Activin A impairs insulin action in cardiomyocytes via up-regulation of miR-143

Marcel Blumensatt¹, Sabrina Greulich¹, Daniella Herzfeld de Wiza¹, Heidi Mueller¹, Bujar Maxhera², Martijn J. Rabelink³, Rob C. Hoeben³, Payam Akhyari², Hadi Al-Hasani¹, Johannes B. Ruige⁴, and D. Margriet Ouwens¹,⁴*

¹Institute for Clinical Biochemistry and Pathobiology, German Diabetes Center, Auf'm Hennekamp 65, D-40225 Duesseldorf, Germany; ²Department of Cardiovascular Surgery, Heinrich-Heine-University, Duesseldorf, Germany; ³Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands; and ⁴Department of Endocrinology, Ghent University Hospital, Ghent, Belgium

Received 26 December 2012; revised 18 June 2013; accepted 25 June 2013; online publish-ahead-of-print 28 June 2013

Time for primary review: 28 days

Aims
Enhanced activin A release from epicardial adipose tissue (EAT) has been linked to the development of cardiac dysfunction in type 2 diabetes (T2D). This study examined whether the inhibition of insulin action induced by epicardial adipokines in cardiomyocytes can be ascribed to alterations in miRNA expression.

Methods and results
Expression levels of miRNAs were assessed by real-time PCR in primary adult rat cardiomyocytes (ARC) exposed to conditioned media generated from EAT biopsies (CM-EAT) from patients with and without T2D. CM-EAT-T2D altered the expression of eight miRNAs in ARC vs. CM-EAT from patients without T2D. Of these, only expression of the miR-143/145 cluster was affected by activin A in the same direction as CM-EAT-T2D. Accordingly, activin A neutralizing antibodies prevented the induction of the miR-143/145 cluster by CM-EAT-T2D. Subsequently, the impact of the miR-143/145 cluster on insulin action was investigated. Transfection of HL-1 cells with precursor-miR-143 (pre-miR-143), but not pre-miR-145, blunted the insulin-mediated phosphorylation of Akt and its substrate proline-rich Akt substrate of 40 kDa (PRAS40), and reduced insulin-stimulated glucose uptake. Also lentivirus-mediated expression of pre-miR-143 in ARC reduced insulin-induced Akt phosphorylation. These effects were ascribed to down-regulation of the miR-143 target and regulator of insulin action, the oxysterol-binding protein-related protein 8 (ORP8) in both ARC and HL-1 cells. Finally, LNA-anti-miR-143 protected against the detrimental effects of CM-EAT-T2D on insulin action in ARC.

Conclusion
Activin A released from EAT-T2D inhibits insulin action via the induction of miR-143 in cardiomyocytes. This miRNA inhibits the Akt pathway through down-regulation of the novel regulator of insulin action, ORP8.

Keywords
Epicardial adipose tissue • Activin A • Diabetic cardiomyopathy • Insulin resistance • miRNA

1. Introduction
Diabetic cardiomyopathy is a common complication and a major cause of mortality in patients with type 2 diabetes (T2D).¹⁻² Myocardial insulin resistance is closely linked to the pathophysiology of diabetic cardiomyopathy.³⁻⁴ Alterations in adipokine secretion by epicardial adipose tissue (EAT) have been linked to the development of cardiac dysfunction in patients with T2D.⁵⁻⁶ We previously showed that conditioned media (CM) generated from EAT from high-fat diet fed guinea pigs as well as patients with T2D induce cardiomyocyte dysfunction as illustrated by reductions in contractile function and induction of insulin resistance.⁸⁻⁹ This detrimental effect induced by CM from EAT from patients with T2D was not observed when exposing cardiomyocytes to CM from other fat depots, such as subcutaneous and pericardial adipose tissue.⁸⁻⁹ Profiling experiments could ascribe part of the cardio-depressant activity present in EAT from patients with T2D to an enhanced secretion of activin A.⁸⁻⁹ However, the molecular mechanism via which the epicardial adipokines, including activin A, impact on myocardial insulin signalling is still incompletely understood.

Accumulating evidence links microRNAs (miRNA) to the development of insulin resistance and pathogenesis of cardiometabolic diseases.¹⁰⁻¹² These small non-coding RNA molecules regulate the
expression levels of target proteins through degradation of the mRNA encoding the protein, through repression of mRNA translation, or both. Among the proteins targeted by miRNAs are components of the insulin signalling system, such as insulin receptor substrate 1 (IRS-1), as well as regulators of activity of the Akt-pathway, such as phosphatase and tensin homologue (PTEN) and oxyester-binding protein-related protein 8 (ORP8). This study aimed at investigating whether alterations in miRNA expression participate in the induction of insulin resistance resulting from enhanced secretion of activin A from EAT from patients with T2D. Therefore, we profiled the alterations in miRNA expression in primary adult rat cardiomyocytes (ARC) that were exposed to CM from EAT biopsies from patients with and without T2D. Subsequently, we analysed whether the observed alterations in miRNA expression could be ascribed to activin A. Finally, we analysed the impact of differentially regulated miRNAs on insulin action in HL-1 cells and ARC expressing precursor forms or LNA-anti-miR for the deregulated miRNA species.

2. Methods

An extended version of the methods applied in this study is provided in the Supplementary material online.

2.1 Conditioned media from adipose tissue

Adipose tissue biopsies collected from the epicardial, pericardial, and subcutaneous adipose tissue depots were collected from patients undergoing open-heart surgery for coronary artery bypass grafting and/or valve replacement procedures after written informed consent. The procedure to obtain adipose tissue samples was approved by the Ethics Committee of the Heinrich-Heine-University (Duesseldorf, Germany). Biopsies were used to generate CM as described. Activin A content in the CM was determined using a Quantikine activin A immunoassay (R&D systems, Minneapolis, MN, USA).

2.2 Isolation and culture of primary adult rat cardiomyocytes

Animal experiments were performed in accordance with the ‘Principle of laboratory animal care’ (NIH publication No. 85-23, revised 1996) and the current version of the German Law on the protection of animals. Cardiomyocytes were isolated from Lewis rats (Lew/Crl) as described. Briefly, rats were killed following anaesthesia with ketamine (100 mg/kg; Ratiopharm, Ulm, Germany) and xylazine (Rompun, 5 mg/kg) (Bayer Healthcare, Leverkusen, Germany). The isolated heart was retrograde perfused using a buffer containing collagenase (Worthington, Lakewood, NJ, USA) and hyaluronidase (Applichem, Darmstadt, Germany). Isolated cardiomyocytes were seeded and cultured on laminin-coated six-well plates before exposure to the CM or control adipocyte medium (AM). When indicated, the CM were incubated for 30 min with 2.5 M of the p38 inhibitor SB203580 (Promega, Mannheim, Germany) before the addition of the CM.

2.3 Whole genome miRNA profiling in rat cardiomyocytes

To assess the effects on miRNA expression, ARC were harvested 16 h after the addition of the CM (1:4 diluted with AM). Data were collected during seven independent experiments for CM from EAT and four independent experiments for CM from the other fat depots. Total RNA was isolated from the cardiomyocytes using the miRNeasy mini kit (Qiagen, Hilden, Germany), reverse transcribed using the miScript I RT kit (Qiagen), wherein miRNA expression levels were determined with the rat miScript Assay 384 set based on miRBase V13.0 (Qiagen) and miScript SYBR Green (Qiagen) on a StepOne Plus real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). Taqman assays were used to validate miR-143 and miR-145 expression levels as described.

2.4 Culture and transfection of HL-1 cells

The cardiac mouse cell line HL-1 was kindly provided by professor Dr. W.C. Claycomb (Louisiana State University, New Orleans, LA, USA). Cells were transfected in six-well dishes with 30 mmol/L Cy3 labelled pre-miR (negative control) or pre-miR precursor (Ambion, Life Technologies, Darmstadt, Germany) at 70–80% confluence using Lipofectamine 2000 (Invitrogen, Life Technologies, Darmstadt, Germany). To silence ORP8, cells were transfected with five validated MISSION® shRNA constructs (TRCN0000105245–249) targeting mouse ORP8 (NM175489) (Sigma Aldrich) or empty vector. Comparable data were obtained for all five distinct ORP8 shRNAs evaluated. One day after transfection, cells were serum-starved for 16 h on Dulbecco’s modified Eagle medium containing 5 mM glucose (Invitrogen) and supplemented with 2 mmol/L l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Thereafter, the cells were stimulated with 200 nM insulin for 30 min or kept untreated, and harvested.

2.5 Lentiviral transduction of primary adult rat cardiomyocytes

Isolated ARC were transduced at an MOI of 5 with lentiviruses expressing pre-miR-143 or empty vector. Two days after transduction, cells were serum-starved and kept untreated or incubated for 10 min with 100 nM insulin, and harvested.

2.6 LNA-anti-miR silencing of primary adult rat cardiomyocytes

Isolated ARC were transfected with 60 nM miCURY LNA inhibitor for miR-143 or miCURY LNA inhibitor control (Exiqon, Vedbæk, Denmark) for 6 h using lipofectamine. Then, cells were exposed to CM or AM overnight. Thereafter, the cells were stimulated with 100 nM insulin for 10 min or kept untreated, and harvested.

2.7 Analysis of protein expression and insulin signalling

Cells were lysed for 2 h at 4°C in 50 mmol/L HEPES (pH 7.4), 1% Triton X-100, supplemented with protease and phosphatase inhibitor cocktails (Complete, PhosStop; Roche Diagnostics, Mannheim, Germany) under gentle rotation. Protein content was determined using Bradford reagent (Biorad Laboratories, Munich, Germany). Effects on protein expression and insulin action were analysed via western blotting as described.

2.8 Glucose uptake in HL-1 cardiomyocytes

Glucose uptake was measured in serum-starved HL-1 cells, either kept untreated or exposed for 30 min to 200 nM insulin. Then 0.12 mM deoxy-D-glucose with 0.055 μCi 2-deoxy-O-[14C]glucose was added to the cells. After a 10 min incubation, incorporated glucose was measured by scintillation counting of the cell lysates.

2.9 Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Significant differences between experimental conditions were evaluated as described in the legends to Table 1 and Figures 1–6 using Graphpad Prism5 (GraphPad, LA Jolla, CA, USA) software. P-values of <0.05 were considered as statistically significant.
3. Results

3.1 Patient characteristics

Adipose tissue biopsies to generate CM were collected from patients with and without T2D. Pre-operative blood glucose levels were higher in T2D patients vs. ND patients. Age and BMI were similar between patients groups (Table S1). Type of surgery and medication use is listed in Supplementary material online, Table S1.

3.2 Effect of epicardial adipokines on miRNA expression in cardiomyocytes

In the absence of CM, 191 out of the 343 miRNAs profiled were detected in primary ARC, including the muscle-specific miR-1, miR-133, and the cardiac-specific miR-208 (Supplementary material online, Figure S1 and Table S2).20 Supplementary material online, Table S2 lists the 152 miRNA species that were not detected in primary ARC. Of the expressed miRNAs, miR-133 was the most abundant in ARC (Supplementary material online, Table S2). Furthermore, the expression of eight miRNA species was changed in ARC exposed to CM from EAT-T2D (CM-EAT-T2D) vs. CM-EAT-ND (Figure 1). Specifically, CM-EAT-T2D reduced the expression of miR-26a, miR-191, miR-218, and miR-425 (Figure 1A–D). The levels of the miRNA cluster miR-143/145, miR-208, and of let-7c were increased in ARC exposed to CM-EAT-T2D vs. CM-EAT-ND (Figure 1E–H). Exposing ARC to CM generated from subcutaneous or pericardial adipose tissue from patients with or without T2D did not alter the expression levels of these miRNAs (Figure 1).

3.3 Effect of activin A on miRNA expression in cardiomyocytes

Previously, we could ascribe an inhibition of insulin action in ARC exposed to CM-EAT-T2D to activin A.9 The amount of activin A in CM was 1.7 ± 0.4 ng/mL in CM-EAT-T2D (n = 14) vs. 0.55 ± 0.15 ng/mL in CM-EAT-ND (n = 10) (P < 0.02) (Supplementary material online, Figure S2). Using Taqman-based assays, we showed that the expression of the miR-143/145 cluster in ARC was dose-dependently increased by recombinant activin A, and that this effect became significant at 1 ng/mL (Figure 2A/B). Furthermore, the response between 1 and 10 ng/mL activin A was not significantly different. Expression levels of the other miRNAs tested were either not affected by activin A, or in the case of miR-191 alterations were observed in the opposite direction (Supplementary material online, Figure S3). To substantiate the involvement of activin A in the induction of the miR-143/145 cluster,

neutralizing activin A antibodies were used. As shown in Figure 2C/D, pre-incubation of activin A or CM-EAT-T2D with activin A neutralizing antibodies prevented the induction of miR-143 and miR-145.

3.4 The induction of the miR-143/145 cluster is mediated by the MAP kinase p38

Activin A is a member of the transforming growth factor β family. Because the induction of the miR-143/145 cluster in response to transforming growth factor β involves the MAP kinase family member p38 in smooth muscle cells, we examined whether p38 participates in the induction of the miR-143/145 cluster by CM-EAT-T2D in ARC. Exposure of ARC to CM-EAT-T2D increased the phosphorylation of p38 when compared with CM-EAT-ND or control medium (Figure 3A). The induction of p38 phosphorylation was significantly blunted when CM-EAT-T2D was incubated with neutralizing antibodies against activin A (Figure 3B). Accordingly, activin A caused a dose-dependent increase in p38 phosphorylation in ARC (Figure 3C). To investigate the involvement of p38 in the induction of the miR-143/145 cluster in ARC, the pharmacological p38-inhibitor SB203580 was used. As shown in Figure 3D/E, the presence of SB203580 abolished the induction of the miR-143/145 cluster by both activin A and CM-EAT-T2D.

Table I: Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>ND</th>
<th>T2D</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>Gender</td>
<td>8 males, 2 females</td>
<td>13 males, 1 female</td>
</tr>
<tr>
<td>Age</td>
<td>65 ± 3.7</td>
<td>73 ± 2.5</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>29.1 ± 1.1</td>
<td>31.3 ± 1.8</td>
</tr>
<tr>
<td>Glucose before operation, mg/dL</td>
<td>110 ± 3.8</td>
<td>150 ± 11</td>
</tr>
</tbody>
</table>

Anthropometric characteristics are expressed as mean ± standard deviation, and differences between the disease groups were evaluated using a Mann–Whitney U-test. ND, non-diabetic; T2D, type 2 diabetes; BMI, body mass index; NS, not significant.

3.5 Effect of miR-143 and miR-145 on insulin action

The mouse cardiac muscle cell line HL-1 was used to assess the impact of the miR-143/145 cluster on insulin action because of better viability following pre-miR expression when compared with primary ARC. This is particularly relevant for the quality of the glucose transport experiments, and HL-1 cells are insulin-sensitive and display the insulin-regulated glucose transporter GLUT4.22 Transfecting HL-1 cells with pre-miR-143 reduced the insulin-mediated phosphorylations of Akt-Thr308, Akt-Ser473, and of the Akt-substrate PRAS40-Thr246 by 35, 25, and 20%, respectively, vs. cells transfected with pre-miR-145 or control pre-miRNA (Figure 4A–C). Insulin-mediated glucose uptake was blunted by 25% in cells expressing pre-miR-143 vs. control miRNA (Figure 4D). Importantly, lentivirus-mediated expression of pre-miR-143 in ARC also led to the inhibition of insulin-mediated Akt-Ser473 phosphorylation (Figure 4F).

The inhibition of insulin action in cells expressing pre-miR-143 could not be ascribed to alterations in the protein expression of key signalling molecules, such as the insulin receptor, IRS-1, PTEN, Akt, PRAS40, or GLUT4 (Figure 4E, Supplementary material online, Figure S4). In mice, down-regulation of the miR-143 target ORP8 associates with hepatic insulin resistance.14 Expression of pre-miR-143 reduced ORP8 protein levels in HL-1 cells and primary ARC by 20 and 25%, respectively (Figure 5A/B). In contrast, control pre-miR or pre-miR-145 did not affect ORP8 abundance in HL-1 cells (Figure 5A). Also exposing ARC to CM-EAT-T2D lowered ORP8 abundance when compared with cells exposed to CM-EAT-ND or control medium (Figure 5C). Finally, lowering ORP8 protein levels in HL-1 cells using distinct ORP8 shRNA constructs was paralleled by reductions in insulin-stimulated Akt phosphorylation and glucose uptake vs. cells transfected with a control shRNA (Figure 5D–G).

To substantiate these observations, the expression of miR-143 was silenced using LNA-anti-miR-143, which is efficiently taken up by primary ARC. In the absence of CM, LNA-anti-miR-143 increased ORP8 protein levels and enhanced insulin-mediated Akt phosphorylation without affecting insulin receptor, PTEN, Akt, and GLUT4 protein levels.
Furthermore, LNA-anti-miR-143 protected against the detrimental effects of CM-EAT-T2D on insulin-mediated Akt phosphorylation and ORP8 protein levels in ARC (Figure 6A–C).

4. Discussion

The present study shows that CM-EAT-T2D alters the expression levels of eight miRNAs in ARC. We previously reported that CM-EAT-T2D or CM-EAT from high-fat diet fed guinea pigs induces ARC dysfunction as illustrated by reductions in contractile function and insulin action.8,9 These effects could be largely ascribed to an increased abundance of activin A in the CM.8,9 Of the miRNAs affected by CM-EAT-T2D, only the levels of miR-143 and miR-145 were affected by activin A in the same direction as CM-EAT-T2D. In addition, the induction of miR-143 and miR-145, which are processed from the same transcript under the control of the same promoter,23,24 was dependent on p38. Moreover, the observed up-regulation of miR-143 in response to CM-EAT-T2D

(Supplementary material online, Figure S5). Furthermore, LNA-anti-miR-143 protected against the detrimental effects of CM-EAT-T2D on insulin-mediated Akt phosphorylation and ORP8 protein levels in ARC (Figure 6A–C).
or activin A resulted in inhibition of insulin action in cardiomyocytes through the down-regulation of a recently identified miR-143 target and mediator of insulin action, ORP8. Finally, the knockdown of miR-143 protected against the inhibition of insulin action and down-regulation of ORP8 in ARC exposed to CM-EAT-T2D. A key finding of the present study is the unravelling of a novel signalling pathway via which alterations in the secretory profile of EAT may result in insulin resistance in the heart of patients with T2D. In patients with T2D, aberrant myocardial energy substrate metabolism coexists with alterations in cardiac structure and function, even in the absence of coronary artery disease or hypertension. Whereas the healthy heart capable of switching between these substrates according to the most favourable energetic yield needed for the prevailing cardiac condition, the heart of patients with T2D displays an impaired metabolic flexibility and myocardial energy substrate metabolism is shifted toward enhanced fatty acid utilization at the expense of glucose metabolism. Studies in humans and rodent models indicate that this metabolic inflexibility is closely associated with myocardial insulin resistance. In particular, the insulin-stimulated phosphorylation of Akt, which mediates cardiac glucose uptake by promoting the translocation of the glucose transporter GLUT4 to the sarcolemma, is impaired. Importantly, the factors responsible for deregulation of this signalling pathway are largely unclear. This study reveals that an elevated secretion of activin A by the EAT contributes to the inhibition of the Akt/GLUT4 pathway through up-regulation of miR-143.

Although we did not assess diabetic rat models for alterations in cardiac miR-143 expression, other studies confirmed a function for miR-143 in obesity and insulin resistance. Feeding mice an obesity-inducing high-fat diet increased the expression of miR-143 in adipose tissue, whereas another study reported up-regulation of both miR-143 and miR-145 in the liver. Furthermore, elevated miR-143 levels have been reported in the liver, heart, skeletal muscle, and pancreas from db/db mice and ob/ob mice when compared with tissues isolated from control animals. In these studies, the factors responsible for the up-regulation of miR-143 remained unidentified. Here, we identify activin A as regulator of miR-143 expression. We could show that the induction of this miRNA cluster by CM-EAT-T2D is abolished upon pretreating the CM with neutralizing activin A antibodies. Furthermore, exposing ARC to the amount of activin A present in CM-EAT-T2D increased the expression of miR-143. Finally, in line with a study examining the regulation of miR-143 expression by transforming growth factor β1 in smooth muscle cells, we observed that the induction of this cluster by activin A and CM-EAT-T2D in cardiomyocytes could be abolished by inhibition of p38.

Our data further link the induction miR-143 to inhibition of insulin action. As in other target tissues for insulin action, insulin signalling in cardiomyocytes is initiated by tyrosine phosphorylation of IRS-1 by the activated insulin receptor. This facilitates the binding and activation of phosphatidylinositol-3′-kinase, thus catalysing the formation of phosphatidylinositol 3,4,5-trisphosphate and providing a platform for the activation of Akt. Therefore, the down-regulation of miR-143 by activin A resulted in increased insulin action in cardiomyocytes through the up-regulation of ORP8.
binding and activation of Akt and subsequent translocation of GLUT4.\textsuperscript{5} The phosphatase PTEN counteracts this signalling pathway by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate to produce phosphatidylinositol 4,5-bisphosphate, and thereby inhibiting the activation of Akt. However, in this study, the observed inhibition of insulin action in cells expressing pre-miR-143 could not be ascribed to alterations in the expression of key components of insulin action, such as the insulin receptor, IRS-1, PTEN, Akt, and GLUT4. This is in accordance with a report on mice with an inducible expression of miR-143 in the liver.\textsuperscript{14} Conversely, the absence of the miR-143/145 cluster was found to protect against high-fat diet induced insulin resistance and hepatic Akt inhibition.\textsuperscript{14} Using proteomics-based approaches, these authors

Figure 3 Regulation of the miR-143/145 expression by the MAP kinase p38. Phosphorylation of p38 in ARC exposed to CM-EAT from patients with (T2D) or without (ND) type 2 diabetes (A), to CM-EAT-T2D in the presence and absence of activin A neutralizing antibodies (B), or to increasing amounts of activin A (C) for 30 min. Experiments are performed using cardiomyocytes from different rats and CM from different donors. Shown are representative blots and quantifications for which the phosphorylation data were normalized for insulin receptor β (IR-β) levels. Data are expressed as mean ± SEM (A: n = 6, B/C: n = 4). (D/E) Effect of inhibition of p38 using SB203580 on the induction of miR-143 (D) and miR-145 (E) in response to a 16 h exposure of ARC to 10 ng/mL activin A or CM-EAT-T2D. All data were collected in four independent experiments using cardiomyocytes from different rats and CM from different donors. # indicates \(P < 0.001\) vs. control AM (control) as determined using ANOVA followed by post hoc Bonferroni analysis; * \(P < 0.05\) for the effect of the neutralizing activin A antibody as determined by a Student’s t-test.
Figure 4 Effect of miR-143 and miR-145 on insulin action. HL-1 cells were transfected with control precursor miRNA or precursors for miR-143 or miR-145. Shown are representative blots and quantifications for the phosphorylation of Akt on Thr308 (A) and Ser473 (B), and of PRAS40 (C) on Thr246 in serum-starved cells (-) or following insulin stimulation (30 min; 200 nM) (+). (D) Rate of glucose uptake in HL-1 cells transfected with control precursor miRNA (control) or a pre-miR-143 in serum-starved cells (-), or following insulin stimulation (30 min; 200 nM) (+). Data were collected in six independent experiments each performed in triplicate and are expressed as mean ± SEM. (E) Effect of miR-143 and miR-145 vs. control pre-miR on the protein levels of the IRβ-subunit, IRS-1, PTEN, and GLUT4. The blot for GAPDH served as a housekeeper control for protein expression. (F) Representative blot and quantification of the effect of miR-143 expression on insulin-induced Akt-Ser473 phosphorylation in primary adult cardiomyocytes kept untreated (-) or following insulin stimulation (10 min; 100 nM) (+). All phosphorylation data were normalized for GAPDH levels, and expressed as mean ± SEM (A/B: n = 8; C: n = 5; F: n = 6). Differences among the experimental conditions were evaluated using ANOVA followed by post hoc Bonferroni analysis. ***P < 0.001, **P < 0.01, and *P < 0.05.
Figure 5  Inhibition of insulin action by CM-EAT-T2D and miR-143 involves down-regulation of ORP8. Representative blots and quantification of ORP8 abundance in HL-1 cells (A) transfected with control precursor miRNA or precursors for miR-143 or miR-145, primary ARC (B) transduced with empty vector or vector expressing miR-143 (B), or ARC exposed to CM-EAT from patients with (T2D) or without (ND) type 2 diabetes (C). ORP8 protein levels were normalized for GAPDH expression, and expressed as mean ± SEM (A: n = 8; B: n = 6; C: n = 4). Differences among the experimental conditions were evaluated using ANOVA followed by post hoc Bonferroni analysis. ***P < 0.001; *P < 0.05. (D–F) Effect of reduced expression of ORP8 on insulin action in HL-1 cells. HL-1 cells transfected with shRNAs for ORP8 were analysed for ORP8 expression (D), induction of Akt-phosphorylation (E/F), and glucose uptake (G) in untreated cells (−), and after insulin stimulation (30 min; 200 nM) (+). Signals were normalized for GAPDH (D) and Akt (E/F), respectively. Similar data were obtained with five distinct shRNA constructs. Data are expressed as mean ± SEM collected in 10 independent experiments conducted with distinct shRNA constructs. *P < 0.05 as determined using a Student’s t-test.
further identified ORP8 as the miR-143 target responsible for the abrogation of insulin action in the liver.\textsuperscript{14} We extend these observations to cardiomyocytes by showing that incubation of ARC with CM-EAT-T2D as well as expression of pre-miR-143 in ARC and HL-1 cells results in a down-regulation of ORP8 expression, and that the absence of miR-143 protects against the induction of insulin resistance by CM-EAT-T2D in ARC. Furthermore, down-regulation of ORP8 expression was associated with inhibition of insulin-mediated Akt signaling. However, the molecular mechanism via which ORP8 regulates Akt phosphorylation remains to be elucidated.

In contrast to miR-143, expression of pre-miR-145 had no effect on insulin action in HL-1 cells. This is in contrast to studies on colon cancer, where IRS-1 was identified as miR-145 regulated target, but in agreement with observations on mice with an inducible expression of miR-145 in the liver.\textsuperscript{14,29} Importantly, up-regulation of miR-145 has been reported in human samples from subjects with dilated cardiomyopathy and end-stage heart failure, thus highlighting the need for characterization of target genes regulated by miR-145.\textsuperscript{30,31}

Another question that remains to be addressed is the contribution of the activin A-p38-miR-143/145-ORP8 pathway in the pathophysiology of diabetic cardiomyopathy. A limitation of this study is that we could not analyse cardiac ventricular biopsies from patients with diabetic cardiomyopathy for alterations in the expression of the miR-143/145 cluster or ORP8. Furthermore, although we obtained comparable results for activin release from EAT from high-fat diet fed guinea pigs, confounding effects of medication use on adipokine release in human EAT cannot be fully excluded. However, it should be noted that in a separate clinical study, activin A levels were found to be inversely associated with the myocardial metabolic rate of glucose uptake as measured using $[^{18}F]$-fluoro-2-deoxy-D-glucose during an euglycaemic–hyperinsulinaemic clamp in men with uncomplicated T2D.\textsuperscript{32} Furthermore, activin A released from EAT has been linked to the development of atrial

**Figure 6** Silencing miR-143 reverses the abrogation of insulin action by CM-EAT-T2D in cardiomyocytes. Primary adult rat cardiomyocytes were transfected with LNA-anti-miR-143 (+) or LNA inhibitor control (−), and exposed to CM-EAT-T2D or control AM. Shown are representative blots and quantification for induction of Akt-Thr308 (A) and Akt-Ser473 (B) phosphorylation following insulin stimulation (10 min; 100 nM) and ORP8 expression (C). Signals were normalized for Akt (A/B) or IR-β (C) protein levels and are expressed as mean ± SEM from four independent experiments conducted on cardiomyocyte preparations from different rats. Data were analysed by ANOVA and Bonferroni multiple comparison analysis. *P < 0.05 for the effect of CM-EAT-T2D vs. control AM; #P < 0.05 for the difference between LNA-anti-miR-143 vs. LNA-inhibitor control.
fibrosis. Collectively, these data support an involvement of activin A in the pathophysiology of diabetic cardiomyopathy.

In conclusion, this study identified a mechanism via which an enhanced secretion of activin A by EAT-T2D induces myocardial insulin resistance. Specifically, the p38-dependent induction of mir-143 inhibits the activation of the Akt pathway regulating glucose uptake by insulin through down-regulation of a novel regulator of insulin action, ORP8. The elucidation of this signalling pathway may contribute to the recognition of more specific therapeutic targets for the prevention and treatment of diabetic cardiomyopathy.

Supplementary Material
Supplementary Material is available at Cardiovascular Research online.

Acknowledgements
We thank Dr Robert Schwenk (DIFE, Potsdam, Germany), and Prof. Dr Juergen Eckel (both German Diabetes Center, Duesseldorf) for helpful and critical discussions on this study.

Conflict of interest: none declared.

Funding
This work was supported by the Federal Ministry of Health, the Ministry of Innovation, Science, Research and Technology of the German State of North-Rhine Westphalia and the German Centre for Diabetes Research (Deutsches Zentrum für Diabetesforschung, DZD).

References