HHS Public Access

Author manuscript

Arterioscler Thromb Vasc Biol. Author manuscript; available in PMC 2017 May 01.

Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2016 May ; 36(5): 864–873. doi:10.1161/ATVBAHA.115.306770.

Regulation of Vascular Smooth Muscle Cell Dysfunction under Diabetic Conditions by miR-504

Marpadga A. Reddy[*], **Sadhan Das**, **Chen Zhuo**, **Wen Jin**, **Mei Wang**, **Linda Lanting**, and **Rama Natarajan**[*]

Department of Diabetes Complications and Metabolism, Beckman Research Institute of City of Hope, Duarte, CA

Abstract

Objectives—Diabetes accelerates pro-atherogenic and pro-inflammatory phenotype of VSMC associated with vascular complications. Evidence shows that microRNAs (miRNAs) play key roles in VSMC functions, but their role under diabetic conditions is unclear. We profiled miRNAs in VSMC from diabetic mice and examined their role in VSMC dysfunction.

Approach and Results—High throughput small RNA-sequencing identified 135 differentially expressed miRNAs in VSMC from type-2 diabetic db/db mice (db/dbVSMC) versus non-diabetic db/+ mice. Several of these miRNAs were known to regulate VSMC functions. We further focused on miR-504, because it was highly upregulated in db/dbVSMC, and its function in VSMC is unknown. miR-504 and its host gene *Fgf13* were significantly increased in db/dbVSMC and in aortas from db/db mice. Bioinformatics analysis predicted that miR-504 targets including signaling adaptor Grb10 and transcription factor Egr2 could regulate growth factor signaling. We experimentally validated *Grb10* and *Egr2* as novel targets of miR-504. Overexpression of miR-504 in VSMC inhibited contractile genes, and enhanced ERK1/2 activation, proliferation and migration. These effects were blocked by miR-504 inhibitors. Grb10 knockdown mimicked miR-504 functions and increased inflammatory genes. Egr2 knockdown inhibited anti-inflammatory *Socs1* and increased pro-inflammatory genes. Furthermore, high-glucose and palmitic acid upregulated miR-504 and inflammatory genes, but downregulated *Grb10*.

Conclusions—Diabetes mis-regulates several miRNAs including miR-504 that can promote VSMC dysfunction. Since changes in many of these miRNAs are sustained in diabetic VSMC even after *in vitro* culture, they may be involved in metabolic memory of vascular complications. Targeting such mechanisms could offer novel therapeutic strategies for diabetic complications.

Keywords

vascular smooth muscle cells; microRNA; diabetes; atherosclerosis; inflammation

[*]Correspondence to: Rama Natarajan, PhD, Department of Diabetes Complications and Metabolism, Beckman Research Institute of City of Hope, 1500 East Duarte Road, Duarte, CA 91010, Ph: 626-256-4673; rnatarajan@coh.org. Marpadga A. Reddy, PhD, Department of Diabetes Complications and Metabolism, Beckman Research Institute of City of Hope, 1500 East Duarte Road, Duarte, CA 91010, Ph: 626-256-4673; mreddy@coh.org.

Disclosures

None.

INTRODUCTION

Vascular smooth muscle cells (VSMC) in the vessel wall have key functions in vascular remodeling in response to injury[1–3]. Phenotypic switching of VSMC from contractile to synthetic states plays an important role in these processes and the development of vascular diseases such as atherosclerosis and restenosis[4–6] which are significantly accelerated in diabetes[7–9]. VSMC contribute to atherosclerotic lesion formation by migration to the sites of injury where they proliferate, produce extracellular matrix and undergo lipid uptake.[5, 6] VSMC also produce inflammatory cytokines and adhesion molecules to promote monocyte retention, survival and differentiation into foam cells, key events in lesion formation and progression of atherosclerosis[8, 10]. Key pathological factors associated with diabetes including high glucose (HG), advanced glycation end products, growth factors and oxidized lipids promote VSMC dysfunction by enhancing inflammatory gene expression, migration and proliferation via activation of multiple signal transduction pathways and downstream transcription factors[5, 7, 11–15]. Recent studies have also identified the role of epigenetic mechanisms and microRNAs (miRNA) in VSMC dysfunction[16–18]. However, much less is known about the role of miRNAs in VSMC dysfunction implicated in diabetic vascular disease.

miRNAs are small non-coding RNAs (~22-nucleotides long) which regulate several biological processes by down-regulating their target genes via post-transcriptional mechanisms. Dysregulation of their expression has been implicated in several patho-physiological conditions including vascular diseases[18–20]. VSMC-specific deletion of Dicer, an RNase III endonuclease essential for the biogenesis of miRNAs, is associated with internal hemorrhage and defective blood vessels, reduced VSMC proliferation and contractile phenotype, suggesting important functions for miRNAs in VSMC during development[21]. Furthermore, Dicer knockdown in adult mice reduced global miRNA levels and blood pressure suggesting critical role of miRNA in VSMC functions[21, 22]. Recent studies demonstrated that miR-145, miR-21, let-7g, miR-29, miR-221, miR-222, miR-126, miR-132, miR-146a and miR-155 have roles in VSMC dysfunction associated with atherosclerosis and restenosis. Vascular injury was found to downregulate miR-143/145, but upregulate miR-21, miR-146a, and miR-222 and promote synthetic phenotype[18, 23–30]. Angiotensin II induced miR-132 in VSMC which was associated with increased inflammatory gene expression[28]. Some miRNAs have been implicated in inflammatory and fibrotic gene regulation related to diabetic vascular complications[31, 32]. For example, miR-125b and miR-200 family members are upregulated in VSMC from type 2 diabetic db/db mice (db/dbVSMC) relative to control db/+ mice (db/+VSMC). These miRNAs could promote an inflammatory phenotype in VSMC via targeting a histone methyl transferase Suv39h1 and a transcription repressor Zeb1 respectively[33, 34]. Changes in other miRNAs including miR-138 under diabetic conditions have been reported, which also regulate proliferation and migration[35–37]. However the role of many other miRNAs dysregulated in diabetic VSMC is still unknown.

In this study we identified several differentially expressed miRNAs in VSMC under diabetic conditions by performing small RNA-sequencing (smRNA-seq) in db/dbVSMC versus db/+

mice (db/+VSMC). We further characterized the function of miR-504 which was up-regulated in db/dbVSMC. Our results demonstrated that miR-504 targets Grb10 and Egr2 to dysregulate growth factor signaling and reduce genes associated with contractile phenotype and enhance inflammatory gene expression, proliferation and migration in VSMC. We also demonstrated upregulation of miR-504 under diabetic conditions *in vitro* (HG, and Palmitic acid treatment) in cultured VSMC. These data identified a novel function for miR-504 in VSMC associated with sustained diabetic vascular complications.

MATERIALS AND METHODS

Detailed Materials and Methods are available in the online only Data Supplement. Aortic VSMC were isolated from 10–12 weeks old type 2 diabetic db/db mice and control db/+ mice (Jackson laboratories, Bar Harbor, ME) and cultured as described[12, 33]. RNA extraction and gene expression analysis were performed by quantitative Realtime PCR (RT-qPCR)[28, 33]. Small RNA-seq (smRNA-seq) was performed once, with one sample per group using Illumina protocols. Reads were aligned to mouse genome (mm10) and differentially expressed miRNAs defined in miRbase (V21) identified as described in Online Supplement (Fig. SI). Transient transfection of VSMC, immunoblotting, luciferase assays, cell proliferation and migration assays were performed as described earlier with some modifications[28, 33]. All the experiments were repeated at least twice with replicates. Statistical analysis of data was performed using Graphpad Prism. Two groups were compared using unpaired Student's t-tests and multiple groups using One-way ANOVA followed by multiple comparison tests. Statistical significance was detected at $p< 0.05$ level. Results were expressed as Mean ± SEM of multiple experiments.

RESULTS

Profiling diabetes induced miRNAs in db/dbVSMC

To determine the profiles of diabetes induced miRNAs in VSMC, we performed smRNA-seq of RNA isolated from db/dbVSMC and db/+VSMC (Fig. SI, Online Supplement) on the Illumina platform. Sequencing reads were aligned to mouse genome GRCm38/mm10 using Novoalign, sequences of mature miRNAs were retrieved using mirBase release 21 and reads were normalized by trimmed-mean of M values (TMM). (Figure SI and Tables SI–SII, online Supplement). Differentially expressed mature miRNAs were identified by comparing db/db and db/+ samples using criteria of minimum reads ≥ 50 reads in either samples and fold change ≥1.5 (Fig SI, Online Supplement). Results showed that let-7 family members, miR-140, miR-29, miR-329, and miR-10a were among the top15 expressed miRNAs in db/+ and db/db VSMC (Fig. SII, online Supplement). smRNA-seq data analysis also revealed that 58 miRNAs were upregulated and 77 were downregulated in db/dbVSMC relative to db/+VSMC (Fig. 1A–B and Table SIII, online Supplement). The top 20 differentially regulated miRNAs are shown in Fig. 1C. Validation of some of the differentially expressed miRNAs by RT-qPCR is shown in Fig-1D–K. Differentially expressed miRNAs included those with known functions in vascular cells such as phenotypic switching, proliferation, angiogenesis, and inflammation[18, 23–30, 38]. These results suggest that diabetes mis-regulates various miRNA levels in VSMC, which could regulate VSMC functions implicated in accelerated

vascular complications. However, the function of many of these miRNAs in VSMC remains unknown. Interestingly, dysregulated expression of miRNAs persisted in the diabetic VSMC even after *in vitro* culture for several passages indicating that they may participate in metabolic memory of vascular dysfunction in which deleterious effects of prior hyperglycemic exposure remain sustained long after return to normoglycemia[39–41].

Increased expression of miR-504 and its host gene Fibroblast growth factor 13 (Fgf13) in VSMC and aortas from db/db mice

Next, to determine the functional roles of diabetes induced miRNAs, we further characterized the expression and function of miR-504, one of the highly upregulated miRNAs in db/dbVSMC (Fig. 1C). This miRNA was reported to promote tumorigenecity by targeting p53[42], but its function in VSMC and diabetes complications is unknown. The gene coding for miR-504 is located in the third intron of the *Fgf13* gene on X-chromosome (Fig. 2A). Therefore, we examined levels of both miR-504 and *Fgf13* in VSMC cultured *in vitro* and in aortas isolated from db/db and db/+ mice. RT-qPCR results showed that levels of both miR-504 and its host gene *Fgf13* were significantly upregulated in db/dbVSMC compared with db/+VSMC (Fig. 2B–C), and also in de-endothelialized aortas from db/db mice relative to db/+ mice (Fig. 2D–E), demonstrating *in vivo* relevance. These results clearly showed that miR-504 and its host gene *Fgf13* are upregulated by diabetes in VSMC.

Targets of miR-504 regulate growth factor signaling

Bioinformatics analysis with publicly available target prediction tools such as Targetscan and miRANDA revealed that miR-504 can target growth factor receptors and downstream signaling components. Accordingly, RNA-seq data set obtained from db/dbVSMC and db/+ VSMC (full dataset not shown) showed that 211 potential targets were downregulated in db/dbVSMC versus db/+VSMC and several of them were found to be involved in growth factor signaling (Fig 2F, and Table SIV, Online Supplement). Ingenuity Pathway Analysis (IPA) revealed that miR-504 target genes could potentially regulate pathways involved in cell growth, inflammation and cardiovascular functions (Fig. 2G), supporting the notion that, increased miR-504 could promote VSMC dysfunction by altering growth factor signaling and inflammatory responses associated with diabetic vascular complications.

Grb10 and Egr2 are direct targets of miR-504

Next, to characterize top candidate targets of miR-504, we transfected db/+VSMC with miR-504 mimic (504M) and negative control (NCM) oligonucleotides. Potential candidate miR-504 target genes were analyzed by RT-qPCR 48 hours post transfection. Transfection of db/+VSMC with 504M significantly increased miR-504 levels (Fig. 3A) and downregulated expression of potential targets *Grb10*, *Egr2, Tsc1 and Pdgfra* (Fig. 3B–E). But, miR-504 did not inhibit all the targets tested including *Trp53,* the mouse homolog of human tumor suppressor *TP53, Cdkn2a*, cell cycle regulator and *Crtc1*, downstream mediator of PI3K signaling (Fig. 3F–H). Previous studies showed that miR-504 targets *TP53* in human cells[42]. But, mouse homolog *Trp53* was not inhibited because miR-504 binding sites are not conserved in its 3′-UTR. Other targets *Cdkn2a* and *Crtc1* mRNAs that were not inhibited, might be regulated at the translational level in VSMC.

We further characterized the functions of Grb10 and Egr2 in VSMC, because they play key roles in growth factor signaling and inflammation[43–45], however, their functions in VSMC relevant to diabetes complications are not well understood. To demonstrate that Grb10 and Egr2 are bona-fide targets of miR-504, we cloned the 3′-UTRs of mouse and human *Grb10* and mouse *Egr2* mRNAs downstream of Renilla luciferase in pSICHECK2 vector, which also expresses firefly luciferase used as an internal control. Co-transfection of these reporter plasmids with 504M or NCM oligonucleotides showed that 504M significantly downregulated activity of luciferase fused with 3′UTRs of mouse and human Grb10 (mGrb10 and hGrb10) and mouse Egr2 (Fig. 3I), but control pSICHECK2 (CHECK) vector was unaffected (Fig 3I). Immunoblotting also confirmed downregulation of Grb10 protein in db/+VSMC transfected with 504M versus NCM (Fig. 3J). Downregulation of Egr2 protein levels could not be verified due to lack of specific antibodies. Next, we tested if inhibition of endogenous miR-504 can increase activity of Grb10 and Egr2 3′-UTRs. Results showed that co-transfection of 3′-UTR reporter plasmids with 504 inhibitor oligonucleotides (504I) significantly increased Grb10 and Egr2 3′-UTR activities relative to control (NCI) oligonucleotides (Fig. 3K–L). Similar results were obtained using plasmids expressing miR-504 inhibitors (p504I) vs control plasmid (pNCI) expressing scramble sequence (Fig. 3M–N) These results demonstrate that Grb10 and Egr2 are bonafide targets of miR-504 in VSMC.

Grb10 gene silencing inhibits Egr2 expression and enhances inflammatory gene expression in VSMC

We next examined the involvement of miR-504 targets in inflammatory gene regulation using gene silencing approaches. We transfected db/+VSMC with two siRNA oligonucleotides targeting mouse Grb10 (siGrb10a and siGrb10b) and a control non-targeting siRNA (siNC), and analyzed gene expression 72 h post transfection. As shown in Fig. 4A, siGrb10a and siGrb10b significantly inhibited *Grb10* expression compared to siNC transfected cells. Immunoblotting experiments also confirmed downregulation of Grb10 protein by these siRNAs (Fig. 4B). Furthermore, Grb10 gene silencing significantly upregulated inflammatory genes *Il6* and *Ccl2* relative to siNC transfected cells (Fig. 4C–D). Interestingly, Grb10 downregulation also inhibited *Egr2* expression (Fig. 4E), suggesting Egr2 may be downstream of Grb10 in VSMC. These results demonstrate that Grb10 signaling negatively regulates inflammatory gene expression by acting as a brake, and its downregulation by miR-504 under diabetic conditions could upregulate inflammatory genes in VSMC.

Egr2 downregulation increases inflammatory gene expression in VSMC

Next, we examined the effect of Egr2 downregulation on inflammatory genes by transfecting db/+VSMC with two siRNAs targeting *Egr2* (siEgr2a and siEgr2b) or control siNC oligonucleotides. Gene expression analysis showed that siEgr2a and siEgr2b significantly inhibited *Egr2* expression in VSMC (Fig. 4F). Furthermore, *Egr2* downregulation led to upregulation of pro-inflammatory genes *Il6*, *Ccl2* and Cyclooxygenase-2 (*Ptgs2*) in non-diabetic db/+VSMC (Fig. 4G–I). We also examined the mechanisms involved in pro-inflammatory phenotype conferred by *Egr2* gene silencing in VSMC. Previous reports in myeloid cells showed that Egr2 induces suppressor of cytokine signaling-1 (*Socs1)* and *Socs*

2, negative regulators of cytokine signaling, to inhibit inflammation[45, 46]. Because our RNA-seq data revealed downregulation of *Socs*1 in db/dbVSMC (Fig. 2F), we examined its expression after *Egr2* gene silencing. RT-qPCR results showed that *Egr2* gene silencing significantly downregulated *Socs1* expression in db+VSMC (Fig. 4J) demonstrating that downregulation of Socs family members might be one of the mechanisms involved in increased inflammatory genes via Egr2 downregulation by miR-504 in diabetes.

Overexpression of miR-504 or Grb10 gene silencing enhances ERK1/2 activation in VSMC

Next, we determined the role of miR-504 and Grb10 in growth factor signaling in VSMC. We transfected control db/+VSMC with 504M or NCM and 48 h later treated with PDGF (10 ng/ml). At indicated time points, cell lysates were subjected to imunoblotting with phospho-specific antibodies. Results showed that PDGF induced ERK1/2 activation was greatly enhanced in db/+VSMC transfected with 504M relative to NCM transfected cells. In contrast, other signaling pathways including PI3K (p85 and Akt phosphorylation) and S6 kinase were not affected (Fig. 5A). Interestingly, we obtained similar results, i.e., enhanced PDGF induced ERK1/2 activation, after Grb10 gene silencing with siGrb10a or siGrb10b vs siNC in db/+VSMC (Fg. 5H). These results demonstrate that miR-504 might enhance ERK1/2 activation by targeting Grb10 and thereby impact VSMC growth related functions.

Regulation of VSMC dysfunction by miR-504 and Grb10

Next, we examined whether miR-504 overexpression regulates VSMC proliferation, phenotypic switching and migration as functional outcomes. Results showed that db/+VSMC transfected with miR-504 mimics (504M) showed significantly increased proliferation (Fig-5B) and downregulation of contractile phenotype genes including *Tagln*, *Acta2* and *Cnn1* versus NCM transfected cells (Fig. 5C). Furthermore, 504M also significantly enhanced PDGF induced db/+VSMC migration relative to NCM transfected cells (Fig. 5D). To further confirm the role of miR-504, we performed cell proliferation and migration assays after transfection with 504I and NCI oligonucleotides. 504I significantly inhibited VSMC cell proliferation (Fig. 5E) and PDGF-induced migration (Fig. 5G). In addition, transfection with plasmids expressing miR-504 inhibitor (p504I) also significantly blocked cell proliferation versus control plasmid pNCI (Fig. 5F). Furthermore, Grb10 gene silencing also inhibited contractile genes (Fig. 5I) and augmented PDGF induced db/+VSMC migration (Fig. 5J), but did not significantly affect proliferation (data not shown). These results demonstrate that miR-504 can promote VSMC proliferation, synthetic phenotype and migration, and that Grb10 downregulation is one of the key mechanisms underlying miR-504 mediated VSMC dysfunction.

Regulation of miR-504 under diabetic conditions

We next examined whether miR-504 expression is altered under diabetic conditions *in vitro* in VSMC. Because, type 2 diabetes is associated with hyperglycemia and elevated free fatty acids, db/+VSMC were treated with high (25 mmol/L) glucose (HG), palmitic acid (100 umol/L, PA), a combination of HG + PA (HP), and normal (5.5 mmol/L) glucose (NG) as control. RT-qPCR analysis showed that HG and PA alone increased miR-504 levels, while combination of HG and PA (HP) further augmented miR-504 expression in db/+VSMC relative to NG treated cells (Fig. 6A). This was associated with downregulation of miR-504

target *Grb10* (Fig. 6B) and upregulation of inflammatory genes *Ccl2, Il6 and Ptgs2* (Fig. 6D–F). However, *Egr2* was not significantly altered under these conditions (Fig. 6C). These results demonstrate that key factors associated with diabetes pathogenesis can induce miR-504 implicated in VSMC dysfunction.

DISCUSSION

In this study, we identified several miRNAs that were differentially expressed under diabetic conditions in VSMC and demonstrated that one of the highly induced miRNAs, miR-504, promotes proliferation, migration and inflammatory gene expression by targeting growth factor signaling. Some of the differentially expressed miRNAs were already known to regulate VSMC functions relevant to phenotypic switch, proliferation and inflammatory gene regulation via targeting growth factor signaling and key transcription factors[18, 23–30]. However, their regulation under diabetic conditions was not known. Dysregulation of these miRNAs by diabetes further supports the important role of miRNAs in vascular diseases. We further characterized the function of miR-504 in VSMC. In mice miR-504 is located on X-chromosome in the 3rd intron of *Fgf13*. Our results showed that host gene *Fgf13* was also increased in db/dbVSMC, but further studies are needed to determine whether increases in miR-504 are due to altered transcription or posttranscriptional mechanisms such as miRNA biogenesis. The only known function of miR-504 is to promote tumorigenesis by targeting tumor suppressor p53 in cancer cells[42], but its function and targets in VSMC are unknown. MiR-504 does not target p53 in mice due to lack of miR-504 binding sites in its 3′-UTR. We used bioinformatics approaches to identify novel potential targets of miR-504, and found that miR-504 can target several key components of growth factor signaling. Our RNA-seq analysis revealed downregulation of 211 potential miR-504 targets in db/dbVSMC, with many of them related to growth factor signaling (Fig. 2F and Table SIV, online Supplement). Diabetes can promote VSMC proliferation, inflammation and migration via modulation of growth factor induced signal transduction[7, 13, 47–52], but the mechanisms involved remain unclear. Our results showed that increased levels of miR-504 enhanced ERK1/2 activation, VSMC proliferation and migration, and inhibited contractile gene expression in db/+VSMC, all events associated with VSMC dysfunction in atherosclerosis and restenosis. Furthermore, miR-504 inhibitors could block miR-504 mediated inhibition of target gene 3′-UTR activities, VSMC proliferation and migration. Thus upregulation of miR-504 under diabetic conditions may be one of the mechanisms involved in augmented growth factor signaling and VSMC dysfunction implicated in vascular diseases.

We demonstrated that Grb10 and Egr2 are two bonafide targets of miR-504. Furthermore, we also showed their function related to vascular diseases. Grb10 belongs to a family of adapter proteins Grb7 and Grb14 that regulate growth factor signaling. Grb10 has an N-terminal Pleckstrin homology (PH) domain, a C-terminal Src homology 2 (SH2) domain, and in between a region of about 50 amino acids known as the BPS (between PH and SH2) domain[53]. These domains allow it to interact with diverse groups of molecules including growth factor receptors and downstream signaling components. Therefore, Grb10 exhibits diverse effects depending on cell types and growth factors tested[53–58]. Recent phosphoproteomic studies identified Grb10 as an mTORC1 substrate and as a negative regulator of insulin signaling. Grb10 knockdown enhanced growth factor induced PI3K and

ERK1/2 activation and inhibited apoptosis[59]. Further studies established tumor suppressor function of Grb10 and suggested its role in fine tuning growth factor signaling in mammalian cells [43, 60–63]. But, its role in VSMC proliferation, migration and inflammatory gene regulation has not been previously examined. Our data demonstrate that Grb10 knockdown with siRNAs or by miR-504 mimics, enhances PDGF induced ERK1/2 activation. Furthermore, Grb10 knockdown increased expression of inflammatory genes *Ccl2* and *Il6*, which promote pro-atherogenic phenotype of VSMC. Grb10 gene silencing also enhanced VSMC migration, and inhibited *Egr2* as well as contractile gene expression, effects similar to miR-504 overexpression. These results indicate that Grb10 is a key downstream mediator of miR-504 functions in VSMC. Activation of ERK1/2 by growth factors is an important event in the inhibition of contractile genes to promote synthetic phenotype and increase pro-inflammatory gene expression, proliferation and migration in VSMC[64, 65]. Previous studies showed that ERK1/2 activation is enhanced in db/dbVSMC and VSMC cultured under diabetic conditions[12, 66, 67], but, the molecular mechanisms were unclear. Here we show that Grb10 downregulation by miR-504 could enhance ERK1/2 activation, thus illustrating a novel mechanism involved in enhanced growth factor signaling and ERK1/2 activation under diabetic conditions leading to VSMC dysfunction.

Egr2 (Krox-20) is a C(2)H(2)-type zinc-finger transcription factor and plays an important role in hindbrain development, monocyte and macrophage differentiation[68–71]. Egr2 is regulated by growth factors[72] and exhibits anti-proliferative effects in cancer cells[44]. Furthermore, in myeloid cells Egr2 mediates anti-inflammatory effects via upregulation of *Socs1 and Socs2* genes[45]. However, the function of Egr2 in VSMC has not been evaluated. Our RNA-seq data showed downregulation of *Egr2* in db/dbVSMC which exhibit a pro-inflammatory phenotype. Furthermore, we demonstrated that Egr2 was a direct target of miR-504 and its inhibition reduced anti-inflammatory *Socs1* and increased expression of several pro-inflammatory genes, supporting an anti-inflammatory function of Egr2 in VMSC. Egr2 gene silencing however had no effect on contractile gene expression. Thus, inhibition of Egr2 by miR-504 in diabetes might enhance inflammation in VSMC. Together these data establish pro-atherogenic and pro-inflammatory functions of miR-504 in VSMC via downregulation of key signaling adapters and transcription factors in diabetes (Fig-6G).

We further demonstrated that HG, PA and a combination of HG and PA, major pathological factors in diabetes, could increase miR-504 and downregulate Grb10 in VSMC. This was accompanied by increases in inflammatory genes, further supporting the involvement of miR-504 in diabetes induced VSMC dysfunction. Palmitate was reported to modulate VSMC phenotype by inhibiting contractile genes and increasing inflammatory genes[73]. Our results suggest that palmitate induced miR-504 might play a role in these events.

Epigenetic mechanisms and non-coding RNAs have been implicated in the phenomenon of “metabolic memory”, in which prior periods of exposure to hyperglycemia leads to sustained long-term vascular complications despite subsequent glycemic control[32, 39]. Previous studies showed that the memory of persistently increased levels of inflammatory genes, VSMC migration and dysfunction under diabetic conditions can be related to variations in epigenetic histone modifications and miRNA dependent mechanisms[33, 34, 39, 40]. Interestingly, increases in miR-504 were observed not only in aortas

from db/db mice, but also in db/dbVSMC cultured *in vitro* for several passages relative to db/+ VSMC cultured under similar conditions, suggesting that this miRNA may contribute to metabolic memory implicated in sustained diabetic vascular dysfunction. Other differentially expressed miRNAs in db/dbVSMC may also contribute in a synergistic fashion via changes in the their specific targets. Identifying the mechanisms involved, or targeting miRNAs such as miR-504, could lead to novel therapeutic approaches to attenuate metabolic memory and thereby alleviate diabetes induced progression of vascular complications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research reported in this publication included work the Animal Research Core supported by the National Cancer Institute of the NIH under award number P30CA33572.

Sources of Funding

This work was supported by grants from the National Institutes of Health (NIH) R01 HL106089-01 and NIH R01 DK 065073 to RN.

Nonstandard Abbreviations and Acronyms

ERK1/2	extracellular signal-regulated kinase1/2
VSMC	vascular smooth muscle cells
db/+VSMC	VSMC from db/+ mice
db/dbVSMC	VSMC from db/db mice
PDGF	platelet derived growth factor
HG	high glucose
PA	palmitic acid
miRNA	microRNA
NCM	negative control mimic
504M	miR-504 mimic
NCI	negative control inhibitor
504I	miR-504 inhibitor

References

1. Ross R. The pathogenesis of atherosclerosis: A perspective for the 1990s. Nature. 1993; 362:801–809. [PubMed: 8479518]
2. Schwartz SM. Smooth muscle migration in atherosclerosis and restenosis. J Clin Invest. 1997; 100:S87–89. [PubMed: 9413408]
3. Libby P. Inflammation in atherosclerosis. Nature. 2002; 420:868–874. [PubMed: 12490960]

4. Gomez D, Owens GK. Smooth muscle cell phenotypic switching in atherosclerosis. Cardiovasc Res. 2012; 95:156–164. [PubMed: 22406749]
5. Doran AC, Meller N, McNamara CA. Role of smooth muscle cells in the initiation and early progression of atherosclerosis. Arterioscler Thromb Vasc Biol. 2008; 28:812–819. [PubMed: 18276911]
6. Wall VZ, Bornfeldt KE. Arterial smooth muscle. Arterioscler Thromb Vasc Biol. 2014; 34:2175–2179. [PubMed: 25231633]
7. Natarajan R, Nadler JL. Lipid inflammatory mediators in diabetic vascular disease. Arterioscler Thromb Vasc Biol. 2004; 24:1542–1548. [PubMed: 15166011]
8. Natarajan R, Cai Q. Monocyte retention in the pathology of atherosclerosis. Future Cardiol. 2005; 1:331–340. [PubMed: 19804116]
9. Rask-Madsen C, King GL. Proatherosclerotic mechanisms involving protein kinase c in diabetes and insulin resistance. Arterioscler Thromb Vasc Biol. 2005; 25:487–496. [PubMed: 15637306]
10. Allahverdian S, Chehroudi AC, McManus BM, Abraham T, Francis GA. Contribution of intimal smooth muscle cells to cholesterol accumulation and macrophage-like cells in human atherosclerosis. Circulation. 2014; 129:1551–1559. [PubMed: 24481950]
11. Schmidt AM, Yan SD, Wautier JL, Stern D. Activation of receptor for advanced glycation end products: A mechanism for chronic vascular dysfunction in diabetic vasculopathy and atherosclerosis. Circ Res. 1999; 84:489–497. [PubMed: 10082470]
12. Li SL, Reddy MA, Cai Q, Meng L, Yuan H, Lanting L, Natarajan R. Enhanced proatherogenic responses in macrophages and vascular smooth muscle cells derived from diabetic db/db mice. Diabetes. 2006; 55:2611–2619. [PubMed: 16936211]
13. Meng L, Park J, Cai Q, Lanting L, Reddy MA, Natarajan R. Diabetic conditions promote binding of monocytes to vascular smooth muscle cells and their subsequent differentiation. Am J Physiol Heart Circ Physiol. 2010; 298:H736–745. [PubMed: 20008269]
14. Geraldes P, King GL. Activation of protein kinase c isoforms and its impact on diabetic complications. Circ Res. 2010; 106:1319–1331. [PubMed: 20431074]
15. Reddy MA, Li SL, Sahar S, Kim YS, Xu ZG, Lanting L, Natarajan R. Key role of src kinase in s100b-induced activation of the receptor for advanced glycation end products in vascular smooth muscle cells. J Biol Chem. 2006; 281:13685–13693. [PubMed: 16551628]
16. Alexander MR, Owens GK. Epigenetic control of smooth muscle cell differentiation and phenotypic switching in vascular development and disease. Annu Rev Physiol. 2012; 74:13–40. [PubMed: 22017177]
17. Albinsson S, Sessa WC. Can micrornas control vascular smooth muscle phenotypic modulation and the response to injury? Physiol Genomics. 2011; 43:529–533. [PubMed: 20841497]
18. Maegdefessel L, Rayner KJ, Leeper NJ. Microrna regulation of vascular smooth muscle function and phenotype: Early career committee contribution. Arterioscler Thromb Vasc Biol. 2015; 35:2–6. [PubMed: 25520518]
19. Mendell JT, Olson EN. Micrornas in stress signaling and human disease. Cell. 2012; 148:1172–1187. [PubMed: 22424228]
20. Urbich C, Kuehbacher A, Dimmeler S. Role of micrornas in vascular diseases, inflammation, and angiogenesis. Cardiovasc Res. 2008; 79:581–588. [PubMed: 18550634]
21. Albinsson S, Suarez Y, Skoura A, Offermanns S, Miano JM, Sessa WC. Micrornas are necessary for vascular smooth muscle growth, differentiation, and function. Arterioscler Thromb Vasc Biol. 2010; 30:1118–1126. [PubMed: 20378849]
22. Albinsson S, Skoura A, Yu J, DiLorenzo A, Fernandez-Hernando C, Offermanns S, Miano JM, Sessa WC. Smooth muscle mirnas are critical for post-natal regulation of blood pressure and vascular function. PLoS One. 2011; 6:e18869. [PubMed: 21526127]
23. Wei Y, Schober A, Weber C. Pathogenic arterial remodeling: The good and bad of micrornas. Am J Physiol Heart Circ Physiol. 2013; 304:H1050–1059. [PubMed: 23396454]
24. Zhou J, Li YS, Nguyen P, Wang KC, Weiss A, Kuo YC, Chiu JJ, Shyy JY, Chien S. Regulation of vascular smooth muscle cell turnover by endothelial cell-secreted microrna-126: Role of shear stress. Circ Res. 2013; 113:40–51. [PubMed: 23603512]

25. Ding Z, Wang X, Schnackenberg L, Khaidakov M, Liu S, Singla S, Dai Y, Mehta JL. Regulation of autophagy and apoptosis in response to ox-ldl in vascular smooth muscle cells, and the modulatory effects of the microrna hsa-let-7 g. Int J Cardiol. 2013; 168:1378–1385. [PubMed: 23305858]
26. Zhao J, Imbrie GA, Baur WE, Iyer LK, Aronovitz MJ, Kershaw TB, Haselmann GM, Lu Q, Karas RH. Estrogen receptor-mediated regulation of microrna inhibits proliferation of vascular smooth muscle cells. Arterioscler Thromb Vasc Biol. 2013; 33:257–265. [PubMed: 23175673]
27. Davis-Dusenbery BN, Chan MC, Reno KE, Weisman AS, Layne MD, Lagna G, Hata A. Down-regulation of kruppel-like factor-4 (klf4) by microrna-143/145 is critical for modulation of vascular smooth muscle cell phenotype by transforming growth factor-beta and bone morphogenetic protein 4. J Biol Chem. 2011; 286:28097–28110. [PubMed: 21673106]
28. Jin W, Reddy MA, Chen Z, Putta S, Lanting L, Kato M, Park JT, Chandra M, Wang C, Tangirala RK, Natarajan R. Small rna sequencing reveals micrornas that modulate angiotensin ii effects in vascular smooth muscle cells. J Biol Chem. 2012; 287:15672–15683. [PubMed: 22431733]
29. Leung A, Trac C, Jin W, Lanting L, Akbany A, Saetrom P, Schones DE, Natarajan R. Novel long noncoding rnas are regulated by angiotensin ii in vascular smooth muscle cells. Circ Res. 2013; 113:266–278. [PubMed: 23697773]
30. Sun SG, Zheng B, Han M, Fang XM, Li HX, Miao SB, Su M, Han Y, Shi HJ, Wen JK. Mir-146a and kruppel-like factor 4 form a feedback loop to participate in vascular smooth muscle cell proliferation. EMBO Rep. 2011; 12:56–62. [PubMed: 21109779]
31. Kato M, Castro NE, Natarajan R. Micrornas: Potential mediators and biomarkers of diabetic complications. Free Radic Biol Med. 2013; 64:85–94. [PubMed: 23770198]
32. Reddy MA, Natarajan R. Epigenetic mechanisms in diabetic vascular complications. Cardiovasc Res. 2011; 90:421–429. [PubMed: 21266525]
33. Reddy MA, Jin W, Villeneuve L, Wang M, Lanting L, Todorov I, Kato M, Natarajan R. Pro-inflammatory role of microrna-200 in vascular smooth muscle cells from diabetic mice. Arterioscler Thromb Vasc Biol. 2012; 32:721–729. [PubMed: 22247255]
34. Villeneuve LM, Kato M, Reddy MA, Wang M, Lanting L, Natarajan R. Enhanced levels of microrna-125b in vascular smooth muscle cells of diabetic db/db mice lead to increased inflammatory gene expression by targeting the histone methyltransferase suv39h1. Diabetes. 2010; 59:2904–2915. [PubMed: 20699419]
35. Chen TC, Sung ML, Kuo HC, Chien SJ, Yen CK, Chen CN. Differential regulation of human aortic smooth muscle cell proliferation by monocyte-derived macrophages from diabetic patients. PLoS One. 2014; 9:e113752. [PubMed: 25409512]
36. Li T, Yang GM, Zhu Y, Wu Y, Chen XY, Lan D, Tian KL, Liu LM. Diabetes and hyperlipidemia induce dysfunction of vsmcs: Contribution of the metabolic inflammation/mirna pathway. Am J Physiol Endocrinol Metab. 2015; 308:E257–269. [PubMed: 25425000]
37. Xu J, Li L, Yun HF, Han YS. Mir-138 promotes smooth muscle cells proliferation and migration in db/db mice through down-regulation of sirt1. Biochem Biophys Res Commun. 2015; 463:1159–1164. [PubMed: 26079878]
38. Bazzoni F, Rossato M, Fabbri M, Gaudiosi D, Mirolo M, Mori L, Tamassia N, Mantovani A, Cassatella MA, Locati M. Induction and regulatory function of mir-9 in human monocytes and neutrophils exposed to proinflammatory signals. Proc Natl Acad Sci U S A. 2009; 106:5282–5287. [PubMed: 19289835]
39. Villeneuve LM, Reddy MA, Lanting LL, Wang M, Meng L, Natarajan R. Epigenetic histone h3 lysine 9 methylation in metabolic memory and inflammatory phenotype of vascular smooth muscle cells in diabetes. Proc Natl Acad Sci U S A. 2008; 105:9047–9052. [PubMed: 18579779]
40. Reddy MA, Zhang E, Natarajan R. Epigenetic mechanisms in diabetic complications and metabolic memory. Diabetologia. 2015; 58:443–455. [PubMed: 25481708]
41. Keating ST, El-Osta A. Epigenetics and metabolism. Circ Res. 2015; 116:715–736. [PubMed: 25677519]
42. Hu W, Chan CS, Wu R, Zhang C, Sun Y, Song JS, Tang LH, Levine AJ, Feng Z. Negative regulation of tumor suppressor p53 by microrna mir-504. Mol Cell. 2010; 38:689–699. [PubMed: 20542001]

43. Kabir NN, Kazi JU. Grb10 is a dual regulator of receptor tyrosine kinase signaling. Mol Biol Rep. 2014; 41:1985–1992. [PubMed: 24420853]
44. Unoki M, Nakamura Y. Growth-suppressive effects of bpoz and egr2, two genes involved in the pten signaling pathway. Oncogene. 2001; 20:4457–4465. [PubMed: 11494141]
45. Li S, Miao T, Sebastian M, Bhullar P, Ghaffari E, Liu M, Symonds AL, Wang P. The transcription factors egr2 and egr3 are essential for the control of inflammation and antigen-induced proliferation of b and t cells. Immunity. 2012; 37:685–696. [PubMed: 23021953]
46. Sumitomo S, Fujio K, Okamura T, Yamamoto K. Egr2 and egr3 are the unique regulators for systemic autoimmunity. JAKSTAT. 2013; 2:e23952. [PubMed: 24058814]
47. Sobel BE. Acceleration of restenosis by diabetes: Pathogenetic implications. Circulation. 2001; 103:1185–1187. [PubMed: 11238257]
48. Faries PL, Rohan DI, Takahara H, Wyers MC, Contreras MA, Quist WC, King GL, Logerfo FW. Human vascular smooth muscle cells of diabetic origin exhibit increased proliferation, adhesion, and migration. J Vasc Surg. 2001; 33:601–607. [PubMed: 11241133]
49. Hsueh WA, Jackson S, Law RE. Control of vascular cell proliferation and migration by ppar-gamma: A new approach to the macrovascular complications of diabetes. Diabetes Care. 2001; 24:392–397. [PubMed: 11213897]
50. Wang CC, Gurevich I, Draznin B. Insulin affects vascular smooth muscle cell phenotype and migration via distinct signaling pathways. Diabetes. 2003; 52:2562–2569. [PubMed: 14514641]
51. Natarajan R, Reddy MA, Malik KU, Fatima S, Khan BV. Signaling mechanisms of nuclear factor-kappab-mediated activation of inflammatory genes by 13-hydroperoxyoctadecadienoic acid in cultured vascular smooth muscle cells. Arterioscler Thromb Vasc Biol. 2001; 21:1408–1413. [PubMed: 11557664]
52. Natarajan R, Gu JL, Rossi J, Gonzales N, Lanting L, Xu L, Nadler J. Elevated glucose and angiotensin ii increase 12-lipoxygenase activity and expression in porcine aortic smooth muscle cells. Proc Natl Acad Sci U S A. 1993; 90:4947–4951. [PubMed: 8506339]
53. Desbuquois B, Carre N, Burnol AF. Regulation of insulin and type 1 insulin-like growth factor signaling and action by the grb10/14 and sh2b1/b2 adaptor proteins. FEBS J. 2013; 280:794–816. [PubMed: 23190452]
54. Lim MA, Riedel H, Liu F. Grb10: More than a simple adaptor protein. Front Biosci. 2004; 9:387–403. [PubMed: 14766376]
55. Holt LJ, Siddle K. Grb10 and grb14: Enigmatic regulators of insulin action--and more? Biochem J. 2005; 388:393–406. [PubMed: 15901248]
56. Morrione A, Valentinis B, Resnicoff M, Xu S, Baserga R. The role of mgrb10alpha in insulin-like growth factor i-mediated growth. J Biol Chem. 1997; 272:26382–26387. [PubMed: 9334212]
57. Dufresne AM, Smith RJ. The adapter protein grb10 is an endogenous negative regulator of insulin-like growth factor signaling. Endocrinology. 2005; 146:4399–4409. [PubMed: 16037382]
58. Giorgetti-Peraldi S, Murdaca J, Mas JC, Van Obberghen E. The adapter protein, grb10, is a positive regulator of vascular endothelial growth factor signaling. Oncogene. 2001; 20:3959–3968. [PubMed: 11494124]
59. Yu Y, Yoon SO, Poulogiannis G, Yang Q, Ma XM, Villen J, Kubica N, Hoffman GR, Cantley LC, Gygi SP, Blenis J. Phosphoproteomic analysis identifies grb10 as an mtorc1 substrate that negatively regulates insulin signaling. Science. 2011; 332:1322–1326. [PubMed: 21659605]
60. Illert AL, Albers C, Kreutmair S, Leischner H, Peschel C, Miething C, Duyster J. Grb10 is involved in bcr-abl-positive leukemia in mice. Leukemia. 2015; 29:858–868. [PubMed: 25249015]
61. Mokbel N, Hoffman NJ, Girgis CM, Small L, Turner N, Daly RJ, Cooney GJ, Holt LJ. Grb10 deletion enhances muscle cell proliferation, differentiation and glut4 plasma membrane translocation. J Cell Physiol. 2014; 229:1753–1764. [PubMed: 24664951]
62. Liu B, Liu F. Feedback regulation of mtorc1 by grb10 in metabolism and beyond. Cell Cycle. 2014; 13:2643–2644. [PubMed: 25486344]
63. Kebache S, Ash J, Annis MG, Hagan J, Huber M, Hassard J, Stewart CL, Whiteway M, Nantel A. Grb10 and active raf-1 kinase promote bad-dependent cell survival. J Biol Chem. 2007; 282:21873–21883. [PubMed: 17535812]

64. Kawai-Kowase K, Owens GK. Multiple repressor pathways contribute to phenotypic switching of vascular smooth muscle cells. Am J Physiol Cell Physiol. 2007; 292:C59–69. [PubMed: 16956962]

65. Takahashi E, Berk BC. Map kinases and vascular smooth muscle function. Acta Physiol Scand. 1998; 164:611–621. [PubMed: 9887983]

66. Natarajan R, Bai W, Rangarajan V, Gonzales N, Gu JL, Lanting L, Nadler JL. Platelet-derived growth factor bb mediated regulation of 12-lipoxygenase in porcine aortic smooth muscle cells. J Cell Physiol. 1996; 169:391–400. [PubMed: 8908207]

67. Natarajan R, Scott S, Bai W, Yerneni KK, Nadler J. Angiotensin ii signaling in vascular smooth muscle cells under high glucose conditions. Hypertension. 1999; 33:378–384. [PubMed: 9931133]

68. Wilkinson DG, Bhatt S, Chavrier P, Bravo R, Charnay P. Segment-specific expression of a zinc-finger gene in the developing nervous system of the mouse. Nature. 1989; 337:461–464. [PubMed: 2915691]

69. Laslo P, Spooner CJ, Warmflash A, Lancki DW, Lee HJ, Sciammas R, Gantner BN, Dinner AR, Singh H. Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. Cell. 2006; 126:755–766. [PubMed: 16923394]

70. Pospisil V, Vargova K, Kokavec J, Rybarova J, Savvulidi F, Jonasova A, Necas E, Zavadil J, Laslo P, Stopka T. Epigenetic silencing of the oncogenic mir-17-92 cluster during pu.1-directed macrophage differentiation. EMBO J. 2011; 30:4450–4464. [PubMed: 21897363]

71. Pham TH, Benner C, Lichtinger M, Schwarzfischer L, Hu Y, Andreesen R, Chen W, Rehli M. Dynamic epigenetic enhancer signatures reveal key transcription factors associated with monocytic differentiation states. Blood. 2012; 119:e161–171. [PubMed: 22550342]

72. Lemaire P, Revelant O, Bravo R, Charnay P. Two mouse genes encoding potential transcription factors with identical DNA-binding domains are activated by growth factors in cultured cells. Proc Natl Acad Sci U S A. 1988; 85:4691–4695. [PubMed: 3133658]

73. Shen H, Eguchi K, Kono N, Fujiu K, Matsumoto S, Shibata M, Oishi-Tanaka Y, Komuro I, Arai H, Nagai R, Manabe I. Saturated fatty acid palmitate aggravates neointima formation by promoting smooth muscle phenotypic modulation. Arterioscler Thromb Vasc Biol. 2013; 33:2596–2607. [PubMed: 23968977]

Significance

Diabetes is associated with significantly accelerated vascular complications like atherosclerosis and restenosis which are linked with vascular smooth muscle cell (VSMC) dysfunction. Because microRNAs regulate diverse VSMC functions, we profiled diabetes induced microRNAs and examined their functions in VSMC. Our results showed that several microRNAs including miR-504 were dysregulated in VSMC derived from type 2 diabetic mice. Functional studies revealed that miR-504 downregulated two novel targets Grb10 and Egr2, and enhanced growth factor induced signaling. MiR-504 also reduced the expression of contractile genes and promoted a pro-atherogenic phenotype in VSMC. Furthermore, miR-504 was induced by high glucose and palmitic acid, key pathological factors in type 2 diabetes. These results suggest that dysregulation of miRNAs including miR-504 is a key mechanism underlying VSMC dysfunction in diabetes. Targeting miRNA dependent mechanisms might provide novel and much needed therapeutic approaches for sustained vascular complications of diabetes.

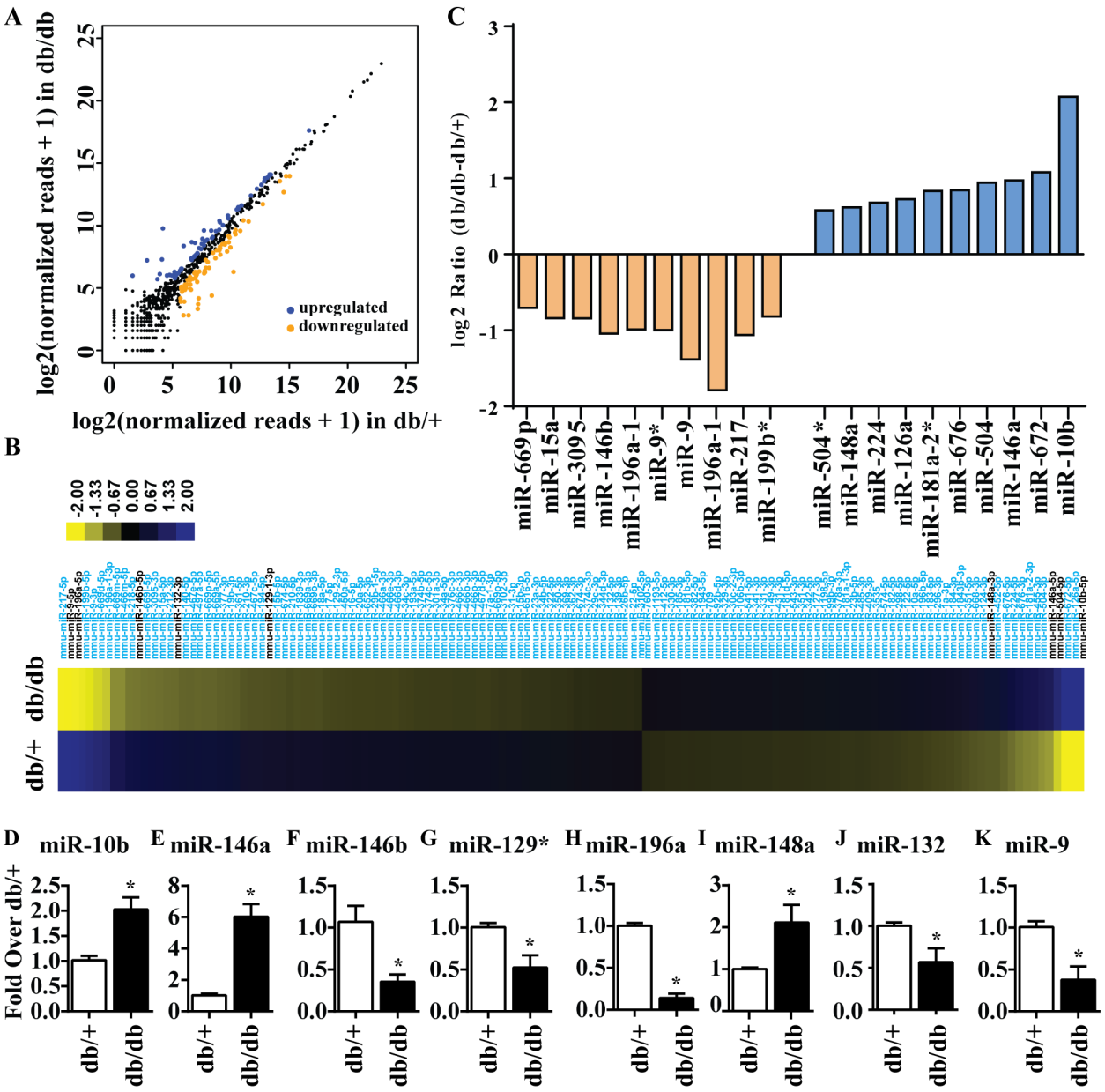

Fig. 1. Regulation of miRNAs by type 2 diabetes in VSMC

A. Scatter plot showing 135 differentially expressed miRNAs in db/dbVSMC versus db/+VSMC. Small RNA-seq was performed on Illumina platform with one sample each of db/+VSMC and db/dbVSMC. Differentially expressed miRNAs were identified as described in Methods section (using criteria of miminum reads ≥50 reads in either samples and fold change ≥1.5). **B.** Heatmap showing unsupervised hierarchical clustering of miRNA expression data in db/dbVSMC and db/+VSMC. For a given miRNA, expression levels in log2 scale of the two samples were mean-centered and presented with yellow color representing lower than mean expression and blue representing higher than mean expression level. **C.** Top 20 differentially expressed miRNAs in db/dbVSMC versus db/+VSMC. **D–K.** RT-qPCR validation of differentially expressed miRNAs in db/dbVSMC compared with db/

+VSMC. RT-qPCR data was normalized with internal control U6 and results were expressed as fold over db/+VSMC (Mean±SEM; *, $p<0.05$ vs db/+VSMC; n = 6, from two different batches of VSMC cultures run in triplicate).

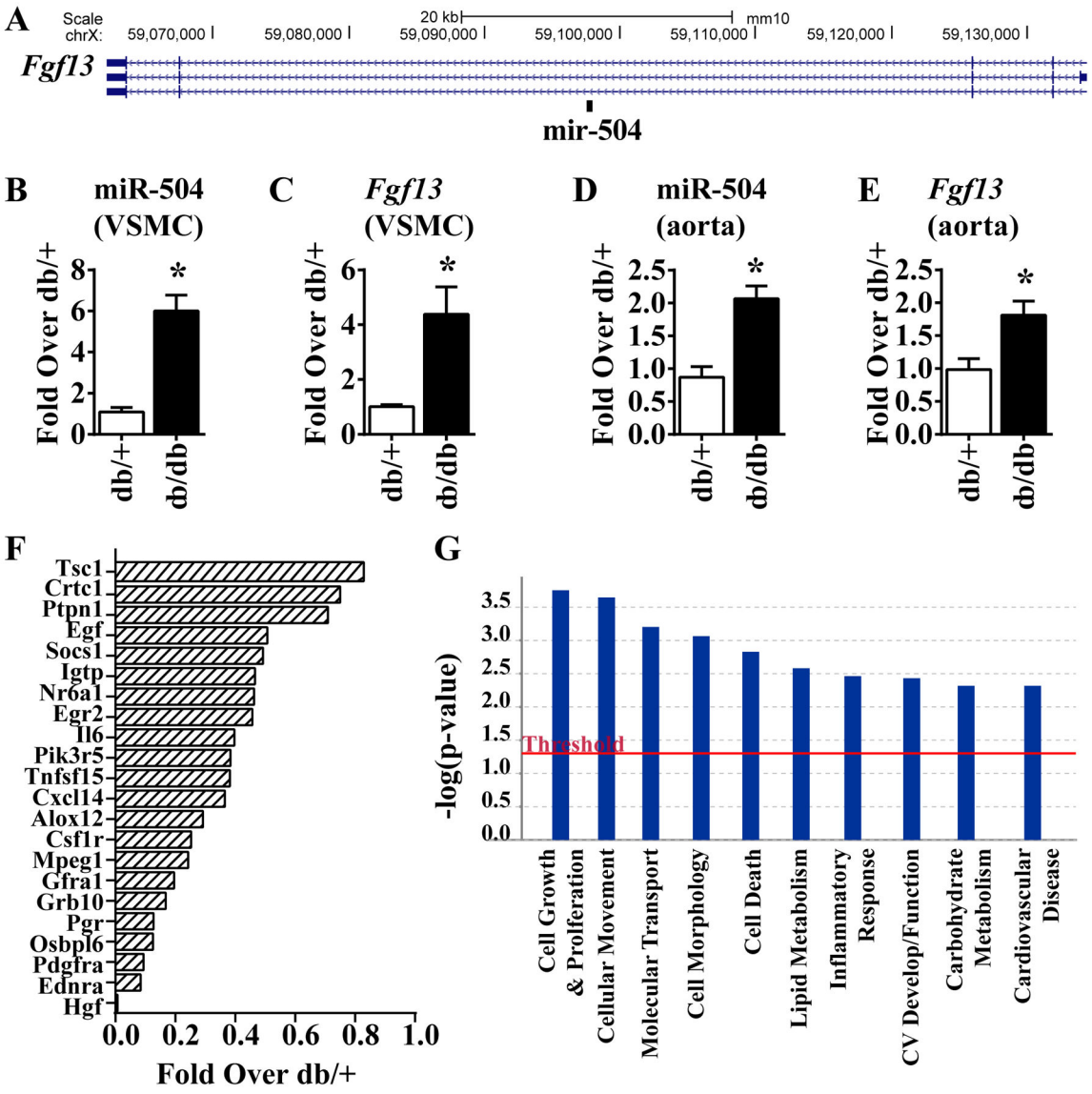

Fig. 2. Increased expression of mir-504 and its host gene *Fgf13* in VSMC and aortas from db/db mice

A. Schematic diagram showing location of miR-504 and host gene *Fgf13* on mouse genome (modified from UCSC genome browser, mouse genome mm10). **B–E.** RT-qPCRs were performed to validate increased expression of miR-504 and *Fgf13* in VSMC (B–C) and aortas (D–E) from db/db mice. RT-qPCR results were expressed as fold over db/+ samples. Mean±SEM. *, $p<0.05$ vs db/+ samples, n = 6 for VSMC (B–C); n=5 db/+ and 7 db/db mice for aortas (D–E). **F.** Expression levels of potential miR-504 targets in db/dbVSMC relative to db/+VSMC. Total RNA was analyzed by RNA-seq on Illumina platform and the relative expression levels (log2 ratio, db/dbVSMC-db/+VSMC) was shown as Fold over db/+VSMC (one sample each). **G.** Top 10 biological functions of miR-504 potential targets identified using Ingenuity pathway analysis (IPA) software. Y-axis shows –log (p-value) and the horizontal red line inside the bar graph represents the threshold value ($p<0.05$).

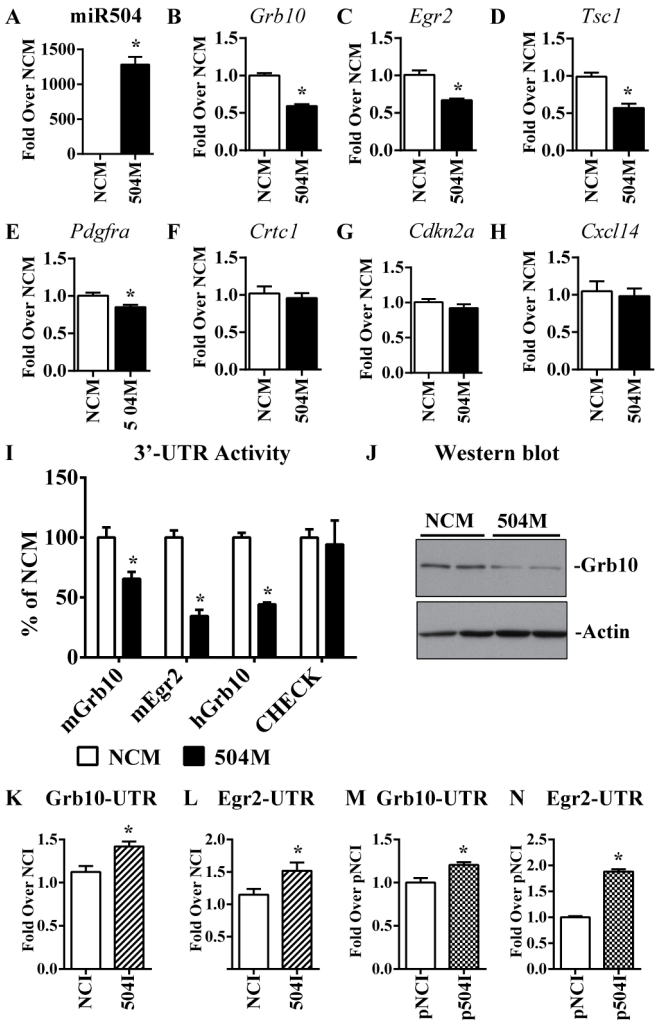

Fig. 3. Grb10 and Egr2 are direct targets of miR-504 in VSMC

A–H. Validation of miR-504 targets in VSMC. db/+VSMC were transfected with miR-504 mimics (504M) and negative control (NCM) oligonucleotides by Nucleofection and 48 h post transfection expression of indicated genes was analyzed by RT-qPCR. Results were expressed as fold over NCM transfected cells (Mean±SEM. *, $p<0.05$ vs NCM, n = 6, except H, n=3). **I.** Inhibition of 3′-UTR activity by miR-504. psiCHECK plasmids with Renilla luciferase containing 3′-UTRs of indicated mouse (m) and human (h) genes were co-transfected with 504M or NCM into db/+VSMC. Activity of Renilla and internal control firefly luciferase in cell lysates were determined 48 h post transfection. Results normalized to firefly luciferase were expressed as % of NCM transfected cells. Data represents Mean ±SEM. *, $p<0.05$ vs NCM, n=6. **J.** Immunoblot showing downregulation of Grb10 in cell lysates from db/+VSMC transfected with 504M relative to NCM (Upper panel). Lower panel shows internal control β-Actin. **K–L.** miR-504 inhibitors (504I) increase Grb10 and Egr2 3′-UTR activities. Grb10 and Egr2 3′-UTR reporter plasmids were co-transfected with miR-504 inhibitor (504I) or control inhibitor (NCI) oligonucleotides, and luciferase activity in cell lysates was determined 48 h post transfection. Mean±SEM. *$p<0.05$ vs NCI, n=6.

M–N. Transfection of plasmids expressing miR-504 inhibitor (p504I) also increased activity of 3′-UTRs relative to control plasmid expressing scramble sequence (pNCI). Data represents Mean±SEM. *p<0.05 vs NCI, n=6.

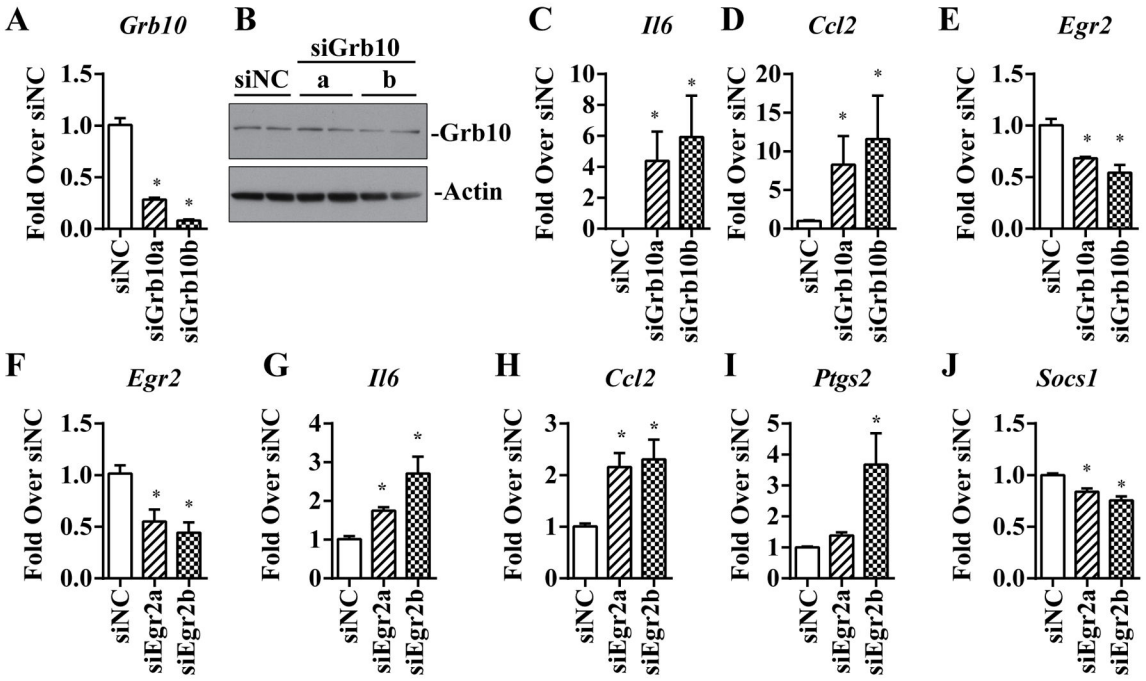

Fig. 4. Silencing of Grb10 and Egr2 with siRNAs induces inflammatory gene expression in db/+VSMC

A–E. Grb10 gene silencing (A–B) increases inflammatory genes (C–D) and inhibits *Egr2* expression (E). db/+VSMC were transfected with two siRNAs targeting Grb10 (siGrb10a and siGrb10b) and control siNC oligonucleotides. Expression of the indicated genes was analyzed 72 h post transfection by RT-qPCR, normalized with internal control *Actb* and expressed as Fold over siNC (Mean±SEM, *, $p<0.05$ vs siNC, n=6). Panel B shows immunoblotting with indicated antibodies to confirm inhibition of Grb10 protein levels by siGrb10a and siGrb10b versus siNC. **F–J.** *Egr2* gene silencing (F) increased expression of inflammatory genes (G–I) and inhibited anti-inflammatory *Socs1* (J) in db/+VSMC. RT-qPCR analysis of indicated genes was performed using RNA extracted from db/+VSMC with siEgr2a, siEgr2b and siNC oligonucleotides, 72 h after transfection (Mean±SEM. *, $p<0.05$ vs siNC, n=6).

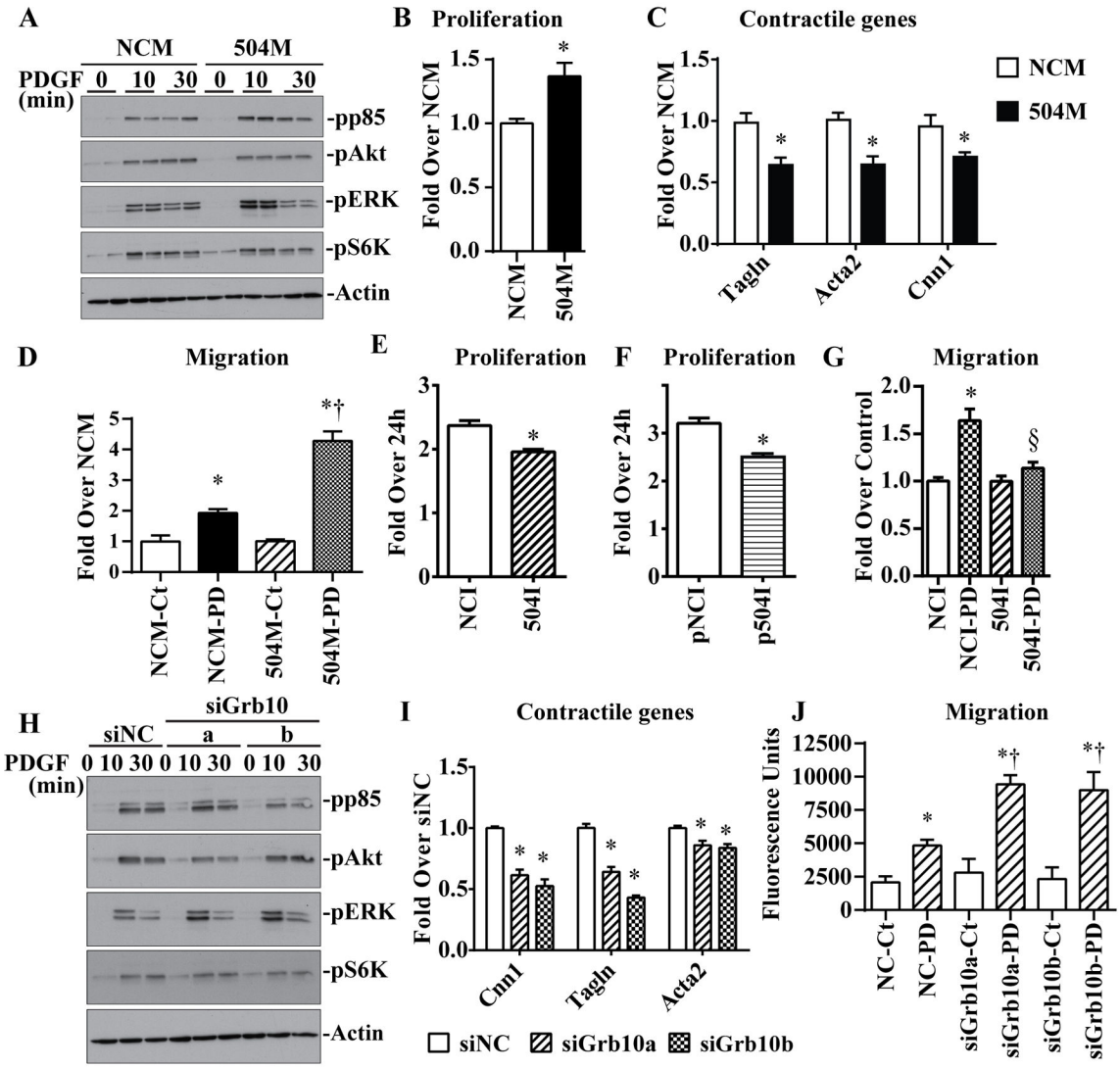

Fig. 5. Overexpression of miR-504 or Grb10 gene silencing enhances ERK activation and VSMC dysfunction

A. Increased levels of miR-504 mimic (504M) enhanced ERK activation. db/+VSMC were transfected with 504M or NCM, and 48 h later stimulated with serum depletion medium (SD) alone (0) or SD containing PDGF (10 ng/ml) for indicated time points. Total cell lysates were immunoblotted with antibodies against indicated phosphorylated (p)-proteins and internal control β-Actin. Similar results were obtained in another experiment**. B.** Cell proliferation assays were performed with db/+VSMC transfected with NCM or 504M and results were expressed as Fold over NCM (Mean±SEM. *, $p<0.05$ vs NCM, †, $p<0.05$ vs NCM-PD, n=4). **C.** Inhibition of contractile genes by miR-504. Expression of indicated genes was determined by RT-qPCR in 504M and NCM transfected db/+VSMC, 48–72 h post transfection. Results were expressed as Fold over NCM (Mean±SEM. *, $p<0.05$ vs NCM, n=6). D. miR-504 promotes VSMC migration. Migration assays were performed with db/+VSMC transfected with NCM or 504M in 96-transwell plates (in triplicate wells) using SD without (Ct) or with 1 ng/mL of PDGF (PD) as stimulant in the bottom chamber.

Migrated VSMC were labeled with Calcein, fluorescence was determined using a fluorescent plate reader and results expressed as fold over NCM transfected cells (Mean ±SEM. *, $p<0.05$ vs NCM, n=3). Results shown are representative of two separate experiments. **E–F.** Inhibition of VSMC proliferation by miR-504 inhibitors. Proliferation assays were performed using VSMC transfected with control (NCI) and miR-504 inhibitor (504I) oligonucleotides (E) or plasmids pNCI and p504I expressing scrambled sequences respectively. Results were expressed as fold over 24 h. (Mean±SEM. *, $p<0.05$ vs 24 h, n=12). **G.** Inhibition of migration by miR-504 inhibitors. PDGF induced migration assays were performed with VSMC transfected with NCI and 504I oligonucleotides. (*, $p<0.05$ vs NCI-control; §, $p<0.05$ vs NCI-PD, n=12). **H**. Grb10 knockdown enhanced ERK1/2 activation. db/+VSMC were transfected with Grb10 siRNAs (siGrb10a and siGrb10b) or siNC, 72 h post transfection cells were stimulated with SD (0) or SD with PDGF (10 ng/ml) and cell lysates were immunoblotted with indicated antibodies. Results shown are representative of two separate experiments. **I.** Grb10 knockdown inhibited genes associated with contractile phenotype. Expression of indicated genes in siNC, siGrb10a and siGrb10b transfected MVSMC was analyzed by RT-qPCR. (Mean±SEM. *, $p<0.05$ vs siNC-Cont, n=5). **J**. Grb10 gene silencing enhanced PDGF induced VSMC migration. db/+VSMC were transfected with siGrb10a, siGrb10b or siNC and 72 h post transfection migration assays were performed in 96-transwells (quadruplicate wells) with SD medium (Cont) or with PDGF (1 ng/mL). Bar graph shows the fluorescence units (Mean±SEM. *, $p<0.05$ vs siNC-Cont; †, $p<0.05$ vs siNC-PDGF, n=4).

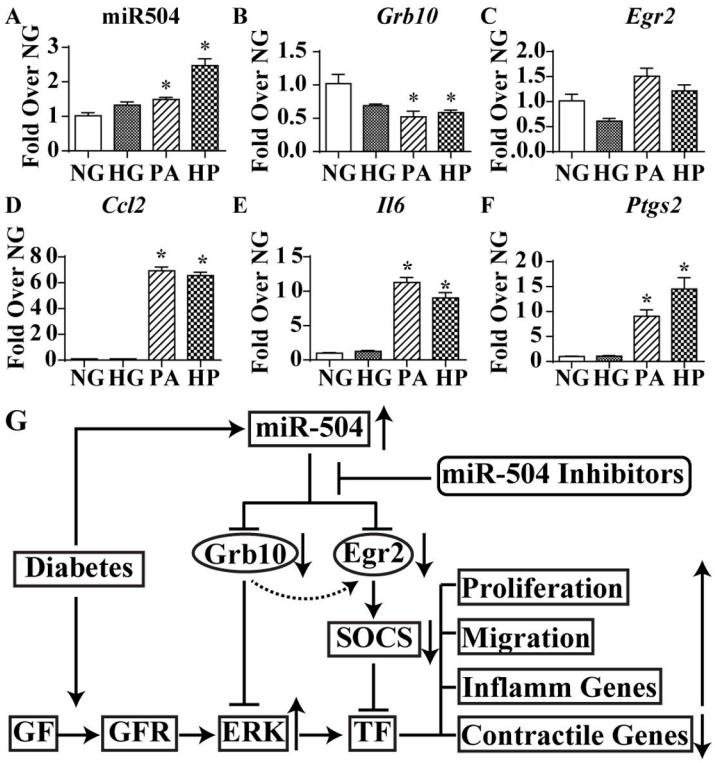

Fig. 6. Regulation of miR-504 under diabetic conditions *in vitro* in VSMC

A–F. VSMC were treated with M199 medium containing 5.5 mmol/L glucose (NG), 25 mmol/L glucose (HG), 100 μmol/L palmitic acid (PA) or HG+PA for 48 h. Expression of miR-504 (A), target genes (B–C) and inflammatory genes (D–F) was analyzed by RT-qPCR. Results were expressed as Fold over NG after normalization with let-7b for miR-504 and *Actb* for other genes (Mean±SEM. *, $p<0.05$ vs NG, n=3–6). **G.** Schematic diagram showing mechanisms of miR-504 mediated VSMC dysfunction. Diabetes increases miR-504, which targets Grb10 and Egr2. Grb10 downregulation enhances growth factor (GF) induced ERK1/2 activation, while Egr2 downregulation reduces anti-inflammatory genes. Thus, miR-504 and its targets promote VSMC dysfunction by inhibiting contractile genes, and augmenting inflammatory genes, proliferation and migration, key steps involved in restenosis and atherosclerosis. Arrows indicate the direction of changes in expression or function. GFR-GF receptor.