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MicroRNA-dependent regulation of KLF4 by glucose in vascular smooth muscle

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Abstract

Diabetes is a major risk factor for cardiovascular disease and this is in part due to the effects of hyperglycemia on vascular smooth muscle cells. Small non-coding microRNAs are known to control smooth muscle phenotype and arterial contractility and are dysregulated in diabetes. The effect of microRNAs on smooth muscle differentiation is in part mediated by the transcription factor KLF4 but the role of this mechanism in diabetic vascular disease is not fully understood. Herein, we have investigated the importance of hyperglycemia and diabetes for the expression of KLF4 in vascular smooth muscle and the involvement of miRNAs in this regulation.

Hyperglycemia down-regulated KLF4 in vascular smooth muscle cells and similar results were found in arteries of diabetic mice and patients. This correlated with a Foxa2-dependent up-regulation of miR-29c, which targeted KLF4 in vascular smooth muscle cells. Importantly, by preventing downregulation of KLF4, the induction of smooth muscle contractile protein markers by glucose was inhibited.

In conclusion, miR-29 mediated inhibition of KLF4 in hyperglycemic conditions contributes to increased expression of contractile markers in vascular smooth muscle cells. Further studies are warranted to determine the therapeutic implications of miR-29 inhibition in diabetic vascular disease.

Introduction

Cardiovascular complications are the leading cause of death in diabetic patients, which have more than two-fold risk of cardiovascular disease compared to non-diabetic subjects [1]. These complications include microvascular disease such as retinopathy, neuropathy and nephropathy, as well as macrovascular disease including myocardial infarction, peripheral vascular disease and stroke. The vascular effects in diabetic patients can in some cases be attributed to alterations in smooth muscle phenotype. Unlike striated muscle, smooth muscle cells are not terminally differentiated and exhibit phenotypic plasticity in response to extracellular cues, becoming more or less contractile [2]. Dedifferentiation into a less contractile phenotype is often associated with an increased production of extracellular matrix proteins as well as increased migratory and proliferative capacity. However, separate signaling pathways control smooth muscle proliferation and contractility and independent reports suggest that these pathways are not mutually exclusive [3-5]. An example of such co-existence has been demonstrated under diabetic conditions, when smooth muscle cells were shown to differentiate into a more contractile phenotype and hence exhibit increased contractility [6,7] and simultaneously exhibit an increased capacity to transition into a proliferative state [8].

One factor that is known to negatively influence both contractile differentiation and proliferation of smooth muscle cells is the transcription factor, krüppel-like factor 4 (KLF4) [4]. We recently found that KLF4 is downregulated in smooth muscle cells by hyperglycemia in a microarray screen [9]. It is well established that KLF4 is a potent inhibitor of cell proliferation, primarily via regulation of p21^{WAF1} and may play an important role in carcinogenesis [10]. The KLF4-dependent repression of smooth muscle specific gene expression occurs via both inhibition of myocardin/SRF-dependent transcription as well as transcriptional silencing via epigenetic changes of smooth muscle marker loci [11]. Consistent with these results, conditional deletion of KLF4 in smooth muscle, results in delayed loss of smooth muscle differentiation, but accelerated neointimal growth following vascular injury [4]. The importance of KLF4 in vascular disease is further emphasized by its role in trans-differentiation of smooth muscle cells to macrophage- or mesenchymal stem cell-like phenotypes [12]. Notably, recent evidence suggests that approximately 16% of macrophage-like cells in atherosclerotic lesions are derived from smooth muscle cells [13].

In addition to transcriptional regulation, the expression of KLF4 is controlled at the post-transcriptional level by small non-coding microRNAs (miRNAs) [14-16]. These miRNAs bind with partial complementarity to the KLF4 encoding mRNA and induce RNAi mediated silencing of KLF4 protein expression. Elevated expression of KLF4 is observed in smooth muscle following conditional deletion of Dicer, which is essential for miRNA biogenesis [17]. Several specific miRNAs have been demonstrated to regulate KLF4, including the smooth muscle enriched miR-143/145 cluster [14,15] and the aneurysm-associated mir-29 cluster [16]. Interestingly, miR-29 is upregulated in multiple tissues in diabetes including skeletal and cardiac muscle [18,19]. The effects of diabetes on miR-29 expression in smooth muscle remain to be determined.

The aim of the present study was to determine if hyperglycemia and diabetes regulate the expression of KLF4 in vascular smooth muscle and to identify the possible involvement of miRNAs in this regulation. The results suggest that KLF4 is down-regulated by hyperglycemia both *in vitro* and *in vivo*, including arteries from diabetic mice and patients. Furthermore, we demonstrate that miR-29 was upregulated in diabetic conditions and that the effect of hyperglycemia on KLF4 expression is dependent on miR-29 in cultured vascular smooth muscle cells.

Results

Regulation of KLF4 by hyperglycemia and diabetes

In a previous study, we screened for genes that were differentially regulated in cultured smooth muscle cells exposed to 25 mM glucose (high glucose; HG), compared to cells cultured in 1.7 mM glucose (low glucose; LG), for 1 week [6]. The microarray analysis revealed a significant down-regulation of KLF4 by hyperglycemia. To confirm these data at both mRNA and protein level, we herein performed qPCR analysis to examine the KLF4 expression in smooth muscle cells exposed to LG, normal glucose (5,5 mM; NG) or HG conditions for 1 week. KLF4 mRNA expression was significantly decreased in both NG and HG conditions compared to LG (Fig 1A). A small but significant decrease in KLF4 expression was also found in the osmotic control (LG plus 23,3 mM mannitol) compared to LG. Western blot analysis confirmed the effect of glucose on KLF4 protein expression (Fig 1B and D). Furthermore, Wnt5a, a direct transcriptional target of KLF4 [23], was significantly down-regulated in hyperglycemic conditions (Fig 1C-D).

To determine the effect of hyperglycemia on KLF4 expression *in vivo*, we used arteries from the hyperglycemic Ins2(Akita) mouse model at the age of 12 weeks. These mice have a single amino acid substitution in the insulin 2 gene resulting in protein misfolding followed by beta cell apoptosis [24]. Heterozygous male mice become hyperglycemic after approximately 3 weeks of age and the glucose levels are stabilized at about 30 mM at 9 weeks of age. Quantitative PCR and Western blot analysis was performed on tail artery and small mesenteric arteries, respectively. This revealed significant downregulation of mRNA and protein expression of KLF4 in the vascular wall of hyperglycemic mice (Fig 1E-G). Similar results were obtained using a KLF4 antibody with a different epitope (Suppl fig 1).

The potential involvement of KLF4 in diabetic vascular disease was further investigated by analyzing KLF4 mRNA and protein expression in left internal mammary arteries from diabetic and non-diabetic patients. As shown in figure 1H-J, both mRNA and protein levels of KLF4 were found to be reduced in arteries from diabetic patients. Taken together, these results demonstrate a decrease of KLF4 expression in smooth muscle cells of the vascular wall under diabetic conditions.

Regulation of KLF4-targeting miRNAs by hyperglycemia

Several miRNAs have been demonstrated to target KLF4 in smooth muscle including miR-143, miR-145 [14,15,25] and miR-29 [16,26]. To investigate the regulation of these miRNAs by glucose we incubated smooth muscle cells in LG, NG, HG or LG+mannitol conditions for 1 week (Fig 2A-D). As shown in figure 2B, miR-29c was positively regulated in the hyperglycemic condition, while the expression levels of miR-29a, miR-29b and miR-143/145 were unchanged compared to the LG condition at this time point (Fig 2A, C-D and suppl fig 2b). We next analyzed if mir-29 family members were upregulated in mammary arteries from diabetic patients where both miR-29b and miR-29c were induced in diabetic patients with borderline significance (Fig 2E-G).

Regulation of miR-29 by FoxA2 in vascular smooth muscle cells

Previous findings suggest that miR-29 in liver is regulated by the transcription factor Foxa2 in diabetes [27]. We first investigated the expression levels of Foxa2 in smooth muscle cells cultured under HG and LG conditions for 1 week and found a significant upregulation in HG conditions (Fig 3A). We next transfected smooth muscle cells with Foxa2 siRNA, which caused a significant decrease in both Foxa2 (Fig 3B) and miR-29c (Fig 3C) expression, suggesting that miR-29 is transcriptionally regulated by Foxa2 during hyperglycemia.

Regulation of KLF4 by miR-29 in vascular smooth muscle cells

In silico analysis of the 3'-UTR of KLF4 using TargetScanHuman (release 7.1) revealed a conserved binding site for miR-29 at position 482-488 of the KLF4 3' UTR (Suppl fig 3). This site is evolutionary conserved in at least 42 species from X. tropicalis to humans. To identify the role of miR-29 for KLF4 regulation, miR-29c mimic was transfected into cultured smooth muscle cells. This resulted in a significant decrease of KLF4 mRNA and protein expression in LG conditions and loss of glucose sensitivity (fig 4A-C). To test if endogenous levels of miR-29c were sufficient to regulate KLF4, we next transfected cells with a miR-29c inhibitor. This caused an increased KLF4 expression in HG conditions and, again, loss of glucose sensitivity of KLF4 (Fig 4D-F). Similar results were observed at the mRNA level suggesting that miR-29 promotes both translational inhibition and mRNA degradation of KLF4. Although, miR-29c inhibitor primarily reduced miR-29c expression, a minor effect was also observed on miR-29a and miR-29b expression (Suppl fig 2).

To confirm the interaction of miR-29 with KLF4 mRNA, we transfected smooth muscle cells with a pMIR translation reporter, containing the 3-UTR and the entire coding sequence of KLF4 cloned into the firefly luciferase transgene [28]. The luciferase activity is inhibited by miRNAs targeting KLF4 and, accordingly, HG significantly decreased KLF4 luciferase activity compared to LG conditions (Fig 4G). To pinpoint the role of miR-29c for this effect we used a miR-29c inhibitor and found that this nearly abolished the decrease in KLF4 luciferase activity by high glucose. MiR-199, which is not predicted to target KLF4 was used as a negative control and inhibition of this miRNA did not affect luciferase activity. Mannitol (23.3 mM) was used as osmotic control in a separate experiment and had no effect on luciferase activity compared to LG control (data not shown). Thus, these results suggest that elevated glucose conditions down-regulates KLF4 expression via miR-29c.

Overexpression of KLF4 can prevent glucose-induced expression of contractile smooth muscle markers

We have previously demonstrated that elevated glucose conditions can promote the expression of contractile smooth muscle markers [6]. To test the possibility that part of this effect is mediated by KLF4, we overexpressed KLF4 in cultured smooth muscle cells, using adenoviral-mediated transduction, under high and low glucose conditions. A significant increase in KLF4 expression was observed after Ad.KLF4 transduction (Fig 5A-B). Interestingly, similar to endogenous KLF4, the expression of transduced KLF4 tended to be reduced in HG conditions, further supporting a posttranscriptional regulation of KLF4. Similar to previous observations, we found increased expression of smooth muscle markers calponin and SM22 in HG vs LG conditions (Fig 5A-C). Overexpression of KLF4 decreased calponin and SM22 to LG levels, suggesting that low expression of KLF4 is necessary for the effects of glucose on contractile protein markers in smooth muscle.

Discussion

In the present study, we have performed a detailed analysis of the posttranscriptional regulation of the transcription factor KLF4 in smooth muscle. We found that both hyperglycemic and diabetic conditions result in downregulation of KLF4 in isolated smooth muscle cells as well as in mouse and human arteries. In cultured smooth muscle cells, this effect is partly mediated by upregulation of the FoxA2 transcription factor and its transcriptional target miR-29, which directly interacts with KLF4 mRNA. Importantly, our results demonstrate that KLF4 downregulation is crucial for the effects of glucose on smooth muscle marker expression.

Our results suggest that KLF4 is down regulated in smooth muscle cells by hyperglycemia as well as in in arteries from diabetic mice and patients. These results are consistent with earlier findings in other tissues including adipose tissue and kidney [20-22,29] but in conflict with recent results in smooth muscle [30] [31]. We are unsure as to the reason for these discrepancies, but they may be due to differences in diabetes models or the time point of when KLF4 expression is analyzed.

One important mechanism for KLF4 regulation is posttranscriptional processing by miRNAs, and several specific miRNAs have been experimentally validated to target KLF4 [14-16,32]. Some of these miRNAs coincide with miRNAs that are known to be upregulated in diabetes. In our model of hyperglycemia in cultured smooth muscle cells, we found upregulation of miR-29 in the vasculature of type II diabetic patients and in cultured vascular smooth muscle cells cultured under hyperglycemic conditions.

The miR-29 family consists of four miRNAs (miR-29a/b-1/b-2/c) with identical seed sequences (3'-ACCACGA-5') that are transcribed from two genomic clusters, miR29a/b-1 from the miR-29a cluster and miR-29b-2/c from the miR-29c cluster [33]. MicroRNAs generally target multiple genes by complementarity binding of the seed region to the 3'-UTR of the mRNA sequence. Several targets of miR-29 have been identified with particular focus on the regulation of extracellular matrix, cell proliferation and apoptosis [33-36]. Using *in silico* target prediction it is evident

that KLF4 is a predicted and highly conserved target of miR-29. Experimental evidence also supports this prediction in breast cancer cells [26] and pulmonary artery smooth muscle cells [16]. Using a miR-29c inhibitor, we could prevent downregulation of KLF4 by glucose. However, since the miR-29c inhibitor may affect miR-29a and miR-29b expression, we cannot exclude a minor contribution of these miRNAs. Furthermore, multiple miRNAs are known to interact with KLF4 including smooth muscle enriched miR-143/145 and miR-1 [32], and we do not exclude regulation by these miRNAs in diabetic conditions. Conversely, miR-29 targets multiple transcripts other than KLF4 in smooth muscle, which may be involved in the effects observed in this study. Another limitation of the present study is that the functional importance of miR-29c was not tested *in vivo*. Although *in vitro* experiments point towards an important role of glucose-induced miR-29c for KLF4 expression and smooth muscle phenotype regulation, further studies are warranted to demonstrate the *in vivo* relevance of this finding.

The induction of miR-29 in diabetes is rather well established in non-smooth muscle cell types considering that members of the miR-29 family are upregulated in plasma [37], skeletal muscle [18], pancratic islets [38], liver [27] and cardiomyocytes [19]. MiR-29c is also increased in podocytes under diabetic conditions and knock-down of miR-29c prevents the progression of diabetic nephropathy [39]. Interestingly, global and liver specific knockout of the miR-29 family has revealed that miR-29a is a positive regulator of insulin secretion from the pancreas, and that all members of miR-29 family promotes obesity and insulin resistance via regulation of insulin signaling in the liver and appetite control [40]. Thus it appears that miR-29 can play an important role in different aspects of diabetes including diabetic vascular disease.

KLF4 is a negative regulator of both proliferation and smooth muscle differentiation and it is thus possible that a reduced expression of this transcription factor in diabetic conditions can result in both hypercontractility and increased growth of the smooth muscle cells in the vascular wall. In accordance with this hypothesis, diabetes is associated with increased contractile smooth muscle function in both humans and animal models [41,42]. Furthermore, diabetic conditions can promote smooth muscle proliferation although this effect may be independent of hyperglycemia [43,44]. Accordingly, high glucose does not affect smooth muscle proliferation rates in our culture model [9]. In the present study, we show that overexpression of KLF4 in cells exposed to hyperglycemia can prevent the upregulation of contractile smooth muscle markers suggesting that low expression of KLF4 is essential for the effects on smooth muscle differentiation by high glucose. Our earlier findings have revealed that nuclear translocation of the myocardin related transcription factor (MRTF), via activation of the PKC/Rho signaling pathway, is required for activation of smooth muscle differentiation by glucose [6,45]. Since, KLF4 is a negative regulator of MRTF/SRF activity [46], it is likely that downregulation of KLF4 and activation of MRTF cooperate to promote expression of smooth muscle markers in response to hyperglycemia.

Although elevated contractile differentiation is often considered beneficial in smooth muscle cells, this likely depends on the clinical situation and vascular bed. In resistance arteries, hypercontractility can result in local changes in blood flow and ischemia due to vasospasm as well as global elevation on systemic blood pressure. An

increased risk of both vasospasm and hypertension has been reported in diabetic patients and it is possible that enhanced smooth muscle contractility is involved in this effect [47]. Conversely, diabetes is known to be protective against abdominal aortic aneurysm [48]. Although the molecular mechanisms underlying this phenomenon are not completely understood, it is notable that knockout of smooth muscle KLF4 in animal models also protects against aneurysm development [49]. Deletion of KLF4 also attenuated the downregulation of contractile markers in smooth muscle. Since mutations in genes involved in smooth muscle contraction are known to predispose to aortic aneurysms, it is likely that maintaining or increasing smooth muscle contractility confers protection against the development and dissection of aortic aneurysms [50]. In support of this hypothesis, we recently reported a reduced expression of action polymerization and MRTF-regulated miRNA expression in mildly dilated human aorta [51]. Taken together, our data supports the view that hyperglycemia and diabetes protects against abdominal aneurysm via downregulation of KLF4 and increased contractile differentiation of smooth muscle. However, it is important to note that increased expression of miR-29 *per se* is considered to be detrimental for aneurysm development, due to its negative effect on extracellular matrix proteins [52,53]. The overall importance of miR-29-KLF4 regulation by glucose for aneurysm development is therefore difficult to predict.

In conclusion, we have demonstrated that hyperglycemia results in upregulation of miR-29 and subsequent inhibition of its target KLF4 in vascular smooth muscle cells. Further, this effect is involved in mediating elevated expression of smooth muscle markers in cells under hyperglycemic condition. Future studies are required to fully understand the involvement of miR-29 and KLF4 in diabetic vascular disease.

Methods

Animals - Akita type 1 diabetic mice (C57BL/6-*Ins2*^{Akita}/J) were obtained from the Jackson laboratory and bred at Lund University. Mice were euthanized by cervical dislocation. Small mesenteric arteries and aorta from male adult 12 week old heterozygous Akita and littermate control mice were dissected free from fat and surrounding tissue and snap frozen in liquid nitrogen. All experiments were approved by the Malmö/Lund animal ethics committee and performed in accordance with relevant guidelines and regulations (M9-15, M61-16).

Human samples – Left internal mammary arteries were obtained from patients undergoing coronary artery by-pass surgery as described previously [6]. Samples were dissected free from fat and surrounding tissue in sterile, calcium-free, HEPES buffer (135.5 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl2, 11.6 mM glucose, and 11.6 mM HEPES, pH 7.4) and then snap frozen in liquid nitrogen. The study was performed in accordance with relevant guidelines and regulations including the Declaration of Helsinki. Informed consent was obtained from all patients and the study was approved by the regional ethical review board in Lund 2014/202. Due to ethical constraints, the collected patient parameter was limited to diabetic status in patients during initial parts of the study. For the remaining patients (10 non-diabetics and 6 diabetics) additional parameters including blood pressure, blood glucose, age and gender was also obtained (Table 1).

Cell culture and transfection - Mouse aortic smooth muscle cells (mAoSMCs) were isolated from mouse aorta by enzymatic digestion and maintained in culture as described [17]. Cells were used at passages 3–5 for experiments and cultured in DMEM (Gibco #11966-025) supplemented with low glucose (LG; 1.7 mM glucose), normal glucose (NG; 5.5 mM glucose), high glucose (HG; 25 mM glucose) or low glucose plus mannitol (LG+Man; 1.7 mM glucose + 23.3 mM mannitol) for 1 week. The medium was routinely exchanged every 48h. All media were supplemented with 10% fetal bovine serum (Biochrom #S0115) and 50U/50µg/ml penicillin/streptomycin (Biochrom, A2212).

For miRNA transfections, cells were transfected with miR-29c inhibitor (Thermo Scientific; 20nM), Inhibitor control (Sigma-Aldrich; 20 nM), miR-29c mimic (Thermo Scientific; 20nM) or mimic control (Sigma-Aldrich; 20 nM, respectively) for 4 or 8 days. Transfections were performed at day 0 and day 4 using oligofectamine transfection reagent (Invitrogen, #12252-011) according to the manufacturer's instructions. Foxa2 siRNA (Thermo Scientific, 20mM) and control siRNA (Thermo Scientific, 20mM) were transfected using the same protocol as for miRNAs but only for 96h. Cells were then cultured for 1 week in LG or HG conditions with 10% fetal bovine serum and 50U/50µg/ml penicillin/streptomycin.

For adenoviral transduction, cells were incubated with Ad-m-KLF4 adenovirus 20 MOI (Vector Biolabs, #1791) or Ad-CMV-Null 20 MOI (Vector Biolabs, #1300) for 96 hr in normal glucose. Cells were then changed to LG or HG conditions for 1 week.

Luciferase activity- pMIR-Report-Luc-KLF4-FL was a gift from Michael Ruppert (Addgene plasmid # 34597) [28]. A dual-luciferase reporter assay system (Promega, Madison, WI) was used to determine gene promoter activity according to the manufacturer's instructions. Briefly, vascular smooth muscle cells were plated in 12-well plates and transfected with pMIR-Report-Luc-KLF4-FL and renilla plasmids (hRenilla luciferase expression for normalization; Promega) using fugene (2 µl/well) reagent (Promega, #E2691). Firefly and hRenilla luciferase activities in the cell lysates were measured using a Multiscan GO microplate spectrophotometer (Thermo Scientific). The relative luciferase activity was calculated by normalizing the promoter-driven firefly luciferase activity to the hRenilla luciferase activity.

For co-transfection with miR-29c inhibitor, after 96hr transfection of Klf4-luc and renilla, cells were with miR-29c inhibitor (Thermo Scientific, Pittsburgh, PA, USA; 20nM), or negative control (Mission miRNA: Sigma-Aldrich, St. Louis, MO, USA; 20 nM, respectively) for 8 days with double transfection using Oligofectamine transfection reagent (Life Technologies).

Quantitative real-time PCR (qRT-PCR) - Total RNA was isolated using miRNeasy mini kit (Qiagen, #217004) according to the manufacturer's instructions. Purity and concentration were determined with an ND-1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA). The expression of mRNA was analyzed by real-time qPCR (StepOnePlus qPCR cycler, Applied Biosystems) using QuantiFast SYBR Green RT-PCR Kit Qiagen, #204156) and

QuantiTect primer assays: Mouse - Mm_Klf4, QT00104174; Mm_FoxA2, QT00242809; Mm_GADPH, QT01658692; Hs_Klf4, QT00061033; Hs_18s, QT00199367. Primer sequences are proprietary of Qiagen.

The expression of miRNAs was analyzed by real-time qPCR (StepOnePlus qPCR cycler, Applied Biosystems) using miScript SYBR Green PCR Kit (Qiagen, # 218073) and miScript Primer Assays: Mm_miR-29a, # MS00001372; Mm_miR-29b, # MS00005936, Mm_miR-29c, # MS00001379; Mm_miR-143, #MS00001617; Mm_miR-145, # Ms00001631; Hs_SNORD-95, # MS00033726.

Protein extraction and Western Blotting - Cells grown on 6-well plates were washed twice with ice-cold PBS and lysed on ice directly in the wells with 75 µl of 1x Laemmli sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol). Frozen tissue was pulverized using liquid nitrogen as described previously [54]. Proteins were detected using commercially available primary antibodies: Klf4 (1:500, Cell Signaling, #4038S; Thermo Fisher Scientific, # PA5-20897), calponin (1:1000, Abcam, #ab46784), SM22α (1:5000, Abcam, #ab14106), Wnt5a (1:1000, Abcam, ab72583), HSP90 (1:1000, BD Transduction Laboratories, #610418. Secondary mouse or rabbit HRP-conjugated antibodies (1:5000 or 1:10000, Cell Signaling, #7074, #7076) were used. Bands were visualized using ECL (Pierce West Femto) and images were acquired using the Odyssey Fc Imager (LI-COR Biosciences).

Statistics - Values are presented as mean ± S.E.M. unless otherwise stated. P-values were calculated by Student's test ore one-way analysis of variance (ANOVA) followed by Boneferroni's multiple comparison tests using GraphPad Prism 5 (GraphPad Software Inc.). $P<0.05$ was considered statistically significant. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

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Author contributions

Conception and design (THT, KGS, MFG and SA), acquisition of data (THT), providing samples (JN, JS, EGV, MG), analysis and interpretation of data (THT, KGS, MFG and SA), drafting or revising the article (THT, KGS, MFG, JN, MG and SA).

Competing financial interests

None

Figure legends

Figure 1. KLF4 expression is reduced by hyperglycemia and diabetes. KLF4 mRNA (A, n=4-6) and protein (B, n=6) expression was evaluated in aortic smooth muscle cells cultured under high (HG) and low (LG) glucose conditions for 1 week. For mRNA analysis, culture with normal glucose (NG) and LG+mannitol (Man) was also included. The known KLF4 target Wnt5a (C, n=6) was analyzed by western blot under the same conditions as in B. Representative western blots are shown in D. KLF4 mRNA (E, n=17-19, tail artery) and protein expression (F, n=23-26, small mesenteric arteries) was analyzed in arteries from hyperglycemic Akita mice and littermate controls. Representative western blots are shown in G. KLF4 mRNA (H, n=12-13) and protein expression (I, n=11-12) was analyzed in left internal mammary arteries from diabetic and non-diabetic patients. Representative western blots are shown in J. Full blots are shown in supplementary figure 4-6. Data are presented as mean +/-S.E.M. Statistical significance was analyzed using one-way ANOVA with Bonferroni post-hoc test (A) or student's *t*-test. * $p<0.05$, *** $p<0.001$

Figure 2. Expression of miR-29 is increased by hyperglycemia and diabetes. Expression levels of miR-29a (A, n=3-7), miR-29c (B, n=3-7), miR-143 (C, n=6), and miR-145 (D, n=6) were evaluated in aortic smooth muscle cells cultured under and low glucose (LG), normal glucose (NG), high glucose (HG) and LG+mannitol (Man) conditions for 1 week. Expression levels of miR-29a (E, n=17), miR-29b (F, n=17), miR-29c (G, n=17) were analyzed in left internal mammary arteries from diabetic and non-diabetic patients. Data are presented as mean +/-S.E.M. Statistical significance was analyzed using one-way ANOVA with Bonferroni post-hoc test (A-D) or student's *t*-test (E-J). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 3. The transcription factor Foxa2 is induced by glucose and regulates glucose-induced miR-29c expression. Expression levels of Foxa2 mRNA were analyzed in smooth muscle cells cultured under low (LG) and high glucose (HG) conditions for 1 week (A, n=8-9). Foxa2 (B, n=5-6) and miR-29 (C, n=5-6) expression levels and evaluated in cells transfected with Foxa2 siRNA under HG or LG conditions (B, n=5-6). Data are presented as mean +/-S.E.M. Statistical significance was analyzed using student's *t*-test (A) or one-way ANOVA with Bonferroni post-hoc test (B-C). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 4. Expression of KLF4 is regulated by miR-29 in vascular smooth muscle cells. Expression levels of KLF4 mRNA (A, n=6) and protein (B, n=4) were analyzed in smooth muscle cells treated with miR-29c mimic for 8 days. Representative western blots are shown in C. Expression levels of KLF4 mRNA (D, n=6) and protein (E, n=4) were analyzed in smooth muscle cells treated with miR-29c inhibitor for 8 days. Representative western blots are shown in E. Luciferase activity was analyzed in smooth muscle cells co-transfected with a p-miR-KLF4-luc plasmid and miR-29c or miR-199 inhibitor, and cultured under LG or HG conditions (G, n=6). Full blots are shown in supplementary figure 7-8. Data are presented as mean +/-S.E.M. Statistical significance was analyzed using one-way ANOVA with Bonferroni post-hoc test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 5. Overexpression of KLF4 prevents glucose-dependent induction of smooth muscle marker expression. Cultured smooth muscle cells were transduced with Ad.KLF4 and then subjected to high (HG) or low (LG) glucose conditions for one week. The protein expression of KLF4 (A, n=4), Calponin (B, n=4) and SM22 (C, n=4) was analyzed by western blotting. Representative western blots are shown in E. Full blots are shown in supplementary figure 9. Data are presented as mean +/-S.E.M. Statistical significance was analyzed using one-way ANOVA with Bonferroni post-hoc test. ** $p<0.01$, *** $p<0.001$.

Table 1. Clinical parameters of left internal mammary artery donors

Clinical parameters were obtained for patients undergoing coronary artery bypass graft surgery (n = 6–10 patients).

	Non-diabetics	Diabetics	t-test
Age	70.27 ± 3.578	69.50 ± 1.821	p=0.8813
Blood glucose (mM)	6.545 ± 0.3566	9.050 ± 0.7442	p=0.0035
Systolic blood pressure (mmHg)	131.4 ± 7.436	145.8 ± 7.635	p=0.2230
Diastolic blood pressure (mmHg)	74.40 ± 3.833	82.50 ± 4.161	p=0.193

Figure 1

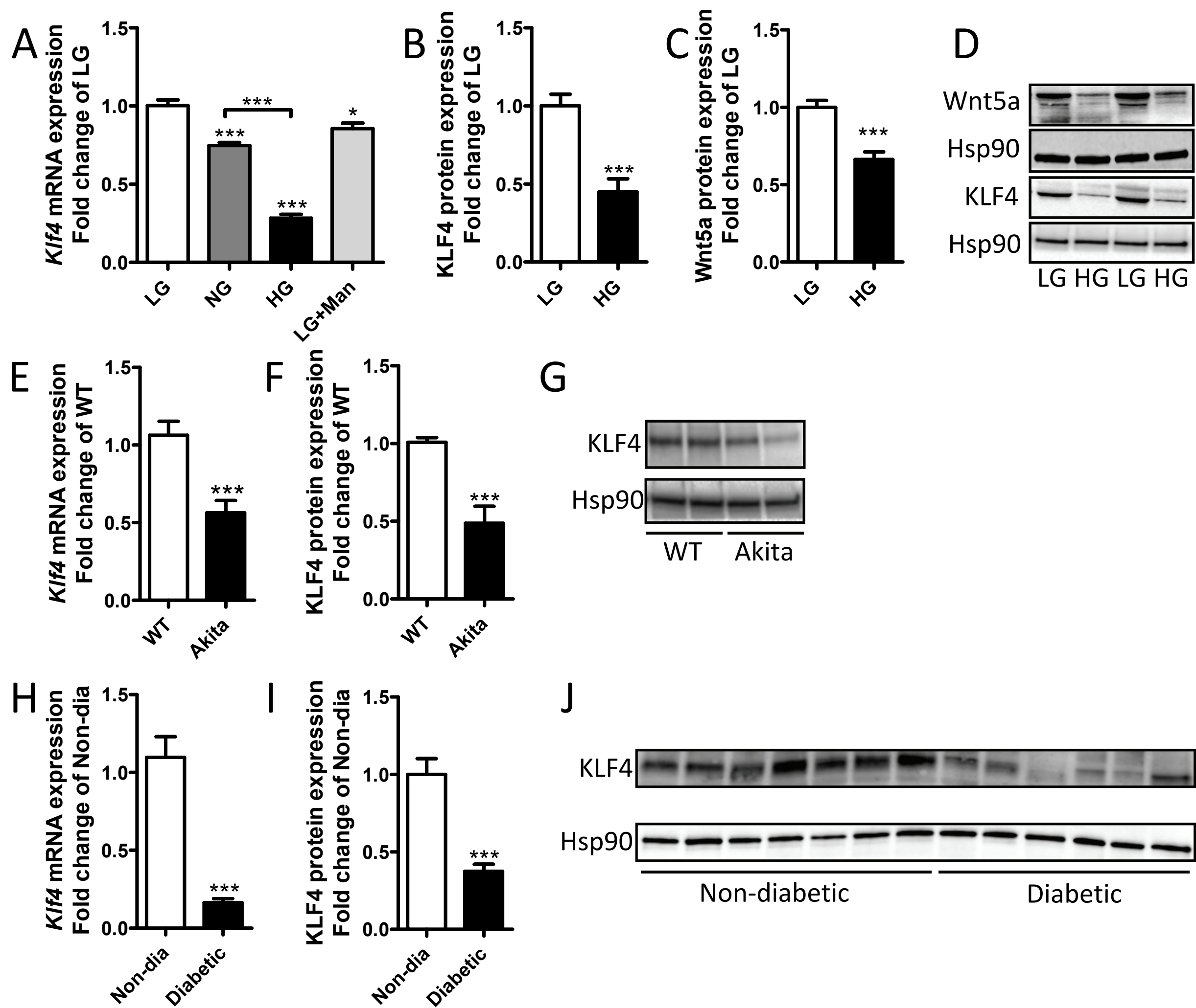


Figure 2

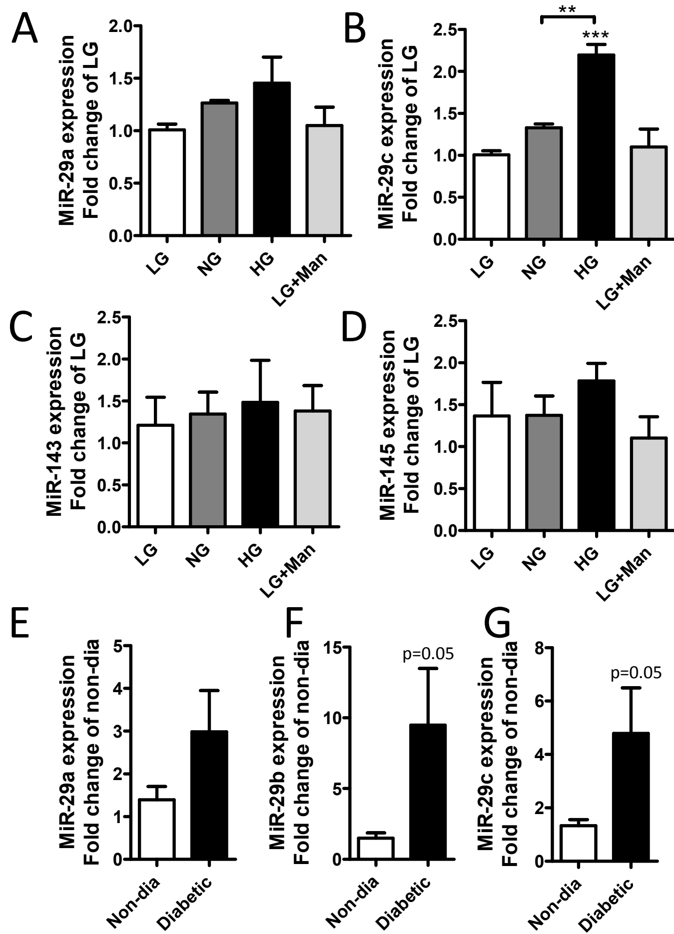


Figure 3

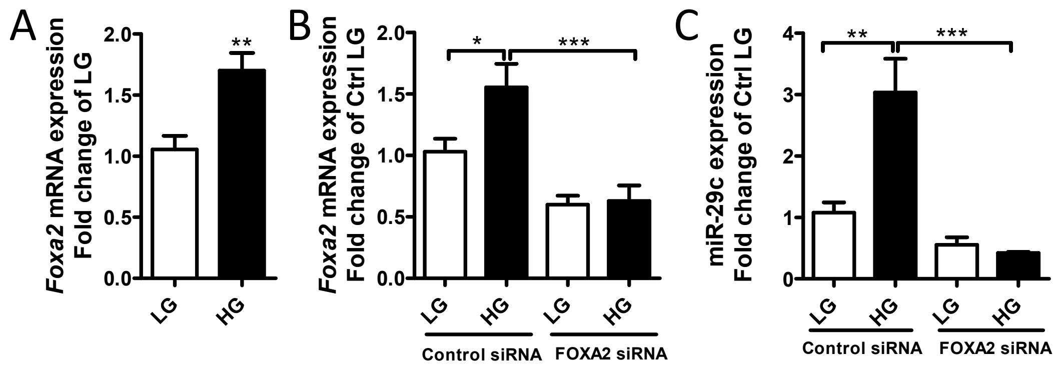
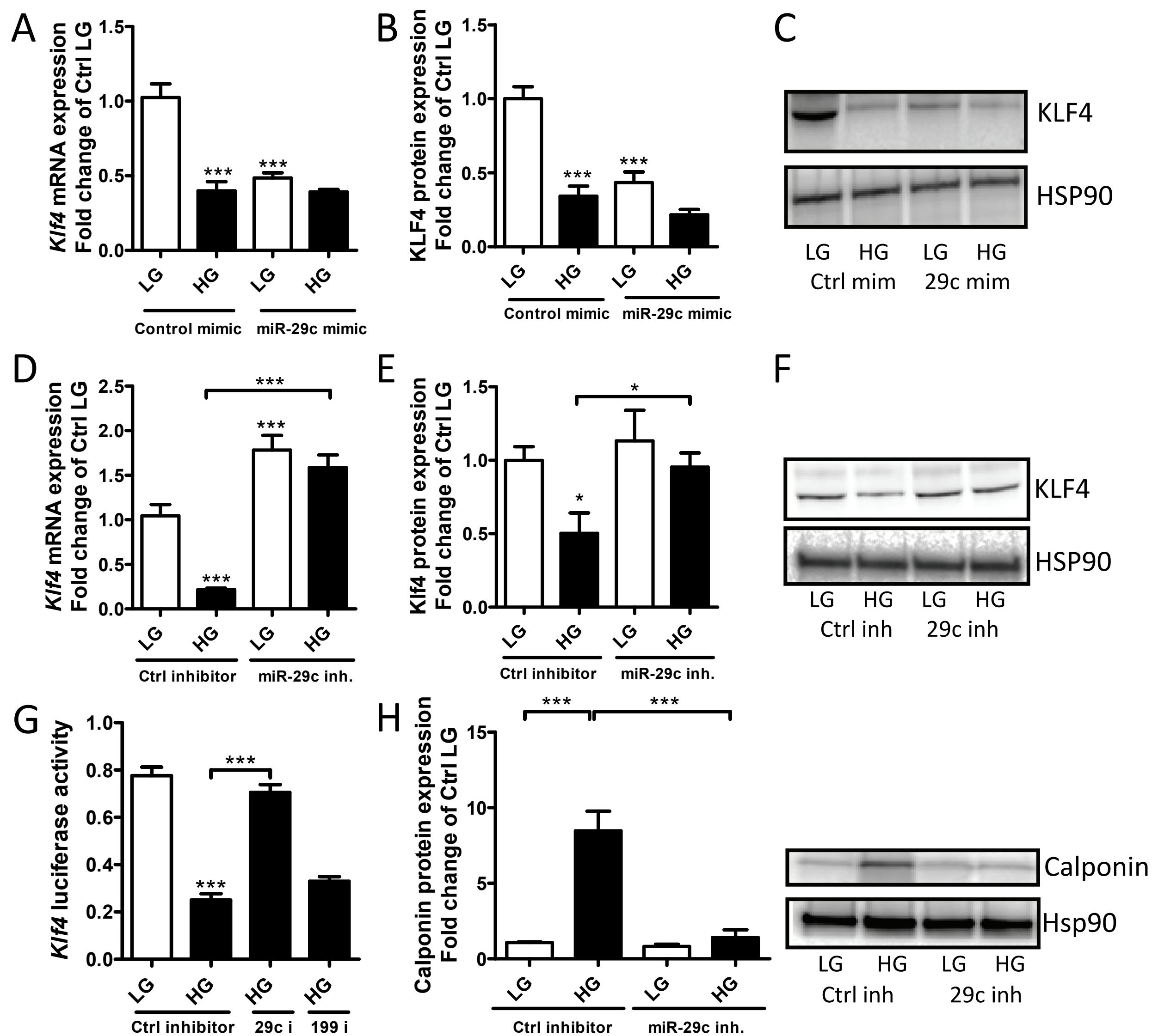


Figure 4



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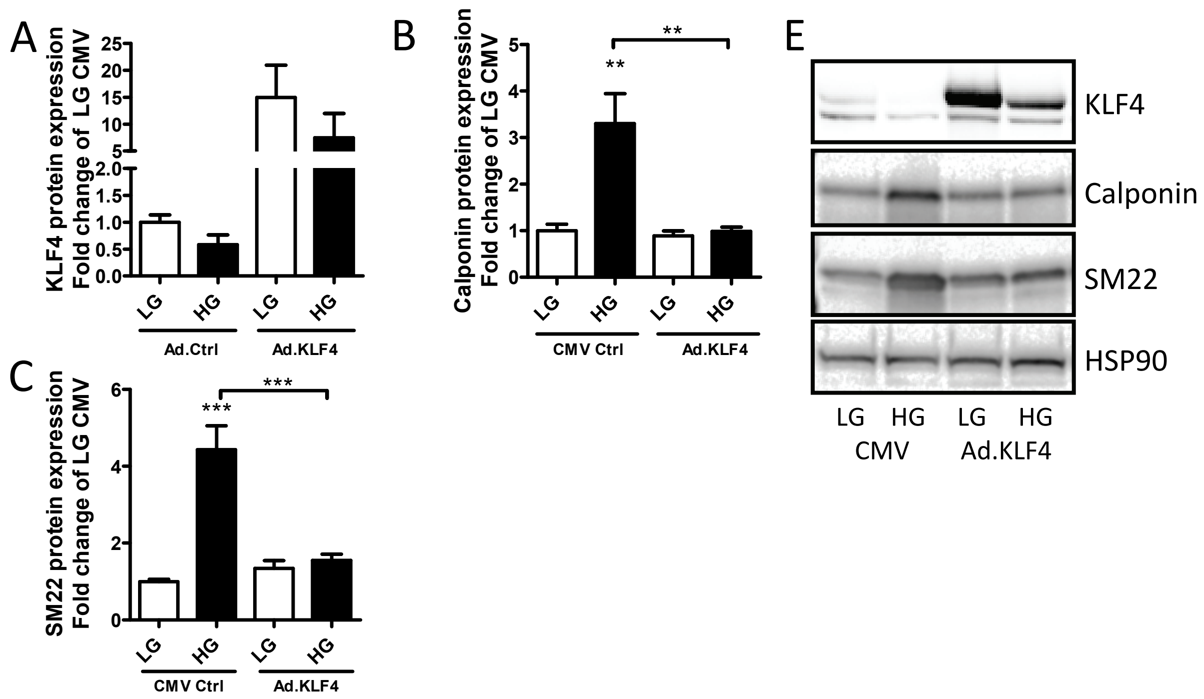
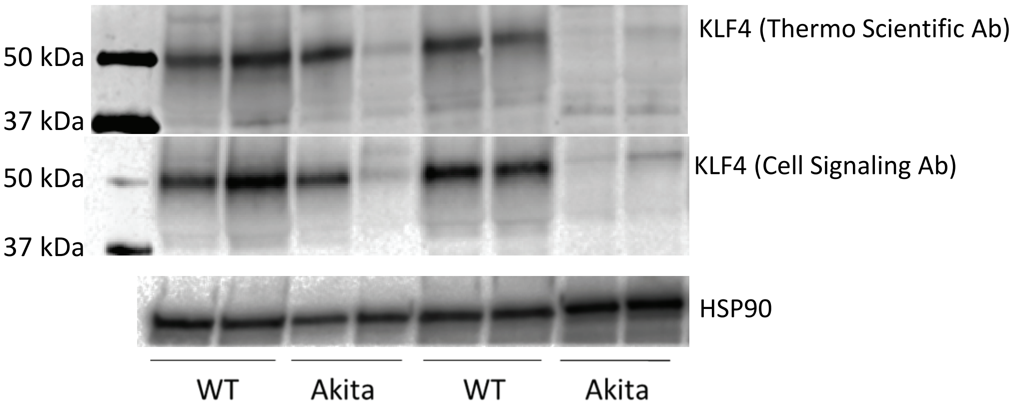


Supplementary figures for ”MicroRNA-dependent regulation of KLF4 by glucose in vascular smooth muscle”

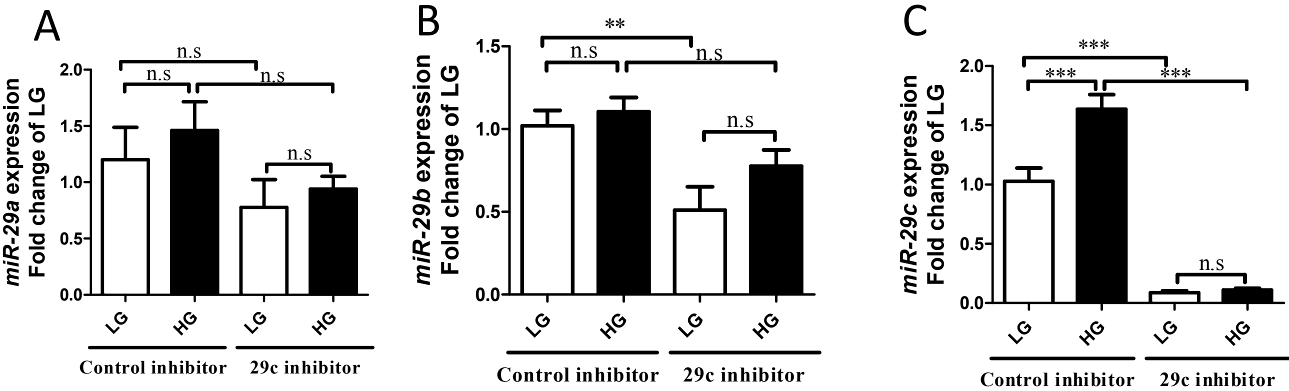
Tran Thi Hien, Eliana Garcia-Vaz, Karin G. Stenkula , Johan Sjögren ,Johan Nilsson, Maria F. Gomez and Sebastian Albinsson

Supplementary figure 1



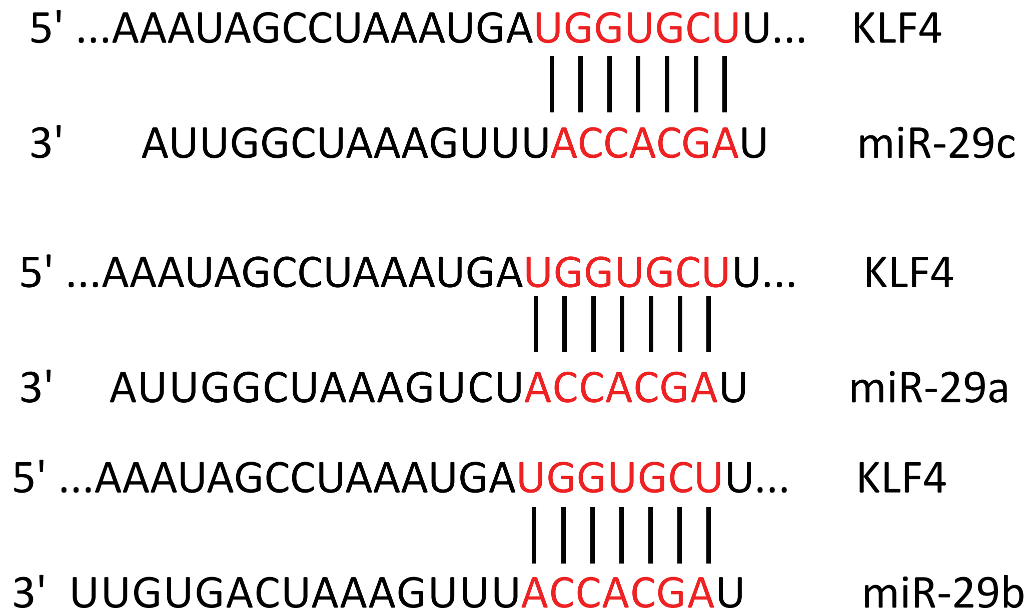
Supplementary figure 1. Comparison of KLF4 antibody from Cell Signaling (targeting the amino terminus of KLF4) and Thermo Scientific (targeting the carboxy terminus of KLF4). Small mesenteric arteries from hyperglycemic Akita mice and normoglycemic controls (WT) were evaluated by western blotting.

Supplementary figure 2



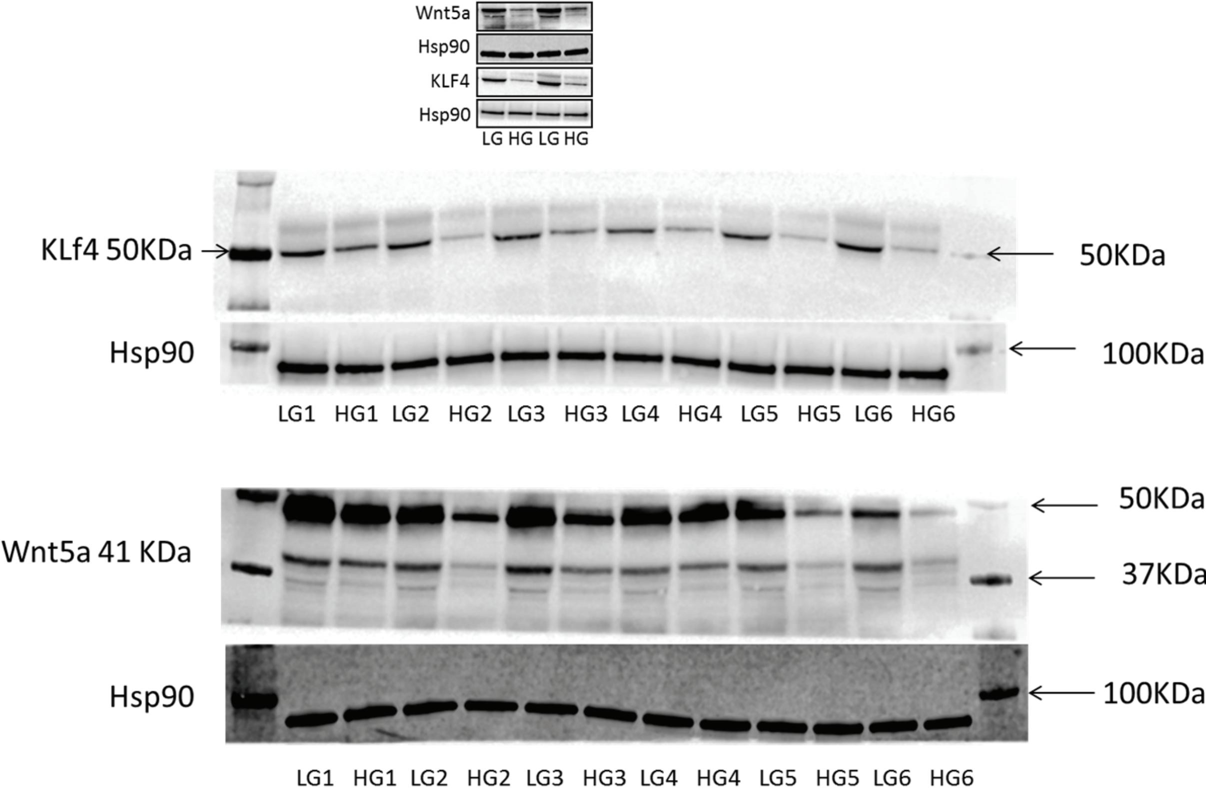
Supplementary figure 2. Effect of miR-29 inhibitor on miR-29 a/b/c expression. Expression of miR-29a (A), miR-29b (B) and miR-29c (C) was analyzed using qPCR in smooth muscle cells after transfection with miR-29c inhibitor.

Supplementary figure 3



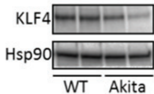
Supplementary figure 3. MicroRNA-29a/b/c binds to a consencus sequence in the KLF4 3'UTR. The interaction between the seed region of miR-29 and KLF4 3'UTR is shown in red.

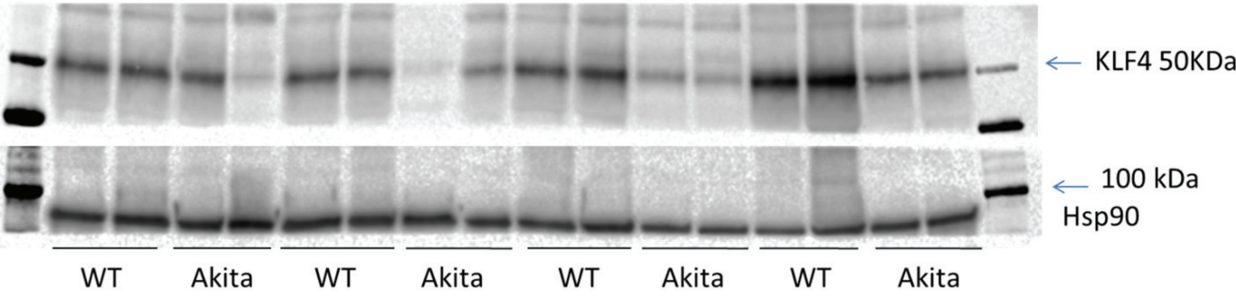
Supplementary figure 4

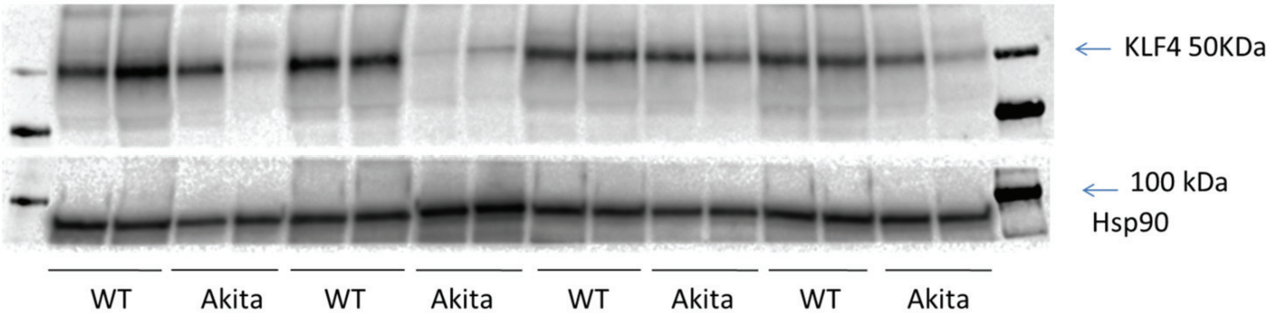


Supplementary figure 4. Original blots for Figure 1D

Supplementary figure 5

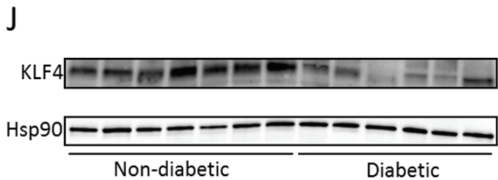


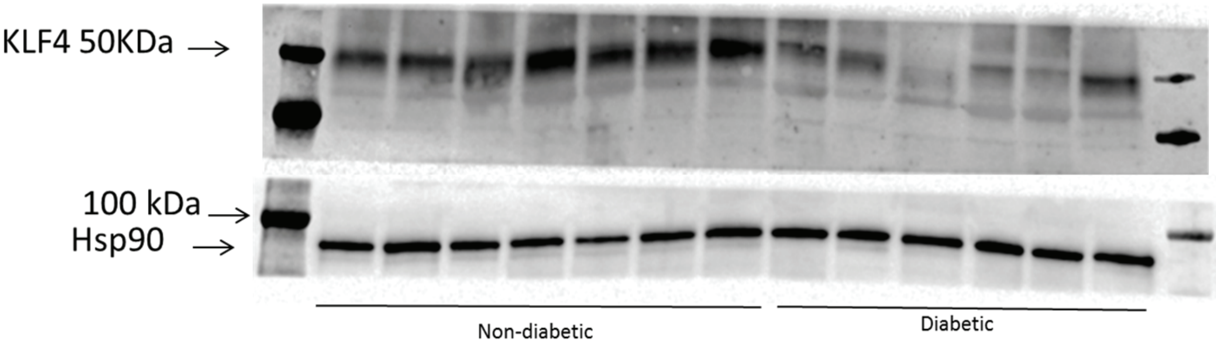




Supplementary figure 5. Original blots for Figure 1G

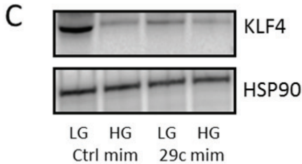
Supplementary figure 6

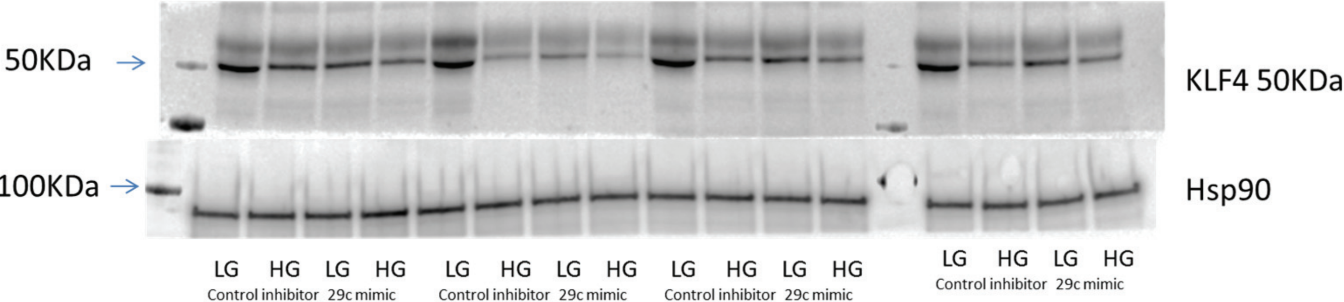




Supplementary figure 6. Original blots for Figure 1J

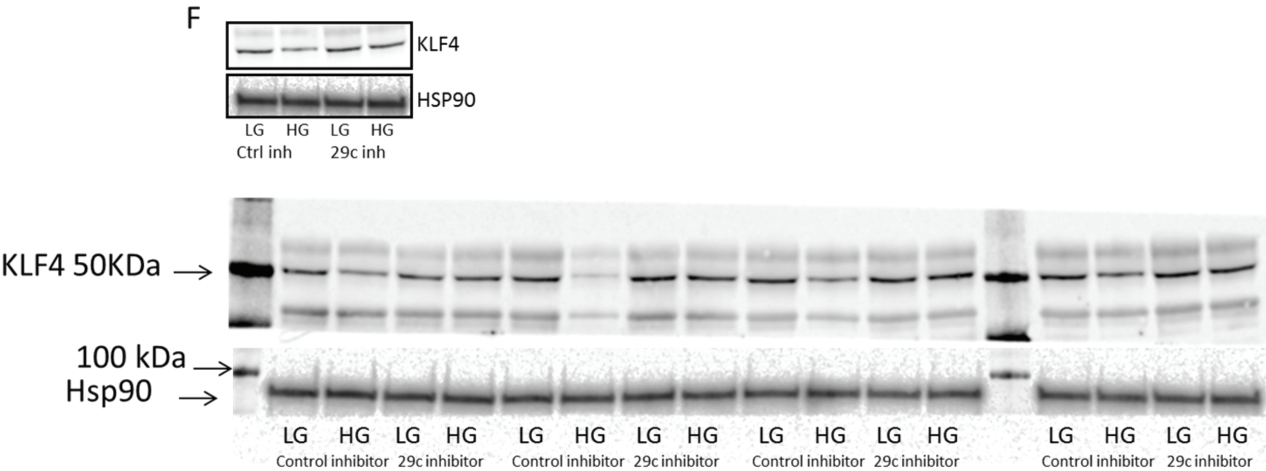
Supplementary figure 7





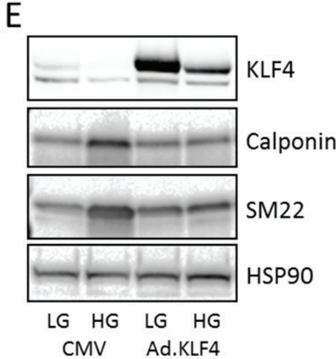
Supplementary figure 7. Original blots for Figure 4C.

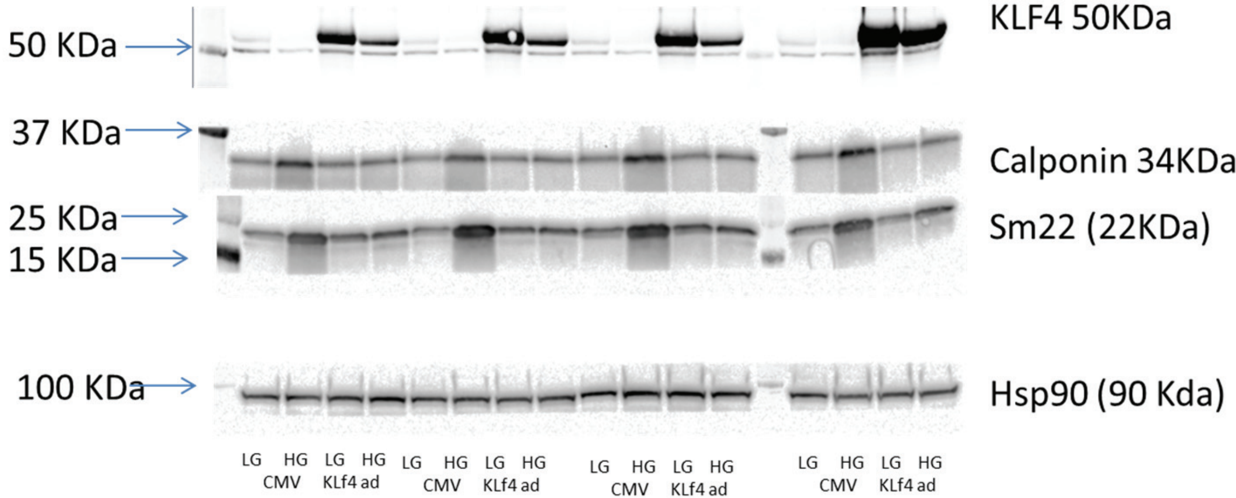
Supplementary figure 8



Supplementary figure 8. Original blots for Figure 4F

Supplementary figure 9





Supplementary figure 9. Original blots for Figure 5E