MicroRNA-22 regulates smooth muscle cell differentiation from stem cells by targeting methyl CpG binding protein 2

By Hanqing Zhao

Supervisors: Dr Qingzhong Xiao

Professor Shu Ye

Submitted for the Degree of Doctor of Philosophy

Centre for Clinical Pharmacology

William Harvey Research Institute

Barts and the London School of Medicine and Dentistry

Queen Mary University of London

2015

Declaration of originality

I confirm that all research included within this thesis is my own work or is properly acknowledged below when carried out in collaboration with or through the support of others, with my contribution indicated. Previously published papers are also acknowledged below.

I declare that all work in my project is original. In addition, my work does not break any UK law, infringe upon any third party's copyright or other Intellectual Property Right, or contain any confidential material.

To prove the originality, I accept that the university has the right to use plagiarism detection software to check the electronic version of the thesis.

Moreover, I affirm that this thesis has not been submitted for the award of a degree by this or any other university.

Signature: Hanqing Zhao

Date: 15/07/2015

Collaboration

During my PhD study, I received assistance and valuable suggestions from my group. The flow cytometry and cell sorting experiments were carried out by Dr Guanmei Wen. Dr Qingzhong Xiao and Dr Guanmei Wen helped me perform the *in vivo* Matrigel experiments. ChIP assays were carried out with help from Dr Le Anh Luong. Experiments related to vascular stem/progenitor cell isolation and differentiation were performed with the help of Dr Li Zhang's group at Zhejiang University, China. Dr Xiaotian Yu and Dr Yuan Huang assisted with the creation of miRNA reporters and with cell culture.

Publications list and conferences

Journal publications

1. **Zhao H**, Wen G, Huang Y, Yu X, Chen Q, Afzal TA, Luong le A, Zhu J, Shu Y, Zhang L, Xiao Q. (2015). "MicroRNA-22 Regulates Smooth Muscle Cell Differentiation From Stem Cells by Targeting Methyl CpG-Binding Protein 2." Arterioscler Thromb Vasc Biol 35(4): 918-929.

2. Yu X, Zhang L, Wen G, **Zhao H**, Luong LA, Chen Q, Huang Y, Zhu J, Ye S, Xu Q, Wang W, Xiao Q. (2015). "Upregulated sirtuin 1 by miRNA-34a is required for smooth muscle cell differentiation from pluripotent stem cells." Cell Death Differ 22:1170-80.

3. Huang Y, Lin L, Yu X, Wen G, Pu X, **Zhao H**, Fang C, Zhu J, Ye S, Zhang L, Xiao Q. (2013). "Functional involvements of heterogeneous nuclear ribonucleoprotein A1 in smooth muscle differentiation from stem cells in vitro and in vivo." Stem Cells 31(5): 906-917.

Conferences

1. 22th - 25th March 2015: European Atherosclerosis Society (EAS) & Congress, Glasgow, **Oral presentation**

2. 25th - 26th September 2014: British Atherosclerosis Society (BAS) & Congress, Cambridge, **Poster presentation**

3. 1st July 2014, William Harvey Research Institute Annual Research Review Day, **Poster presentation**

4. 16th October 2013: William Harvey Day, London, **Poster presentation**

5. 29th -30th August 2014: The 20th Annual Meeting of the Chinese Life Science Society, London, UK

6. Abstract was selected as **Oral presentation** by the Arteriosclerosis, Thrombosis, and Vascular Biology | Peripheral Vascular Disease 2015 Scientific Sessions, San Francisco, California, USA, 5th -7th May 2015

Acknowledgements

First and foremost, I would like to express my deep appreciation to Dr Qingzhong Xiao, my principal supervisor, for his guidance and unreserved mentorship during my four-year PhD study. Dr Qingzhong is a gentleman with a noble personality. He is an expert in navigating and sailing our research-ship in the wild ocean of knowledge to the discovery of new land, but also a trusted friend who has shared precious advice from his colourful personal life experiences, as well as his love of family, humour, and academic research.

Sincere thanks also go to my dear second supervisor Professor Shu Ye, who is an excellent teacher, giving generous helps and useful suggestions. Meanwhile, I would like to express my gratitude to my tutor in China, Professor Shujun Zhang, who first showed me the biological world when I was an undergraduate. I am indebted to our group's collaborator, Dr Li Zhang from the Department of Cardiology at Zhejiang University, for his valuable input and accessibility. The fieldwork would not have been completed without help and advice from my examiners, Professor Andrew Tinker and Dr Claudio Mauro. Special thanks are also extended to my two laboratory managers, Mrs Sue Shaw-Hawkins and Mr Ian Joy, for lending me their expertise and insight with regard to the best lab materials.

Many thanks to our group members, including Dr Xiaotian Yu, Dr Guanmei Wen, Dr Yuan Huang, Dr Qishan Chen, Dr Le Anh Luong, and Dr Changchun Fang, who gave expert suggestions and support to my work.

In addition, I appreciate other current and former group members for the happiness and warmth you have given me during my PhD study, including Miss Rossie Zhang, Miss Meixia Ren, Dr Fu Ng, Dr Kate Witowska, Dr Xiangyuan Pu, Dr Andrew Moore from Professor Shu Ye's group; and Dr Changcun Fang, Mr Luyang Lin, Dr Cheng Zhang, Dr Feng Yang, and Mr Tayyab Adeel Afza from Dr Qingzhong Xiao's group.

Moreover, I am grateful for the financial support from the British Heart Foundation and China Scholarship Council, which allowed me to concentrate on my studies without needing to worry about basic living expenses. Furthermore, thanks to the William Harvey Research Institute, Bart, and the London School of Medicine and Dentistry for providing an excellent environment in which to carry out my research.

Many people who have kindly helped me who cannot be listed here individually. Thank you all very much.

My four years in London were enjoyable in large part owing to the many friends that became a part of my life. I am grateful for the time spent with Dr Zheng Li, Mr Fei Jiao, Miss Sandy Chu, and Mr Rui Yang and for many other people and memories.

My endless love goes to my mother Mrs Yazhi Wang and my father Professor Yongren Zhao, who have given me their patronage and passionate encouragement. The meaning of my life is of their love and for their love. Finally, I would like to thank my extended family in China for providing unconditional love and support throughout my life.

Abstract

In recent years, microRNAs have emerged as important regulators in various biological processes, as a new class of biomarkers, and as novel therapeutic drugs for many diseases, including cardiovascular diseases. Tumour suppressor microRNA-22 (miRNA-22 or miR-22) has been reported to regulate cardiac aging and to play a role in hematopoietic cell differentiation and maturation. Moreover, DNA methylation, a major modification of eukaryotic genomes, plays an essential role in mammalian development. Methyl CpG-binding protein 2 (MECP2) is capable of binding specifically to methylated DNA and is involved in gene silencing. The main objectives of this PhD project were to determine the functional impact of miRNA-22 and its target gene, MECP2, in smooth muscle cell (SMC) differentiation and to delineate the molecular mechanism involved.

Mouse embryonic stem (ES) cells were seeded on collagen-coated flasks in differentiation medium to promote SMC differentiation. MiRNA-22 was significantly upregulated during SMC differentiation from ES cells. Enforced expression of miRNA-22 by its mimic or knockdown of miR-22 by its antagomiR promoted or inhibited SMC differentiation from ES cells, respectively. As expected, miRNA-22 overexpression in stem cells promoted SMC differentiation *in vivo*. Consistently, a similar change in miR-22 expression and a similar functional role for miRNA-22 were observed during SMC differentiation from adventitia stem/progenitor cells isolated from murine blood vessels. MECP2, the founding member of the family of methyl CpG binding domain proteins, was identified by several computational miRNA target prediction tools as one of the top targets of miR-22. Methyl CpG binding domain proteins bind specifically to methylated and unmethylated DNA and recruit distinct interacting protein partners to establish a repressive or active chromatin

environment. Interestingly, expression of the gene encoding MECP2 decreased significantly during SMC differentiation. MECP2 decreased dramatically in miRNA-22–overexpressing cells, but significantly increased after miRNA-22 knockdown in differentiating stem cells. Moreover, luciferase assays showed that miR-22 substantially inhibited wild-type, but not mutant, MECP2-3′-UTR luciferase activity. In addition, modulation of MECP2 expression levels affected the expression of multiple SMC-specific marker genes in differentiated ES cells. At the mechanistic level, our data showed that MECP2 transcriptionally repressed SMC gene expression by modulating various SMC transcription factors as well as several established SMC differentiation regulators. Additionally, enrichment of H3K9 trimethylation around the promoter regions of SMC transcription factors and other known differentiation regulator genes increased after MECP2 overexpression, suggesting that modulation of DNA methylation is another mechanism underlying MECP2-mediated gene expression during SMC differentiation from stem cells. Finally, miR-22 was upregulated by platelet-derived growth factor-BB and transforming growth factor-β through a transcriptional mechanism during SMC differentiation.

Taken together, the findings obtained from my PhD project strongly suggest that miR-22 plays an important role in SMC differentiation from both embryonic and adventitial stem cells and that epigenetic regulation through MECP2 is required for miR-22–mediated SMC differentiation.

List of Abbreviation and Acronyms

2D	2-dimensional
3D	3-dimensional
ACTA2	Actin alpha 2
AGO	Argonaute
APS	Ammonium persulfate
ASC	Adult stem cell
ASVD	Arteriosclerotic vascular disease
At-RA	All-trans retinoic acid
BM	Basic medium
BrdU	5-bromo-2'-deoxyuridine
CAM	Cell adhesion molecule
Cbx3	Chromobox protein homolog 3
ChIP	Chromatin immunoprecipitation
CM	ES cell culture medium
CSC	Cancer stem cell

CVD	Cardiovascular disease
DAPI	4’,6-diamidino-2-phenylindole
db-cAMP	Dibutyryl-cAMP
DGCR8	DiGeorge syndrome critical region gene 8
DM	SMC differentiation medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EB	Embryoid-body
ECM	Extracellular matrix
ES cell	Embryonic stem cell
FBS	Fetal bovine serum
GFP	Green fluorescent protein
h	Hour/hours
H3K9me3	Histone h3 trimethyl lys9
HEK293	Human embryonic kidney 293 cell
HDAC7	Histone deacetylase 7
HnRNPA1	Heterogeneous nuclear ribonucleoprotein A1

HnRNPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1
ICAM	Intracellular cell adhesion molecule
ICM	Inner cell mass
iPSC	Induced pluripotent stem cell
KLF4	Kruppel-like factor 4
Lefty1	Left-right determination factor 1
LIF	Leukemia inhibitory factor
MECP2	Methyl CpG binding protein 2
MEF2C	Myocyte-specific enhancer factor 2C
MEFs	Mouse embryonic fibroblasts
miR	Micro RNA
miR-22	Micro RNA-22
miRNA	Micro RNA
Myocd	Myocardin
Nox4	NADPH oxidase 4
Nrf3	Nuclear factor erythroid 2-related factor 3
Oct4	Octamer-binding transcription factor 4

PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
Pla2g7	Phospholipase A2, Group VII (Platelet-Activating Factor Acetylhydrolase, Plasma)
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
RA	Retinoic acid
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
s	Second/seconds
Sca-1+ cell	Stem cell antigen-1positive cell
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SirT1	Deacetylase sirtuin1
SM22α	Smooth muscle 22 α

SMC	Smooth muscle cell
SM-MHC	Smooth muscle myosin heavy chain
SMαA	Smooth Muscle α-Actin
SOX4	(Sex Determining Region Y)-box 4
SRF	Serum response factor
SSC	Somatic stem cell
TGF	Transforming growth factor
TPA	12-O-tetradecanoylphorbol-13-acetate
TRBP	Tar RNA binding protein
UTR	Untranslated region
VSMC	Vascular smooth muscle cell
WHO	World Health Organization

Table of Contents

Declaration of originality ..... **- 2 -**

Collaboration ..... **- 3 -**

Publications list and conferences ..... **- 4 -**

Acknowledgements ..... **- 6 -**

Abstract ..... **- 8 -**

List of Abbreviation and Acronyms ..... **- 10 -**

List of Figures ..... **- 21 -**

List of Tables ..... **- 23 -**

Chapter 1

1. Introduction ..... **- 24 -**

1.1. Cardiovascular disease ..... - 24 -

1.1.1. Atherosclerosis, vascular smooth muscle cells, and stem cells ..... - 24 -

1.1.2 Regenerative medicine techniques in cardiovascular disease ..... - 31 -

1.2. Stem cell ..... - 32 -

1.2.1. Characteristics and classification of stem cells ..... - 32 -

1.2.2. Embryonic stem (ES) cells ..... - 35 -

1.2.3. ES cell differentiation ..... - 40 -

1.3. Smooth muscle cell (SMC) differentiation ..... - 42 -

1.3.1. Smooth muscle cells ..... - 42 -

1.3.2. SMC differentiation from stem cells and the mechanisms involved ..... - 43 -

1.4. MicroRNA ..... - 47 -

1.4.1. MiRNA and its biogenesis ..... - 47 -

1.4.2. MiRNAs and their targets ..... - 51 -

1.4.3. MiRNAs, SMC differentiation, and cardiovascular development ..... - 53 -

1.4.4. MiRNA-22 and its regulatory roles ..... - 56 -

1.5. Methyl CpG binding protein 2 (MECP2) ..... - 59 -

1.5.1. Gene regulation by MECP2 ..... - 59 -

1.5.2. MECP2 and human diseases ..... - 61 -

1.6 Hypothesis, Project Aims, and Objectives ..... - 63 -

Chapter 2

2. Materials and Methods ..... - 65 -

2.1. Materials ..... - 65 -

2.2. ES Cell Culture ..... - 67 -

2.2.1. Medium preparation ..... - 67 -

2.2.2. Mouse ES cell maintenance ..... - 67 -

2.2.3. Mouse ES cell passaging/sub-culturing ..... - 68 -

2.2.4. Mouse ES cell freezing and defrosting ..... - 69 -

2.3. Smooth muscle cell *in vitro* differentiation from ES cells ..... - 70 -

2.3.1. SMC differentiation medium (DM) ..... - 70 -

2.3.2. SMC differentiation from ES cell ..... - 70 -

2.4. SMC differentiation from vascular stem/progenitor cells ..... - 71 -

2.5. Transfection ..... - 72 -

2.5.1. Plasmid transfection ..... - 72 -

2.5.2. Transfection of miRNA precursors or inhibitors ..... - 73 -

2.5.3. MiR-22 and MECP2 co-overexpression ..... - 73 -

2.5.4. MECP2 siRNA transfection ..... - 74 -

2.6. SMC *in vivo* differentiation ..... - 75 -

2.6.1. Generation of lentiviral particles for miR-22 overexpression ..... - 75 -

2.6.2. Lentiviral infection and cell sorting ..... - 83 -

2.6.3. SMC differentiation *in vivo* from miRNA-22-overexpressing ES cells ..... - 83 -

2.6.4. Immunofluorescence staining for sections ..... - 84 -

2.7. RNA extraction and analysis ..... - 85 -

2.7.1. Total RNA extraction from cells ..... - 85 -

2.7.2. Small RNA extraction from cells ..... - 86 -

2.7.3. Normal RT-PCR ..... - 88 -

2.7.4. Real-time PCR (qPCR) ..... - 93 -

2.8. Protein extraction and analysis ..... - 94 -

2.8.1. Chemicals and buffers ..... - 94 -

2.8.2. Protein extraction from cells ..... - 97 -

2.8.3. Western blotting ..... - 98 -

2.9. Gene cloning and mutation ..... - 100 -

2.9.1. MECP2 3′ -UTR cloning and miR-22 binding site mutation ..... - 100 -

2.9.2. MECP2 gene clone ..... - 102 -

2.9.3. MiR-22 precursor clone ..... - 103 -

2.9.4. Plasmid amplification and extraction ..... - 103 -

2.10. Luciferase assay ..... - 105 -

2.10.1. Gene promoter activity assays ..... - 105 -

2.10.2. pmiR-Luc-MECP2 reporter activity assays ..... - 107 -

2.11. Chromatin immunoprecipitation (ChIP) assay ..... - 108 -

2.12. Flow cytometry ..... - 110 -

2.13. PDGF-BB, TGF-beta, and ActD treatment ..... - 111 -

2.14. Statistical analysis ..... - 111 -

Chapter 3

3. Results ..... - 112 -

3.1. SMC differentiation from ES cell ..... - 112 -

3.1.1. SMC-specific genes were upregulated during ES cell differentiation ..... - 112 -

3.2. The role of miR-22 in SMC differentiation from stem cells ..... - 114 -

3.2.1. Functional involvement of miR-22 in SMC differentiation from ES cells *in vitro* and *in vivo* - 114 -

3.3. MiR-22 target gene in SMC differentiation from ES cells ..... - 132 -

3.3.1. MECP2 is a predicted miR-22 target gene..... - 132 -

3.3.2. MECP2 is repressed during SMC differentiation ..... - 135 -

3.3.3. MECP2 is a miR-22 target gene in SMC differentiation ..... - 138 -

3.3.4. MECP2 is a specific target of miR-22..... - 141 -

3.3.5. MECP2 is negatively regulated by miR-22 through binding sites in the 3′ -UTR. - 143 -

3.4. MECP2 inhibition is required for miR-22-mediated SMC differentiation from ES cells- 146 -

3.4.1. MECP2 knockdown increases SMC gene expression..... - 146 -

3.4.2. MECP2 overexpression inhibits SMC gene expression..... - 148 -

3.4.3. MECP2 overexpression abolishes SMC gene expression induced by miR-22. ..... - 151 -

3.5. Mechanism underlying MECP2-mediated SMC gene repression..... - 154 -

3.5.1. Functional importance of the SRF binding site within SMC-specific gene promoters in MECP2-mediated SMC gene expression..... - 154 -

3.5.2. MECP2 represses SMC transcription factors ..... - 158 -

3.6. Other SMC differentiation regulators are regulated by miR-22 and MECP2 ..... - 162 -

3.7. MECP2 represses SMC gene expression by increasing H3K9 methylation within gene promoters ..... - 166 -

3.8. PDGF-BB and TGF-β upregulate miR-22 during SMC differentiation through a transcriptional mechanism ..... - 171 -

Chapter 4

4. Discussion, Conclusion, and Future Plans ..... - 174 -

4.1. SMC differentiation from ES cells ..... - 174 -

4.2. MiR-22 and SMC differentiation from ES cell and adventitia stem/progenitor cell ..... - 176 -

4.3. MECP2 is a downstream target gene of miR-22 during SMC differentiation ..... - 178 -

4.4. MECP2 regulates the expression of genes involved in SMC differentiation through a transcriptional mechanism ..... - 181 -

4.5. Epigenetic regulation of SMC genes by MECP2 during SMC differentiation ..... - 182 -

4.6. Conclusion ..... - 184 -

4.7. Future Plans ..... - 187 -

References ..... - 189 -

List of Figures

Figure 1 Distribution of major causes of death including CVD, in 2012. ..... - 26 -

Figure 2 MiRNA biogenesis pathway. ..... - 50 -

Figure 3 Gene activation or repression by MECP2 ..... - 60 -

Figure 4 Gene expression of SMC-specific genes during stem cell differentiation toward SMCs ..... - 113 -

Figure 5 Gene expression of miRNA-22 during stem cell differentiation. ..... - 116 -

Figure 6 MiRNA-22 is involved in SMC differentiation from stem cell ..... - 117 -

Figure 7 Generation of miRNA-22-overexpressing ES cells and differentiation towards SMCs ..... - 122 -

Figure 8 MiRNA-22 promotes SMC differentiation *in vivo*. ..... - 124 -

Figure 9 Modulation of miR-22 expression in differentiating ES cells regulates SMC transcription factors. ..... - 127 -

Figure 10 Functional importance of miR-22 in adventitia stem/progenitor cell differentiation towards SMCs. ..... - 130 -

Figure 11 Schematic illustration of MECP2 3′-UTR regions and pmiR-Luc-MECP2 reporters. ..... - 134 -

Figure 12 Downregulation of MECP2 during SMC differentiation from ES cells. ..... - 136 -

Figure 13 MECP2 is a miR-22 target gene during SMC differentiation. ..... - 139 -

Figure 14 MECP2 protein expression is specifically inhibited by miR-22 overexpression. ... - 142 -

Figure 15 Binding sites within the 3′-UTR are required for MECP2 gene repression by miR-22..... - 144 -

Figure 16 MECP2 knockdown increases SMC gene expression. ..... - 147 -

Figure 17 MECP2 overexpression inhibits SMC gene and protein expression. ..... - 149 -

Figure 18 MECP2 overexpression abolishes SMC gene expression induced by miR-22. ..... - 152 -

Figure 19 MECP2 overexpression abolishes the gene expression of SMC transcription factors and other reported SMC differentiation regulators mediated by miR-22. ..... - 153 -

Figure 20 SRF binding site is required for MECP2-mediated SMC gene expression..... - 156 -

Figure 21 MECP2 negatively regulates SMC transcription factor gene expression..... - 160 -

Figure 22 MECP2 negatively regulates other reported SMC differentiation regulators. ..... - 164 -

Figure 23 MECP2 increases the binding of H3K9me3 to the gene promoter regions of SRF, Myocd, and Pla2g7. ..... - 168 -

Figure 24 MiR-22 overexpression decreases H3K9 methylation within the SRF, Myocd, and Pla2g7 gene promoters..... - 170 -

Figure 25 PDGF-BB and TGF-β upregulate miR-22 through a transcriptional mechanism. .. - 172 -

Figure 26 Proposed model of miRNA-mediated SMC differentiation. ..... - 186 -

List of Tables

Table 1 Antibodies, enzymes, and buffers used for gene research ..... - 66 -

Table 2 Primer sets used in the present study ..... - 82 -

Table 3 Normal PCR conditions ..... - 92 -

Table 4 Percentage of agarose according to DNA or RNA size ..... - 92 -

Table 5 Recipes of buffers used in protein analysis ..... - 96 -

Chapter 1

1. Introduction

1.1. Cardiovascular disease

1.1.1. Atherosclerosis, vascular smooth muscle cells, and stem cells

Cardiovascular disease (CVD) is a class of diseases that involve the heart or blood vessels. According to the latest data from the World Health Organization (WHO), CVD is the leading cause of death globally (31.4%), accounted for around 17.5 million deaths in 2012 (**Figure 1**) (WHO, 2014). Moreover, it causes 4.35 million deaths in Europe annually (Mendis et al., 2011). Most instances of CVD could be prevented through population-wide strategies that address behavioural risk factors such as tobacco use, unhealthy diet, obesity, physical inactivity, and the harmful use of alcohol (Mendis et al., 2011). There are four main types of CVD: coronary heart disease (CHD) that leads to myocardial infarction (Buschdorf and Stratling), stroke, peripheral arterial disease, and aortic disease (Mendis et al., 2011, Sheridan et al., 2009).

The underlying cause of CVD, atherosclerosis, also known as arteriosclerotic vascular disease, is a chronic inflammatory disease initiated by endothelial dysfunction (Kaperonis et al., 2006), and it usually occurs inside medium to large size of arteries as a

result of plaque building up from fat, cholesterol, calcium and other substances found in the blood and vascular wall (Go et al., 2014, Ross and Glomset, 1973).

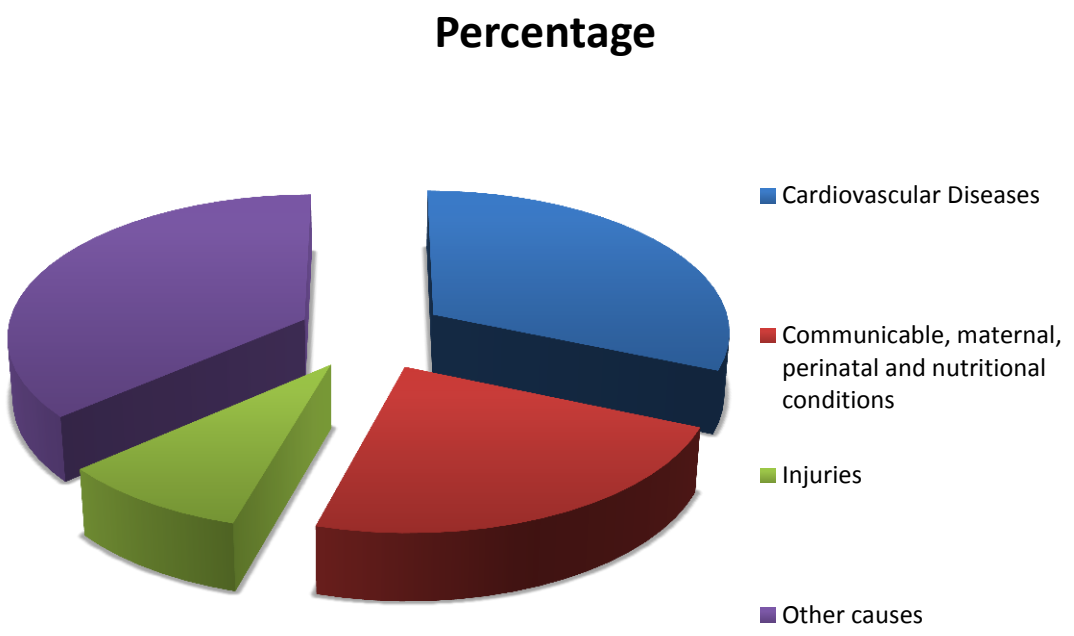


Figure 1 Distribution of major causes of death including CVD, in 2012.

CVD was a major cause of death in 2012, accounting for 31.4% of all deaths as this pie chart described. The data shown in the pie chart were retrieved from WHO published statistical results in 2014 (WHO, 2014).

As mentioned above, atherosclerosis is a progressive disease in which lipids, inflammatory cells, and vascular smooth muscle cells (VSMCs) accumulate in the neointima layer (the inner layer of the artery) of the artery and form a plaque with fibrous cap. Neointima formation is a common feature of atherosclerosis and restenosis after balloon angioplasty. The neointima is a new or thickened layer of arterial intima formed by the migration and proliferation of cells from the blood vessel media or other surrounding tissues. After an artery is injured, leukocytes and monocytes enter the intima, where they become foam cells and express scavenger receptors that internalize modified lipoproteins and form the fatty streak. The region of the intima with the fatty streak is called the neointima. A high-fat Western diet combined with other risk factors damages the lining of the artery, known as the endothelium. Endothelium has already been proved that it is not a simple lining of cells on the inner arterial wall (Galley and Webster, 2004). Healthy endothelium plays an important role in the free passage process of molecules and cells into the underlying interstitium. It is also a dynamic endocrine organ that secretes a numerous vascular protective molecules such as nitric oxide and prostaglandin. Together, they play an important role in inhibiting leukocyte adhesion and migration, platelet adhesion and aggregation and vascular smooth muscle cell proliferation and migration(Landmesser et al., 2004). However, endothelium dysfunction, resulting from smoking, hypertension, diabetes, genetic alterations, elevated plasma homocysteine concentrations and infectious microorganisms, can cause an overexpression of some cell adhesion molecules (CAMs) such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular cell adhesion molecules (ICAMs). This upregulation of CAMs is the first step of leukocyte recruitment to the site of lesion that allows more leukocytes to adhere onto the endothelium and enter the intima through a process known as diapedesis (Libby et al., 2010, Orlandi and Bennett, 2010, Libby, 2002), initiating local inflammation. The

recruited leukocytes ingest the oxidised low-density cholesterol in the intimal layer of the vascular wall, causing foam cell formation (Johnson, 2014).

Clinical manifestations occur once the atheroma has evolved beyond the formation of the fatty streak. At this point, VSMCs accumulate in the plaque, where they adopt a synthetic phenotype. They then proliferate, secrete copious amounts of extracellular matrix (Djarmati et al.), increase the size of the atherosclerotic lesion. The VSMCs in the plaque, which differ from medial VSMCs, are capable to engulf aggregated low-density-lipoprotein (LDL) that trapped in the intima. Increasing evidences suggesting that the elevated levels of LDL cholesterol in the blood leads to lipid accumulation in vascular wall (Galkina and Ley, 2009, Dushkin, 2012). Hence, intimal VSMCs contribute further to foam cell formation.

VSMCs that reside in the middle layer of the vessel, the media, have a contractile phenotype with an elongated, spindle-shaped morphology. Synthetic VSMCs alternatively have cobblestone morphology and a secretory function. The synthetic phenotype constitutes the majority of phenotypes displayed by VSMCs in embryological development. Traditionally, atherosclerotic VSMCs were thought to originate solely from VSMCs within the media layer. In recent years, this assumption has been questioned (Wang et al., 2015, Tsai et al., 2012, Hu and Xu, 2011, Bai et al., 2010, Torsney and Xu, 2011, Campagnolo et al., 2011, Kirton and Xu, 2010, Xu, 2008, Zampetaki et al., 2008, Adams et al., 2007b, Adams et al., 2007a, Xu, 2007, Xu, 2006, Roberts et al., 2005, Torsney et al., 2005).

VSMCs may directly or indirectly participate in the formation of the vascular lesions. Transplant arteriosclerotic is an example of vascular lesion that results from organ

transplantation (Xu, 2006). Xu's group has further demonstrated that VSMCs contribute to transplant arteriosclerosis partly through progenitor cell differentiation in lesions on the vessel wall. And the accelerated arteriosclerosis transplant is the major barrier to long-term survival of patients (Xu, 2006, Xu, 2008). According to the traditional view, intimal SMCs in transplant arteriosclerotic lesions were originally thought to be derived from the donor vessels rather than recipient vessel (Ross, 1986). However, recent data obtained from different laboratories demonstrated a different view. Hillebrands et al used the rat model to show that all neointimal VSMCs in both aortic and cardiac allografts were originated from the recipient (Hillebrands et al., 2001). Hu et al. also found that 40% of VSMCs in arteriosclerotic lesions were derived from recipients and 60% from the grafted vessel in mouse model (Hu et al., 2002). In contrast, transplant arteriosclerosis of cardiac allografts varies in human model and VSMCs with a low percentage were found to be recipient derived (Glaser et al., 2002). These results suggested an important role of VSMC migration and proliferation in arteriosclerosis, and without a doubt the role of VSMCs in transplant arteriosclerosis is valuable for medical researches and discussions, regardless of the origination of the VSMCs.

Circulating or vascular stem/progenitor cells have recently been identified as a source of atherosclerotic/neointima VSMCs. They are thought to derive from the arterial wall (Sainz et al., 2006, Hu et al., 2004) or the circulation (Simper et al., 2002). Torsney and colleagues confirmed the presence of vascular progenitor cells in human atherosclerotic vessels by detecting stem/progenitor cell markers such as CD34, stem cell antigen-1 (Sca-1), c-kit and VEGF receptor 2 (VEGFR2) in normal and diseased human arteries from patients undergoing coronary artery bypass surgery (Torsney et al., 2007). Moreover, for treating damaged vasculature, Scott et al. documented that stem cells have

great potential in the field of regenerative medicine, and hence could provide solutions to the problem of vascular repair and regeneration (Scott et al., 2013). Recent evidence indicates that stem/progenitor cells are abundant in the vessel wall, in which laminar shear stress can stimulate these cells to differentiate towards the endothelial lineage, while cyclic strain results in smooth muscle differentiation (Zhang et al., 2013). In samples taken from the proximal ascending aorta, progenitor cell number was two- to three-fold higher in the adventitia than in the internal mammary artery (Torsney et al., 2007). These vascular progenitors are important in atherosclerosis, as demonstrated in study by using animal models of vein grafts (Hu et al., 2002). Importantly, using the same vein graft model, the same group also demonstrated that stem/progenitor cells derived from adventitia could differentiate into SMCs *in vitro* and/or *in vivo* and contribute to atherosclerotic lesion formation and progression (Hu et al., 2004). In addition, Zhang et al. speculated that human smooth muscle progenitors in the human-circulating blood contribute to the pathogenesis of vascular diseases, and vascular stem cells are closely related to vascular repair and disease development (Zhang et al., 2013). On other hand, progenitor cells in the circulation and adventitia were confirmed that it contribute to endothelial repair and SMC accumulation (Xu, 2006).

Rapid advancements in stem/progenitor cell research in recent years have paved the way to novel therapies in cardiovascular disease, although questions remain (Sheng et al., 2013). Nevertheless, the molecular mechanisms behind stem/progenitor cell differentiation into VSMCs and the implications for CVD will undoubtedly lead to the development of new drugs for the prevention of CVD.

1.1.2 Regenerative medicine techniques in cardiovascular disease

Regenerative medicine is a rapidly developing field with the ultimate goal of repairing, replacing, or regenerating cells, tissues, or organs lost or damaged because of disease, injury, or ageing. For cardiovascular regeneration, growing evidence suggests that impaired cardiac and vascular functions resulting from CVD can be restored/improved by endogenous or exogenous stem/progenitor cells. Findings from clinical trials also demonstrate the potential capacity of adult and embryonic stem cells to regenerate damaged tissues/organs and improve their respective functions (Packer et al., 2013).

Furthermore, adult and embryonic stem cells are under intense evaluation for use in regenerative medicine because they have the potential for unlimited self-renewal; the ability to differentiate into a wide range of specialized cell types, including vascular endothelial cells (Zeng et al., 2006, Xiao et al., 2006) and smooth muscle cells (SMCs) (Xiao et al., 2007b, Xiao et al., 2012, Pepe et al., 2010, Huang et al., 2013, Yu et al., 2015); and the ability to secrete cytokines and growth factors to support endogenous tissue/organ repair (Murata et al., 2010). The exact mechanism of action behind stem and progenitor cell regeneration is still uncertain, but does not appear to occur solely through the replacement or regeneration of lost cells. Strong evidence suggests that transplanted stem cells promote endogenous cellular repair by inducing paracrine cell-to-cell signalling, such as the production of cytokines or other factors (Kinkaid et al., 2010).

Although the exact benefit of regenerative medicine to patients has yet to be determined and the translation of ongoing research into clinical treatments is some way off, no one

doubts that regenerative medicine will eventually offer new, additional, or complementary treatment options for patients with CVD.

1.2. Stem cell

1.2.1. Characteristics and classification of stem cells

Stem cells are undifferentiated cells that can differentiate into specialized cells and divide (through mitosis) to produce more stem cells. Two important properties of all stem cells are the capability of unlimited self-renewal and the capacity to differentiate into various cell lineages (also known as pluripotency) of many tissue types (Cogle et al., 2003). Although this dual functionality has been much studied, the search for molecular signatures of 'stemness' and pluripotency is only now beginning to gather momentum. Stem cells are classified into four types: two physiological types present at different stages of life, embryonic stem cells (ES cells) and adult stem cells (ASCs); one engineered or 'induced' type, induced pluripotent stem (iPS) cells; and one pathological type present in cancers with some stem properties, cancer stem cells (CSCs) (Herreros-Villanueva et al., 2014). In the past decades, the majority of stem cell studies have focused on ES cells, and the knowledge generated from ES cell studies has guided investigations of the other three types of stem cells.

The second class of stem cells is ASCs, also known as somatic stem cells (SSCs), are thought to be postnatal derivatives of ES cells present throughout the body, including the skin, brain, bone marrow, intestine, and neural tissue. At least three of the core

ES cell transcription factors (OCT4, KLF4, and SOX2) have been reported to be regulated in ASCs (Alvarez et al., 2012). ASCs are primarily involved in maintaining and repairing/regenerating specific tissues; under certain circumstances, they may contribute to disease development. The ASCs for potential use in stem cell therapy are autologous; because they are usually harvested from the patient, there are almost no immunologic barriers. However, unlike ES cells, ASCs do not fully retain their proliferative and multi-lineage differentiation capabilities in aging humans (Boyette and Tuan, 2014), meaning they only maintain the capacity to differentiate into specific cell lineages from a given germ layer based on their origin. Furthermore, the same group also proved that ASCs are limited in quantity because of their lower capacity to divide during *in vitro* culture (Boyette and Tuan, 2014). Hematopoietic stem cells (HSCs) have been used in clinical settings for years to reconstitute the immune system in cancer and other illnesses, while other ASCs have been used as gene delivery vectors to enhance tissue regeneration, destroy cancer cells, and regenerate cartilage and bone (Boyette and Tuan, 2014).

Fifty years ago, Sir John Gurdon succeeded in a ground-breaking work to generate cloned frogs by transferring the nucleus of a tadpole's somatic cell into an oocyte, thus demonstrating that cells retain genetic information and can be rejuvenated by artificial treatment to again acquire pluripotency (Gurdon, 1962). Numerous other studies of nuclear reprogramming have since been conducted. In 2006, Shinya Yamanaka’s lab first generated the induced pluripotent stem cells (iPSCs) by transducing four key transcription factors, OCT4, SOX2, c-Myc, and KLF4, which are artificially engineered into adult cells (Takahashi and Yamanaka, 2006). iPSC generation is a milestone achievement in stem cell research, not only breaking the dogma that somatic

cell differentiation is an irreversible process, but also creating new avenues in disease modelling and regenerative medicine that avoid the controversial use or destruction of embryos required for ESC generation.

The final type of stem cell, CSC, is found within tumours. CSCs can self-renew, produce differentiated progeny, and drive tumorigenesis. The ability of cancer cells to form non-adherent spheroids in *in vitro* culture is frequently used as a surrogate marker for the stemness of CSCs (Alvarez et al., 2012).

Since the first report in 1998, human ESCs have been viewed as a key component of future treatments for diseases such as Parkinson's disease and spinal cord injury (Thomson, 1998). However, clinical applications and research using human ESCs face several barriers, including ethical concerns about the use of human embryos and scientific concerns about immune rejection after transplantation, which could be overcome by iPSC technology. Since Yamanaka's group discovered iPSCs in 2006, accumulating evidence has demonstrated that iPSCs have properties similar to those of ES cells (unlimited self-renewal and the ability to differentiate into different cell types derived from the three germ layers: endoderm, mesoderm, and ectoderm), indicating that iPS cell technologies can be applied in medical science, in particular for regenerative medicine and human disease modelling (Takahashi and Yamanaka, 2006, Okano and Yamanaka, 2014).

Pluripotent stem cells have been used as a critical tool in regenerative medicine and tissue engineering for clinical cell therapies to treat devastating and incurable diseases, including type 1 diabetes, blindness, damaged articular cartilage, neurological diseases, Parkinson's disease (PD), corneal diseases, and terminal cardiovascular

diseases (Boyette and Tuan, 2014, Hayashi et al., 2012, Kikuchi et al., 2011). Furthermore, the use of iPSCs in regenerative medicine to treat other diseases, including CVD, is under investigation worldwide (Chi et al., 2015, Wong et al., 2013, Egashira et al., 2011).

One important concern for iPS cell generation is the cellular source. Ideally, human iPSCs should be obtained using minimally invasive procedures associated with the lowest possible risk. In 2007, human iPSCs were first established from dermal fibroblasts by Yamanaka's group (Takahashi et al., 2007). Thereafter, cord blood (CB) stem cells were reprogrammed to iPSCs by retroviral transduction with only two factors (OCT4 and SOX2) in 2 weeks and without the need for additional chemical compounds (Giorgetti et al., 2010). Moreover, another report has suggested that iPSCs can be generated easily from $CD34^+$ cord blood cells by repressing p53 expression through the addition of a lentiviral p53 short-hairpin RNA expression vector (Takenaka et al., 2010).

1.2.2. Embryonic stem (ES) cells

Embryogenesis is often viewed as a progressive loss of developmental capacity from a 'totipotent' zygote. However, in reality, the mammalian egg is a highly specialized and restricted cell, and its developmental potential is unlocked through the formation of epiblast cells in the inner cell mass (ICM) of the blastocyst (Selwood and Johnson, 2006).

ES cells have unlimited self-renewal capacity and pluripotency, defined as the ability to

differentiate into most cell types within an organism. Thus, ES cells hold great promise for regenerative medicine and serve as an excellent *in vitro* model system with which to study early development in mammals. Because ES cells can give rise to functional gametes, they have been used extensively to create genetically engineered lines of mice for developmental, genetic, and biomedical research (Capecchi, 2005). ES cells are pluripotent stem cells derived from the ICM of a mammalian blastocyst, an early-stage embryo approximately 3.5 days post coitum (Nichols and Smith, 2012). ES cells were first derived in 1981 by explanting blastocysts or ICMs from mice, and their pluripotency was demonstrated by their ability to contribute to the development of all tissues in mice after the injection of isolated ES cells into host blastocysts (Evans and Kaufman, 1981, Martin, 1981). Human ES cells, first isolated in 1998 by Thomson and colleagues, have the potential to form derivatives of all three embryonic germ layers (Thomson, 1998). In 2004, a new standard protocol was first established to generate human ES cells efficiently by supplementing FGF/activin into the cell culture system (Cowan et al., 2004).

Studies in experimental mammalian embryology have mainly focused on mouse embryos. Structures in the mouse embryo, including the placenta, extra-embryonic membranes, and the egg cylinder, are very similar to the corresponding structures in the human embryo. Nonetheless, human ES cells will be particularly valuable for investigating human embryonic development.

For ES cell studies, the initial challenge scientists need to overcome is maintaining the undifferentiated status of ES cells and their two major properties, self-renewal and pluripotency, during *in vitro* culture and maintenance. As mentioned earlier, mouse ES cell lines were initially obtained from the blastocysts of the Sv129 strain as round colonies on a layer of chemically arrested mouse embryonic fibroblasts (MEFs), known as a

'feeder layer' (Evans and Kaufman, 1981), indicating that MEFs might provide essential factors for maintaining undifferentiated ES cells. ES cell lines have also been established from single cells isolated following epiblast microdissection at embryonic day 4.5 (E4.5) (Brook and Gardner, 1997). Leukaemia inhibitory factor (LIF) derived from feeder layer cells is the critical factor in inhibiting ES differentiation and supporting the proliferation of undifferentiated stem cells (Williams et al., 1988). LIF is a cytokine produced by the endometrium that promotes blastocyst implantation (Stewart et al., 1992). GP130, the receptor for LIF, is expressed in ES cells and mediates ES cell self-renewal and pluripotency by phosphorylating the transcription factor STAT3 (Williams et al., 1988). Using a constitutively activated form of STAT3, Yokota's group has shown that activation of this transcription factor alone is sufficient to support ES cell self-renewal at a high density in serum-supplemented medium (Matsuda et al., 1999). Although STAT3 signalling is sufficient to support mouse ES cell self-renewal, this pathway is not responsible for the self-renewal of human ES cells (Smith, 2001, Thomson, 1998, Sato et al., 2004). Instead, Sato et al. found that activation of the Wnt pathway, mediated by 6-bromoindirubin-3′-oxime (BIO), a specific pharmacological inhibitor of glycogen synthase kinase-3 (GSK-3), can support the undifferentiated phenotype of both human and mouse ES cells and sustain the expression of some ES cell-specific transcription factors, such as Oct-3/4, Rex-1, and Nanog (Sato et al., 2004).

Evidence suggests that not only GP130, but also a class I cytokine receptor, the low-affinity LIF receptor (LIF-R), can mediate the actions of LIF via heterodimerization (Gearing et al., 1991, Gearing and Bruce, 1992, Davis et al., 1993). Utilising self-renewal and pluripotency as surrogate markers to evaluate whether ES cells remain in the undifferentiated state, studies have shown that oncostatin M (OSM), cardiotrophin (CT-1),

and ciliary neurotrophic factor (CNTF), which are LIF-related cytokines, can act through the same receptor complex (in the case of CNTF, additionally including the CNTF-Rα subunit) and similarly sustain ES cell self-renewal (Conover et al., 1993, Rose et al., 1994, Wolf et al., 1994, Yoshida et al., 1994, Pennica et al., 1995). Furthermore, ES cells can also be derived and maintained *in vitro* using a combination of interleukin-6 and soluble interleukin-6 receptor (IL-6/sIL-6R) (Nichols et al., 1994, Yoshida et al., 1994). Hitoshi et al. found that granulocyte colony-stimulating factor receptor (G-CSF-R) could signal ES cell self-renewal (Niwa et al., 1998). Surprisingly, G-CSF-R is a cytokine receptor that is evolutionarily related to GP130 and LIF-R, but is not expressed in ES cells (Gearing et al., 1991, Chambers et al., 1997).

Because pluripotency is considered one of the most important ES cell properties, it is critical to retain pluripotency by preventing differentiation. Studies over the past few years have revealed the role of some transcription factor networks and epigenetic processes in the maintenance of ES cell pluripotency; these include OCT3/4, Nanog, SOX2, Esrrb, and Klf4 (Pesce and Scholer, 2001, Pesce et al., 1998, Ambrosetti et al., 1997, Niwa et al., 2000, Mitsui et al., 2003, Boyer et al., 2005, Boyer et al., 2006, Kaji et al., 2009, Kalmar et al., 2009). In addition, a paper published in *Nature Genetics* suggested that DNA modification, histone or chromatin structure changing also can more or less affect the activities of the genes targeted by these transcription factors (Jaenisch and Bird, 2003).

Oct3/4, a member of the POU transcription factor family, is encoded by *Pou5f1* and is a central player in ES cell self-renewal and differentiation into specific lineages (Pardo et al., 2010, Nichols et al., 1998). It has been reported that Oct3/4 can directly inhibit ES cell differentiation towards the trophectoderm by interacting with Cdx2, an inducer of trophectoderm differentiation, to form a repressor complex (Niwa et al., 2005). Sox2 is

widely believed to cooperate with Oct3/4 in activating Oct3/4 target genes (Yuan et al., 1995). Nevertheless, OCT3/4 can associate with SOX2 to form a complex that suppresses the expression of Cdx2 (Niwa et al., 2005). Interestingly, Oct3/4 and Sox2 are transcriptionally regulated by an enhancer containing a composite element recognized by Oct4 and Sox2 (Chew et al., 2005, Okumura-Nakanishi et al., 2005). Nanog, the variant homeodomain-containing protein, which was isolated in a functional cDNA screen in ES cells, cooperates with STAT3 to drive ES cell self-renewal (Chambers et al., 2003). As mentioned earlier, three essential transcription factors, OCT3/4, SOX2, and Nanog, have essential roles in early development and are required for the propagation of undifferentiated ES cells in culture. In this respect, some studies have provided new evidence to support that OCT3/4, Nanog, and SOX2 co-regulate stem cell pluripotency by co-occupying a substantial portion of their target genes, including *STAT3*, *HESX1*, *FGF-2*, and *TCF* (Pan and Thomson, 2007, Boyer et al., 2005, Chew et al., 2005, Pan et al., 2006). Estrogen-related-receptor beta (Esrrb) has also been found to coordinate with two master regulators of the ES cell genetic program, Oct4 and Nanog, by binding to the proximal 5′-untranslated region of the *Oct4* gene (van den Berg et al., 2008, Zhang et al., 2008). Kruppel-like factor 4 (*KLF4*) has been identified as a direct target of STAT3. Importantly, regulation of *KLF4* by either LIF or Stat3 supports LIF-independent ES cell self-renewal (Hall et al., 2009, Niwa et al., 2009). KLF4 can also bind to the promoters of Oct3/4 target genes, such as left-right determination factor 1 (Lefty1) which is a related members of the TGF-β family of growth factors (Meno et al., 1996), and regulate ES cell pluripotency in co-operation with Oct3/4 and Sox2 (Nakatake et al., 2006).

1.2.3. ES cell differentiation

The potential to differentiate into multiple cell lineages is the main feature of ES cells. ES cells can differentiate into any kind of cell under the appropriate culture conditions after removal of LIF and/or basic fibroblast growth factor. To date, three major culture conditions have been used for *in vitro* ES cell differentiation: embryoid body (EB) formation, which mimics *in vivo* embryonic development by culturing ES cells in a suspension condition for more than 14 days and allowing ES cells to differentiate into multiple cell lineages and form a very compact cellular aggregate in floating conditions; a feeder cell co-culture method in which feeder cells provide ES cells an intimate cell contact and secrete factor(s) that promote ES cell proliferation and differentiation; and an extracellular matrix-coated condition that supports the differentiation of ES cells.

EBs, three-dimensional cell structures formed in suspension culture by ES cells, comprise cells from three embryonic germ layers (Thomson, 1998, Doetschman et al., 1985). EB formation used a number of approaches, such as bacterial-grade dish, methylcellulose-coated plate, low-adherent micro-well plate and the hanging drops method.

The first supporting evidence for a feeder-cell culture approach was reported in 1977. Bone marrow-derived adherent cells were able to support the prolonged proliferation and differentiation of genetically incompatible stem cells and precursor cells (Dexter et al., 1977). Adherent growth-arrested but viable and bioactive cells (primary cells or immobilized cell lines) have typically been used as feeder layer cells. Specifically, Thomson's paper reported that ES cells attached to a mouse embryonic fibroblast feeder layer displayed a continuously undifferentiated status, suggesting that the feeder layer cells provided an excellent environment for ES cell proliferation and long-term

maintenance *in vitro* (Thomson, 1998). Moreover, OP9 stromal cells, derived from new-born bone marrow of the mouse calvaria model, can induce ES cell differentiation toward blood cells of the erythroid, myeloid, and B cell lineages (Nakano et al., 1994). In addition to OP9 co-cultures, stromal cells from the aorta-gonad-mesonephros (AGM) region have also been used to support the haematopoiesis of mouse and human ES cells, suggesting that AGM stromal cells are involved in early stem cell development and hematopoietic differentiation *(Weisel et al., 2006)*. Such a notion was further supported by the finding that co-culturing GM stromal cells with mouse ES cells induces ES cell differentiation into Mac-1^+ myeloid cells and $B220^+$ B cells (Lim et al., 2013).

Found within mammalian tissues, the extracellular matrix (Djarmati et al.) is a complex structural entity that surrounds and supports cells (Tien and Nelson, 2014). The ECM regulates various cellular processes, including cell differentiation (Adams and Watt, 1993, Badylak, 2005), growth, adhesion, proliferation, morphology, and gene expression (Kleinman et al., 2003). In recent years, a considerable effort has been put into the research to study how ECM components regulate stem cell behaviours; increasing evidence suggests that the ECM complex can induce differentiation from ES cells into SMCs and endothelial cells (Luo et al., 2013, Xiao et al., 2006, Xiao et al., 2007a). Many ECM molecules, the respective receptors, and their functional significance in stem cell biology have been characterized by different groups. One of the major challenges in ECM research is establishing the specific differentiation conditions appropriate for different cell types. Another challenge in ECM research is unravelling the signalling pathways through which the ECM regulates cell-lineage–specific gene transcription.

1.3. Smooth muscle cell (SMC) differentiation

1.3.1. Smooth muscle cells

The accumulation of SMCs in the intima plays a crucial role in the pathogenesis of vascular lesions, including atherosclerosis and restenosis (Yoshida and Owens, 2005). Therefore, understanding the cellular and molecular mechanisms that underlie VSMC differentiation is essential for developing new approaches to the prevention and treatment of these diseases.

VSMCs originate from at least seven different progenitor sources during embryonic development: the neural crest, proepicardium, serosal mesothelium, secondary heart field, somite, mesoangioblast, and stem/progenitor cells (Majesky, 2007). Importantly, SMCs with different embryological origins display a distinct cellular function. Moreover, unlike other terminally differentiated somatic cells, SMCs can undergo phenotypic changes *in vitro* and *in vivo*, switching between secretory and contractile phenotypes, thus obscuring our conceptual understanding of terminal differentiation in these cells (Majesky, 2007, Yoshida and Owens, 2005).

1.3.2. SMC differentiation from stem cells and the mechanisms involved

Accumulating evidence indicates that vascular stem/progenitor cells play a major role in various cardiovascular diseases, including atherosclerosis and angioplasty restenosis (Adams et al., 2007a). SMC differentiation is a critical process during cardiovascular system formation and development, and SMC proliferation is related to cardiovascular disease, as in atherosclerosis. Understanding the transcriptional regulatory circuitry of SMC differentiation from stem cells is fundamental to understanding human cardiovascular system development and realizing the therapeutic potential of these cells (Xiao et al., 2010, Kane et al., 2011). To understand the mechanism of SMC differentiation from ES cells, researchers have established several *in vitro* differentiation strategies to induce SMC differentiation from stem cells (Xie et al., 2007, Xie et al., 2011b, Xiao et al., 2007a).

The first reported method for SMC differentiation used P19 cells, an embryonic carcinoma cell line derived from an embryo-derived teratocarcinoma in mice. The P19 cell line has properties similar to those of normal ES cells, including unlimited self-renewal ability and pluripotency (Rideg et al., 1994). P19 cells can differentiate into fibroblast-like cells that express high levels of smooth muscle actin protein in the presence of either 5×10^{7} M retinoic acid (RA) for 2 days or 1% DMSO for 4 days (Rudnicki et al., 1990). Furthermore, Owens's group reported that P19 cells treated for 48 h with 1×10^{6} M RA exhibited a profound change in cell morphology and expressed a high level of smooth muscle α-actin. Additionally, in the same study, the authors

observed that a clonal line derived from RA-treated P19 cells stably expressed multiple SMC specific markers, such as SMαA and SM-MHC (Blank et al., 1995).

To improve the efficiency of SMC differentiation from P19 cells, A404 cells, a P19-derived clonal cell line with an actin alpha 2 (ACTA2) promoter/intron-driven puromycin resistance gene, has been used (Manabe and Owens, 2001). In cells incubated with all-trans RA (atRA) for two days and screened for 5 days with puromycin, more than 90% of the differentiating A404 cells expressed ACTA2, calponin (CNN1), and/or smooth muscle myosin heavy chain (SM-MHC), suggesting that most of the cells were SMCs (Manabe and Owens, 2001, Spin et al., 2004).

Unsurprisingly, a similar method has been successfully applied to SMC differentiation from ES cells. Undifferentiated ES cells were induced to differentiate into EBs for 6 days and then incubated with 1×10^{-8} mol/L atRA and 0.5×10^{-3} mol/L dibutyryl-cAMP (db-cAMP) for 4 days. Data showed that ~67% of the cells within EBs were SMC-like cells, compared to 10% in untreated EBs (Drab et al., 1997, Sinha et al., 2004). Via a similar strategy, SMCs were also successfully derived from human ES cells. $CD34^+$ cells, intermediate vascular stem/progenitor cells, were first isolated from differentiating human ES cells and then induced to differentiate into SMCs by platelet-derived growth factor-BB (PDGF-BB). However, it is noteworthy to mention that apart from the SMC genes, the differentiated SMC-like cells also expressed the endothelial markers angiopoietin-2 and Tie2, indicating that the SMC differentiation process was incomplete (Ferreira et al., 2007, Xie et al., 2007).

To increase SMC differentiation efficiency and simplify the SMC differentiation protocol, our group has established a two-dimensional (2D) *in vitro* SMC differentiation model

from ES cells using type IV collagen as a cellular base membrane (Xiao et al., 2007a). Applying this simple but efficient SMC differentiation model, our group has identified and isolated an intermediate vascular stem/progenitor cell population, which are positive for stem cell antigen-1–positive cells (Sca-1^+) from day 3–4 differentiating ES cells. Such vascular stem/progenitor cells can be induced to differentiate into both functional SMCs (Xiao et al., 2007b) and endothelial cells (Xiao et al., 2006, Zeng et al., 2006) by PDGF-BB and VEGF/shear stress, respectively. A highly purified SMC population (more than 95% of cells express high levels of various SMC markers) can be derived from Sca-1^+ cells by culturing them on collagen IV pre-coated flasks with SMC differentiation medium for several passages (Xiao et al., 2007b). Interestingly, another cell population, VEGFR2+ progenitor cells, has also been identified and isolated from the differentiating ES cells using similar methods (Yamashita et al., 2000, Sone et al., 2003), providing another vascular stem/progenitor cell population with the potential to differentiate into SMCs and endothelial cells.

Importantly, with our SMC differentiation model, our group has uncovered a fairly complicated SMC differentiation regulatory network comprising integrin/receptor pathways, redox signalling molecules, transcription factors, nuclear/chromatin proteins, histone deacetylases (HDACs), and microRNAs. Specifically, Xiao's group has demonstrated for the first time that collagen IV plays a crucial role in the early stage of SMC differentiation and that integrin (α1, β1, and αv)-FAK/ paxillin -PI 3-kinase-mitogen-activated protein kinase and PDGF receptor-beta signalling pathways are involved in SMC differentiation (Xiao et al., 2007b). Xiao and his colleagues were the first to report that the differentiation of mouse ES cells towards the SMC lineage is mediated by Nox4-produced H_2O_2 (Xiao et al., 2009). The group also showed that

PDGF-BB promotes the differentiation of ES cells towards the SMC lineage via the upregulation of histone deacetylase 7 (HDAC7) transcription/splicing (Margariti et al., 2009) and that transcription factor Sp1 is required for PDGF-BB-regulated HDAC7 activation (Zhang et al., 2010b). Moreover, Xiao's group was the first to report that reactive oxygen species (ROS) balance and transcription factor nuclear factor erythroid 2-related factor 3 (Nrf3) play a crucial role in SMC differentiation from stem cells (Pepe et al., 2010) and that the fine interactions between Nrf3 and another ROS mediator, phospholipase A2, group 7 (Pla2g7), are essential for SMC lineage specification from stem/progenitor cells *in vitro* and *in vivo* (Xiao et al., 2012). Using a nuclear proteomics approach, Xiao's group has further defined important roles for three nuclear/chromatin proteins, chromo box protein homolog 3 (Cbx3), heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNPA2B1), and hnRNPA1, in SMC differentiation from stem/progenitor cells and in chick embryonic arteriogenesis (Wang et al., 2012, Xiao et al., 2011, Huang et al., 2013). Xiao's group has also investigated the functional implications of microRNAs in SMC differentiation from stem cells (Yu et al., 2015, Zhao et al., 2015), as discussed in detail in the next section.

Other researchers have identified other signalling molecules and pathways involved in VSMC differentiation, proliferation, and development, including Notch, Wnt, Akt/FoxO/Myocd, RhoA/ROCK, ERK 1/2, p38MAPKα, and the G protein-mediated pathway (Chang et al., 2012, Marinou et al., 2012, Althoff et al., 2012, Zhang et al., 2012b, Long et al., 2013, Pagiatakis et al., 2012, Yang et al., 2013b). Although these studies have significantly increased our understanding of the complicated process of SMC differentiation, the full molecular mechanisms underlying SMC differentiation from stem cells remain to be elucidated.

1.4. MicroRNA

1.4.1. MiRNA and its biogenesis

MicroRNAs (miRNAs) are endogenous, highly conserved, short, non-coding, 22-nucleotide RNAs that constitute a novel class of gene expression regulators with important roles in various aspects of development, homeostasis, and disease (Wightman et al., 1991, Wightman et al., 1993, Vasudevan et al., 2007, Vasudevan et al., 2008). The noncoding miRNAs were initially discovered over 30 years ago in the nematode *Caenorhabditis elegans* as a pair of RNAs derived from the *Lin-4* gene that specifically bound to a partially complementary target in the 3′-untranslated region (UTR) of Lin-14 mRNA (Ambros and Horvitz, 1984). Subsequent findings showed that the amount of LIN-14 protein was reduced by Lin-4 miRNA without a noticeable change in the level of Lin-14 mRNA (Wightman et al., 1991). Since the let-7 miRNA was found, mature miRNA are named using the prefix 'miR' followed by a unique identifying number (e.g. miRNA-34). An additional lower case letter is added after the number if the same miRNA precursor gives rise to miRNAs with different nucleotide sequences (e.g. miRNA-34a, miRNA-34b, and miRNA-34c). Perron's and Provost's group has suggested that up to 90% of human genes are regulated by miRNAs (Perron and Provost, 2008). As of July 2014, approximately 35,828 mature miRNAs from 223 species (including 2588 miRNAs from *Homo sapiens* and 1915 from *Mus muscles*) had been reported and included in 'miRBase: the microRNA database' (http://www.mirbase.org/), which includes published microRNA sequences with associated annotations and provides a gene naming and nomenclature function in the miRBase Registry. Growing evidence suggests that miRNAs are involved in critical developmental and cellular processes, including cell cycle, cell

survival, apoptosis, migration, and differentiation (Zhao and Srivastava, 2007, Fazi and Nervi, 2008).

As shown in **Figure 2**, the primary miRNA (pri-miRNA), which has a stem-loop structure containing the mature miRNA sequence, is first transcribed in the nucleus from the miRNA gene by ribonuclease III (RNase III) (Winter et al., 2009). The Drosha and DGCR8/Pasha complex, also called the microprocessor complex, cleaves the stem-loop of the pri-miRNA and generates the miRNA precursor (pre-miRNA), a small hairpin-shaped RNA of approximately 65 nucleotides. Thereafter, the pre-miRNA is exported to the cytoplasm in a Ran-GTP/Exportin-5-dependent mechanism (**Figure 2)** (Winter et al., 2009). In the cytoplasm, the pre-miRNA is processed by another RNase III enzyme, Dicer, an endonuclease of ~200 kDa specific for dsRNAs that plays an important role in controlling development and metabolism. Dicer cleaves the stem-loop structure and generates the mature microRNA (about 20–22 nucleotides) as part of a short RNA duplex (Fu et al., 2015b). Like Drosha, Dicer associates with an RNA-binding protein, the double-stranded RNA-binding domain protein called Tar RNA binding protein (TRBP) in humans, to stabilize and enhance the cleavage of the pre-miRNA (Chendrimada et al., 2005). Yuanshuai et al. reported that Dicer plays diverse roles in development and metabolism in various tissues and that Dicer expression is higher in the brain than in other tissues examined (Fu et al., 2015a, Bernstein et al., 2001, Hammond, 2015).

The double-stranded miRNA duplex is separated into two strands by a helicases complex [e.g. p68, p72, RNA helicase A (RHA), RCK/p54, TNRC6B, Gemin3/4, and human Mov10 or its *D. melanogaster* orthologue Armitage] (Winter et al., 2009). The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs

through mRNA cleavage, translational repression, or deadenylation. The passenger strand (star strand) is degraded (Winter et al., 2009). Normally, the functional strand of the mature miRNA is fully incorporated with active RISC and participates in gene regulation. However, studies suggest that the star strand can also be loaded into RISC to regulate target gene expression (Ghildiyal et al., 2010, Okamura et al., 2009). Although multiple studies have been conducted, the exact mechanism by which the miRNA strand is selected from the double stranded duplex is still not clear.

Traditionally, mature miRNAs have been thought to suppress gene expression by inducing mRNA cleavage (Yekta et al., 2004) or mRNA decay (Giraldez et al., 2006, Wu et al., 2006) or by inhibiting mRNA translation (Filipowicz et al., 2008) by targeting/binding a specific RNA sequence within the 3′-UTR. However, recent evidence suggests that miRNAs can also target the 5′-UTR or coding region of their target genes (Tay et al., 2008). Moreover, in some cases, miRNAs can upregulate gene expression (Vasudevan et al., 2007).

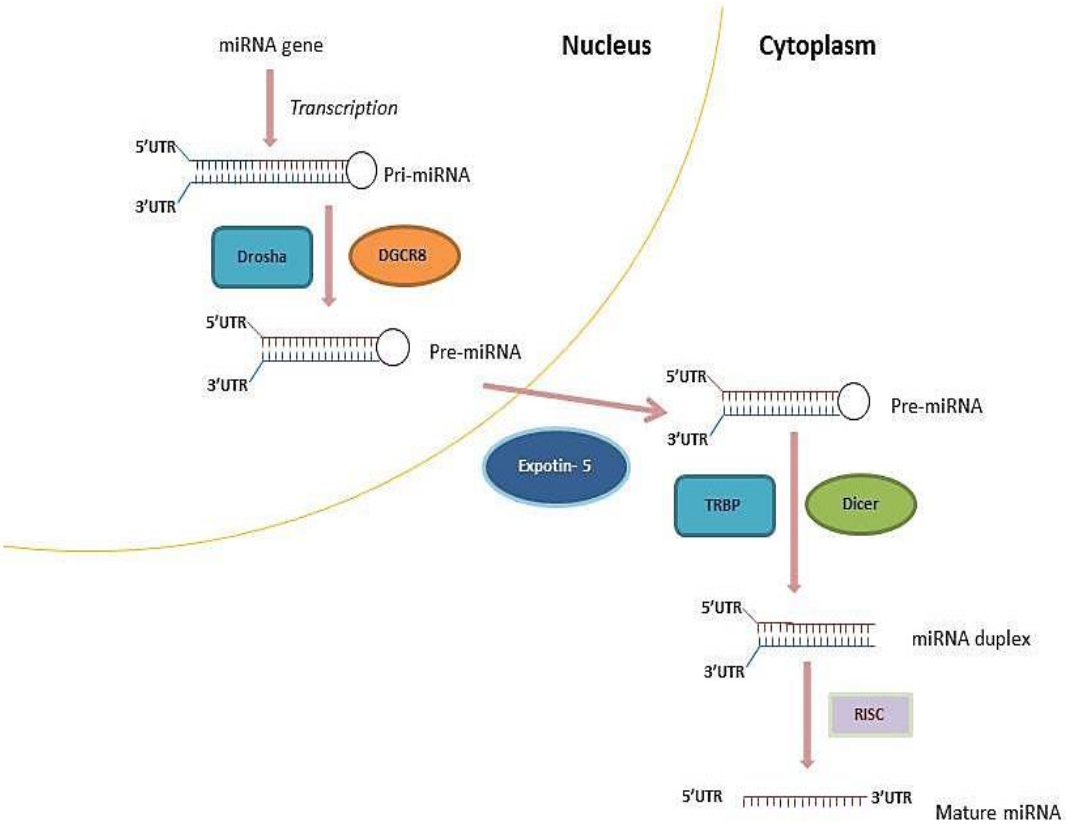


Figure 2 MiRNA biogenesis pathway.

The general miRNA biogenesis pathway begins with primary miRNA (pri-miRNA) transcription by RNA polymerase II. The pri-miRNA is converted to pre-miRNA by the Drosha and DGCR8 complex. After export from nucleus, the stem-loop structure of the pre-miRNA is cleaved by Dicer and its partners TRBP/PACT complex to form the double-stranded miRNA duplex (Winter et al., 2009).

1.4.2. MiRNAs and their targets

Defining miRNA target genes is a critical step in all miRNA studies because it is key to understanding the biological role of the miRNA. Moreover, validated targets provide the best biomarker(s) for determining the efficacy of an miRNA mimic or inhibitor in clinical miRNA therapeutics. Several general approaches were used in this PhD project to predict and identify miRNA targets: bioinformatics target prediction, miRNA/mRNA interactions, and the influence of miRNA on a target protein translation (Thomson et al., 2011, Kuhn et al., 2008).

Using bioinformatics tools for predicting target genes of miRNAs is a relatively straightforward and commonly used approach through pairing the miRNAs with target mRNAs using standard Watson-Crick rules. Generally, the most important determinant for target gene binding is the seed sequence of the miRNA, which is normally the first 6-8 nucleotides at 5-terminus of miRNAs. This will lead to a great number of candidate targets being predicted, many of which are false positives. Therefore, all bioinformatics target prediction algorithms also use additional factors to improve accuracy of target gene prediction (Hammond, 2015, Ekimler and Sahin, 2014). The most popular target prediction algorithms in miRNA research are TargetScan, miRanda and PicTar (Kuhn et al., 2008).

Gene-specific experimental validation of miRNAs is a series of methods commonly used to verify individual miRNA/mRNA interactions using the well-established techniques of qRT-PCR, luciferase reporter assays, and western blot (Thomson et al., 2011). Generally, the upregulation/downregulation of target genes by a given miRNA is observed at the protein or mRNA level using western blot or qRT-PCR, respectively. Luciferase reporter

assays have been employed extensively to demonstrate a direct link whereby expression of a 3′-UTR luciferase reporter is altered through manipulation of a regulatory miRNA. MiRNA binding site mutation is also applied to provide direct evidence that the miRNA binding site(s) are required for target gene regulation by miRNAs. The reporter assay is a rapid and reproducible assay that quickly eliminates any predicted miRNA binding site that is not functional. In contrast, the disadvantages of reporter assays are that they are labour intensive, dependent on the region selected for cloning, and sensitive to variations in protocol (Lytle et al., 2007, Kong et al., 2008, Hendrickson et al., 2009).

Several new technologies and methods have also been used to study potential miRNA targets, including biochemical isolation of miRNA/mRNA complexes and proteomic analysis (Hammond, 2015). Biochemical target identification is a method used to isolate and identify the mRNA targets of miRNAs through the association of miRNA/RISC complexes. RISC is immunoprecipitated using anti-Argonaute antibodies, with or without prior RNA crosslinking, and bound target RNAs are identified through microarray analysis or next-generation sequencing. Quantitative proteomic analysis is used to directly detect the effect of a miRNA on target protein production; it is possibly more reflective of the true target set, but is also technically challenging (Ekimler and Sahin, 2014).

As mentioned earlier, miRNAs mainly target homologous sites in 3′-UTR to suppress target gene translation and/or degrade target mRNA through a mechanism known as RNA interference (Liu et al., 2012). However, evidence also indicates that miRNAs can positively regulate gene expression. In 2007, Vasudevan et al. were the first to report that miRNAs directly associate with Argonaute (AGO) and fragile X mental

retardation-related protein 1 (FXR1) and interact with AU-rich elements (AREs) within the 3′-UTR of target genes to activate target gene translation in HEK293 cells. Importantly, miR-369-3 regulated translational activity only under cell cycle arrest conditions, while traditional translational repression was observed when the cells were switched back to the proliferative state (Vasudevan et al., 2007).

1.4.3. MiRNAs, SMC differentiation, and cardiovascular development

Tuschl's group first reported that many miRNAs are expressed in a tissue-specific manner, suggesting that certain miRNAs are important for cell/tissue specification (Landgraf et al., 2007). The contribution of miRNAs to ES cell differentiation was confirmed in later studies using ES cells deficient in Dicer and Drosha, the two enzymes important for miRNA biogenesis. Murchison and colleagues reported that Dicer-deficient mice died at embryonic day 7.5 with an undetectable level of multipotent stem cells, decreased expression of mature microRNAs, and a defect in gene silencing (Murchison et al., 2005). Importantly, the authors observed that Dicer deficiency compromised the proliferation of ES cells, suggestion that miRNAs affect ES cell proliferation (Murchison et al., 2005). Other studies using Dicer- or Drosha-deficient ES cells also suggest that miRNAs play a role in ES cell self-renewal and differentiation (Wang et al., 2007, Oberdoerffer et al., 2005). Accordingly, deletion of Dicer in SMCs results in widespread loss of miRNA expression in SMCs and contractile function in resistance arteries (Albinsson et al., 2011). In another study, disruption of Drosha in VSMCs of mice not only led to the

dysregulation of miRNA expression, but also resulted in embryonic lethality and a reduction in cell proliferation (Fan et al., 2013), suggesting a functional role of miRNAs in VSMC functions and development. An essential role of miRNAs in cardiovascular development was also demonstrated in a study of Dicer-deficient mice; the loss of miRNAs severely impaired heart and blood vessel development (Yang et al., 2005). Furthermore, it has been shown that deletion of Dicer in vascular smooth muscle caused late embryonic lethality at embryonic day 16 to 17 owing to decreased SMC proliferation and differentiation, which resulted in thinner vessel walls, impaired contractility, and haemorrhage (Albinsson et al., 2010). Taken together, these findings clearly suggest that miRNAs play an important role in SMC function and cardiovascular development.

A detailed understanding of the involvement of any given miRNA in SMC function and cardiovascular development remains to be established through the study of the functional importance of individual miRNAs in these processes. In this regard, growing evidence suggests that several miRNAs (e.g. miR-1, miR-10a, miR-34a, miR-133, miR-638, miR-143, miR-145, miR-206, miR-221, miR-222, and miR-223) are involved in SMC proliferation, phenotype switching, differentiation, actin remodelling, and migration *in vitro* or *in vivo*. Specifically, it is well established that miR-221 and miR-222 levels, assessed via microarray analysis, are upregulated in vascular walls with neointimal lesion formation (Ji et al., 2007). Davis and colleagues showed that miRNA-221 modulates VSMC proliferation and phenotype switching in response to PDGF signalling, leading to downregulation of targets, a tyrosine kinase receptor for the peptide growth factor (c-Kit) and a tumour suppressor (p27Kip1) (Davis et al., 2009). In addition, miRNA-222 was also found to be involved in VSMC proliferation and neointimal hyperplasia by targeting p27 (Kip1) and p57 (Kip2) (Liu et al., 2009). Moreover, Chen's group uncovered at first time

that miRNA-10 regulates SMC differentiation from ES cells by targeting histone deacetylase 4 (HDAC4). The authors identified NF-kappaB (NF-κB) signalling as a requirement for miR-10 activation by RA during SMC differentiation (Huang et al., 2010). The study, published by Davis and colleagues, demonstrated that TGF-β promotes VSMC differentiation by increasing the expression of miRNA-21 and miRNA-199a, that TGF-β modulates miRNA-21 by promoting the processing of pri-miRNA-21 into pre-miRNA-21 by the Drosha complex, and that programmed cell death 4 (PDCD4) is the target gene of miRNA-21 during VSMC differentiation (Davis et al., 2008). Moreover, clear evidence suggests that the mouse miR-143/145 cluster is involved in neointimal lesions and the contractile phenotype of VSMCs *in vivo* (Boettger et al., 2009) and that miRNA-145 modulates VSMC differentiation and proliferation, alone or in combination with miRNA-143 *in vitro* (Rangrez et al., 2011, Cheng et al., 2009, Cordes et al., 2009). Additionally, Yamaguchi et al. showed that miRNA-145 regulates the differentiation and proliferation of SMCs derived from human ES cells by directly targeting Kruppel-like factor 4 and 5 (KLF4 and 5) (Yamaguchi et al., 2011). Studies from other groups have also demonstrated that miRNA-143/145 acts as an integral component of the regulatory network controlling SMC cytoskeletal remodelling and phenotypic switching during vascular disease (Rangrez et al., 2011). Furthermore, miRNA-143/145 is implicated in impaired VSMC differentiation in an animal model (Norata et al., 2012).

Results presented by Torella et al. showed that miR-133 is a key regulator of vascular smooth muscle cell phenotypic switching in both *in vitro* and *in vivo*, suggesting a potential therapeutic application for vascular diseases (Torella et al., 2011). Using a highly efficient *in vitro* model of retinoid acid (RA)-induced ESC/SMC differentiation, Chen's group showed that miRNA-1 promotes SMC differentiation from ES cells through the

inhibition of KLF4 (Xie et al., 2011a). In 2012, Metzinger's group reported that overexpression of miRNA-223 increased VSMC proliferation and markedly enhanced VSMC migration. As a complement to the *in vitro* findings, the authors also observed vascular calcification in ApoE knockout mice, with significant downregulation of miR-143 and miR-145 and upregulation of miR-223 in samples (Rangrez et al., 2012). The results suggest that altered expression of miR-223 plays a part in VSMC migration and calcification in *in vivo* and *in vitro*.

Recently, a role for miRNA-206 as a regulator of pulmonary arterial SMC proliferation, apoptosis, and differentiation was suggested (Jalali et al., 2012). Most recently, miR-34a was identified by our group as another important miRNA involved in SMC differentiation from stem cells *in vivo* and *in vitro*. Importantly, we provided compelling evidence to demonstrate that miR-34a mediates SMC differentiation by upregulating its target gene *SirT1*, and that *SirT1* is a transcriptional activator of SMC differentiation genes (Yu et al., 2015).

Despite enormous efforts, the significance and exact role of other individual miRNAs in SMC differentiation and cardiovascular system development are incompletely understood. Further investigation is required.

1.4.4. MiRNA-22 and its regulatory roles

1.4.4.1. Role in cell cycle regulation and cancers

MiRNA-22 is a highly evolutionarily conserved microRNA expressed in various primary

tissues and cell lines (Landgraf et al., 2007). MYC, the human homologue of a retroviral oncogene, has a well-established role in regulating cell cycle progression and cell survival (Meyer and Penn, 2008). MiRNA-22 has been suggested to function as a tumour suppressor by modulating cell cycle arrest through 1) the direct repression of MYC binding protein (MYCBP) expression and subsequent reduction of oncogenic c-Myc activity (Xiong et al., 2010); 2) the direct suppression of EVI-1 oncogene expression (Patel et al., 2010); and 3) the direct reduction of estrogen receptor alpha (ERα) level (Pandey and Picard, 2009).

Moreover, miRNA-22 potently inhibits cancer cell proliferation and growth by targeting the c-Myc binding partner Max. MiRNA-22 causes cell cycle arrest in the G1 phase and reduces the amount of Max available for c-Myc binding, thereby altering the transcriptional output of the Myc-Max complex (Ting et al., 2010). Furthermore, miRNA-22 negatively regulates cell proliferation and increases cell cycle length in cerebellar granular neuronal precursors by reducing Max protein level and N-Myc/Max-dependent promoter activity (Berenguer et al., 2013). Guo et al. have suggested that miRNA-22 acts as tumour suppressor by targeting a specific target site within the 3′-UTR of the *Sp1* gene and inhibiting gastric cancer cell migration and invasion (Guo et al., 2013). MiRNA-22 was also found to suppress gastric cancer cell growth, proliferation, and invasion, in part by inhibiting cluster of differentiation 151 (CD151) (Wang et al., 2014). Concomitantly, miR-22 associated with miR-200C can target the proto-oncogene protein Wnt-1 to inhibit gastric cancer growth, suggesting miRNA-200C and miRNA-22 as potential gene therapy targets for gastric cancer (Tang et al., 2013). Additionally, another study reported that knockdown of miRNA-22 was significantly associated with the malignant development of gastric cancer and that this microRNA functioned as an

independent prognostic factor for patients, hinting that miRNA-22 might have therapeutic potential for patients with gastric cancer (Wang et al., 2013).

Phosphatase and tensin homolog (PTEN), another well-known tumour suppressor that modulates cell cycle progression and cell survival in many cell types, is repressed by miRNA-22, which directly targets its 3′-UTR, resulting in hyperactivation of AKT and transformation of bronchial epithelial cells (Poliseno et al., 2010, Bar and Dikstein, 2010). Notably, miRNA-22 itself can be upregulated by AKT, suggesting that miRNA-22 forms a feed-forward circuit with AKT in its regulation.

MiRNA-22 has also been implicated in a number of diseases, such as lung cancer and colorectal cancer (Ling et al., 2012, Zhang et al., 2012a, Xu et al., 2011). Moreover, miRNA-22 has been suggested to represent a novel predictive biomarker for pemetrexed-based treatment of non-small cell lung cancer (Franchina et al., 2014).

1.4.4.2. Other functions

In addition to decreasing cell motility and inhibiting cell invasion *in vitro*, miRNA-22 also restores the cellular senescence program in cancer cells and acts as a tumour suppressor by directly targeting three genes involved in the senescence programme, CDK6, SIRT1, and Sp1 (Xu et al., 2011). A recent study found that miRNA-22 modulated the survival and longevity of dendritic cells through the YWHAZ signalling pathway (Min et al., 2013)). Furthermore, miRNA-22 has also been described as a regulator of cardiac protection. In one study, exosomes enriched with miR-22 and

secreted from mesenchymal stem cells played a protective role in cardiomyocytes after myocardial infarction (Feng et al., 2014). Finally, a study reported in 2013 suggests that miR-22 plays a role in cardiac ageing, cardiomyocyte hypertrophy, or cardiac decompensation by regulating the target gene mimecan (osteoglycin, OGN) (Jazbutyte et al., 2013).

1.5. Methyl CpG binding protein 2 (MECP2)

1.5.1. Gene regulation by MECP2

DNA methylation is a major determinant in the epigenetic silencing of genes. Post-translational modification of core histones serves to lay down epigenetic states at the level of the genetic blueprint (DNA), whereas nucleosome positioning and non-coding RNAs (ncRNAs) facilitate the formation of epigenetic states (Du et al., 2015). MECP2 is the founding member of a family of methyl CpG-binding proteins that contain a methyl-CpG-binding domain (MBD). The main reported function of these proteins is to silence gene expression by preventing the binding of activating transcription factors or by recruiting enzymes that catalyse histone post-translational modifications into chromatin-remodelling complexes that in turn alter the structure of chromatin and actively promote transcriptional repression (Miranda and Jones, 2007, Cohen et al., 2008). However, studies suggest that that MeCP2 plays a complex role, coordinating either transcriptional repression or activation, depending on the molecular context (**Figure 3**) (Cohen et al., 2008, Chahrour et al., 2008).

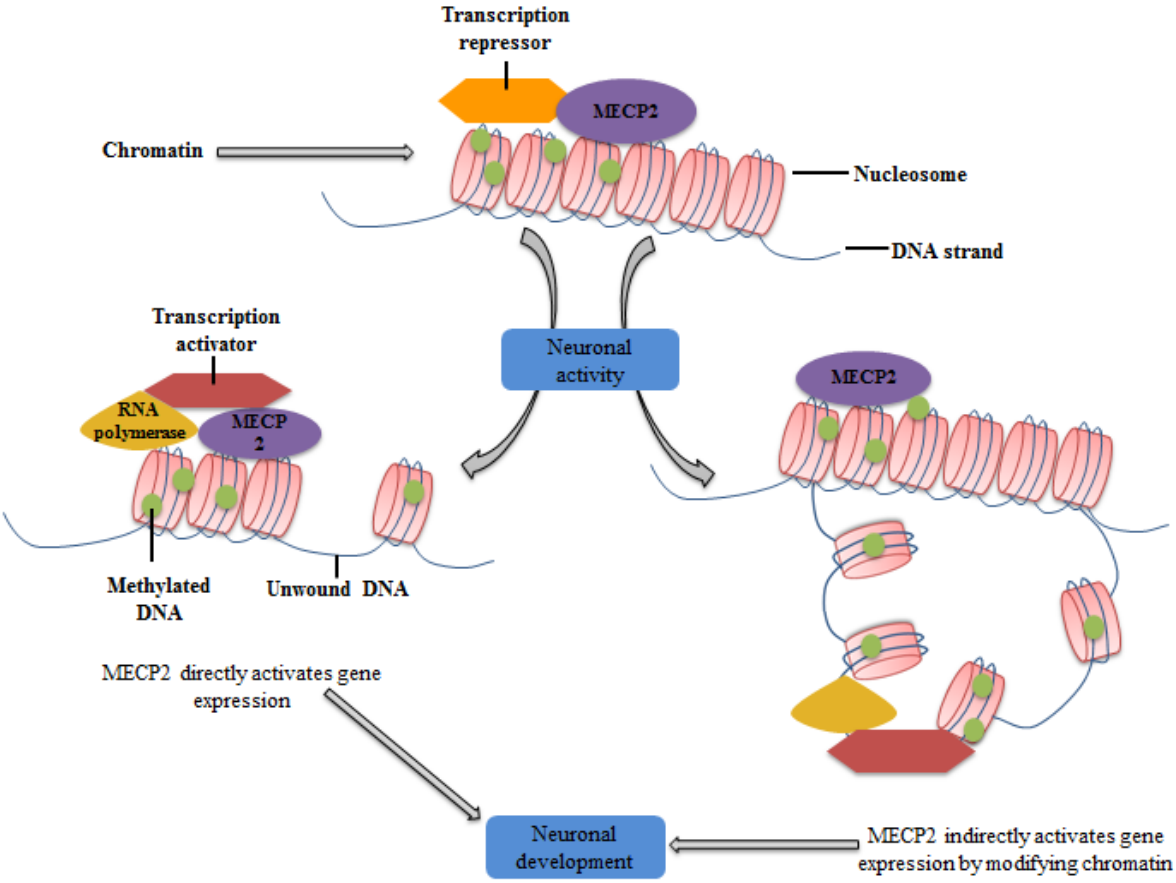


Figure 3 Gene activation or repression by MECP2

MECP2 can function as an activator or a repressor to regulate the expression of various genes. The functions of MECP2 are dependent on the DNA methylation status of the relevant genomic region (Cohen et al., 2008, Chahrour et al., 2008).

1.5.2. MECP2 and human diseases

Genetic mutations of MECP2 have been found in a numerous human diseases. One well-documented disease is Rett syndrome (Coughlan et al.), an X-linked disorder that affects the development of the brain. It can cause severe physical and mental disability in children and lead to severe mental retardation in females (Pohodich and Zoghbi, 2015). Mutations in the X-linked gene encoding MECP2 are the primary genetic causes of RTT. New evidence from animal studies suggests that, apart from genetic mutations, various post-translational modifications, such as phosphorylation, regulate the function of MECP2 in learning and memory, drug addiction, depression-like behaviour, and the response to anti-depressant treatment (Yang and Pan, 2014, Zimmermann et al., 2015). Genetic evidence to support the causal role of MECP2 in RTT comes from the MECP2+/- mouse model. Johnson and colleagues found that ~20% of female MECP2+/- mice exhibited RTT-like breathing abnormalities that began as early as 3 weeks of age. Interestingly, breathing abnormalities were also present in male mice with the MECP2 gene deletion (Johnson et al., 2015).

Moreover, accumulating evidence indicates that MECP2 is also involved in other diseases. A recent study showed that MECP2 regulates lipid metabolism reprogramming via repression of the key gene peroxisome proliferator-activated receptor gamma (Tsukamoto, 2015). X-ray-induced inhibition of lung cancer cells is mediated by enhanced expression of the Axin gene via genomic DNA demethylation and histone acetylation. Mechanically, it has been shown that MECP2 represses histone deacetylase, leading to transcriptional activation of the Axin gene via histone acetylation, thus suggesting a role for MECP2 in cancer prevention (Yang et al., 2013a, Esteller, 2005).

MECP2 has also been reported to interact with lens epithelium-derived growth factor p75 (LEDGF/p75), a transcriptional co-activator and binding partner of human immunodeficiency virus (HIV) integrase (Aqil et al., 2015). MECP2 expression is highly upregulated in human monocytic cells expressing HIV-1 Nef protein during the early phase of the viral replication cycle, suggesting that MECP2 indirectly activates HIV integrase via its interaction with LEDGF/p75 (Aqil et al., 2015). MECP2 has also been implicated in Parkinson's disease (Xie et al., 2013) and autonomic cardiovascular control (Bissonnette et al., 2007). Additionally, MECP2 was identified as a reversibly repressed gene in mouse hearts in response to transverse aortic constriction (TAC); its expression normalized after removal of the constriction. Data from patients in the same study showed that MECP2 mRNA was significantly repressed in failing human hearts; its expression normalized after unloading by a ventricular assist device (LVAD) (Mayer et al., 2015), confirming an important role of MECP2 in heart disease.

1.6 Hypothesis, Project Aims, and Objectives

Hypothesis:

As discussed above, a growing number of studies suggest that miRNA-22 is a critical regulator of cell cycle progression and/or arrest. Given that cell cycle arrest or exit from cell cycle progression is a critical process during cell differentiation (Kim et al., 2006, Cardinali et al., 2009), miRNA-22 could be a major regulator of cell differentiation from stem cells. Importantly, Ruohola-Baker's group has shown that miRNA-22 is upregulated during human ES cell differentiation, implying a role for miRNA-22 in stem cell differentiation (Stadler et al., 2010). Furthermore, our previous study showed that miR-22 is one of the top upregulated miRNAs in our SMC differentiation model (Yu et al., 2015). Therefore, we hypothesized that miR-22 and its target gene(s) play a part in SMC differentiation from stem cells.

Aims:

This PhD project investigated the functional involvement of miRNA-22 and its potential target gene(s) in SMC differentiation from ES cells *in vivo* and *in vitro* and the molecular mechanisms involved.

Objectives:

1) Use real-time qPCR to confirm previous results obtained from miRNA microarray analyses showing that miR-22 is upregulated during SMC differentiation from stem cells.

2) Use our group's well-established *in vitro* SMC differentiation model to study the functional importance of miRNA-22 in ES cell differentiation towards SMCs.

3) Determine if miR-22 plays a similar role in SMC differentiation *in vivo* by generating miR-22-overexpressing and control ES cell lines and using a Matrigel implant model.

4) Confirm the functional importance of miR-22 in SMC differentiation from adult blood vessel stem/progenitor cells.

5) Identify and validate the target gene(s) of miR-22 in SMC differentiation.

6) Elucidate the underlying molecular mechanisms by which miR-22 and its target gene(s) regulate genes involved in SMC differentiation.

Chapter 2

2. Materials and Methods

2.1. Materials

Name	Company
Antibodies against MECP2 (Goat, N-17, sc-5755)	Santa Cruz Biotech, USA
Antibodies against SRF (Rabbit, G-20, sc-335)	Santa Cruz Biotech, USA
Antibody against Smooth Muscle Myosin Heavy Chain (SM-MHC) (Rabbit, AHP1117)	AbD Serotec
Antibodies against calponin (Rabbit, Ab46794)	Abcam, UK
Antibodies against MECP2 (Rabbit, ab2828, CHIP grade)	Abcam, UK
Antibodies against SM22α (Rabbit, Ab14106)	Abcam, UK

Monoclonal anti-α smooth muscle actin (SMαA) (Clone 1A4, A5228)	Sigma
Antibodies against GFP (G6539)	Sigma
Antibody against H3K9me3 (Mouse, 05-1250)	Millipore
Anti-rabbit IgG (whole molecular)-Peroxidase antibody produced in goat (A9169)	Sigma
Antibodies against α-tubulin (mouse)	Sigma
Anti-trimethyl Histone H3(Lys9) (CMA308)	Millipore
Majority secondary antibodies	Sigma, and Santa Cruz Biotech, USA
Majority enzymes and buffers for gene clone and mutation	Promega, New England Biolabs® Ins. And Invitrogen

Table 1 Antibodies, enzymes, and buffers used for gene research

Other materials used in this study were purchased from Sigma unless specifically indicated.

2.2. ES Cell Culture

2.2.1. Medium preparation

2.2.1.1. ES cell basic medium (BM)

ES cell basic medium (BM), the main medium used for ES cell culture, consisted of KnockOut™ D-MEM (Gibco/Invitrogen), 100 U/ml penicillin, and 100 µg/ml streptomycin. BM was typically stored at 4°C in a refrigerator.

2.2.1.2. ES cell complete culture medium (CM)

ES cell complete culture medium (CM) was prepared in small amounts (usually 50 ml) and placed at 4°C in a refrigerator for short-term storage. ES cell complete CM contained 90% BM, 10% foetal bovine serum (FBS) for stem cells (Invitrogen), 1% 100× MEM Non-Essential Amino Acids Solution (MEM-NEAA; Invitrogen), 0.1% recombinant human leukaemia inhibitory factor (LIF, 10 µg/ml; ProSpec), and 0.01% 1 M 2-mercaptoethanol (Sigma-Aldrich).

2.2.2. Mouse ES cell maintenance

Detailed protocols for mouse ES cell (ES-D3 cell line, CRL-1934; ATCC, Manassas,

USA) culture and SMC differentiation have been described in our previous reports (Huang et al., 2013, Xiao et al., 2012, Wang et al., 2012, Xiao et al., 2011, Zhang et al., 2010b, Pepe et al., 2010, Margariti et al., 2009, Xiao et al., 2009, Xiao et al., 2007a). Briefly, mouse ES cells were usually cultured in T25 flask with 5 ml ES cell complete CM and maintained in an incubator at 37°C with 5% CO_2. The growth status and morphology of the ES cells were checked daily. Once cell confluence (coverage) reached ~80% (normally every 2–3 days), ES cells were sub-cultured at a ratio of 1:6 to 1:10 as described below.

2.2.3. Mouse ES cell passaging/sub-culturing

Before ES cell passaging/sub-culturing, a new T25 flask was coated with pre-warmed 0.04% gelatin (Sigma-Aldrich) in PBS and incubated at 37°C for at least 30 min. After removal of the old CM, ES cells were washed once with pre-warmed 1× PBS and then rinsed with 2 ml trypsin-EDTA for 10 s. Excess trypsin-EDTA (1.8 ml) was removed from the ES cells, and the cells were incubated in the incubator for less than 2 min. Pre-warmed CM was then added to the flask to neutralize the reaction, and the ES cells were dissociated into a single-cell suspension by pipetting several times. To achieve a typical ES cell passage ratio of 1:4 to 1:10, a suitable amount of ES cell suspension was added to the pre-coated T25 flask. Additional fresh ES cell CM (up to 5 ml) was added to the T25 flask, and the cells were cultured at 37°C in a 5% CO_2 incubator for 2–3 days.

2.2.4. Mouse ES cell freezing and defrosting

Mouse ES cells were frozen in freezing medium (FM), which consisted of 50% DMEM (Sigma-Aldrich), 40% FBS, and 10% dimethyl sulphoxide (DMSO; Sigma-Aldrich). The ES cells were washed with 1× PBS once after removal of the culture medium from the flask and then treated with trypsin-EDTA (Sigma-Aldrich) for about 1 min in a 37°C incubator. BM containing 10% FBS was then quickly added to the flasks to neutralize the reaction. The cell suspension was transferred into a new 15 ml tube and spun at 1,200 × *g* for 3 min. The supernatant was discarded, and the ES cells were re-suspended in 4 ml of FM. The ES cell suspension was split into several cryovials at a ratio of 1:4. The vials were placed into a freezing container (Mr Frosty) and stored in a -80°C freezer overnight. All cryovials were transferred to a liquid nitrogen vessel the day after for long-term storage.

For ES cell defrosting/recovery, the cryovial containing the cells was warmed in a 37°C water bath until the cells were defrosted completely. The thawed cells were transferred into a 15-ml conical centrifuge tube, and 4 ml DMEM was added to the tube. Cells were spun at 1,200 × *g* for 3 min and re-suspended in 5 ml fresh ES cell CM. Cells were transferred to a T25 culture flask pre-coated with 0.04% gelatin and cultured in the incubator at 37°C with 5% CO_2 until passaging. The ES cells were usable when the growth rate became normal.

2.3. Smooth muscle cell *in vitro* differentiation from ES cells

2.3.1. SMC differentiation medium (DM)

The MEM, alpha modification (α-MEM) was the basal medium used to prepare the SMC differentiation medium (DM). In brief, 1 vial of Minimum Essential Medium Eagle (M0644, Sigma) and 2.2 mg $NaHCO_3$ were dissolved with 1 litre warmed, autoclaved ddH_2O. The mixture was then filtered with a Nalgene® Disposable Bottle Top Filter (500 ml; Thermo Scientific) into a new autoclaved bottle. The fresh α-MEM was stored at 4°C in a refrigerator for later use.

Fresh SMC differentiation medium, known as DM, composed of α-MEM, 10% FBS (Invitrogen), 0.05 mM 2-mecraptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin, was stored at 4°C in a refrigerator for up to 2 weeks.

2.3.2. SMC differentiation from ES cell

Detailed protocols for SMC differentiation from ES cells have been described in our previous reports (Huang et al., 2013, Xiao et al., 2012, Wang et al., 2012, Xiao et al., 2011, Zhang et al., 2010b, Pepe et al., 2010, Margariti et al., 2009, Xiao et al., 2009, Xiao et al., 2007a). Briefly, before cell seeding, cell culture flasks or plates were coated with 5 μg/ml collagen I/IV in cold PBS for more than 2 h at room temperature in a clean hood.

Undifferentiated ES cells were washed once with pre-warmed 1× PBS and trypsinized into single cells with 0.05% trypsin-EDTA. Cells were counted and seeded onto pre-coated flasks or plates at the optimal cell density and cultured in warm DM in an incubator at 37°C with 5% CO_2 for the indicated times. DM was refreshed every other day from day 2.

2.4. SMC differentiation from vascular stem/progenitor cells

Detailed protocols for mouse adventitia stem cell antigen 1-positive (AdSca-1+) cell isolation and culture have been described in our previous reports (Hu et al., 2004, Xiao et al., 2012). Briefly, thoracic arteries were harvested, and the periadventitial fat was carefully removed. The arteries were then washed with PBS and digested for 10–15 min in a digestion solution containing 1 mg/ml collagenase (Sigma, C0130-1G). The adventitia layer was carefully peeled away from the media layer under a stereomicroscope and cut into small pieces. The second digestion was applied to the small adventitia pieces in a mixed solution with 3 mg/ml of collagenase and 500 μg/ml elastase. Next, the digested cell suspension was placed onto a shaker at 37°C for 2–2.5 h, with vortexing every 20–30 min. The suspension was then collected and filtered through a 70-μm BD Falcon® cell strainer to obtain single cells. Fresh cells isolated from 8–10 mice were combined and sorted using the Anti-Sca-1 MicroBead Kit (MACS Miltenyi Biotec). Isolated AdSca-1+ cells were induced to SMCs in the SMC differentiation medium (DMEM, 10% FBS, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 ng/ml TGF-β) for 2 to 6 days before further treatment. The medium was refreshed every other day.

2.5. Transfection

2.5.1. Plasmid transfection

To determine the optimal transfection efficiency, plasmids and related controls were transfected into cells using two transfection reagents, FuGENE-6 (Roche) or TurboFect transfection agent (Thermo), according to the manufacturers' instructions. As suggested, the optimal confluence for adherent cells was 70–90% at the time of transfection. Fresh α-MEM containing 5% FBS was added to the culture flasks or plates to replace the old culture medium 1 h before the transfection. The plasmids and respective controls were first diluted in serum-free DMEM or other serum-free growth medium such as Opti-MEM. The appropriate volume of transfection reagent was added to the diluted DNA mixture and mixed immediately by pipetting. The best ratio of transfection agent to DNA was 3:1 for FuGENE-6 and 2:1 for TurboFect. After incubation for 15–20 min at room temperature, the complex was pipetted several times and added to the cell culture in a drop-wise manner. The plate was shaken gently to distribute the transfection complexes evenly before placing the plate in the incubator. After incubation for 5 to 8 h, the same volume of cell culture medium (α-MEM) with 15% FBS was added to restore the level of FBS to 10%, and the cells were incubated at 37°C in an incubator with 5% CO_2 overnight. The next day, the old medium was replaced with fresh normal differentiation medium (DM), and the cells were harvested at the indicated times.

2.5.2. Transfection of miRNA precursors or inhibitors

MicroRNA reverse transfection, which involves simultaneously transfecting and plating cells, is similar to the procedure used for suspension cell transfection. It was performed according to the protocol provided by the manufacture. In brief, ES cells were first trypsinized into a single-cell suspension using 0.05% trypsin-EDTA. After counting the cell number with a haemocytometer chamber (Hauser Scientific), normal growth medium (DM) was used to dilute the cells to the appropriate concentration. The diluted cells were placed in an incubator at 37°C with 5% CO_2. As the first step of reverse transfection, siPORT™ NeoFX™ Transfection Agent (Invitrogen) was diluted in OPTI-MEM® I Reduced Serum Medium 1× (Gibco) and incubated for 10 min in the clean hood at room temperature. MicroRNA mimics or inhibitors were diluted into the appropriate volume of OPTI-MEM® I medium and mixed with an equal volume of pre-diluted siPORT™ NeoFX™ Transfection Agent mixture. After incubating the mixture at room temperature for another 10 min, the transfection reagent/microRNA complex was dispensed onto a culture plate. Finally, the cell suspension was overlaid onto the transfection complexes, and the plate was gently tilted back and forth to distribute the complexes and cells evenly. According to our preliminary data, the optimised final concentration for Anti-miR™ miRNA Inhibitor and Pre-miR™ miRNA Precursor was 30 nM. After overnight incubation, transfected cells were refreshed with fresh DM and incubated at 37°C under normal cell culture conditions until ready to assay.

2.5.3. MiR-22 and MECP2 co-overexpression

The pre-generated miR-22 overexpression ES cells (pLL3.7-GFP-miR-22) or control ES

cells (pLL3.7-GFP) were seeded on culture flasks or plates coated with 5ug/ml collagen I/IV to induce to SMCs in fresh DM for 2-3 days prior to transfection. After washed with warm 1×PBS, miR-22 overexpression ES cells or control ES cells was transfected with MECP2 overexpression plasmid (pCMV5-MECP2) or control plasmid, respectively, by using TurboFect Transfection Reagent (Thermo Fisher Scientific Inc.). The flaks or plates were gently tilted back and forth to evenly distribute transfection complex and cells, and incubated in 37°C and 5% CO_2 overnight. The culture medium was replaced with fresh DM every other day prior to cell collection for analysis.

2.5.4. MECP2 siRNA transfection

A pool of small interfering RNAs (siRNAs) for MECP2 (MISSION® esiRNA, esiRNA targeting mouse Mecp2, EMU085661-20UG) and MISSION® siRNA Universal Negative Control #1 (SIC001-10NMOL) were purchased from Sigma. A suitable amount of ES cells was cultured on collagen I-coated 6-well plates with fresh made DM for 2–3 days before transfection. As usual, differentiating ES cells were washed with warm 1× PBS once. For each well of the 6-well plate, 5 µl siIMPORTER™ reagent was diluted into 25 µl serum-free medium in a 1.6-ml microcentrifuge tube, and the mixture was pipetted gently, according to the manual. Meanwhile, 25 µl siRNA Diluent was diluted into 10 µl serum-free medium in a fresh microcentrifuge tube and then mixed with 6 µl 20 µM siRNA, followed by the siIMPORTER™ reagent mixture. After incubation for 5–30 min, the siRNA/siIMPORTER™ mixture was overlaid onto cells, and the plate was gently tilted back and forth to distribute the transfection complex evenly. The transfected cells were incubated at 37°C for 48–72 h before harvesting.

2.6. SMC in vivo differentiation

2.6.1. Generation of lentiviral particles for miR-22 overexpression

The lentiviral particles for miR-22 overexpression were produced using a miR-22 plasmid (pLL3.7-GFP-miR-22), cloned by us, and the pLL-3.7-GFP vector (Addgene, Plasmid 11795). Approximate 606 bp of a genomic fragment containing the mmu-miR-22 precursor (95 bp) and its flanking sequence (256 bp and 261 bp, respectively) was amplified by PCR with a specific primer set as shown in **Table 2** from differentiating stem cells and cloned into the Hpa-I/Xho-I sites of the pLL3.7-GFP expression vector, generating pLL3.7-GFP-miR-22. All vectors were verified by DNA sequencing, and the vectors with the correct sequence were further amplified and used to produce lentiviral particles. Briefly, 2–2.5 × 10^6 293T cells were plated on a 10-cm plate or T75 flask 1 day before transfection. The pLL3.7-GFP (control) or pLL3.7-GFP-miR-22 (miR-22 overexpression) was co-transfected with the 3rd generation packaging plasmids pMDLg/pRRE (Addgene, 12251) and pRsv-Rev (Addgene, 12253) and the envelope plasmid pMD2.G (Addgene, 12259) into 293T cells using FuGENE-6, according to the manufacturer's instructions. After incubation at 37°C in 5% CO_2 overnight, the old medium was replaced with normal medium (DMEM) containing 10% FBS. The supernatant containing the lentivirus was harvested 48 h later, filtered, aliquoted, and stored at -80°C for future use.

Gene names	Forward (5'-3')	Reverse (5'-3')	Application
U6 snoRNA	GATGACACGCAAATTCGTG	miRNA universal reverse primer (Invitrogen, A11193-051)	Real-time RT-PCR (RT-qPCR)
18s	CCCAGTAAGTGCGGGTCATAA	CCGAGGGCCTCACTAAACC	RT-qPCR
miR-22 (mu/hu)	AAGCTGCCAGTTGAAGAACTGT	miRNA universal reverse primer (Invitrogen, A11193-051)	RT-qPCR
miR-22 precursor (mu)	ACCTGGCTGAGCCGCAGTAG	AGGGGCAGAGGGCAACAGTTC	RT-qPCR to detect miR-22 precursor RNA
miR-22 Primary (mu)	AAAGGGGCACAAAGCAAGTG	CAGGAAAGCTGGGTGACAGG	RT-qPCR to detect miR-22 primary RNA

SMαA	TCCTGACGCTGAAGTATCCGAT	GGCCACACGAAGCTCGTTATAG	RT-qPCR
SM22α	GATATGGCAGCAGTGCAGAG	AGTTGGCTGTCTGTGAAGTC	RT-qPCR
h1-Calponin	GGTCCTGCCTACGGCTTGTC	TCGCAAAGAATGATCCCGTC	RT-qPCR
SM-myh11	AAGCAGCCAGCATCAAGGAG	AGCTCTGCCATGTCCTCCAC	RT-qPCR
SRF	CCTACCAGGTGTCGGAATCTGA	TCTGGATTGTGGAGGTGGTACC	RT-qPCR
Myocd	TCAATGAGAAGATCGCTCTCCG	GTCATCCTCAAAGGCGAATGC	RT-qPCR
MEF2C	AAGCCAAATCTCCTCCCCCTAT	TGATTCACTGATGGCATCGTGT	RT-qPCR
MECP2	GGCTGTGGTAAAACCCGTCCG	GGCTTGTCTCTGAGGCCCTGGA	RT-qPCR
Nox4	ATTTGCCTGGAAGAACCCAAG	CATCGGTAAAGTCTCTCCGCA	RT-qPCR

HDAC7	CCCAGTGTGCTCTACATTTCCC	CACGTTGACATTGAAGCCCTC	RT-qPCR
Nrf3	TGCCAGATGCAGGCGGATGC	TTGCCTGGGCTGACACCCCT	RT-qPCR
Cbx3	GAACGAATAATCGGCGCCA	ATGTTCGCCTCCTTTGCCA	RT-qPCR
hnRNPA1	TTCATCCAGTCAGAGAGGTCGC	TGAAGTTCCCTCCTCGACCAA	RT-qPCR
hnRNPA2B1	CTGCAAGCAAAAGATCAAGAGG	GCTCAACTACCCTGCCATCAA	RT-qPCR
Pla2g7	CACTGGCAAGACACATCTTC	ATCAGATCTGTACAACCGAC	RT-qPCR
SMαA-P1	CATAACGAGCTGAGCTGCCTC	CCAAACAAGGAGCAAAGACG	CHIP assay (with CArG region)
SMαA-P2	GATCAGAGCAAGGGGCTATA	CTACTTACCCTGACAGCGAC	CHIP assay (without CArG region)
SM22α-	GCAGGTTCCTTTGTCGG	CTGCTTGGCTCACCAC	CHIP assay

P1	GCCA	CCCG	(with CArG region)
SM22α-P2	CTTTAAACCCCTCACCCAGC	ATGACTTGCACTTACAAGG	CHIP assay (without CArG region)
SRF-P_F1/R1	GGCTGGGCCCTCCCCCATTT	TGGCTGGTTTGCTGGTTTGGCA	CHIP assay
SRF-P_F3/R3	TCAGGCCTGTGCTTTAGCCTCG	GATGGGGGCAGGGCGGAAAG	CHIP assay (Adjacent region)
Myocd-P_F2/R2	ACGTGGGACCCTGTCACCCC	GGATTCGGTGGCCTGGGCAAG	CHIP assay
Myocd-P_F3/R3	CGGGAGTTGCAAGCCAACCCA	TCCCCAGCTTACTGCAGGGCT	CHIP assay (Adjacent region)
Pla2g7-	GGGCTCCTAGCTGGCAC	TCTCCACCCCAACCCA	CHIP assay

p2	GTC	CCCC	
Pla2g7-p10	GGGATGGGCACAGCTCGTCG	CTCGACCCTCCCCTCCTCCG	CHIP assay (Adjacent region)
Nox4-p	CCATTGCACACTCCTCACCT	GAAGCTCAGATTCCCTCTAGGA	CHIP assay
Nox4-p-adj	TGGACCATGGCTTCAGTGTT	CAGCACACCGGGCTTTGAA	CHIP assay (Adjacent region)
HDAC7-p	CACTGGCAGGTGAATCCTGT	GGACAGAGGATTGTGCAGGT	CHIP assay
HDAC7-p-adj	TCCAGGACACTCAAGAAGGG	GCCTGGGGTGTCCCTTTATC	CHIP assay (Adjacent region)
mus miR-22 precursor	GTGCTCGTTAACCTGCCCTTTGAATGCCGAAG	GTGCTCCTCGAGGGGGAGGTGGAGTCACCTAT	pLL3.7-GFP-miR22 clone
Mus	CTCGTCACGCGTTTGGC	CTCCACTCTAGATCAGCTAACTCTCTCG	pCMV5-MECP2

MECP2	CGCCGCTGCCGCCAC	GTCACGG	clone
pmiR-Luc-MECP2-A	GCTGTCACGCGTGCGGATTGCAAAGCAAACCAACA	GTCGACGAGCTCACCTGGCACTGGCAATGGGA	MECP2 3'UTR reporter-A clone
pmiR-Luc-MECP2-B	CTACTGACGCGTCCCAACCTGCCCCA TGCACTC	TCCTCAGAGCTCTGCACACCAAGGGCAGCAGTT	MECP2 3'UTR reporter-B clone
pmiR-Luc-MECP2-C	CTGCTGACGCGTCCGGCATGAGATGGGGGCAGA	CTGTGTGAGCTCTCCTTTCCCTCCTGGCACTCCTA	MECP2 3'UTR reporter-C clone
pmiR-Luc-MECP2-A- bs2^{mu}	TCTTCTGTTCCATTTGAAGGCAGTGCTGAACC	GGTTCAGCACTGCCTTCAAATGGAACAGAAGA	miR-22 binding site 2 mutation in reporter-A
pmiR-	CCAACAAGAATAAATT	TCTTCTGTTCCATTTG	miR22

Luc-MEC P2-A-bs1/2 mu	TGAAGGTTG TCTCTTCTCC	AAGGCAGTGCTGAA CC	binding site 1 and 2 combination al mutation in reporter-A
pmiR-Luc-MEC P2-C-bs mu	ATGTTTCTGTTTGAAGG GACAATGGAGTGC	GCACTCCATTGTCCCTT CAAACAGAAACAT	miR-22 binding site mutation in reporter-C

Table 2 Primer sets used in the present study

2.6.2. Lentiviral infection and cell sorting

For lentiviral infection, mouse ES cells were seeded on 0.04% gelatin- coated T25 flaks 24 hours early and incubated in 37°C and 5% CO_2 incubator. On following day, the old culture medium was replaced with 4 ml of fresh ES cell complete medium, followed by adding 5µl of 10µg/ml of Polybrene (Hexadimethrine Bromide, H9268; Sigma-Aldrich) and 1ml of respective lentiviral particles. Medium containing viral constructs was replaced with fresh complete medium after 24 hours of infection. Infected cells were cultured for further 2 to 3 day, and the GFP-positive cells were sorted out by using FITC-anti-GFP antibody and anti-FITC microbeads (Miltenyi Biotec Ltd). GFP-positive ES cells were cultured in ES cell medium for 2 to 3 passages before using.

2.6.3. SMC differentiation *in vivo* from miRNA-22-overexpressing ES cells

Control (pLL3.7-GFP) or miRNA-22 over-expression (pLL3.7-GFP-miRNA-22) ES cells were generated as described earlier and induced to SMCs differentiate from ES cells. The procedures and principles for SMCs differentiation *in vivo* were similar to that as described in our previous study (Xiao et al., 2012, Huang et al., 2013). Briefly, control or miRNA-22 over-expression ES cells (1×10^6 cells in 50µl medium) were mixed with 50µl of Matrigel (Becton Dickinson Labware) and PDGF-BB (100ng/ml) on ice, and subcutaneously injected into C57BL/6J mice. After 10~13 days, mice were sacrificed and the implants (Matrigel plugs) were harvested from relate mice before stored in liquid nitrogen for future

using. Nearly half of each Matrigel plug were sectioned for detection of cell markers and the other were lysed and extracted total RNA to examine related gene expression levels, respectively. All animal experiments were performed basing on the protocols and principles approved by the Institutional Committee for Use and Care of Laboratory Animals.

2.6.4. Immunofluorescence staining for sections

For the optimum cutting temperature (Harvey et al.) compound–embedded Matrigel implants, all sections were cut to a thickness of 8 μm, every 40 μm along the longitudinal axis of the Matrigel plug. Every section was numbered using an oil pen and stored at -20°C for future use. Numbered sections (for instance, sections 5, 15, and 25) were subjected to immunohistological analysis with the appropriate antibody. Briefly, frozen sections were air-dried for at least 30 min and fixed in cold acetone for 15 min. After using a PARA Pen to circle the tissue zone, all section slides were placed in a staining chamber containing 1× PBS and washed three times with PBS for 10 min each. All sections were blocked with 5% BSA in PBS (Sigma) for 1 h at room temperature in a humidified chamber and then incubated with primary antibodies (SM-MHC and GFP) or IgG controls at a dilution of 1:400 in blocking buffer in a cold room (4°C) overnight. The sections were washed three times with 1× PBS for 10 min each and then incubated with the appropriate FITC- or TRITC-conjugated secondary antibodies. Sections were incubated with DAPI (1:1000; Sigma) for 5 min and mounted with anti-fade mounting medium (Fluoroshield™ with 1,4-diazabicyclo[2.2.2]octane; Sigma, F6937). Images were taken and assessed with an Axioplan 2 Imaging Microscope (Plan-NEOFLUAR 20×, NA 0.5, objective lenses, AxioCam camera) and AxioVision software (Carl Zeiss MicroImaging, Inc.) at room

temperature. Photoshop software (Adobe) was used for further image analyses. Two well-trained independent investigators blinded to the treatments determined the percentage of GFP-labelled SM-MHC-positive cells per field. Four random high-power fields (200×) in each section, with three sections from each implant and four implants for each group, were analysed.

2.7. RNA extraction and analysis

2.7.1. Total RNA extraction from cells

To avoid contamination when extracting RNA, all work areas were cleaned with 70% ethanol; whole cell scrapers were sterilized in 75% ethanol for 15 min and then washed with sterilized cold 1× PBS. The cell culture medium from flasks or plates was removed by pipet or vacuum pump, and cells were washed with warm 1× PBS once and cold 1× PBS twice. Cells were scraped in cold PBS to minimum enzyme activity, and the collected cells in cold PBS were transferred to a sterilized 1.5-ml tube. The cells were centrifuged for 2 min at 4°C and 5,000 rpm, and the cell pellet at the bottom of the tube was stored at -80°C for future use.

Total RNA was isolated from cells using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, before RNA extraction, an appropriate amount of Lysis Solution was freshly prepared by adding the suggested amount of 2-mercaptoethanol (2-ME), required to fully inactivate RNases, to the lysis buffer provided in the kit. For each RNA preparation, 250 or 500 µL of the Lysis

Solution/2-ME mixture was added to cell pellets containing less than 5×10^6 cells or 5×10^6 to 1×10^7 cells, respectively. The cell solution was vortexed until all clumps disappeared. The mixture was then transferred to a GenElute™ Filtration Column placed in a 2-ml collection tube and centrifuged at room temperature for 2 min at maximum speed. To precipitate the RNA, an equal volume of 70% ethanol was added to the flow-through solution and mixed thoroughly. The lysate/ethanol mixture (~700 µL) was transferred to a clear binding column and centrifuged at maximum speed for 15 s. The column was returned to the same collection tube after removal of the flow-through. If the volume of lysate/ethanol mixture was greater than 700 µl, the previous step was repeated to allow all RNA to bind to the column. Thereafter, the column was respectively washed twice with 500 µL Wash Solution 1 and 2 by centrifuging at maximum speed for 15 s. After the final wash, the flow-through was removed from the collection tube, and the sample column was centrifuged at maximum speed for 2 min to fully remove the ethanol from the column. The RNA binding column was placed in a fresh collection tube, and 50 µl of Elution Solution was added to the column. After incubation for 1 min at room temperature, the column was centrifuged at maximum speed for 1 min to elute the RNA. The RNA concentration was measured using a NanoDrop spectrophotometer. RNA samples were used immediately or stored at -80°C. The RNA was kept on ice after thawing.

2.7.2. Small RNA extraction from cells

Total RNA containing small RNA (microRNA) was extracted from cells ($1–5 \times 10^6$) using the mirVana™ Protein and RNA Isolation System™ Kit (Applied Biosystems, Ambion Inc.) or TRI Reagent (Sigma) according to the manufacturers' instructions.

For RNA extraction using the mirVana™ Protein and RNA Isolation System™ Kit, cell pellets were mixed with 625 µl ice-cold cell disruption buffer. Samples were vortexed to completely lyse the cells and to obtain a homogeneous lysate. An equal volume of 2× denaturing solution at room temperature was immediately added to the lysate to prevent RNA degradation. After mixing, 1250 µl acid-phenol:chloroform was added to the mixture and vortexed for 1 min to mix. To separate the mixture into aqueous (upper) and organic (lower) phases, the mixture was centrifuged for 5 min at 13,200 rpm at room temperature. The upper aqueous phase was carefully removed and transferred into a fresh centrifuge tube. The volume of 100% ethanol (room temperature) added to the aqueous phase was 1.25 times the volume of the recovered aqueous phase. Each lysate/ethanol sample was mixed thoroughly and aliquoted onto a filter cartridge placed in one of the collection tubes. The mixture was centrifuged at 13,200 rpm for 30 s. The flow-through was discarded, and centrifugation was repeated until all of the lysate/ethanol samples had filtered through the cartridge. MiRNA Wash Solution 1 (700 µl) was added to the filter cartridge and centrifuged for roughly 30 s. The flow-through was discarded, and the filter cartridge was washed with 500 µl Wash Solution 2/3. A repeat wash with 500 µl Wash Solution 2/3 was carried out, and the flow-through was discarded. The residual fluid was removed from the filter cartridge by centrifuging the assembly for 1 min. The filter cartridge was transferred into a fresh collection tube, and 100 µl Elution Solution, pre-heated to 95°C, was aliquoted to the centre of the filter. The miRNA eluate was recovered by centrifugation for 30 s, and the concentration of miRNA in each sample was determined using a spectrophotometer. The samples were either stored at -80ºC or further processed.

For RNA extraction using TRI Reagent, 1 ml TRIzol® Reagent (for 1–5 × 10^6 trypsinized cells) was added to cell pellet and mixed by pipetting. For cells cultured in 6-well plates,

12-well plates, or 24-well plates, TRIzol® Reagent was added directly to each well after a wash with 1× PBS, and the cell lysate was drawn repeatedly through the pipette tip to form a homogeneous lysate. The homogeneous samples were incubated for 5 min at room temperature to permit complete dissociation of nucleoprotein complexes. The lysate was centrifuged for 1 min at 13,200 rpm, and the suspension was transferred to a new RNase-free Eppendorf tube. Chloroform (0.2 ml; Sigma-Aldrich) was added to each tube, vortexed vigorously for 15 s, and incubated at room temperature for 2 to 3 min. The sample was centrifuged at 12,000 × g for 15 min at 2–8°C. Subsequently, the colourless upper aqueous phase containing RNA was transferred to a new tube, and isopropanol (volume equal to that of TRIzol® Reagent) was added to each tube and mixed for 5 s. Samples were left at room temperature for 10 min and then centrifuged at 12,000 × g for 10 min at 2–8°C. The supernatant was carefully removed, and the RNA precipitate at the bottom of the tube was washed with 75% ethanol (volume equal to that of TRIzol® Reagent) twice by centrifuging at 7,500 × g for 5 min at 2–8°C. The supernatant was discarded after the final wash. The pellet was air dried for 10 min and then dissolved in 50–100 µl RNase-free distilled water. The RNA concentration was measured using a NanoDrop machine, and the RNA samples were stored at -80°C for future use.

2.7.3. Normal RT-PCR

2.7.3.1. Normal reverse transcription (RT)

For normal cDNA synthesis, RevertAid Reverse Transcriptase (Thermo Scientific) or the ImProm-II™ RT Kit (Promega, Madison, WI, USA) was used.

When using RevertAid Reverse Transcriptase, the RNA sample was removed from the -80°C freezer and thawed on ice. In accordance with the RevertAid Reverse Transcriptase product manual, 0.1 ng–5 µg template RNA was mixed gently with 1 µl Random Primer (C118A, Promega) and up to 12.5 µl DEPC-treated water. When the RNA template was GC-rich or known to contain secondary structures, the mixture was centrifuged briefly at maximum speed at 2–8°C and incubated at 65°C for 5 min. Subsequently, the treated RNA/primer mixture was mixed with the following components: 4 µl 5× Reaction Buffer, 0.5 µl Thermo Scientific RiboLock RNase Inhibitor (EO0381), 2 µl dNTP mixture (10 mM), and 1 µl RevertAid Reverse Transcriptase. The final volume of each reaction was 20 µl. The RNA was converted to cDNA by incubating the mixture at 25°C for 10 min, 42°C for 60 min, and 70°C for 10 min in the PCR thermocycler. The cDNA product was diluted to a working concentration of 5 ng/µl and stored at -20°C for future use.

When using the ImProm-II™ Reverse Transcription System, up to 1 µg template RNA was mixed with random primer, denatured at 70°C for 5 min, and chilled on ice for 5 min. Subsequently, the reverse transcription reaction mixture containing 4.5 µl nuclease-free water, 5 µl ImProm-II™ Reaction Buffer, 1 µl ImProm-II™ Reverse Transcriptase, 3 µl magnesium chloride ($MgCl_2$), 1 µl dNTPs, and 0.5 µl Ribonuclease Inhibitor was added in each reaction. Following an initial annealing step at 25°C for 5 min, the reaction complex was incubated at 42°C for 1 hour. The cDNA product was diluted to a working concentration of 5 ng/µl and stored at -20°C for future use.

2.7.3.2. MiRNA reverse transcription

The miRNA cDNA was synthesized using the NCode™ VILO™ miRNA cDNA Synthesis Kit (A11169, Invitrogen) by following the instructions provided with the kit. Briefly, up to 2 µg RNA was mixed with the 10× SuperScript® Enzyme Mix, 5× Reaction Mix, and DEPC-treated water. The final volume of the mixture was 20 µl. The miRNA was then transcribed into cDNA by incubating the mixture at 37°C for 60 min and 95°C for 5 min in a PCR thermocycler. The final cDNA product was diluted to 5 ng/ml for immediate use or placed in a -20°C freezer for long-term storage.

2.7.3.3. Polymerase chain reaction (PCR)

To set up parallel reactions and to minimize the possibility of pipetting errors, the PCR mixture was prepared by mixing 13 µl nuclease-free water, 2.5 µl 10× DreamTaq Buffer, 2.5 µl dNTP mix (final concentration 2 mM), 2 µl primers including the respective forward primer and reverse primer (**Table 2**), and 0.14 µl DreamTaq DNA Polymerase for each PCR reaction. The PCR mixture was vortexed, centrifuged, and aliquoted to individual PCR tubes. The template DNA was then added. Finally, the reactions were placed in a Peltier thermal cycler (DNA Engine Tetrad 2; MJ Research), and the PCR programme was performed using the recommended thermal cycling conditions outlined in **Table 3**.

The final PCR product was detected by performing UltraPure™ Agarose (Invitrogen) gel electrophoresis. The percentage of agarose gel typically used for different purposes is shown below (**Table 4**). Accordingly, the appropriate amount of agarose powder was

dissolved in 60–70 ml 1× TBE buffer (or TAE) in a beaker and boiled in a microwave oven. The breaker was shaken gently every 30 s to prevent the formation of agarose clumps. The agarose was completely dissolved by microwaving the solution for 1–3 min. Next, an appropriate amount of GelRed Nucleic Acid Stain (10,000× in DMSO; Biotium) was added to the agarose gel solution when the agarose gel had cooled to 50–60°C (assessed by touch). The gel solution was poured into the casting tray of the gel box with the well comb in place. After the gel solution had completely solidified, 20–30 µl of each sample mixed with 6× DNA loading buffer or 10–15 µl of DNA ladder was added to each well of the gel, and the gel box was filled with 1× TAE buffer (or TBE) until the gel was covered. The gel was run at a maximum of 70 V until the dye front reached the end of the gel. The gel was transferred to the AlphaImager® HP System (Alpha Innotech) with dual wavelength ultraviolet illumination (365 nm or 302 nm). The image was captured and analysed using Automatic Image Capture software (Alpha Innotech).

Step	Temperature, °C	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 3 Normal PCR conditions

Fragment Size	% Agarose (in 1X TBE)
100bp-2kb	2
200bp-4kb	1.5
400bp-10kb	1

Table 4 Percentage of agarose according to DNA or RNA size

2.7.4. Real-time PCR (qPCR)

2.7.4.1. mRNA

To avoid potential contamination, the working area was cleaned with 70% ethanol, and only sterilized pipette tips and tubes were used to prepare the real-time PCR (qPCR) mixtures. cDNA samples were thawed from -20°C and placed on ice.

A KAPA SYBR® FAST qPCR Kit Master Mix (KAPA Biosystems) was used in qPCR. The qPCR master mix containing the appropriate volume of the following components was first prepared: 2× KAPA SYBR® FAST qPCR Master Mix, 2 μM forward primer, 2 μM reverse primer, and DEPC-treated water. The final volume in each well of a 384-well plate was 10 μl, comprising 8 μl qPCR master mix and 2 μl cDNA sample. The 384-well plate was then sealed with MicroAmp® PCR film, vortexed briefly, and centrifuged at 1,200 × *g* for 2 min. An Applied Biosystems 7900 HT TaqMan Real-Time PCR System was used to run the qPCR reactions with the following program: 95°C for 3 min and 40 cycles of 95°C for 1–3 s and 60°C for 1 min. Once the reaction finished, the raw Ct values were automatically acquired with SDS 2.3 software (Applied Biosystems). The relative expression abundance of the mRNAs of interest were analysed and calculated using relative quantification methods (ΔΔC methods). The respective control treatment was set at 1.0. GAPDH, 18S rRNA, or β-actin was used as the endogenous control in mRNA detection.

2.7.4.2. MiRNA

For microRNA detection, EXPRESS SYBR® Green™ qPCR SuperMix (Invitrogen) was used to amplify the microRNAs of interest. For each qPCR reaction, the qPCR mixture consisted of 1.5 µl RNase-free water, 5 µl NCode™ EXPRESS SYBR® Green™ qPCR SuperMix, 0.75 µl 2 µM miRNA-specific forward primer, and 0.75 µl 2 µM miRNA Universal Primer (Invitrogen). The mixture was vortexed, and 8 µl aliquots were added to each well of a 384-well plate (MicroAmp® Optical 384-well Plate). Subsequently, 2 µl of each cDNA sample was added to the appropriate well. Each sample was assessed in duplicate. Real-time qPCR was performed using default conditions (50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min) in 10-µl reactions using an Applied Biosystems 7900 HT TaqMan Real-Time PCR System. Data were analysed using relative quantification methods (ΔΔ C methods) as described above. U6 snRNA was used as the endogenous control to normalise the expression levels of small RNAs.

2.8. Protein extraction and analysis

2.8.1. Chemicals and buffers

All chemicals/reagents used in the following buffers (**Table 5**) were purchased from Sigma-Aldrich unless otherwise indicated.

Name	Components
RIPA buffer	Tris-HCl: 50 mM, PH 7.4. NP-40:1 % Na-deoxycholate: 0.25 % NaCl: 150 mM
5× SDS loading buffer	Tris-Cl (0.25 M, 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, 1 litre	Tris base: 30.3 *g* Glycine:144.1 *g* ***Distilled Water to 1 litre***
1× Running buffer, 1 litre	10 × Tris-glycine buffer: 100 ml 10% SDS: 10 ml

	Distilled Water to 1 litre
1 × Transfer buffer, 1 litre	10 × Tris-glycine buffer: 100 ml Methanol : 200 ml ***Distilled Water to 1 litre***
10 × TBS buffer, 1 litre	Tris HCl: 24 *g* Tris Base: 5.6 *g* NaCl: 88 *g* ***Distilled Water to 1 litre***
1 × TBST, 1 litre	10 × TBS: 100 ml Tween® 20:500 µl ***Distilled Water to 1 litre***

Table 5 Recipes of buffers used in protein analysis

2.8.2. Protein extraction from cells

As with the total RNA extraction protocol, all work areas were cleaned with 70% ethanol to avoid contamination. To the same end, all cell scrapers used for protein extraction were sterilized in 75% ethanol diluted in distilled water for about 15 min and then washed in cold 1× PBS.

The cell culture medium from flasks was removed by pipet and vacuum pump. Cells were washed with warm 1× PBS once and cold 1× PBS twice. Cells were scraped from T25 or T75 flasks with cold 1× PBS and transferred to a sterilized 1.5-ml tube. Cells were centrifuged for 2 min at 4°C and 5,000 rpm. The cell pellet was lysed with 100 µl cold lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8.0) supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich) and 0.5% Triton on ice. The lysate was vortexed briefly and then sonicated for 3–5 s at 4°C to disrupt the cell membrane and release the protein contents. For complete cell lysis, the lysates were placed on a shaker with gently stirring in the cold room for 1 hour. The lysed samples were then centrifuged at 13,200 rpm for 10 min at 2–8°C. Up to 100 µl of supernatant from each sample was transferred to a new 0.5-ml Eppendorf tube and placed on ice. Bio-Rad Protein Assay Reagent (Bio-Rad) was used to measure the protein concentration. The assay solution mixture was first diluted at a ratio of 1:5 using distilled water. To each protein sample (2 µl), 998 µl diluted Bio-Rad Protean assay solution was added. The samples were vortexed and incubated for 10 min at room temperature. A negative control was prepared by adding 2 µl lysis buffer to 998 µl diluted protein assay solution and used to normalize the final readings. The protein concentration was measured with a spectrophotometer (SmartSpec™ 3000; Bio-Rad), and the OD value was measured at 595 nm. Lysis buffer was added to

each sample to adjust the protein concentration to equal levels, and a suitable amount of pre-warmed 5× sodium dodecyl sulphate (SDS) protein loading buffer was added to each sample. The protein samples were placed in a heat block, denatured at 95°C for 10 min, vortexed, and centrifuged briefly. When the samples had cooled to room temperature after several minutes, they were used for western blotting analyses immediately or placed in a -80°C freezer for long-term storage.

2.8.3. Western blotting

Detail protocols for western blot electrophoresis have been described in our previous reports (Yu et al., 2015, Huang et al., 2013). Briefly, western blot electrophoresis was carried out using a Mini-PROTEAN® Tetra Cell system (Bio-Rad, UK), and protein was separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with 6–10% Tris-glycine gels. The 10% separating gel (4 ml) and 6% stacking gel (2.5 ml) were freshly prepared. The 10% separating gel consisted of 1.6 ml double-distilled water, 1.635 ml 30% acrylamide (National Diagnostics, USA), 1 ml 1.5 M Tris-HCl buffer (pH 8.8), 40 µl 10% SDS, 40 µl 10% APS, and 4 µl TEMED (Sigma). The 6% stacking gel contained 1.3 ml double-distilled water, 0.5 ml 30% acrylamide, 0.625 ml 0.5 M Tris-HCl buffer (pH 6.8), 25 µl 10% SDS, 25 µl 10% APS, and 2.5 µl TEMED. The 10% separating gel was prepared first and added to the glass casting chamber. The gel surface was levelled by layering 1 ml butanol (Sigma-Aldrich) onto the 10% separating gel. The upper butanol was discarded from the chamber, and the solidified separating gel was washed with distilled water to remove the remaining butanol. Subsequently, 2.5 ml of the 6% stacking gel was added onto the 10% separating gel, and a plastic comb was inserted immediately

to create 10 wells per gel. The stacking gel was incubated at room temperature for 10–15 min until it solidified. Thereafter, the chamber was placed in the electrophoresis running system, and a suitable amount of 1× Running buffer was poured into the chamber. ColorPlus Prestained Protein Ladder (15 µl; New England BioLabs® Inc.) was loaded into the first well of the gel, and 40 µl of each protein sample was added to the remaining wells. The gel was typically run at a fixed voltage of 160 V for 70–90 min at room temperature until the indicator dye reached the bottom of gel.

Before the gel transfer procedure, a polyvinylidene difluoride (PVDF) membrane (GE Healthcare) was placed in a box containing methanol (Sigma) for 15 min to activate the membrane. The used methanol was discarded, and the membrane was soaked in cold 1× Transfer buffer for another 15 min. Meanwhile, the western blot sponges were washed with distilled water and soaked in cold 1× Transfer buffer with filters for 15 min. After the completion of electrophoresis, the gel was assembled with the pre-coated membranes, pre-wetted blotting sponges, and filter papers in the correct order/direction and transferred to a western blotting cassette. Importantly, air bubbles within the cassette were removed by rolling a roller over the surface of the gel before transfer. The blotting cassettes were placed into a Mini Trans-Blot Central Core (Bio-Rad) filled with cold 1× Transfer buffer. The whole transfer procedure was performed in a cold room for about 150 min at 60 V. After the transfer, the membrane was blocked in 5% skim milk diluted in 1× TBST buffer on a 2D rocker, with the protein facing upwards, for 1 h at room temperature. The membrane was then incubated with the appropriate primary antibody on a slow-speed roller for 12–16 h or overnight in the cold room.

On the following day, the membrane was removed from the bottle containing the primary antibody and washed three times with 1× TBST for 10 min each on a 2D shaker. The

membrane was then incubated with horseradish peroxidase-conjugated secondary antibody with gentle shaking for 1 h at room temperature, followed by three washes with 1× TBST (10 min each). The membrane was then incubated with ECL-PLUS Reagent (Amersham Biosciences, Stockholm, Sweden) for 1–3 min at room temperature. Excess ECL reagent was removed, and the membrane was placed in an autoradiographic cassette and covered with a piece of X-ray film in the dark room. Eventually, the film was developed with an X-Ray Film Processor (SRX-101A; Konica Minolta, USA) using Fix Buffer and Develop Buffer in the dark room for 2 s to 15 min, depending on the signal strength of the examined proteins.

2.9. Gene cloning and mutation

2.9.1. MECP2 3′-UTR cloning and miR-22 binding site mutation

As shown in **Figure 11**, four miR-22 binding sites (1577, 2797, 4537, and 8347) have been found within the MECP2 3′-UTR. Three murine MECP2 gene fragments (MECP2-A: 1567–2698, MECP2-B: 4142–4968, and MECP2-C: 8173–9137; NM-001081979) containing miR-22 binding sites 1 and 2 (Reporter-A), 3 (Reporter-B), and 4 (Reporter-C), respectively, were amplified with PCR using the respective primers, as shown in **Table 2**. The insert DNA fragments and the pmiR-Luc-report vector (Ambion, Applied Biosystems) were first digested with MluI (Promega) at 37°C overnight. After purification, the purified inserts and vectors were digested with HindIII or SacⅠ (Promega)

at 37°C for 12 h. On the following day, 1 µl bacterial alkaline phosphatase (BAP; Invitrogen) was added to the digested vector, mixed by pipetting, and incubated at 65°C for 1–2 h for vector dephosphorylation. The DNA inserts and vectors were purified with agarose gel electrophoresis, and the DNA concentration of the inserts and vectors was measured using a 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 of vector was used as a control. After the overnight incubation, the ligation mixture was transformed into JM109 competent cells. The resulting clones were picked and identified by PCR. The correct clones were further amplified, and plasmid DNA was extracted using a GenElute™ Plasmid Miniprep Kit (Sigma). The DNA was sequenced for final verification. The resulting vectors were designated pmiR-Luc-MECP2-A [harbouring miR-22 binding sites 1 (~1577 bp) and 2 (~2797 bp)], -B (harbouring binding site 3, ~4537 bp), and -C (harbouring binding site 4, ~8347 bp).

Mutations in miR-22 binding sites 1 and 2, alone or combination with reporter A or C, were introduced into the pmiR-Luc-MECP2 reporter using the QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies). According to manufacturer's instructions, the pmiR-Luc-MECP2 reporter was removed from the -20°C freezer and thawed on ice. The site-directed mutagenesis reactions containing 10× QuikChange Multi Reaction Buffer, double-distilled H_2O, QuikSolution, dsDNA template, mutagenic primers, dNTP mix, and QuikChange Multi Enzyme Blend were prepared for thermal cycling with the following parameters: 95°C for 2 min; 30 cycles of 95°C for 30 s, 55°C for 30 s, and 65°C for 5 min (1 min/1 kb); 10 min at 65°C; and 2 min at 4°C. Just before digesting the amplification products with 1.5 µl DpnI at 37°C for 5 min, XL10-Gold

ultracompetent cells were thawed from -80°C on the ice for transformation. XL10-Gold ultracompetent cells (45 μl) were aliquoted to a pre-chilled 1.5-ml tube and mixed with 2 μl β-ME. The tube was placed on ice and swirled gently every 2 min five times. DpnI-treated DNA (2 μl) from each mutagenesis reaction was added to the ultracompetent cells. The mixture was incubated on ice for 30 min, heated for 30 s at 42°C in a water bath, and chilled for 2 min at 2–8°C. The treated mixture was mixed with 250 μl pre-heated Luria Broth (LB) medium without ampicillin and incubated for 1 h on a shaker at 37°C and 200 rpm. The bacteria were spread on a culture dish [LB medium containing 1000× ampicillin (50 μg/ml)] and incubated at 37°C for 16–24 h. The resultant vectors were designated pmiR-Luc-MECP2-A-bs2mu, pmiR-Luc-MECP2-A-bs1/2mu, and pmiR-Luc-MECP2-C-bsmu. All mutants were verified by both PCR and DNA sequencing. Vectors with the correct mutations were amplified and used in the following experiments.

2.9.2. MECP2 gene clone

Two MECP2 overexpression plasmids were generated in my project. The protocol was similar to that described above. Briefly, the target DNA was amplified by PCR with two primers as shown in **Table 2** and cloned into the pCMV5-HA vector (Addgene). After double digestions of the DNA inserts and pCMV5-HA vector with the MluI and XbaI enzymes (Promega) in a 37°C water bath overnight, the digested pCMV5-HA vector DNA was treated with BAP to remove the phosphate group. The purified insert DNAs were ligated into the pCMV5-HA vector using T4 DNA ligase as described above and transformed into bacteria. The final products with the correct DNA sequence were named pCMV5-MECP2-TV1 and pCMV5-MECP2-TV2. The pCMV5-MECP2-TV2 was used

to overexpress MECP2 because this is the main isoform of MECP2 detected in differentiating ES cells.

2.9.3. MiR-22 precursor clone

Using similar procedures, miR-22 precursor (~557 bp) was cloned into the pLL3.7 vector (~7650 bp; Addgene) to generate a miR-22-overexpressing ES cell line, as described above. The amplified inserts and pLL3.7 vector DNA were digested by HpaI and XhoI (Promega). The remaining procedure was similar to that described above. After confirmation with DNA sequence analysis, the resulting vector was designated pLL3.7-mmu-miR-22.

2.9.4. Plasmid amplification and extraction

2.9.4.1. Plasmid amplification

Plasmid transformation was used to amplify the plasmid DNA. The plasmid, stored in the -80°C freezer, was thawed on the ice. Plasmid/DNA (1 µl) or negative control (ddH_2O) was added to 50 µl of aliquoted JM109 bacteria (Promega) for each amplification reaction. The plasmids and JM109 cells were vortexed gently, and the mixture was incubated on ice for 30 min, at 42°C for 1 min, and at 2–8°C for 1 min. The plasmid/bacteria mixture was then added to 500 µl LB medium without antibiotics (ampicillin), incubated on a floor shaker for 1 h at 37°C and 210 rpm, and then centrifuged for 5 min at 5,000 rpm. After discarding

400–450 µl of supernatant, the remaining liquid (~50 µl) containing the plasmid/bacteria was resuspended and dispensed onto the surface of a culture dish in a drop-wise manner and incubated overnight in a 37°C incubator. Distinct single clones were picked and incubated with 5 ml LB with ampicillin in a 14-ml polystyrene round-bottom tube (Falcon) for 12–16 h on a floor shaker. The samples were then centrifuged at 4,000 × *g* for 10 min, and the supernatant was discarded to collect the plasmids.

2.9.4.2. Plasmid extraction

The plasmids were isolated and extracted from bacteria according to the manufacturer's instructions using a GenElute™ Plasmid Miniprep Kit (Sigma), which included Resuspension Solution, Lysis Solution, Neutralization Solution, Column Preparation Solution, Wash Solutions 1 and 2, and Elution Solution. All steps in the protocol described below were carried out at room temperature.

Briefly, the collected bacterial pellet obtained as described in the previous section was resuspended in 200 µl Resuspension Solution containing RNaseA Solution in a 14-ml polystyrene round-bottom tube (Falcon), pipetted thoroughly to homogeneity, and transferred to a 1.5-ml microcentrifuge tube. The resuspended mixture was lysed by adding 200 µl Lysis Solution and inverted gently to mix the samples. To avoid prolonged lysis that permanently denatures supercoiled plasmid DNA and renders it unsuitable for most downstream applications, the lysis procedure was terminated within 5 min. The lysed bacterial debris was mixed with 350 µl Neutralization Solution to precipitate the cell debris; the tube was inverted and centrifuged at 12,000 × *g* for 10 min. To collect the DNA

precipitate, the clear lysate containing the DNA plasmid was transferred to a binding column with a 2-ml collection tube, which had been pretreated with 500 µl Column Preparation Solution. The flow-through in the collection tube was discarded after centrifuging the sample briefly at 13,000 rpm. Optional Wash Solution (500 µl) was added to the column to avoid nuclease contamination of the final plasmid product when the bacterial strains used contained the wild-type $EndA^+$ gene. After the addition of 750 µl Wash Solution diluted with ethanol, the column was centrifuged at 12,000 × *g* for 1 min, and the flow-through was discarded prior to the final elution. The column was placed into a new 2-ml collection tube. Elution Solution (75 µl) was added, incubated for 1 min, and centrifuged at 12,000 × *g* for 1 min. The plasmid DNA product in the eluate was placed at -20°C for long-term storage.

2.10. Luciferase assay

2.10.1. Gene promoter activity assays

Differentiating ES cells (Day 2 or Day 3) cultured in a 24-well plate coated with collagen were co-transfected with individual gene reporters (pGL3-Luc-SMαA, pGL3-Luc-SM22α, pGL3-Luc-SMαA-SRF^{mu}, pGL3-Luc-SM22α-SRF^{mu}, pGL3-Luc-SRF, pGL3-Luc-MEF2c, pGL3-Luc-Myocd, pGL3-Luc-Pla2g7-P2, pGL3-Luc-Pla2g7-P10, pGL3-Luc-HDAC7, pGL3-Luc-Nox4, 150-200ng/well) and control (pCMV5-HA) or MECP2 overexpression (pCMV5-HA-MECP2) plasmids (200 ng/well), as indicated in the figure legends, using FuGENE-6 (Roche) or TurboFect transfection agent (Thermo) according

to the manufacturers' instructions. pShuttle2-LacZ (200 ng/well) or pRenilla (20 ng/well) was included as a control for normalisation. The differentiation medium (DM) was refreshed the day after transfection, and the luciferase assay was processed 48–72 h post transfection.

For luciferase analysis used the Renilla gene as control, the cells were washed with warm 1× PBS once and cold 1× PBS once after removal of the old culture medium from the wells. Reporter Lysis Buffer (100 µl; Promega) was added to each well. The culture plate was then placed on a 2D rocker for 30 min (40–50 rpm), incubated in a -80°C freezer for at least 2 h, and shaken for 30 min at room temperature. The cell lysates were harvested into 1.5-ml Eppendorf tubes, and the supernatant obtained after a 5-min centrifugation at 4°C and 13,200 rpm was discarded. A single-tube luminometer (Turner BioSystem, Sunnyvale, CA, USA) was turned on before the luciferase analysis was conducted. For luciferase activity measurement, 15 µl supernatant from each lysate was mixed with 100 µl luciferase assay substrate (E151A, Promega) diluted in luciferase assay buffer (E152A, Promega) for detection with a luminometer. The procedure for measurement of Renilla activity was similar to that for measurement of luciferase activity. The same amount of clear lysate (15 µl) was added to 100 µl Renilla assay substrate [Coelenterazine (S200A, Promega, 2000×)] diluted in Renilla substrate buffer (25 mM Tris-HCl, pH 7.5, 120 mM NaCl). A relative luciferase unit (RLU) was defined as the ratio of luciferase activity to Renilla activity, with the activity of the control set at 1.0.

The activity of LacZ was detected using a β-Gal Kit (Invitrogen) when the pShuttle2-LacZ was used as the internal control for DNA transfection. The early steps of the β-Gal assay were similar to those described for the luciferase assay. Briefly, 15 µl cell lysate was used to measure luciferase activity, as described previously. For β-galactosidase

activity analysis, 1–10 µl cell lysate was diluted to a final volume of 30 µl with distilled water and transferred to a fresh 1.5-ml microcentrifuge tube. Ortho-nitrophenyl-β-D-galactopyranoside (ONPG; 70 µl) and 1× cleavage buffer with β-mercaptoethanol (β-Me; 200 µl) were added to the diluted cell lysate, vortexed, and then centrifuged briefly at maximum speed (13,200 rpm). The mixture was incubated in a water bath at 37°C for 30 min. Because hydrolysis of ONPG to the ONP anion by β-galactosidase produces a bright yellow colour, yellow colour was observed when β-galactosidase was present in the samples. Finally, 500 µl of stop buffer was added to the mixture to stop the reaction, and the absorbance was read at 420 nm against a blank containing lysis buffer, ONPG, and cleavage buffer. A relative luciferase unit (RLU) was defined as the ratio of luciferase activity to β-galactosidase activity, with the activity of the control set at 1.0.

2.10.2. pmiR-Luc-MECP2 reporter activity assays

The pmiR-Luc-MECP2 (3′-UTR) reporter activity assay was similar to that described for the gene promoter activity analysis. Briefly, differentiating ES cells (Day 2 or Day 3) cultured in a 24-well plate coated with collagen were co-transfected with individual pmiR-Luc-MECP2 3′-UTR reporters (pmiR-Luc-MECP2-A, pmiR-Luc-MECP2-B, pmiR-Luc-MECP2-C, pmiR-Luc-MECP2-A-miR-22 $BS1^{mu}$, pmiR-Luc-MECP2-A-miR-22 $BS1/2^{mu}$, pmiR-Luc-MECP2-C-miR-22 BS^{mu}; 150–200 ng/well) and control (precursor: control) or miR-22 precursor/mimics (precursor: miR-22) (30 nM), as indicated in the figure legends, using siPORT™ NeoFX™ Transfection Agent (Invitrogen), according to the manufacturer's instructions. pShuttle2-LacZ (200 ng/well) or pRenilla (20 ng/well) was included as a control for normalisation. The differentiation

medium (DM) was refreshed the day after transfection, and the luciferase assay was processed 48–72 h post transfection using methods similar to those described for the gene promoter activity analyses.

2.11. Chromatin immunoprecipitation (ChIP) assay

Differentiating ES cells were co-transfected with control (pCMV5) or MECP2 overexpression (pCMV5-MECP2) plasmids in T75 flasks using TurboFect transfection agent (Thermo) and cultured for 48 h with one medium change at 24 h. The culture medium was refreshed before the ChIP assay. Formaldehyde (1%) was added to the culture medium, and the flask was swirled gently to mix the solution before incubation at room temperature for 10 min. To each flask, 1 ml 10× glycine was added to quench any unreacted formaldehyde. The flask was swirled and incubated for 10 min at room temperature. The cells on the surface of the flask were scraped directly into a 15-ml tube and then centrifuged for 2 min at 1,000 rpm and 4°C. After discarding the supernatant, the cell pellets were washed twice with 3 ml 1× PBS containing Protease Inhibitor Cocktail II and then precipitated with 1 ml 1× PBS containing Protease Inhibitor Cocktail II by centrifuging. After removal of the supernatant, the cells were resuspended in 1 ml SDS lysis buffer containing Protease Inhibitor Cocktail II, and 300–400 µl aliquots were added to each microfuge tube. All samples were placed on ice before sonication. The cells were sheared eight times for 20 s each using an EpiShear™ Probe Sonicator (Active Modif® Inc.). The sheared samples were diluted into 500 µl Dilution Buffer containing Protease Inhibitor Cocktail II. Dynabeads® Protein G (30–50 µl; Novex) was pre-washed three

times with Dilution Buffer containing Protease Inhibitor Cocktail II, added to the sheared samples, and incubated on a roller in the cold room for 1–2 h to pre-clear the sheared samples. After centrifugation of the mixture, the clear supernatant was transferred to a new 1.5-ml tube and mixed with antibodies (2 µg/immunoprecipitation) raised against MECP2 (rabbit, ab2828, Abcam), SRF (rabbit, G-20, sc-335, Santa Cruz), or H3K9me3 (mouse, 05-1250, Millipore). An equal amount of normal rabbit IgG or mouse IgG was used as control. A suitable amount of dilution buffer was added to the tube to bring the final volume of each sample to 1 ml, and the sample was incubated on a roller in the cold room overnight. The following day, 30–50 µl Dynabeads® Protein G (Novex) was pre-washed three times with dilution buffer containing Protease Inhibitor Cocktail II, added to each immunoprecipitation sample, and incubated on a roller in the cold room for 1–2 h to pull down the chromatin/protein complexes. After removal of the supernatant, the remaining precipitate was washed with Low Salt Immune Complex Wash Buffer (Catalogue #20-154), High Salt Immune Complex Wash Buffer (Catalogue #20-155), and TE Buffer (Catalogue #20-157). The washed immunoprecipitation reactions were then eluted from the beads using 200 µl Elution Buffer (50 mM $NaHCO_3$, 1% SDS) and incubated at room temperature for 30 min. The supernatant was collected, mixed with 8 µl 5 M NaCl, and incubated at 65°C for 4–5 h to reverse the DNA-protein crosslinks. After the addition of 1 µl RNase A to sample and incubation for 30 min at 37°C, 1 µl of proteinase K solution was added to each sample and incubated in a heat block at 45°C for 1–2 h. Immunoprecipitated DNA was extracted, purified, and used to amplify target DNA sequences in real-time PCR using the specific primers shown in **Table 2**. Promoter DNA enrichment with a specific antibody was defined as the ratio of promoter DNA to input, with that of the control sample (pCMV5) set at 1.0. PCR amplification of the adjacent

promoter regions or a promoter without a CArG region was used as an additional control for specific promoter DNA enrichment.

2.12. Flow cytometry

Differentiated ES cells were dissociated into single cells with trypsin-EDTA (Gibco/Invitrogen) after washing the cells once with warm 1× PBS. The cells were washed with cold 1× PBS containing 10% FBS once after collection, mixed with 1 ml cold 4% paraformaldehyde (PFA; 1×10^5 to 5×10^5 cells/ml), and incubated on ice for 15 min. After centrifugation, the supernatant was discarded, and the cell pellet was re-suspended in 1 ml cold permeabilization buffer (PBS/0.1% Triton X-100, 1×10^5 to 5×10^5 cells/ml) and incubated on ice for 5–10 min for intracellular marker detection. After removal of the permeabilization buffer with centrifugation, the cell pellets were resuspended in 10% FBS diluted in 1× PBS to bring the cell concentration to 1×10^7 cells/ml. After incubation on ice for 20 min to block non-specific antibody binding, 100 µl aliquots of the single cell suspension were added to tubes and incubated for 1 h at room temperature in a dark box with antibodies against GFP or SM-MHC or IgG as a negative control. The samples were washed with 1 ml cold 1× PBS and centrifuged. The supernatant was removed completely. The cell pellet was mixed with an appropriate amount of secondary antibody (1:50 to 1:100) diluted in 1× PBS containing 10% normal FBS and incubated for 30 min in a dark box. Cold 1× PBS was then added to wash the sample. PBS was removed, and the cells were resuspended in 400 µl 1% PFA. A FACSCalibur sorting system (Becton Dickinson) was used to analyse the data.

2.13. PDGF-BB, TGF-beta, and ActD treatment

Platelet-derived growth factor-BB (PDGF-BB) and transforming growth factor beta 1 (TGF-β1) have been widely reported as two important SMC differentiation inducers (Kumar and Owens, 2003, Donovan et al., 2013, Sone et al., 2003, Xiao et al., 2010). Undifferentiated ES cells were seeded on cell culture flasks or plates and cultured with differentiation medium (DM) for 2–3 days to initiate SMC differentiation. The cells were then treated with different amounts of PDGF-BB or TGF-β for 12 h and 3 h, respectively. The final concentration of PDGF-BB and TGF-β used in the following experiments was 5 ng/ml and 2 ng/ml, respectively.

For actinomycin D (ActD) treatment, 2–3 days pre-differentiated ES cells were treated for 6 h with PDGF-BB or TGF-β in the absence or presence of 1 μg/ml ActD.

2.14. Statistical analysis

GraphPad Prism 5 (GraphPad Software Inc.) was used for statistical analysis. Data were presented as the mean ± SEM of ≥3 independent experiments. A two-tailed Student's t-test was used to compare two groups. One-way ANOVA was used to compare different groups. A value of $P < 0.05$ was considered statistically significant.

Chapter 3

3. Results

3.1. SMC differentiation from ES cell

3.1.1. SMC-specific genes were upregulated during ES cell differentiation

Our previous studies have shown that two extracellular matrix proteins (Djarmati et al.), collagen type I and IV, promote SMC differentiation by activating several signalling pathways (Xiao et al., 2012, Huang et al., 2013, Pepe et al., 2010, Xiao et al., 2011, Xiao et al., 2007a). To confirm these findings, undifferentiated ES cells were plated on flasks coated with collagen I and cultured in SMC differentiation medium (DM) to promote SMC differentiation. Undifferentiated ES cells (Day 0) and differentiated ES cells (Day 2, 4, 6, and 8) were harvested respectively at different time points. The mRNA expression of SMC-specific genes, including smooth muscle alpha actin (SmαA) and smooth muscle myosin heavy chain (SM-MHC), were significantly upregulated from Day 4 to Day 8 (compared to undifferentiated ES cells, Day 0). SmαA expression reached a maximum level at Day 6, and SM-myh11 expression peaked at Day 8 (**Figure 4**).

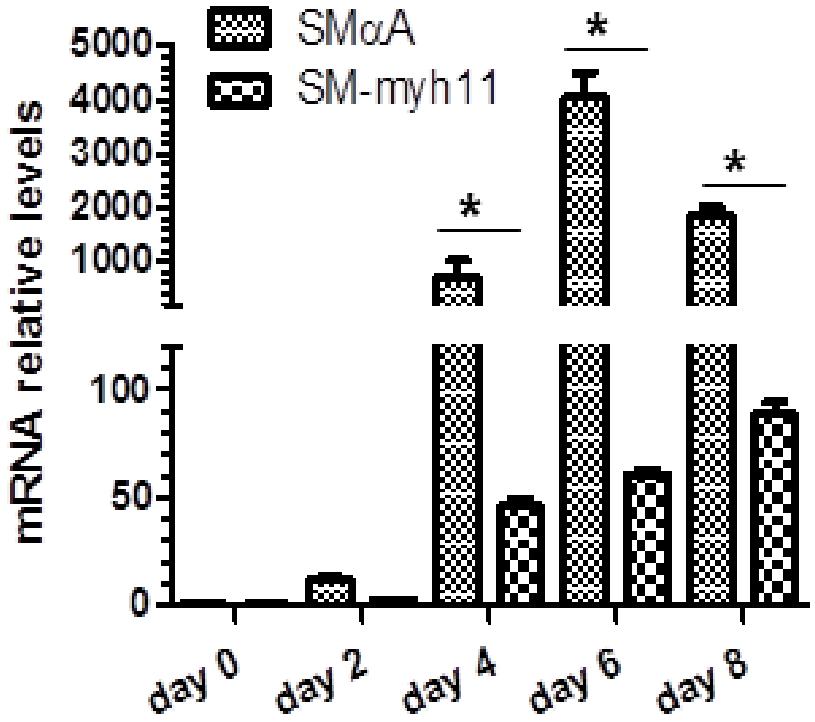


Figure 4 Gene expression of SMC-specific genes during stem cell differentiation toward SMCs.

Undifferentiated ES cells were cultured on pre-coated flasks with 5 μg/ml collagen and cultured for 2, 4, 6, and 8 days. Day 0 represents undifferentiated ES cells. Cells were harvested at the indicated time points. Total RNA was extracted and transcribed to cDNA. The cDNA were subjected to real-time PCR analysis with SMC-specific primers for SmαA and SM-MHC. The data represent the mean ± SEM of three independent experiments. The mRNA abundance was normalized to the 18S rRNA level and presented to expression on Day 0. Significant difference from control (Day 0), *$P < 0.05$.

3.2. The role of miR-22 in SMC differentiation from stem cells

3.2.1. Functional involvement of miR-22 in SMC differentiation from ES cells *in vitro* and *in vivo*

3.2.1.1. Previous studies: miRNAs and SMC differentiation

To identify potential miRNA candidates involved in SMC differentiation, total RNA including small RNA was harvested from undifferentiated ES cells (Day 0), differentiating SMCs (Day 4), and differentiated SMCs (Day 8) and subjected to miRNA microarray analysis (Miltenyi Biotec GmbH) in our previous study. Data from the microRNA microarray analysis revealed that miR-22 was upregulated during SMC differentiation from Day 0 to Day 8 (Yu et al., 2015).

Our microarray results also identified miRNAs other than miR-22 that were also upregulated in SMC differentiation from ES cells, such as miR-34a. Some miRNAs related to SMC differentiation, such as miR-143, miR-145, and miR-133, were upregulated in the early stage of SMC differentiation (Day 4) when compared with expression in undifferentiated ES cells (Day 0). In contrast, miR-21, which is involved in SMC proliferation, was undetectable at the early stage of differentiation (Day 4) but upregulated at the late stage (Day 8). MiR-146a, miR-203, miR-126-3p, and miR-34b-5p or miR-214 were increased at the early stage of differentiation and undetectable or downregulated at the late stage.

3.2.1.2. The role of miR-22 in SMC differentiation *in vitro*

Data from our miRNA microarray analyses showed that miR-22 was one of the top upregulated miRNAs during SMC differentiation from mouse ES cells *in vitro*. To further confirm the finding that miRNA-22 was induced during SMC differentiation, the gene expression of miRNA-22 during SMC differentiation was detected with RT-qPCR. The results confirmed our microarray results, showing that miRNA-22 gene expression was upregulated during ES cell differentiation toward SMCs (**Figure 5**). MiRNA-22 gene expression reached a maximum level at the differentiated SMC time point (Day 8).

In addition, to investigate whether miR-22 induction was important for SMC differentiation, gain-of-function experiments using Pre-miR™ mmu-miR-22 miRNA Precursor (Ambion) were performed in differentiating ES cells. The data showed that the gene expression (**Figure 6A**) of four smooth muscle differentiation specific markers, SMαA, smooth muscle 22 alpha (SM22α), h1-calponin, and SM-myh11, were significantly upregulated by miR-22 overexpression. Western blot results also showed that miRNA-22 overexpression increased the protein expression of SMC-specific markers (**Figure 6B**). On the other hand, data from loss-of-function experiments using Anti-miR™ miR-22 inhibitor (Ambion) clearly revealed that the gene expression of the same SMC-specific markers was inhibited by miR-22 knockdown (**Figure 6E**). The western blot results showed the same trend (**Figure 6F**). ImageJ software was used to quantify and compare protein expression. We observed that the protein level of SMC-specific markers was higher or lower in gain-of-function or loss-of-function miR-22 experiments, respectively, when compared with the level in the respective negative control. Both miRNA experiments suggest a critical role for miR-22 in SMC differentiation from stem cells.

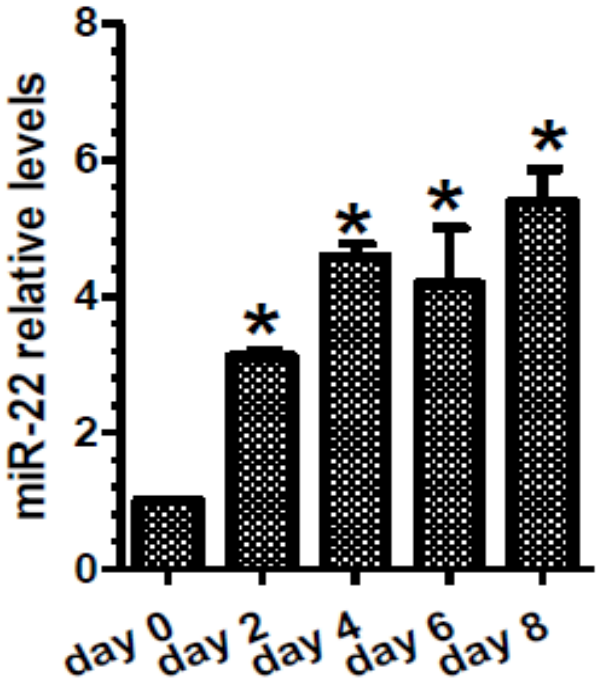


Figure 5 Gene expression of miRNA-22 during stem cell differentiation.

Undifferentiated ES cells were seeded on T25 flasks coated with 5 µg/ml collagen I and cultured for 2, 4, 6, or 8 days in normal differentiation medium (DM). Undifferentiated ES cells (Day 0), differentiating ES cells (Day 2, 4, and 6), and differentiated SMCs (Day 8) were harvested at different time points (Day 0, 2, 4, 6, and 8). Total mRNA/miRNA was extracted with TRIzol reagent and subjected to RT-qPCR analysis. U6 snRNA was used as an internal control for normalisation. The data represent the mean ± SEM of three independent experiments. *$P < 0.05$ (vs. Day 0)

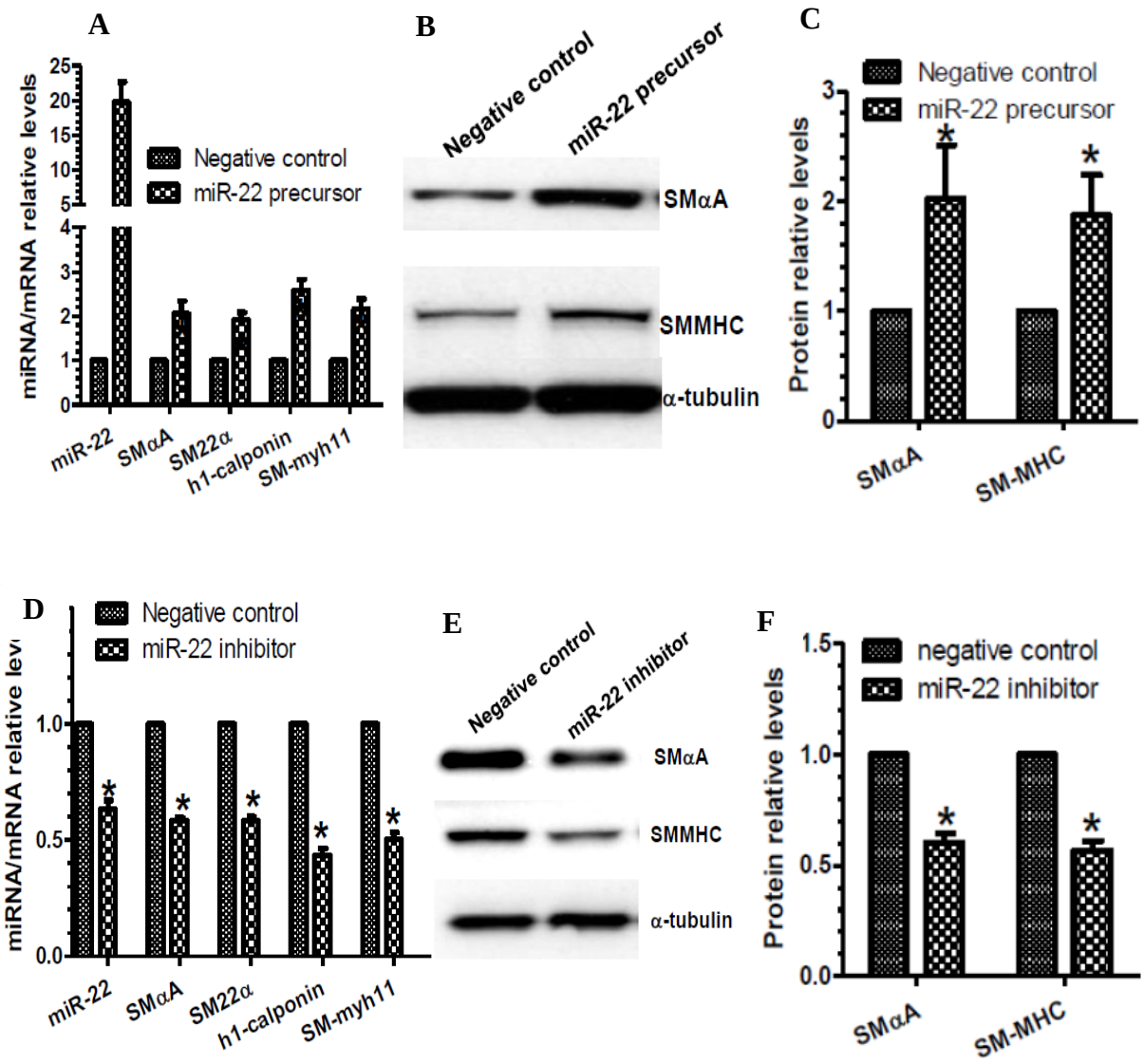


Figure 6 MiRNA-22 is involved in SMC differentiation from stem cell.

Undifferentiated ES cells were placed in T25 flasks coated with 5 µg/ml collagen I and differentiated to SMCs. (**A–C**) MiR-22 overexpression promotes SMC marker expression at the gene and protein levels. ES cells were transfected with miR-22 precursor or negative control and cultured in SMC differentiation medium for 48–72 h. (**D–F**) Knockdown of miR-22 impairs SMC marker expression. Day 3 differentiating ES cells were transfected with miR-22 inhibitor or negative control and cultured in SMC differentiation medium for 48 h. Total RNA and protein were harvested and subjected to RT-qPCR and western blot

analyses, respectively. **(C and F)** Blots were subjected to densitometric analysis with ImageJ software. The data presented here are representative or the mean ± SEM of three independent experiments. *P < 0.05 (vs. respective control).

3.2.1.3. The role of miR-22 in SMC differentiation *in vivo*

The data presented above suggest that miRNA-22 plays an important role during SMC differentiation from ES cells *in vitro*. To determine whether miR-22 has a similar role in SMC differentiation *in vivo*, miR-22-overexpressing (pLL3.7-GFP-miR-22) and control (pLL3.7-GFP) ES cells were generated, sorted, and characterised. As shown in **Figure 7A**, up to 91.2% of cells were GFP-positive (**Figure 7A**) in the sorted miR-22-overexpressing and control ES cell populations, indicating that the pLL3.7-GFP lentivirus and pLL3.7-GFP-miR-22 lentivirus were successfully infected into the undifferentiated ES cells. No significant differences were detected between the sorted cells and their parent ES cells in terms of morphology, self-renewal, and pluripotency when cells were cultured in ES cell culture medium for up to at least five passages (**data not shown**). Compared with the expression of miR-22 in the differentiated ES cells (Day 8), the expression of miR-22 was low in the parental ES cells, control cells, and miR-22-overexpressing ES cells. Additionally, the expression in control and miR-22-overexpressing ES cells was similar under ES cell culture conditions (Day 0, undifferentiated) (**Figure 7B**), indicating that the expression machinery for miR-22 was inhibited under stem cell culture conditions. After differentiation in DM for 8 days, the parental ES cells, control ES cells (pLL3.7-GFP), and miR-22-overexpressing ES cells (pLL3.7-GFP-miR-22) were collected for further analysis. Similar to expression in the parent cells, the expression of miR-22 in control and miR-22-overexpressing cells significantly increased during differentiation. In contrast to expression in the control ES cell group (pLL3.7-GFP), expression of miR-22 in miR-22-overexpressing ES cells (pLL3.7-GFP-miR-22) was further upregulated at Day 8 of differentiation (**Figure 7B**), suggesting that the mechanism inhibiting miR-22 expression under stem cell culture conditions had been removed, enabling upregulation of miR-22 in

these cells during SMC differentiation. Additional quantitative data from flow cytometry analysis showed an increased number of SmαA-positive cells among the parental ES cell (65.6% ± 10.1%), control cell (pLL3.7-GFP) (69.9% ± 7.3%), and miR-22-overexpressing cell (87.69% ± 15.6%) populations at Day 8 of differentiation, when compared with the number in undifferentiated cell populations at Day 0 (3.25% ± 2.3%, 2.5% ± 1.5%, and 3.5% ± 2.1%, respectively). The results provided additional evidence that the ES cells differentiated to SMCs successfully (**Figure 7C**). Importantly, more SmαA-positive cells were observed in the miR-22-overexpressing cell population than in the control ES cell population at Day 8 of differentiation (**Figure 7C**), suggesting that a greater number of SMCs differentiated from miR-22-overexpressing ES cells and confirming that miR-22 promotes SMC differentiation.

To investigate the functional relevance of miR-22 in SMC differentiation from stem cells *in vivo*, pLL3.7-GFP and pLL3.7-GFP-miR-22 cells were mixed with Matrigel and PDGF-BB and injected into mice. The Matrigel implants were collected two weeks later for immunofluorescence staining analysis. We observed a higher percentage of GFP-positive SMCs in miR-22-overexpressing Matrigel grafts than in control Matrigel implants (**Figure 8A and B)**. As expected, the majority of cells in the Matrigel implants were GFP-positive (**Figure 8A**), implying an exogenous origin. Moreover, total RNA was extracted from control and miR-22-overexpressing implants, and the expression in the implants of two SMC differentiation markers (SmαA and SM-MHC) and miR-22 was detected with RT-qPCR. Our data showed that the expression of miR-22, SmαA, and SM-MHC was higher in the Matrigel implants of pLL3.7-GFP-miR-22 ES cells than in those of control cells (**Figure 8C**), confirming the efficiency of miR-22 overexpression and the importance of

miR-22 in SMC differentiation *in vivo*. Taken together, our data clearly support a regulatory role of miR-22 in SMC differentiation from stem cells *in vitro* and *in vivo*.

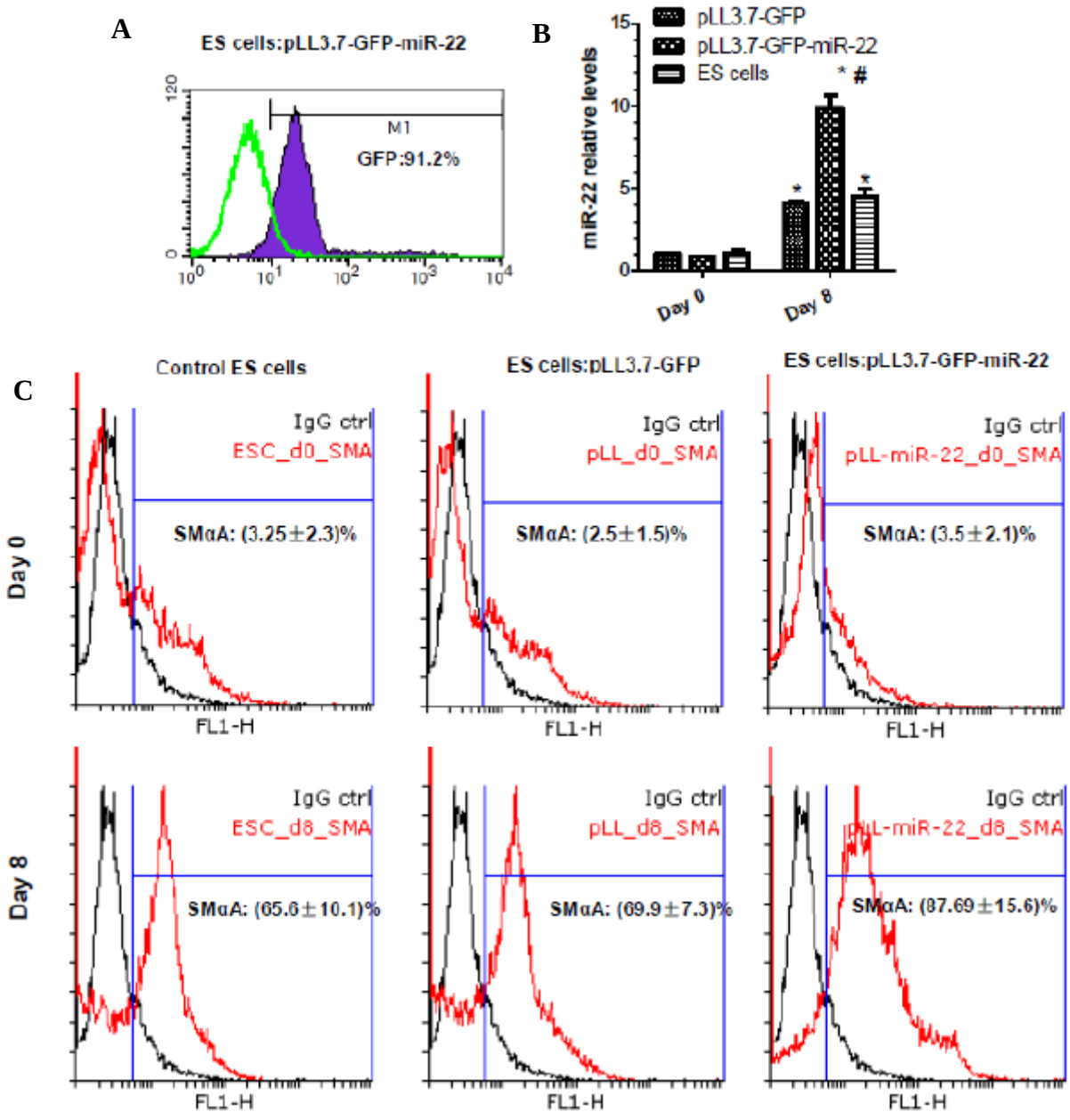


Figure 7 Generation of miRNA-22-overexpressing ES cells and differentiation towards SMCs.

Parental ES cells (control ES cells), control (pLL3.7-GFP) cells, and miR-22-overexpressing (pLL3.7-GFP-miR-22) ES cells were seeded on collagen-coated flasks to induce SMC differentiation. Control and miR-22-overexpressing cells were harvested after 8 days of culture in SMC differentiation medium (DM). Day 0 (undifferentiated) and Day 8

(differentiated) cells were then subjected to flow cytometry analysis using an SMαA antibody. (**A**) Sorted ES cells were GFP-positive. (**B**) MiR-22 increased significantly during SMC differentiation *in vitro*. *P < 0.05 (vs. Day 0), #P < 0.05 (pLL3.7-GFP-miR-22 vs. pLL3.7-GFP). (**C**) A greater number of SMCs differentiated from miR-22-overexpressing ES cells. Representative flow cytometry histograms and the mean ± SEM of three independent experiments are shown here.

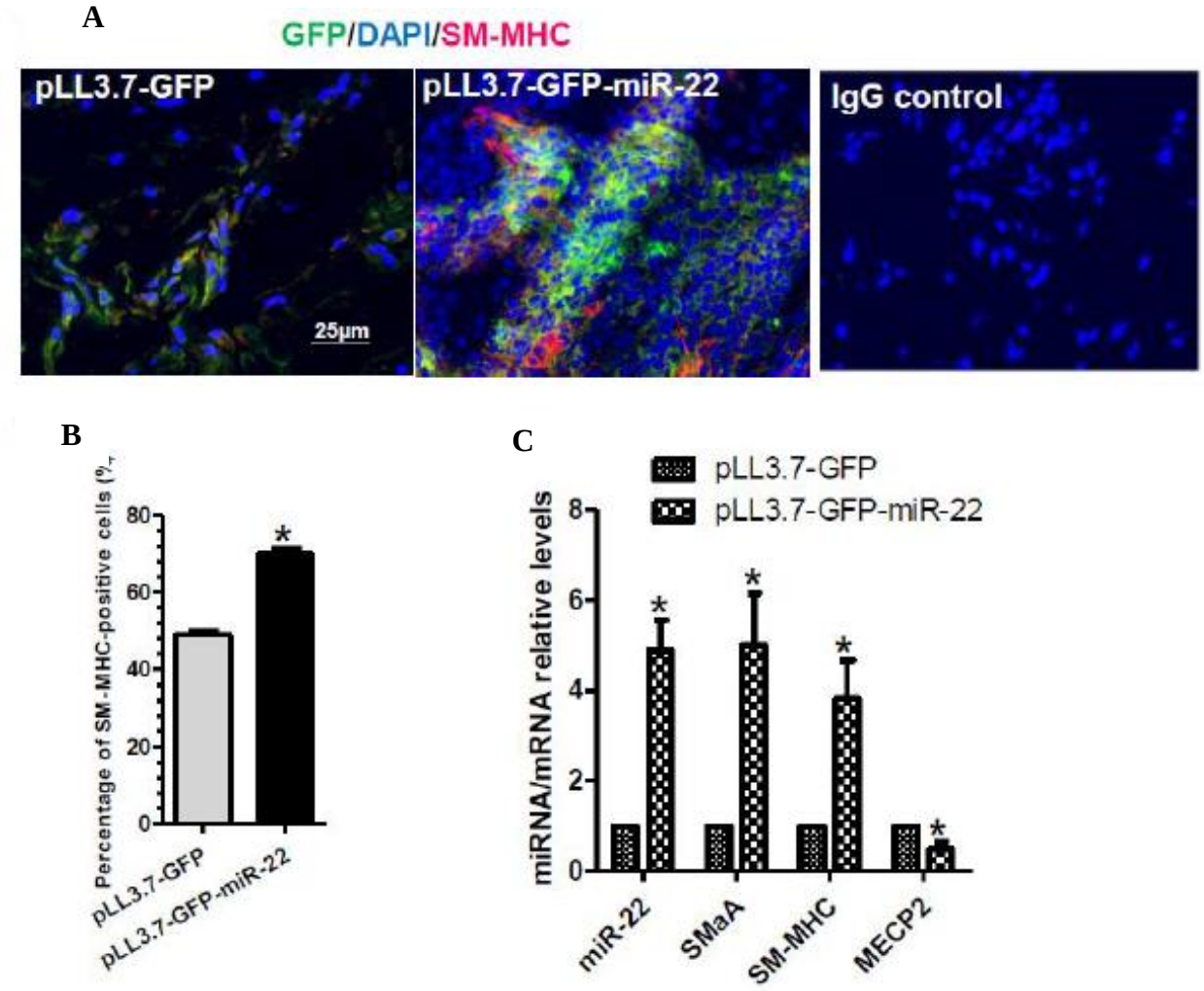


Figure 8 MiRNA-22 promotes SMC differentiation *in vivo*.

pLL3.7-GFP and pLL3.7-GFP-miR-22 ES cells were subcutaneously injected into C57BL/6J mice with Matrigel containing 100 ng/ml of PDGF-BB to promote *in vivo* SMC differentiation. Matrigel plugs implanted with control (pLL3.7-GFP) or miR-22 overexpressing (pLL3.7-GFP-miR-22) ES cells were harvested, sectioned, and stained with antibodies against GFP and SM-MHC. Representative images (**A**) and quantitative data (**B**) showing the percentage of SM-MHC-positive cells are shown. Cells with green fluorescence signal are GFP-positive cells (implanted cells) within the Matrigel plugs. The percentage of GFP-labelled SM-MHC-positive cells per field in four random high-power

fields (200×) in each section was examined by two well-trained independent investigators blinded to the treatments. Three sections from each implant and four implants for each group were analysed. *P < 0.05. (**C**) Gene expression within Matrigel implants. Total RNA samples were extracted from partial Matrigel implants and subjected to RT-qPCR analysis. The data presented here are the mean ± SEM of four Matrigel implants. *P < 0.05.

3.2.1.4. MiR-22 upregulates SMC transcription factors

Using the miRNA-22 overexpression and inhibition experiments described in **Figure 6,** we also investigated the relationship between miRNA-22 and some smooth muscle cell transcription factors (**Figure 6**). The SMC transcription factors examined were serum response factor (SRF), myocardin (Myocd), and myocyte enhancer factor 2C (MEF2c). Undifferentiated ES cells were placed on a collagen-coated T25 flask in DM. MiR-22 precursor, miR-22 inhibitor, or negative controls were transfected into differentiating ES cells at Day 2. RNA was collected, and cDNA was synthesized. In qPCR analysis, we found that the gene expression of SRF and Myocd increased when cells were transfected with miR-22 precursor (**Figure 9A**), but decreased when cells were transfected miR-22 inhibitor (**Figure 9B**) during SMC differentiation, indicating that SRF and Myocd were regulated by miR-22 in a manner similar to that of SMC-specific genes. However, as shown in **Figure 9**, the gene expression of the third transcription factor, MEF2C, did not differ from that of the negative control group in the miR-22 loss-of-function and gain-of-function experiments, suggesting that miR-22 modulation in differentiating ES cells has no effect on the expression of MEF2C. The above data suggest that miR-22 works in concert with SRF and Myocd, but not MEF2C, during SMC differentiation from stem cells.

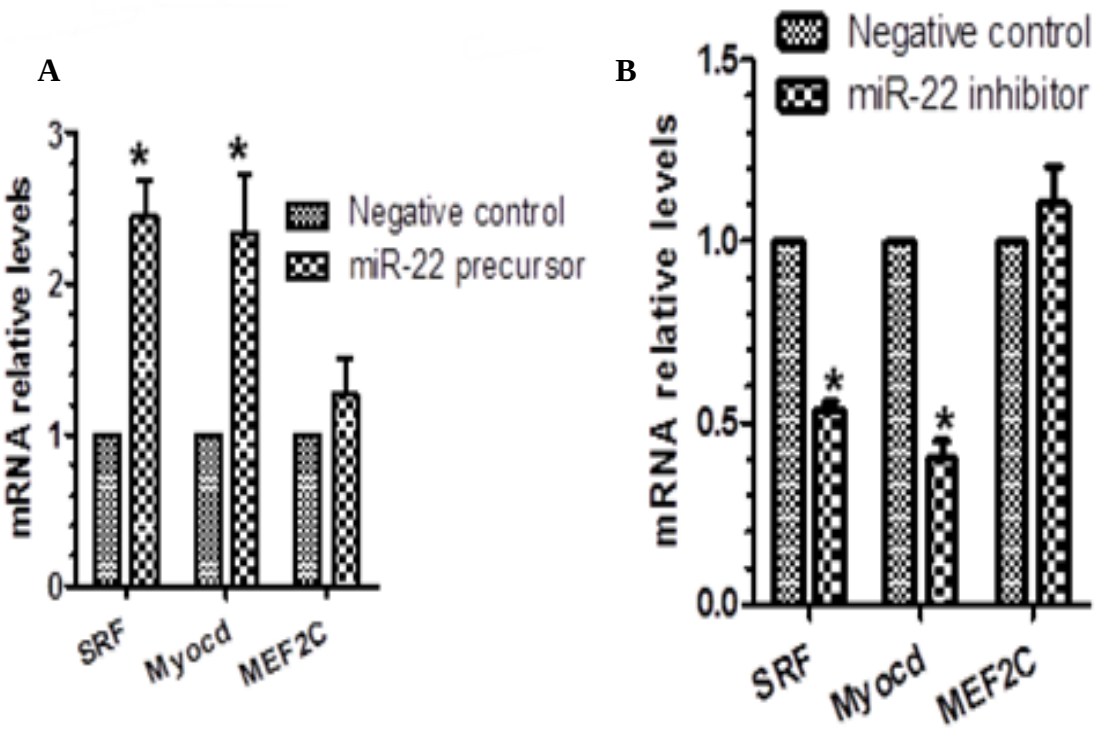


Figure 9 Modulation of miR-22 expression in differentiating ES cells regulates SMC transcription factors.

Total RNA was harvested as described in **Figure 5** and subjected to real-time PCR with specific primers for SRF, Myocd, and MEF2C. MiR-22 overexpression (**A**) increased and miR-22 inhibition (**B**) reduced SRF and Myocd expression. Data represent the mean ± SEM of three independent experiments (n = 3). The mRNA abundance was normalized to 18S rRNA levels and presented relative to the respective control. Significant difference from the negative control, *P < 0.05.

3.2.1.5. Functional importance of miR-22 in adventitia stem/progenitor cell differentiation towards SMCs

Stem cell antigen 1-positive (Sca-1^+) cells can accelerate re-endothelialisation of injured arteries and reduce neointima formation through differentiation into functional endothelial cells (ECs) (Xiao et al., 2006). In addition, differentiation of Sca-1^+ cells, one of the major blood vessel residential stem/progenitor cell populations, into SMCs that contribute to vein graft atherosclerosis has been reported (Hu et al., 2004). Furthermore, our group demonstrated that collagen IV plays a crucial role in SMC differentiation from Sca-1^+ progenitor cells (Xiao et al., 2007a). Adventitia Sca-1^+ cells isolated from vessel adventitia, described in our previous study (Xiao et al., 2012), were induced to differentiate into SMCs in order to investigate the role of miR-22 in SMC differentiation. Real-time qPCR analyses showed that the expression of various SMC differentiation genes (SMαA and SM-myh11) increased as cells transitioned from undifferentiated cells (Day 0) to differentiated cells (Day 6), indicating that Sca-1^+ cells successfully differentiated into SMCs. Importantly, the gene expression of miR-22 was upregulated during SMC differentiation from adventitia stem cell antigen 1-positive cells from Day 2 to Day 4, but not to Day 6 (**Figure 10A**). To clarify the functional role of miR-22 in Sca-1^+ progenitor cell differentiation to SMCs, miR-22 overexpression and knockdown experiments were performed. The results showed that enforced expression of miR-22 by its precursor notably increased all SMC genes, including those encoding SMαA, SM22α, h1-calponin, and SM-myh11, while knockdown of miR-22 by its inhibitor markedly decreased SMC gene expression, suggesting functional involvement of miR-22 in SMC specification of vascular residential stem/progenitor cells (**Figure 10B and C**). Interestingly, the gene expression levels of methyl CpG binding protein 2 (MECP2), a putative target gene of miR-22, was

significantly downregulated and upregulated by overexpression and inhibition of miR-22, respectively, in differentiating Sca-1^{+} progenitor cells, indicating a negative correlation between miR-22 and MECP2 expression in adventitia stem/progenitor cell differentiation towards SMCs (**Figure 10B and C**).

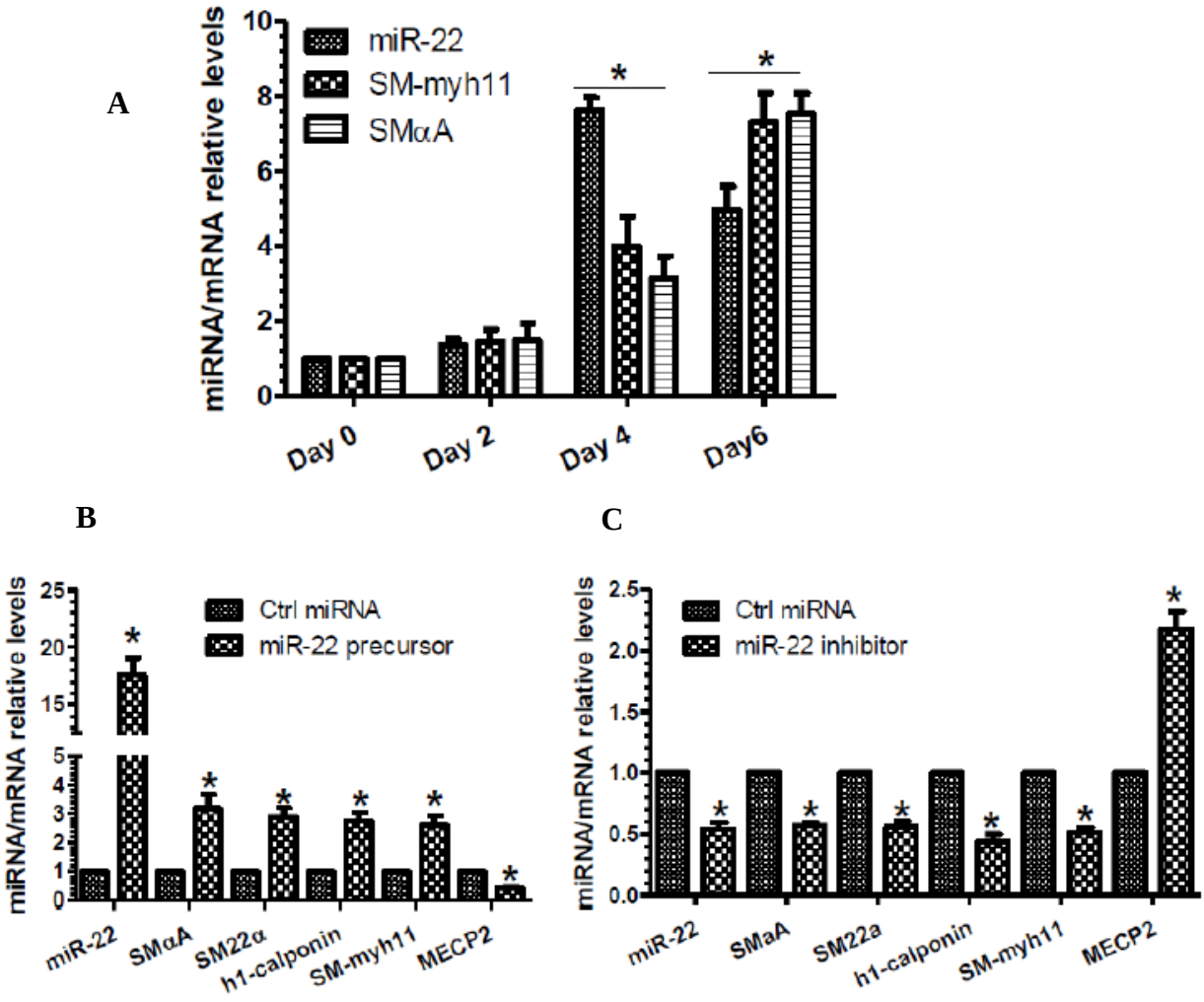


Figure 10 Functional importance of miR-22 in adventitia stem/progenitor cell differentiation towards SMCs.

(A) Induction of miR-22 during SMC differentiation from mouse adventitia stem cell antigen 1-positive (AdSca-1$^+$) cells. Freshly isolated Day 0 samples served as undifferentiated controls. **(B)** The expression of a number of SMC markers was upregulated by miR-22 overexpression. AdSca-1$^+$ cells were transfected with miR-22 precursor or a negative control and cultured in SMC differentiation medium for 48–72 h. **(C)** MiR-22 inhibition reduced SMC marker expression in differentiating AdSca-1$^+$ cells. MiR-22 inhibitor or a negative control was transfected into Day 2 differentiating AdSca-1$^+$

cells, which were then cultured for 2 days before collection. Total RNA was harvested and subjected to RT-qPCR analysis. The data presented here are the mean ± SEM of three independent experiments. *$P < 0.05$.

3.3. MiR-22 target gene in SMC differentiation from ES cells

3.3.1. MECP2 is a predicted miR-22 target gene

A critical step in miRNA research is identifying the target gene(s) of a specific miRNA. In searching for potential target genes of miR-22, several computational algorithmic databases were utilised, including PicTar (www.pictar.mdc-berlin.de), miRanda (www.microrna.org), and microRNA target (www.GeneCopoeia.com). Methyl CpG Binding Protein 2 (MECP2) was predicted as one of the top targets of miR-22. MECPT2 is the founding member of a family of methyl-CpG binding proteins, which repress gene transcription directly, prevent the binding of activating trans factors, or recruit enzymes that catalyse histone post-translational modifications and chromatin-remodelling complexes that alter the structure of chromatin and actively promote transcriptional repression (Miranda and Jones, 2007).

Moreover, the information retrieved from the online computational algorithmic databases revealed that the seed sequence of miR-22 is predicted to hybridize with several regions of the MECP2 3′-UTR, which is evolutionarily conserved among different vertebrate species. At least four conserved binding sites for miR-22 have been identified within the MECP2 3′-UTR (**Figure 11**).

Finally, a favourable minimum loop-free energy in the formation of the miR-22:MECP2 3′-UTR duplex stem loop for all four examined miR-22 binding sites was acquired using

mfold (http://mfold.rna.albany.edu/?q=DINAMelt/Two-state-melting), suggesting that miR-22 is involved in the translational repression of MECP2.

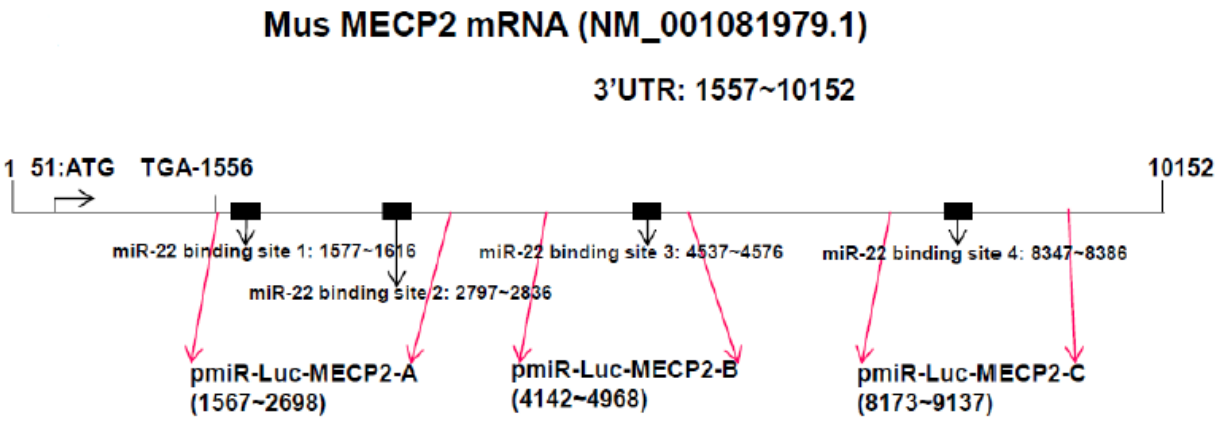


Figure 11 Schematic illustration of MECP2 3′-UTR regions and pmiR-Luc-MECP2 reporters.

The positions of four miR-22 binding sites (BS1–4, black rectangles) within the 3′-UTR of the MECP2 gene are shown, along with the individual 3′-UTR segments of MECP2 in pmiR-Luc-MECP2 reporter-A, -B, and -C.

3.3.2. MECP2 is repressed during SMC differentiation

To establish that MECP2 is a target gene of miRNA-22, we examined MECP2 expression patterns during SMC differentiation from ES cells. As expected, MECP2 gene expression was downregulated, as demonstrated by RT-qPCR analyses (**Figure 12A**). Consistently, western blot analysis showed decreased production of MECP2 protein during SMC differentiation from ES cells (**Figure 12B).** Together, these results indicate a negative relationship between miR-22 and MECP2 expression during SMC differentiation.

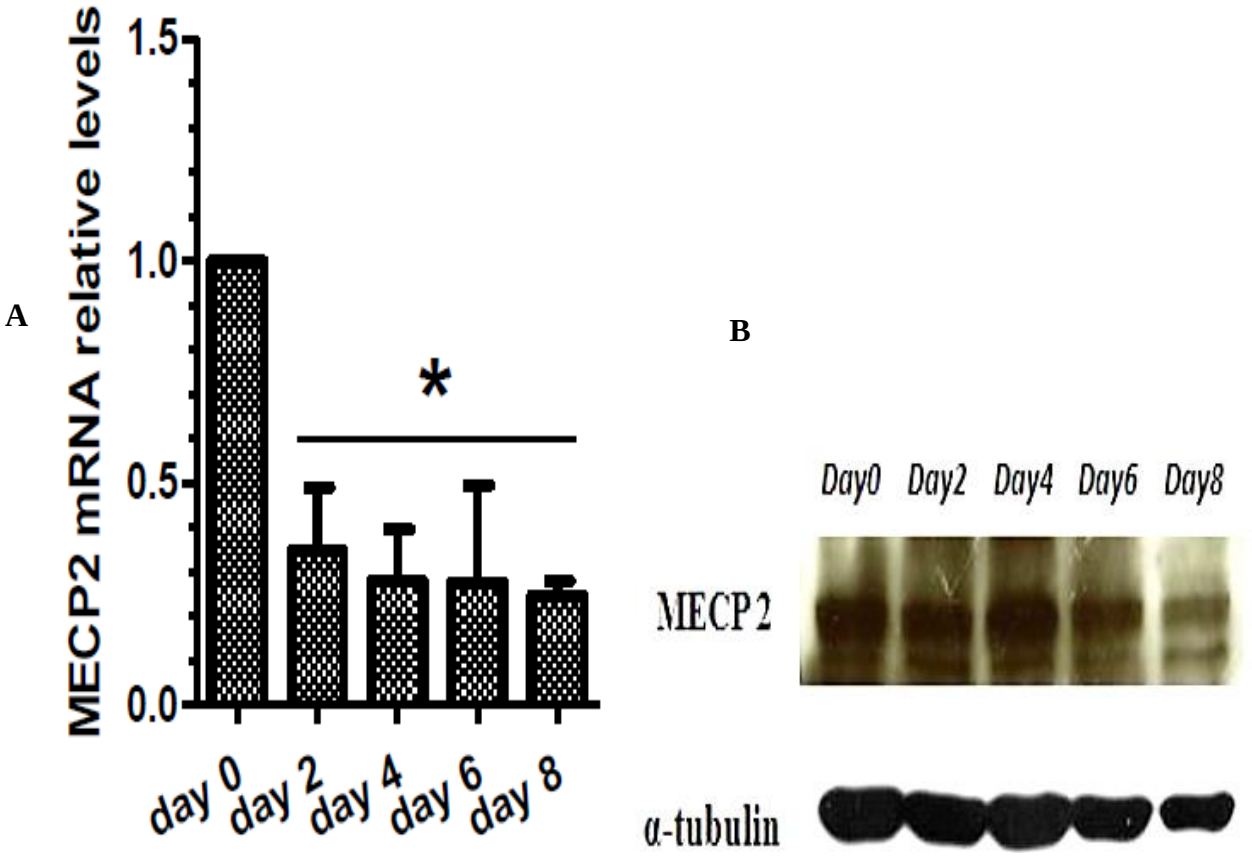


Figure 12 Downregulation of MECP2 during SMC differentiation from ES cells.

Undifferentiated ES cells were seeded onto T25 flasks pre-coated with 5 µg/ml collagen and cultured in DM for 2, 4, 6, and 8 days in a 37°C incubator. Cells were harvested at the indicated time points, and RNA and protein were extracted. (**A**) Total RNA from these cells was immediately isolated using the TRIzol method, as described in the *Materials and Methods*. RNA from the different collection time points was reverse transcribed into cDNA, and RT-PCR was performed using MECP2 and 18S rRNA specific primers. The mRNA abundance was normalized to the mRNA level of 18S rRNA and presented relative to expression on Day 0 (undifferentiated ES cells). The data presented here are the average of three independent experiments. *P < 0.05. (**B**) Total protein was harvested at the

indicated time points and detected by western blot with a MECP2 antibody. α-Tubulin was included as an internal control.

3.3.3. MECP2 is a miR-22 target gene in SMC differentiation

The causal relationships between MECP2 and miR-22 were first studied in miR-22 overexpression and inhibition experiments using RT-PCR and western blot analysis. Pre-miR™ mmu-miR-22 miRNA precursor (Ambion) or Anti-miR™ miR-22 inhibitor (Ambion) was transfected into differentiating cells at Day 2. The cells for gain-of-function and loss-of-function experiments were harvested 48 h after transfection and analysed with RT-qPCR. The data showed that miR-22 overexpression downregulated MECP2 gene expression and that miR-22 knockdown upregulated MECP2 gene expression, indicating a negative correlation between the expression of miR-22 and MECP2 during SMC differentiation (**Figure 13A**).

To confirm these observations, a miR-22 overexpression plasmid (pLL3.7-miR-22) and its control (pLL3.7 vector) were generated and transfected at the same time point (Day 2) into differentiating cells. Western blot analysis was performed to assess the protein expression of MECP2. The analysis showed that MECP2 protein expression was reduced when cells were transfected with pLL3.7-miR-22 (**Figure 13B and C**). These findings indicate that MECP2 is negatively regulated by miR-22 and strongly suggest that MECP2 is a miR-22 target gene during SMC differentiation.

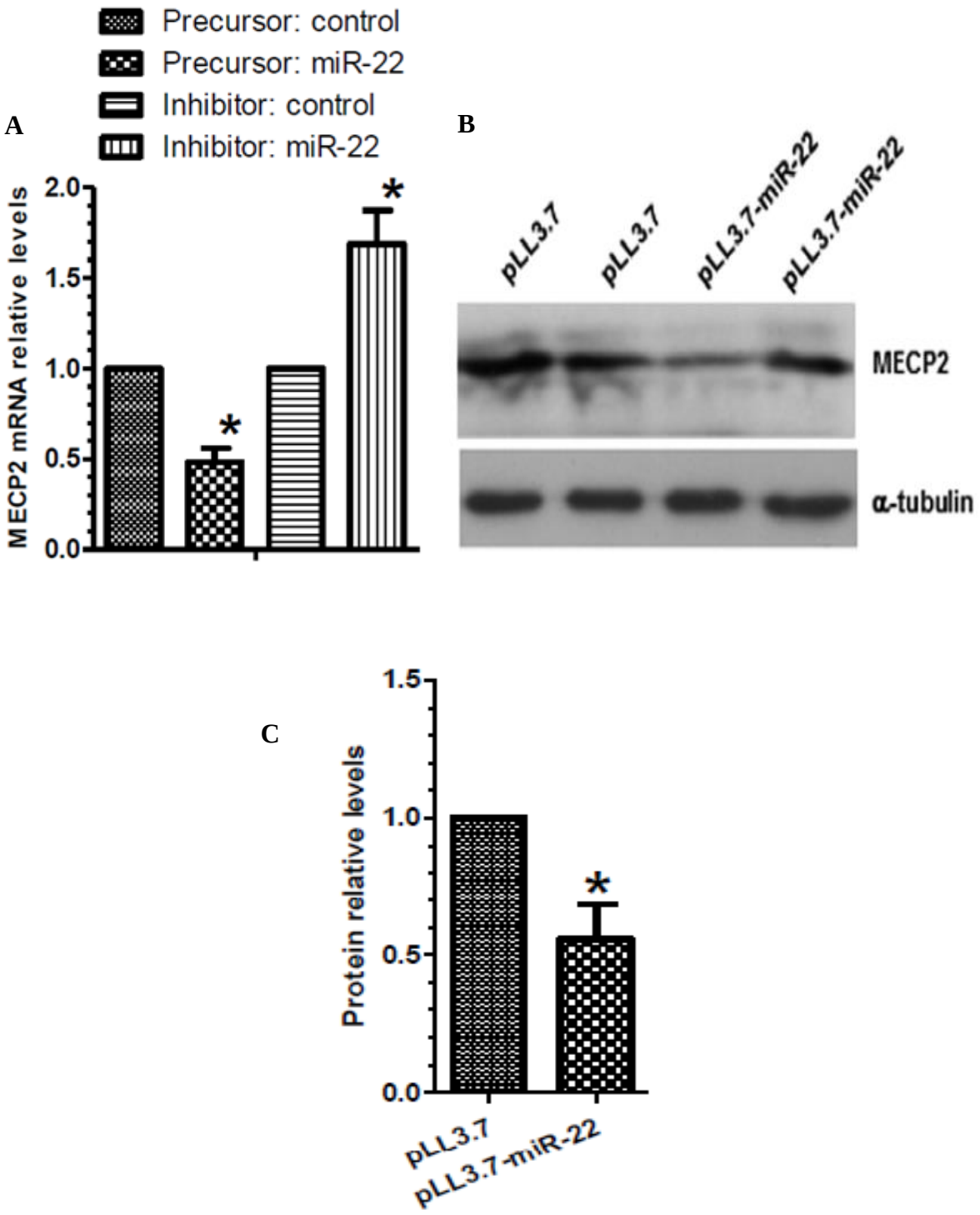


Figure 13 MECP2 is a miR-22 target gene during SMC differentiation.

(**A**) The gene expression of MECP2 after transfection of either Pre-miR™ mmu-miR-22 miRNA precursor (Ambion) or Anti-miR™ miR-22 inhibitor (Ambion) into differentiating

cells at Day 2. The data presented here are the mean ± SEM of three independent experiments. *P < 0.05 (vs. the respective negative control). (**B**) pLL3.7-miR-22 or a control plasmid was transfected into differentiating cells at Day 2. The samples were analysed by western blot with a MECP2 antibody (goat, N-17, sc-5755). The data presented here are representative of two independent experiments. (**C**) The blots were subjected to densitometric analysis with ImageJ software. *P < 0.05 (vs. control).

3.3.4. MECP2 is a specific target of miR-22

Our data suggest that MECP2 is a target of miR-22 during SMC differentiation from ES cells. We further wondered whether the MECP2 gene is a specific target gene of miR-22. To address this question, the precursors of miR-22, miR-34a (another SMC differentiation miRNA reported by our group (Yu et al., 2015)), and miR-150 (an endothelial cell differentiation miRNA reported by our group (Luo et al., 2013)) were transfected into HEK293 cell using TurboFect transfection agent (Thermo) as described in the *Materials and Methods*. Western blot results showed that MECP2 expression was clearly inhibited by miR-22 overexpression, but not by overexpression of miR-34a and miR-150, suggesting that MECP2 is a specific target of miR-22 (**Figure 14**).

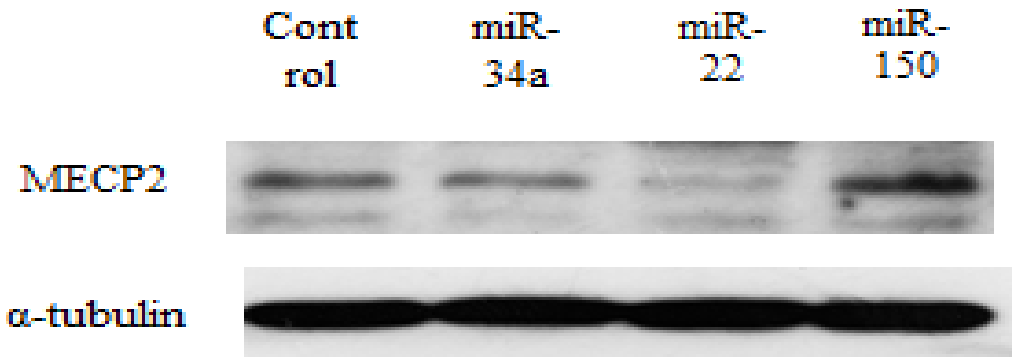


Figure 14 MECP2 protein expression is specifically inhibited by miR-22 overexpression.

The precursors of miR-34a, miR-22, and miR-150 and controls were transfected into HEK293 cells. At 48 h after transfection, total protein was harvested and subjected to western blot analyses with a MECP2 antibody. α-Tubulin was included in the western blot as an internal control.

3.3.5. MECP2 is negatively regulated by miR-22 through binding sites in the 3′-UTR

As mentioned previously, four conserved miR-22 binding sites with high scores have been identified in the 3′-UTR of the MECP2 gene using TargetScan (www.targetscan.org) (**Figure 15A**). To determine if MECP2 is regulated by miR-22 directly or indirectly, we first attempted to generate a miRNA reporter containing the full-length 3′-UTR of MECP2, which is about 8700 bp. Unfortunately, we were unsuccessful. Instead, we generated three separate MECP2 3′-UTR reporters, as indicated in **Figure 11**. The three reporters contained the first two miR-22 binging sites (~21 bp and 1241 bp), the third binding site (~2981 bp), and the fourth binding site (~6791 bp), and they were respectively designated pmiR-Luc-MECP2-A, -B, and -C (**Figure 15A**). A miRNA reporter assay demonstrated that the luciferase activity of reporters A and C, but not reporter B, was downregulated by miR-22 overexpression (**Figure 15B**). To determine which binding site within the 3′-UTR is the specific and direct binding site for miR-22, mutations in the miR-22 binding sites within the MECP2 3′-UTR were introduced, in accordance with the QuikChange Multi Site-Directed Mutagenesis Kit manual (Agilent Technologies). Luciferase activity analyses using the mutated miR-22 binding sites showed that the second binding site (within reporter A) and the forth binding site (within reporter C) were required for miR-22-mediated inhibition of MECP2 3′-UTR reporter activity (**Figure 15C**).

A

MECP2 3'UTR-A/bs1: 21 (5')-AAAGCAAACCAACAAGAATAAAGGCAGCTG TTGTCTCTTC-60 (3')

miR-22 : 3'-GUCAAGAAGUUGACCGUCGAA-5'

MECP2 3'UTR-A/bs1^{mu}: 21 (5')-AAAGCAAACCAACAAGAATA AA**TTTGAAG**GTTGTCTCTTC-60 (3')

MECP2 3'UTR-A/bs2:1241 (5')-TCTAGTTGTT TCT TCTGT TCCAGGCAGCTG CAGTGCTGAA-1280 (3')

miR-22 : 3'-GUCAAGAAGUUGACCGUCGAA-5'

MECP2 3'UTR-A/bs2^{mu} :1241 (5')-TCTAGTTGTTTCT TCTGTTCCA**TTTGAAG**G CAGTGCTGAA-1280 (3')

MECP2 3'UTR-B/bs:2981 (5')-GTCAGTCTCC TTTAAGCAGGCAGCTTTGGT GGGGTTTTTC-3020 (3')

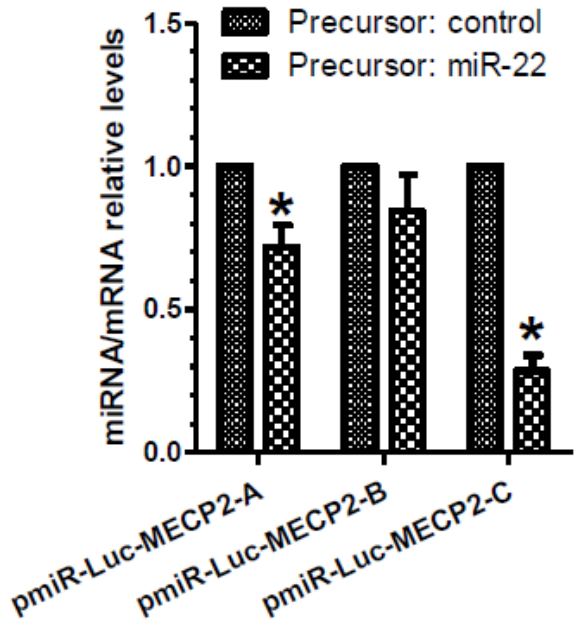
miR-22 : 3'-GUCAAGAAGUUGACCGUCGAA-5'

MECP2 3'UTR-C/bs: 6791 (5')-ACCTGGTTCA ATGT TTCTGGGCAGCTGGACAATGGAGTGC -6830 (3')

miR-22 : 3'-GUCAAGAAGUUGACCGUCGAA-5'

MECP2 3'UTR-C/bs^{mu} :6791 (5')-ACCTGGTTCA ATGT TTCTG**TTTGAAG**GGACAATGGAGTGC -6830 (3')

B



C

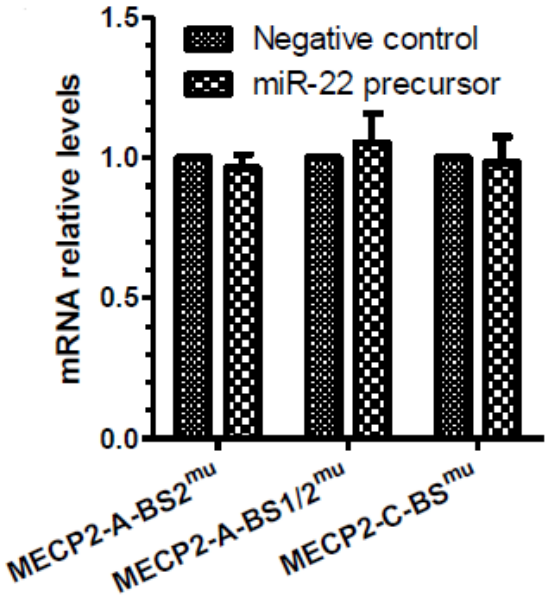


Figure 15 Binding sites within the 3′-UTR are required for MECP2 gene repression by miR-22.

(**A**) The potential binding sites of miR-22 within the MECP2 3′-UTR as predicted by TargetScan are depicted. (**B and C**) Binding sites located around ~1241 and ~6791 of the

MECP2 3′-UTR are required for miR-22-mediated MECP2 gene repression. Undifferentiated ES cells were seeded in flasks coated with 5 µg/ml collagen and cultured for 2 days in DM before transfection. According to the co-transfection protocol, miR-22 and an individual reporter were co-transfected into Day 2 differentiating cells, and the luciferase activities were measured at 48 h post transfection. (**B**) MiR-22 precursor or a negative control and three wild-type MECP2 3′-UTR reporters (pmiR-Luc-MECP2-A, -B, and -C) were co-transfected into differentiating ES cells, and their luciferase activity was assayed. (**C**) The luciferase activities of the three indicated mutants [pmiR-Luc-MECP2-A bindings site 2 (bs2-mu), combined mutations (bs1/2-mu), and pmiR-Luc-MECP2-C bindings site (bs-mu)] were analysed when miR-22 was overexpressed at the same time. 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 Renilla activity in the control samples was set at 1.0.

3.4. MECP2 inhibition is required for miR-22-mediated SMC differentiation from ES cells

3.4.1. MECP2 knockdown increases SMC gene expression

We have provided solid evidence to support that MECP2 is an authentic miR-22 target during SMC differentiation. Therefore, MECP2 was knocked down in differentiating ES cells using a specific MECP2 small interfering RNA (MISSION® esiRNA, EMU085661-20UG) to investigate the functional involvement of MECP2 in the differentiation of ES cells toward SMCs. qPCR was used to analyse the gene expression of various SMC markers (SMαA, SM22α, h1-calponin, and SM-myh11) and MECP2. As expected, MECP2 gene expression was downregulated by MECP2-specific siRNA (**Figure 16)**. Meanwhile, the gene expression of four SMC differentiation markers was markedly increased by MECP2 knockdown, suggesting that MECP2 inhibition can recapitulate the effect of miR-22 in the differentiation of ES cells to SMCs (**Figure 16**).

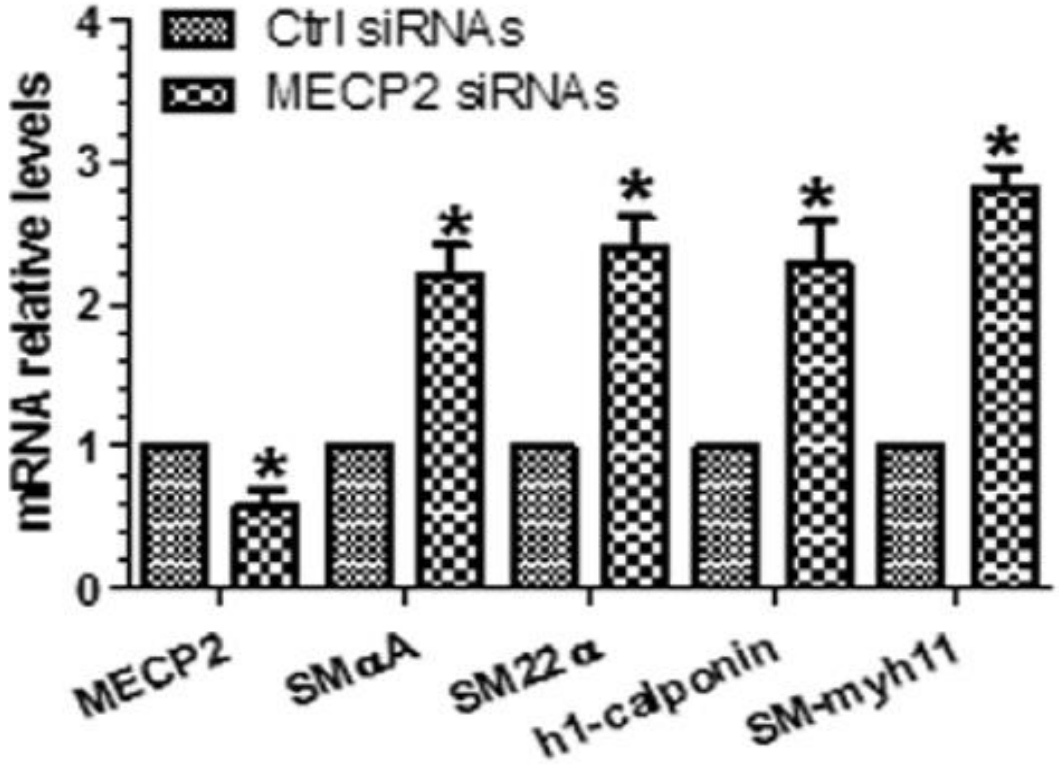


Figure 16 MECP2 knockdown increases SMC gene expression.

Day 2–3 differentiating embryonic stem (ES) cells were respectively transfected with control small interfering RNA (ctrl siRNAs) or MECP2-specific siRNA (MECP2 siRNAs) and cultured for 48 or 72 h in SMC differentiation medium (DM). Total RNA was harvested and subjected to RT-qPCR analyses. The data presented here are the mean ± SEM of three to four independent experiments. *P < 0.05 (MECP2 siRNA vs. control siRNA).

3.4.2. MECP2 overexpression inhibits SMC gene expression

As mentioned earlier, MECP2 knockdown increased SMC gene expression. To investigate the function of MECP2 in the regulation of SMC genes, control (pCMV5) and MECP2 overexpression (pCMV5-MECP2) vectors were generated and transfected into Day 2 or Day 3 differentiating ES cells, respectively. The data showed that overexpression of MECP2 increased MECP2 expression at the gene and protein levels (**Figure 17)**. The gene expression of four SMC specific markers (SMαA, SM22α, SM-myh11, and h1-calponin) was significantly repressed by MECP2 overexpression (**Figure 17A**), suggesting that MECP2 acts as a repressor of SMC differentiation genes during SMC differentiation. Western blot data showed a similar trend for MECP2, SMαA, and SM-MHC expression at the protein level (**Figure 17B and C**).

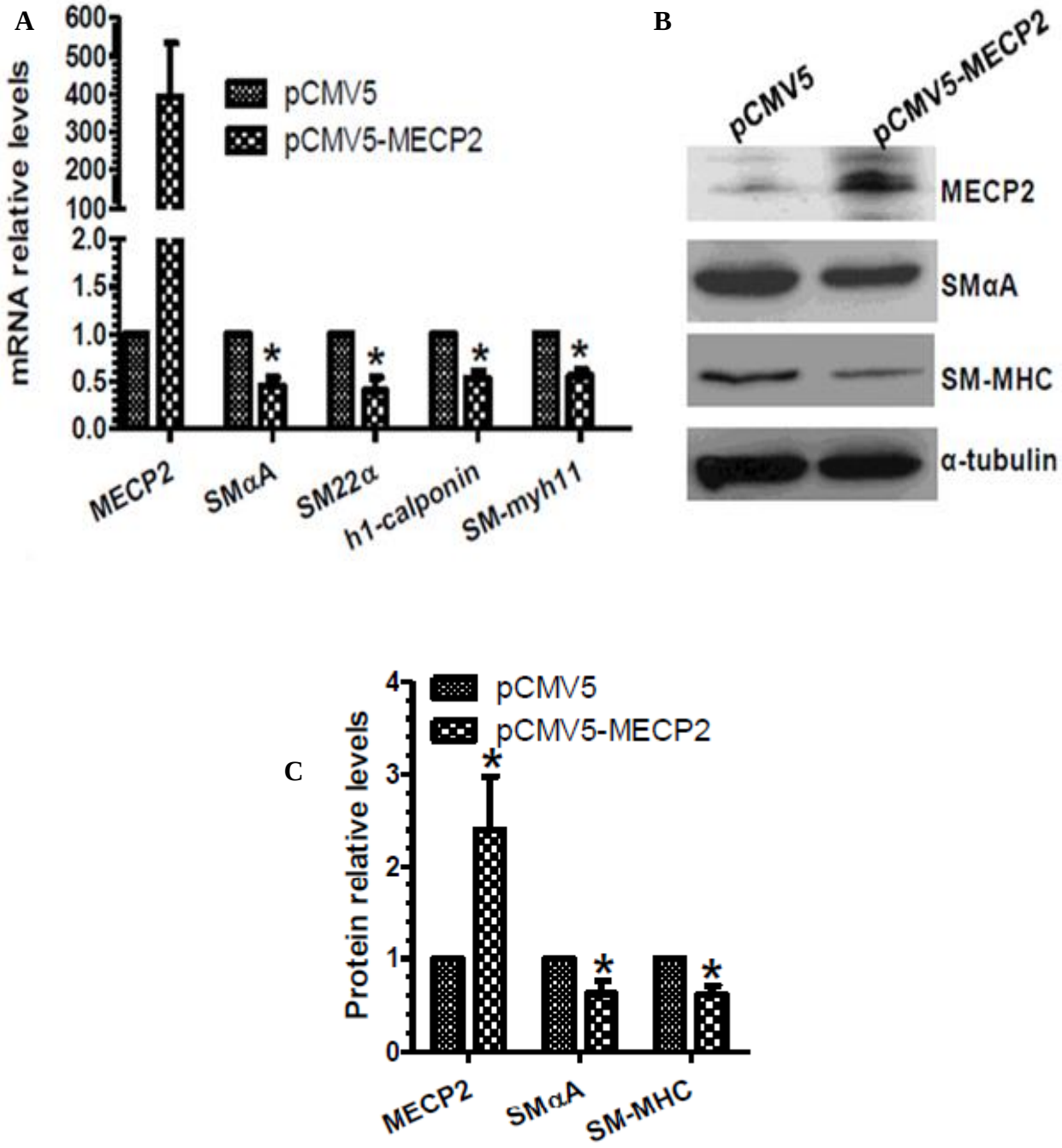


Figure 17 MECP2 overexpression inhibits SMC gene and protein expression.

Day 2–3 differentiating ES cells were transfected with a control (pCMV5) or MECP2 overexpression (pCMV5-MECP2) plasmid per the manufacturer's instructions and cultured in SMC differentiation medium (DM) for 48 or 72 h at 37°C in a 5% CO_2 incubator. Total RNA and protein were harvested and subjected to RT-qPCR (**A**) and

western blotting (**B and C**) analyses, respectively. (**B**) α-Tubulin was used as a control. (**C**) The blots were subjected to densitometric analysis with ImageJ software. The data presented here are representative or the mean ± SEM of three independent experiments, *P < 0.05 vs. control (pCMV5 vector).

3.4.3. MECP2 overexpression abolishes SMC gene expression induced by miR-22.

To investigate further the functional importance of MECP2 in miR-22-mediated SMC differentiation, a control or MECP2 overexpression vector was transfected into Day 2–3 differentiating control (pLL3.7-GFP) or miR-22-overexpressing (pLL3.7-GFP-miR-22) ES cells, and the gene expression of MECP2, miR-22, SMC differentiation genes (SMαA and SM-MHC), SMC transcription factors (SRF, Myocd, and MEF2C), and other reported SMC differentiation regulators (Nox4, HDAC7, and Pla2g7) were analysed with RT-qPCR. Data showed that the MECP2 expression was successfully downregulated and upregulated by miR-22 (2^{nd} columns) and MECP2 (3^{rd} columns) overexpression in differentiating ES cells, respectively (**Figure 18A**). Consistent with our previous observation, SMαA and SM-MHC gene expression was upregulated and downregulated by miR-22 (2^{nd} columns) and MECP2 (3^{rd} columns) overexpression, respectively (**Figure 18B**). Importantly, our co-transfection data (4^{th} columns) showed that re-activation of MECP2 almost completely abolished the SMC-specific gene (SMαA and SM-MHC) upregulation induced by miR-22 overexpression (compare 4^{th} columns with 2^{nd} columns) (**Figure 18B**), implying that MECP2 inhibition/repression is required for miR-22-mediated SMC gene expression. Additional analyses with these samples revealed a similar trend for SMC transcription factors (SRF and Myocd) and other SMC differentiation regulators (Nox4, HDAC7, and Pla2g7) (**Figure 19)**, suggesting that miR-22 and MECP2 work in concert with these transcription factors and SMC differentiation regulators to modulate SMC differentiation from stem cells.

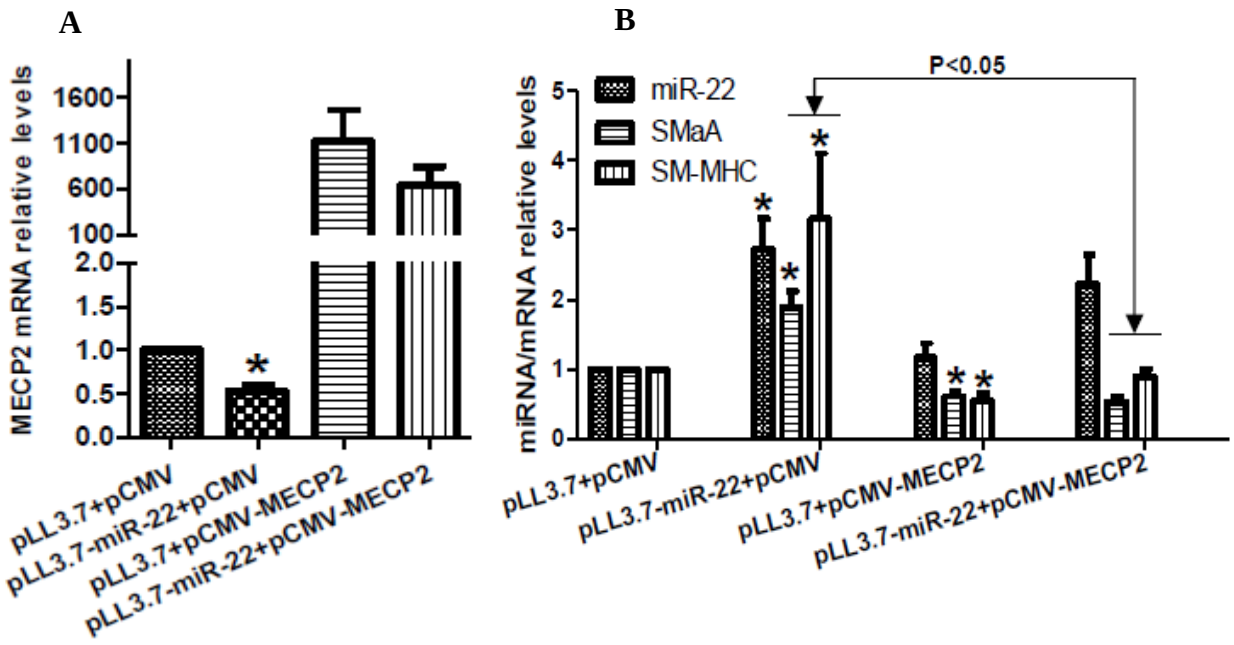


Figure 18 MECP2 overexpression abolishes SMC gene expression induced by miR-22.

Day 2 or 3 differentiating control ES cells (pLL3.7-GFP) and miR-22-overexpressing ES cells (pLL3.7-GFP-miR-22) were transfected with control (pCMV5) or MECP2 overexpression (pCMV5-MECP2) plasmids, respectively. The transfected cells were cultured in normal SMC differentiation medium (DM) for 48 to 72 h before cell collection. Total RNA was harvested and subjected to RT-qPCR analysis with primers for MECP2 (**A**) and miR-22, SmαA, and SM-MHC (**B**). The data presented here are the mean ± SEM of three independent experiments. *P < 0.05 (vs. control group/1st columns).

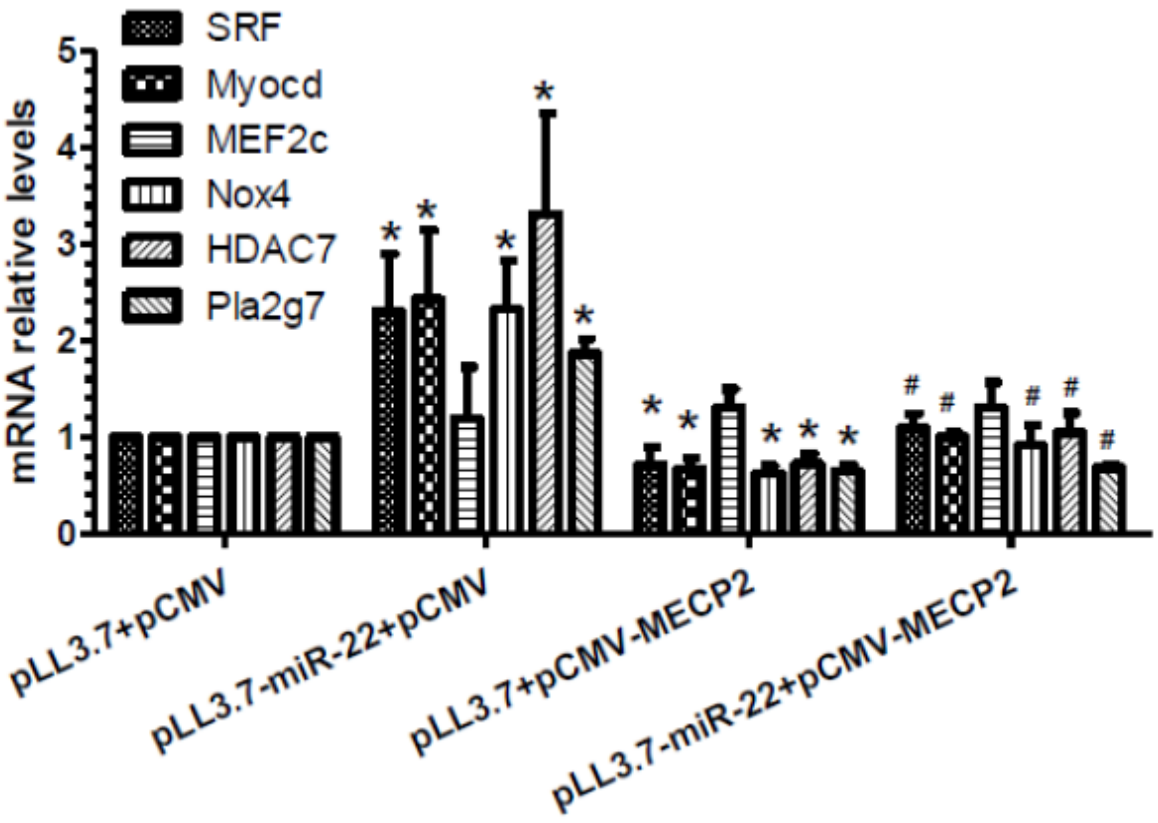


Figure 19 MECP2 overexpression abolishes the gene expression of SMC transcription factors and other reported SMC differentiation regulators mediated by miR-22.

Total RNA and cDNA were obtained from the experiments described in **Figure 18** and subjected to RT-qPCR analysis with specific primers as indicated. The data presented here are the mean ± SEM of three independent experiments. *P < 0.05 (vs. control group/1st columns), #P < 0.01 (vs. 2nd columns).

3.5. Mechanism underlying MECP2-mediated SMC gene repression

3.5.1. Functional importance of the SRF binding site within SMC-specific gene promoters in MECP2-mediated SMC gene expression

As shown above, we have demonstrated clearly that SMC-specific gene expression is repressed by MECP2 gene activation. To understand the underlying molecular mechanism of MECP2-mediated SMC gene expression, two SMC gene promoter reporters, used in our group's previous study (Huang et al., 2013), pGL3-Luc-SMαA and pGL3-Luc-SM22α, were transfected into Day 2 differentiating ES cells. The luciferase activities of the reporters were measured according to the protocol described in the *Materials and Methods*. Overexpression of MECP2 significantly inhibited SMαA and SM22α gene promoter activities in differentiating ES cells (**Figure 20A**), indicating that overexpression of MECP2 represses specific SMC gene expression at the transcriptional level.

It has been widely reported that the SRF binding element (CArG) within the promoter region of SMC-specific genes is important not only for SMC gene regulation but also for SMC differentiation from vascular $CD34^{+}$ stem/progenitor cells and normal stem/progenitor cells (Miano et al., 2004, Wu et al., 2015, Huang et al., 2013). Therefore, we asked whether the SRF element also plays a role in MECP2-mediated SMC gene repression. To answer this question, two SMC-specific gene promoters with SRF binding site mutations (pGL3-Luc-SMαA-SRF^{mu} and pGL3-Luc-SM22α-SRF^{mu}), produced in our

previous study (Huang et al., 2013), were transfected into differentiating ES cells. Luciferase assays were performed 48 to 72 h post transfection. Mutation of the SRF binding element within the SMC gene promoters almost completely nullified the inhibitory effects of MECP2 overexpression on SMC gene transcriptional activity (**Figure 20A**), suggesting that the SRF binding site within the promoters is required for MECP2-mediated SMC gene repression. To determine whether MECP2 bound directly to the promoters of the SMC genes during SMC differentiation, a control (pCMV5) or MECP2 overexpression (pCMV5-MECP2) plasmid was transfected into differentiating ES cells, and binding was analysed with the ChIP assay, as described in the *Materials and Methods*. We observed no significant enrichment of MECP2 within the promoter regions of the SMC genes (SMαA and SM22α) in ChIP assays using a MECP2-specific antibody (Abcam), suggesting no direct binding of MECP2 to SMC gene promoters (**Figure 20B**).

Furthermore, another set of ChIP assay using an SRF antibody (Sigma) was performed to assess whether MECP2 modulates SRF binding to SMC-specific gene promoters. As expected, we observed a significant enrichment of SRF within SMαA and SM22α gene promoters (up to 10-fold enrichment). However, no significant difference in SRF enrichment at either SMC gene promoter was observed in control and MECP2 overexpressing cells (**Figure 20C**), indicating that MEPC2 plays no major role in regulating the direct binding of SRF to SMC gene promoters.

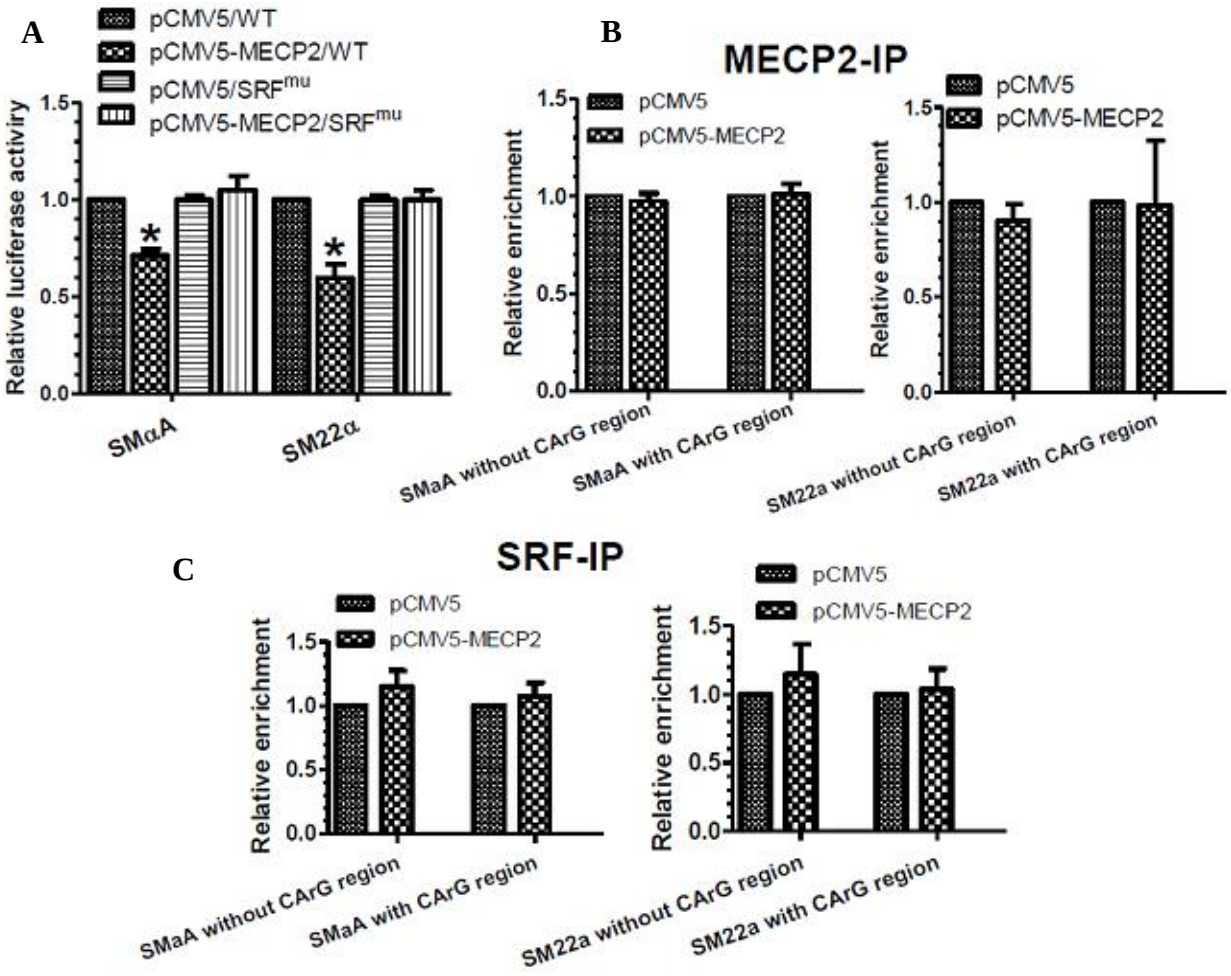


Figure 20 SRF binding site is required for MECP2-mediated SMC gene expression.

(**A**) SRF binding site mutations abolish the promoter activity of SMC differentiation genes induced by MECP2 overexpression. Undifferentiated ES cells were cultured in 24-well plates. Plasmids and mutants were transfected into the cells at 48 h or 72 h post differentiation. The data presented here are normalized from three independent experiments. *P < 0.05 (vs. pCMV5 control group). (**B and C**) Undifferentiated ES cells were cultured in T75 flasks. Control (pCMV5) or MECP2 overexpression (pCMV5-MECP2) plasmids were transfected into Day 2 or 3 differentiating ES cells. ChIP assays were performed using antibodies against MECP2 and SRF, as well as respective normal IgG, as described in the *Materials and Methods*. PCR amplification of the non-CArG

regions was included as an additional control for specific promoter DNA enrichment. (**B**) The ChIP assay showed no direct binding of MECP2 to the promoter regions of SMC differentiation genes. (**C**) The binding capacity of SRF at the promoter regions of SMC differentiation genes was not affected by MECP2 overexpression.

3.5.2. MECP2 represses SMC transcription factors

Our previous data showed that the gene expression of SRF and Myocd, but not MEF2c, was upregulated by miR-22 overexpression and downregulated by miR-22 knockdown (**Figure 9)**, suggesting that miR-22 regulates SMC differentiation from stem cells by regulating these two SMC transcription factors. Therefore, we wondered whether MECP2, as a miR-22 target, regulates SMC transcription factors during SMC differentiation. To answer this question, the pCMV5 (control) or pCMV5-MECP2 (MECP2 overexpression) plasmid was transfected into differentiating cells, and the gene expression of SRF, Myocd, and MEF2C was analysed with RT-qPCR. The RT-qPCR results showed that two transcription factors, SRF and Myocd, were regulated by MECP2 in a manner opposite to that induced by miR-22, implying an important role for MECP2 in the regulation of these two transcription factors during SMC differentiation (**Figure 21A**). Consistently, no distinct change in the expression of another transcription factor, MEF2C, was observed in cells overexpressing MECP2, suggesting that neither miR-22 nor its target MECP2 regulates MEF2C gene expression during SMC differentiation. To confirm the findings, luciferase activity assays were performed using the SRF, MEF2C, and Myocd gene promoter reporter plasmids (pGL3-Luc-SRF, pGL3-Luc-MEF2c, and pGL3-Luc-Myocd) generated in our previous study (Huang et al., 2013) under MECP2 overexpression conditions. The luciferase activities of the SRF and Myocd reporters were downregulated by MECP2 overexpression. On the other hand, MECP2 overexpression did not affect the luciferase activity of the MEF2C reporter (**Figure 21B**), confirming that MECP2 regulates SMC differentiation through modulation of the transcription factors SRF and Myocd.

CHIP assays with a MECP2-specific antibody were conducted to examine whether MECP2 directly interacts with the SRF and/or Myocd gene promoters. In our previous study (Huang et al., 2013), four pairs of specific primers spanning the promoter regions of the SRF, MEF2c, and Myocd genes were designed and used to detect the regions within the promoters of these genes that potentially interact with hnRNPA1. In this study, the four primer pairs for the SRF and Myocd gene promoters were assessed in a preliminary study, and the best primer pairs were chosen for use in the following ChIP experiments. As shown in **Figure 21C**, a significant enrichment of MECP2 within the promoter regions of SRF (up to 3-fold) and Myocd (up to 20-fold) was observed, and the enrichment were enhanced by MECP2 overexpression (**Figure 21C**), suggesting that MECP2 directly binds to the SRF and Myocd gene promoters. Taken together, our data demonstrate that MECP2 transcriptionally represses the gene expression of two SMC transcription factors (SRF and Myocd) during SMC differentiation from stem cells through its direct binding to the promoter regions of SRF and Myocd.

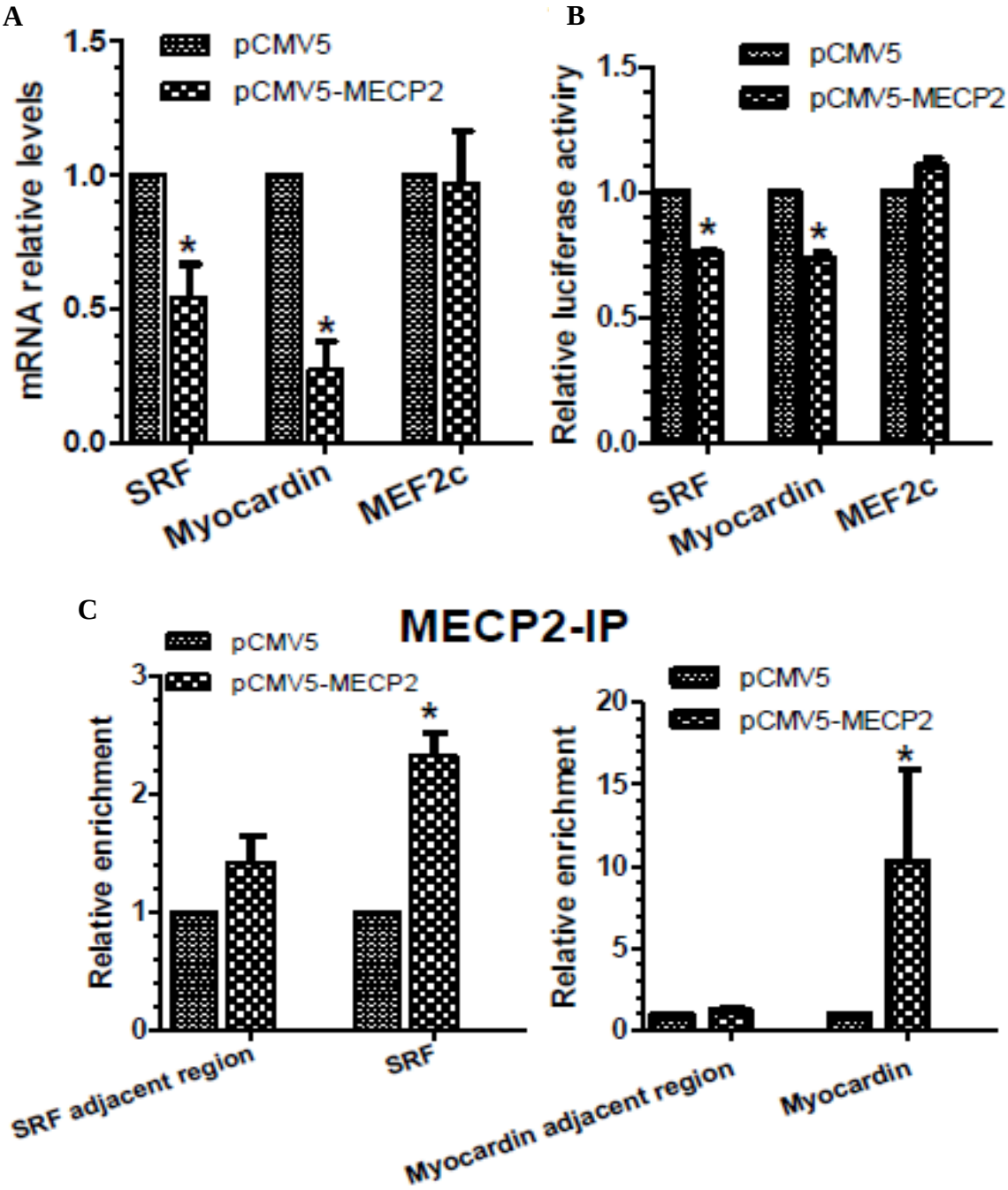


Figure 21 MECP2 negatively regulates SMC transcription factor gene expression.

(**A**) The expression of SRF and Myocd, but not MEF2c, is significantly downregulated by MECP2 overexpression. Total RNA was harvested as described in **Figure 17A**. (**B**) The promoter activities of the SRF and Myocd genes are modulated by MECP2. Day 2–3 differentiating ES cells were transfected with the luciferase reporter plasmid pGL3-Luc-

SRF, pGL3-Luc-MEF2c, or pGL3-Luc-Myocd (0.15 μg/2.5 × 10^4 cells) together with pCMV5 or pCMV5-MECP2 (0.2 μg/2.5 × 10^4 cells). pShuttle-LacZ (0.2 μg/2.5 × 10^4 cells) was included as a control. Luciferase and β-galactosidase activities were detected 48 h after transfection. The data presented here are the mean ± SEM of four independent experiments. *P < 0.05 (vs. control). (**C**) MECP2 binds directly to the promoter regions of the SRF and Myocd genes. ChIP assays were performed using antibodies against MECP2 or normal IgG as described in the *Materials and Methods*. PCR amplification of the adjacent regions was included as an additional control for specific promoter DNA enrichment. The data presented here are the mean ± SEM of three independent experiments. *P < 0.05 (vs. control).

3.6. Other SMC differentiation regulators are regulated by miR-22 and MECP2

In our previous studies, several genes were identified as SMC differentiation regulators, including nuclear factor erythroid 2-related factor 3 (*Nrf3*) (Pepe et al., 2010), NADPH oxidase 4 (*Nox4*) (Xiao et al., 2009), platelet-activating factor acetylhydrolase (*Pla2g7*) (Xiao et al., 2012), histone deacetylase 7 (*HDAC7*) (Margariti et al., 2009) and DNA/RNA binding proteins [heterochromatin protein 1γ (*Cbx3*) (Xiao et al., 2011), heterogeneous nuclear ribonucleoprotein (*hnRNP*) A2B1 (Wang et al., 2012) and A1(Huang et al., 2013)]. Thus, we wondered whether miR-22 and MECP2 play a role in the regulation of these genes. To this aim, the gene expression of the aforementioned SMC differentiation regulators was therefore examined in miR-22- or MECP2-overexpressing cells. RT-qPCR data showed that the expression of Nox4, HDAC7, hnRNPA2B1, and Pla2g7, but not Cbx3, hnRNPA1, and Nrf3, was significantly upregulated in differentiating cells with miR-22 overexpression (**Figure 22A**). Importantly, only Nox4, HDAC7, and Pla2g7 were downregulated by MECP2 overexpression (**Figure 22B**). Taken together, our data showed that three SMC differentiation factors, Nox4, HDAC7, and Pla2g7, were inversely regulated by miR-22 (**Figure 22A**) and MECP2 (**Figure 22B**), suggesting that miR-22 works in concert with MECP2 in the regulation of these three genes. Interestingly, these three genes were co-regulated by miR-22 and MECP2 in a manner similar to that of other SMC differentiation genes, such as SRF and Myocd (**Figure 19)**. In summary, we demonstrated that miR-22 and MECP2 mediate SMC differentiation at least partially through regulation of the SMC differentiation modulators Nox4, HDAC7, and Pla2g7.

To study the relationship between MECP2 and Pla2g7, luciferase assays were carried out in Day 2–3 differentiating ES cells using a functional Pla2g7 gene promoter reporter (pGL3-Luc-Pla2g7-P2, harbouring 387 bp of a Pla2g7 gene promoter fragment located ~2.4 to 2.0 kb upstream of the translation start site of the Pla2g7 gene) and a control reporter (pGL3-Luc-Pla2g7-P10, located within exon 1 of the Pla2g7 gene) generated in our previous study (Huang et al., 2013). The data from the luciferase assays showed that the promoter activity of pGL3-Luc-Pla2g7-P2, but not pGL3-Luc-Pla2g7-P10, was regulated by MECP2 overexpression (**Figure 22C)**.

Furthermore, ChIP assays were conducted using an antibody against MECP2 in differentiating ES cells to verify that MECP2 regulates Pla2g7 gene expression by binding directly to it promoter. The results showed MECP2 enrichment (up to 4-fold) at the Pla2g7 gene promoter (region 2); MECP2 overexpression increased the accumulation of MECP2 at the Pla2g7 gene promoter, while no apparent enrichment of MECP2 at an adjacent promoter area (region 10) was observed (**Figure 22D)**. Taken together, these findings demonstrate that MECP2 regulates Pla2g7 gene expression through direct interaction with region 2 (-2.4 to -2.0 kb) of the Pla2g7 gene promoter. Likewise, enrichment of MECP2 observed in the promoter regions of Nox4 and HDAC7 was increased by MECP2 enforced expression (**Figure 22F**), suggesting that MECP2 also binds directly to the promoters of Nox4 and HDAC7. The findings indicate that MECP2 binds directly to the promoter regions of three SMC differentiation regulators. Luciferase activity analysis using the gene promoter reporters for Nox4 and HDAC7 demonstrated that MECP2 overexpression inhibited not only the promoter activity of Pla2g7, but also that of Nox4 and HDAC7 (**Figure 22E**).

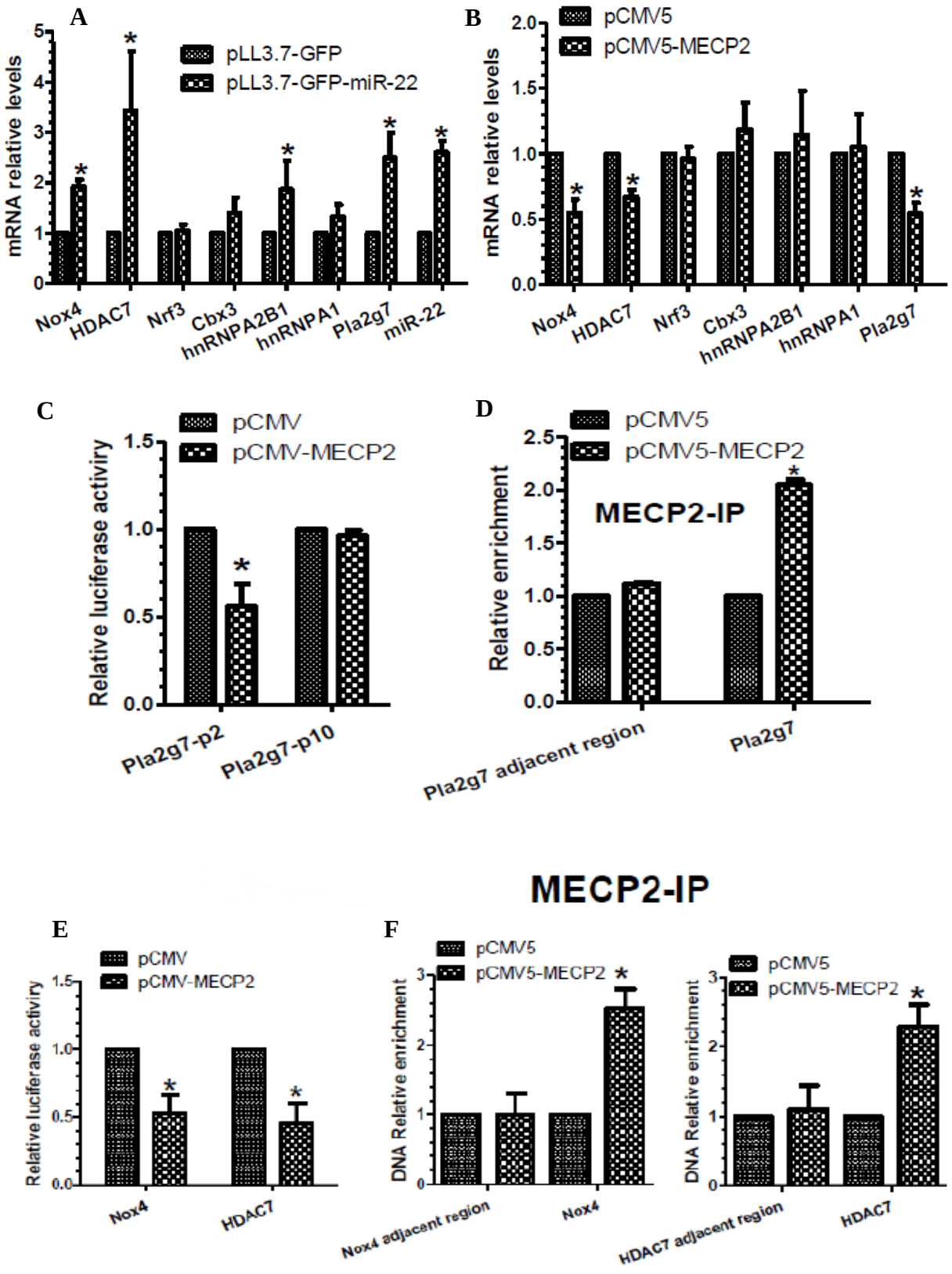


Figure 22 MECP2 negatively regulates other reported SMC differentiation regulators.

(**A**) Undifferentiated control (pLL3.7-GFP) cells or miR-22-overexpressing (pLL3.7-GFP-miR-22) cells were cultured in differentiation medium for 2–3 days. RNA was harvested, and qPCR was performed using primers for SMC regulators (Nox4, Nrf3, HDAC7, Cbx3, hnRNPA1, hnRNPA2B1, and Pla2g7) and miR-22. (**B**) Three reported SMC differentiation regulators were regulated by MECP2. A control (pCMV5) or MECP2 overexpression (pCMV5-MECP2) plasmid was transfected into Day 2–3 differentiating ES cells, and total RNA was harvested after 48 h culture as described in **Figure 17A**. (**C**) The promoter activity of the Pla2g7 gene was repressed by MECP2 overexpression. Day 2–3 differentiating ES cells were transfected with the luciferase reporter plasmids pGL3-Luc-Pla2g7-2 or pGL3-Luc-Pla2g7-10 (0.15 μg/2.5 × 10^4 cells) together with a control (pCMV5) or MECP2 overexpression (pCMV5-MECP2) plasmid at a concentration of 0.2 μg/2.5 × 10^4 cells. The data presented here are the mean ± SEM of four independent experiments. (**D**) MECP2 binds directly to the promoter (region 2) of the Pla2g7 gene. ChIP assays were performed as usual. PCR amplification of the adjacent region (region 10) was included as an additional control for specific promoter DNA enrichment. The data presented here are the mean ± SEM of three independent experiments. (**E**) The promoter activities of the Nox4 and HDAC7 genes were suppressed by MECP2 overexpression. Day 2–3 differentiating ES cells were transfected with the luciferase reporter plasmids pGL3-Nox4-Luc or pGL3-HDAC7-Luc (0.15 μg/2.5 × 10^4 cells) together with pCMV5 or pCMV5-MECP2 (0.2 μg/2.5 × 10^4 cells). pShuttle-LacZ (0.2 μg/2.5 × 10^4 cells) was used as a control. Luciferase and β-galactosidase activities were measured 48 h after transfection. (**F**) MECP2 binds directly to the promoter regions of the Nox4 and HDAC7 genes. PCR amplification of the adjacent regions was included as an additional control for specific promoter DNA enrichment. The data presented here are the mean ± SEM of four independent experiments. *$P < 0.05$ (vs. control).

3.7. MECP2 represses SMC gene expression by increasing H3K9 methylation within gene promoters

It has been well-documented that MECP2 participates in Rett syndrome (Coughlan et al.) by binding specifically to methylated DNA, and methylation of lysine H3K9 is closely associated with gene transcriptional repression (Rosenfeld et al., 2009, Chen et al., 2015). In addition, MECP2, a member of the methyl CpG-binding domain (MBD) family, is a candidate for the readout of DNA methylation because members of the family recruit chromatin remodellers, histone deacetylases, and methylases to methylated DNA associated with gene repression (Du et al., 2015).

To elucidate the molecular mechanism by which MECP2 represses SMC gene expression during SMC differentiation from ES cells, we first examined whether MECP2 regulates H3K9me3 levels using western blot. Data from MECP2 overexpression experiments showed that the levels of H3K9me3 in control and MECP2-overexpressing cells were similar (**Figure 23A**), suggesting that H3K9me3 levels were not significantly affected by MECP2 overexpression. However, ChIP assays using an H3K9me3-specific antibody showed variations in H3K9me3 enrichment within the promoter regions of the examined genes: up to 2.5-fold for SmαA and SM22α, 23-fold for SRF, 40-fold for Myocd, and 96-fold for Pla2g7. Importantly, MECP2 overexpression enhanced H3K9me3 enrichment within the gene promoters of SRF, Myocd, and Pla2g7 (**Figure 23C and D**), consistent with the enrichment of MECP2 within the promoters of these genes as shown in the CHIP assays with a MECP2 antibody (**Figure 21C** and **Figure 22D**). On the other hand, no further enrichment of H3K9me3 with MECP2 overexpression was observed in the promoters of SmαA and SM22α (**Figure 23B**). As described earlier, both MECP2 and

miR-22 are involved in the regulation of some SMC differentiation regulators, such as SRF, Myocd, and Pla2g7. Therefore, additional ChIP assays using an H3K9me3 antibody or IgG were performed in cells with or without miR-22 enforced expression. The ChIP assay data showed that miR-22 overexpression did not affect H3K9me3 enrichment levels in the promoters of the SMαA (**Figure 24A**) and SM22α genes (**Figure 24B**). As expected, enrichment of H3K9me3 within the promoter regions of SRF (**Figure 24C**), Myocd (**Figure 24D**), and Pla2g7 (**Figure 24E**) was decreased by miR-22 overexpression, indicating that enforced miR-22 expression significantly suppressed the enrichment of H3K9me3 within the three promoter regions.

In summary, our data demonstrate that MECP2 inhibits SMC-specific gene expression partially by increasing H3K9 trimethylation within the gene promoters of SMC-specific transcription factors (SRF and Myocd) and differentiation modulators (e.g. Pla2g7).

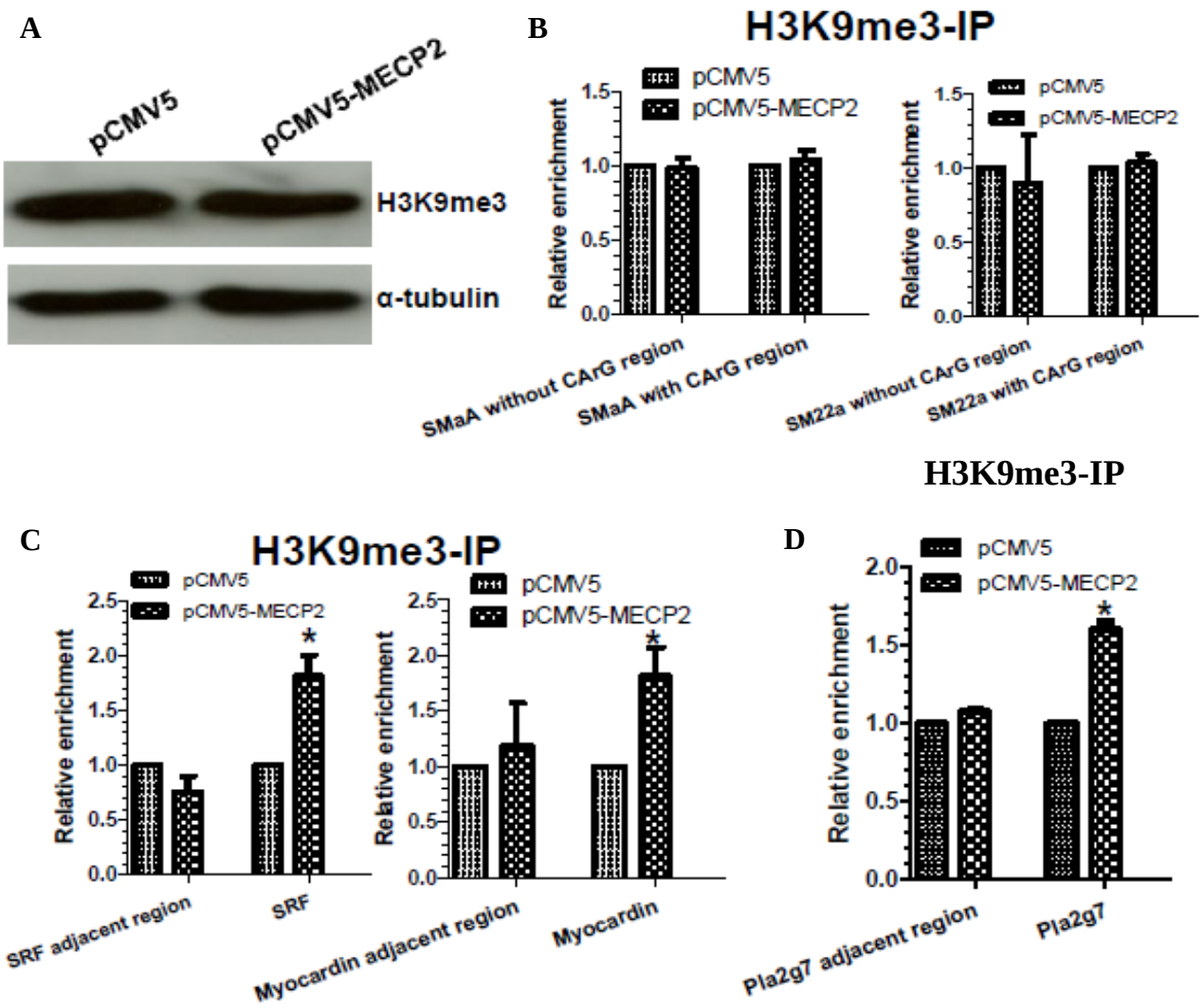


Figure 23 MECP2 increases the binding of H3K9me3 to the gene promoter regions of SRF, Myocd, and Pla2g7.

(**A**) H3K9me3 protein levels were not affected by MECP2 overexpression. Total protein was harvested as described in the *Material and Methods*. α-Tubulin was included as an internal control. Data presented here are representative of three experiments. (**B–D**) H3K9me3 CHIP assays. ChIP assays were performed using an antibody against H3K9me3 (Millipore) or normal mouse IgG. The data presented here are the mean ± SEM of three independent experiments. *P < 0.05 (vs. control). (**B**) The enrichment of H3K9me3 within the promoter regions of the SMαA and SM22α genes is not affected by MECP2

overexpression. (**C**) MECP2 overexpression increases H3K9me3 binding to the promoter regions of the SRF and Myocd genes. (**D**) The enrichment of H3K9me3 within the promoter region of the Pla2g7 gene was significantly increased by overexpression of MECP2.

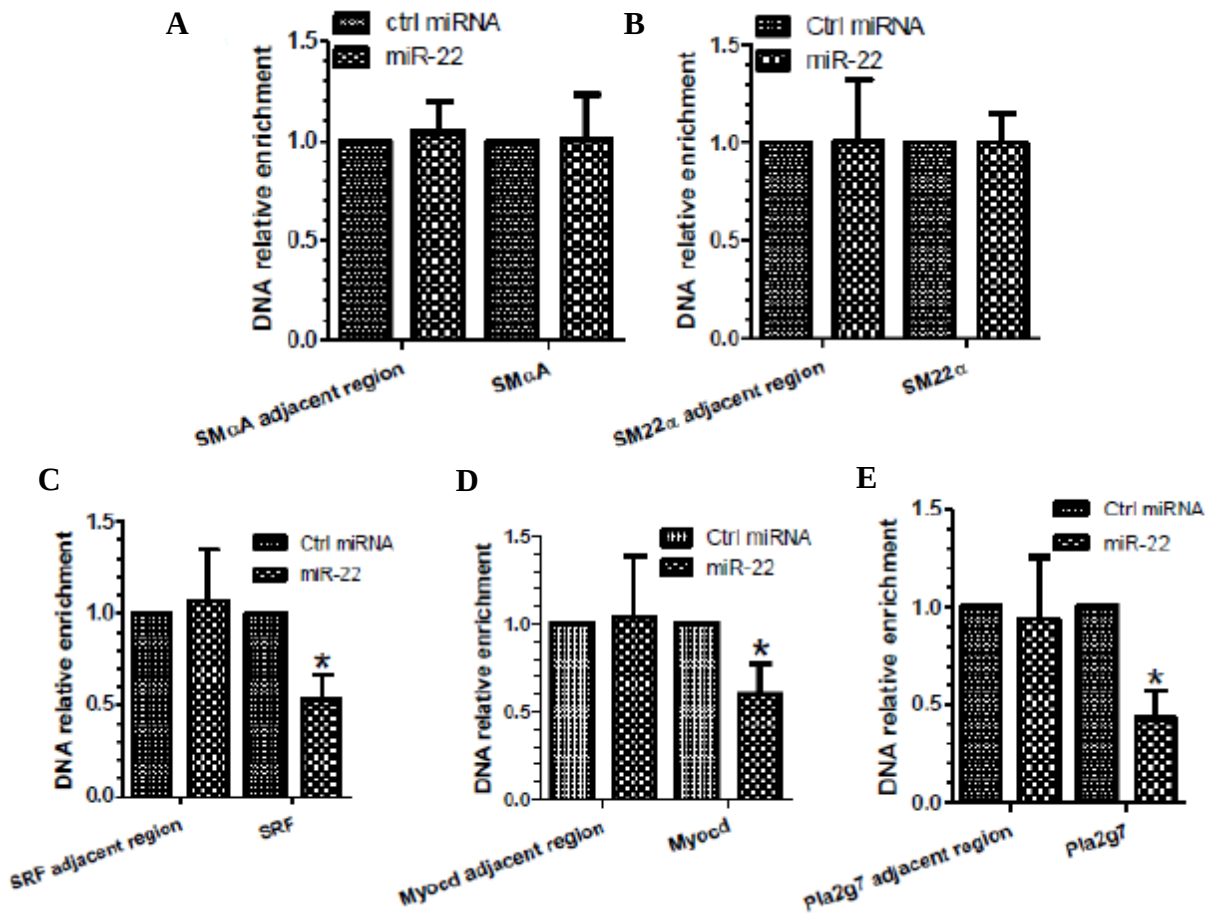


Figure 24 MiR-22 overexpression decreases H3K9 methylation within the SRF, Myocd, and Pla2g7 gene promoters.

ChIP assays were performed using an antibody against H3K9me3 or normal mouse IgG, following the protocol described previously. The enrichment of H3K9me3 within the promoter regions of the SMαA (**A**), SM22α (**B**), SRF (**C**), Myocd (**D**), and Pla2g7 (**E**) genes was examined using two pairs of primers specific for the regions of interest and adjacent regions of each individual gene as indicated. The data presented here are the mean ± SEM of three independent experiments. *P < 0.05 (vs. control).

3.8. PDGF-BB and TGF-β upregulate miR-22 during SMC differentiation through a transcriptional mechanism

Our previous studies suggested that, during stem cell differentiation, an auto-secreted growth factor, PDGF-BB, and transforming growth factor β (TGF-β) from differentiating cells activate their respective downstream signal pathways (e.g. Nox4), which in turn trigger the SMC differentiation program (Xiao et al., 2009, Zhang et al., 2010b). Thus, we wondered whether miR-22 is involved in signalling during SMC differentiation. To address this question, Day 2 or 3 differentiating ES cells were treated with four different doses of PDGF-BB or TGF-β as indicated to determine the best concentrations for subsequent experiments. Data showed that PDGF-BB and TGF-β upregulated miR-22 expression in a dose-dependent manner. Importantly, the expression of miR-22 reached the highest levels with 2.5 ng/ml PDGF-BB and 1 ng/ml TGF-β (**Figure 25A** and **B**), suggesting that these concentrations should be used in the following studies. Moreover, qPCR data showed that both PDGF-BB and TGF-β treatments increased the expression of miR-22 precursor and primary miR-22 (**Figure 25C** and **D**), suggesting that miR-22 is regulated by PDGF-BB and TGF-β at the transcriptional level. This notion was confirmed by the finding that incubation of differentiating ES cells with the RNA synthesis inhibitor actinomycin D (ActD) (1 μg/mL) nearly abolished the effect of PDGF-BB and TGF-β treatments on the expression of miR-22 (**Figure 25E** and **F**).

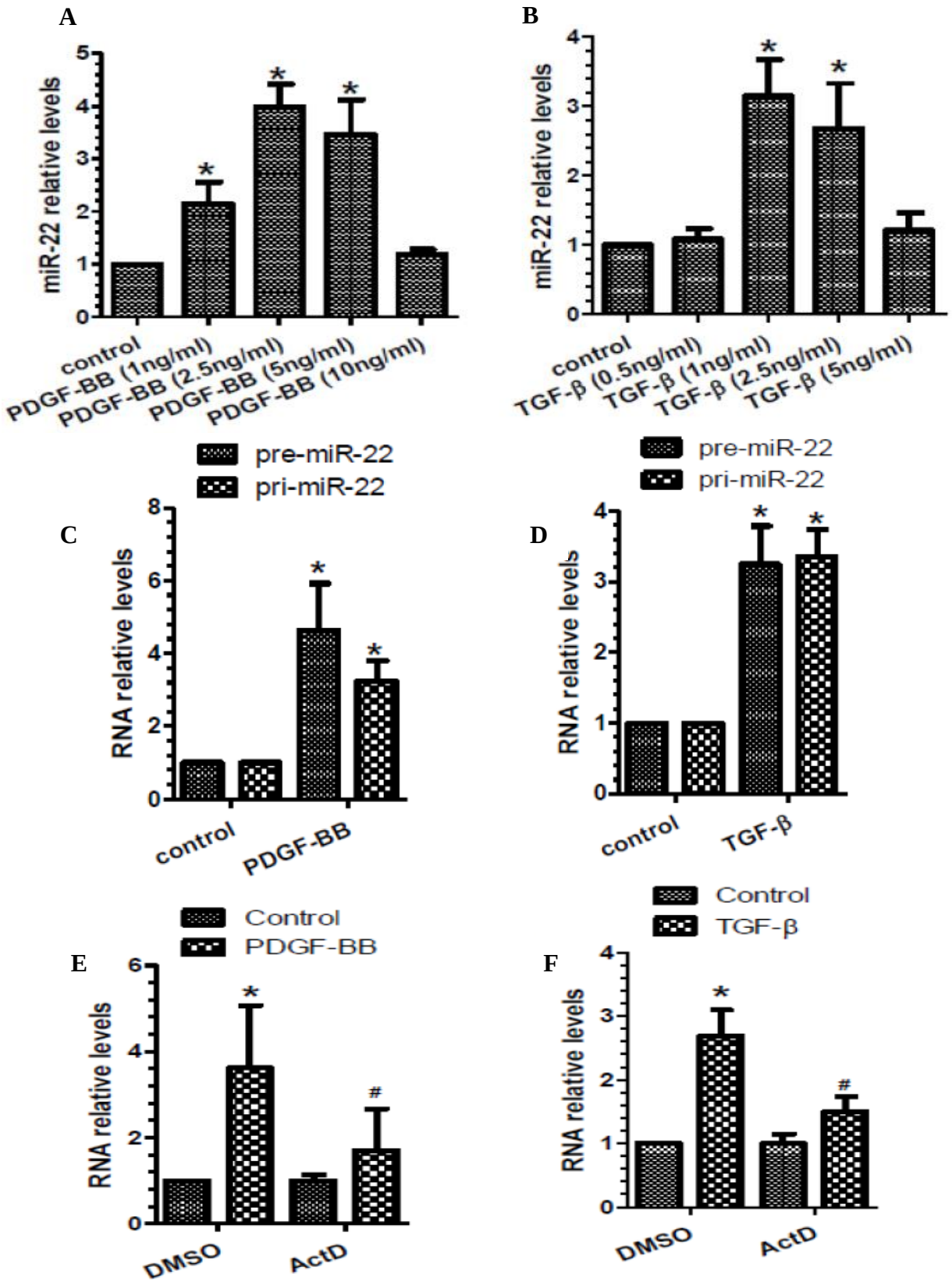


Figure 25 PDGF-BB and TGF-β upregulate miR-22 through a transcriptional mechanism.

(**A**) PDGF-BB upregulates miR-22 expression. Pre-differentiating ES cells (Day 2-3) were incubated with the indicated dose of PDGF-BB as indicated for 12 h. (**B**) The gene expression of miR-22 is also upregulated by TGF-β. The differentiating cells were incubated for 3 h with different concentrations of TGF-β. (**C)** MiR-22 precursor and primary miR-22 levels are upregulated by PDGF-BB treatment. Day 2–3 differentiating ES cells were incubated with PDGF-BB (2.5 ng/ml) for 12 h prior to cell collection. (**D**) In cells treated with 1 ng/ml TGF-β, expression of miR-22 precursor and primary miR-22 also increase. (**E**) Actinomycin D (ActD) represses the effect of PDGF-BB treatment on the expression of miR-22. Day 2–3 differentiating ES cells were incubated with 2.5 ng/ml PDGF-BB in the presence or absence of ActD in DMSO (1 μg/ml) for 6 h. Total RNA was harvested and subjected to RT-qPCR analysis with specific primers for primary miR-22. (**F**) ActD also suppresses the effect of TGF-β treatment on the expression of miR-22. Differentiating ES cells were treated with or without TGF-β (1 ng/ml) in the presence or absence of ActD in DMSO (1 μg/ml) for 6 h. The data presented here are the mean ± SEM of three independent experiments. *$P < 0.05$ (vs. control/DMSO), #$P < 0.05$ (ActD vs. DMSO in the presence of PDGF-BB or TGF-β).

Chapter 4

4. Discussion, Conclusion, and Future Plans

4.1. SMC differentiation from ES cells

SMCs are normally quiescent and are programmed for contraction under normal physiological conditions. However, in response to local inflammation, they migrate from the media to the intima where they can proliferate and synthesize extracellular matrix proteins as well as inflammatory cytokines, causing vascular lesion formation and/or intima thickening. As such, it is well accepted that SMCs play critical roles in cardiovascular diseases such as stroke, hypertension, and atherosclerosis. Moreover, there is a rate-limiting step in constructing autologous human vessels *in vitro* to replace diseased or injured vasculature because of the limited lifespan of adult vascular smooth muscle cells (SMCs) and rare sources of adult artery. Therefore, it would be helpful and important to be able to generate vascular tissue or grafts *in vitro* if alternative cell sources can be used to obtain large amounts of functional SMCs. To resolve such a problem, major efforts have been put into stem/progenitor cell research in the past decades, and significant achievements have been obtained in the field of stem/progenitor cell research.

Accumulating evidence has shown that the gene regulatory program of SMC differentiation from pluripotent stem cells is orchestrated by a coordinated molecular network comprising various signalling pathways and molecules, such as the Myocd-SRF complex, extracellular matrix, integrins, retinoid receptor, TGF family, notch family, ROS,

microRNAs (such as 34a and 22), HDACs, and others (e.g. paired-like homeodomain 2 and protein inhibitor of activated STAT-1) (Xie et al., 2011b, Xiao et al., 2010, Huang et al., 2013, Zhao et al., 2015, Chen et al., 2015, Yu et al., 2015). Despite enormous efforts have been put into this field in the past decades, our understanding of the molecular mechanisms underlying SMC differentiation are far from complete because the SMC differentiation procedure is complicated and only a few processes have been found to regulate several signalling pathways. In the present study, we have advanced our understanding of this topic by uncovering an important role for miR-22 in regulating SMC-specific gene expression and SMC differentiation from murine ES cells *in vitro* and *in vivo*. Furthermore, we present evidence for a functional role of MECP2 in SMC differentiation and SMC-specific gene regulation. Importantly, we have provided compelling evidence to support that the identified target gene, MECP2, functions as an important repressor of SMC differentiation genes during SMC differentiation from stem cells.

As described in our previous studies, we have successfully established a simple but very efficient model for the differentiation of ES cell towards SMCs. In 2007, Xiao et al. reported that collagen type IV stimulated ES cells to differentiate into Sca-1^+ cells and further differentiate into SMCs, implying that signalling pathways mediated by collagen type IV are important for SMC lineage specification (Xiao et al., 2007a). Our group also reported that collagen type I could regulate SMC differentiation from ES cells through a pathway similar to that used by collagen type IV. In our studies, SMC-specific markers such as SMαA, SM22α, h1-calponin, and SM-MHC were examined to verify that ES cells had been successfully induced to differentiate into SMCs (Huang et al., 2013, Yu et al., 2015). Therefore, the same panel of SMC differentiation markers was used in this project

to study SMC differentiation and the molecular mechanisms involved. As expected, the gene expression of SMαA and SM-MHC was upregulated during SMC differentiation and reached a maximum level at Day 6 (SMαA) or Day 8 (SM-MHC) (**Figure 4**), confirming SMC lineage specification from ES cells. In 2004, Owens's group suggested that SMαA and SM22α are markers for the early SMC differentiation stage, while calponin and SM-MHC represent late/mature SMC differentiation markers (Owens et al., 2004). This classification provides an explanation for our finding that the levels SMαA and SM-MHC gene expression peak at different times during SMC differentiation. Moreover, data from our time course study also suggest that the best period for modulating SMC differentiation or investigating the underlying mechanisms of SMC differentiation in our model is Day 2 to Day 6. Hence, most treatments during SMC differentiation in this project were conducted during this period to investigate the mechanisms by which miR-22 and MECP2 regulate SMC differentiation from ES cells.

4.2. MiR-22 and SMC differentiation from ES cell and adventitia stem/progenitor cell

The human miR-22 gene is located in a minimal loss of heterozygosis region between markers D17S1866 and D17S1574 on chromosome 17 (17p13.3) (close to TP53) in cancer cells, overlapping the exon 2 region of the spliced non-coding C17orf91 transcript (Rodriguez et al., 2004). Primary miR-22 is processed from a capped polyadenylated transcript (Cai et al., 2004). The mouse counterpart also maps to a cancer-associated genomic region (Xiong et al., 2010), implying an important role for miR-22 in cancers.

Indeed, several studies have independently identified miR-22 as a tumour suppressor (Ling et al., 2012, Zhang et al., 2012a, Xu et al., 2011). Moreover, it was recently reported that miR-22 contributes to cardiac aging by inducing cellular senescence and promoting the migratory activity of cardiac fibroblasts through its targeting of osteoglycin (Jazbutyte et al., 2013), suggesting that miR-22 plays a role in cardiovascular disease. Furthermore, miR-22 is upregulated during human ES cell differentiation (Stadler et al., 2010), induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) during monocytic differentiation from HL-60 leukaemia cell lines (Ting et al., 2010), and associated with erythroid maturation (Choong et al., 2007), implying that miR-22 could play a role in hematopoietic cell differentiation and maturation. However, the functional role of miR-22 in SMC differentiation from pluripotent stem cells remained unclear.

In a previous study, miR-22 was identified in microRNA microarray experiments as one of the top miRNA candidates with high expression during SMC differentiation from ES cells (Yu et al., 2015). The gene expression of miR-22 was measured with qPCR during SMC differentiation, from the undifferentiated stage (Day 0) to a late stage (Day 8). The results, described in **Figure 5**, showed that miR-22 was upregulated in the SMC differentiation process (**Figure 5**). Importantly, utilizing miRNA gain/loss-of-function analyses, we confirmed a critical role for miR-22 in SMC differentiation from ES cells *in vitro* (**Figure 6**).

Furthermore, to determine whether miR-22 regulates SMC differentiation from ES cells *in vivo*, miR-22-overexpressing and control ES cell lines were generated and used with our well-established *in vivo* SMC differentiation model (Matrigel-stem cells-PDGF-BB complex implantation) (Huang et al., 2013, Xiao et al., 2012, Yu et al., 2015). Data from *in vitro* experiments with these cell lines showed that more SMCs derived from the miR-22-

overexpressing ES cells (pLL3.7-GFP-miR-22 ES cells) than from control ES cells (**Figure 7**), further supporting a role for miR-22 in promoting SMC differentiation. Importantly, additional supporting evidence was obtained from *in vivo* experiments using the aforementioned miR-22-overexpressing cell lines combined with the *in vivo* Matrigel implantation model. Data from immunofluorescence staining and RT-qPCR analyses showed that more SMCs and higher levels of SMC-specific genes were detected in the Matrigel implants with miR-22-overexpressing ES cells than in those with control ES cells (**Figure 8**). These data provide the first evidence to support that miR-22 plays an important role in embryonic SMC differentiation *in vitro* and *in vivo*. They also demonstrate that miR-22 is an important SMC differentiation regulator.

We have also provided evidence that miR-22 plays a similar role in SMC differentiation from adult vascular stem/progenitor cells, Sca-1^+ cells, (**Figure 10**), which could translate into a vascular disease setting.

4.3. MECP2 is a downstream target gene of miR-22 during SMC differentiation

The fundamental and most difficult step of miRNA studies is identifying and validating *bona fide* mRNA target(s) that mediate a given function of the examined miRNA(s). It has been suggested that an average miRNA has approximately 100 target genes and regulates a large fraction of protein-coding genes, which form a regulatory network (Brennecke et al., 2005). Therefore, it is not easy to identify the actual targets of any miRNA. Having

demonstrated that miR-22 can regulate SMC differentiation *in vitro* and *in vivo*, we sought to identify the downstream mRNA target(s) responsible for miR-22-mediated SMC differentiation from stem cells. MECP2 was identified as a top target gene of miR-22 during SMC differentiation using several computational algorithmic databases, including TargetScan (www.targetscan.org), PicTar (www.pictar.mdc-berlin.de), and miRanda (www.microrna.org). Interestingly, MECP2 mRNA has a long 3′-UTR of about 8.7 kb that bears many evolutionarily conserved miRNA target sites, suggesting that it might be regulated by miRNAs. Using information from computer databases, we identified four highly conserved binding sites for miR-22 within the 3′-UTR of MECP2, as shown in **Figure 11**, implying that MECP2 is a potential mRNA target of miR-22. To establish that our prediction was correct, several experiments involving miR-22 loss/gain-of-function were conducted in differentiating ES cells. As shown in **Figure 12,** MECP2 gene and protein expression decreased during SMC differentiation from stem cells (**Figure 12A**) and showed a negative correlation with miR-22 expression levels (**Figure 5**). Moreover, MECP2 gene and protein expression was negatively regulated by miR-22, as demonstrated in miR-22 overexpression and inhibition experiments (**Figure 13A and B**). Importantly, miR-22, but not miR-34a and miR-150, were shown to downregulate MECP2 (**Figure 14**), confirming that MECP2 is a specific target of miR-22 during SMC differentiation. Furthermore, data from luciferase activity assays with MECP2 3′-UTR reporters containing wild-type and mutant versions of the miR-22 binding site demonstrated that, of the four miR-22 binding sites, two mediated MECP2 gene repression by miR-22 (**Figure 15**). Interestingly, the two binding sites located around ~1241 bp and ~6791 bp were responsible for miR-22-mediated MECP2 gene regulation in this study, but not the one located in the middle of the 3′-UTR (~2891 bp), consistent with an essential rule of microRNA–mRNA interactions, in which the site efficacy improves markedly for some

genes with long 3′-UTRs when the position of the miRNA binding site is not too distal from the poly(A) tail or the termination codon (Filipowicz et al., 2008). However, because we failed to obtain a mutant in which with only the 1st miR-22 binding site (~21 bp) was affected, we cannot exclude the importance of this binding site in mediating the effects of miR-22 on MECP2 3′-UTR activity. Nonetheless, our data demonstrate that MECP2 is a genuine mRNA target of miR-22. Finally, but importantly, the MECP2 gene expression levels in the Matrigel plugs implanted with miR-22-overexpressing ES cells were much lower than in the Matrigel plugs implanted with control ES cells (**Figure 8C**), suggesting that MECP2 gene expression is negatively regulated by miR-22 and that MECP2 is a true mRNA target of miR-22 during *in vivo* SMC differentiation from stem cells.

Studies to determine if miR-22 has other mRNA targets during SMC differentiation are warranted. Several other miR-22 mRNA targets, including oncogene EVI-1 (Patel et al., 2011), HDAC4 (Zhang et al., 2010a), PTEN (Bar and Dikstein, 2010), estrogen receptor α (ERα) (Pandey and Picard, 2009), c-Myc binding protein (MYCBP) (Xiong et al., 2010), MYC-associated factor X (Mann et al.) (Ting et al., 2010), and TET2 (Song et al., 2013), have been reported in cancer cells. However, none has been shown to be a true miR-22 mRNA target during SMC differentiation. Among them, only the expression of EVI-1 was negatively associated with miR-22 expression in the miR-22 overexpression and/or inhibition experiments, but overexpression of miR-22 failed to downregulate EVI-1 3′-UTR luciferase activity. These data suggest that target gene regulation by miR-22 is dependent on the cellular context or that miR-22 plays different role under various physiological and pathological conditions by targeting distinct target gene(s).

4.4. MECP2 regulates the expression of genes involved in SMC differentiation through a transcriptional mechanism

Two novel mechanistic findings in the present study are that MECP2 repression is required for miR-22-mediated SMC differentiation from stem cells and that MECP2 inhibits the gene expression of SMC differentiation. MECP2 negatively regulated the expression of SMC-specific markers such as SMαA, SM22α, SM-myh11, and h1-calponin (**Figure 16** and **Figure 17**), suggesting that MECP2 acts as a repressor of SMC differentiation genes during SMC differentiation. Furthermore, as shown in **Figure 17**, miR-22 or MECP2 overexpression alone in differentiating ES cells upregulated or downregulated the expression of various SMC-specific genes, respectively. In addition, re-activation of MECP2 almost completely abolished the SMC-specific gene upregulation induced by miR-22 overexpression, suggesting that MECP2 repression is required for miR-22-mediated SMC gene expression during SMC differentiation from ES cells (**Figure 18**). Moreover, it has been reported that MECP2 plays different roles in gene regulation, including transcriptional repression, activation of transcription, nuclear organization, and splicing (Bogdanovic and Veenstra, 2009). Our data showed that MECP2 is a transcriptional repressor not only for SMC differentiation genes (SMαA, SM22α), but also for SMC transcription factors (SRF and Myocd) and other genes that regulate SMC differentiation (e.g. Nox4, HDAC7, and Pla2g7) (**Figure 18, Figure 19, Figure 20, Figure 21 and Figure 22**). Taken together, our data suggest that MECP2 is a repressor of SMC differentiation whose inhibitory effect on SMC differentiation is de-repressed by the upregulation of miR-22 during SMC differentiation from stem cells.

4.5. Epigenetic regulation of SMC genes by MECP2 during SMC differentiation

MECP2 is the founding member of the methyl CpG-binding domain protein family, whose members specifically bind to methylated and unmethylated DNA and recruit distinct interacting protein partners to establish a repressive or active chromatin environment (Bogdanovic and Veenstra, 2009), respectively. MECP2 is involved in a variety of biological processes and diseases, such as Rett syndrome and neural development (Chen et al., 2001, Guy et al., 2001), modulation of human iNOS gene expression (Chan et al., 2005), regulation of myofibroblast differentiation during pulmonary fibrosis (Hu et al., 2011), myogenesis (Agarwal et al., 2007), neural differentiation from ES cells (Okabe et al., 2010) or neural precursors (Tsujimura et al., 2009), adult neurogenesis (Szulwach et al., 2010), neuron electrophysiological properties (Zhang et al., 2010c), and embryonic development (Tate et al., 1996). At the molecular level, MECP2 recognizes and binds to epigenetic modifications such as DNA methylation and chromatin marks and recruits various protein complexes that can modify epigenetic states to regulate gene expression (Zimmermann et al., 2015).

DNA methylation is associated with gene silencing. However, the mechanisms by which DNA methylation inhibits transcription have been uncovered only recently. Numerous processes by which DNA methylation can influence transcription have been proposed. One model suggests that DNA methylation directly inhibits the binding of transcription factors to their target sites, thus prohibiting transcription. Other proposed mechanisms are based on the idea that methylation of CpG sequences alters chromatin structure by affecting histone modifications and nucleosome occupancy within the promoter regions of genes

(Miranda and Jones, 2007). Importantly, a recent study suggests that MECP2 can switch transcriptional activity in the epigenetic regulation of neural chromatin and gene expression by binding hydroxymethylcytosine (5hmC)- or 5-methylcytosine (5mC)-containing DNA with a similar affinity. It has been reported that MECP2 binding to 5hmC can facilitate transcription in neural cell types while at the same time acting as a repressor when bound to 5mC-containing DNA (Mellen et al., 2012).

Another important finding of the present study is that MECP2 acts as a potential transcriptional repressor of SMC gene regulation by modulating the epigenetic modification of SMC-specific transcription factors and/or SMC differentiation modulators. DNA methylation and histone modifications are the two major epigenetic mechanisms implicated in the regulation of gene transcription in mammals. It is widely accepted that hypomethylation of DNA surrounding the proximal promoter region is a prerequisite for gene activation, whereas hypermethylation leads to gene silencing. In addition, MECP2 recognizes and binds to epigenetic modifications such as DNA methylation and chromatin marks and recruits various protein complexes that can modify epigenetic states to regulate gene expression at the molecular level (Zimmermann et al., 2015). A study published in 2003 reported that MECP2 is associated with histone methyltransferase activity *in vivo* and that such activity is directed against Lys9 of histone H3 (Fuks et al., 2003). Moreover, our group found that H3K9 methylation was enriched within SMC-specific gene promoter regions in differentiating stem cells (Xiao et al., 2011). These studies prompted us to investigate whether DNA methylation, especially H3K9, regulated MECP2 repression via SMC-specific gene markers during ES cell differentiation to SMCs.

The data shown in **Figure 23** and **Figure 24** revealed that the expression level of trimethylation on Lys 9 of histone 3 (H3K9me3) was not regulated by MECP2. Instead,

enrichment of H3K9me3 within the promoters of several SMC-specific transcription factors (e.g. SRF and Myocd) and other SMC differentiation regulators (e.g. Pla2g7) was modulated by MECP2 as well as miR-22, implying that epigenetic regulation of SMC gene expression by miR-22 and/or MECP2 is a major mechanism involved in miR-22-mediated SMC differentiation from stem cells.

4.6. Conclusion

In conclusion, this project has established a novel role for miR-22 in mouse ES cell differentiation toward SMCs *in vitro* and *in vivo*. It has also provided compelling evidence to support the conclusion that MECP2 is a genuine mRNA target of miR-22 during SMC differentiation and that repression of MECP2 in differentiating ES cells is required for miR-22-mediated SMC differentiation. Additionally, our data indicate that MECP2 regulates SMC gene expression through a transcriptional mechanism as well as an epigenetic signalling pathway. Furthermore, we have demonstrated that both miR-22 and MECP2 regulate SMC-specific gene expression by modulating other SMC differentiation regulator (Pla2g7, Nox4, and HDAC7) and that MECP2 transcriptionally regulates the gene expression of several transcriptional regulators (SRF and Myocd) through direct binding within their promoter regions. Finally, MECP2 increases H3K9 trimethylation within gene promoters. On the basis of these findings and those of our previous studies, we propose an SMC differentiation mechanism model mediated by miRNAs (Yu et al., 2015, Zhao et al., 2015). During stem cell differentiation, PDGF-BB and/or TGF-β, auto-secreted from differentiating cells, upregulate the expression of miR-22 and/or miR-34a in

SMC differentiation. On one hand, upregulation of miR-22 attenuates the inhibitory effects of MECP2 on SMC-specific transcription factors (SRF and Myocd) and other master regulators of SMC differentiation (e.g. Pla2g7, Nox4 and HDAC7), thus triggering an SMC-specific gene expression programme and promoting SMC differentiation. On the other hand, miR-34a upregulates its target gene, SirT1, in an unusual manner, which in turn regulates the transcription of three SMC transcription factors (SRF, Myocd, and MEF2C), resulting in the activation of SMC differentiation genes and SMC differentiation (Yu et al., 2015). Undoubtedly, the findings presented in this study significantly increase our understanding of the molecular mechanisms of SMC differentiation and will benefit clinical research in cardiovascular disease.

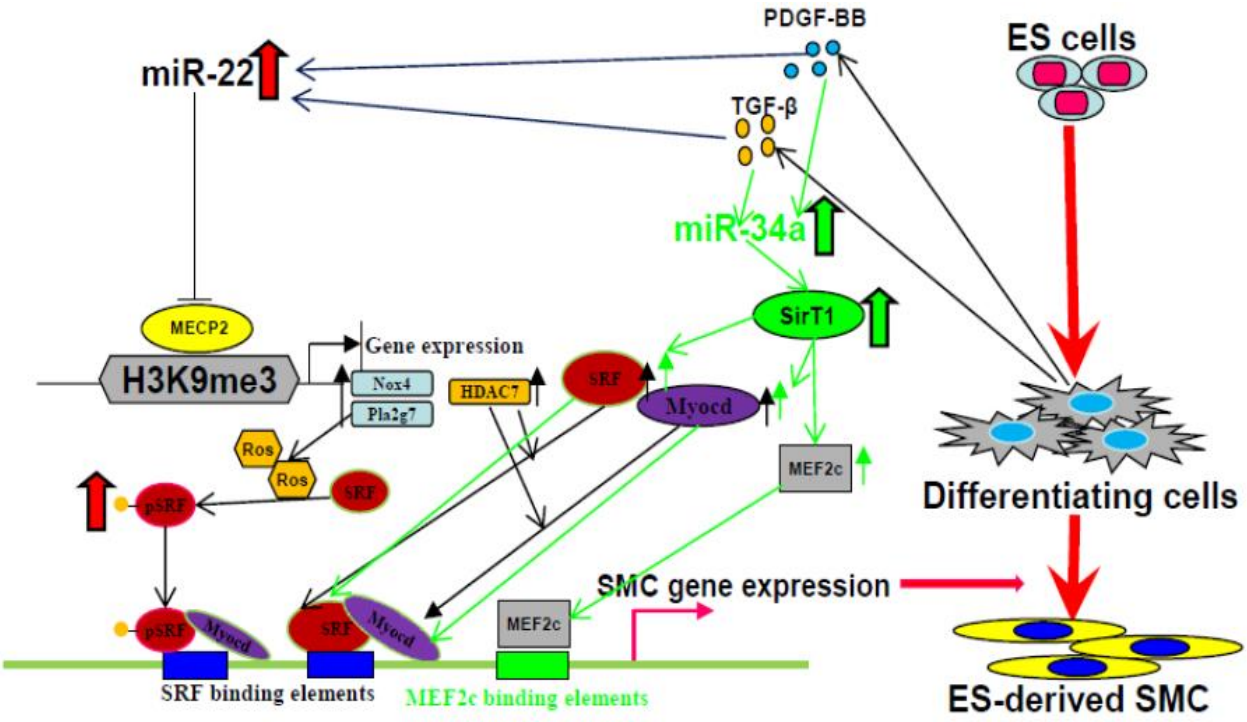


Figure 26 Proposed model of miRNA-mediated SMC differentiation.

4.7. Future Plans

We have presented compelling evidence in this study to support that miR-22 acts as an important SMC differentiation regulator *in vitro* and *in vivo*. However, the functional importance of miR-22 in embryonic SMC differentiation and cardiovascular system development are still unclear. Currently, little is known about the pathological role of miR-22 in cardiovascular diseases. All of them warrant further investigation.

First, it is unclear if the findings presented in this study translated into humans. To this end, the functional role of miR-22 in human SMC differentiation and the related mechanisms need to be investigated further using a human SMC differentiation model (e.g. human ES cells, human iPS cells, or human adult vascular stem/progenitor cells).

Second, despite demonstrating that the modulation of H3K9 methylation within the gene promoters of SMC transcription factors and other SMC differentiation regulators is one of the mechanisms responsible for MECP2-mediated SMC differentiation from stem cells, the functional involvements of MECP2 in the regulation of other epigenetic modifications (e.g. monomethylation of H3K4 and H3K79 and/or trimethylation of H3K27 and H3K9) remain to be fully elucidated.

Third, although we have provided compelling evidence to support that MECP2 is one of the functional target genes of miR-22 during SMC differentiation, it is widely accepted that a single miRNA can target multiple mRNAs, referred to as a 'targetome', to regulate gene expression transcriptionally and/or post-transcriptionally and thereby play physiological and/or pathological roles in embryonic development and human diseases. The miR-22 'targetome' during SMC differentiation remains to be fully defined.

Fourth, global and/or VSMC-specific miR-22 knockout mice are required to study the functional relevance of miR-22 in VSMC differentiation and embryonic cardiovascular development.

Finally, the potential importance of miR-22 in adult VSMC functions (e.g. proliferation, migration, adhesion, apoptosis/senescence, and extracellular matrix protein synthesis/remodelling) and vascular diseases (atherosclerosis, post-angioplasty restenosis and/or in-stent restenosis) remains to be investigated.

References

ADAMS, B., XIAO, Q. & XU, Q. 2007a. Stem cell therapy for vascular disease. *Trends Cardiovasc Med,* 17**,** 246-51.

ADAMS, B., XIAO, Q. & XU, Q. 2007b. Vascular progenitor cells and atherosclerosis. *Future Cardiol,* 3**,** 635-45.

ADAMS, J. C. & WATT, F. M. 1993. Regulation of development and differentiation by the extracellular matrix. *Development,* 117**,** 1183-98.

AGARWAL, N., HARDT, T., BRERO, A., NOWAK, D., ROTHBAUER, U., BECKER, A., LEONHARDT, H. & CARDOSO, M. C. 2007. MeCP2 interacts with HP1 and modulates its heterochromatin association during myogenic differentiation. *Nucleic Acids Res,* 35**,** 5402-8.

ALBINSSON, S., SKOURA, A., YU, J., DILORENZO, A., FERNANDEZ-HERNANDO, C., OFFERMANNS, S., MIANO, J. M. & SESSA, W. C. 2011. Smooth muscle miRNAs are critical for post-natal regulation of blood pressure and vascular function. *PLoS One,* 6**,** e18869.

ALBINSSON, S., SUAREZ, Y., SKOURA, A., OFFERMANNS, S., MIANO, J. M. & SESSA, W. C. 2010. MicroRNAs are necessary for vascular smooth muscle growth, differentiation, and function. *Arterioscler Thromb Vasc Biol,* 30**,** 1118-26.

ALTHOFF, T. F., JUAREZ, J. A., TROIDL, K., TANG, C., WANG, S., WIRTH, A., TAKEFUJI, M., WETTSCHURECK, N. & OFFERMANNS, S. 2012. Procontractile G protein-mediated signaling pathways antagonistically regulate smooth muscle differentiation in vascular remodeling. *J Exp Med,* 209**,** 2277-90.

ALVAREZ, C. V., GARCIA-LAVANDEIRA, M., GARCIA-RENDUELES, M. E., DIAZ-RODRIGUEZ, E., GARCIA-RENDUELES, A. R., PEREZ-ROMERO, S., VILA, T. V., RODRIGUES, J. S., LEAR, P. V. & BRAVO, S. B. 2012. Defining stem cell types: understanding the therapeutic potential of ESCs, ASCs, and iPS cells. *J Mol Endocrinol,* 49**,** R89-111.

AMBROS, V. & HORVITZ, H. R. 1984. Heterochronic mutants of the nematode Caenorhabditis elegans. *Science,* 226**,** 409-16.

AMBROSETTI, D. C., BASILICO, C. & DAILEY, L. 1997. Synergistic activation of the fibroblast growth factor 4 enhancer by Sox2 and Oct-3 depends on protein-protein interactions facilitated by a specific spatial arrangement of factor binding sites. *Mol Cell Biol,* 17**,** 6321-9.

AQIL, M., MALLIK, S., BANDYOPADHYAY, S., MAULIK, U. & JAMEEL, S. 2015. Transcriptomic Analysis of mRNAs in Human Monocytic Cells Expressing the HIV-1 Nef Protein and Their Exosomes. *Biomed Res Int,* 2015**,** 492395.

BADYLAK, S. F. 2005. Regenerative medicine and developmental biology: the role of the extracellular matrix. *Anat Rec B New Anat,* 287**,** 36-41.

BAI, X., WANG, X. & XU, Q. 2010. Endothelial damage and stem cell repair in atherosclerosis. *Vascul Pharmacol,* 52**,** 224-9.

BAR, N. & DIKSTEIN, R. 2010. miR-22 forms a regulatory loop in PTEN/AKT pathway and modulates signaling kinetics. *PLoS One,* 5**,** e10859.

BERENGUER, J., HERRERA, A., VUOLO, L., TORROBA, B., LLORENS, F., SUMOY, L. & PONS, S. 2013. MicroRNA 22 regulates cell cycle length in cerebellar granular neuron precursors. *Mol Cell Biol,* 33**,** 2706-17.

BERNSTEIN, E., CAUDY, A. A., HAMMOND, S. M. & HANNON, G. J. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature,* 409**,** 363-6.

BISSONNETTE, J. M., KNOPP, S. J., MAYLIE, J. & THONG, T. 2007. Autonomic cardiovascular control in methyl-CpG-binding protein 2 (Mecp2) deficient mice. *Auton Neurosci,* 136**,** 82-9.

BLANK, R. S., SWARTZ, E. A., THOMPSON, M. M., OLSON, E. N. & OWENS, G. K. 1995. A retinoic acid-induced clonal cell line derived from multipotential P19 embryonal carcinoma cells expresses smooth muscle characteristics. *Circ Res,* 76**,** 742-9.

BOETTGER, T., BEETZ, N., KOSTIN, S., SCHNEIDER, J., KRUGER, M., HEIN, L. & BRAUN, T. 2009. Acquisition of the contractile phenotype by murine arterial smooth muscle cells depends on the Mir143/145 gene cluster. *J Clin Invest,* 119**,** 2634-47.

BOGDANOVIC, O. & VEENSTRA, G. J. 2009. DNA methylation and methyl-CpG binding proteins: developmental requirements and function. *Chromosoma,* 118**,** 549-65.

BOYER, L. A., LEE, T. I., COLE, M. F., JOHNSTONE, S. E., LEVINE, S. S., ZUCKER, J. P., GUENTHER, M. G., KUMAR, R. M., MURRAY, H. L., JENNER, R. G., GIFFORD, D. K., MELTON, D. A., JAENISCH, R. & YOUNG, R. A. 2005. Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell,* 122**,** 947-56.

BOYER, L. A., PLATH, K., ZEITLINGER, J., BRAMBRINK, T., MEDEIROS, L. A., LEE, T. I., LEVINE, S. S., WERNIG, M., TAJONAR, A., RAY, M. K., BELL, G. W., OTTE, A. P., VIDAL, M., GIFFORD, D. K., YOUNG, R. A. & JAENISCH, R. 2006. Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature,* 441**,** 349-53.

BOYETTE, L. B. & TUAN, R. S. 2014. Adult Stem Cells and Diseases of Aging. *J Clin Med,* 3**,** 88-134.

BRENNECKE, J., STARK, A., RUSSELL, R. B. & COHEN, S. M. 2005. Principles of microRNA-target recognition. *PLoS Biol,* 3**,** e85.

BROOK, F. A. & GARDNER, R. L. 1997. The origin and efficient derivation of embryonic stem cells in the mouse. *Proc Natl Acad Sci U S A,* 94**,** 5709-12.

BUSCHDORF, J. P. & STRATLING, W. H. 2004. A WW domain binding region in methyl-CpG-binding protein MeCP2: impact on Rett syndrome. *J Mol Med (Berl),* 82**,** 135-43.

CAI, X., HAGEDORN, C. H. & CULLEN, B. R. 2004. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA,* 10**,** 1957-66.

CAMPAGNOLO, P., WONG, M. M. & XU, Q. 2011. Progenitor cells in arteriosclerosis: good or bad guys? *Antioxid Redox Signal,* 15**,** 1013-27.

CAPECCHI, M. R. 2005. Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. *Nat Rev Genet,* 6**,** 507-12.

CARDINALI, B., CASTELLANI, L., FASANARO, P., BASSO, A., ALEMA, S., MARTELLI, F. & FALCONE, G. 2009. Microrna-221 and microrna-222 modulate differentiation and maturation of skeletal muscle cells. *PLoS One,* 4**,** e7607.

CHAHROUR, M., JUNG, S. Y., SHAW, C., ZHOU, X., WONG, S. T., QIN, J. & ZOGHBI, H. Y. 2008. MeCP2, a key contributor to neurological disease, activates and represses transcription. *Science,* 320**,** 1224-9.

CHAMBERS, I., COLBY, D., ROBERTSON, M., NICHOLS, J., LEE, S., TWEEDIE, S. & SMITH, A. 2003. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell,* 113**,** 643-55.

CHAMBERS, I., COZENS, A., BROADBENT, J., ROBERTSON, M., LEE, M., LI, M. & SMITH, A. 1997. Structure of the mouse leukaemia inhibitory factor receptor gene: regulated expression of mRNA encoding a soluble receptor isoform from an alternative 5' untranslated region. *Biochem J,* 328 (Pt 3)**,** 879-88.

CHAN, G. C., FISH, J. E., MAWJI, I. A., LEUNG, D. D., RACHLIS, A. C. & MARSDEN, P. A. 2005. Epigenetic basis for the transcriptional hyporesponsiveness of the human inducible nitric oxide synthase gene in vascular endothelial cells. *J Immunol,* 175**,** 3846-61.

CHANG, L., NOSEDA, M., HIGGINSON, M., LY, M., PATENAUDE, A., FULLER, M., KYLE, A. H., MINCHINTON, A. I., PURI, M. C., DUMONT, D. J. & KARSAN, A. 2012. Differentiation of vascular smooth muscle cells from local precursors during embryonic and adult arteriogenesis requires Notch signaling. *Proc Natl Acad Sci U S A,* 109**,** 6993-8.

CHEN, L., CHEN, K., LAVERY, L. A., BAKER, S. A., SHAW, C. A., LI, W. & ZOGHBI, H. Y. 2015. MeCP2 binds to non-CG methylated DNA as neurons mature, influencing transcription and the timing of onset for Rett syndrome. *Proc Natl Acad Sci U S A,* 112**,** 5509-14.

CHEN, R. Z., AKBARIAN, S., TUDOR, M. & JAENISCH, R. 2001. Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. *Nat Genet,* 27**,** 327-31.

CHENDRIMADA, T. P., GREGORY, R. I., KUMARASWAMY, E., NORMAN, J., COOCH, N., NISHIKURA, K. & SHIEKHATTAR, R. 2005. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature,* 436**,** 740-4.

CHENG, Y., LIU, X., YANG, J., LIN, Y., XU, D. Z., LU, Q., DEITCH, E. A., HUO, Y., DELPHIN, E. S. & ZHANG, C. 2009. MicroRNA-145, a novel smooth muscle cell phenotypic marker and modulator, controls vascular neointimal lesion formation. *Circ Res,* 105**,** 158-66.

CHEW, J. L., LOH, Y. H., ZHANG, W., CHEN, X., TAM, W. L., YEAP, L. S., LI, P., ANG, Y. S., LIM, B., ROBSON, P. & NG, H. H. 2005. Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. *Mol Cell Biol,* 25**,** 6031-46.

CHI, H. J., GAO, S., YANG, X. C., CAI, J., ZHAO, W. S., SUN, H. & GENG, Y. J. 2015. Clinical Application of Induced Pluripotent Stem Cells in Cardiovascular Medicine. *Cardiology,* 131**,** 236-244.

CHOONG, M. L., YANG, H. H. & MCNIECE, I. 2007. MicroRNA expression profiling during human cord blood-derived CD34 cell erythropoiesis. *Exp Hematol,* 35**,** 551-64.

COGLE, C. R., GUTHRIE, S. M., SANDERS, R. C., ALLEN, W. L., SCOTT, E. W. & PETERSEN, B. E. 2003. An overview of stem cell research and regulatory issues. *Mayo Clin Proc,* 78**,** 993-1003.

COHEN, S., ZHOU, Z. & GREENBERG, M. E. 2008. Medicine. Activating a repressor. *Science,* 320**,** 1172-3.

CONOVER, J. C., IP, N. Y., POUEYMIROU, W. T., BATES, B., GOLDFARB, M. P., DECHIARA, T. M. & YANCOPOULOS, G. D. 1993. Ciliary neurotrophic factor maintains the pluripotentiality of embryonic stem cells. *Development,* 119**,** 559-65.

CORDES, K. R., SHEEHY, N. T., WHITE, M. P., BERRY, E. C., MORTON, S. U., MUTH, A. N., LEE, T. H., MIANO, J. M., IVEY, K. N. & SRIVASTAVA, D. 2009. miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. *Nature,* 460**,** 705-10.

COUGHLAN, L., UUSI-KERTTULA, H., MA, J., DEGG, B. P., PARKER, A. L. & BAKER, A. H. 2014. Retargeting adenovirus serotype 48 fiber knob domain by peptide incorporation. *Hum Gene Ther,* 25**,** 385-94.

COWAN, C. A., KLIMANSKAYA, I., MCMAHON, J., ATIENZA, J., WITMYER, J., ZUCKER, J. P., WANG, S., MORTON, C. C., MCMAHON, A. P., POWERS, D. & MELTON, D. A. 2004. Derivation of embryonic stem-cell lines from human blastocysts. *N Engl J Med,* 350**,** 1353-6.

DAVIS, B. N., HILYARD, A. C., LAGNA, G. & HATA, A. 2008. SMAD proteins control DROSHA-mediated microRNA maturation. *Nature,* 454**,** 56-61.

DAVIS, B. N., HILYARD, A. C., NGUYEN, P. H., LAGNA, G. & HATA, A. 2009. Induction of microRNA-221 by platelet-derived growth factor signaling is critical for modulation of vascular smooth muscle phenotype. *J Biol Chem,* 284**,** 3728-38.

DAVIS, S., ALDRICH, T. H., STAHL, N., PAN, L., TAGA, T., KISHIMOTO, T., IP, N. Y. & YANCOPOULOS, G. D. 1993. LIFR beta and gp130 as heterodimerizing signal transducers of the tripartite CNTF receptor. *Science,* 260**,** 1805-8.

DEXTER, T. M., MOORE, M. A. & SHERIDAN, A. P. 1977. Maintenance of hemopoietic stem cells and production of differentiated progeny in allogeneic and semiallogeneic bone marrow chimeras in vitro. *J Exp Med,* 145**,** 1612-6.

DJARMATI, A., DOBRICIC, V., KECMANOVIC, M., MARSH, P., JANCIC-STEFANOVIC, J., KLEIN, C., DJURIC, M. & ROMAC, S. 2007. MECP2 mutations in Serbian Rett syndrome patients. *Acta Neurol Scand,* 116**,** 413-9.

DOETSCHMAN, T. C., EISTETTER, H., KATZ, M., SCHMIDT, W. & KEMLER, R. 1985. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J Embryol Exp Morphol,* 87**,** 27-45.

DONOVAN, J., SHIWEN, X., NORMAN, J. & ABRAHAM, D. 2013. Platelet-derived growth factor alpha and beta receptors have overlapping functional activities towards fibroblasts. *Fibrogenesis Tissue Repair,* 6**,** 10.

DRAB, M., HALLER, H., BYCHKOV, R., ERDMANN, B., LINDSCHAU, C., HAASE, H., MORANO, I., LUFT, F. C. & WOBUS, A. M. 1997. From totipotent embryonic stem cells to spontaneously contracting smooth muscle cells: a retinoic acid and db-cAMP in vitro differentiation model. *FASEB J,* 11**,** 905-15.

DU, Q., LUU, P. L., STIRZAKER, C. & CLARK, S. J. 2015. Methyl-CpG-binding domain proteins: readers of the epigenome. *Epigenomics***,** 1-23.

DUSHKIN, M. I. 2012. Macrophage/foam cell is an attribute of inflammation: mechanisms of formation and functional role. *Biochemistry (Mosc),* 77**,** 327-38.

EGASHIRA, T., YUASA, S. & FUKUDA, K. 2011. Induced pluripotent stem cells in cardiovascular medicine. *Stem Cells Int,* 2011**,** 348960.

EKIMLER, S. & SAHIN, K. 2014. Computational Methods for MicroRNA Target Prediction. *Genes (Basel),* 5**,** 671-83.

ESTELLER, M. 2005. Dormant hypermethylated tumour suppressor genes: questions and answers. *J Pathol,* 205**,** 172-80.

EVANS, M. J. & KAUFMAN, M. H. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature,* 292**,** 154-6.

FAN, P., CHEN, Z., TIAN, P., LIU, W., JIAO, Y., XUE, Y., BHATTACHARYA, A., WU, J., LU, M., GUO, Y., CUI, Y., GU, W. & YUE, J. 2013. miRNA Biogenesis Enzyme Drosha Is Required for Vascular Smooth Muscle Cell Survival. *PLoS One,* 8**,** e60888.

FAZI, F. & NERVI, C. 2008. MicroRNA: basic mechanisms and transcriptional regulatory networks for cell fate determination. *Cardiovasc Res,* 79**,** 553-61.

FENG, Y., HUANG, W., WANI, M., YU, X. & ASHRAF, M. 2014. Ischemic preconditioning potentiates the protective effect of stem cells through secretion of exosomes by targeting Mecp2 via miR-22. *PLoS One,* 9**,** e88685.

FERREIRA, L. S., GERECHT, S., SHIEH, H. F., WATSON, N., RUPNICK, M. A., DALLABRIDA, S. M., VUNJAK-NOVAKOVIC, G. & LANGER, R. 2007. Vascular progenitor cells isolated from human embryonic stem cells give rise to endothelial and smooth muscle like cells and form vascular networks in vivo. *Circ Res,* 101**,** 286-94.

FILIPOWICZ, W., BHATTACHARYYA, S. N. & SONENBERG, N. 2008. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet,* 9**,** 102-14.

FRANCHINA, T., AMODEO, V., BRONTE, G., SAVIO, G., RICCIARDI, G. R., PICCIOTTO, M., RUSSO, A., GIORDANO, A. & ADAMO, V. 2014. Circulating miR-22, miR-24 and miR-34a as novel predictive biomarkers to pemetrexed-based chemotherapy in advanced non-small cell lung cancer. *J Cell Physiol,* 229**,** 97-9.

FU, Y., ZHANG, J., SHI, Z., WANG, G., LI, W. & JIA, L. 2015a. A key gene of the small RNA pathway in the flounder, Paralichthys olivaceus: identification and functional characterization of dicer. *Fish Physiol Biochem.*

FU, Y., ZHANG, J., SHI, Z., WANG, G., LI, W. & JIA, L. 2015b. A key gene of the small RNA pathway in the flounder, Paralichthys olivaceus: identification and functional characterization of dicer. *Fish Physiol Biochem,* 41**,** 1221-31.

FUKS, F., HURD, P. J., WOLF, D., NAN, X., BIRD, A. P. & KOUZARIDES, T. 2003. The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *J Biol Chem,* 278**,** 4035-40.

GALKINA, E. & LEY, K. 2009. Immune and inflammatory mechanisms of atherosclerosis (*). *Annu Rev Immunol,* 27**,** 165-97.

GALLEY, H. F. & WEBSTER, N. R. 2004. Physiology of the endothelium. *Br J Anaesth,* 93**,** 105-13.

GEARING, D. P. & BRUCE, A. G. 1992. Oncostatin M binds the high-affinity leukemia inhibitory factor receptor. *New Biol,* 4**,** 61-5.

GEARING, D. P., THUT, C. J., VANDEBOS, T., GIMPEL, S. D., DELANEY, P. B., KING, J., PRICE, V., COSMAN, D. & BECKMANN, M. P. 1991. Leukemia inhibitory factor receptor is structurally related to the IL-6 signal transducer, gp130. *EMBO J,* 10**,** 2839-48.

GHILDIYAL, M., XU, J., SEITZ, H., WENG, Z. & ZAMORE, P. D. 2010. Sorting of Drosophila small silencing RNAs partitions microRNA* strands into the RNA interference pathway. *RNA,* 16**,** 43-56.

GIORGETTI, A., MONTSERRAT, N., RODRIGUEZ-PIZA, I., AZQUETA, C., VEIGA, A. & IZPISUA BELMONTE, J. C. 2010. Generation of induced pluripotent stem cells from human cord blood cells with only two factors: Oct4 and Sox2. *Nat Protoc,* 5**,** 811-20.

GIRALDEZ, A. J., MISHIMA, Y., RIHEL, J., GROCOCK, R. J., VAN DONGEN, S., INOUE, K., ENRIGHT, A. J. & SCHIER, A. F. 2006. Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science,* 312**,** 75-9.

GLASER, R., LU, M. M., NARULA, N. & EPSTEIN, J. A. 2002. Smooth muscle cells, but not myocytes, of host origin in transplanted human hearts. *Circulation,* 106**,** 17-9.

GO, A. S., MOZAFFARIAN, D., ROGER, V. L., BENJAMIN, E. J., BERRY, J. D., BLAHA, M. J., DAI, S., FORD, E. S., FOX, C. S., FRANCO, S., FULLERTON, H. J., GILLESPIE, C., HAILPERN, S. M., HEIT, J. A., HOWARD, V. J., HUFFMAN, M. D., JUDD, S. E., KISSELA, B. M., KITTNER, S. J., LACKLAND, D. T., LICHTMAN, J. H., LISABETH, L. D., MACKEY, R. H., MAGID, D. J., MARCUS, G. M., MARELLI, A., MATCHAR, D. B., MCGUIRE, D. K., MOHLER, E. R., 3RD, MOY, C. S.,

MUSSOLINO, M. E., NEUMAR, R. W., NICHOL, G., PANDEY, D. K., PAYNTER, N. P., REEVES, M. J., SORLIE, P. D., STEIN, J., TOWFIGHI, A., TURAN, T. N., VIRANI, S. S., WONG, N. D., WOO, D. & TURNER, M. B. 2014. Executive summary: heart disease and stroke statistics--2014 update: a report from the American Heart Association. *Circulation,* 129**,** 399-410.

GUO, M. M., HU, L. H., WANG, Y. Q., CHEN, P., HUANG, J. G., LU, N., HE, J. H. & LIAO, C. G. 2013. miR-22 is down-regulated in gastric cancer, and its overexpression inhibits cell migration and invasion via targeting transcription factor Sp1. *Med Oncol,* 30**,** 542.

GURDON, J. B. 1962. The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. *J Embryol Exp Morphol,* 10**,** 622-40.

GUY, J., HENDRICH, B., HOLMES, M., MARTIN, J. E. & BIRD, A. 2001. A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. *Nat Genet,* 27**,** 322-6.

HALL, J., GUO, G., WRAY, J., EYRES, I., NICHOLS, J., GROTEWOLD, L., MORFOPOULOU, S., HUMPHREYS, P., MANSFIELD, W., WALKER, R., TOMLINSON, S. & SMITH, A. 2009. Oct4 and LIF/Stat3 additively induce Kruppel factors to sustain embryonic stem cell self-renewal. *Cell Stem Cell,* 5**,** 597-609.

HAMMOND, S. M. 2015. An overview of microRNAs. *Adv Drug Deliv Rev.*

HARVEY, C. G., MENON, S. D., STACHOWIAK, B., NOOR, A., PROCTOR, A., MENSAH, A. K., MNATZAKANIAN, G. N., ALFRED, S. E., GUO, R., SCHERER, S. W., KENNEDY, J. L., ROBERTS, W., SRIVASTAVA, A. K., MINASSIAN, B. A. & VINCENT, J. B. 2007. Sequence variants within exon 1 of MECP2 occur in females with mental retardation. *Am J Med Genet B Neuropsychiatr Genet,* 144B**,** 355-60.

HAYASHI, R., ISHIKAWA, Y., ITO, M., KAGEYAMA, T., TAKASHIBA, K., FUJIOKA, T., TSUJIKAWA, M., MIYOSHI, H., YAMATO, M., NAKAMURA, Y. & NISHIDA, K. 2012. Generation of corneal epithelial cells from induced pluripotent stem cells derived from human dermal fibroblast and corneal limbal epithelium. *PLoS One,* 7**,** e45435.

HENDRICKSON, D. G., HOGAN, D. J., MCCULLOUGH, H. L., MYERS, J. W., HERSCHLAG, D., FERRELL, J. E. & BROWN, P. O. 2009. Concordant regulation of translation and mRNA abundance for hundreds of targets of a human microRNA. *PLoS Biol,* 7**,** e1000238.

HERREROS-VILLANUEVA, M., BUJANDA, L., BILLADEAU, D. D. & ZHANG, J. S. 2014. Embryonic stem cell factors and pancreatic cancer. *World J Gastroenterol,* 20**,** 2247-54.

HILLEBRANDS, J. L., KLATTER, F. A., VAN DEN HURK, B. M., POPA, E. R., NIEUWENHUIS, P. & ROZING, J. 2001. Origin of neointimal endothelium and alpha-actin-positive smooth muscle cells in transplant arteriosclerosis. *J Clin Invest,* 107**,** 1411-22.

HU, B., GHARAEE-KERMANI, M., WU, Z. & PHAN, S. H. 2011. Essential role of MeCP2 in the regulation of myofibroblast differentiation during pulmonary fibrosis. *Am J Pathol,* 178**,** 1500-8.

HU, Y., MAYR, M., METZLER, B., ERDEL, M., DAVISON, F. & XU, Q. 2002. Both donor and recipient origins of smooth muscle cells in vein graft atherosclerotic lesions. *Circ Res,* 91**,** e13-20.

HU, Y. & XU, Q. 2011. Adventitial biology: differentiation and function. *Arterioscler Thromb Vasc Biol,* 31**,** 1523-9.

HU, Y., ZHANG, Z., TORSNEY, E., AFZAL, A. R., DAVISON, F., METZLER, B. & XU, Q. 2004. Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice. *J Clin Invest,* 113**,** 1258-65.

HUANG, H., XIE, C., SUN, X., RITCHIE, R. P., ZHANG, J. & CHEN, Y. E. 2010. miR-10a contributes to retinoid acid-induced smooth muscle cell differentiation. *J Biol Chem,* 285**,** 9383-9.

HUANG, Y., LIN, L., YU, X., WEN, G., PU, X., ZHAO, H., FANG, C., ZHU, J., YE, S., ZHANG, L. & XIAO, Q. 2013. Functional involvements of heterogeneous nuclear ribonucleoprotein A1 in smooth muscle differentiation from stem cells in vitro and in vivo. *Stem Cells,* 31**,** 906-17.

JAENISCH, R. & BIRD, A. 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet,* 33 Suppl**,** 245-54.

JALALI, S., RAMANATHAN, G. K., PARTHASARATHY, P. T., ALJUBRAN, S., GALAM, L., YUNUS, A., GARCIA, S., COX, R. R., JR., LOCKEY, R. F. & KOLLIPUTI, N.

2012. Mir-206 regulates pulmonary artery smooth muscle cell proliferation and differentiation. *PLoS One,* 7**,** e46808.

JAZBUTYTE, V., FIEDLER, J., KNEITZ, S., GALUPPO, P., JUST, A., HOLZMANN, A., BAUERSACHS, J. & THUM, T. 2013. MicroRNA-22 increases senescence and activates cardiac fibroblasts in the aging heart. *Age (Dordr),* 35**,** 747-62.

JI, R., CHENG, Y., YUE, J., YANG, J., LIU, X., CHEN, H., DEAN, D. B. & ZHANG, C. 2007. MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion formation. *Circ Res,* 100**,** 1579-88.

JOHNSON, C. M., CUI, N., ZHONG, W., OGINSKY, M. F. & JIANG, C. 2015. Breathing abnormalities in a female mouse model of Rett syndrome. *J Physiol Sci,* 65**,** 451-9.

JOHNSON, J. L. 2014. Emerging regulators of vascular smooth muscle cell function in the development and progression of atherosclerosis. *Cardiovasc Res,* 103**,** 452-60.

KAJI, K., NORRBY, K., PACA, A., MILEIKOVSKY, M., MOHSENI, P. & WOLTJEN, K. 2009. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature,* 458**,** 771-5.

KALMAR, T., LIM, C., HAYWARD, P., MUNOZ-DESCALZO, S., NICHOLS, J., GARCIA-OJALVO, J. & MARTINEZ ARIAS, A. 2009. Regulated fluctuations in nanog expression mediate cell fate decisions in embryonic stem cells. *PLoS Biol,* 7**,** e1000149.

KANE, N. M., XIAO, Q., BAKER, A. H., LUO, Z., XU, Q. & EMANUELI, C. 2011. Pluripotent stem cell differentiation into vascular cells: a novel technology with promises for vascular re(generation). *Pharmacol Ther,* 129**,** 29-49.

KAPERONIS, E. A., LIAPIS, C. D., KAKISIS, J. D., DIMITROULIS, D. & PAPAVASSILIOU, V. G. 2006. Inflammation and atherosclerosis. *Eur J Vasc Endovasc Surg,* 31**,** 386-93.

KIKUCHI, T., MORIZANE, A., DOI, D., ONOE, H., HAYASHI, T., KAWASAKI, T., SAIKI, H., MIYAMOTO, S. & TAKAHASHI, J. 2011. Survival of human induced pluripotent stem cell-derived midbrain dopaminergic neurons in the brain of a primate model of Parkinson's disease. *J Parkinsons Dis,* 1**,** 395-412.

KIM, H. K., LEE, Y. S., SIVAPRASAD, U., MALHOTRA, A. & DUTTA, A. 2006. Muscle-specific microRNA miR-206 promotes muscle differentiation. *J Cell Biol,* 174**,** 677-87.

KINKAID, H. Y., HUANG, X. P., LI, R. K. & WEISEL, R. D. 2010. What's new in cardiac cell therapy? Allogeneic bone marrow stromal cells as "universal donor cells". *J Card Surg,* 25**,** 359-66.

KIRTON, J. P. & XU, Q. 2010. Endothelial precursors in vascular repair. *Microvasc Res,* 79**,** 193-9.

KLEINMAN, H. K., PHILP, D. & HOFFMAN, M. P. 2003. Role of the extracellular matrix in morphogenesis. *Curr Opin Biotechnol,* 14**,** 526-32.

KONG, Y. W., CANNELL, I. G., DE MOOR, C. H., HILL, K., GARSIDE, P. G., HAMILTON, T. L., MEIJER, H. A., DOBBYN, H. C., STONELEY, M., SPRIGGS, K. A., WILLIS, A. E. & BUSHELL, M. 2008. The mechanism of micro-RNA-mediated translation repression is determined by the promoter of the target gene. *Proc Natl Acad Sci U S A,* 105**,** 8866-71.

KUHN, D. E., MARTIN, M. M., FELDMAN, D. S., TERRY, A. V., JR., NUOVO, G. J. & ELTON, T. S. 2008. Experimental validation of miRNA targets. *Methods,* 44**,** 47-54.

KUMAR, M. S. & OWENS, G. K. 2003. Combinatorial control of smooth muscle-specific gene expression. *Arterioscler Thromb Vasc Biol,* 23**,** 737-47.

LANDGRAF, P., RUSU, M., SHERIDAN, R., SEWER, A., IOVINO, N., ARAVIN, A., PFEFFER, S., RICE, A., KAMPHORST, A. O., LANDTHALER, M., LIN, C., SOCCI, N. D., HERMIDA, L., FULCI, V., CHIARETTI, S., FOA, R., SCHLIWKA, J., FUCHS, U., NOVOSEL, A., MULLER, R. U., SCHERMER, B., BISSELS, U., INMAN, J., PHAN, Q., CHIEN, M., WEIR, D. B., CHOKSI, R., DE VITA, G., FREZZETTI, D., TROMPETER, H. I., HORNUNG, V., TENG, G., HARTMANN, G., PALKOVITS, M., DI LAURO, R., WERNET, P., MACINO, G., ROGLER, C. E., NAGLE, J. W., JU, J., PAPAVASILIOU, F. N., BENZING, T., LICHTER, P., TAM, W., BROWNSTEIN, M. J., BOSIO, A., BORKHARDT, A., RUSSO, J. J., SANDER, C., ZAVOLAN, M. & TUSCHL, T. 2007. A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell,* 129**,** 1401-14.

LANDMESSER, U., HORNIG, B. & DREXLER, H. 2004. Endothelial function: a critical determinant in atherosclerosis? *Circulation,* 109**,** II27-33.

LIBBY, P. 2002. Inflammation in atherosclerosis. *Nature,* 420**,** 868-74.

LIBBY, P., DICARLI, M. & WEISSLEDER, R. 2010. The vascular biology of atherosclerosis and imaging targets. *J Nucl Med,* 51 Suppl 1**,** 33S-37S.

LIM, W. F., INOUE-YOKOO, T., TAN, K. S., LAI, M. I. & SUGIYAMA, D. 2013. Hematopoietic cell differentiation from embryonic and induced pluripotent stem cells. *Stem Cell Res Ther,* 4**,** 71.

LING, B., WANG, G. X., LONG, G., QIU, J. H. & HU, Z. L. 2012. Tumor suppressor miR-22 suppresses lung cancer cell progression through post-transcriptional regulation of ErbB3. *J Cancer Res Clin Oncol,* 138**,** 1355-61.

LIU, H., SARNAIK, S. M., MANOLE, M. D., CHEN, Y., SHINDE, S. N., LI, W., ROSE, M., ALEXANDER, H., CHEN, J., CLARK, R. S., GRAHAM, S. H. & HICKEY, R. W. 2012. Increased cytochrome c in rat cerebrospinal fluid after cardiac arrest and its effects on hypoxic neuronal survival. *Resuscitation,* 83**,** 1491-6.

LIU, X., CHENG, Y., ZHANG, S., LIN, Y., YANG, J. & ZHANG, C. 2009. A necessary role of miR-221 and miR-222 in vascular smooth muscle cell proliferation and neointimal hyperplasia. *Circ Res,* 104**,** 476-87.

LONG, X., COWAN, S. L. & MIANO, J. M. 2013. Mitogen-activated protein kinase 14 is a novel negative regulatory switch for the vascular smooth muscle cell contractile gene program. *Arterioscler Thromb Vasc Biol,* 33**,** 378-86.

LUO, Z., WEN, G., WANG, G., PU, X., YE, S., XU, Q., WANG, W. & XIAO, Q. 2013. MicroRNA-200C and -150 play an important role in endothelial cell differentiation and vasculogenesis by targeting transcription repressor ZEB1. *Stem Cells,* 31**,** 1749-62.

LYTLE, J. R., YARIO, T. A. & STEITZ, J. A. 2007. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. *Proc Natl Acad Sci U S A,* 104**,** 9667-72.

MAJESKY, M. W. 2007. Developmental basis of vascular smooth muscle diversity. *Arterioscler Thromb Vasc Biol,* 27**,** 1248-58.

MANABE, I. & OWENS, G. K. 2001. Recruitment of serum response factor and hyperacetylation of histones at smooth muscle-specific regulatory regions during

differentiation of a novel P19-derived in vitro smooth muscle differentiation system. *Circ Res,* 88**,** 1127-34.

MANN, J., CHU, D. C., MAXWELL, A., OAKLEY, F., ZHU, N. L., TSUKAMOTO, H. & MANN, D. A. 2010. MeCP2 controls an epigenetic pathway that promotes myofibroblast transdifferentiation and fibrosis. *Gastroenterology,* 138**,** 705-14, 714 e1-4.

MARGARITI, A., XIAO, Q., ZAMPETAKI, A., ZHANG, Z., LI, H., MARTIN, D., HU, Y., ZENG, L. & XU, Q. 2009. Splicing of HDAC7 modulates the SRF-myocardin complex during stem-cell differentiation towards smooth muscle cells. *J Cell Sci,* 122**,** 460-70.

MARINOU, K., CHRISTODOULIDES, C., ANTONIADES, C. & KOUTSILIERIS, M. 2012. Wnt signaling in cardiovascular physiology. *Trends Endocrinol Metab,* 23**,** 628-36.

MARTIN, G. R. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A,* 78**,** 7634-8.

MATSUDA, T., NAKAMURA, T., NAKAO, K., ARAI, T., KATSUKI, M., HEIKE, T. & YOKOTA, T. 1999. STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *EMBO J,* 18**,** 4261-9.

MAYER, S., GILSBACH, R., PREISSL, S., MONROY ORDONEZ, E. B., SCHNICK, T., BEETZ, N., LOTHER, A., ROMMEL, C., IHLE, H. M., BUGGER, H., RUHLE, F., SCHREPPER, A., SCHWARZER, M., HEILMANN, C., BONISCH, U., GUPTA, S. K., WILPERT, J., KRETZ, O., VON ELVERFELDT, D., ORTH, J., AKTORIES, K., BEYERSDORF, F., BODE, C., STILLER, B., KRUGER, M., THUM, T., DOENST, T., STOLL, M. & HEIN, L. 2015. Adrenergic Repression of the Epigenetic Reader MeCP2 Facilitates Cardiac Adaptation in Chronic Heart Failure. *Circ Res*.

MELLEN, M., AYATA, P., DEWELL, S., KRIAUCIONIS, S. & HEINTZ, N. 2012. MeCP2 binds to 5hmC enriched within active genes and accessible chromatin in the nervous system. *Cell,* 151**,** 1417-30.

MENDIS, S., PUSKA, P. & NORRVING, B. 2011. *Global atlas on cardiovascular disease prevention and control,* Geneva, World Health Organization in collaboration with the World Heart Federation and the World Stroke Organization.

MENO, C., SAIJOH, Y., FUJII, H., IKEDA, M., YOKOYAMA, T., YOKOYAMA, M., TOYODA, Y. & HAMADA, H. 1996. Left-right asymmetric expression of the TGF beta-family member lefty in mouse embryos. *Nature,* 381**,** 151-5.

MEYER, N. & PENN, L. Z. 2008. Reflecting on 25 years with MYC. *Nat Rev Cancer,* 8**,** 976-90.

MIANO, J. M., RAMANAN, N., GEORGER, M. A., DE MESY BENTLEY, K. L., EMERSON, R. L., BALZA, R. O., JR., XIAO, Q., WEILER, H., GINTY, D. D. & MISRA, R. P. 2004. Restricted inactivation of serum response factor to the cardiovascular system. *Proc Natl Acad Sci U S A,* 101**,** 17132-7.

MIN, S., LIANG, X., ZHANG, M., ZHANG, Y., MEI, S., LIU, J., SU, X., CAO, S., ZHONG, X., LI, Y., SUN, J., LIU, Q., JIANG, X., CHE, Y. & YANG, R. 2013. Multiple tumor-associated microRNAs modulate the survival and longevity of dendritic cells by targeting YWHAZ and Bcl2 signaling pathways. *J Immunol,* 190**,** 2437-46.

MIRANDA, T. B. & JONES, P. A. 2007. DNA methylation: the nuts and bolts of repression. *J Cell Physiol,* 213**,** 384-90.

MITSUI, K., TOKUZAWA, Y., ITOH, H., SEGAWA, K., MURAKAMI, M., TAKAHASHI, K., MARUYAMA, M., MAEDA, M. & YAMANAKA, S. 2003. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell,* 113**,** 631-42.

MURATA, M., TOHYAMA, S. & FUKUDA, K. 2010. Impacts of recent advances in cardiovascular regenerative medicine on clinical therapies and drug discovery. *Pharmacol Ther,* 126**,** 109-18.

MURCHISON, E. P., PARTRIDGE, J. F., TAM, O. H., CHELOUFI, S. & HANNON, G. J. 2005. Characterization of Dicer-deficient murine embryonic stem cells. *Proc Natl Acad Sci U S A,* 102**,** 12135-40.

NAKANO, T., KODAMA, H. & HONJO, T. 1994. Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science,* 265**,** 1098-101.

NAKATAKE, Y., FUKUI, N., IWAMATSU, Y., MASUI, S., TAKAHASHI, K., YAGI, R., YAGI, K., MIYAZAKI, J., MATOBA, R., KO, M. S. & NIWA, H. 2006. Klf4

cooperates with Oct3/4 and Sox2 to activate the Lefty1 core promoter in embryonic stem cells. *Mol Cell Biol,* 26**,** 7772-82.

NICHOLS, J., CHAMBERS, I. & SMITH, A. 1994. Derivation of germline competent embryonic stem cells with a combination of interleukin-6 and soluble interleukin-6 receptor. *Exp Cell Res,* 215**,** 237-9.

NICHOLS, J. & SMITH, A. 2012. Pluripotency in the embryo and in culture. *Cold Spring Harb Perspect Biol,* 4**,** a008128.

NICHOLS, J., ZEVNIK, B., ANASTASSIADIS, K., NIWA, H., KLEWE-NEBENIUS, D., CHAMBERS, I., SCHOLER, H. & SMITH, A. 1998. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell,* 95**,** 379-91.

NIWA, H., BURDON, T., CHAMBERS, I. & SMITH, A. 1998. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Dev,* 12**,** 2048-60.

NIWA, H., MIYAZAKI, J. & SMITH, A. G. 2000. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet,* 24**,** 372-6.

NIWA, H., OGAWA, K., SHIMOSATO, D. & ADACHI, K. 2009. A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells. *Nature,* 460**,** 118-22.

NIWA, H., TOYOOKA, Y., SHIMOSATO, D., STRUMPF, D., TAKAHASHI, K., YAGI, R. & ROSSANT, J. 2005. Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. *Cell,* 123**,** 917-29.

NORATA, G. D., PINNA, C., ZAPPELLA, F., ELIA, L., SALA, A., CONDORELLI, G. & CATAPANO, A. L. 2012. MicroRNA 143-145 deficiency impairs vascular function. *Int J Immunopathol Pharmacol,* 25**,** 467-74.

OBERDOERFFER, P., KANELLOPOULOU, C., HEISSMEYER, V., PAEPER, C., BOROWSKI, C., AIFANTIS, I., RAO, A. & RAJEWSKY, K. 2005. Efficiency of RNA interference in the mouse hematopoietic system varies between cell types and developmental stages. *Mol Cell Biol,* 25**,** 3896-905.

OKABE, Y., KUSAGA, A., TAKAHASHI, T., MITSUMASU, C., MURAI, Y., TANAKA, E., HIGASHI, H., MATSUISHI, T. & KOSAI, K. 2010. Neural development of methyl-

CpG-binding protein 2 null embryonic stem cells: a system for studying Rett syndrome. *Brain Res,* 1360**,** 17-27.

OKAMURA, K., LIU, N. & LAI, E. C. 2009. Distinct mechanisms for microRNA strand selection by Drosophila Argonautes. *Mol Cell,* 36**,** 431-44.

OKANO, H. & YAMANAKA, S. 2014. iPS cell technologies: significance and applications to CNS regeneration and disease. *Mol Brain,* 7**,** 22.

OKUMURA-NAKANISHI, S., SAITO, M., NIWA, H. & ISHIKAWA, F. 2005. Oct-3/4 and Sox2 regulate Oct-3/4 gene in embryonic stem cells. *J Biol Chem,* 280**,** 5307-17.

ORLANDI, A. & BENNETT, M. 2010. Progenitor cell-derived smooth muscle cells in vascular disease. *Biochem Pharmacol,* 79**,** 1706-13.

OWENS, G. K., KUMAR, M. S. & WAMHOFF, B. R. 2004. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev,* 84**,** 767-801.

PACKER, C., BODDICE, B. & SIMPSON, S. 2013. Regenerative medicine techniques in cardiovascular disease: where is the horizon? *Regen Med,* 8**,** 351-60.

PAGIATAKIS, C., GORDON, J. W., EHYAI, S. & MCDERMOTT, J. C. 2012. A novel RhoA/ROCK-CPI-17-MEF2C signaling pathway regulates vascular smooth muscle cell gene expression. *J Biol Chem,* 287**,** 8361-70.

PAN, G., LI, J., ZHOU, Y., ZHENG, H. & PEI, D. 2006. A negative feedback loop of transcription factors that controls stem cell pluripotency and self-renewal. *FASEB J,* 20**,** 1730-2.

PAN, G. & THOMSON, J. A. 2007. Nanog and transcriptional networks in embryonic stem cell pluripotency. *Cell Res,* 17**,** 42-9.

PANDEY, D. P. & PICARD, D. 2009. miR-22 inhibits estrogen signaling by directly targeting the estrogen receptor alpha mRNA. *Mol Cell Biol,* 29**,** 3783-90.

PARDO, M., LANG, B., YU, L., PROSSER, H., BRADLEY, A., BABU, M. M. & CHOUDHARY, J. 2010. An expanded Oct4 interaction network: implications for stem cell biology, development, and disease. *Cell Stem Cell,* 6**,** 382-95.

PATEL, J. B., APPAIAH, H. N., BURNETT, R. M., BHAT-NAKSHATRI, P., WANG, G., MEHTA, R., BADVE, S., THOMSON, M. J., HAMMOND, S., STEEG, P., LIU, Y. & NAKSHATRI, H. 2010. Control of EVI-1 oncogene expression in metastatic breast cancer cells through microRNA miR-22. *Oncogene*.

PATEL, J. B., APPAIAH, H. N., BURNETT, R. M., BHAT-NAKSHATRI, P., WANG, G., MEHTA, R., BADVE, S., THOMSON, M. J., HAMMOND, S., STEEG, P., LIU, Y. & NAKSHATRI, H. 2011. Control of EVI-1 oncogene expression in metastatic breast cancer cells through microRNA miR-22. *Oncogene,* 30**,** 1290-301.

PENNICA, D., SHAW, K. J., SWANSON, T. A., MOORE, M. W., SHELTON, D. L., ZIONCHECK, K. A., ROSENTHAL, A., TAGA, T., PAONI, N. F. & WOOD, W. I. 1995. Cardiotrophin-1. Biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex. *J Biol Chem,* 270**,** 10915-22.

PEPE, A. E., XIAO, Q., ZAMPETAKI, A., ZHANG, Z., KOBAYASHI, A., HU, Y. & XU, Q. 2010. Crucial role of nrf3 in smooth muscle cell differentiation from stem cells. *Circ Res,* 106**,** 870-9.

PERRON, M. P. & PROVOST, P. 2008. Protein interactions and complexes in human microRNA biogenesis and function. *Front Biosci,* 13**,** 2537-47.

PESCE, M., GROSS, M. K. & SCHOLER, H. R. 1998. In line with our ancestors: Oct-4 and the mammalian germ. *Bioessays,* 20**,** 722-32.

PESCE, M. & SCHOLER, H. R. 2001. Oct-4: gatekeeper in the beginnings of mammalian development. *Stem Cells,* 19**,** 271-8.

POHODICH, A. E. & ZOGHBI, H. Y. 2015. Rett syndrome: disruption of epigenetic control of postnatal neurological functions. *Hum Mol Genet*.

POLISENO, L., SALMENA, L., RICCARDI, L., FORNARI, A., SONG, M. S., HOBBS, R. M., SPORTOLETTI, P., VARMEH, S., EGIA, A., FEDELE, G., RAMEH, L., LODA, M. & PANDOLFI, P. P. 2010. Identification of the miR-106b~25 microRNA cluster as a proto-oncogenic PTEN-targeting intron that cooperates with its host gene MCM7 in transformation. *Sci Signal,* 3**,** ra29.

RANGREZ, A. Y., M'BAYA-MOUTOULA, E., METZINGER-LE MEUTH, V., HENAUT, L., DJELOUAT, M. S., BENCHITRIT, J., MASSY, Z. A. & METZINGER, L. 2012.

Inorganic phosphate accelerates the migration of vascular smooth muscle cells: evidence for the involvement of miR-223. *PLoS One,* 7**,** e47807.

RANGREZ, A. Y., MASSY, Z. A., METZINGER-LE MEUTH, V. & METZINGER, L. 2011. miR-143 and miR-145: molecular keys to switch the phenotype of vascular smooth muscle cells. *Circ Cardiovasc Genet,* 4**,** 197-205.

RIDEG, K., HIRKA, G., PRAKASH, K., BUSHAR, L. M., NOTHIAS, J. Y., WEINMANN, R., ANDREWS, P. W. & GONCZOL, E. 1994. DNA-binding proteins that interact with the 19-base pair (CRE-like) element from the HCMV major immediate early promoter in differentiating human embryonal carcinoma cells. *Differentiation,* 56**,** 119-29.

ROBERTS, N., JAHANGIRI, M. & XU, Q. 2005. Progenitor cells in vascular disease. *J Cell Mol Med,* 9**,** 583-91.

RODRIGUEZ, A., GRIFFITHS-JONES, S., ASHURST, J. L. & BRADLEY, A. 2004. Identification of mammalian microRNA host genes and transcription units. *Genome Res,* 14**,** 1902-10.

ROSE, T. M., WEIFORD, D. M., GUNDERSON, N. L. & BRUCE, A. G. 1994. Oncostatin M (OSM) inhibits the differentiation of pluripotent embryonic stem cells in vitro. *Cytokine,* 6**,** 48-54.

ROSENFELD, J. A., WANG, Z., SCHONES, D. E., ZHAO, K., DESALLE, R. & ZHANG, M. Q. 2009. Determination of enriched histone modifications in non-genic portions of the human genome. *BMC Genomics,* 10**,** 143.

ROSS, R. 1986. The pathogenesis of atherosclerosis--an update. *N Engl J Med,* 314**,** 488-500.

ROSS, R. & GLOMSET, J. A. 1973. Atherosclerosis and the arterial smooth muscle cell: Proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. *Science,* 180**,** 1332-9.

RUDNICKI, M. A., SAWTELL, N. M., REUHL, K. R., BERG, R., CRAIG, J. C., JARDINE, K., LESSARD, J. L. & MCBURNEY, M. W. 1990. Smooth muscle actin expression during P19 embryonal carcinoma differentiation in cell culture. *J Cell Physiol,* 142**,** 89-98.

SAINZ, J., AL HAJ ZEN, A., CALIGIURI, G., DEMERENS, C., URBAIN, D., LEMITRE, M. & LAFONT, A. 2006. Isolation of "side population" progenitor cells from healthy arteries of adult mice. *Arterioscler Thromb Vasc Biol,* 26**,** 281-6.

SATO, N., MEIJER, L., SKALTSOUNIS, L., GREENGARD, P. & BRIVANLOU, A. H. 2004. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med,* 10**,** 55-63.

SCOTT, E., LOYA, K., MOUNTFORD, J., MILLIGAN, G. & BAKER, A. H. 2013. MicroRNA regulation of endothelial homeostasis and commitment-implications for vascular regeneration strategies using stem cell therapies. *Free Radic Biol Med,* 64**,** 52-60.

SELWOOD, L. & JOHNSON, M. H. 2006. Trophoblast and hypoblast in the monotreme, marsupial and eutherian mammal: evolution and origins. *Bioessays,* 28**,** 128-45.

SHENG, C. C., ZHOU, L. & HAO, J. 2013. Current stem cell delivery methods for myocardial repair. *Biomed Res Int,* 2013**,** 547902.

SHERIDAN, S. L., BEHREND, L., VU, M. B., MEIER, A., GRIFFITH, J. M. & PIGNONE, M. P. 2009. Individuals' responses to global CHD risk: a focus group study. *Patient Educ Couns,* 76**,** 233-9.

SIMPER, D., STALBOERGER, P. G., PANETTA, C. J., WANG, S. & CAPLICE, N. M. 2002. Smooth muscle progenitor cells in human blood. *Circulation,* 106**,** 1199-204.

SINHA, S., HOOFNAGLE, M. H., KINGSTON, P. A., MCCANNA, M. E. & OWENS, G. K. 2004. Transforming growth factor-beta1 signaling contributes to development of smooth muscle cells from embryonic stem cells. *Am J Physiol Cell Physiol,* 287**,** C1560-8.

SMITH, A. G. 2001. Embryo-derived stem cells: of mice and men. *Annu Rev Cell Dev Biol,* 17**,** 435-62.

SONE, M., ITOH, H., YAMASHITA, J., YURUGI-KOBAYASHI, T., SUZUKI, Y., KONDO, Y., NONOGUCHI, A., SAWADA, N., YAMAHARA, K., MIYASHITA, K., PARK, K., SHIBUYA, M., NITO, S., NISHIKAWA, S. & NAKAO, K. 2003. Different

differentiation kinetics of vascular progenitor cells in primate and mouse embryonic stem cells. *Circulation,* 107**,** 2085-8.

SONG, S. J., ITO, K., ALA, U., KATS, L., WEBSTER, K., SUN, S. M., JONGEN-LAVRENCIC, M., MANOVA-TODOROVA, K., TERUYA-FELDSTEIN, J., AVIGAN, D. E., DELWEL, R. & PANDOLFI, P. P. 2013. The oncogenic microRNA miR-22 targets the TET2 tumor suppressor to promote hematopoietic stem cell self-renewal and transformation. *Cell Stem Cell,* 13**,** 87-101.

SPIN, J. M., NALLAMSHETTY, S., TABIBIAZAR, R., ASHLEY, E. A., KING, J. Y., CHEN, M., TSAO, P. S. & QUERTERMOUS, T. 2004. Transcriptional profiling of in vitro smooth muscle cell differentiation identifies specific patterns of gene and pathway activation. *Physiol Genomics,* 19**,** 292-302.

STADLER, B., IVANOVSKA, I., MEHTA, K., SONG, S., NELSON, A., TAN, Y., MATHIEU, J., DARBY, C., BLAU, C. A., WARE, C., PETERS, G., MILLER, D. G., SHEN, L., CLEARY, M. A. & RUOHOLA-BAKER, H. 2010. Characterization of microRNAs involved in embryonic stem cell states. *Stem Cells Dev,* 19**,** 935-50.

STEWART, C. L., KASPAR, P., BRUNET, L. J., BHATT, H., GADI, I., KONTGEN, F. & ABBONDANZO, S. J. 1992. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature,* 359**,** 76-9.

SZULWACH, K. E., LI, X., SMRT, R. D., LI, Y., LUO, Y., LIN, L., SANTISTEVAN, N. J., LI, W., ZHAO, X. & JIN, P. 2010. Cross talk between microRNA and epigenetic regulation in adult neurogenesis. *J Cell Biol,* 189**,** 127-41.

TAKAHASHI, K., TANABE, K., OHNUKI, M., NARITA, M., ICHISAKA, T., TOMODA, K. & YAMANAKA, S. 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell,* 131**,** 861-72.

TAKAHASHI, K. & YAMANAKA, S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell,* 126**,** 663-76.

TAKENAKA, C., NISHISHITA, N., TAKADA, N., JAKT, L. M. & KAWAMATA, S. 2010. Effective generation of iPS cells from CD34+ cord blood cells by inhibition of p53. *Exp Hematol,* 38**,** 154-62.

TANG, H., KONG, Y., GUO, J., TANG, Y., XIE, X., YANG, L. & SU, Q. 2013. Diallyl disulfide suppresses proliferation and induces apoptosis in human gastric cancer through Wnt-1 signaling pathway by up-regulation of miR-200b and miR-22. *Cancer Lett,* 340**,** 72-81.

TATE, P., SKARNES, W. & BIRD, A. 1996. The methyl-CpG binding protein MeCP2 is essential for embryonic development in the mouse. *Nat Genet,* 12**,** 205-8.

TAY, Y., ZHANG, J., THOMSON, A. M., LIM, B. & RIGOUTSOS, I. 2008. MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. *Nature,* 455**,** 1124-8.

THOMSON, D. W., BRACKEN, C. P. & GOODALL, G. J. 2011. Experimental strategies for microRNA target identification. *Nucleic Acids Res,* 39**,** 6845-53.

THOMSON, P. 1998. Wolf-Hirschhorn syndrome. Review of the literature and three case studies. *J Am Podiatr Med Assoc,* 88**,** 192-7.

TIEN, J. & NELSON, C. M. 2014. Microstructured extracellular matrices in tissue engineering and development: an update. *Ann Biomed Eng,* 42**,** 1413-23.

TING, Y., MEDINA, D. J., STRAIR, R. K. & SCHAAR, D. G. 2010. Differentiation-associated miR-22 represses Max expression and inhibits cell cycle progression. *Biochem Biophys Res Commun,* 394**,** 606-11.

TORELLA, D., IACONETTI, C., CATALUCCI, D., ELLISON, G. M., LEONE, A., WARING, C. D., BOCHICCHIO, A., VICINANZA, C., AQUILA, I., CURCIO, A., CONDORELLI, G. & INDOLFI, C. 2011. MicroRNA-133 controls vascular smooth muscle cell phenotypic switch in vitro and vascular remodeling in vivo. *Circ Res,* 109**,** 880-93.

TORSNEY, E., HU, Y. & XU, Q. 2005. Adventitial progenitor cells contribute to arteriosclerosis. *Trends Cardiovasc Med,* 15**,** 64-8.

TORSNEY, E., MANDAL, K., HALLIDAY, A., JAHANGIRI, M. & XU, Q. 2007. Characterisation of progenitor cells in human atherosclerotic vessels. *Atherosclerosis,* 191**,** 259-64.

TORSNEY, E. & XU, Q. 2011. Resident vascular progenitor cells. *J Mol Cell Cardiol,* 50**,** 304-11.

TSAI, T. N., KIRTON, J. P., CAMPAGNOLO, P., ZHANG, L., XIAO, Q., ZHANG, Z., WANG, W., HU, Y. & XU, Q. 2012. Contribution of stem cells to neointimal formation of decellularized vessel grafts in a novel mouse model. *Am J Pathol,* 181**,** 362-73.

TSUJIMURA, K., ABEMATSU, M., KOHYAMA, J., NAMIHIRA, M. & NAKASHIMA, K. 2009. Neuronal differentiation of neural precursor cells is promoted by the methyl-CpG-binding protein MeCP2. *Exp Neurol,* 219**,** 104-11.

TSUKAMOTO, H. 2015. Metabolic reprogramming and cell fate regulation in alcoholic liver disease. *Pancreatology,* 15**,** S61-5.

VAN DEN BERG, D. L., ZHANG, W., YATES, A., ENGELEN, E., TAKACS, K., BEZSTAROSTI, K., DEMMERS, J., CHAMBERS, I. & POOT, R. A. 2008. Estrogen-related receptor beta interacts with Oct4 to positively regulate Nanog gene expression. *Mol Cell Biol,* 28**,** 5986-95.

VASUDEVAN, S., TONG, Y. & STEITZ, J. A. 2007. Switching from repression to activation: microRNAs can up-regulate translation. *Science,* 318**,** 1931-4.

VASUDEVAN, S., TONG, Y. & STEITZ, J. A. 2008. Cell-cycle control of microRNA-mediated translation regulation. *Cell Cycle,* 7**,** 1545-9.

WANG, G., JACQUET, L., KARAMARITI, E. & XU, Q. 2015. Origin and differentiation of vascular smooth muscle cells. *J Physiol,* 593**,** 3013-30.

WANG, G., XIAO, Q., LUO, Z., YE, S. & XU, Q. 2012. Functional impact of heterogeneous nuclear ribonucleoprotein A2/B1 in smooth muscle differentiation from stem cells and embryonic arteriogenesis. *J Biol Chem,* 287**,** 2896-906.

WANG, W., LI, F., ZHANG, Y., TU, Y., YANG, Q. & GAO, X. 2013. Reduced expression of miR-22 in gastric cancer is related to clinicopathologic characteristics or patient prognosis. *Diagn Pathol,* 8**,** 102.

WANG, X., YU, H., LU, X., ZHANG, P., WANG, M. & HU, Y. 2014. MiR-22 suppresses the proliferation and invasion of gastric cancer cells by inhibiting CD151. *Biochem Biophys Res Commun,* 445**,** 175-9.

WANG, Y., MEDVID, R., MELTON, C., JAENISCH, R. & BLELLOCH, R. 2007. DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nat Genet,* 39**,** 380-5.

WEISEL, K. C., GAO, Y., SHIEH, J. H. & MOORE, M. A. 2006. Stromal cell lines from the aorta-gonado-mesonephros region are potent supporters of murine and human hematopoiesis. *Exp Hematol,* 34**,** 1505-16.

WHO. 2014. *World Health Organization methods and data sources for global causes of death 2000-2012* [Online]. Geneva, Swizerland. Available: www.who.int/healthinfo/global.../GHE_DALY_Global_2000_2012.xls [Accessed October 2015.

WIGHTMAN, B., BURGLIN, T. R., GATTO, J., ARASU, P. & RUVKUN, G. 1991. Negative regulatory sequences in the lin-14 3'-untranslated region are necessary to generate a temporal switch during Caenorhabditis elegans development. *Genes Dev,* 5**,** 1813-24.

WIGHTMAN, B., HA, I. & RUVKUN, G. 1993. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell,* 75**,** 855-62.

WILLIAMS, R. L., HILTON, D. J., PEASE, S., WILLSON, T. A., STEWART, C. L., GEARING, D. P., WAGNER, E. F., METCALF, D., NICOLA, N. A. & GOUGH, N. M. 1988. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature,* 336**,** 684-7.

WINTER, J., JUNG, S., KELLER, S., GREGORY, R. I. & DIEDERICHS, S. 2009. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol,* 11**,** 228-34.

WOLF, E., KRAMER, R., POLEJAEVA, I., THOENEN, H. & BREM, G. 1994. Efficient generation of chimaeric mice using embryonic stem cells after long-term culture in the presence of ciliary neurotrophic factor. *Transgenic Res,* 3**,** 152-8.

WONG, W. T., SAYED, N. & COOKE, J. P. 2013. Induced pluripotent stem cells: how they will change the practice of cardiovascular medicine. *Methodist Debakey Cardiovasc J,* 9**,** 206-9.

WU, L., FAN, J. & BELASCO, J. G. 2006. MicroRNAs direct rapid deadenylation of mRNA. *Proc Natl Acad Sci U S A,* 103**,** 4034-9.

WU, Y., SHEN, Y., KANG, K., ZHANG, Y., AO, F., WAN, Y. & SONG, J. 2015. Effects of estrogen on growth and smooth muscle differentiation of vascular wall-resident CD34(+) stem/progenitor cells. *Atherosclerosis,* 240**,** 453-61.

XIAO, J., YANG, B., LIN, H., LU, Y., LUO, X. & WANG, Z. 2007a. Novel approaches for gene-specific interference via manipulating actions of microRNAs: examination on the pacemaker channel genes HCN2 and HCN4. *J Cell Physiol,* 212**,** 285-92.

XIAO, Q., LUO, Z., PEPE, A. E., MARGARITI, A., ZENG, L. & XU, Q. 2009. Embryonic stem cell differentiation into smooth muscle cells is mediated by Nox4-produced H2O2. *Am J Physiol Cell Physiol,* 296**,** C711-23.

XIAO, Q., PEPE, A. E., WANG, G., LUO, Z., ZHANG, L., ZENG, L., ZHANG, Z., HU, Y., YE, S. & XU, Q. 2012. Nrf3-Pla2g7 interaction plays an essential role in smooth muscle differentiation from stem cells. *Arterioscler Thromb Vasc Biol,* 32**,** 730-44.

XIAO, Q., WANG, G., LUO, Z. & XU, Q. 2010. The mechanism of stem cell differentiation into smooth muscle cells. *Thromb Haemost,* 104**,** 440-8.

XIAO, Q., WANG, G., YIN, X., LUO, Z., MARGARITI, A., ZENG, L., MAYR, M., YE, S. & XU, Q. 2011. Chromobox protein homolog 3 is essential for stem cell differentiation to smooth muscles in vitro and in embryonic arteriogenesis. *Arterioscler Thromb Vasc Biol,* 31**,** 1842-52.

XIAO, Q., ZENG, L., ZHANG, Z., HU, Y. & XU, Q. 2007b. Stem cell-derived Sca-1+ progenitors differentiate into smooth muscle cells, which is mediated by collagen IV-integrin alpha1/beta1/alphav and PDGF receptor pathways. *Am J Physiol Cell Physiol,* 292**,** C342-52.

XIAO, Q., ZENG, L., ZHANG, Z., MARGARITI, A., ALI, Z. A., CHANNON, K. M., XU, Q. & HU, Y. 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**,** 2244-51.

XIE, C., HUANG, H., SUN, X., GUO, Y., HAMBLIN, M., RITCHIE, R. P., GARCIA-BARRIO, M. T., ZHANG, J. & CHEN, Y. E. 2011a. MicroRNA-1 regulates smooth

muscle cell differentiation by repressing Kruppel-like factor 4. *Stem Cells Dev,* 20**,** 205-10.

XIE, C., RITCHIE, R. P., HUANG, H., ZHANG, J. & CHEN, Y. E. 2011b. Smooth muscle cell differentiation in vitro: models and underlying molecular mechanisms. *Arterioscler Thromb Vasc Biol,* 31**,** 1485-94.

XIE, C. Q., ZHANG, J., VILLACORTA, L., CUI, T., HUANG, H. & CHEN, Y. E. 2007. A highly efficient method to differentiate smooth muscle cells from human embryonic stem cells. *Arterioscler Thromb Vasc Biol,* 27**,** e311-2.

XIE, T., ZHANG, J., YUAN, X., YANG, J., DING, W., HUANG, X. & WU, Y. 2013. Is X-linked methyl-CpG binding protein 2 a new target for the treatment of Parkinson's disease. *Neural Regen Res,* 8**,** 1948-57.

XIONG, J., DU, Q. & LIANG, Z. 2010. Tumor-suppressive microRNA-22 inhibits the transcription of E-box-containing c-Myc target genes by silencing c-Myc binding protein. *Oncogene,* 29**,** 4980-8.

XU, D., TAKESHITA, F., HINO, Y., FUKUNAGA, S., KUDO, Y., TAMAKI, A., MATSUNAGA, J., TAKAHASHI, R. U., TAKATA, T., SHIMAMOTO, A., OCHIYA, T. & TAHARA, H. 2011. miR-22 represses cancer progression by inducing cellular senescence. *J Cell Biol,* 193**,** 409-24.

XU, Q. 2006. The impact of progenitor cells in atherosclerosis. *Nat Clin Pract Cardiovasc Med,* 3**,** 94-101.

XU, Q. 2007. Progenitor cells in vascular repair. *Curr Opin Lipidol,* 18**,** 534-9.

XU, Q. 2008. Stem cells and transplant arteriosclerosis. *Circ Res,* 102**,** 1011-24.

YAMAGUCHI, S., YAMAHARA, K., HOMMA, K., SUZUKI, S., FUJII, S., MORIZANE, R., MONKAWA, T., MATSUZAKI, Y., KANGAWA, K. & ITOH, H. 2011. The role of microRNA-145 in human embryonic stem cell differentiation into vascular cells. *Atherosclerosis,* 219**,** 468-74.

YAMASHITA, J., ITOH, H., HIRASHIMA, M., OGAWA, M., NISHIKAWA, S., YURUGI, T., NAITO, M. & NAKAO, K. 2000. Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature,* 408**,** 92-6.

YANG, L. H., HAN, Y., LI, G., XU, H. T., JIANG, G. Y., MIAO, Y., ZHANG, X. P., ZHAO, H. Y., XU, Z. F., STOECKER, M., WANG, E., XU, K. & WANG, E. H. 2013a. Axin gene methylation status correlates with radiosensitivity of lung cancer cells. *BMC Cancer,* 13**,** 368.

YANG, W. & PAN, H. 2014. Regulation mechanism and research progress of MeCP2 in Rett syndrome. *Yi Chuan,* 36**,** 625-30.

YANG, W. J., YANG, D. D., NA, S., SANDUSKY, G. E., ZHANG, Q. & ZHAO, G. 2005. Dicer is required for embryonic angiogenesis during mouse development. *J Biol Chem,* 280**,** 9330-5.

YANG, X., GONG, Y., TANG, Y., LI, H., HE, Q., GOWER, L., LIAW, L. & FRIESEL, R. E. 2013b. Spry1 and Spry4 differentially regulate human aortic smooth muscle cell phenotype via Akt/FoxO/myocardin signaling. *PLoS One,* 8**,** e58746.

YEKTA, S., SHIH, I. H. & BARTEL, D. P. 2004. MicroRNA-directed cleavage of HOXB8 mRNA. *Science,* 304**,** 594-6.

YOSHIDA, K., CHAMBERS, I., NICHOLS, J., SMITH, A., SAITO, M., YASUKAWA, K., SHOYAB, M., TAGA, T. & KISHIMOTO, T. 1994. Maintenance of the pluripotential phenotype of embryonic stem cells through direct activation of gp130 signalling pathways. *Mech Dev,* 45**,** 163-71.

YOSHIDA, T. & OWENS, G. K. 2005. Molecular determinants of vascular smooth muscle cell diversity. *Circ Res,* 96**,** 280-91.

YU, X., ZHANG, L., WEN, G., ZHAO, H., LUONG, L. A., CHEN, Q., HUANG, Y., ZHU, J., YE, S., XU, Q., WANG, W. & XIAO, Q. 2015. Upregulated sirtuin 1 by miRNA-34a is required for smooth muscle cell differentiation from pluripotent stem cells. *Cell Death Differ,* 22**,** 1170-80.

YUAN, H., CORBI, N., BASILICO, C. & DAILEY, L. 1995. Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. *Genes Dev,* 9**,** 2635-45.

ZAMPETAKI, A., KIRTON, J. P. & XU, Q. 2008. Vascular repair by endothelial progenitor cells. *Cardiovasc Res,* 78**,** 413-21.

ZENG, L., XIAO, Q., MARGARITI, A., ZHANG, Z., ZAMPETAKI, A., PATEL, S., CAPOGROSSI, M. C., HU, Y. & XU, Q. 2006. HDAC3 is crucial in shear- and VEGF-induced stem cell differentiation toward endothelial cells. *J Cell Biol,* 174**,** 1059-69.

ZHANG, C., ZENG, L., EMANUELI, C. & XU, Q. 2013. Blood flow and stem cells in vascular disease. *Cardiovasc Res*.

ZHANG, G., XIA, S., TIAN, H., LIU, Z. & ZHOU, T. 2012a. Clinical significance of miR-22 expression in patients with colorectal cancer. *Med Oncol,* 29**,** 3108-12.

ZHANG, J., YANG, Y., YANG, T., LIU, Y., LI, A., FU, S., WU, M., PAN, Z. & ZHOU, W. 2010a. microRNA-22, downregulated in hepatocellular carcinoma and correlated with prognosis, suppresses cell proliferation and tumourigenicity. *Br J Cancer,* 103**,** 1215-20.

ZHANG, L., JIN, M., MARGARITI, A., WANG, G., LUO, Z., ZAMPETAKI, A., ZENG, L., YE, S., ZHU, J. & XIAO, Q. 2010b. Sp1-dependent activation of HDAC7 is required for platelet-derived growth factor-BB-induced smooth muscle cell differentiation from stem cells. *J Biol Chem,* 285**,** 38463-72.

ZHANG, X., CUI, N., WU, Z., SU, J., TADEPALLI, J. S., SEKIZAR, S. & JIANG, C. 2010c. Intrinsic membrane properties of locus coeruleus neurons in Mecp2-null mice. *Am J Physiol Cell Physiol,* 298**,** C635-46.

ZHANG, X., ZHANG, J., WANG, T., ESTEBAN, M. A. & PEI, D. 2008. Esrrb activates Oct4 transcription and sustains self-renewal and pluripotency in embryonic stem cells. *J Biol Chem,* 283**,** 35825-33.

ZHANG, Z., WANG, M., FAN, X. H., CHEN, J. H., GUAN, Y. Y. & TANG, Y. B. 2012b. Upregulation of TRPM7 channels by angiotensin II triggers phenotypic switching of vascular smooth muscle cells of ascending aorta. *Circ Res,* 111**,** 1137-46.

ZHAO, H., WEN, G., HUANG, Y., YU, X., CHEN, Q., AFZAL, T. A., LUONG LE, A., ZHU, J., SHU, Y., ZHANG, L. & XIAO, Q. 2015. MicroRNA-22 regulates smooth muscle cell differentiation from stem cells by targeting methyl CpG-binding protein 2. *Arterioscler Thromb Vasc Biol,* 35**,** 918-29.

ZHAO, Y. & SRIVASTAVA, D. 2007. A developmental view of microRNA function. *Trends Biochem Sci,* 32**,** 189-97.

ZIMMERMANN, C. A., HOFFMANN, A., RAABE, F. & SPENGLER, D. 2015. Role of mecp2 in experience-dependent epigenetic programming. *Genes (Basel),* 6**,** 60-86.