Insulin-likegrowthfactor2mRNA-bindingprotein2OS=Musmusculus GN=Igf2bp2PE=1SV=1
H13_MOUSE	0.627204	0	1.544569		HistoneH1.3OS=Musmusculus GN=Hist1h1dPE=1SV=2
RANB3_MOUSE	0.630777	0.3	1.548399		Ran-bindingprotein3OS=Mus musculusGN=Ranbp3PE=1SV=2
SPF27_MOUSE	0.635218	0.3	1.553173		Pre-mRNA-splicingfactorSPF27 OS=MusmusculusGN=Bcas2PE=2 SV=1
P20L1_MOUSE	0.637010	0	1.555103		PHDfingerprotein20-likeprotein1 OS=MusmusculusGN=Phf20l1 PE=2SV=2
HMGA2_MOUSE	0.643604	0.1	1.562227		HighmobilitygroupproteinHMGI-C OS=MusmusculusGN=Hmga2 PE=1SV=1
EDF1_MOUSE	0.643840	0.3	1.562482		Endothelialdifferentiation-related factor1OS=MusmusculusGN=Edf1 PE=1SV=1
TECR_MOUSE	0.644426	0.2	1.563117		Very-long-chainenoyl-CoA reductaseOS=Musmusculus GN=TecrPE=1SV=1
ITAL_MOUSE	0.649337	0.1	1.568448		Integrinalpha-LOS=Musmusculus GN=ItgalPE=1SV=2
ABCE1_MOUSE	0.650618	0.1	1.56984		ATP-bindingcassettesub-familyE member1OS=Musmusculus GN=Abce1PE=2SV=1
C1QBP_MOUSE	0.665730	0.2	1.586371		Complementcomponent1Q subcomponent-bindingprotein, mitochondrialOS=Musmusculus GN=C1qbpPE=1SV=1
AT2B2_MOUSE	0.671810	0.2	1.59307		Plasmamembranecalcium-transportingATPase2OS=Mus musculusGN=Atp2b2PE=1SV=2
DVL3_MOUSE	0.674132	0.3	1.595637		Segmentpolarityproteindishevelled homologDVL-3OS=Musmusculus GN=Dvl3PE=1SV=2
RFPLA_MOUSE	0.691521	0.2	1.614985		Retfingerprotein-like4AOS=Mus musculusGN=Rfpl4aPE=2SV=1
WNK1_MOUSE	0.703097	0.6	1.627996		Serine/threonine-protein kinase WNK1OS=Musmusculus GN=Wnk1PE=1SV=2
NDUS5_MOUSE	0.703673	0.1	1.628646		NADHdehydrogenase[ubiquinone] iron-sulfurprotein5OS=Mus musculusGN=Ndufs5PE=1SV=3
FKB11_MOUSE	0.704716	0.6	1.629824		Peptidyl-prolylcis-transisomerase FKBP11OS=Musmusculus GN=Fkbp11PE=2SV=1
SC22B_MOUSE	0.707687	0.1	1.633183		Vesicle-traffickingproteinSEC22b OS=MusmusculusGN=Sec22b PE=1SV=3
PUR8_MOUSE	0.718084	0.1	1.644996		AdenylosuccinatelyaseOS=Mus
					musculusGN=AdslPE=2SV=2
BIEA_MOUSE	0.734828	0	1.664199		BiliverdinreductaseAOS=Mus musculusGN=BlvraPE=2SV=1
ADT4_MOUSE	0.752110	0	1.684254		ADP/ATPtranslocase4 OS=Mus musculusGN=Slc25a31PE=2SV=1

H2AY_MOUSE	0.761745	0.3	1.69554		Corehistonemacro-H2A.1OS=Mus musculusGN=H2afyPE=1SV=3
GSLG1_MOUSE	0.771952	0.3	1.707579		Golgiapparatusprotein1 OS=Mus musculusGN=Glg1PE=1SV=1
EVL_MOUSE	0.778213	0	1.715005		Ena/VASP-likeproteinOS=Mus musculusGN=EvlPE=1SV=2
RPC2_MOUSE	0.780418	0	1.717628		DNA-directedRNApolymeraseIII subunitRPC2OS=Musmusculus GN=Polr3bPE=2SV=2
SLK_MOUSE	0.789592	0.1	1.728586		STE20-likeserine/threonine-protein kinaseOS=MusmusculusGN=Slk PE=1SV=2
LARP7_MOUSE	0.790086	0	1.729178		La-relatedprotein7OS=Mus musculusGN=Larp7PE=1SV=2
ATPG_MOUSE	0.849791	0	1.80224		ATPsynthasesubunitgamma, mitochondrialOS=Musmusculus GN=Atp5c1PE=1SV=1
H2A2B_MOUSE	0.867560	0	1.824574		HistoneH2Atype2-BOS=Mus musculusGN=Hist2h2abPE=1 SV=3
ZBT43_MOUSE	0.883939	0.1	1.845407		Zincfingerand BTBdomain-containingprotein43OS=Mus musculusGN=Zbtb43PE=2SV=2
HECD1_MOUSE	0.898640	0.3	1.864307		E3ubiquitin-proteinligaseHECTD1 OS=MusmusculusGN=Hectd1 PE=1SV=2
ALBU_MOUSE	0.907684	0.3	1.876031		SerumalbuminOS=Musmusculus GN=AlbPE=1SV=3
MEP50_MOUSE	0.921289	0.4	1.893807		Methylosomeprotein50 OS=Mus musculusGN=Wdr77PE=1SV=1
MAP6_MOUSE	0.960701	0	1.946255		Microtubule-associatedprotein6 OS=MusmusculusGN=Map6PE=1 SV=2
PLP2_MOUSE	0.968741	0.4	1.957132		Proteolipidprotein2OS=Mus musculusGN=Plp2PE=2SV=1
M4K4_MOUSE	1.004881	0.1	2.006777		Mitogen-activatedproteinkinase kinasekinasekinase4OS=Mus musculusGN=Map4k4PE=1SV=1
EHD3_MOUSE	1.008355	0	2.011615		EHdomain-containingprotein3 OS=MusmusculusGN=Ehd3PE=1 SV=2
FBLN3_MOUSE	1.010373	0.1	2.014431		EGF-containingfibulin-like extracellularmatrixprotein1 OS=MusmusculusGN=Efemp1 PE=2SV=1
PI3R4_MOUSE	1.022615	0.2	2.031597		Phosphoinositide3-kinaseregulatory subunit4OS=Musmusculus GN=Pik3r4PE=1SV=3
HAP28_MOUSE	1.123091	0.7	2.178132		28kDaheat-andacid-stable phosphoproteinOS=Musmusculus GN=Pdap1PE=1SV=1
ATPO_MOUSE	1.164437	0.6	2.241457		ATPsynthasesubunitO, mitochondrialOS=Musmusculus
					GN=Atp5oPE=1SV=1
NUFP2_MOUSE	1.298795	0.1	2.460232		NuclearfragileX mentalretardation-interactingprotein2OS=Mus musculusGN=Nufip2PE=1SV=1
MRCKB_MOUS					Serine/threonine-protein kinase MRCKbetaOS=Musmusculus

E					GN=Cdc42bpbPE=1SV=2
CPPED_MOUSE	1.474910	0	2.779663		Serine/threonine-protein phosphataseCPPED1OS=Mus musculusGN=Cpped1PE=2SV=1
ABRA_MOUSE	1.561250	0.3	2.951095		
H2AX_MOUSE	1.567391	0.1	2.963682		HistoneH2AXOS=Musmusculus GN=H2afxPE=1SV=2
BRCC3_MOUSE	1.613716	0.4	3.060391		Lys-63-specificdeubiquitinase BRCC36OS=Musmusculus GN=Brcc3PE=2SV=1
ARP3B_MOUSE	1.795556	0.6	3.471492		Actin-relatedprotein3BOS=Mus musculusGN=Actr3bPE=2SV=1
FRIL2_MOUSE	2.195888	0.4	4.581715		Ferritinlightchain2OS=Mus musculusGN=Ftl2PE=2SV=2
OtherreportedtargetsofmiR-214					
CTNB1_MOUSE	-0.181716	0.6	0.881654		Cateninbeta-1OS=Musmusculus GN=Ctnnb1PE=1SV=1
PCBP2_MOUS					Poly(rC)-bindingprotein2 OS=Mus

Table 16. . Proteomics data.Complete list of Proteins regulated by miR-214 in VSMCs.

Note: Proteins/genes with yellow-highlighted have been implicated in regulation of cell migration, proliferation, adhesion, actin filament reorganization and actin polymerization, cell cycle, and gene expression, etc.

9 Reference:

Abdullah, A., et al.
2011 The number of years lived with obesity and the risk of all-cause and cause-specific mortality. Int J Epidemiol 40(4):985-96.

Ahmed, F. W., et al.
2016 Metformin improves circulating endothelial cells and endothelial progenitor cells in type 1 diabetes: MERIT study. Cardiovasc Diabetol 15(1):116.

Ahmed, M. I., et al.
2014 MicroRNA-214 controls skin and hair follicle development by modulating the activity of the Wnt pathway. J Cell Biol 207(4):549-67.

Akhtar, S., et al.
2013 CXCL12 promotes the stabilization of atherosclerotic lesions mediated by smooth muscle progenitor cells in Apoe-deficient mice. Arterioscler Thromb Vasc Biol 33(4):679-86.

Albinsson, S., et al.
2010 MicroRNAs are necessary for vascular smooth muscle growth, differentiation, and function. Arterioscler Thromb Vasc Biol 30(6):1118-26.

Allahverdian, S., P. S. Pannu, and G. A. Francis
2012 Contribution of monocyte-derived macrophages and smooth muscle cells to arterial foam cell formation. Cardiovasc Res 95(2):165-72.

Allen, S. J., S. E. Crown, and T. M. Handel
2007 Chemokine: receptor structure, interactions, and antagonism. Annu Rev Immunol 25:787-820.

Alpert, M. A., et al.
2014 Obesity and heart failure: epidemiology, pathophysiology, clinical manifestations, and management. Transl Res 164(4):345-56.

Ambros, V.
2004 The functions of animal microRNAs. Nature 431(7006):350-5.

Ambros, V., et al.
2003 A uniform system for microRNA annotation. RNA 9(3):277-9.

Ambros, V., and H. R. Horvitz
1984 Heterochronic mutants of the nematode Caenorhabditis elegans. Science 226(4673):409-16.

Aoyama, T., et al.
2000 LOX-1 mediates lysophosphatidylcholine-induced oxidized LDL uptake in smooth muscle cells. FEBS Lett 467(2-3):217-20.

Atkins, G. B., and D. I. Simon
2013 Interplay between NF-kappaB and Kruppel-like factors in vascular inflammation and atherosclerosis: location, location, location. J Am Heart Assoc 2(3):e000290.

Auge, N., et al.
2002 Oxidized LDL-induced smooth muscle cell proliferation involves the EGF receptor/PI-3 kinase/Akt and the sphingolipid signaling pathways. Arterioscler Thromb Vasc Biol 22(12):1990-5.

Aurora, A. B., et al.
2012 MicroRNA-214 protects the mouse heart from ischemic injury by controlling Ca^{2+} overload and cell death. J Clin Invest 122(4):1222-32.

Barnes, M. J., and R. W. Farndale
1999 Collagens and atherosclerosis. Exp Gerontol 34(4):513-25.

Bartel, D. P.
2004 MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116(2):281-97.

Bellezza, I., et al.

2018 Nrf2-Keap1 signaling in oxidative and reductive stress. Biochim Biophys Acta Mol Cell Res 1865(5):721-733.

Ben-Shoshan, J., and J. George

2007 Endothelial progenitor cells as therapeutic vectors in cardiovascular disorders: from experimental models to human trials. Pharmacol Ther 115(1):25-36.

Berk, B. C.

2008 Atheroprotective signaling mechanisms activated by steady laminar flow in endothelial cells. Circulation 117(8):1082-9.

Bhaumik, S. R., E. Smith, and A. Shilatifard

2007 Covalent modifications of histones during development and disease pathogenesis. Nat Struct Mol Biol 14(11):1008-16.

Bitterli, L., et al.

2016 Endothelial progenitor cells as a biological marker of peripheral artery disease. Vasc Med 21(1):3-11.

Bjornheden, T., et al.

1999 Evidence of hypoxic areas within the arterial wall in vivo. Arterioscler Thromb Vasc Biol 19(4):870-6.

Blann, A. D., et al.

2005 Circulating endothelial cells. Biomarker of vascular disease. Thromb Haemost 93(2):228-35.

Blaszczyk, J., et al.

2001 Crystallographic and modeling studies of RNase III suggest a mechanism for double-stranded RNA cleavage. Structure 9(12):1225-36.

Bleul, C. C., et al.

1996 A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). J Exp Med 184(3):1101-9.

Bobryshev, Y. V.

2006 Monocyte recruitment and foam cell formation in atherosclerosis. Micron 37(3):208-22.

Bobryshev, Y. V., and R. S. Lord

1996 Langhans cells of human arterial intima: uniform by stellate appearance but different by nature. Tissue Cell 28(2):177-94.

Boettger, T., et al.

2009 Acquisition of the contractile phenotype by murine arterial smooth muscle cells depends on the Mir143/145 gene cluster. J Clin Invest 119(9):2634-47.

Bohlen, H. G.

1983 Tissue PO2 in the intestinal muscle layer of rats during chronic diabetes. Circ Res 52(6):677-82.

Boisvert, W. A., et al.

2006 Up-regulated expression of the CXCR2 ligand KC/GRO-alpha in atherosclerotic lesions plays a central role in macrophage accumulation and lesion progression. Am J Pathol 168(4):1385-95.

Bonello, L., et al.

2012 Mobilization of CD34+ KDR+ endothelial progenitor cells predicts target lesion revascularization. J Thromb Haemost 10(9):1906-13.

Bonetti, P. O., L. O. Lerman, and A. Lerman

2003 Endothelial dysfunction: a marker of atherosclerotic risk. Arterioscler Thromb Vasc Biol 23(2):168-75.

Braunwald, E.

2001 Changing the practice of cardiovascular medicine. Atheroscler Suppl 2(1):27-30.

Bryan, H. K., et al.

2013 The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation. Biochem Pharmacol 85(6):705-17.

Bucha, S., D. Mukhopadhyay, and N. P. Bhattacharyya
2015 Regulation of mitochondrial morphology and cell cycle by microRNA-214 targeting Mitofusin2. Biochem Biophys Res Commun 465(4):797-802.
Buchwalter, G., C. Gross, and B. Wasylyk
2004 Ets ternary complex transcription factors. Gene 324:1-14.
Burger, D., and R. M. Touyz
2012 Cellular biomarkers of endothelial health: microparticles, endothelial progenitor cells, and circulating endothelial cells. J Am Soc Hypertens 6(2):85-99.
Butini, L., et al.
1994 Intercellular adhesion molecules (ICAM)-1 ICAM-2 and ICAM-3 function as counter-receptors for lymphocyte function-associated molecule 1 in human immunodeficiency virus-mediated syncytia formation. Eur J Immunol 24(9):2191-5.
Camino-Lopez, S., et al.
2007 Tissue factor induction by aggregated LDL depends on LDL receptor-related protein expression (LRP1) and Rho A translocation in human vascular smooth muscle cells. Cardiovasc Res 73(1):208-16.
Cao, D., et al.
2005 Modulation of smooth muscle gene expression by association of histone acetyltransferases and deacetylases with myocardin. Mol Cell Biol 25(1):364-76.
Cao, S., et al.
2006 NF-kappaB1 (p50) homodimers differentially regulate pro- and anti-inflammatory cytokines in macrophages. J Biol Chem 281(36):26041-50.
Caplan, A. I.
2009 Why are MSCs therapeutic? New data: new insight. J Pathol 217(2):318-24.
Caplice, N. M., et al.
2003 Smooth muscle cells in human coronary atherosclerosis can originate from cells administered at marrow transplantation. Proc Natl Acad Sci U S A 100(8):4754-9.
Carè, A., et al.
2007 MicroRNA-133 controls cardiac hypertrophy. Nat Med 13(5):613-8.
Cercek, B., et al.
1997 Nuclear factor-kappaB activity and arterial response to balloon injury. Atherosclerosis 131(1):59-66.
Chahine, M. N., et al.
2009 Oxidized LDL affects smooth muscle cell growth through MAPK-mediated actions on nuclear protein import. J Mol Cell Cardiol 46(3):431-41.
Chan, L. S., et al.
2009 Role of microRNA-214 in ginsenoside-Rg1-induced angiogenesis. Eur J Pharm Sci 38(4):370-7.
Chan, M. C., et al.
2010 Molecular basis for antagonism between PDGF and the TGFbeta family of signalling pathways by control of miR-24 expression. EMBO J 29(3):559-73.
Chan, M. C., et al.
2007 A novel regulatory mechanism of the bone morphogenetic protein (BMP) signaling pathway involving the carboxyl-terminal tail domain of BMP type II receptor. Mol Cell Biol 27(16):5776-89.
Chandrasekar, B., et al.
2003 Fractalkine (CX3CL1) stimulated by nuclear factor kappaB (NF-kappaB)-dependent inflammatory signals induces aortic smooth muscle cell proliferation through an autocrine pathway. Biochem J 373(Pt 2):547-58.
Chapple, S. J., X. Cheng, and G. E. Mann
2013 Effects of 4-hydroxynonenal on vascular endothelial and smooth muscle cell redox signaling and function in health and disease. Redox Biol 1:319-31.

Charo, I. F., and R. M. Ransohoff
2006 The many roles of chemokines and chemokine receptors in inflammation. N Engl J Med 354(6):610-21.
Chen, B., et al.
2015a The role of Nrf2 in oxidative stress-induced endothelial injuries. J Endocrinol 225(3):R83-99.
Chen, D. L., et al.
2014 Identification of microRNA-214 as a negative regulator of colorectal cancer liver metastasis by way of regulation of fibroblast growth factor receptor 1 expression. Hepatology 60(2):598-609.
Chen, J., et al.
2011a Induction of microRNA-1 by myocardin in smooth muscle cells inhibits cell proliferation. Arterioscler Thromb Vasc Biol 31(2):368-75.
Chen, K. C., et al.
2011b Negative feedback regulation between microRNA let-7g and the oxLDL receptor LOX-1. J Cell Sci 124(Pt 23):4115-24.
Chen, K. C., et al.
2011c OxLDL up-regulates microRNA-29b, leading to epigenetic modifications of MMP-2/MMP-9 genes: a novel mechanism for cardiovascular diseases. FASEB J 25(5):1718-28.
Chen, L., et al.
2015b Suppression of fibrogenic signaling in hepatic stellate cells by Twist1-dependent microRNA-214 expression: Role of exosomes in horizontal transfer of Twist1. Am J Physiol Gastrointest Liver Physiol 309(6):G491-9.
Chen, L., et al.
2013a 4-HNE increases intracellular ADMA levels in cultured HUVECs: evidence for miR-21-dependent mechanisms. PLoS One 8(5):e64148.
Chen, Q., et al.
2013b Matrix metalloproteinases: inflammatory regulators of cell behaviors in vascular formation and remodeling. Mediators Inflamm 2013:928315.
Chen, Q., et al.
2015c miRNA-34a reduces neointima formation through inhibiting smooth muscle cell proliferation and migration. J Mol Cell Cardiol 89(Pt A):75-86.
Chen, Q., et al.
2015d miRNA-34a reduces neointima formation through inhibiting smooth muscle cell proliferation and migration. J Mol Cell Cardiol 89(Pt A):75-86.
Chen, T., et al.
2009 MicroRNA-125a-5p partly regulates the inflammatory response, lipid uptake, and ORP9 expression in oxLDL-stimulated monocyte/macrophages. Cardiovasc Res 83(1):131-9.
Chen, X., et al.
2012 Genome-wide association study validation identifies novel loci for atherosclerotic cardiovascular disease. J Thromb Haemost 10(8):1508-14.
Chen, Z. G., et al.
2015e Upregulated microRNA-214 enhances cardiac injury by targeting ITCH during coxsackievirus infection. Mol Med Rep 12(1):1258-64.
Cheng, Y., et al.
2009 MicroRNA-145, a novel smooth muscle cell phenotypic marker and modulator, controls vascular neointimal lesion formation. Circ Res 105(2):158-66.
Chien, S.
2003 Molecular and mechanical bases of focal lipid accumulation in arterial wall. Prog Biophys Mol Biol 83(2):131-51.
Chinetti-Gbaguidi, G., S. Colin, and B. Staels
2015 Macrophage subsets in atherosclerosis. Nat Rev Cardiol 12(1):10-7.

Chinetti-Gbaguidi, G., and B. Staels
2009 Lipid ligand-activated transcription factors regulating lipid storage and release in human macrophages. Biochim Biophys Acta 1791(6):486-93.
Chistiakov, D. A., A. N. Orekhov, and Y. V. Bobryshev
2015 Vascular smooth muscle cell in atherosclerosis. Acta Physiol (Oxf) 214(1):33-50.
Chiu, J. J., et al.
2003 Analysis of the effect of disturbed flow on monocytic adhesion to endothelial cells. J Biomech 36(12):1883-95.
Chiu, J. J., and S. Chien
2011 Effects of disturbed flow on vascular endothelium: pathophysiological basis and clinical perspectives. Physiol Rev 91(1):327-87.
Choi, H. Y., et al.
2009a ATP-binding cassette transporter A1 expression and apolipoprotein A-I binding are impaired in intima-type arterial smooth muscle cells. Circulation 119(25):3223-31.
Choi, S. H., et al.
2009b Lipoprotein accumulation in macrophages via toll-like receptor-4-dependent fluid phase uptake. Circ Res 104(12):1355-63.
Choi, Y. C., et al.
2011 Regulation of vascular endothelial growth factor signaling by miR-200b. Mol Cells 32(1):77-82.
Chotani, M. A., K. Touhalisky, and I. M. Chiu
2000 The small GTPases Ras, Rac, and Cdc42 transcriptionally regulate expression of human fibroblast growth factor 1. J Biol Chem 275(39):30432-8.
Choudhary, S., et al.
2006 Quantitation and localization of matrix metalloproteinases and their inhibitors in human carotid endarterectomy tissues. Arterioscler Thromb Vasc Biol 26(10):2351-8.
Clarke, M. C., et al.
2008 Chronic apoptosis of vascular smooth muscle cells accelerates atherosclerosis and promotes calcification and medial degeneration. Circ Res 102(12):1529-38.
Clarke, M. C., et al.
2010 Vascular smooth muscle cell apoptosis induces interleukin-1-directed inflammation: effects of hyperlipidemia-mediated inhibition of phagocytosis. Circ Res 106(2):363-72.
Clinton, S. K., et al.
1992 Macrophage colony-stimulating factor gene expression in vascular cells and in experimental and human atherosclerosis. Am J Pathol 140(2):301-16.
Cooke, J. P.
2003 Flow, NO, and atherogenesis. Proc Natl Acad Sci U S A 100(3):768-70.
Cordes, K. R., et al.
2009 miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. Nature 460(7256):705-10.
Cory, G. O., and A. J. Ridley
2002 Cell motility: braking WAVEs. Nature 418(6899):732-3.
Crisan, M., et al.
2008 Purification and long-term culture of multipotent progenitor cells affiliated with the walls of human blood vessels: myoendothelial cells and pericytes. Methods Cell Biol 86:295-309.
Cui, Y., et al.
2014 Platelet-derived growth factor-BB induces matrix metalloproteinase-2 expression and rat vascular smooth muscle cell migration via ROCK and ERK/p38 MAPK pathways. Mol Cell Biochem 393(1-2):255-63.
Curtiss, L. K., and P. S. Tobias
2009 Emerging role of Toll-like receptors in atherosclerosis. J Lipid Res 50 Suppl:S340-5.
Cushing, S. D., et al.

1990 Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. Proc Natl Acad Sci U S A 87(13):5134-8.

Dai, X. Y., et al.

2012 Increased stability of phosphatase and tensin homolog by intermedin leading to scavenger receptor A inhibition of macrophages reduces atherosclerosis in apolipoprotein E-deficient mice. J Mol Cell Cardiol 53(4):509-20.

Dandre, F., and G. K. Owens

2004 Platelet-derived growth factor-BB and Ets-1 transcription factor negatively regulate transcription of multiple smooth muscle cell differentiation marker genes. Am J Physiol Heart Circ Physiol 286(6):H2042-51.

Davis-Dusenbery, B. N., and A. Hata

2010 Mechanisms of control of microRNA biogenesis. J Biochem 148(4):381-92.

Davis, B. N., et al.

2008 SMAD proteins control DROSHA-mediated microRNA maturation. Nature 454(7200):56-61.

Davis, B. N., et al.

2009 Induction of microRNA-221 by platelet-derived growth factor signaling is critical for modulation of vascular smooth muscle phenotype. J Biol Chem 284(6):3728-38.

de Nigris, F., et al.

2003 Beneficial effects of antioxidants and L-arginine on oxidation-sensitive gene expression and endothelial NO synthase activity at sites of disturbed shear stress. Proc Natl Acad Sci U S A 100(3):1420-5.

Deng, M., et al.

2013 miR-214 promotes tumorigenesis by targeting lactotransferrin in nasopharyngeal carcinoma. Tumour Biol 34(3):1793-800.

Dentelli, P., et al.

2010 microRNA-222 controls neovascularization by regulating signal transducer and activator of transcription 5A expression. Arterioscler Thromb Vasc Biol 30(8):1562-8.

Derfoul, A., et al.

2011 Decreased microRNA-214 levels in breast cancer cells coincides with increased cell proliferation, invasion and accumulation of the Polycomb Ezh2 methyltransferase. Carcinogenesis 32(11):1607-14.

Desmouliere, A., et al.

1993 Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. J Cell Biol 122(1):103-11.

Diacovo, T. G., et al.

1994 A functional integrin ligand on the surface of platelets: intercellular adhesion molecule-2. J Clin Invest 94(3):1243-51.

Duan, Q., et al.

2015 MicroRNA-214 Is Upregulated in Heart Failure Patients and Suppresses XBP1-Mediated Endothelial Cells Angiogenesis. J Cell Physiol 230(8):1964-73.

Dufourcq, P., et al.

2002 Vitronectin is up-regulated after vascular injury and vitronectin blockade prevents neointima formation. Cardiovasc Res 53(4):952-62.

Duling, B. R., W. Kuschinsky, and M. Wahl

1979 Measurements of the perivascular PO2 in the vicinity of the pial vessels of the cat. Pflugers Arch 383(1):29-34.

Dwivedi, A., S. C. Slater, and S. J. George

2009 MMP-9 and -12 cause N-cadherin shedding and thereby beta-catenin signalling and vascular smooth muscle cell proliferation. Cardiovasc Res 81(1):178-86.

Dzau, V. J., R. C. Braun-Dullaeus, and D. G. Sedding

2002 Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. Nat Med 8(11):1249-56.

Eden, S., et al.

2002 Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck. Nature 418(6899):790-3.

Ehara, S., et al.

2004 Spotty calcification typifies the culprit plaque in patients with acute myocardial infarction: an intravascular ultrasound study. Circulation 110(22):3424-9.

el Azzouzi, H., et al.

2013 The hypoxia-inducible microRNA cluster miR-199a∼214 targets myocardial PPARδ and impairs mitochondrial fatty acid oxidation. Cell Metab 18(3):341-54.

Elia, L., et al.

2009 The knockout of miR-143 and -145 alters smooth muscle cell maintenance and vascular homeostasis in mice: correlates with human disease. Cell Death Differ 16(12):1590-8.

Esterbauer, H., R. J. Schaur, and H. Zollner

1991 Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 11(1):81-128.

Fabunmi, R. P., et al.

1998 Expression of tissue inhibitor of metalloproteinases-3 in human atheroma and regulation in lesion-associated cells: a potential protective mechanism in plaque stability. Circ Res 83(3):270-8.

Fang, Y., and P. F. Davies

2012 Site-specific microRNA-92a regulation of Kruppel-like factors 4 and 2 in atherosusceptible endothelium. Arterioscler Thromb Vasc Biol 32(4):979-87.

Febbraio, M., et al.

2000 Targeted disruption of the class B scavenger receptor CD36 protects against atherosclerotic lesion development in mice. J Clin Invest 105(8):1049-56.

Feinberg, M. W., et al.

2005 Kruppel-like factor 4 is a mediator of proinflammatory signaling in macrophages. J Biol Chem 280(46):38247-58.

Feinberg, M. W., et al.

2004 Essential role for Smad3 in regulating MCP-1 expression and vascular inflammation. Circ Res 94(5):601-8.

Filipowicz, W.

2005 RNAi: the nuts and bolts of the RISC machine. Cell 122(1):17-20.

Fischer, C., M. Schneider, and P. Carmeliet

2006 Principles and therapeutic implications of angiogenesis, vasculogenesis and arteriogenesis. Handb Exp Pharmacol (176 Pt 2):157-212.

Galis, Z. S., and J. J. Khatri

2002 Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. Circ Res 90(3):251-62.

Gambillara, V., et al.

2008 Effects of reduced cyclic stretch on vascular smooth muscle cell function of pig carotids perfused ex vivo. Am J Hypertens 21(4):425-31.

Gawaz, M., et al.

1998 Activated platelets induce monocyte chemotactic protein-1 secretion and surface expression of intercellular adhesion molecule-1 on endothelial cells. Circulation 98(12):1164-71.

Geng, Y. J., and G. K. Hansson

1992 Interferon-gamma inhibits scavenger receptor expression and foam cell formation in human monocyte-derived macrophages. J Clin Invest 89(4):1322-30.

Giacco, F., and M. Brownlee

2010 Oxidative stress and diabetic complications. Circ Res 107(9):1058-70.

Gimbrone, M. A., Jr., et al.
1997 Hemodynamics, endothelial gene expression, and atherogenesis. Ann N Y Acad Sci 811:1-10; discussion 10-1.
Gimona, M., et al.
2003 Calponin repeats regulate actin filament stability and formation of podosomes in smooth muscle cells. Mol Biol Cell 14(6):2482-91.
Girerd, X., et al.
1996 Remodeling of the radial artery in response to a chronic increase in shear stress. Hypertension 27(3 Pt 2):799-803.
Goldman, J., L. Zhong, and S. Q. Liu
2007 Negative regulation of vascular smooth muscle cell migration by blood shear stress. Am J Physiol Heart Circ Physiol 292(2):H928-38.
Gomez, D., and G. K. Owens
2012 Smooth muscle cell phenotypic switching in atherosclerosis. Cardiovasc Res 95(2):156-64.
Grarup, N., T. Sparso, and T. Hansen
2010 Physiologic characterization of type 2 diabetes-related loci. Curr Diab Rep 10(6):485-97.
Green, J., et al.
2014 Flow patterns regulate hyperglycemia-induced subendothelial matrix remodeling during early atherogenesis. Atherosclerosis 232(2):277-84.
Griffiths-Jones, S.
2004 The microRNA Registry. Nucleic Acids Res 32(Database issue):D109-11.
—
2006 miRBase: the microRNA sequence database. Methods Mol Biol 342:129-38.
Groenewegen, H. C., et al.
2008 Non-bone marrow origin of neointimal smooth muscle cells in experimental in-stent restenosis in rats. J Vasc Res 45(6):493-502.
Gu, C., et al.
2015 MicroRNA-214 induces dendritic cell switching from tolerance to immunity by targeting β-Catenin signaling. Int J Clin Exp Pathol 8(9):10050-60.
Gutierrez, N. C., et al.
2010 Deregulation of microRNA expression in the different genetic subtypes of multiple myeloma and correlation with gene expression profiling. Leukemia 24(3):629-37.
Ha, M., and V. N. Kim
2014 Regulation of microRNA biogenesis. Nat Rev Mol Cell Biol 15(8):509-24.
Haase, A. D., et al.
2005 TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. EMBO Rep 6(10):961-7.
Han, J., et al.
2004 The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev 18(24):3016-27.
Han, J., et al.
2006 Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. Cell 125(5):887-901.
Han, Y., et al.
2008 CREG promotes a mature smooth muscle cell phenotype and reduces neointimal formation in balloon-injured rat carotid artery. Cardiovasc Res 78(3):597-604.
Hanna, A. K., et al.
1997 Antisense basic fibroblast growth factor gene transfer reduces neointimal thickening after arterial injury. J Vasc Surg 25(2):320-5.
Hansson, G. K.
2005 Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 352(16):1685-95.
Hara, M. R., M. B. Cascio, and A. Sawa
2006 GAPDH as a sensor of NO stress. Biochim Biophys Acta 1762(5):502-9.

Heino, J.
2000 The collagen receptor integrins have distinct ligand recognition and signaling functions. Matrix Biol 19(4):319-23.
Heldin, C. H., B. Westermark, and A. Wasteson
1981 Platelet-derived growth factor. Isolation by a large-scale procedure and analysis of subunit composition. Biochem J 193(3):907-13.
Hillebrands, J. L., et al.
2002 Bone marrow does not contribute substantially to endothelial-cell replacement in transplant arteriosclerosis. Nat Med 8(3):194-5.
Hu, W., and Y. Huang
2015 Targeting the platelet-derived growth factor signalling in cardiovascular disease. Clin Exp Pharmacol Physiol 42(12):1221-4.
Hu, Y., et al.
1998 Activation of PDGF receptor alpha in vascular smooth muscle cells by mechanical stress. FASEB J 12(12):1135-42.
Hu, Y., et al.
2002 Smooth muscle cells in transplant atherosclerotic lesions are originated from recipients, but not bone marrow progenitor cells. Circulation 106(14):1834-9.
Hu, Y., et al.
2004 Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice. J Clin Invest 113(9):1258-65.
Huang, H., et al.
2010 miR-10a contributes to retinoid acid-induced smooth muscle cell differentiation. J Biol Chem 285(13):9383-9.
Huang, P., et al.
2013 Association of the KLF14 rs4731702 SNP and serum lipid levels in the Guangxi Mulao and Han populations. Biomed Res Int 2013:231515.
Huang, V., et al.
2012 Upregulation of Cyclin B1 by miRNA and its implications in cancer. Nucleic Acids Res 40(4):1695-707.
Ibarra, N., et al.
2006 Nap1 regulates Dictyostelium cell motility and adhesion through SCAR-dependent and -independent pathways. Curr Biol 16(7):717-22.
Ighodaro, O. M.
2018 Molecular pathways associated with oxidative stress in diabetes mellitus. Biomed Pharmacother 108:656-662.
Ikesue, M., et al.
2011 Syndecan-4 deficiency limits neointimal formation after vascular injury by regulating vascular smooth muscle cell proliferation and vascular progenitor cell mobilization. Arterioscler Thromb Vasc Biol 31(5):1066-74.
Inoguchi, T., et al.
2000 High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C--dependent activation of NAD(P)H oxidase in cultured vascular cells. Diabetes 49(11):1939-45.
Inoue, T., et al.
1998 Lower expression of neutrophil adhesion molecule indicates less vessel wall injury and might explain lower restenosis rate after cutting balloon angioplasty. Circulation 97(25):2511-8.
Iorio, M. V., et al.
2007 MicroRNA signatures in human ovarian cancer. Cancer Res 67(18):8699-707.
Ishii, T., et al.
2004 Role of Nrf2 in the regulation of CD36 and stress protein expression in murine macrophages: activation by oxidatively modified LDL and 4-hydroxynonenal. Circ Res 94(5):609-16.

Iwasaki, H., et al.
2000 Mechanical stretch stimulates growth of vascular smooth muscle cells via epidermal growth factor receptor. Am J Physiol Heart Circ Physiol 278(2):H521-9.
Iwata, H., et al.
2010 Bone marrow-derived cells contribute to vascular inflammation but do not differentiate into smooth muscle cell lineages. Circulation 122(20):2048-57.
Jagadeesha, D. K., et al.
2012 Nox1 transactivation of epidermal growth factor receptor promotes N-cadherin shedding and smooth muscle cell migration. Cardiovasc Res 93(3):406-13.
Ji, R., et al.
2007 MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion formation. Circ Res 100(11):1579-88.
Johnson, J. L.
2014 Emerging regulators of vascular smooth muscle cell function in the development and progression of atherosclerosis. Cardiovasc Res 103(4):452-60.
Johnson, J. L., et al.
2005 Divergent effects of matrix metalloproteinases 3, 7, 9, and 12 on atherosclerotic plaque stability in mouse brachiocephalic arteries. Proc Natl Acad Sci U S A 102(43):15575-80.
Jyrkkanen, H. K., et al.
2008 Nrf2 regulates antioxidant gene expression evoked by oxidized phospholipids in endothelial cells and murine arteries in vivo. Circ Res 103(1):e1-9.
Kappert, K., et al.
2001 Integrins alphavbeta3 and alphavbeta5 mediate VSMC migration and are elevated during neointima formation in the rat aorta. Basic Res Cardiol 96(1):42-9.
Karwowski, W., et al.
2012 The mechanism of vascular calcification - a systematic review. Med Sci Monit 18(1):RA1-11.
Katakami, N., et al.
2004 Role of pim-1 in smooth muscle cell proliferation. J Biol Chem 279(52):54742-9.
Kawai-Kowase, K., and G. K. Owens
2007 Multiple repressor pathways contribute to phenotypic switching of vascular smooth muscle cells. Am J Physiol Cell Physiol 292(1):C59-69.
Keeley, T. P., and G. E. Mann
2019 Defining Physiological Normoxia for Improved Translation of Cell Physiology to Animal Models and Humans. Physiol Rev 99(1):161-234.
Kerger, H., et al.
1995 Systemic and subcutaneous microvascular oxygen tension in conscious Syrian golden hamsters. Am J Physiol 268(2 Pt 2):H802-10.
Keskek, S. O., et al.
2017 High Levels of Circulating Endothelial Progenitor Cells Are Associated with Acrotism in Patients with Takayasu Arteritis. Med Princ Pract 26(2):132-138.
Khvorova, A., A. Reynolds, and S. D. Jayasena
2003 Functional siRNAs and miRNAs exhibit strand bias. Cell 115(2):209-16.
Kieffer, P., et al.
2001 Activation of metalloproteinase-2, loss of matrix scleroprotein content and coronary artery calcification. Atherosclerosis 157(1):251-4.
Kim, A. S., et al.
2000 Autoinhibition and activation mechanisms of the Wiskott-Aldrich syndrome protein. Nature 404(6774):151-8.
Kim, H. Y., et al.
2008 Upregulation of interleukin-8/CXCL8 in vascular smooth muscle cells from spontaneously hypertensive rats. Hypertens Res 31(3):515-23.

Kim, M., et al.
2012 Laminar flow activation of ERK5 protein in vascular endothelium leads to atheroprotective effect via NF-E2-related factor 2 (Nrf2) activation. J Biol Chem 287(48):40722-31.
Kim, S. A., et al.
2017 Laminar shear stress suppresses vascular smooth muscle cell proliferation through nitric oxide-AMPK pathway. Biochem Biophys Res Commun 490(4):1369-1374.
Kim, V. N., J. Han, and M. C. Siomi
2009 Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol 10(2):126-39.
Kitamura, T., et al.
1996 Molecular cloning of p125Nap1, a protein that associates with an SH3 domain of Nck. Biochem Biophys Res Commun 219(2):509-14.
Kizub, I. V., K. I. Klymenko, and A. I. Soloviev
2014 Protein kinase C in enhanced vascular tone in diabetes mellitus. Int J Cardiol 174(2):230-42.
Klaassen, I., et al.
2015 The role of CTGF in diabetic retinopathy. Exp Eye Res 133:37-48.
Kobayashi, A., et al.
2004 Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. Mol Cell Biol 24(16):7130-9.
Kuehbacher, A., et al.
2007 Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis. Circ Res 101(1):59-68.
Kunjathoor, V. V., et al.
2002 Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. J Biol Chem 277(51):49982-8.
Kwak, B. R., et al.
2014 Biomechanical factors in atherosclerosis: mechanisms and clinical implications. Eur Heart J 35(43):3013-20, 3020a-3020d.
Lacolley, P., et al.
2012 The vascular smooth muscle cell in arterial pathology: a cell that can take on multiple roles. Cardiovasc Res 95(2):194-204.
Landry, D. B., et al.
1997 Activation of the NF-kappa B and I kappa B system in smooth muscle cells after rat arterial injury. Induction of vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1. Am J Pathol 151(4):1085-95.
Landsman, L., et al.
2009 CX3CR1 is required for monocyte homeostasis and atherogenesis by promoting cell survival. Blood 113(4):963-72.
Latronico, M. V., and G. Condorelli
2010 MicroRNAs and cardiac conduction. Curr Drug Targets 11(8):907-12.
Lavie, C. J., et al.
2013 Impact of obesity and the obesity paradox on prevalence and prognosis in heart failure. JACC Heart Fail 1(2):93-102.
Lavie, C. J., et al.
2014 Obesity and cardiovascular diseases: implications regarding fitness, fatness, and severity in the obesity paradox. J Am Coll Cardiol 63(14):1345-54.
Lee, C. W., et al.
2006a Transcriptional regulation of VCAM-1 expression by tumor necrosis factor-alpha in human tracheal smooth muscle cells: involvement of MAPKs, NF-kappaB, p300, and histone acetylation. J Cell Physiol 207(1):174-86.
Lee, G. L., et al.

2012 TLR 2 induces vascular smooth muscle cell migration through cAMP response element-binding protein-mediated interleukin-6 production. Arterioscler Thromb Vasc Biol 32(11):2751-60.

Lee, J. H., et al.

2013 Interaction of NADPH oxidase 1 with Toll-like receptor 2 induces migration of smooth muscle cells. Cardiovasc Res 99(3):483-93.

Lee, J. S., et al.

2006b Klf2 is an essential regulator of vascular hemodynamic forces in vivo. Dev Cell 11(6):845-57.

Lee, M. J.

2018 Transforming growth factor beta superfamily regulation of adipose tissue biology in obesity. Biochim Biophys Acta Mol Basis Dis 1864(4 Pt A):1160-1171.

Lee, R. C., and V. Ambros

2001 An extensive class of small RNAs in Caenorhabditis elegans. Science 294(5543):862-4.

Lee, Y. B., et al.

2009 Twist-1 regulates the miR-199a/214 cluster during development. Nucleic Acids Res 37(1):123-8.

Lee, Y., et al.

2006c The role of PACT in the RNA silencing pathway. EMBO J 25(3):522-32.

Leeper, N. J., et al.

2011 MicroRNA-26a is a novel regulator of vascular smooth muscle cell function. J Cell Physiol 226(4):1035-43.

Leitinger, N., and I. G. Schulman

2013 Phenotypic polarization of macrophages in atherosclerosis. Arterioscler Thromb Vasc Biol 33(6):1120-6.

Li, D., et al.

2010a MicroRNA-125a/b-5p inhibits endothelin-1 expression in vascular endothelial cells. J Hypertens 28(8):1646-54.

Li, E., T. H. Bestor, and R. Jaenisch

1992 Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69(6):915-26.

Li, G., et al.

2000 Direct in vivo evidence demonstrating neointimal migration of adventitial fibroblasts after balloon injury of rat carotid arteries. Circulation 101(12):1362-5.

Li, H., H. Xu, and B. Sun

2012 Lipopolysaccharide regulates MMP-9 expression through TLR4/NF-kappaB signaling in human arterial smooth muscle cells. Mol Med Rep 6(4):774-8.

Li, K., et al.

2015a Apolipoprotein E enhances microRNA-146a in monocytes and macrophages to suppress nuclear factor-kappaB-driven inflammation and atherosclerosis. Circ Res 117(1):e1-e11.

Li, K., et al.

2015b MicroRNA-214 suppresses gluconeogenesis by targeting activating transcriptional factor 4. J Biol Chem 290(13):8185-95.

Li, N., et al.

2010b ERK is integral to the IFN-gamma-mediated activation of STAT1, the expression of key genes implicated in atherosclerosis, and the uptake of modified lipoproteins by human macrophages. J Immunol 185(5):3041-8.

Li, P., et al.

2013 MicroRNA-638 is highly expressed in human vascular smooth muscle cells and inhibits PDGF-BB-induced cell proliferation and migration through targeting orphan nuclear receptor NOR1. Cardiovasc Res 99(1):185-93.

Li, Z., et al.

1996 Increased expression of 72-kd type IV collagenase (MMP-2) in human aortic atherosclerotic lesions. Am J Pathol 148(1):121-8.

Liang, K. C., et al.

2007 Interleukin-1beta induces MMP-9 expression via p42/p44 MAPK, p38 MAPK, JNK, and nuclear factor-kappaB signaling pathways in human tracheal smooth muscle cells. J Cell Physiol 211(3):759-70.

Libby, P.

2002 Inflammation in atherosclerosis. Nature 420(6917):868-74.

—

2013 Collagenases and cracks in the plaque. J Clin Invest 123(8):3201-3.

Libby, P., and S. K. Clinton

1992 Cytokines as mediators of vascular pathology. Nouv Rev Fr Hematol 34 Suppl:S47-53.

Liehn, E. A., A. Schober, and C. Weber

2004 Blockade of keratinocyte-derived chemokine inhibits endothelial recovery and enhances plaque formation after arterial injury in ApoE-deficient mice. Arterioscler Thromb Vasc Biol 24(10):1891-6.

Lin, C. C., et al.

2008 Tumor necrosis factor-alpha induces MMP-9 expression via p42/p44 MAPK, JNK, and nuclear factor-kappaB in A549 cells. Toxicol Appl Pharmacol 229(3):386-98.

Lin, Y., et al.

2009 Involvement of MicroRNAs in hydrogen peroxide-mediated gene regulation and cellular injury response in vascular smooth muscle cells. J Biol Chem 284(12):7903-13.

Linder, S., and M. Aepfelbacher

2003 Podosomes: adhesion hot-spots of invasive cells. Trends Cell Biol 13(7):376-85.

Linton, M. F., et al.

2000 The Role of Lipids and Lipoproteins in Atherosclerosis. *In* Endotext. L.J. De Groot, G. Chrousos, K. Dungan, K.R. Feingold, A. Grossman, J.M. Hershman, C. Koch, M. Korbonits, R. McLachlan, M. New, J. Purnell, R. Rebar, F. Singer, and A. Vinik, eds. South Dartmouth (MA).

Lipskaia, L., et al.

2014 Synergistic role of protein phosphatase inhibitor 1 and sarco/endoplasmic reticulum Ca2+ -ATPase in the acquisition of the contractile phenotype of arterial smooth muscle cells. Circulation 129(7):773-85.

Lipskaia, L., et al.

2005 Sarco/endoplasmic reticulum Ca2+-ATPase gene transfer reduces vascular smooth muscle cell proliferation and neointima formation in the rat. Circ Res 97(5):488-95.

Liu, G., et al.

2011a Mechanical stretch augments insulin-induced vascular smooth muscle cell proliferation by insulin-like growth factor-1 receptor. Exp Cell Res 317(17):2420-8.

Liu, S. Q., et al.

2003 Pattern formation of vascular smooth muscle cells subject to nonuniform fluid shear stress: role of PDGF-beta receptor and Src. Am J Physiol Heart Circ Physiol 285(3):H1081-90.

Liu, X., et al.

2011b MicroRNA-31 regulated by the extracellular regulated kinase is involved in vascular smooth muscle cell growth via large tumor suppressor homolog 2. J Biol Chem 286(49):42371-80.

Liu, X., et al.

2012 Cell-specific effects of miR-221/222 in vessels: molecular mechanism and therapeutic application. J Mol Cell Cardiol 52(1):245-55.

Liu, X., et al.

2009 A necessary role of miR-221 and miR-222 in vascular smooth muscle cell proliferation and neointimal hyperplasia. Circ Res 104(4):476-87.

Liu, Y., et al.

2005 Kruppel-like factor 4 abrogates myocardin-induced activation of smooth muscle gene expression. J Biol Chem 280(10):9719-27.

Liu, Y., et al.

2014 TLR2 and TLR4 in autoimmune diseases: a comprehensive review. Clin Rev Allergy Immunol 47(2):136-47.

Loebel, D. A., et al.

2005 A conserved noncoding intronic transcript at the mouse Dnm3 locus. Genomics 85(6):782-9.

Long, H., et al.

2015 microRNA-214 promotes epithelial-mesenchymal transition and metastasis in lung adenocarcinoma by targeting the suppressor-of-fused protein (Sufu). Oncotarget 6(36):38705-18.

Lovren, F., et al.

2012 MicroRNA-145 targeted therapy reduces atherosclerosis. Circulation 126(11 Suppl 1):S81-90.

Lu, H. Q., et al.

2013 Circulating miR-214 is associated with the severity of coronary artery disease. J Geriatr Cardiol 10(1):34-8.

Lu, W., and X. Li

2018 Vascular stem/progenitor cells: functions and signaling pathways. Cell Mol Life Sci 75(5):859-869.

Lund, E., et al.

2004 Nuclear export of microRNA precursors. Science 303(5654):95-8.

Luo, Z., et al.

2013 MicroRNA-200C and -150 play an important role in endothelial cell differentiation and vasculogenesis by targeting transcription repressor ZEB1. Stem Cells 31(9):1749-62.

Lv, G., et al.

2014 MicroRNA-214 protects cardiac myocytes against H2O2-induced injury. J Cell Biochem 115(1):93-101.

Machesky, L. M., et al.

1999 Scar, a WASp-related protein, activates nucleation of actin filaments by the Arp2/3 complex. Proc Natl Acad Sci U S A 96(7):3739-44.

Mack, C. P.

2011 Signaling mechanisms that regulate smooth muscle cell differentiation. Arterioscler Thromb Vasc Biol 31(7):1495-505.

Maeda, H., et al.

1995 TGF-beta enhances macrophage ability to produce IL-10 in normal and tumor-bearing mice. J Immunol 155(10):4926-32.

Magne, D., et al.

2005 Cartilage formation in growth plate and arteries: from physiology to pathology. Bioessays 27(7):708-16.

Makinen, P. I., et al.

2010 Silencing of either SR-A or CD36 reduces atherosclerosis in hyperlipidaemic mice and reveals reciprocal upregulation of these receptors. Cardiovasc Res 88(3):530-8.

Malek, A. M., S. L. Alper, and S. Izumo

1999 Hemodynamic shear stress and its role in atherosclerosis. JAMA 282(21):2035-42.

Maltese, G., et al.

2017 The anti-ageing hormone klotho induces Nrf2-mediated antioxidant defences in human aortic smooth muscle cells. J Cell Mol Med 21(3):621-627.

Marcola, M., and C. E. Rodrigues

2015 Endothelial progenitor cells in tumor angiogenesis: another brick in the wall. Stem Cells Int 2015:832649.

Marleau, S., et al.

2005 EP 80317, a ligand of the CD36 scavenger receptor, protects apolipoprotein E-deficient mice from developing atherosclerotic lesions. FASEB J 19(13):1869-71.

Martin, M. M., et al.

2007 The human angiotensin II type 1 receptor +1166 A/C polymorphism attenuates microRNA-155 binding. J Biol Chem 282(33):24262-9.

Mayr, M., et al.

2002 Mechanical stress-induced DNA damage and rac-p38MAPK signal pathways mediate p53-dependent apoptosis in vascular smooth muscle cells. FASEB J 16(11):1423-5.

Maziere, C., et al.

2013 Oxidized low density lipoprotein increases RANKL level in human vascular cells. Involvement of oxidative stress. Biochem Biophys Res Commun 440(2):295-9.

McConnell, B. B., and V. W. Yang

2010 Mammalian Kruppel-like factors in health and diseases. Physiol Rev 90(4):1337-81.

McDonald, O. G., and G. K. Owens

2007 Programming smooth muscle plasticity with chromatin dynamics. Circ Res 100(10):1428-41.

McDonald, O. G., et al.

2006 Control of SRF binding to CArG box chromatin regulates smooth muscle gene expression in vivo. J Clin Invest 116(1):36-48.

McLaren, J. E., et al.

2011 Cytokines, macrophage lipid metabolism and foam cells: implications for cardiovascular disease therapy. Prog Lipid Res 50(4):331-47.

Merlet, E., et al.

2013 miR-424/322 regulates vascular smooth muscle cell phenotype and neointimal formation in the rat. Cardiovasc Res 98(3):458-68.

Miano, J. M.

2003 Serum response factor: toggling between disparate programs of gene expression. J Mol Cell Cardiol 35(6):577-93.

Minami, T., et al.

2006 Thrombin-induced autoinhibitory factor, Down syndrome critical region-1, attenuates NFAT-dependent vascular cell adhesion molecule-1 expression and inflammation in the endothelium. J Biol Chem 281(29):20503-20.

Mitra, S., et al.

2011 Oxidized low-density lipoprotein and atherosclerosis implications in antioxidant therapy. Am J Med Sci 342(2):135-42.

Miyata, T., et al.

2005 Platelet-derived growth factor-BB (PDGF-BB) induces differentiation of bone marrow endothelial progenitor cell-derived cell line TR-BME2 into mural cells, and changes the phenotype. J Cell Physiol 204(3):948-55.

Mohanty, M. J., and X. Li

2002 Stretch-induced Ca(2+) release via an IP(3)-insensitive Ca(2+) channel. Am J Physiol Cell Physiol 283(2):C456-62.

Moiseeva, E. P.

2001 Adhesion receptors of vascular smooth muscle cells and their functions. Cardiovasc Res 52(3):372-86.

Molnár, V., et al.

2008 Changes in miRNA expression in solid tumors: an miRNA profiling in melanomas. Semin Cancer Biol 18(2):111-22.

Moon, S. K., H. M. Kim, and C. H. Kim

2004 PTEN induces G1 cell cycle arrest and inhibits MMP-9 expression via the regulation of NF-kappaB and AP-1 in vascular smooth muscle cells. Arch Biochem Biophys 421(2):267-76.

Moore, K. J., and M. W. Freeman

2006 Scavenger receptors in atherosclerosis: beyond lipid uptake. Arterioscler Thromb Vasc Biol 26(8):1702-11.
Moore, K. J., et al.
2005 Loss of receptor-mediated lipid uptake via scavenger receptor A or CD36 pathways does not ameliorate atherosclerosis in hyperlipidemic mice. J Clin Invest 115(8):2192-201.
Moore, K. J., F. J. Sheedy, and E. A. Fisher
2013 Macrophages in atherosclerosis: a dynamic balance. Nat Rev Immunol 13(10):709-21.
Morgan, A. R., et al.
2004 Differences in matrix metalloproteinase-1 and matrix metalloproteinase-12 transcript levels among carotid atherosclerotic plaques with different histopathological characteristics. Stroke 35(6):1310-5.
Morrow, D., et al.
2005 Cyclic strain inhibits Notch receptor signaling in vascular smooth muscle cells in vitro. Circ Res 96(5):567-75.
Mosser, D. M., and J. P. Edwards
2008 Exploring the full spectrum of macrophage activation. Nat Rev Immunol 8(12):958-69.
Mullins, R. D.
2000 How WASP-family proteins and the Arp2/3 complex convert intracellular signals into cytoskeletal structures. Curr Opin Cell Biol 12(1):91-6.
Myllärniemi, M., et al.
1997 Inhibition of platelet-derived growth factor receptor tyrosine kinase inhibits vascular smooth muscle cell migration and proliferation. FASEB J 11(13):1119-26.
Nakamura, K., J. J. Fuster, and K. Walsh
2014 Adipokines: a link between obesity and cardiovascular disease. J Cardiol 63(4):250-9.
Nakao, S., et al.
2008 Contact-dependent promotion of cell migration by the OL-protocadherin-Nap1 interaction. J Cell Biol 182(2):395-410.
Natsume, Y., et al.
2018 Combined Analysis of Human and Experimental Murine Samples Identified Novel Circulating MicroRNAs as Biomarkers for Atrial Fibrillation. Circ J 82(4):965-973.
Newby, A. C., and A. B. Zaltsman
1999 Fibrous cap formation or destruction--the critical importance of vascular smooth muscle cell proliferation, migration and matrix formation. Cardiovasc Res 41(2):345-60.
Nicholls, C., H. Li, and J. P. Liu
2012 GAPDH: a common enzyme with uncommon functions. Clin Exp Pharmacol Physiol 39(8):674-9.
Nie, M., et al.
2003 Transcriptional regulation of cyclooxygenase 2 by bradykinin and interleukin-1beta in human airway smooth muscle cells: involvement of different promoter elements, transcription factors, and histone h4 acetylation. Mol Cell Biol 23(24):9233-44.
Nigro, P., J. Abe, and B. C. Berk
2011 Flow shear stress and atherosclerosis: a matter of site specificity. Antioxid Redox Signal 15(5):1405-14.
Niinikoski, J., C. Heughan, and T. K. Hunt
1973 Oxygen tensions in the aortic wall of normal rabbits. Atherosclerosis 17(3):353-9.
Nishizuka, Y.
1995 Protein kinase C and lipid signaling for sustained cellular responses. FASEB J 9(7):484-96.
Okada, H., et al.
2015 Inhibition of microRNA-214 ameliorates hepatic fibrosis and tumor incidence in platelet-derived growth factor C transgenic mice. Cancer Sci 106(9):1143-52.
Okamura, K., et al.

2007 The mirtron pathway generates microRNA-class regulatory RNAs in Drosophila. Cell 130(1):89-100.

Okamura, K., et al.

2004 Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. Genes Dev 18(14):1655-66.

Ooi, B. K., B. H. Goh, and W. H. Yap

2017 Oxidative Stress in Cardiovascular Diseases: Involvement of Nrf2 Antioxidant Redox Signaling in Macrophage Foam Cells Formation. Int J Mol Sci 18(11).

Orr, A. W., et al.

2009 Molecular mechanisms of collagen isotype-specific modulation of smooth muscle cell phenotype. Arterioscler Thromb Vasc Biol 29(2):225-31.

Ortega, F. B., C. J. Lavie, and S. N. Blair

2016 Obesity and Cardiovascular Disease. Circ Res 118(11):1752-70.

Owens, G. K.

1995 Regulation of differentiation of vascular smooth muscle cells. Physiol Rev 75(3):487-517.

Owens, G. K., M. S. Kumar, and B. R. Wamhoff

2004 Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol Rev 84(3):767-801.

Panousis, C. G., G. Evans, and S. H. Zuckerman

2001 TGF-beta increases cholesterol efflux and ABC-1 expression in macrophage-derived foam cells: opposing the effects of IFN-gamma. J Lipid Res 42(5):856-63.

Parathath, S., et al.

2013 Hypoxia in murine atherosclerotic plaques and its adverse effects on macrophages. Trends Cardiovasc Med 23(3):80-4.

Parmar, K. M., et al.

2006 Integration of flow-dependent endothelial phenotypes by Kruppel-like factor 2. J Clin Invest 116(1):49-58.

Parto, P., and C. J. Lavie

2017 Obesity and CardiovascularDiseases. Curr Probl Cardiol 42(11):376-394.

Pashkow, F. J.

2011 Oxidative Stress and Inflammation in Heart Disease: Do Antioxidants Have a Role in Treatment and/or Prevention? Int J Inflam 2011:514623.

Passerini, A. G., et al.

2004 Coexisting proinflammatory and antioxidative endothelial transcription profiles in a disturbed flow region of the adult porcine aorta. Proc Natl Acad Sci U S A 101(8):2482-7.

Pedersen, T. X., et al.

2013 Osteopontin deficiency dampens the pro-atherogenic effect of uraemia. Cardiovasc Res 98(3):352-9.

Penna, E., et al.

2011 microRNA-214 contributes to melanoma tumour progression through suppression of TFAP2C. EMBO J 30(10):1990-2007.

Penna, E., F. Orso, and D. Taverna

2015 miR-214 as a key hub that controls cancer networks: small player, multiple functions. J Invest Dermatol 135(4):960-9.

Pfeffer, S., et al.

2005 Identification of microRNAs of the herpesvirus family. Nat Methods 2(4):269-76.

Pflederer, T., et al.

2010 Characterization of culprit lesions in acute coronary syndromes using coronary dual-source CT angiography. Atherosclerosis 211(2):437-44.

Pidkovka, N. A., et al.

2007 Oxidized phospholipids induce phenotypic switching of vascular smooth muscle cells in vivo and in vitro. Circ Res 101(8):792-801.

Pillai, R. S., et al.
2005 Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science 309(5740):1573-6.
Place, R. F., et al.
2008 MicroRNA-373 induces expression of genes with complementary promoter sequences. Proc Natl Acad Sci U S A 105(5):1608-13.
Pluddemann, A., C. Neyen, and S. Gordon
2007 Macrophage scavenger receptors and host-derived ligands. Methods 43(3):207-17.
Qiu, J., et al.
2014 Biomechanical regulation of vascular smooth muscle cell functions: from in vitro to in vivo understanding. J R Soc Interface 11(90):20130852.
Qiu, P., and L. Li
2002 Histone acetylation and recruitment of serum responsive factor and CREB-binding protein onto SM22 promoter during SM22 gene expression. Circ Res 90(8):858-65.
Quilici, J., et al.
2004 Circulating endothelial cell count as a diagnostic marker for non-ST-elevation acute coronary syndromes. Circulation 110(12):1586-91.
Quintavalle, M., et al.
2010 MicroRNA control of podosome formation in vascular smooth muscle cells in vivo and in vitro. J Cell Biol 189(1):13-22.
Qwarnstrom, E. E., et al.
1994 Fibronectin attachment activates the NF-kappa B p50/p65 heterodimer in fibroblasts and smooth muscle cells. J Biol Chem 269(49):30765-8.
Raines, E. W.
2004 PDGF and cardiovascular disease. Cytokine Growth Factor Rev 15(4):237-54.
Raines, E. W., and N. Ferri
2005 Thematic review series: The immune system and atherogenesis. Cytokines affecting endothelial and smooth muscle cells in vascular disease. J Lipid Res 46(6):1081-92.
Rakeman, A. S., and K. V. Anderson
2006 Axis specification and morphogenesis in the mouse embryo require Nap1, a regulator of WAVE-mediated actin branching. Development 133(16):3075-83.
Ramji, D. P., and T. S. Davies
2015 Cytokines in atherosclerosis: Key players in all stages of disease and promising therapeutic targets. Cytokine Growth Factor Rev 26(6):673-85.
Rangrez, A. Y., et al.
2011 miR-143 and miR-145: molecular keys to switch the phenotype of vascular smooth muscle cells. Circ Cardiovasc Genet 4(2):197-205.
Ratert, N., et al.
2013 miRNA profiling identifies candidate mirnas for bladder cancer diagnosis and clinical outcome. J Mol Diagn 15(5):695-705.
Rayner, K. J., et al.
2010 MiR-33 contributes to the regulation of cholesterol homeostasis. Science 328(5985):1570-3.
Reid, A. J., et al.
2010 Diffuse and uncontrolled vascular smooth muscle cell proliferation in rapidly progressing pediatric moyamoya disease. J Neurosurg Pediatr 6(3):244-9.
Remus, E. W., et al.
2013 miR181a protects against angiotensin II-induced osteopontin expression in vascular smooth muscle cells. Atherosclerosis 228(1):168-74.
Rho, S. S., K. Ando, and S. Fukuhara
2017 Dynamic Regulation of Vascular Permeability by Vascular Endothelial Cadherin-Mediated Endothelial Cell-Cell Junctions. J Nippon Med Sch 84(4):148-159.

Richardson, M. R., and M. C. Yoder
2011 Endothelial progenitor cells: quo vadis? J Mol Cell Cardiol 50(2):266-72.
Rodacka, A., J. Gerszon, and M. Puchala
2014 [The biological significance of oxidative modifications of cysteine residues in proteins illustrated with the example of glyceraldehyde-3-phosphate dehydrogenase]. Postepy Hig Med Dosw (Online) 68:280-90.
Rohatgi, R., H. Y. Ho, and M. W. Kirschner
2000 Mechanism of N-WASP activation by CDC42 and phosphatidylinositol 4, 5-bisphosphate. J Cell Biol 150(6):1299-310.
Rohatgi, R., et al.
2001 Nck and phosphatidylinositol 4,5-bisphosphate synergistically activate actin polymerization through the N-WASP-Arp2/3 pathway. J Biol Chem 276(28):26448-52.
Rong, J. X., et al.
2003 Transdifferentiation of mouse aortic smooth muscle cells to a macrophage-like state after cholesterol loading. Proc Natl Acad Sci U S A 100(23):13531-6.
Roque, M., et al.
2002 CCR2 deficiency decreases intimal hyperplasia after arterial injury. Arterioscler Thromb Vasc Biol 22(4):554-9.
Ross, R.
1993 The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 362(6423):801-9.
—
1999 Atherosclerosis--an inflammatory disease. N Engl J Med 340(2):115-26.
Rubin, K., et al.
1988 Induction of B-type receptors for platelet-derived growth factor in vascular inflammation: possible implications for development of vascular proliferative lesions. Lancet 1(8599):1353-6.
Rzucidlo, E. M., K. A. Martin, and R. J. Powell
2007 Regulation of vascular smooth muscle cell differentiation. J Vasc Surg 45 Suppl A:A25-32.
Sage, A. P., Y. Tintut, and L. L. Demer
2010 Regulatory mechanisms in vascular calcification. Nat Rev Cardiol 7(9):528-36.
Saito, T., et al.
2005 Angiotensin II suppresses growth arrest specific homeobox (Gax) expression via redox-sensitive mitogen-activated protein kinase (MAPK). Regul Pept 127(1-3):159-67.
Saiura, A., et al.
2001 Circulating smooth muscle progenitor cells contribute to atherosclerosis. Nat Med 7(4):382-3.
Salmon, M., et al.
2012 Cooperative binding of KLF4, pELK-1, and HDAC2 to a G/C repressor element in the SM22α promoter mediates transcriptional silencing during SMC phenotypic switching in vivo. Circ Res 111(6):685-96.
Sano, H., et al.
2001 Functional blockade of platelet-derived growth factor receptor-beta but not of receptor-alpha prevents vascular smooth muscle cell accumulation in fibrous cap lesions in apolipoprotein E-deficient mice. Circulation 103(24):2955-60.
Santilli, S. M., A. S. Tretinyak, and E. S. Lee
2000 Transarterial wall oxygen gradients at the deployment site of an intra-arterial stent in the rabbit. Am J Physiol Heart Circ Physiol 279(4):H1518-25.
Sarmento, O. F., et al.
2015 A novel role for KLF14 in T regulatory cell differentiation. Cell Mol Gastroenterol Hepatol 1(2):188-202 e4.
Sartore, S., et al.
2001 Contribution of adventitial fibroblasts to neointima formation and vascular remodeling: from innocent bystander to active participant. Circ Res 89(12):1111-21.

Sata, M., et al.
2002 Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. Nat Med 8(4):403-9.
Schaeffer, D. F., et al.
2009 LOX-1 augments oxLDL uptake by lysoPC-stimulated murine macrophages but is not required for oxLDL clearance from plasma. J Lipid Res 50(8):1676-84.
Schober, A., et al.
2002 Deposition of platelet RANTES triggering monocyte recruitment requires P-selectin and is involved in neointima formation after arterial injury. Circulation 106(12):1523-9.
Schober, A., and A. Zernecke
2007 Chemokines in vascular remodeling. Thromb Haemost 97(5):730-7.
Schober, A., et al.
2004 Crucial role of the CCL2/CCR2 axis in neointimal hyperplasia after arterial injury in hyperlipidemic mice involves early monocyte recruitment and CCL2 presentation on platelets. Circ Res 95(11):1125-33.
Schultz, G. S., and A. Wysocki
2009 Interactions between extracellular matrix and growth factors in wound healing. Wound Repair Regen 17(2):153-62.
Schwartz, M. A.
2001 Integrin signaling revisited. Trends Cell Biol 11(12):466-70.
Schwartz, M. A., and R. K. Assoian
2001 Integrins and cell proliferation: regulation of cyclin-dependent kinases via cytoplasmic signaling pathways. J Cell Sci 114(Pt 14):2553-60.
Schwarz, D. S., et al.
2003 Asymmetry in the assembly of the RNAi enzyme complex. Cell 115(2):199-208.
Schwarzenbach, H., et al.
2012 Diagnostic potential of PTEN-targeting miR-214 in the blood of breast cancer patients. Breast Cancer Res Treat 134(3):933-41.
Scott, N. A., et al.
1996 Identification of a potential role for the adventitia in vascular lesion formation after balloon overstretch injury of porcine coronary arteries. Circulation 93(12):2178-87.
Selley, M. L.
1997 Determination of the lipid peroxidation product (E)-4-hydroxy-2-nonenal in clinical samples by gas chromatography--negative-ion chemical ionisation mass spectrometry of the O-pentafluorobenzyl oxime. J Chromatogr B Biomed Sci Appl 691(2):263-8.
Selley, M. L., et al.
1989 Determination of the lipid peroxidation product trans-4-hydroxy-2-nonenal in biological samples by high-performance liquid chromatography and combined capillary column gas chromatography-negative-ion chemical ionisation mass spectrometry. J Chromatogr 488(2):329-40.
SenBanerjee, S., et al.
2004 KLF2 Is a novel transcriptional regulator of endothelial proinflammatory activation. J Exp Med 199(10):1305-15.
Serruys, P. W., et al.
1988 Incidence of restenosis after successful coronary angioplasty: a time-related phenomenon. A quantitative angiographic study in 342 consecutive patients at 1, 2, 3, and 4 months. Circulation 77(2):361-71.
Sevieux, N., et al.
2003 Short-term stretch translocates the alpha-1-subunit of the Na pump to plasma membrane. Cell Biochem Biophys 38(1):23-32.
Shi, G. J., et al.

2018 Involvement of growth factors in diabetes mellitus and its complications: A general review. Biomed Pharmacother 101:510-527.

Shi, K., et al.

2013 MicroRNA-214 suppresses osteogenic differentiation of C2C12 myoblast cells by targeting Osterix. Bone 55(2):487-94.

Shi, Y., et al.

2000 Myofibroblast involvement in glycosaminoglycan synthesis and lipid retention during coronary repair. J Vasc Res 37(5):399-407.

Shi, Y., et al.

1996a Adventitial myofibroblasts contribute to neointimal formation in injured porcine coronary arteries. Circulation 94(7):1655-64.

Shi, Y., et al.

1996b Transforming growth factor-beta 1 expression and myofibroblast formation during arterial repair. Arterioscler Thromb Vasc Biol 16(10):1298-305.

Shi, Y., et al.

1999 Role of matrix metalloproteinases and their tissue inhibitors in the regulation of coronary cell migration. Arterioscler Thromb Vasc Biol 19(5):1150-5.

Shi, Y., et al.

1996c Adventitial remodeling after coronary arterial injury. Circulation 93(2):340-8.

Shih, T. C., et al.

2012 MicroRNA-214 downregulation contributes to tumor angiogenesis by inducing secretion of the hepatoma-derived growth factor in human hepatoma. J Hepatol 57(3):584-91.

Shimada, K.

2009 Immune system and atherosclerotic disease: heterogeneity of leukocyte subsets participating in the pathogenesis of atherosclerosis. Circ J 73(6):994-1001.

Shimizu, K., et al.

2001 Host bone-marrow cells are a source of donor intimal smooth- muscle-like cells in murine aortic transplant arteriopathy. Nat Med 7(6):738-41.

Shiohama, A., et al.

2007 Nucleolar localization of DGCR8 and identification of eleven DGCR8-associated proteins. Exp Cell Res 313(20):4196-207.

Silence, J., et al.

2001 Persistence of atherosclerotic plaque but reduced aneurysm formation in mice with stromelysin-1 (MMP-3) gene inactivation. Arterioscler Thromb Vasc Biol 21(9):1440-5.

Singh, N. N., and D. P. Ramji

2006a The role of transforming growth factor-beta in atherosclerosis. Cytokine Growth Factor Rev 17(6):487-99.

—

2006b Transforming growth factor-beta-induced expression of the apolipoprotein E gene requires c-Jun N-terminal kinase, p38 kinase, and casein kinase 2. Arterioscler Thromb Vasc Biol 26(6):1323-9.

Siow, R. C., C. M. Mallawaarachchi, and P. L. Weissberg

2003 Migration of adventitial myofibroblasts following vascular balloon injury: insights from in vivo gene transfer to rat carotid arteries. Cardiovasc Res 59(1):212-21.

Smith, E. B.

1965 The Influence of Age and Atherosclerosis on the Chemistry of Aortic Intima.2. Collagen and Mucopolysaccharides. J Atheroscler Res 5(2):241-8.

Sobue, K., K. Hayashi, and W. Nishida

1999 Expressional regulation of smooth muscle cell-specific genes in association with phenotypic modulation. Mol Cell Biochem 190(1-2):105-18.

Spiegelman, B. M., and R. Heinrich

2004 Biological control through regulated transcriptional coactivators. Cell 119(2):157-67.

Sprague, E. A., et al.
1985 Stimulation of albumin endocytosis by cationized ferritin in cultured aortic smooth muscle cells. Am J Pathol 121(3):433-43.
Steffen, A., et al.
2004 Sra-1 and Nap1 link Rac to actin assembly driving lamellipodia formation. EMBO J 23(4):749-59.
Sun, Q., et al.
2006 Defining the mammalian CArGome. Genome Res 16(2):197-207.
Sun, S. G., et al.
2011 miR-146a and Kruppel-like factor 4 form a feedback loop to participate in vascular smooth muscle cell proliferation. EMBO Rep 12(1):56-62.
Sun, Y., et al.
2013 MiR-490-3p modulates the proliferation of vascular smooth muscle cells induced by ox-LDL through targeting PAPP-A. Cardiovasc Res 100(2):272-9.
Suzuki, T., et al.
2000 Molecular cloning of a novel apoptosis-related gene, human Nap1 (NCKAP1), and its possible relation to Alzheimer disease. Genomics 63(2):246-54.
Sykiotis, G. P., and D. Bohmann
2010 Stress-activated cap'n'collar transcription factors in aging and human disease. Sci Signal 3(112):re3.
Takabe, W., E. Warabi, and N. Noguchi
2011 Anti-atherogenic effect of laminar shear stress via Nrf2 activation. Antioxid Redox Signal 15(5):1415-26.
Takenawa, T., and H. Miki
2001 WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin filaments and cell movement. J Cell Sci 114(Pt 10):1801-9.
Tanner, F. C., et al.
1998 Expression of cyclin-dependent kinase inhibitors in vascular disease. Circ Res 82(3):396-403.
Teng, G., and F. N. Papavasiliou
2009 Shhh! Silencing by microRNA-155. Philos Trans R Soc Lond B Biol Sci 364(1517):631-7.
Tesfamariam, B.
2016 Endothelial Repair and Regeneration Following Intimal Injury. J Cardiovasc Transl Res 9(2):91-101.
Teslovich, T. M., et al.
2010 Biological, clinical and population relevance of 95 loci for blood lipids. Nature 466(7307):707-13.
Thum, T., et al.
2008 MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. Nature 456(7224):980-4.
Tian, X., et al.
2011 E-cadherin/beta-catenin complex and the epithelial barrier. J Biomed Biotechnol 2011:567305.
Tili, E., et al.
2007 Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. J Immunol 179(8):5082-9.
Torella, D., et al.
2011 MicroRNA-133 controls vascular smooth muscle cell phenotypic switch in vitro and vascular remodeling in vivo. Circ Res 109(8):880-93.
Townsend, N., et al.

2016 Cardiovascular disease in Europe: epidemiological update 2016. Eur Heart J 37(42):3232-3245.

Truty, M. J., et al.
2009 Silencing of the transforming growth factor-beta (TGFbeta) receptor II by Kruppel-like factor 14 underscores the importance of a negative feedback mechanism in TGFbeta signaling. J Biol Chem 284(10):6291-300.

Tsai, A. G., P. C. Johnson, and M. Intaglietta
2007 Is the distribution of tissue pO(2) homogeneous? Antioxid Redox Signal 9(7):979-84.

Tsutsumi, A., et al.
2011 Recognition of the pre-miRNA structure by Drosophila Dicer-1. Nat Struct Mol Biol 18(10):1153-8.

Turner, M., D. Chantry, and M. Feldmann
1990 Transforming growth factor beta induces the production of interleukin 6 by human peripheral blood mononuclear cells. Cytokine 2(3):211-6.

Ueba, H., M. Kawakami, and T. Yaginuma
1997 Shear stress as an inhibitor of vascular smooth muscle cell proliferation. Role of transforming growth factor-beta 1 and tissue-type plasminogen activator. Arterioscler Thromb Vasc Biol 17(8):1512-6.

Ueda, T., et al.
2010 Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis. Lancet Oncol 11(2):136-46.

Urbich, C., and S. Dimmeler
2004 Endothelial progenitor cells: characterization and role in vascular biology. Circ Res 95(4):343-53.

Urbich, C., A. Kuehbacher, and S. Dimmeler
2008 Role of microRNAs in vascular diseases, inflammation, and angiogenesis. Cardiovasc Res 79(4):581-8.

Van Assche, T., et al.
2007 Altered Ca2+ handling of smooth muscle cells in aorta of apolipoprotein E-deficient mice before development of atherosclerotic lesions. Cell Calcium 41(3):295-302.

van Lenten, B. J., and A. M. Fogelman
1992 Lipopolysaccharide-induced inhibition of scavenger receptor expression in human monocyte-macrophages is mediated through tumor necrosis factor-alpha. J Immunol 148(1):112-6.

van Mil, A., et al.
2012 MicroRNA-214 inhibits angiogenesis by targeting Quaking and reducing angiogenic growth factor release. Cardiovasc Res 93(4):655-65.

van Rooij, E., et al.
2006 A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. Proc Natl Acad Sci U S A 103(48):18255-60.

van Rooij, E., et al.
2008 Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc Natl Acad Sci U S A 105(35):13027-32.

van Royen, N., et al.
2002 Exogenous application of transforming growth factor beta 1 stimulates arteriogenesis in the peripheral circulation. FASEB J 16(3):432-4.

Van Vre, E. A., et al.
2012 Apoptotic cell death and efferocytosis in atherosclerosis. Arterioscler Thromb Vasc Biol 32(4):887-93.

Vasudevan, S., Y. Tong, and J. A. Steitz
2007 Switching from repression to activation: microRNAs can up-regulate translation. Science 318(5858):1931-4.

Villarreal, G., Jr., et al.
2010 Defining the regulation of KLF4 expression and its downstream transcriptional targets in vascular endothelial cells. Biochem Biophys Res Commun 391(1):984-9.
Voight, B. F., et al.
2010 Twelve type 2 diabetes susceptibility loci identified through large-scale association analysis. Nat Genet 42(7):579-89.
Volinia, S., et al.
2006 A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A 103(7):2257-61.
von Hundelshausen, P., et al.
2001 RANTES deposition by platelets triggers monocyte arrest on inflamed and atherosclerotic endothelium. Circulation 103(13):1772-7.
Wagenseil, J. E., and R. P. Mecham
2009 Vascular extracellular matrix and arterial mechanics. Physiol Rev 89(3):957-89.
Wahl, S. M., et al.
1993 Transforming growth factor beta enhances integrin expression and type IV collagenase secretion in human monocytes. Proc Natl Acad Sci U S A 90(10):4577-81.
Wahl, S. M., et al.
1987 Transforming growth factor type beta induces monocyte chemotaxis and growth factor production. Proc Natl Acad Sci U S A 84(16):5788-92.
Wang, D. Z., and E. N. Olson
2004 Control of smooth muscle development by the myocardin family of transcriptional coactivators. Curr Opin Genet Dev 14(5):558-66.
Wang, F., et al.
2013a MiR-214 reduces cell survival and enhances cisplatin-induced cytotoxicity via down-regulation of Bcl2l2 in cervical cancer cells. FEBS Lett 587(5):488-95.
Wang, F., et al.
2015 microRNA-214 enhances the invasion ability of breast cancer cells by targeting p53. Int J Mol Med 35(5):1395-402.
Wang, J., et al.
2013b Downregulation of microRNA-214 and overexpression of FGFR-1 contribute to hepatocellular carcinoma metastasis. Biochem Biophys Res Commun 439(1):47-53.
Wang, L., et al.
2010 Cartilage oligomeric matrix protein maintains the contractile phenotype of vascular smooth muscle cells by interacting with alpha(7)beta(1) integrin. Circ Res 106(3):514-25.
Wang, N., et al.
2006 Shear stress regulation of Kruppel-like factor 2 expression is flow pattern-specific. Biochem Biophys Res Commun 341(4):1244-51.
Wang, X., et al.
2014 MicroRNA-214 regulates osteosarcoma survival and growth by directly targeting phosphatase and tensin homolog. Mol Med Rep 10(6):3073-9.
Wang, Y., et al.
2007 DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. Nat Genet 39(3):380-5.
Wang, Y. S., et al.
2012a MicroRNA-152 mediates DNMT1-regulated DNA methylation in the estrogen receptor alpha gene. PLoS One 7(1):e30635.
Wang, Y. S., et al.
2012b MicroRNA-195 regulates vascular smooth muscle cell phenotype and prevents neointimal formation. Cardiovasc Res 95(4):517-26.
Wang, Z., et al.

2004 Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression. Nature 428(6979):185-9.

Warabi, E., et al.

2007 Shear stress stabilizes NF-E2-related factor 2 and induces antioxidant genes in endothelial cells: role of reactive oxygen/nitrogen species. Free Radic Biol Med 42(2):260-9.

Warboys, C. M., et al.

2011 The role of blood flow in determining the sites of atherosclerotic plaques. F1000 Med Rep 3:5.

Watanabe, T., et al.

2008 Dnm3os, a non-coding RNA, is required for normal growth and skeletal development in mice. Dev Dyn 237(12):3738-48.

Wei, X., et al.

2017 A novel role for the Kruppel-like factor 14 on macrophage inflammatory response and atherosclerosis development. Cardiovasc Pathol 27:1-8.

Weinert, S., et al.

2013 The lysosomal transfer of LDL/cholesterol from macrophages into vascular smooth muscle cells induces their phenotypic alteration. Cardiovasc Res 97(3):544-52.

Werner, N., et al.

2005 Circulating endothelial progenitor cells and cardiovascular outcomes. N Engl J Med 353(10):999-1007.

Wernig, F., M. Mayr, and Q. Xu

2003 Mechanical stretch-induced apoptosis in smooth muscle cells is mediated by beta1-integrin signaling pathways. Hypertension 41(4):903-11.

Wightman, B., I. Ha, and G. Ruvkun

1993 Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell 75(5):855-62.

Wilcox, J. N., et al.

1988 Platelet-derived growth factor mRNA detection in human atherosclerotic plaques by in situ hybridization. J Clin Invest 82(3):1134-43.

Williams, H., et al.

2010 MMP-7 mediates cleavage of N-cadherin and promotes smooth muscle cell apoptosis. Cardiovasc Res 87(1):137-46.

Winter, J., et al.

2009 Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol 11(3):228-34.

Worley, L. A., et al.

2008 Micro-RNAs associated with metastasis in uveal melanoma identified by multiplexed microarray profiling. Melanoma Res 18(3):184-90.

Wu, L., J. Fan, and J. G. Belasco

2006 MicroRNAs direct rapid deadenylation of mRNA. Proc Natl Acad Sci U S A 103(11):4034-9.

Wu, W. H., et al.

2011 MicroRNA-130a mediates proliferation of vascular smooth muscle cells in hypertension. Am J Hypertens 24(10):1087-93.

Wu, Y., et al.

2017 MicroRNA-214 regulates smooth muscle cell differentiation from stem cells by targeting RNA-binding protein QKI. Oncotarget 8(12):19866-19878.

Wuttge, D. M., et al.

2004 CXCL16/SR-PSOX is an interferon-gamma-regulated chemokine and scavenger receptor expressed in atherosclerotic lesions. Arterioscler Thromb Vasc Biol 24(4):750-5.

Xia, H., L. L. Ooi, and K. M. Hui

2012 MiR-214 targets beta-catenin pathway to suppress invasion, stem-like traits and recurrence of human hepatocellular carcinoma. PLoS One 7(9):e44206.

Xia, S., et al.
2011 Involvement of Gax gene in hypoxia-induced pulmonary hypertension, proliferation, and apoptosis of arterial smooth muscle cells. Am J Respir Cell Mol Biol 44(1):66-73.
Xiao, Q., et al.
2010 The mechanism of stem cell differentiation into smooth muscle cells. Thromb Haemost 104(3):440-8.
Xiao, Q., et al.
2006 Sca-1+ progenitors derived from embryonic stem cells differentiate into endothelial cells capable of vascular repair after arterial injury. Arterioscler Thromb Vasc Biol 26(10):2244-51.
Xiao, Q., et al.
2014 Matrix metalloproteinase-8 promotes vascular smooth muscle cell proliferation and neointima formation. Arterioscler Thromb Vasc Biol 34(1):90-8.
Xie, C., et al.
2011 MicroRNA-1 regulates smooth muscle cell differentiation by repressing Kruppel-like factor 4. Stem Cells Dev 20(2):205-10.
Xin, M., et al.
2009 MicroRNAs miR-143 and miR-145 modulate cytoskeletal dynamics and responsiveness of smooth muscle cells to injury. Genes Dev 23(18):2166-78.
Xu, C. X., et al.
2012 MicroRNA miR-214 regulates ovarian cancer cell stemness by targeting p53/Nanog. J Biol Chem 287(42):34970-8.
Yanaihara, N., et al.
2006 Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 9(3):189-98.
Yang, F., et al.
2018 miR-22 Is a Novel Mediator of Vascular Smooth Muscle Cell Phenotypic Modulation and Neointima Formation. Circulation 137(17):1824-1841.
Yang, G., et al.
2012 MicroRNA-21 represses human cystathionine gamma-lyase expression by targeting at specificity protein-1 in smooth muscle cells. J Cell Physiol 227(9):3192-200.
Yang, H., et al.
2008 MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN. Cancer Res 68(2):425-33.
Yang, T. S., et al.
2013a MiR-214 regulate gastric cancer cell proliferation, migration and invasion by targeting PTEN. Cancer Cell Int 13(1):68.
Yang, T., et al.
2013b MicroRNA-214 provokes cardiac hypertrophy via repression of EZH2. Biochem Biophys Res Commun 436(4):578-84.
Yang, W. J., et al.
2005a Dicer is required for embryonic angiogenesis during mouse development. J Biol Chem 280(10):9330-5.
Yang, X., et al.
2005b Proinflammatory phenotype of vascular smooth muscle cells: role of efficient Toll-like receptor 4 signaling. Am J Physiol Heart Circ Physiol 289(3):H1069-76.
Yang, X., et al.
2013c Characterization of culprit lesions in acute coronary syndromes compared with stable angina pectoris by dual-source computed tomography. Int J Cardiovasc Imaging 29(4):945-53.
Yang, X., et al.
2016 MicroRNA-214 Inhibits Left Ventricular Remodeling in an Acute Myocardial Infarction Rat Model by Suppressing Cellular Apoptosis via the Phosphatase and Tensin Homolog (PTEN). Int Heart J 57(2):247-50.

Yellaturu, C. R., et al.
2002 A potential role for nuclear factor of activated T-cells in receptor tyrosine kinase and G-protein-coupled receptor agonist-induced cell proliferation. Biochem J 368(Pt 1):183-90.
Yeom, K. H., et al.
2006 Characterization of DGCR8/Pasha, the essential cofactor for Drosha in primary miRNA processing. Nucleic Acids Res 34(16):4622-9.
Yi, R., et al.
2003 Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev 17(24):3011-6.
Yin, B. L., et al.
2009 Downregulating osteopontin reduces angiotensin II-induced inflammatory activation in vascular smooth muscle cells. Inflamm Res 58(2):67-73.
Yin, G., et al.
2010 TWISTing stemness, inflammation and proliferation of epithelial ovarian cancer cells through MIR199A2/214. Oncogene 29(24):3545-53.
Yoshida, T., Q. Gan, and G. K. Owens
2008a Kruppel-like factor 4, Elk-1, and histone deacetylases cooperatively suppress smooth muscle cell differentiation markers in response to oxidized phospholipids. Am J Physiol Cell Physiol 295(5):C1175-82.
Yoshida, T., K. H. Kaestner, and G. K. Owens
2008b Conditional deletion of Krüppel-like factor 4 delays downregulation of smooth muscle cell differentiation markers but accelerates neointimal formation following vascular injury. Circ Res 102(12):1548-57.
Young, J. L., P. Libby, and U. Schonbeck
2002 Cytokines in the pathogenesis of atherosclerosis. Thromb Haemost 88(4):554-67.
Yu, X. H., et al.
2013 Foam cells in atherosclerosis. Clin Chim Acta 424:245-52.
Yu, X. H., X. L. Zheng, and C. K. Tang
2015 Nuclear Factor-kappaB Activation as a Pathological Mechanism of Lipid Metabolism and Atherosclerosis. Adv Clin Chem 70:1-30.
Yu, X., and Z. Li
2014 MicroRNAs regulate vascular smooth muscle cell functions in atherosclerosis (review). Int J Mol Med 34(4):923-33.
Yu, X., et al.
2014 Upregulated sirtuin 1 by miRNA-34a is required for smooth muscle cell differentiation from pluripotent stem cells. Cell Death Differ.
Yu, Z. W., et al.
2010 MicroRNAs contribute to the chemoresistance of cisplatin in tongue squamous cell carcinoma lines. Oral Oncol 46(4):317-22.
Yuasa-Kawase, M., et al.
2012 Patients with CD36 deficiency are associated with enhanced atherosclerotic cardiovascular diseases. J Atheroscler Thromb 19(3):263-75.
Yurchenco, P. D.
2011 Basement membranes: cell scaffoldings and signaling platforms. Cold Spring Harb Perspect Biol 3(2).
Yurdagul, A., Jr., et al.
2013 Altered nitric oxide production mediates matrix-specific PAK2 and NF-kappaB activation by flow. Mol Biol Cell 24(3):398-408.
Zakkar, M., et al.
2009 Activation of Nrf2 in endothelial cells protects arteries from exhibiting a proinflammatory state. Arterioscler Thromb Vasc Biol 29(11):1851-7.
Zeiffer, U., et al.

2004 Neointimal smooth muscle cells display a proinflammatory phenotype resulting in increased leukocyte recruitment mediated by P-selectin and chemokines. Circ Res 94(6):776-84.

Zeng, L., et al.

2006 HDAC3 is crucial in shear- and VEGF-induced stem cell differentiation toward endothelial cells. J Cell Biol 174(7):1059-69.

Zernecke, A., et al.

2009 Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. Sci Signal 2(100):ra81.

Zernecke, A., et al.

2005 SDF-1alpha/CXCR4 axis is instrumental in neointimal hyperplasia and recruitment of smooth muscle progenitor cells. Circ Res 96(7):784-91.

Zhang, H., et al.

2004 Single processing center models for human Dicer and bacterial RNase III. Cell 118(1):57-68.

Zhang, J., et al.

2016a ROS and ROS-Mediated Cellular Signaling. Oxid Med Cell Longev 2016:4350965.

Zhang, L., et al.

2017 Novel Pathological Role of hnRNPA1 (Heterogeneous Nuclear Ribonucleoprotein A1) in Vascular Smooth Muscle Cell Function and Neointima Hyperplasia. Arterioscler Thromb Vasc Biol 37(11):2182-2194.

Zhang, M. J., et al.

2016b An overview of potential molecular mechanisms involved in VSMC phenotypic modulation. Histochem Cell Biol 145(2):119-30.

Zhang, X. J., et al.

2010 Dysregulation of miR-15a and miR-214 in human pancreatic cancer. J Hematol Oncol 3:46.

Zhang, Z. C., et al.

2014 Knockdown of miR-214 promotes apoptosis and inhibits cell proliferation in nasopharyngeal carcinoma. PLoS One 9(1):e86149.

Zhao, H., et al.

2015 MicroRNA-22 regulates smooth muscle cell differentiation from stem cells by targeting methyl CpG-binding protein 2. Arterioscler Thromb Vasc Biol 35(4):918-29.

Zhao, Y., E. Samal, and D. Srivastava

2005 Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. Nature 436(7048):214-20.

Zhao, Y., and D. Srivastava

2007 A developmental view of microRNA function. Trends Biochem Sci 32(4):189-97.

Zheng, X., et al.

2013 Key role of microRNA-15a in the KLF4 suppressions of proliferation and angiogenesis in endothelial and vascular smooth muscle cells. Biochem Biophys Res Commun 437(4):625-31.

Zhou, J., Y. S. Li, and S. Chien

2014 Shear stress-initiated signaling and its regulation of endothelial function. Arterioscler Thromb Vasc Biol 34(10):2191-8.

Zhou, X., et al.

2005 Diverse origin of vascular smooth muscle cells in the neointima. Anadolu Kardiyol Derg 5(3):216-20.

Zoellner, H., et al.

1992 Human arterial smooth muscle cells synthesize granulocyte colony-stimulating factor in response to interleukin-1 alpha and tumor necrosis factor-alpha. Blood 80(11):2805-10.