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Running Title: miRNA-22 in smooth muscle differentiation

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

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ABSTRACT

Objective: In this study, we attempted to uncover the functional impact of miR-22 and its target gene in smooth muscle cell (SMC) differentiation, and delineate the molecular mechanism involved.

Approach and Results: miR-22 was found to be significantly up-regulated during SMC differentiation from embryonic stem (ES) cells and adventitia stem/progenitor cells. Enforced expression of miR-22 by its mimic, while knock-down of miR-22 by its antagomiR, promotes or inhibits SMC differentiation from ES cells and adventitia stem/progenitor cells, respectively. Expectedly, miR-22 overexpression in stem cells promoted SMC differentiation in vivo. MECP2 was predicted as one of the top targets of miR-22. Interestingly, the gene expression levels of MECP2 were significantly decreased during SMC differentiation, and MECP2 was dramatically decreased in miR-22 overexpressing cells, but significantly increased when miR-22 was knockdown in the differentiating stem cells. Importantly, luciferase assay showed miR-22 substantially inhibited wild type, but not mutant MECP2-3'-UTR-luciferase activity. In addition, modulation of MECP2 expression levels affects multiple SMC-specific gene expression in differentiated ES cells. Mechanistically, our data showed that MECP2 could transcriptionally repress SMC gene expression through modulating various SMC transcription factors as well as several proven SMC differentiation regulators. Evidence also revealed that enrichment of H3K9 tri-methylation around the promoter regions of the SMC differentiation regulators genes were significantly increased by MECP2 overexpression. Finally, miR-22 was up-regulated by PDGF-BB and TGF-β through a transcriptional mechanism during SMC differentiation.

Conclusion: miR-22 plays an important role in SMC differentiation, and epigenetic regulation through MECP2 is required for miR-22 mediated SMC differentiation.

Nonstandard Abbreviations and Acronyms:
ES, embryonic stem; SMC, smooth muscle cell; miRs, microRNAs; miR-22, microRNA-22; miR-34a; microRNA-34a; SmαA, smooth muscle alpha actin; SM-MHC, smooth muscle-myosin heave chain; SM22α, smooth muscle 22 alpha; SRF, serum response factor; pSRF, phosphorylated SRF; Myocd, myocardin; MECP2, methyl CpG binding protein 2; H3K9me3, histone H3 trimethyl Lys9; Nox4, NADPH oxidase 4; Pla2g7, phospholipase A2, group VII; HDAC7, histone deacetylase 7; Ros, reactive oxygen species; PDGF-BB, platelet-derived growth factor; TGF-β, transforming growth factor beta; MEF2c, myocyte-specific enhancer factor 2C; SirT1, NAD-dependent deacetylase sirtuin-1; AdSca-1^{+} cells, adventitia stem cell antigen 1-positive cells; CHIP, chromatin immunoprecipitation; RT-qPCR, real time quantitative PCR.

Introduction

Smooth muscle cells (SMCs) that form the walls of blood vessels not only provide the healthy vessel with its structure and ability to contract under normal conditions, they also play a major role in the formation and progression of atherosclerotic plaques. It has been widely accepted that SMC differentiation is a critical process during cardiovascular development, and SMC proliferative related cardiovascular disease, such as atherosclerosis. Therefore, to better understand how vascular SMC differentiation being regulated is essential for improving the treatment or prevention of cardiovascular disorders and realizing the therapeutic potential of stem cells in cardiovascular regenerative medicine. Although recent findings reported from our group and others[1-7] have significantly improved our understanding regarding SMC differentiation and cardiovascular system development, the detailed molecular mechanisms of SMC differentiation from pluripotent stem cells have not been fully clarified.

microRNAs (miRs) are endogenous, highly conserved, short non-coding 22 nucleotide RNAs and constitute a novel class of gene expression regulators which play important roles in various aspects of development, homeostasis, and disease. Interestingly, many miRs are reported to be expressed in a tissue-specific manner, suggesting that certain miRs might be important for cell/tissue specification[8]. In addition, studies using dicer or drosha deficient embryonic stem (ES) cells have suggested that miRs play a role in ES cells self-renewal and differentiation[9-11]. An essential role of miRs in cardiovascular development has been demonstrated in a study of Dicer-deficient mice which showed that the loss of miRs resulted in severe impairment of heart and blood vessel development[12]. Furthermore, it has been shown that conditional deletion of Dicer in vascular smooth muscle caused late embryonic lethality at embryonic day 16 to 17 due to decreased SMC proliferation and differentiation which resulted in thinner vessel walls, impaired contractility, and hemorrhage[13, 14], highlighting the importance of miRs in SMC proliferation and differentiation. Although the disruption of miR processing during embryonic development provides important insights into the understanding of the functional involvements of miRs in cardiovascular development, manipulation of individual miRs may offer more precise answers to the significance and exact role of individual miRs in SMC differentiation because the elimination of virtually all miRs makes it impossible to identify relevant regulatory circuits and related miR targets. microRNA-

22 (miR-22), has been originally suggested as tumour suppressor[15-17], was found to play an important role in cardiovascular disease. It has been recently reported that miR-22 could contribute to cardiac aging by inducing cellular senescence and promoting migratory activity of cardiac fibroblasts trough targeting mimecan (osteoglycin)[18]. Moreover, miR-22 is up-regulated during human embryonic stem (ES) cell differentiation, implying that miR-22 could play a potential role in stem cell differentiation[19]. In the present study, we have demonstrated for the first time that miR-22 plays an important role in SMC differentiation from stem cells *in vitro* and *in vivo* by targeting methyl CpG binding protein 2 (MECP2), a well-known epigenetic regulator. Furthermore, we have also provided compelling evidence to support that MECP2 is a potential transcriptional repressor for SMC-specific gene expression during SMC differentiation through an epigenetic modification mechanism.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

miR-22 mediates SMC differentiation from ES cells in vitro

To induce SMC differentiation, ES cells (ES-D3) were re-seeded into collagen-coated flasks and cultured in SMC differentiation medium for 2 to 8 days as described in our previous studies[1-5, 20-23]. Consistently, SMC specific markers including smooth muscle alpha actin (SMαA) and myosin heave chain (SM-MHC for protein or SM-myh11 for gene) were significantly increased upon cell differentiation (**Figure I**). Our recent study has suggested that miR-34a plays a functional role in SMC differentiation[24]. Interestingly, along with miR-34a another miRNA, miR-22, was emerged as one of the top up-regulated miRNAs during SMC differentiation in our miRNA microarrays analyses[24], which was further confirmed by RT-qPCR analysis (**Figure 1A**), suggesting a role for miR-22 in SMC differentiation. To investigate whether miR-22 induction was important for SMC differentiation, lose-of-function experiments by using Anti-miR™ miR-22 inhibitor (Ambion) were performed in differentiating ES cells. Data showed that both gene and protein levels (**Figure 1B and Figure IIA**) of smooth muscle differentiation specific markers (SMαA, SM22α, h1-calponin and SM-myh11) were significantly inhibited by miR-22 knockdown. On the other hand, data from the gain-of-function experiments using Pre-miR™ mmu-miR-22 miRNA Precursor (Ambion) clearly revealed that miR-22 overexpression could enhance the gene and protein expression of SMC-specific markers (**Figure 1C and Figure IIB**), suggesting a critical role of miR-22 in SMC differentiation from stem cells. Importantly, we also observed that two of important SMC transcription factors, serum response factor (SRF) and myocardin (Myocd), were regulated by miR-22 in a similar manner to SMC specific genes, while modulation of miR-22 expression levels in differentiating ES cells has no effect on the expression level of another transcriptional factor myocyte enhancer factor 2C (MEF2c) (**Figure III**), suggesting that miR-22 works in concert with SRF and Myocd during SMC differentiation from stem cells.

Functional role of miR-22 in SMC differentiation in vivo

miR-22 over-expressing (pLL3.7-GFP-miR-22) and control (pLL3.7-GFP) ES cells were generated to facilitate our *in vivo* SMC differentiation study. GFP-positive cells with high purity (**Figure IVA**) were sorted out from ES cells infected with pLL3.7-GFP- or pLL3.7-GFP-miR-22 lentivirus and maintained in ES cell culture medium.

No significant differences were observed between the sorted cells and their parent ES cells in terms of morphology, self-renew and pluripotency when they were cultured in ES cell culture medium for up to at least five passages (**data not shown**). The expression level of miR-22 was low and similar among parental ES cells, control and miR-22 over-expressing ES cells (**Figure IVB**), indicating the expressional machinery of miR-22 was inhibited under stem cell culture condition. Similar to their parent cells, miR-22 expression was significantly up-regulated during differentiation, and compared to control ES cells (pLL3.7-GFP), the expression levels of miR-22 in miR-22 overexpressing ES cells (pLL3.7-GFP-miR-22) were further up-regulated at day 8 of differentiation (**Figure IVB**), suggesting that the inhibitory mechanism of miR-22 under stem cell culture condition has been removed and miR-22 was successful up-regulated in these cells during SMC differentiation. Consequently, more SMCs were differentiated from miR-22 over-expressing ES cells (**Figure IVC**), further confirming that miR-22 promotes SMC differentiation. To further explore the functional relevance of miR-22 in SMC differentiation *in vivo*, pLL3.7-GFP and pLL3.7-GFP-miR-22 ES cells were subcutaneously injected into C57BL/6J mice with 100ng/ml of PDGF-BB to promote *in vivo* SMC differentiation as described in our previous studies[1, 2]. We observed a higher percentage of cells were SM-MHC-positive SMCs in the implants of miR-22 overexpressing ES cells than that of control cells as demonstrated by immunofluorescence staining with antibody against GFP and SM-MHC (**Figure VA and VB**). As expected, the majority of cells in the Matrigel implants were GFP-positive, implying its exogenous origins (**Figure VA**). Furthermore, our data showed that the expression levels of miR-22, SMαA and SM-MHC in the Matrigel implants of pLL3.7-GFP-miR-22 ES cells were significantly higher than that of control cells (**Figure VC**), further confirming the efficiency of miR-22 over-expression and the importance of miR-22 in SMC differentiation *in vivo*. Taken together, these data firmly suggest a regulatory role of miR-22 in SMC differentiation from stem cells *in vitro* and *in vivo*.

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

Cells positive for Sca-1 has been reported as one of the major blood vessel residential stem/progenitor cells which contribute to atherosclerosis of vein-graft[25]. To further explore its relevance or implication of miR-22 in vascular diseases, adventitia stem cell antigen 1-positive (AdSca-1+) cells isolated from vessel adventitia as described

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previously[2, 25], were induced to differentiate into SMCs. RT-qPCR analyses showed that miR-22 was significantly up-regulated during SMC differentiation from AdSca-1^{+} cells, along with various SMC differentiation genes (**Figure VIA**). Importantly, data from miR-22 over-expression and knockdown experiments (**Figure VIB and VIC**) revealed that enforced expression of miR-22 by its precursor significantly increased all the SMC genes examined, while knock-down of miR-22 by its antagomiR/inhibitor dramatically inhibited these gene expression, respectively, suggesting a functional involvement of miR-22 in SMC specifications of the vascular residential stem/progenitor cells.

Target gene, MECP2, is negatively regulated by miR-22 through the binding sites within 3'UTR

By utilising similar strategies as described in previous study[26], MECP2 was predicted as one of the top targets of miR-22. Firstly, bioinformatics search for putative miR-22 binding sites within the MECP2 mRNA by using several online free accessible computational algorithmic databases (e.g. TargetScan 4.0 and PicTar) revealed that the seed sequence of miR-22 is predicted to hybridize to serval regions of MECP2 3'UTR, which is evolutionarily conserved among vertebrate species. Secondly, at least four highly conserved binding sites for miR-22 have been identified within MECP2 3'UTR (**Figure VIIA and VIIB**). Finally, by using mFold software (http://mfold.rna.albany.edu/?q=DINAMelt/Two-state-melting) we also found a favourable minimum loop-free energy (-10.70 to -12.0 kcal/mol) in the formation of the miR-22:MECP2 3'UTR duplex stem-loop for all four miR-22 binding sites, suggesting that miR-22 is involved in translational repression of MECP2. As expected, MECP2 gene expressions were significantly down-regulated (**Figure 2A**), and displayed a negative correlation with the gene expression levels of miR-22 during SMC differentiation (**Figure 1A**), suggesting that miR-22 may negatively regulate MECP2. Such notion has been firmly supported by the findings that MECP2 gene levels were significantly down-regulated or up-regulated by over-expression or inhibition of miR-22 in the differentiating ES (**Figure 2B**) or AdSca-1^{+} cells (**Figure VIB and VIC**), respectively. Furthermore, as shown in **Figure VC** MECP2 gene expression levels in the Matrigel implants of pLL3.7-GFP-miR-22 ES cells were significantly lower than that of control cells, indicating that MECP2 was also negatively regulated by miR-22 during *in vivo* SMC differentiation. Expectedly, the protein levels of MECP2 were dramatically inhibited by miR-22 over-expression

(**Figure 2C and IIC**), indicating that MECP2 is negatively regulated by miR-22 directly or indirectly. To distinguish these two possibilities, we first attempted to generate the miRNA reporter containing full length of MECP2 3'UTR (~8500bps), but unsuccessful. Instead, three MECP2 3'UTR reporters containing the segments spinning through the first two miR-22 binging sites (1567-2698), the third binding site (4142-4968) and the fourth binding site (8173-9137) were successfully generated in this study, designated as pmiR-Luc-MECP2-A, -B and –C (**Figure VIIA and VIIB**), respectively. Data from our miRNA reporter assay showed that the activity of luciferase of reporter A and C, but not reporter B, was significantly down-regulated by miR-22 over-expression (**Figure 2D**). Most importantly, the site-directed mutagenesis of the predicted miR-22 binding site(s) in the MECP2 3'UTR reporter(s) experiments showed that the 2nd (within reporter A) and 4th (within reporter C) binding sites are required for MECP2 3'UTR reporter activity inhibition mediated by miR-22 (**Figure 2E**).

MECP2 over-expression abolished miR-22 mediated SMC gene expressions

We have provided solid evidence to support that MECP2 is an authentic miR-22 target during SMC differentiation. To investigate the potential role of MECP2 in SMC differentiation, MECP2 knockdown in the differentiating ES cells was conducted by using specific MECP2 siRNA. Data showed that MECP2 knockdown significantly up-regulated SMC specific marker expressions (**Figure 3A**), suggesting that MECP2 inhibition can recapitulate the effects of miR-22 during SMC differentiation from ES cells. Conversely, MECP2 over-expression significantly repressed SMC specific marker expressions (**Figure 3B and IID**), suggesting that MECP2 acts as a repressor of SMC differentiation genes during SMC differentiation. To further explore the functional importance of MECP2 in miR-22-mediated SMC differentiation, control or MECP2 over-expression vector were transfected into day 2~3 differentiating control (pLL3.7-GFP) or miR-22 over-expressing (pLL3.7-GFP-miR-22) ES cells, respectively. RT-qPCR analyses showed that while miR-22 (2nd columns) or MECP2 (3rd columns) over-expression alone in the differentiating ES cells were significantly up-regulated or down-regulated the gene expression levels of various SMC differentiation markers (**Figure 3C**) and specific transcription factors (**Figure VIII**), respectively, re-activation of MECP2 almost completely abolished these gene up-regulations induced by miR-22 over-expression (4th columns),

suggesting that MECP2 repression is required for miR-22 mediates SMC gene expression during SMC differentiation from ES cells.

Functional importance of SRF binding site within SMC-specific genes in MECP2-mediated SMC gene expression

We have demonstrated clearly that SMC specific gene expression was repressed by MECP2 gene activation. To further explore the underlying molecule mechanism by which MECP2 regulates SMC gene expression, luciferase activity assays were conducted in differentiating ES cells using respective SMC gene promoter reporters, pGL3-Luc-SMαA and pGL3-Luc-SM22α. Data showed that the overexpression of MECP2 in differentiating ES cells significantly inhibited SMαA and SM22α gene promoter activities (**Figure IXA**), indicating that MECP2 over-expression can repress specific SMC gene expression at transcriptional level. Since the functional importance of SRF binding element (CArG) within promoter region of SMC-specific genes has been well-documented in SMC gene regulation[27], we thus wondered if such elements also play a role in MECP2-mediated SMC gene repression. For such purpose, another set of luciferase assays using SRF binding site mutants (pGL3-Luc-SMαA-SRF[mu] and pGL3-Luc-SM22α-SRF[mu]) generated in our previous study[1] were carried out in differentiating ES cells. We observed that mutating the SRF binding element within SMC gene promoters almost completely nullified the inhibitory effects of MECP2 over-expression on SMC gene transcriptional activity (**Figure IXA**), suggesting that SRF binding site(s) within the promoters is required for MECP2-mediated SMC gene repression. However, we observed no significant enrichment of MECP2 within the promoter regions of SMC genes as demonstrated by ChIP assays using MECP2 specific antibody (**Figure IXB**) suggesting no direct binding of MECP2 to SMC gene promoters. Furthermore, as expected although we observed a huge degree of SRF enrichment on SMC gene promoter (up to 10 folds enrichment) in separate CHIP assays using SRF specific antibody, such enrichment was not affected by MECP2 over-expression (**Figure IXC**), suggesting that MECP2 plays no significant role in modulation of SRF binding to SMC-specific gene promoters.

SMC transcription factors SRF and Myocd are transcriptionally repressed by MECP2

Our previous data showed that the gene expression levels of SRF and Myocd, but not the MEF2c, were significantly regulated by miR-22 (**Figure III**). Importantly, data

shown in **Figure 4A** revealed that these two transcription factors were regulated by MECP2 in an opposite manner to miR-22, implying an important role of MECP2 in regulation of these two transcription factors during SMC differentiation. Such possibility has been clearly supported by the data obtained from luciferase activity assays using respective SRF, MEF2c and Myocd gene reporter plasmids (pGL3-Luc-SRF, pGL3-Luc-MEF2c and pGL3-Luc-Myocd) generated in our previous study[1] (**Figure 4B**). Interestingly, CHIP assays with MECP2 antibody showed a significant enrichment of MECP2 within the promoter regions of SRF (up to 3 folds) and Myocd (up to 20 folds), and such enrichments were further enhanced by MECP2 over-expression (**Figure 4C**), suggesting that MECP2 directly binds to SRF and Myocd gene promoters. Taken together, our data clearly demonstrated that MECP2 transcriptionally repress SMC transcription factor gene expression during SMC differentiation from stem cells through its direct binding to their promoter regions.

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

Since transcription factor nuclear factor erythroid 2-related factor 3 (Nrf3)[5], NADPH oxidase 4 (Nox4)[21], platelet-activating factor acetylhydrolases (Pla2g7)[2], histone deacetylases 7 (HDAC7)[20] and DNA/RNA binding proteins [heterochromatin Protein 1γ (Cbx3)[3], heterogeneous nuclear ribonucleoprotein (hnRNP) A2B1[4] and A1[1]] have been previously identified as important SMC differentiation regulators in our SMC differentiation system, therefore, we wondered whether miR-22 and/or MECP2 plays any role in regulation of these genes. To this aim, the gene expression levels of above SMC differentiation regulators were examined in the cells over-expressing miR-22 or MECP2. We found the gene expression levels of Nox4, HDAC7 and Pla2g7 were consistently regulated by miR-22 (**Figure 5A**) and MECP2 (**Figure 5B**) in an opposite way. Importantly, these three genes were co-regulated by miR-22 and MECP2 in a similar manner to other SMC differentiation genes (**Figure VIII**), suggesting miR-22 and/or MECP2 mediated SMC differentiation at least partially through regulation of these three reported SMC differentiation modulators. Furthermore, data from luciferase assays using a functional Pla2g7 gene promoter reporter (pGL3-Luc-Pla2g7-P2, harbouring 387bps of Pla2g7 gene promoter fragment located between ~2.4 to 2.0 kb upstream of the translation start site of Pla2g7 gene) and a control reporter (pGL3-Luc-Pla2g7-P10, located within exon 1 of Pla2g7 gene) generated in our previous study[2] showed that the promoter activity of pGL3-Luc-Pla2g7-P2, but not pGL3-Luc-Pla2g7-P10, was significantly regulated by

MECP2 over-expression (**Figure 5C**). Finally, up to 4 times of MECP2 enrichment within Pla2g7 gene promoter (region 2) was observed, and MECP2 over-expression further increased the accumulation of MECP2 within Pla2g7 gene promoter, while no apparent enrichment of MECP2 within the adjacent promoter area (region 10) was observed in the MECP2-CHIP assays (**Figure 5D**), suggesting that MECP2 regulates Pla2g7 gene expression through direct interacting with the region 2 (-2.4 to -2.0 kb) of Pla2g7 gene promoter. Similarly, both Nox4 and HDAC7 were regulated by MECP2 through a transcriptional mechanism as demonstrated in promoter activity analyses (**Figure XA**) and MECP2-CHIP assays (**Figure XB**), respectively.

MECP2 represses SMC gene expression through increasing H3K9 methylation within the gene promoters of SRF, Myocd and Pla2g7

It has been well-documented that MECP2 is capable of binding specifically to methylated DNA and involves in gene silencing, and methylation of lysine H3K9 is closely associated with gene transcriptional repression[28]. To further elucidate the molecular mechanism by which MECP2 represses SMC gene expression, we first examined if H3K9me3 expression levels were regulated by MECP2. Data from over-expression experiments showed that H3K9me3 protein expression levels were not significantly affected by MECP2 over-expression (**Figure 6A**). However, our CHIP assays with H3K9me3 specific antibody showed a variety of degree of H3K9me3 enrichment within the promoter regions of examined genes (up to 2.5 folds for SMαA/SM22α; 23 folds for SRF, 40 times for Myocd and 96 times for Pla2g7, respectively), and importantly such enrichments were further enhanced by MECP2 over-expression for SRF, Myocd and Pla2g7 gene promoters (**Figure 6C and 6D**), but not for SMαA/SM22α (**Figure 6B**), which are consistent with the enrichment of MECP2 within respective gene promoters as observed in our above CHIP assays with MECP2 antibody (**Figures IXB, 4C, and 5D**). Expectedly, miR-22 over-expression significantly inhibited H3K9me3 enrichment within the promoter regions of SRF, Myocd and Pla2g7, but no such inhibition was observed within the promoter regions of SMαA/SM22α (**Figure XI**). Taken together, above data clearly demonstrated that MECP2 represses SMC-specific gene expression, at least partially through increasing H3K9 tri-methylation within the gene promoters of SMC specific transcription factors (SRF and Myocd) and differentiation modulators (eg. Pla2g7).

PDGF-BB and TGF-β up-regulate miR-22 through a transcriptional mechanism

Our previous studies have suggested that during stem cell differentiation, auto-secreted growth factors (e.g. PDGF-BB[20, 23] and TGF-β[21]) from the differentiating cells activate their respective down-dream signal pathways, which in turn trigger SMC differentiation program. We wondered if miR-22 was one of such signal molecules during SMC differentiation. Indeed, RT-qPCR analyses showed that both PDGF-BB and TGF-β were significantly up-regulated miR-22 expression in a dose-dependent pattern (**Figure XIIA and XIIB**). Moreover, PDGF-BB and TGF-β treatments also increased the expression levels of miR-22 precursor and primary RNAs (**Figure XIIC and XIID**), suggesting that miR-22 was regulated by PDGF-BB and TGF-β at transcriptional level. Such a notion was further confirmed by incubating the cells with RNA synthesis inhibitor, actinomycin D (1μg/ml for 6 hours), in which the data showed that Actinomycin D incubation ablated the effect of PDGF-BB and TGF-β treatments on the expression levels of miR-22 (**Figure XIIE and XIIF**).

Discussion

Accumulating evidence in the literature has revealed that the gene regulatory program of SMC differentiation from pluripotent stem cells is orchestrated by a coordinated molecular network composing of various signaling pathways and molecules, such as Myocd–SRF complex, extracellular matrix, integrins, retinoid receptor, TGF family, notch family, reactive oxygen species, microRNAs, HDACs, and others (eg, paired-like homeodomain 2 and protein inhibitor of activated STAT-1)[29, 30]. Despite enormous efforts have been put into this field in the past decades, our understandings into the molecular mechanisms underlying SMC differentiation are still far from complete. In the present study, we have further advanced our knowledge in 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 the first evidence of a functional role for 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 SMC differentiation gene repressor during SMC differentiation from stem cells.

It has been reported that the human miR-22 gene, located in a minimal loss of heterozygosity region between markers D17S1866 and D17S1574 on chromosome 17 (17p13.3) (close to TP53) in cancer cells, overlaps the exon 2 region of the spliced non-coding C17orf91 transcript[31], and the primary miR-22 is processed from a capped, polyadenylated transcript[32], while the mouse counterpart is also mapped to a cancer associated genomic region[33], implying an important role for miR-22 in cancers. Indeed, several studies have independently identified miR-22 as a tumour suppressor[15-17]. Moreover, it has been recently reported that miR-22 could contribute to cardiac aging by inducing cellular senescence and promoting migratory activity of cardiac fibroblasts through targeting osteoglycin[18], suggesting that miR-22 might play a role in cardiovascular disease. Furthermore, it has been documented that miR-22 is up-regulated during human ES cell differentiation[19], induced by TPA during monocytic differentiation from HL-60 leukemia cell lines[34], and is close associated with erythroid maturation[35], implying that miR-22 could play a potential role in hematopoietic cell differentiation and maturation. However, the functional role of miR-22 in SMC differentiation from pluripotent stem cells remains to be explored. In

the present study, miR-22 has been signalled out from our microRNA screening experiments as one of the top potential miRNA candidates with high expression levels during SMC differentiation from ES cells. Importantly, by utilizing miRNA gain/lose-of function analyses, we confirmed a critical role for miR-22 in SMC differentiation from ES cells *in vitro*. Furthermore, by generating miR-22 over-expressing ES cell lines and using our well-established *in vivo* SMC differentiation model (Martigel-stem cells-PDGF-BB complex implantation)[1, 2] we provide first evidence to support that miR-22 plays an important role in embryonic SMC differentiation *in vivo*. These data firmly demonstrated for the first time that miR-22 is an important SMC differentiation regulator.

Identifying and validating the genuine mRNA target(s) that are responsible for or mediate any given functions of examined miRNA(s) is fundamental and most difficult step in miRNA study. Since we have demonstrated clearly that miR-22 can regulate SMC differentiation *in vitro* and *in vivo*, we sought to identify the downstream mRNA target(s) which is responsible for miR-22 mediated SMC differentiation from stem cells. For this purpose, we utilised several computational algorithmic databases including Targetscan (www.targetscan.org), pictar (www.pictar.mdc-berlin.de), and miRanda (www.microrna.org) to predict the putative mRNA targets of miR-22, and found that MECP2 was emerged as a top targets of miR-22. Interestingly, the MECP2 mRNA has a long 3' UTR of about 8.7 kb which bears evolutionarily conserved miRNA target sites, suggesting that it might be regulated by miRNAs. Importantly, we have identified four highly conserved binding sites for miR-22 within MECP2 3'UTR as shown in **Figure VIIA and VIIB,** implying that MECP2 is an mRNA target of miR-22. Indeed, such notion that MECP2 is a bona-fide miR-22 target during SMC differentiation has been supported by several lines of evidence: Firstly, MECP2 gene expression was significantly down-regulated during SMC differentiation from stem cells (**Figure 2A**) which displayed a perfect and negatively association with miR-22 expression levels (**Figure 1A**). Secondly, MECP2 gene and protein expression levels were adversely regulated by miR-22 as demonstrated in miR-22 over-expression and inhibition experiments (**Figure 2B and 2C**). Thirdly, miR-22 over-expression dramatically down-regulates MECP2 3'UTR activity, but such down-regulation was completely abolished when two of the miR-22 binging sites within MECP2 3'UTR were mutated (**Figure 2D and 2E**). Interestingly, the

binding sites that have been proven to be responsible for miR-22 mediated MECP2 gene regulation in this study are the ones located around ~2797bp and ~8347bps, but not the one located in the middle of 3'UTR (~4537bp), which is consistent with one of the principal rules of microRNA–mRNA interactions that for gene with long 3' UTRs, the position of miRNA binding site(s) that is not too far away from the poly(A) tail or the termination codon can significantly improve site efficacy[36], therefore represents a higher possibility of these binding sites being more functional relevance. However, it is noteworthy to mentioned that since we failed to obtain such a mutant with only 1st miR-22 binding site (~1577bp) being mutated in this study, we could not exclude the importance of this binding site in mediating MECP2 3'UTR activity by miR-22. Nonetheless, our data clearly demonstrated that MECP2 is a genuine mRNA target of miR-22. Finally but importantly, comparing with the Matrigel plugs implanted with control ES cells the MECP2 gene expression levels in the Matrigel implants with miR-22 over-expressing ES cells were much lower (**Figure VC**), suggesting that MECP2 gene expression is negatively regulated by miR-22 and MECP2 is also a true mRNA target of miR-22 during *in vivo* SMC differentiation from stem cells.

Apart from MECP2 that has been identified and validated as a bona-fide miR-22 mRNA target during SMC differentiation in the current study, several other miR-22 mRNA targets including oncogene EVI-1[37], HDAC4[38], PTEN[39], estrogen receptor α (ER α)[40], **c**-Myc binding protein (MYCBP)[33], MYC associated factor X (Max)[34], TET2[41], have been reported in cancer cells. However, none of them was proved to be a true miR-22 mRNA target in our SMC differentiation system. Among them, only the expression levels of EVI-1 was negatively associated with miR-22 expression levels in the miR-22 over-expression and/or inhibition experiments, but over-expression of miR-22 failed to down-regulate ZVI-1 3'UTR luciferase activity (**data not shown**). These data suggested such a possibility that some of miR-22 mRNA targets are cell-specific or miR-22 likely plays a divergent role under various physiological and pathological conditions through targeting distinct target gene(s).

One of novel mechanistic findings in the present study is that we provided first time but compelling evidence to support that MECP2 repression is required for miR-22 mediated SMC differentiation from stem cells, and MECP2 functions as an inhibitory regulator in SMC differentiation gene expression. MECP2 is the funder member of

methyl-CpG binding domain proteins that can specifically bind to both methylated and unmethylated DNA and recruit distinct interacting protein partners to establish a repressive or active chromatin environment[42], respectively, and has been reported to be involved in a variety of biological functions and diseases, such as rett syndrome and neural development[43, 44], modulating human iNOS gene expression[45], regulation of myofibroblast differentiation during pulmonary fibrosis[46], myogenesis[47], neural differentiation from ES cells[48] or neural precursors[49], adult neurogenesis[50], neuron electrophysiological properties[51] and embryonic development[52]. Importantly, recent study has suggested that MECP2 can switch their transcriptional activity in the epigenetic regulation of neural chromatin and gene expression through 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 in repression when bound to 5mC containing DNA[53]. In the present study, we have provided compelling evidence to support that MECP2 is an important SMC differentiation mediator by transcriptional regulation of the gene expression of SMC specific markers, transcriptional factors and other SMC differentiation regulators. So far, several molecular roles have been described for MeCP2 in gene regulation: transcriptional repression, activation of transcription, nuclear organization, and splicing[42]. In consistent its roles in gene regulation, we have provided strong evidence in the current study which firmly demonstrated that MECP2 is a transcription repressor to regulate SMC-specific gene expression (SMαA, SM22α, SRF, Myocd, Nox4, HDAC7 and Pla2g7) during SMC differentiation (**Figure 4 and 5, Figure IX and X**). We obtained no evidence to show that MECP2 represses SMC-specific gene expression through direct binding to their gene promoters. Instead, we found that MECP2 could bind directly to the gene promotes of SMC transcription factors (SRF and Myocd) and proven SMC differentiation modulators (e.g. Pla2g7, Nox4 and HDAC7) (**Figure 4C and 5D, Figure IX and X**).

Another important finding of the present study is that we further demonstrate that MECP2 acts as a potential transcriptional repressor for SMC gene regulation through modulating epigenetic modifications of SMC-specific transcription factors and/or SMC differentiation modulators. DNA methylation and histone modifications represent the major epigenetic mechanisms implicated in the regulation of gene transcription in mammals. It has been widely accepted that hypomethylation of the

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DNA surrounding the proximal promoter region is a prerequisite for gene activation, whereas heavy methylation leads to gene silencing. Previous studies reported that MeCP2 was associated with histone methyltransferase activity *in vivo* and such activity is directed against Lys9 of histone H3[54]. Moreover, our previous data also showed that H3K9 methylation was enriched within SMC specific gene promoter regions in the differentiating stem cells[3]. These data prompted us to investigate if MECP2 represses SMC-specific gene expression during stem cell differentiation is through modulating DNA methylation. In the current study we have provided for the first time evidence to support a notion that instead of increasing H3K9me3 protein production in the differentiating stem cells, MECP2 increases H3K9 tri-methylation within the gene promoters of SMC-specific transcription factors and other SMC differentiation regulators, resulting in SMC gene repression during stem cell differentiation towards SMC lineage. However, the functional involvements of MECP2 in the regulation of other epigenetic modifications remain to be fully elucidated.

Finally, we have demonstrated that miR-22 is up-regulated by PDGF-BB and TGF-β through a transcriptional mechanism during SMC differentiation. Interestingly, apart from miR-22, our unpublished data suggests that PDGF-BB and/or TGF-β also transcriptionally up-regulates another reported SMC differentiation miRNA, miR-34a[24]. Taken together, we have successful identified a novel function of miR-22 in SMC differentiation from stem cells *in vitro* and *in vivo,* and provided comprehensive evidence to support that MECP2 is a genuine mRNA target of miR-22 during SMC differentiation, and repression of MECP2 in the differentiating stem cells is required for miR-22 mediated SMC differentiation. Moreover, we have revealed that MECP2 regulates SMC gene expression through a transcriptional mechanism as well as an epigenetic signal pathway. Furthermore, we have found that miR-22 and/or MECP2 modulates SMC-specific gene expression through regulating other proven SMC differentiation masters, and have exemplify demonstrated that MECP2 transcriptionally regulates other SMC differentiation modulators gene expression through its direct binding to their promoter region and increasing H3K9 tri-methylation within the gene promoters. Therefore, based on the findings presented in the current study and our previous study[24] we propose the following molecular mechanism through which miRNAs mediate SMC differentiation (**Figure XIII**): during stem cell differentiation, miR-22 and miR-34a were transcriptionally up-

regulated by PDGF-BB and/or TGF-β auto-secreted from differentiating cells. On the one hand, the inhibitory effects of MECP2 on SMC specific transcription factors (SRF and Myocd) as well as other SMC differentiation master regulators (e.g. Pla2g7, Nox4 and HDAC7) were removed/de-repressed by up-regulated miR-22, triggering SMC-specific gene expression programme and promoting SMC differentiation. On the other hand, increased miR-34a up-regulated its target gene, SirT1, through an unusual manner, which in turn transcriptionally regulated three SMC transcriptional factors (SRF, Myocd and MEF2C), resulting in SMC differentiation gene activation and SMC differentiation. Undoubtedly, the findings presented in this study will significantly increase our knowledge of the molecular mechanisms underlying SMC differentiation and benefit future stem cell application in cardiovascular regenerative medicine.

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Significance

Smooth muscle cell (SMC) differentiation is a complicated process involving many transcription factors and regulators. Studies focused on molecular mechanisms governing SMC differentiation are crucial for improving our understanding of the pathophysiological process of cardiovascular development and various cardiovascular diseases formation/progression, and developing novel therapeutic strategies for these diseases. In this study, we have uncovered a previously unrecognised functional role of miR-22 in stem cell differentiation towards SMCs in vitro and in vivo. We have identified MECP2 is a bona-fide miR-22 target during SMC differentiation, which functions as an inhibitory regulator in SMC differentiation gene expression. Importantly we have further demonstrated that MECP2 acts as a potential transcriptional repressor for SMC gene regulation through modulating epigenetic modifications of SMC-specific transcription factors and/or SMC differentiation modulators. Our data provide novel molecular insights into the regulation of SMC differentiation and enhance our knowledge of SMC differentiation and vascular development.

Figures

Figure 1. miR-22 regulates SMC differentiation from ES cells.

(A) Induction of miR-22 during SMC differentiation from ES cells. Day 0 samples were undifferentiated ES cells and served as negative control. (B) miR-22 inhibition impairs SMC marker expressions. Day 3 differentiating ES cells were transfected with miR-22 inhibitor or negative control, and cultured in SMC differentiation medium for 48 hours. (C) miR-22 overexpression promotes SMC marker expressions. ES cells were transfected with miR-22 precursor or negative control, and cultured in SMC differentiation medium for 48~72 hours. Total RNA and protein were harvested and subjected to RT-qPCR and Western blot analyses, respectively. The data presented here are representative or mean$\pm$S.E.M. of three independent experiments. *P<0.05.
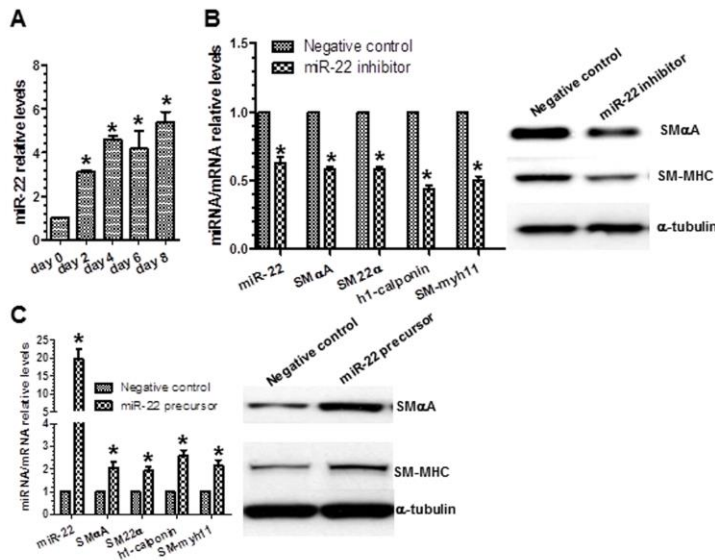
Figure 2. MECP2 was identified as a target gene of miR-22 during SMC differentiation.

(A) Gene expression levels of MECP2 during SMC differentiation from ES cells. (B and C) miR-22 negatively regulates MECP2 expression levels. Total RNA and protein were harvested as described in Figure 1 and subjected to RT-qPCR (B) and Western blot (C) analyses, respectively. (D-E) Binding sites located around ~2797 and ~8347 of MECP2 3'UTR are required for miR-22 mediated MECP2 gene repression. miR-22 precursor or negative control and wild type MECP2 3'UTR reporters (pmiR-Luc-MECP2-A, B, C) or three indicated mutants [pmiR-Luc-MECP2-A bindings site 2 (bs2^{mu}), combinational mutations (bs1/2^{mu}) and pmiR-Luc-MECP2-C bindings site (bs^{mu})] were co-transfected into day 2~3 differentiating ES cells and luciferase activity assay were measured at 48 hours post-transfection. The data presented here are representative or mean $\pm$ S.E.M. of three to four independent experiments. *P<0.05 (treatment versus day 0 or control).
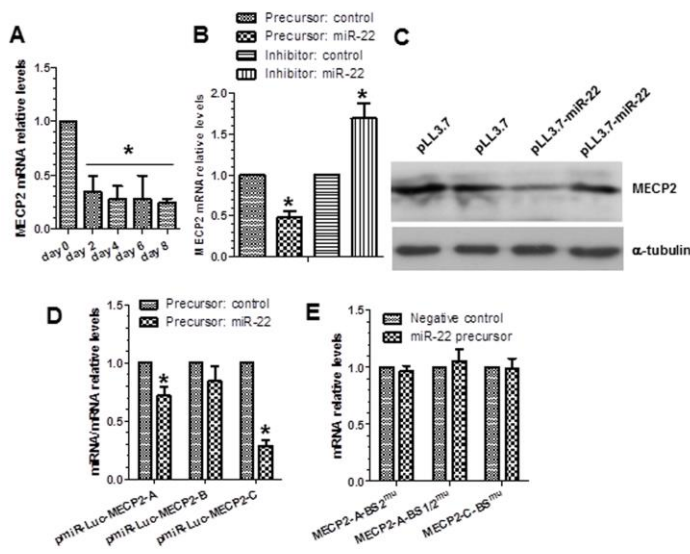
Figure 3. MECP2 repression is required for miR-22 mediated SMC gene expressions.

(A) MECP2 knockdown increases SMC gene expression. Day 2~3 differentiating ES cells were transfected with control siRNA (ctrl siRNAs) or MECP2 specific siRNA (MECP2 siRNAs), and cultured in SMC differentiation medium for another 48 or 72 hours. Total RNAs were harvested and subjected to RT-qPCR analyses. (B) MECP2 over-expression inhibits SMC gene expression. Day 2~3 differentiating ES cells were transfected with control (pCMV5) or MECP2 over-expression plasmid (pCMV5-MECP2), and cultured in SMC differentiation medium for another 48 or 72 hours. Total RNAs and proteins were harvested and subjected to RT-qPCR and Western Blotting analyses, respectively. The data presented here are representative (insert) or mean $\pm$ S.E.M. of three independent experiments, *$P<0.05$. (C) MECP2 over-expression abolished SMC gene expression induced by miR-22. Day 2 differentiating control (pLL3.7-GFP) and miR-22 over-expressing (pLL3.7-GFP-miR-22) ES cells were transfected with respective control (pCMV5) and MECP2 over-expression (pCMV5-MECP2) plasmids, and cultured in SMC differentiation medium for further 48 to 72 hrs. Total RNAs were harvested and subjected to RT-qPCR analyses. The data presented here are mean$\pm$S.E.M. of three independent experiments.
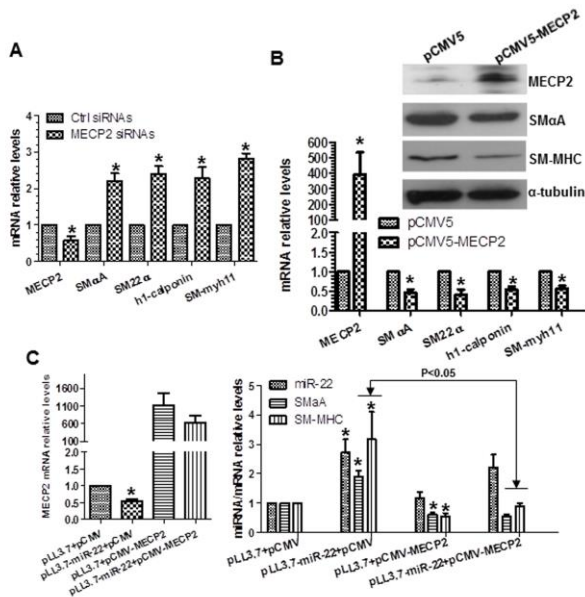
Figure 4. SMC transcription factor gene expressions were negatively regulated by MECP2.

(A) The expression levels of SRF and Myocd, but not the MEF2c, were significantly down-regulated by MECP2 over-expression. Total RNAs were harvested as described in Figure 3A. (B) Promoter activities of SRF and Myocd genes were modulated by MECP2. Day 2~3 differentiating ES cells were transfected with luciferase reporter plasmids pGL3-SRF-Luc, pGL3-MEF2c-Luc or pGL3-Myocd-Luc ($0.15\mu g/2.5\times10^4$ cells) together with pCMV5 or pCMV5-MECP2 ($0.2\mu g/2.5\times10^4$ cells). pShuttle-LacZ ($0.2\mu g/2.5\times10^4$cells) was included as control. Luciferase and β-galactosidase activity assays were detected 48 hours after transfection. The data presented here are mean±S.E.M. of four independent experiments. *$P<0.05$ (vs. control). (C) MECP2 binds directly to the promoter regions of SRF and Myocd genes. ChIP assays were performed using antibodies against MECP2 or normal IgG, respectively, as described in online supplemental data. PCR amplifications of the adjacent regions were included as additional control for specific promoter DNA enrichment. The data presented here are mean±S.E.M. of three independent experiments. *$P<0.05$ (vs. control).
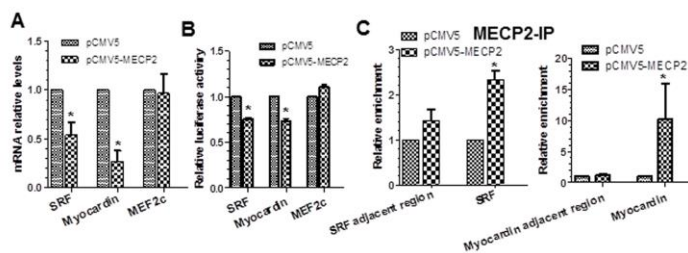

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Figure 5. **O**ther reported SMC differentiation regulators were negatively modulated by MECP2.

(A) Gene expression levels of other reported SMC differentiation regulators in the differentiating cells transfected with control and miR-22 precursor. Total RNAs were harvested as described in Figure 1C. (B) Three reported SMC differentiation regulators were regulated by MECP2. Total RNAs were harvested as described in Figure 3A. (C) Promoter activities of Pla2g7 gene were significantly repressed by MECP2 over-expression. Day 2~3 differentiating ES cells were transfected with luciferase reporter plasmids pGL3-Pla2g7-2-Luc or pGL3-Pla2g7-10-Luc $(0.15\mu g/2.5\times10^4$ cells) together with pCMV5 or pCMV5-MECP2 $(0.2\mu g/2.5\times10^4$ cells). The data presented here are mean±S.E.M. of four independent experiments. *$P<0.05$ (vs. control). (D) MECP2 binds directly to the promoter (region 2) of Pla2g7 gene. ChIP assays were performed as described in Figure 5C. PCR amplifications of the adjacent region (region 10) were included as additional control for specific promoter DNA enrichment. The data presented here are mean±S.E.M. of three independent experiments. *$P<0.05$ (vs. control).
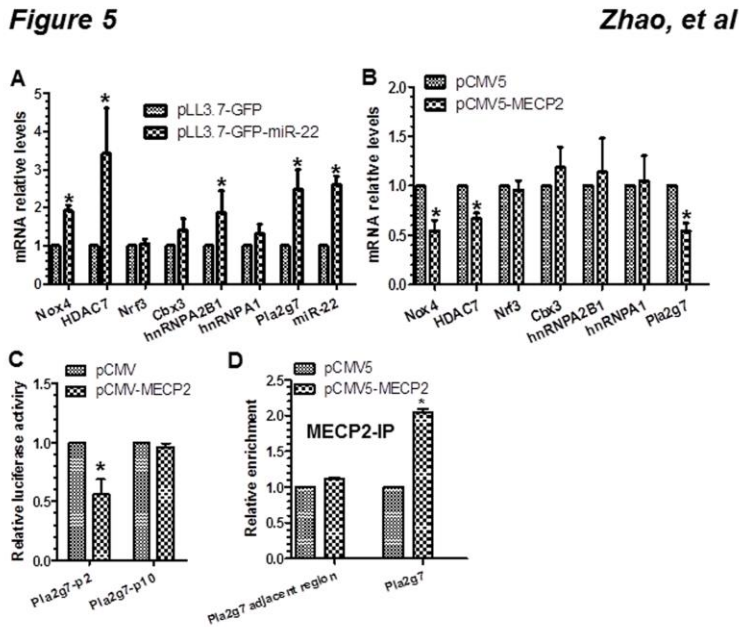
Figure 6. MECP2 modulates H3K9me3 enrichment on the respective gene promoter regions of SRF, Myocd and Pla2g7.

(A) H3K9me3 protein levels were not affected by MECP2 over-expression. Total proteins were harvested as described in Figure 3B. (B) The enrichment of H3K9me3 within the promoter regions of SMαA and SM22α genes were not affected by MECP2 over-expression. (C) MECP2 over-expression increases H3K9me3 bindings to the promoter regions of SRF and Myocd genes. (D) The enrichment of H3K9me3 within the promoter regions of Pla2g7 gene was significantly increased by over-expression of MECP2. ChIP assays were performed using antibody against H3K9me3 or normal mouse IgG, respectively, as described above. The data presented here are mean±S.E.M. of three independent experiments. *$P<0.05$ (vs. control).
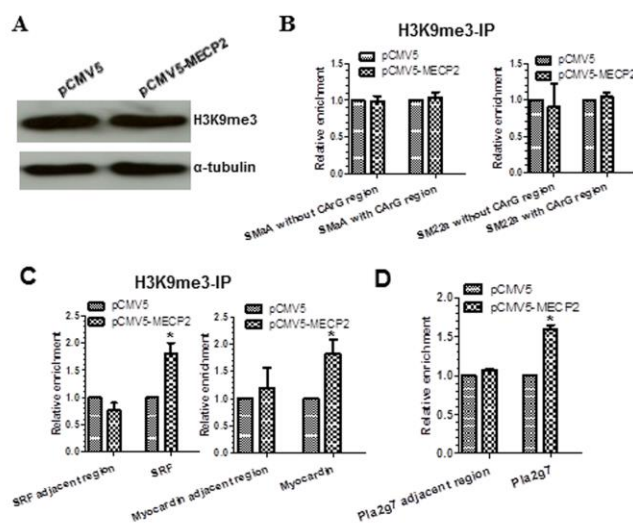
Methods & Materials

Materials. Antibodies against MECP2 (goat, N-17, sc-5755) and SRF (rabbit, G-20, sc-335) were purchased from Santa Cruz Biotech, USA. Antibody against Smooth Muscle Myosin Heavy Chain (SM-MHC) was from AbD Serotec (Rabbit, AHP1117). Antibodies against MECP2 (rabbit, ab2828, CHIP grade), SM22α (rabbit, Ab14106) and calponin (rabbit, Ab46794) were from Abcam, UK. Antibodies against α-tubulin (mouse), monoclonal anti-α smooth muscle actin (SMαA) (Clone 1A4, A5228) and GFP (G6539) were from Sigma. Antibody against H3K9me3 (mouse, 05-1250) was from Millipore. All secondary antibodies were from Dako, Denmark. Other materials used in this study were purchased from Sigma unless specifically indicated.

ES cell culture and smooth muscle cell (SMC) differentiation. Detailed protocols for mouse embryonic stem cells (mESCs) (ES-D3 cell line, CRL-1934; ATCC, Manassas, USA) culture and SMC differentiation were described in our previous studies[1-9]. Briefly, undifferentiated ES cells were dissociated into single cells and seeded onto collagen I/IV (5μg/ml)-coated flasks or plates in differentiation medium [DM, MEM alpha medium (Gibco) supplemented with 10% FBS, 0.05mM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin] for 2 to 8 days prior to further treatment. The medium was refreshed every other day. For PDGF-BB or TGF-β treatment, the pre-differentiated ES cells (day 2~3) were cultured in serum-free basal medium (MEM alpha medium supplemented with 1% BSA, 10ng/ml insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin) for 1hour, followed by the addition of different amounts of PDGF-BB or TGF-β and further incubation for 12 or 3 hours, respectively. In some specific experiments, cells were treated with actinomycin D in DMSO (1μg/ml) for 6 hours in the absence or presence of 2.5ng/ml PDGF-BB or 1ng/ml TGF-β before harvesting for gene expression analysis.

AdSca-1+ cell isolation and differentiation. Detailed protocols for mouse adventitia stem cell antigen 1-positive (AdSca-1+) cell isolation and culture were described in our previous studies[2, 10]. Briefly, thoracic arties were harvested and the periadventitial fat was carefully removed. Arties were rinsed with PBS and pre-digested for 10~15 minutes in a digestion solution containing 1mg/ml collagenase (Sigma, C0130-1G). Adventitia layer was carefully peeled off from media layer under stereo microscope, and cut into small pieces, followed by second digestion in a solution containing 3

mg/ml of collagenase and 500 µg/ml elastase at 37ºC for 2-2.5 hours on the shaker, vortexing every 20-30 min. Digested cell suspension was collected and filtered through a 70µm BD Falcon® cell strainer to obtain single cells. Cells isolated from 8~10 mice were pooled together and sorted using the Anti-Sca-1 MicroBead Kit (MACS Miltenyi Biotec). Freshly isolated AdSca-1+ cells were induced to SMCs in the SMC differentiation medium containing DMEM, 10% FBS, 0.05mM 2-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin and 5ng/ml TGF-β for 2 to 6 days prior to further treatment. The medium was refreshed every other day.

Immunoblotting. Cells were harvested and lysed in lysis buffer (50mM Tris-Cl pH 7.5, 150mM NaCl, 1 mM EDTA pH 8.0) supplemented with protease inhibitors and 0.5% Triton and sonicated to obtain whole cell lysate. 40 µg of protein was separated by SDS-PAGE with 4%~20% Tris-Glycine gel (Invitrogen, Carlsbad, CA, USA) and subjected to standard Western blot analysis. In some experiments, the blots were subjected to densitometric analysis with Image J software. Relative protein expression level was defined as the ratio of target protein expression level to α-tubulin expression level with that of the control sample set as 1.0.

Real time quantitative PCR (RT-qPCR) for mRNA and microRNAs. Real-time quantitative PCR (RT-qPCR) was performed as previously described[11]. Briefly, total RNA containing small RNAs (microRNAs) was extracted from cells using mirVana™ Protein and RNA Isolation System™ Kit (Applied Biosystems, Ambion Inc) or TRI reagent (Sigma) according to the manufacturer's instructions. Reverse transcription for long RNA was performed using an Improm-IITM RT kit (Promega, Madison, WI, USA) with RNase inhibitor (Promega), and Random primers (Promega). The NCode™ VILO™ miRNA cDNA Synthesis Kit (Invitrogen, A11193-051) was used to synthesise poly (A) tails of all the miRNAs followed by cDNA synthesis from the tailed population in a single reaction. The resultant cDNA was diluted to a working concentration of 5ng/µl and stored at -20ºC. Primers were designed using Primer Express software (Applied Biosystems) and the sequence for each primer was shown in supplementary Table I. NCode™ EXPRESS SYBR® GreenER™ qPCR SuperMix Universal was used in miRNA RT-qPCR. Relative mRNA or microRNA expression level was defined as the ratio of target gene expression level or microRNA expression level to 18S or U6snRNA expression level, respectively, with that of the control sample set as 1.0.

Generation of miR-22 overexpressing ES cells. Approximate 606bps of genomic fragment containing mmu-miR-22 precursor (95bps) and its flanking sequence (256bps and 261bps, respectively) was amplified by PCR with specific primer set as shown in Table I from differentiating stem cells, and cloned into Hpa I/Xho I sites of the pLL3.7-GFP (Addgene, Plasmid 11795) expression vector, designated as pLL3.7-GFP-miR-22. All the vectors were verified by DNA sequencing, and the vectors with correct sequence were further amplified and used to produce lentiviral particles. Briefly, 293T cells were transfected with the control (pLL3.7-GFP) or miR-22 (pLL3.7-GFP-miR-22) plasmid and the packaging plasmids, pMDLg/pRRE (Addgene, 12251), pRSV-Rev (Addgene, 12253) and pMD2.G (Addgene, 12259) using TurboFect Transfection Reagent (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. The supernatant containing the lentivirus was harvested 48h later, filtered, aliquoted and stored at −80°C. For lentiviral infection, ES cells were plated 24 hours prior to infection in T25 flasks at 37°C. One millilitre of respective lentiviral particles were added with 10μg/ml hexadimethrine bromide (H9268; Sigma). Viral constructs were incubated 24 hours with the cells before the media was replaced with complete media. Cells were culture for further 2~3 days, and GFP-positive cells were sorted out and cultured in ES cell culture medium for 2~3 passages before using.

In vitro/vivo SMC differentiation of miR-22 over-expressing ES cells and immunofluorescent staining for sections. Control (pLL3.7-GFP) or miR-22 over-expression (pLL3.7-GFP-miR-22) ES cells were induced to differentiate into SMCs as described above. The procedures for in vivo SMC differentiation were similar to that as described in our previous study[1, 2]. Briefly, control or miR-22over-expression ES cells (106 in 50μl) were mixed with 50μl of Matrigel (Becton Dickinson Labware) and PDGF-BB (100ng/ml) at 4°C, and subcutaneously injected into C57BL/6J mice. After 10~13 days, mice were sacrificed and the implants (Matrigel plugs) were harvested and frozen in liquid nitrogen for future using. Half of each Matrigel plug will be sectioned for detection of cell markers and rests were lysed and extracted total RNA to examine related gene expression levels, respectively. All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals.

For immunofluorescent staining, sections were cut at 8 μm for optimum cutting temperature compound–embedded Matrigel implants, every 40 μm along the longitudinal axis of Matrigel plugs, and numbered. Given numbered sections (for instance, sections 5, 15 and 25) were subjected to immunohistological analyses with respective antibody. Briefly, frozen sections were air-dried for at least 30 minutes, followed by fixed in cold acetone for 15 minutes. The sections were then rinsed in PBS and blocked with 5% BSA in PBS (Sigma) for 1 hr at room temperature in a humid chamber. The incubation with primary antibodies (SM-MHC and GFP) or IgG controls diluted in blocking buffer was performed in a cold room (4°C) overnight. Followed by incubation with appropriate FITC or TRITC conjugated secondary antibodies, sections were then incubated with DAPI (1:1000, Sigma) for 5 minutes. Images were assessed with Axioplan 2 imaging microscope with Plan-NEOFLUAR 20×, NA 0.5, objective lenses, AxioCam camera, and Axiovision software (all Carl Zeiss MicroImaging, Inc.) at room temperature, and were processed with Photoshop software (Adobe). The percentage of GFP-labelled SM-MHC-positive cells with clear cell body and strong fluorescence signal per field were counted by two well-trained independent investigators blinded to the treatments, from four random high power fields (200x) in each section, three sections from each implant and four implants for each group.

microRNA and plasmids transfection. Either miRNAs inhibitors or precursors and miRNA negative controls (30nM) were transfected into differentiating ES cells using siPORTTM NeoFXTM transfection agent (Ambion, Applied Biosystems) according to the manufacturer's instructions. Transfected cells were plated into flasks or plates coated with 5μg/ml of collagen and cultured for 48~72 hours in the SMC differentiation medium to allow SMC differentiation. All miRNAs inhibitors or precursors and respective negative controls were purchased from Ambion. Mouse full length of MECP2 gene was amplified by RT-PCR from ES cells with primer set as shown in Table I and cloned into Mlu I/Xba I sites of the pCMV5 expression vector, designated as pCMV5-MECP2. All the vectors were verified by DNA sequencing, and the vectors with correct sequence were further amplified and used in the related experiments. Control (pCMV5) and MECP2 overexpression (pCMV5-MECP2) plasmids were transfected into differentiating ES cells using TurboFect Transfection Reagent (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions.

Flow cytometry analysis. Differentiated cells were dissociated into single cells by trypsin-EDTA (Gibco, Invitrogen) and subjected to flow cytometry analyses using antibodies against GFP or SMαA as described in our previous studies[1, 9].

MECP2 3'UTR clone and miR-22 binding sites mutation. Reporter vector harboring sequences of the murine MECP2 was created using cDNA from ES cells. Three fragments (1567-2698, 4142-4968 and 8173-9137) containing partial 3'-flanking untranslation region (3'UTR) of murine MECP2 gene (NM_001081979) were amplified by PCR with primer sets shown in Table I and cloned into the Mlu I and Sac I sites of the pmiR-reporter-basic vector (Ambion, Applied Biosystems), designated as pmiR-Luc-MECP2-A (harboring miR-22 binding sites 1 1577~1616) and 2 (2797~2836)), B (harboring binding site 3, 4537~4576) and C (containing binding site 4, 8347~8386), respectively (Figure VIIA). miR-22 binding site 1, 2 mutation alone or combination in reporter A or C were introduced into respective pmiR-Luc-MECP2 reporter by using QuikChange™ site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer's instructions. The resultant vectors were designated as pmiR-Luc-MECP2-A-bs2mu, pmiR-Luc-MECP2-A-bs1/2mu, and pmiR-Luc-MECP2-C-bsmu mutants, respectively. All mutants were verified by DNA sequencing.

Transient transfection and luciferase assay. Luciferase assay for WT and mutated MECP2 3'UTR reporters were conducted as previously study[11]. Briefly, day 2~4 differentiating ES cells were co-transfected with individual reporter genes (pmiR-Luc-MECP2-A/B/C, pmiR-Luc-MECP2-A-bs2mu, pmiR-Luc-MECP2-A-bs1/2mu, or pmiR-Luc-MECP2-C-bsmu, 0.15 μg/2.5 x 10⁴ cells) and control or miR-22 precursor (30nM) using siIMPORTER transfection reagents (Millipore), according to the manufacturer's instructions. Gene promoter luciferase activity assays were performed as previously described[4, 5, 8]. Briefly, differentiating cells were co-transfected with respective gene promoter reporter (pGL3-Luc-genes, 0.15 μg/2.5 x 10⁴ cells) and control (pCMV5, 0.20 μg/2.5 x 10⁴ cells) or MECP2 over-expression (pCMV5-MECP2, 0.20 μg/2.5 x 10⁴ cells) plasmid, respectively, using TurboFect Transfection Reagent (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. The gene promoter reporters used in this study were generated in our previous studies, eg. pGL3-Luc-SMαA7, pGL3-Luc-SM22α7, pGL3-Luc-SMαA-

SRFmu[1], pGL3-Luc-SM22α-SRFmu[1], pGL3-Luc-SRF[1], pGL3-Luc-MEF2c[1], pGL3-Luc-Myocardin[1], and pGL3-Luc-HDAC7[5, 7]. pGL3-Luc-Nox4 was purchased from Generay Biotechnology (Shanghai, China). pShuttle2-LacZ (0.20µg/2.5 x 104 cells) was included in all transfection assays as internal control. Luciferase and β-galactosidase activities were detected 48 hours after transfection using a standard protocol. Relative luciferase unit (RLU) was defined as the ratio of Luciferase versus β-galactosidase activity with that of the control (set as 1.0).

MECP2 knockdown by siRNAs. 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. ES cells were cultured on collagen IV-coated 6-well plates for 3~4 days, and 6 µl of 10µM siRNA (final concentration of siRNAs: 60nM) was introduced with siIMPORTER transfection reagents (Millipore) according to the protocol provided. Cells were harvested at 48 or 72 hours after transfection and real-time RT-PCR analyses were performed.

Chromatin immunoprecipitation (ChIP) assays. The ChIP assays were performed as previously described[1-3, 11]. Briefly, differentiating ES cells transfected with control (pCMV5) or MECP2 over-expression (pCMV5-MECP2) plasmids were treated with 1% (v/v) formaldehyde at room temperature for 10 min and then quenched with glycine at room temperature. The medium was removed, cells were harvested and sonicated. The sheared samples were diluted into 1 ml immunoprecipitation buffer, and immunoprecipitations were conducted with antibodies raised against MECP2 (goat, sc-5755; rabbit, ab2828), SRF (rabbit, G-20, sc-335), or H3K9me3 (mouse, 05-1250), respectively. Immunoprecipitation complex was pulled-down using protein-G-Dynabeads. Equal amount (2µg/immunoprecipitation) of normal goat, rabbit or mouse IgG was used as control. The immunoprecipitates were eluted from the beads using 100 µl elution buffer, and immunoprecipitaed DNA was extracted, purified, and then used to amplify target DNA sequences by RT-qPCR using specific primers (Table I). Promoter DNA enrichment with specific antibody was calculated using percent input method with that of the IgG control set as 1.0. The relative level of promoter DNA enrichment was defined as the ratio of promoter DNA enrichments in the samples with treatment(s) (pCMV5-MECP2) to the control samples (pCMV5) with that of the control sample set as 1.0. PCR amplification of the adjacent promoter regions or

regions lacking of SRF binding sites (without CArG region) were included as additional control for specific promoter DNA enrichment. The data was obtained from three to four independent experiments.

Statistical analysis. Data were expressed as mean±SEM and analyzed using a two-tailed student's t-test for two-group comparison or one-way ANOVA followed by Tukey's HSD multiple comparison post-hoc test for comparing different groups. A value of $P < 0.05$ was considered as statistically significant.

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Supplementary Tables and Figures

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

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Supplementary table I: Primer sets used in the present study

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	GAT ATG GCA GCA GTG CAG AG	AGT TGG CTG TCT GTG AAG TC	RT-qPCR
h1-Calponin	GGT CCT GCC TAC GGC TTG TC	TCG CAA AGA ATG ATC CCG TC	RT-qPCR
SM-myh11	AAG CAG CCA GCA TCA AGG AG	AGC TCT GCC ATG TCC TCC AC	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-P1	GCAGGTTCCTTTGTCGGGCCA	CTGCTTGGCTCACCACCCCG	CHIP assay (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-p2	GGGCTCCTAGCTGGCACGTC	TCTCCACCCCAACCCACCCC	CHIP assay
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-miR-22 clone
Mus MECP2	ctcgtcACGCGTttggccgccgctgccgccac	ctccac TCTAGA tcagctaactctctcggtcacgg	pCMV5-MECP2 clone
pmiR-Luc-MECP2-A	GCTGTCACGCGTGCGGATTGCAAAGCAAACCAACA	GTCGACGAGCTCACCTGGCACTGGCAATGGGA	MECP2 3'UTR reporter-A clone
pmiR-Luc-MECP2-B	CTACTGACGCGTCCCAACCTGCCCCATGCACTC	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	TCTTCTGTTCCATTTGAAGGCAGTGCTGAA CC	GGTTCAGCACTGCCTTCAAATGGAACAGAAGA	miR-22 binding site 2 mutation in reporter-A

| pmiR-Luc-MECP2-A-bs1/2[mu]	CCAACAAGAATAAATTTGAAGGTTGTCTCTTCTCC	TCTTCTGTTCCATTTGAAGGCAGTGCTGAA CC	miR22 binding site 1 and 2 combinational mutation in reporter-A
pmiR-Luc-MECP2-C-bs[mu]	ATGTTTCTGTTTGAAGGGACAATGGAGTGC	GCACTCCATTGTCCCTTCAAACAGAAACAT	miR-22 binding site mutation in reporter-C

Supplementary Figures and Legends:

Figure I. Gene expression levels of SMC-specific genes were significantly up-regulated during stem cell differentiation toward SMCs.
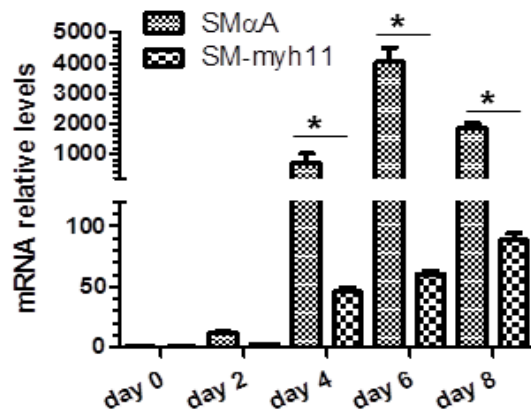
Undifferentiated ES cells were plated into flasks coated with 5µg/ml of collagen and cultured in SMC differentiation medium to allow for SMC differentiation. Total RNA from undifferentiated ES cells (d0) or differentiating ES cells at day 2, 4, 6 and 8 were harvested and subjected to RT-qPCR analyses with primers specific for SMαA and SM-Myh11, respectively. The data presented here are mean±S.E.M. of three independent experiments. Significant difference from control (day 0), *P<0.05.

Figure II. Quantitative analyses of protein relative expression levels.
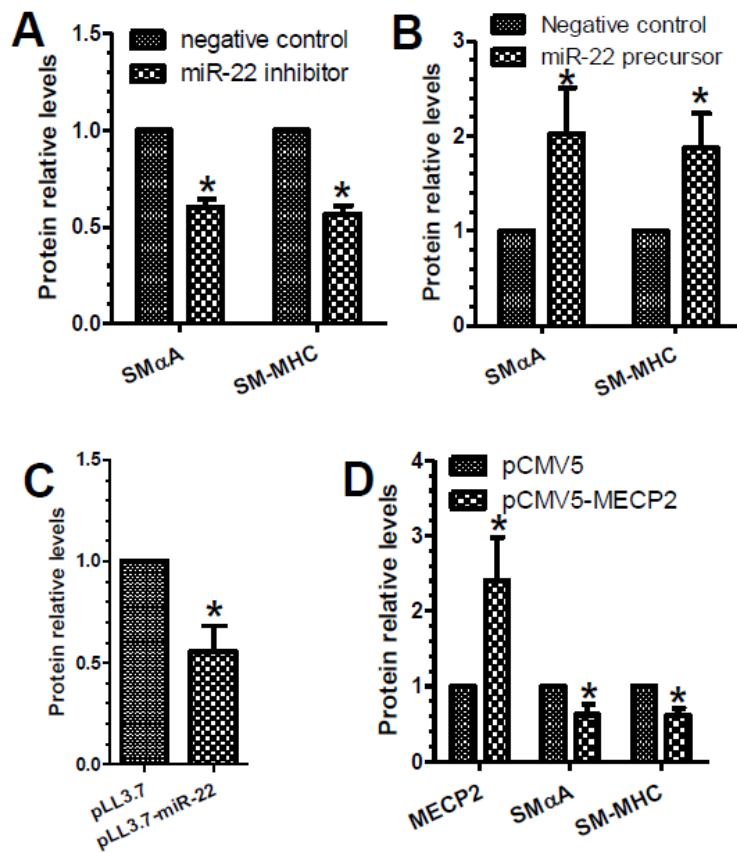
The blots were subjected to densitometric analysis with Image J software. Panel A, B, C and D refer to figure 1B, 1C, 2C and 3B, respectively. *p<0.05 (versus respective control).

Figure III. Modulation of miR-22 expression in differentiating ES cells regulates SMC transcription factors.
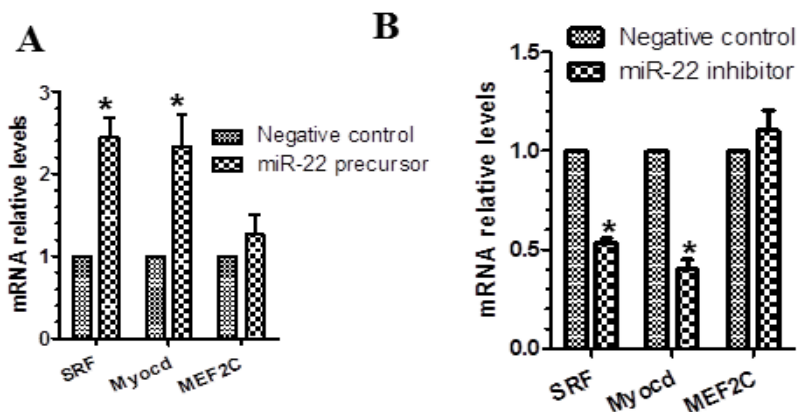
miR-22 over-expression (A) increases, while inhibition (B) reduces SRF and myocardin expressions. Total RNA and protein were harvested as described in Figure 1, and subjected to RT-qPCR analyses. The data presented here are representative or mean±S.E.M. of three independent experiments. *P<0.05.

Figure IV. Generation of miRNA-22 overexpressing ES cells and differentiation towards SMCs.
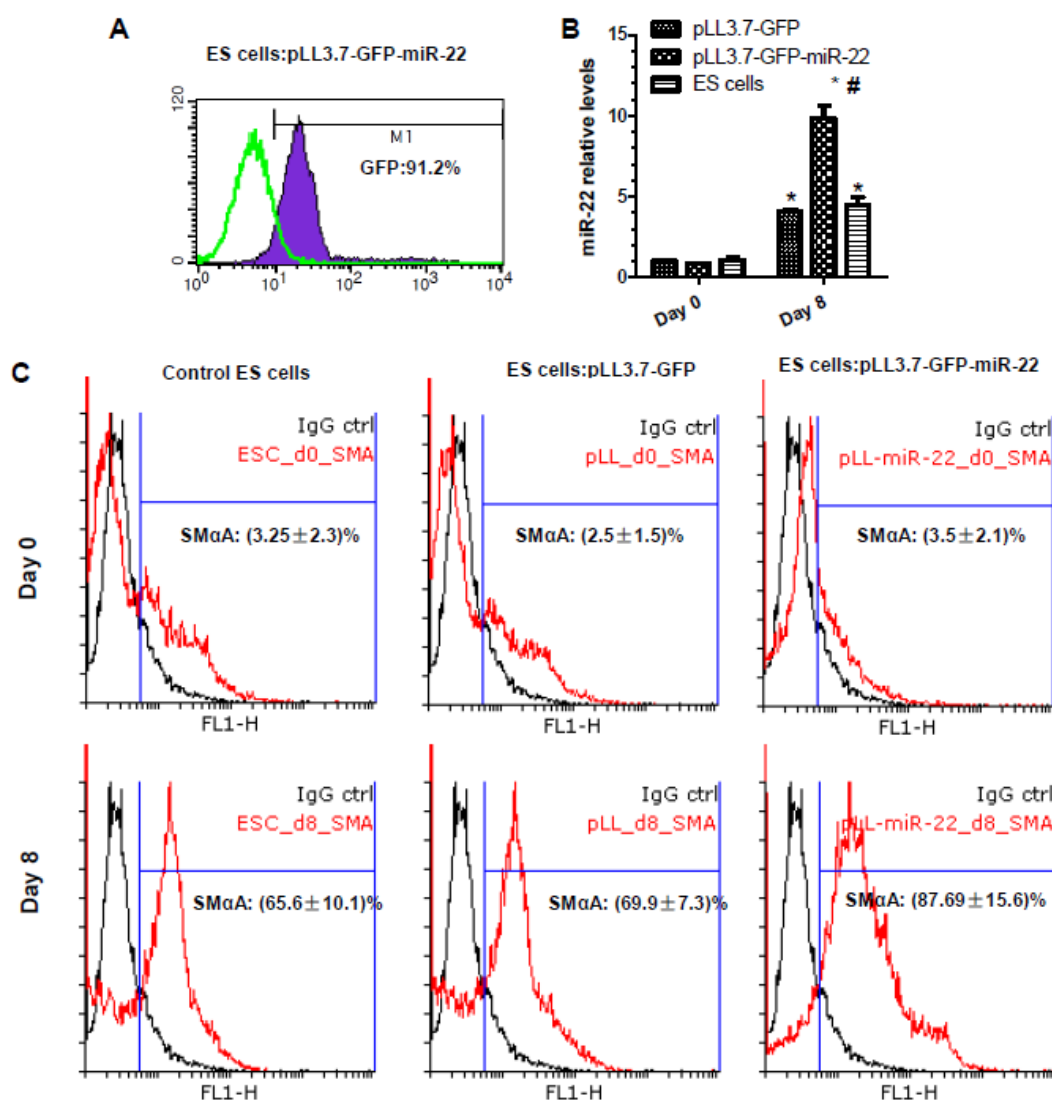
(A) Sorted ES cells were GFP-positive. (B) miR-22 was significantly increased during SMC differentiation. *P<0.05 (versus day 0), #P<0.05 (pLL3.7-GFP-miR-22 versus pLL3.7-GFP). (C) More SMCs were differentiated from miR-22 overexpressing ES cells. Parental ES cells (control ES cells), control (pLL3.7-GFP) and miR-22 over-expressing (pLL3.7-GFP-miR-22) ES cells were induced to differentiate into SMCs for 8 days. Cells were harvested and subjected to flow cytometry analyses using SMαA antibody. Undifferentiated cells (Day 0) were included as differentiation control. Representatives of flow cytometry histogram or mean±S.E.M. of three independent experiments were presented here.

Figure V. miR-22 promotes SMC differentiation in vivo.
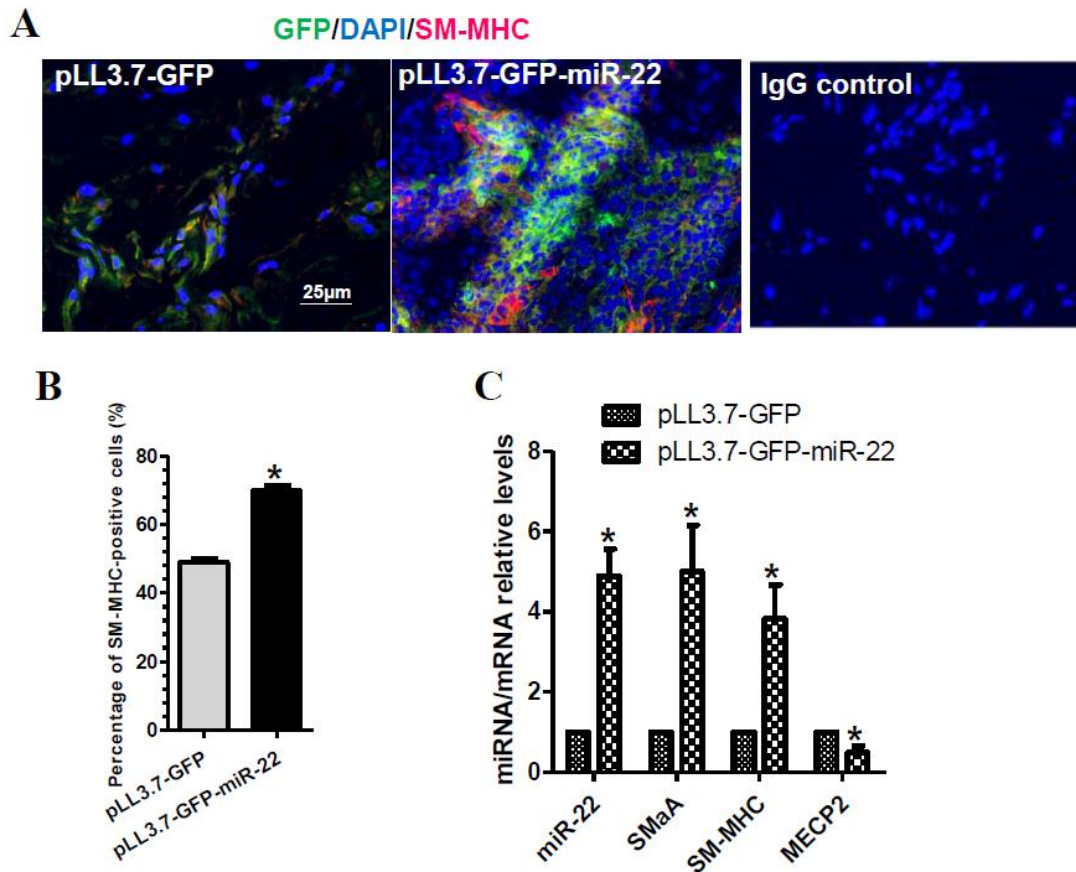
Matrigel plugs implanted with control (pLL3.7-GFP) or miR-22 over-expression (pLL3.7-GFP-miR-22) ES cells were harvested, sectioned and subjected to immunofluorescence staining using antibodies against GFP and SM-MHC. Representative images (A) and quantitative data (B) of the percentage of SM-MHC-positive cells were presented here, respectively. Note: cells with green fluorescence signal indicate GFP-positive cells (implanted cells) within Matrigel plugs. The percentage of GFP-labelled SM-MHC-positive cells with clear cell body and strong fluorescence signal per field were examined by two well-trained independent investigators blinded to the treatments, from four random high power fields (200x) in each section, three sections from each implant and four implants for each group, *p<0.05. (C) Gene expression levels within Matrigel implants. Total RNA samples were extracted from partial Matrigel implants and subjected to RT-qPCR analysis. The data presented here are mean±S.E.M. of four Matrigel implants. *P<0.05.

**Figure VI. Functional importance of miR-22 in adventitia stem/progenitor cell differentiation towards SMCs.**
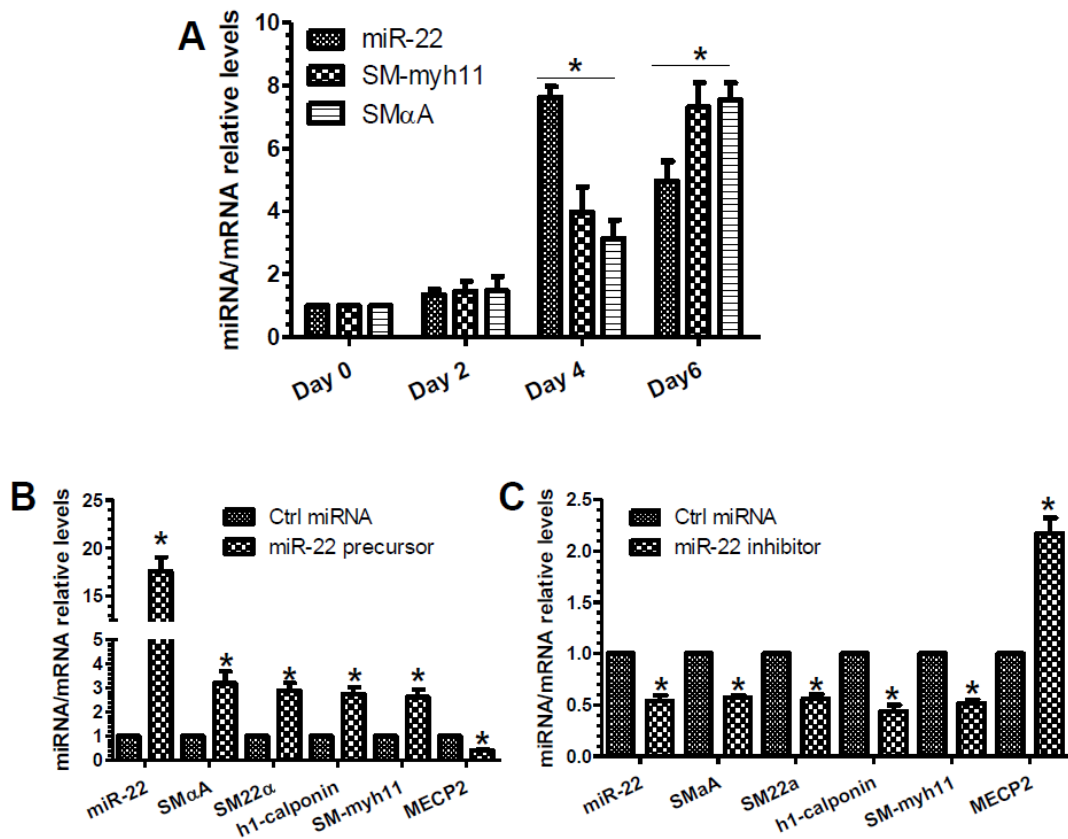
(A) Induction of miR-22 during SMC differentiation from AdSca-1^{+} cells. Day 0 samples were freshly isolated AdSca-1^{+} cells and served as undifferentiated control. (B) miR-22 overexpression up-regulated SMC marker expressions. Cultured AdSca-1^{+} cells were transfected with miR-22 precursor or negative control, and cultured in SMC differentiation medium for 48~72 hours. (C) miR-22 inhibition impairs SMC marker expressions. Day 2 differentiating AdSca-1^{+} cells were transfected with miR-22 inhibitor or negative control, and cultured in SMC differentiation medium for 48 hours. Total RNA was harvested and subjected to RT-qPCR analyses. The data presented here are mean±S.E.M. of three independent experiments. *P<0.05.

Figure VII. Schematic illustration of MECP2 3'UTR regions and pmiR-Luc-MECP2 reporters (wild type and miR-22 binding site mutants)
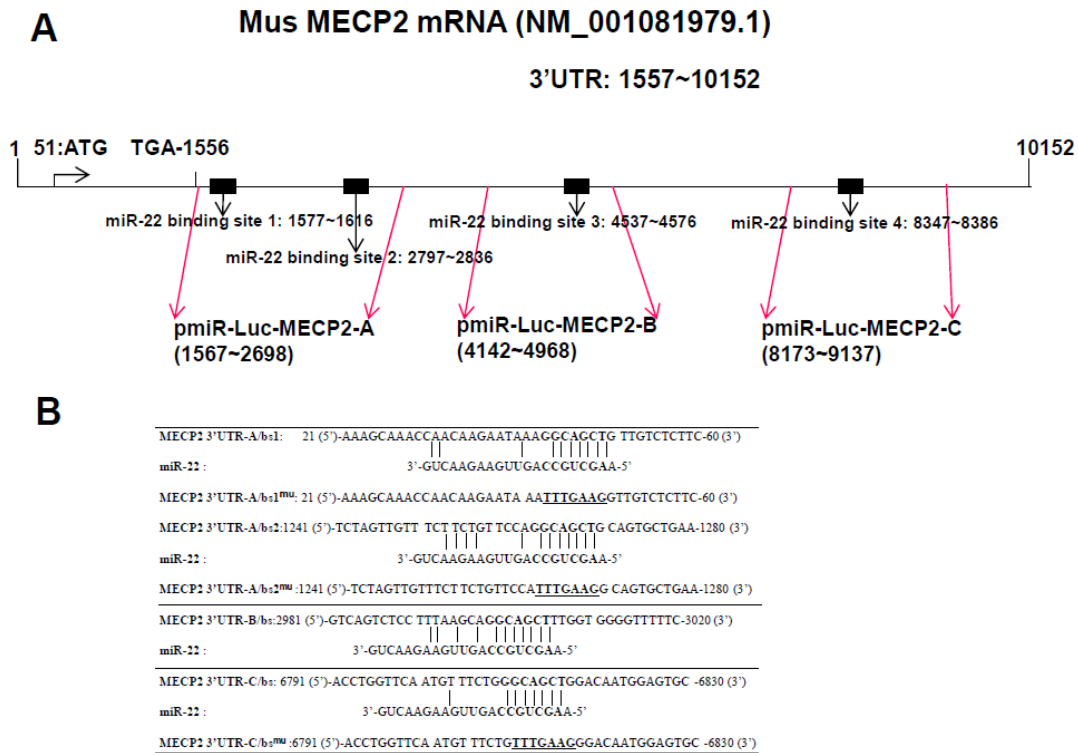

(A) Positions for miR-22 bindings sites (BS1~4, black rectangles) within MECP2 gene, and the individual MECP2 3'UTR segment for respective pmiR-Luc-MECP2 reporter A, B and C are depicted in this illustration. (B) The four miR-22 binding sites were predicted by using Targetscan (A) (www.targetscan.org). The MECP2 3'UTR sequence flanking the respective miR-22 binding sites (wild type and related mutants), and miR-22 sequence were illustrated here, respectively.

Figure VIII. MECP2 over-expression abolished gene expression induced by miR-22.
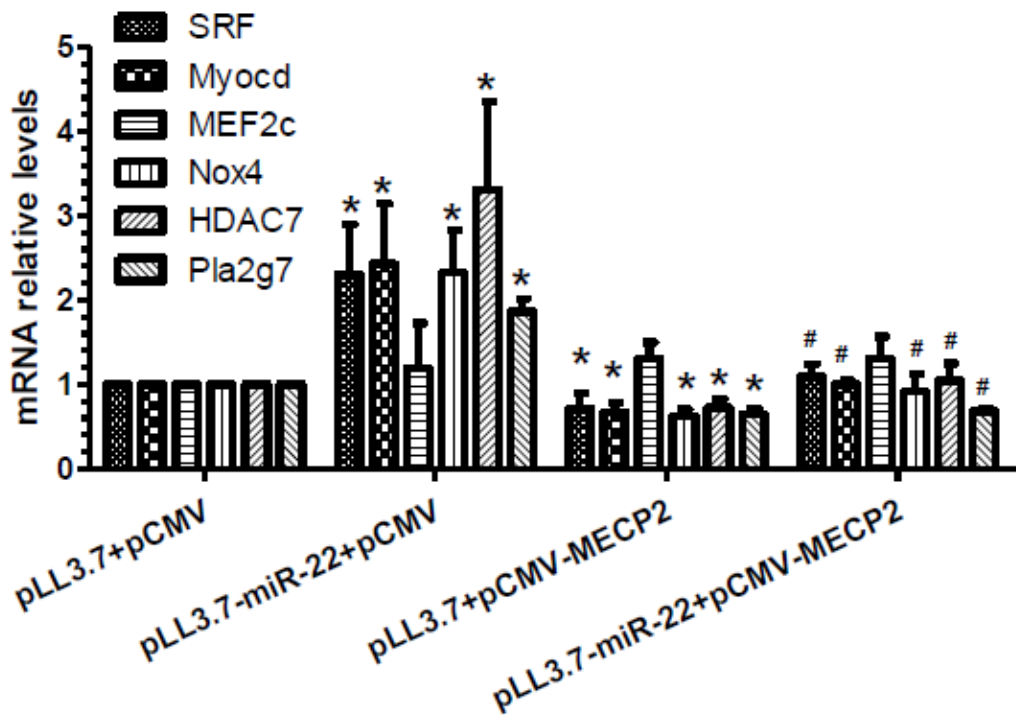

Day 2 differentiating control (pLL3.7-GFP) and miR-22 over-expressing (pLL3.7-GFP-miR-22) ES cells were transfected with respective control (pCMV5) and MECP2 over-expression (pCMV5-MECP2) plasmids, and cultured in SMC differentiation medium for further 48 to 72 hrs. Total RNAs were harvested and subjected to RT-qPCR analyses. The data presented here are mean±S.E.M. of three independent experiments.

**Figure IX. SRF binding site is required for MECP2 mediated SMC gene expression.**
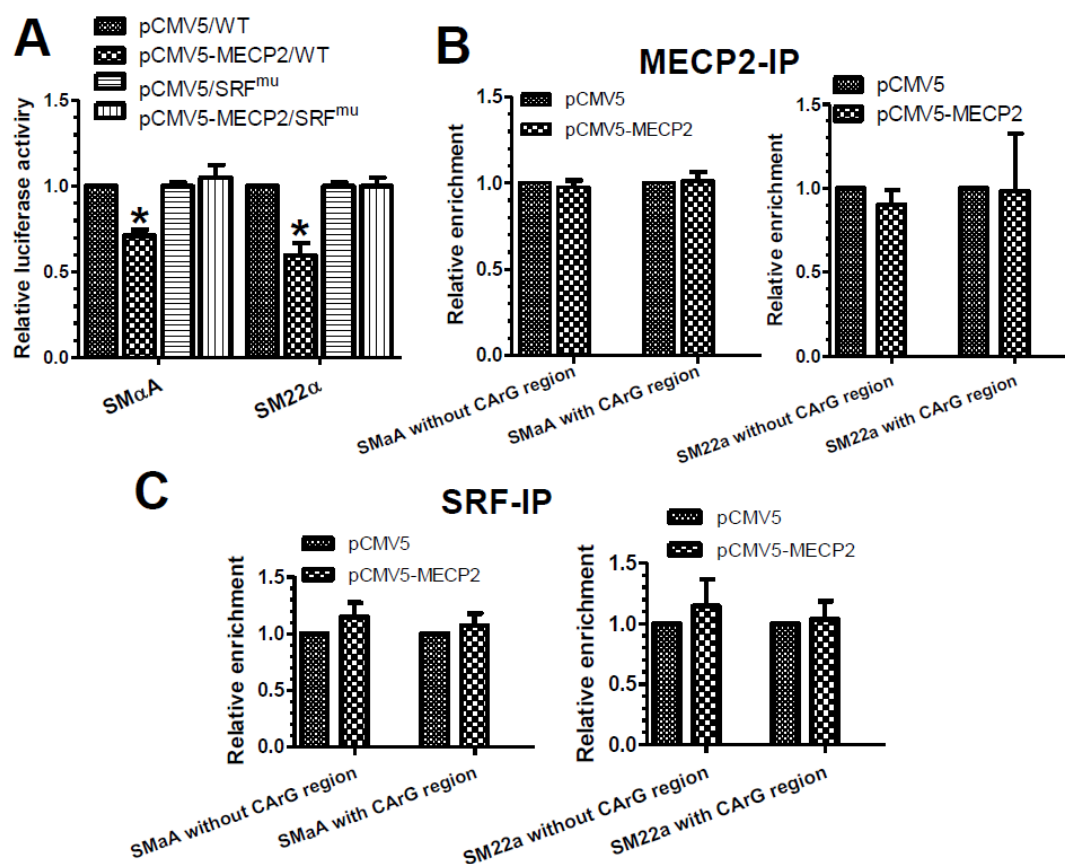
(A) SRF binding site mutation abolished SMC differentiation gene promoter activity induced by MECP2 overexpression. (B) CHIP assay showed no direct binding of MECP2 to the promoter regions of SMC differentiation genes. (C) the binding capacity of SRF to the promoter regions of SMC differentiation genes were not affected by MECP2 over-expression. ChIP assays were performed using antibodies against MECP2 and SRF, as well as its respective normal IgG, as described in online supplemental data. PCR amplifications of the non-CArG regions were included as additional control for specific promoter DNA enrichment. The data presented here are mean±S.E.M. of three independent experiments. *$P<0.05$ (vs. control).

Figure X. Nox4 and HDAC7 gene expressions were transcriptionally regulated by MECP2.
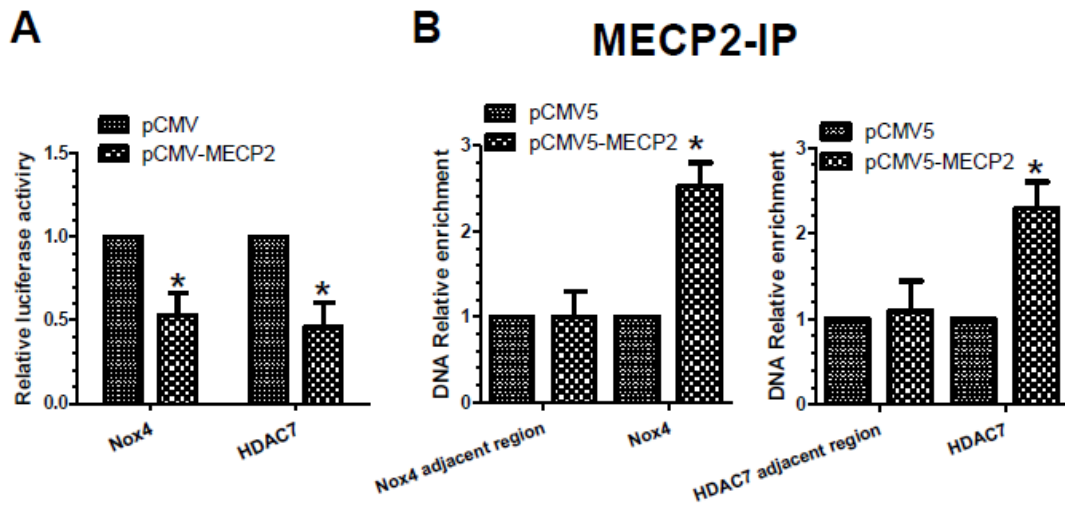

(A) Promoter activities of Nox4 and HDAC7 genes were inhibited by MECP2 over-expression. Day 2~3 differentiating ES cells were transfected with luciferase reporter plasmids pGL3-Nox4-Luc or pGL3-HDAC7-Luc ($0.15\mu g/2.5\times10^4$ cells) together with pCMV5 or pCMV5-MECP2 ($0.2\mu g/2.5\times10^4$ cells). pShuttle-LacZ ($0.2\mu g/2.5\times10^4$ cells) was included as control. Luciferase and β-galactosidase activity assays were detected 48 hours after transfection. The data presented here are mean±S.E.M. of four independent experiments. *$P<0.05$ (vs. control). (B) MECP2 binds directly to the promoter regions of Nox4 and HDAC7 genes. ChIP assays were performed using antibody against MECP2 or normal IgG, respectively. PCR amplifications of the adjacent regions were included as additional control for specific promoter DNA enrichment. The data presented here are mean±S.E.M. of four independent experiments. *$P<0.05$ (vs. control).

Figure XI. miR-22 over-expression decreased the H3K9 methylation within SRF, Myocd and Pla2g7 gene promoters.
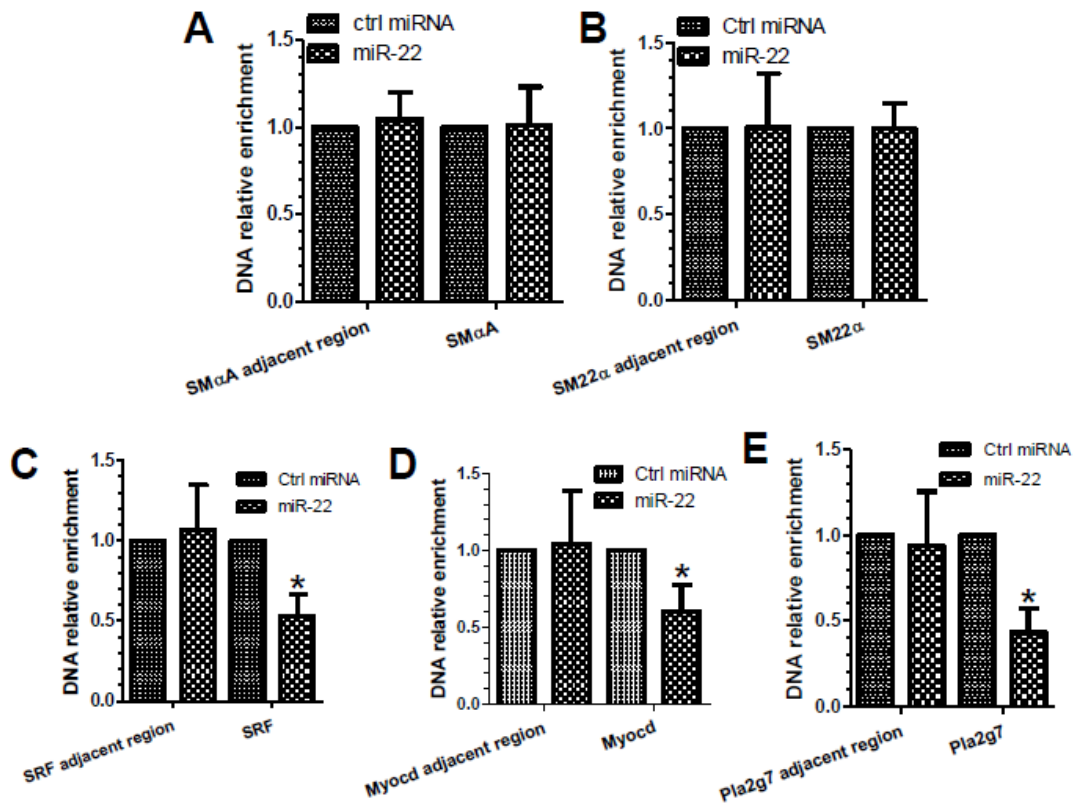

ChIP assays were performed using antibody against H3K9me3 or normal mouse IgG, respectively, as described previously. The enrichment of H3K9me3 within the promoter regions of SMαA (A), SM22α (B), SRF (C), myocd (D) and Pla2g7 (E) were examined using two pairs of primers specific for regions-of-interest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

Figure XII. PDGF-BB and TGF-β up-regulate miR-22 through a transcriptional mechanism.
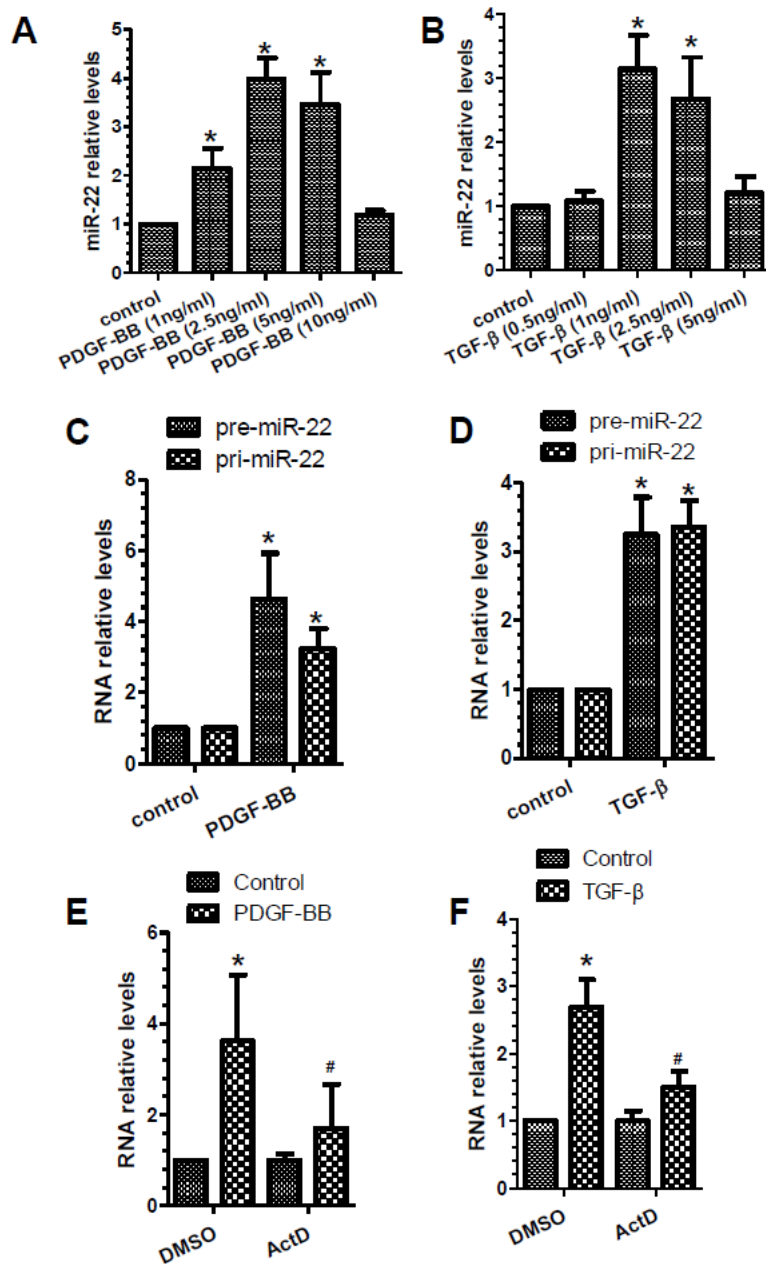
(A and B) Both PDGF-BB and TGF-β up-regulate miR-22 expression. Day 2~3 differentiating ES cells were incubated with the indicated dose of PDGF-BB (A) and TGF-β (B) for 12 hours and 3 hours, respectively. (C and D) Both miR-22 precursor and primary RNA levels were up-regulated by PDGF-BB and TGF-β treatments. Day 2~3 differentiating ES cells were incubated with PDGF-BB (C, 2.5ng/ml) and TGF-β (D, 1ng/ml) for 12 hours and 3 hours, respectively. (E and F) Actinomycin D abolished the effect of PDGF-BB and TGF-β treatments on the expression levels of miR-22. Total RNAs were harvested and subjected to RT-qPCR analyses with respective primers. The data presented here are mean±S.E.M. of three independent experiments. *$P<0.05$ (versus control/DMSO), #$P<0.05$ (ActD versus DMSO in the presence of PDGF-BB/TGF-β).

Figure XIII. Proposed model of miRNAs-mediate SMC differentiation.
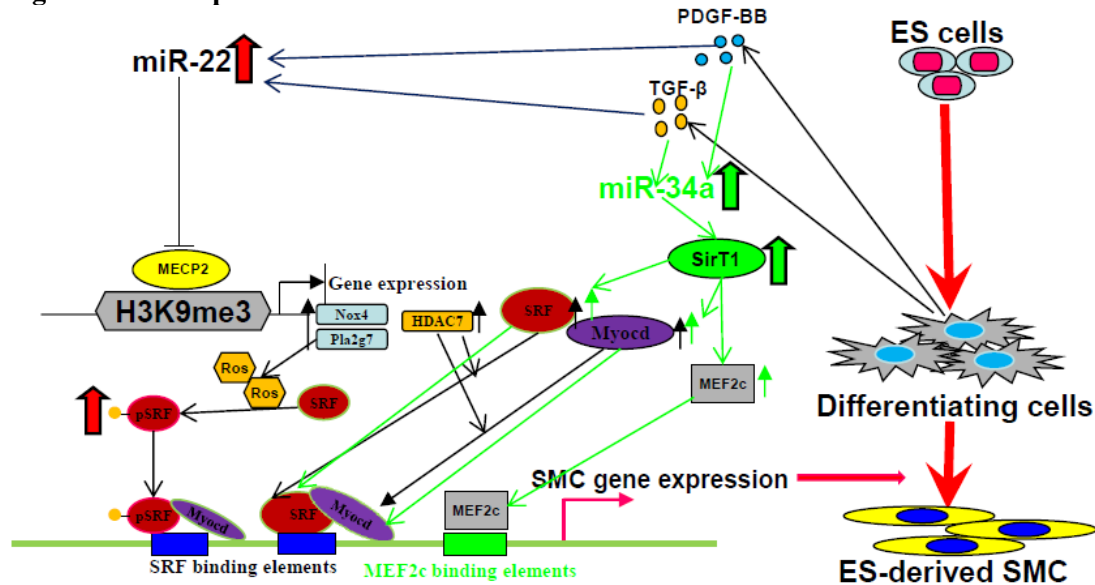
Abbreviations: ESC, embryonic stem cell; SMC, smooth muscle cell; miR-22, microRNA-22; miR-34a; microRNA-34a; SRF, serum response factor; pSRF, phosphorylated SRF; Myocd, myocardin; MECP2, methyl CpG binding protein 2; H3K9me3, histone H3 trimethyl Lys9; Nox4, NADPH oxidase 4; Pla2g7, phospholipase A2, group VII; HDAC7, histone deacetylase 7; Ros, reactive oxygen species; PDGF-BB, platelet-derived growth factor; TGF-β, transforming growth factor beta; MEF2c, myocyte-specific enhancer factor 2C; SirT1, NAD-dependent deacetylase sirtuin-1.