Gestational diabetes induces alterations of sirtuins in fetal endothelial cells

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BACKGROUND: Gestational diabetes (GDM) has long-term consequences for the offspring. Sirtuins (SIRTs) are associated with vascular and metabolic functions. We studied the impact of GDM on SIRT activity and expression in fetal endothelial colony-forming cells (ECFCs) and human umbilical vein endothelial cells (HUVECs) from pregnancies complicated by GDM.

METHODS: ECFCs and HUVECs were isolated from cord and cord blood of 10 uncomplicated pregnancies (NPs) and 10 GDM pregnancies. Nicotinamidadenindinukleotid (NAD$^+$) concentration, SIRT1 and SIRT3 activity, transcription levels of SIRT1, SIRT3, and SIRT4, and protein levels of SIRT1, SIRT3, and SIRT4 were determined *in vitro* with or without SIRT activators resveratrol (RSV) and paeonol.

RESULTS: Fetal ECFCs from GDM pregnancies showed a decreased NAD$^+$ concentration, reduced SIRT1 and SIRT3 activity, and lower transcription levels of SIRT1, SIRT3, and SIRT4. HUVECs from GDM pregnancies had decreased NAD$^+$ concentrations and transcription levels of SIRT1 and SIRT4. RSV markedly enhanced the expression and activity of SIRTs in ECFCs and HUVECs, while paeonol was active only in ECFCs.

CONCLUSION: A reduction of SIRT activity and expression in fetal endothelial cells provides potential mechanistic insights into the pathophysiology of long-term cardiovascular complications observed in the offspring of GDM pregnancies. SIRT activators can increase SIRT activity in ECFCs, which opens perspectives for new therapeutic targets.

Adverse events during fetal life have been implicated to induce fetal programming, which can result in chronic diseases (1). Especially gestational diabetes (GDM) of the mother has been associated with cardiovascular disease, metabolic syndrome, and obesity during later life in the offspring (2,3). The pathophysiological processes responsible for these late complications are largely unknown.

Sirtuins (SIRTs) are important regulators of aging and metabolic diseases (4,5). Mammalian SIRTs belong to the histone deacetylase class III family comprising seven members (SIRT1-7), which require Nicotinamidadenindinukleotid (NAD$^+$) for their enzymatic activity (6). SIRT1 downregulation has been observed in peripheral blood mononuclear cells of individuals with impaired glucose tolerance (7,8). SIRT1 is known to regulate vascular endothelial cell functions (9). Furthermore, *in vitro* and *in vivo* studies demonstrated that a reduction in the number of endothelial progenitor cells (EPCs) under hyperglycemia is associated with reduced SIRT1 levels and activity (8,10). Endothelial cells regulate vascular tone and are the first fetal cells exposed to maternal hyperglycemia.

In recent years, it has become evident that SIRT1 is a key player of EPC dysfunction in insulin resistance and metabolic syndrome (7,10,11). Overexpression of endothelium-specific SIRT1 protects against endothelial dysfunction induced by a high-fat diet and hyperglycemia (12,13). Recently, resveratrol (RSV) and paeonol (2′-Hydroxy-4′-methoxyacetophenone) have been shown to activate SIRTs and thus can be used for pharmacological interventions (14,15). Activation of SIRT1 by RSV can improve vascular functions (16), while paeonol prevented premature senescence induced by oxidative stress in human umbilical vein endothelial cells (HUVECs) by modulating the expression of SIRT1 (15).

EPCs derived from bone marrow contribute to endothelial repair (17). Among them, the specific subtype of endothelial colony-forming cells (ECFCs) harbor clonogenic potential, endothelial phenotype, have the capacity to incorporate into neovessels, and participate in the maintenance of vascular homeostasis *in vivo*. Impairment in the number and function of fetal ECFCs has been observed in preeclampsia and GDM (18,19).

The purpose of this study was to investigate whether fetal endothelial cells including ECFCs and HUVECs from GDM pregnancies show alterations of SIRTs compared with uncomplicated pregnancies (NPs). We further investigated whether paeonol or RSV can activate SIRTs in ECFCs and HUVECs from GDM pregnancies.

RESULTS

Patient Demographics

There were no differences in maternal age, pre-pregnancy body mass index (BMI), gestational age at delivery, cesarean section rate, gender, birth weight, arterial cord blood pH, and

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glycated hemoglobin (HbA1c) levels between women with NPs and pregnancies complicated by GDM (**Table 1**).

Reduction of NAD⁺, SIRT Activity, and Expression in ECFCs From GDM Pregnancies

NAD^+ concentration in ECFCs from GDM pregnancies was significantly lower (0.72 ± 0.24, $P = 0.02$) compared with NPs

Table 1. Clinical and demographic data of patients

Variables	NPs (n = 10)	GDM (n = 10)	P Value
Maternal age (y)	30.20 ± 5.49	34.10 ± 4.63	0.10
Primiparous (%)	40%	60%	0.6
Gestational age at delivery (wk)	39.63 ± 1.18	39.19 ± 1.34	0.44
Maternal pre-pregnancy BMI (kg/m²)	27.86 ± 6.82	30.70 ± 7.34	0.38
Caesarean delivery (%)	50%	40%	1.00
Birth weight (g)	3615 ± 445.1	3679 ± 517.5	0.77
Baby gender, male n (%)	6 (60%)	2 (20%)	0.17
Arterial cord pH	7.27 ± 0.09	7.26 ± 0.05	0.88
HbA1c before delivery	5.09 ± 0.36	5.27 ± 0.26	0.24

Data are given as mean ± SD or number (percentage).
BMI, body mass index; GDM, gestational diabetes; HbA1c, glycated hemoglobin; NPs, uncomplicated pregnancies.

(control = 1), as well as SIRT1 activity (0.87 ± 0.28, $P = 0.004$), SIRT3 activity (0.78 ± 0.48, $P = 0.03$), and relative transcription levels of *SIRT1* (0.65 ± 0.29, $P = 0.03$), *SIRT3* (0.81 ± 0.16, $P = 0.003$), and *SIRT4* (0.75 ± 0.23, $P = 0.003$) when compared with NPs (**Figure 1**). There were no differences in protein levels of SIRT1 ($P = 0.44$), SIRT3 ($P = 0.38$), and SIRT4 ($P = 0.84$) between the two groups.

### Reduction of NAD⁺, SIRT Activity, and Expression in HUVECs From GDM Pregnancies

HUVECs from GDM pregnancies had a significantly lower NAD^+ concentration (0.85 ± 0.21, $P = 0.006$) and relative transcription levels of *SIRT1* (0.85 ± 0.14, $P = 0.04$) and *SIRT4* (0.69 ± 0.11, $P = 0.0039$) when compared with NPs (control = 1). There were no differences in SIRT1 activity (1.11 ± 0.29, $P = 0.34$), SIRT3 activity (1.21 ± 0.38, $P = 0.30$), and relative transcription levels of *SIRT3* (0.94 ± 0.35, $P = 0.70$) (**Figure 2**). The protein levels of SIRT1 ($P = 0.08$), SIRT3 ($P = 0.15$), and SIRT4 ($P = 0.97$) were also not different between the two groups.

Hyperglycemia *In Vitro* Resulted in Elevated NAD⁺ Levels and a Reduction of SIRT Activity and Expression in ECFCs

In ECFCs, the NAD^+ concentration was significantly increased after treatment with 30-mM glucose (15 mM: 1.28 ± 0.22,
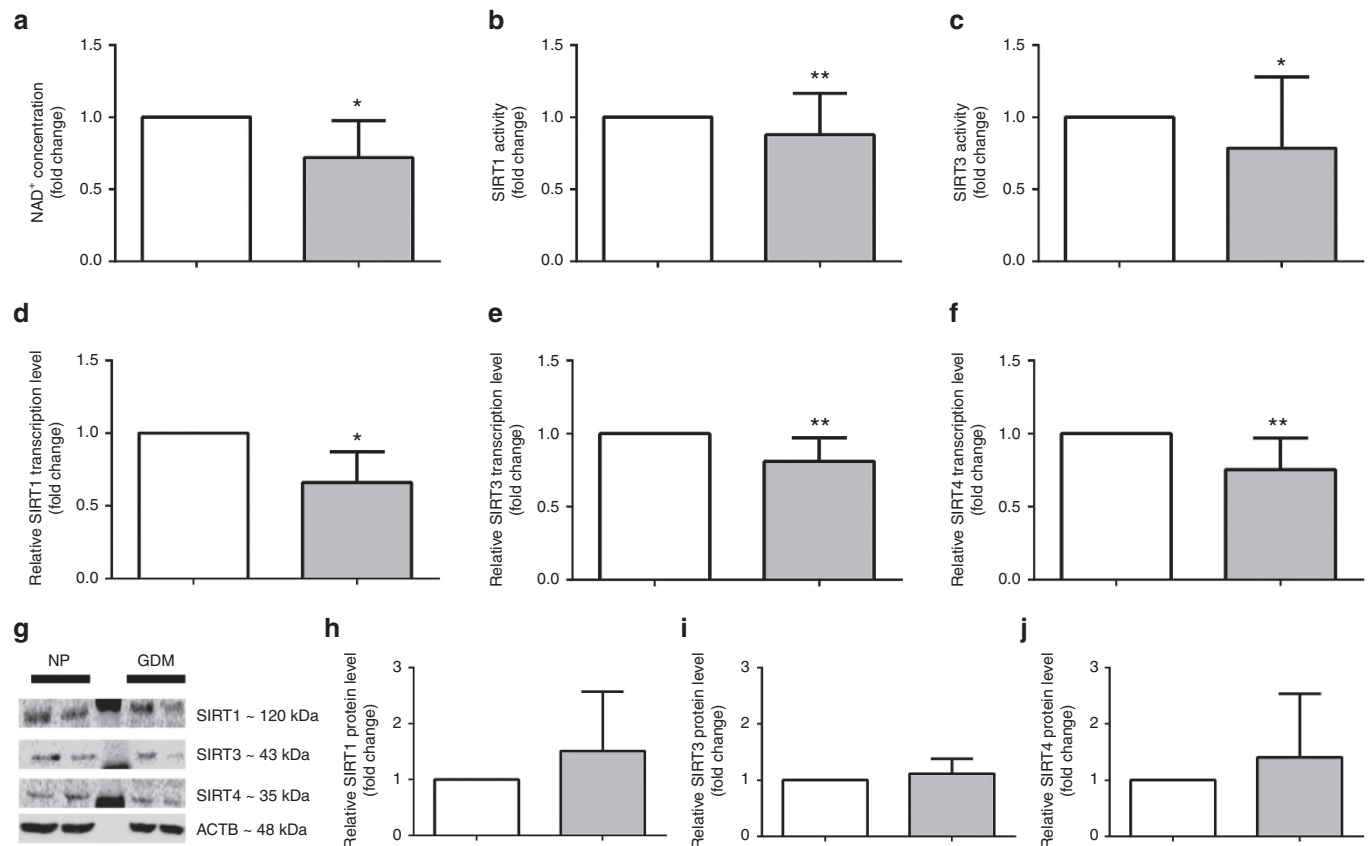
Figure 1. Alterations of sirtuins (SIRTs) in endothelial colony-forming cells (ECFCs) from gestational diabetes (GDM) pregnancies (gray) compared with uncomplicated pregnancies (NPs; white). (**a**) NAD^+ concentration, (**b**) SIRT1 activity, (**c**) SIRT3 activity, relative transcription levels of (**d**) *SIRT1*, (**e**) *SIRT3*, and (**f**) *SIRT4*, and (**g**) representative immunoblots of ECFCs from NPs and GDM pregnancies. Relative protein levels of (**h**) SIRT1, (**i**) SIRT3, and (**j**) SIRT4 of ECFCs from NPs compared with GDM pregnancies were not significantly different. (n = 10). Data are presented as mean fold changes ± SD of GDM pregnancies as compared with NPs (control = 1). *P < 0.05 or **P < 0.01 when compared with NPs.
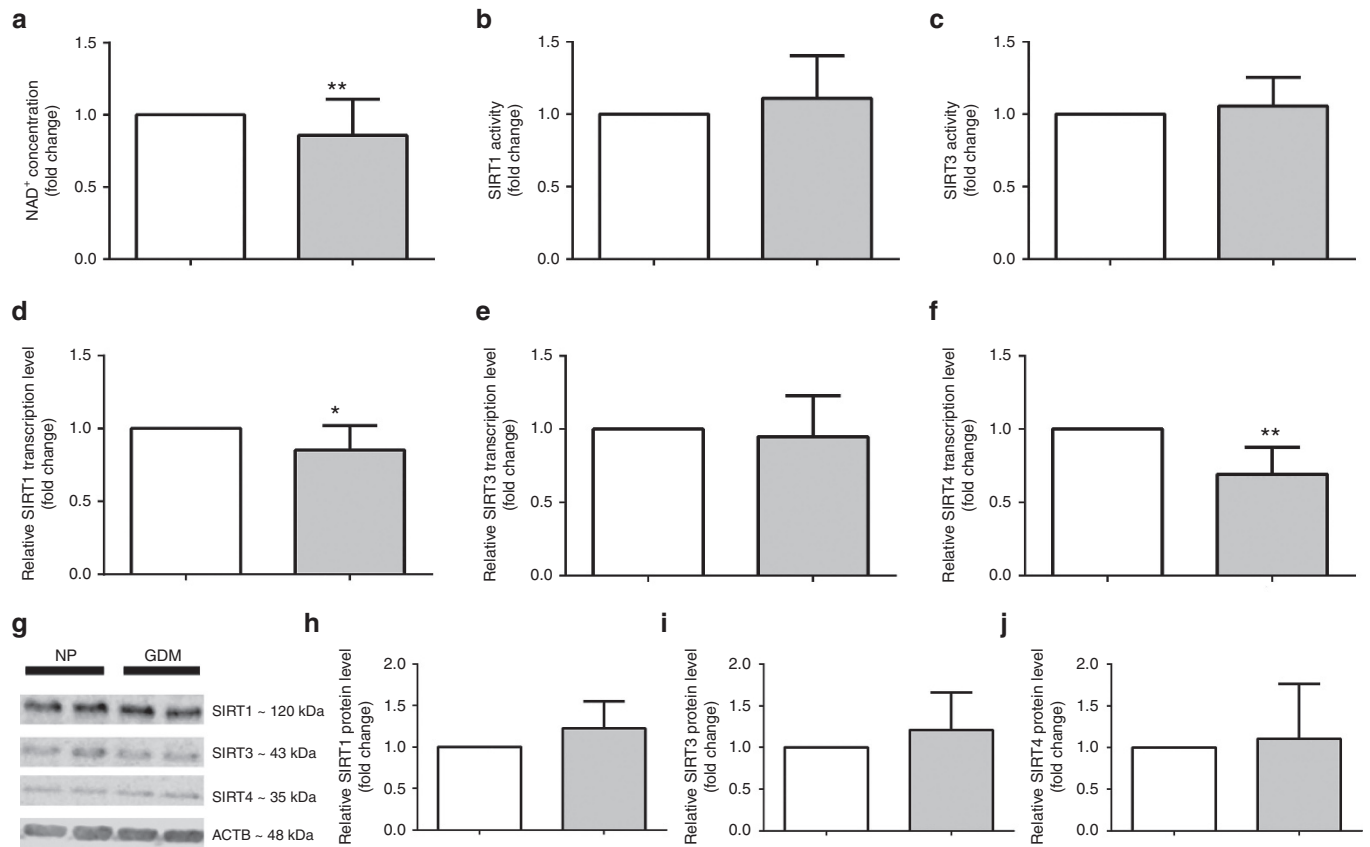
Figure 2. Alterations of sirtuins (SIRTs) in human umbilical vein endothelial cells (HUVECs) from gestational diabetes (GDM) pregnancies (gray) compared with uncomplicated pregnancies (NPs; white). (**a**) NAD$^+$ concentration, (**b**) SIRT1 activity and (**c**) SIRT3 activity, relative transcription levels of (**d**) *SIRT1*, (**e**) *SIRT3*, and (**f**) *SIRT4*, and (**g**) representative immunoblots of HUVECs from NPs and GDM pregnancies. Relative protein levels of (**h**) SIRT1, (**i**) SIRT3, and (**j**) SIRT4 of HUVECs from NPs compared with GDM pregnancies without significant alterations. ($n = 6$). Data are presented as mean fold changes ± SD of GDM pregnancies as compared with NPs (control = 1), *$P < 0.05$ or **$P < 0.01$ when compared with NPs.

$P > 0.05$; 1.64 ± 0.23, $P < 0.05$) compared with standard culture medium containing 5.5-mM glucose (control = 1). Incubation with 30-mM glucose decreased SIRT1 activity (15 mM: 1.13 ± 0.25, $P > 0.05$; 30 mM: 0.55 ± 0.11, $P < 0.05$). We observed no change in SIRT3 activity (15 mM: 1.13 ± 0.18, $P > 0.05$; 30 mM: 0.65 ± 0.26, $P > 0.05$) (**Figure 3**). *SIRT1* (15 mM: 1.10 ± 0.11, $P > 0.05$; 30 mM: 0.77 ± 0.13, $P < 0.05$) and *SIRT4* transcription levels (15 mM: 1.26 ± 0.19, $P > 0.05$; 30 mM: 0.69 ± 0.08, $P < 0.05$) were significantly lower after exposure to 30-mM glucose, while there was no change in *SIRT3* transcription levels (15 mM: 1.15 ± 0.12, $P > 0.05$; 30 mM: 0.83 ± 0.09, $P > 0.05$). There were no differences in protein levels of SIRT1, SIRT3, and SIRT4 between control cells, 15-mM treatment, and 30-mM treatment.

Hyperglycemic Treatment *In Vitro* Resulted in a Reduction of SIRT Activity and Expression in HUVECs

NAD$^+$ concentration in HUVECs remained unaffected after a treatment with 15-mM or 30-mM glucose (15 mM: 1.0 ± 0.10, $P > 0.05$; 30 mM: 1.13 ± 0.18, $P > 0.05$) compared with standard culture medium containing 5.5-mM glucose (control = 1). As opposed to this, exposure to 30-mM glucose resulted in a significant decrease of SIRT1 (15 mM: 0.90 ± 0.03 $P > 0.05$;

30 mM: 0.73 ± 0.08, $P < 0.01$) and SIRT3 activity (15 mM: 0.90 ± 0.02, $P > 0.05$; 30 mM: 0.59 ± 0.13, $P < 0.01$) (**Figure 4**). *SIRT1* (15 mM: 1.04 ± 0.41, $P > 0.05$; 30 mM: 0.77 ± 0.21, $P < 0.05$) and *SIRT4* (15 mM: 0.86 ± 0.16, $P > 0.05$; 30 mM: 0.78 ± 0.09, $P < 0.05$) transcription levels showed a significant reduction after a 30-mM glucose treatment, whereas there were no alterations in *SIRT3* (15 mM: 1.26 ± 0.12, $P > 0.05$; 30 mM: 0.89 ± 0.18, $P > 0.05$) transcription levels. SIRT1, SIRT3, and SIRT4 protein levels showed no differences between control cells, 15-mM glucose treatments, and 30-mM glucose treatments.

Effect of SIRT Activators in ECFCs

In this section, the control group represents cells mock treated with ethanol from each patient group. After treatment with 50-μM paeonol for 24 h, the relative transcription levels of *SIRT 3* (NPs with paeonol: 1.53 ± 0.41, $P < 0.01$; GDM pregnancies with paeonol: 1.22 ± 0.16, $P > 0.05$) and *SIRT4* (NPs with paeonol: 2.18 ± 0.63, $P < 0.01$; GDM pregnancies with paeonol: 1.74 ± 0.35, $P > 0.05$) markedly increased in ECFCs of NPs but not in those of GDM pregnancies. There were no significant differences in NAD$^+$ concentration (NPs with paeonol: 1.21 ± 0.34, $P > 0.05$; GDM pregnancies with
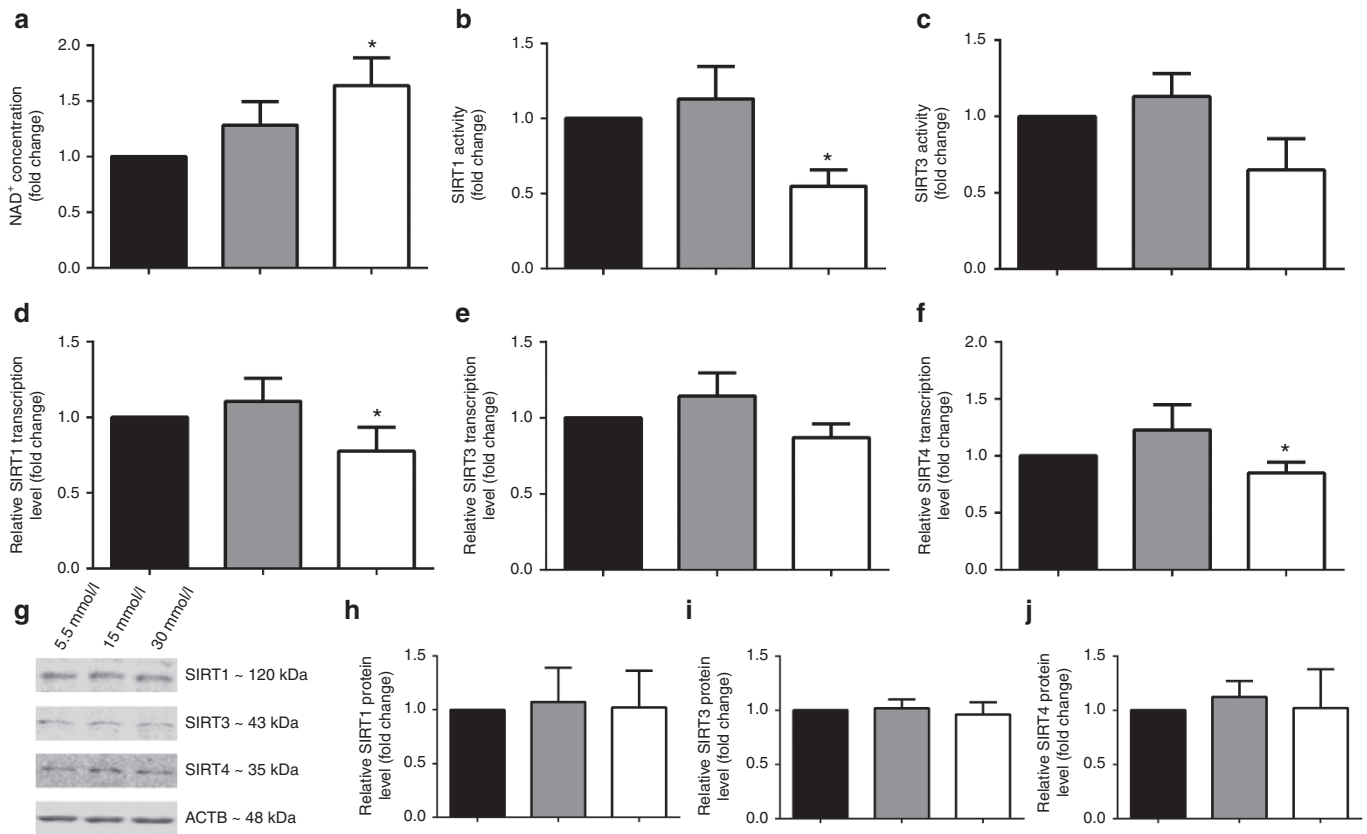
Figure 3. Alterations of NAD⁺ and sirtuins (SIRTs) in endothelial colony-forming cells (ECFCs) from uncomplicated pregnancies (NPs; black) treated with 15-mM (gray) and 30-mM (white) glucose. (**a**) NAD⁺ concentration, (**b**) SIRT1 activity, (**c**) SIRT3 activity, relative transcription levels of (**d**) *SIRT1*, (**e**) *SIRT3*, and (**f**) *SIRT4* in ECFCs from NPs after incubation with 15-mM or 30-mM glucose compared with standard culture medium (5.5 mM). (**g**) Representative immunoblots and relative protein levels of (**h**) SIRT1, (**i**) SIRT3, and (**j**) SIRT4 of ECFCs from NPs treated with 5.5-, 15-, or 30-mM glucose with no significant differences. *n* = 5. Data are shown as mean fold changes ± SD of 15- or 30-mM-glucose–treated ECFCs compared with 5.5-mM-glucose–treated ECFCs (control = 1). *$P < 0.05$ when compared with 5.5-mM-glucose–treated ECFCs.

paeonol: 1.19 ± 0.56, $P > 0.05$), SIRT1 activity (NPs with paeonol: 1.39 ± 0.50, $P > 0.05$; GDM pregnancies with paeonol: 1.56 ± 0.52, $P > 0.05$), SIRT3 activity (NPs with paeonol: 0.99 ± 0.43, $P > 0.05$; GDM pregnancies with paeonol: 1.36 ± 0.41, $P > 0.05$), or *SIRT1* transcription level (NPs with paeonol: 2.48 ± 0.13, $P > 0.05$; GDM pregnancies with paeonol: 0.86 ± 0.17, $P > 0.05$) (**Figure 5**).

After treatment with 1-μM RSV for 24 h, the NAD⁺ concentration significantly increased in ECFCs from NPs but not in those from GDM pregnancies (NPs with RSV: 1.48 ± 0.24, $P < 0.05$; GDM pregnancies with RSV: 1.38 ± 0.26, $P > 0.05$), and SIRT1 activity was stimulated in both NPs and GDM pregnancies (NPs with RSV: 1.68 ± 0.22, $P < 0.05$; GDM pregnancies with RSV: 2.04 ± 0.64, $P < 0.01$). SIRT3 activity markedly increased only in GDM pregnancies (NPs with RSV: 1.00 ± 0.40, $P > 0.05$; GDM pregnancies with RSV: 1.68 ± 0.23, $P < 0.05$). The relative transcription level of *SIRT1* (NPs with RSV: 3.57 ± 0.45, $P < 0.01$; GDM pregnancies with RSV: 1.37 ± 0.20, $P > 0.05$) and *SIRT3* (NPs with RSV: 1.54 ± 0.57, $P < 0.01$; GDM pregnancies with RSV: 1.14 ± 0.18, $P > 0.05$) increased significantly only in ECFCs of NPs but not in those of GDM pregnancies, while there were no changes in *SIRT4* transcription levels (NPs with RSV: 1.72 ± 0.81, $P > 0.05$; GDM pregnancies with RSV:

1.08 ± 0.19, $P > 0.05$). Paeonol and RSV both did not significantly change protein levels of SIRT1, SIRT3, and SIRT4 in NPs and GDM pregnancies ($P > 0.05$) (**Figure 5**).

Effect of SIRT Activators on HUVECs

Control group represents cells mock treated with ethanol from each patient group. Treatment with 50-μM paeonol for 24 h increased the relative transcription level of *SIRT4* (NPs with paeonol: 1.42 ± 0.33, $P < 0.05$; GDM pregnancies with paeonol: 1.37 ± 0.36, $P < 0.05$) in both NPs and GDM pregnancies (**Figure 6**). However, paeonol did not significantly impact NAD⁺ concentration (NPs with paeonol: 1.31 ± 0.23, $P > 0.05$; GDM pregnancies with paeonol: 1.13 ± 0.12, $P > 0.05$), SIRT1 activity (NPs with paeonol: 1.10 ± 0.15, $P > 0.05$; GDM pregnancies with paeonol: 1.07 ± 0.52, $P > 0.05$), SIRT3 activity (NPs with paeonol: 0.98 ± 0.25, $P > 0.05$; GDM pregnancies with paeonol: 0.94 ± 0.20, $P > 0.05$), and the relative transcription levels of *SIRT1* (NPs with paeonol: 1.15 ± 0.38, $P > 0.05$; GDM pregnancies with paeonol: 1.12 ± 0.38, $P > 0.05$) and *SIRT3* (NPs with paeonol: 1.22 ± 0.61, $P > 0.05$; GDM pregnancies with paeonol: 1.03 ± 0.14, $P > 0.05$)

After treatment with 1-μM RSV for 24 h, the NAD⁺ concentration increased in NPs (NPs with RSV: 1.42 ± 0.31, $P < 0.01$;
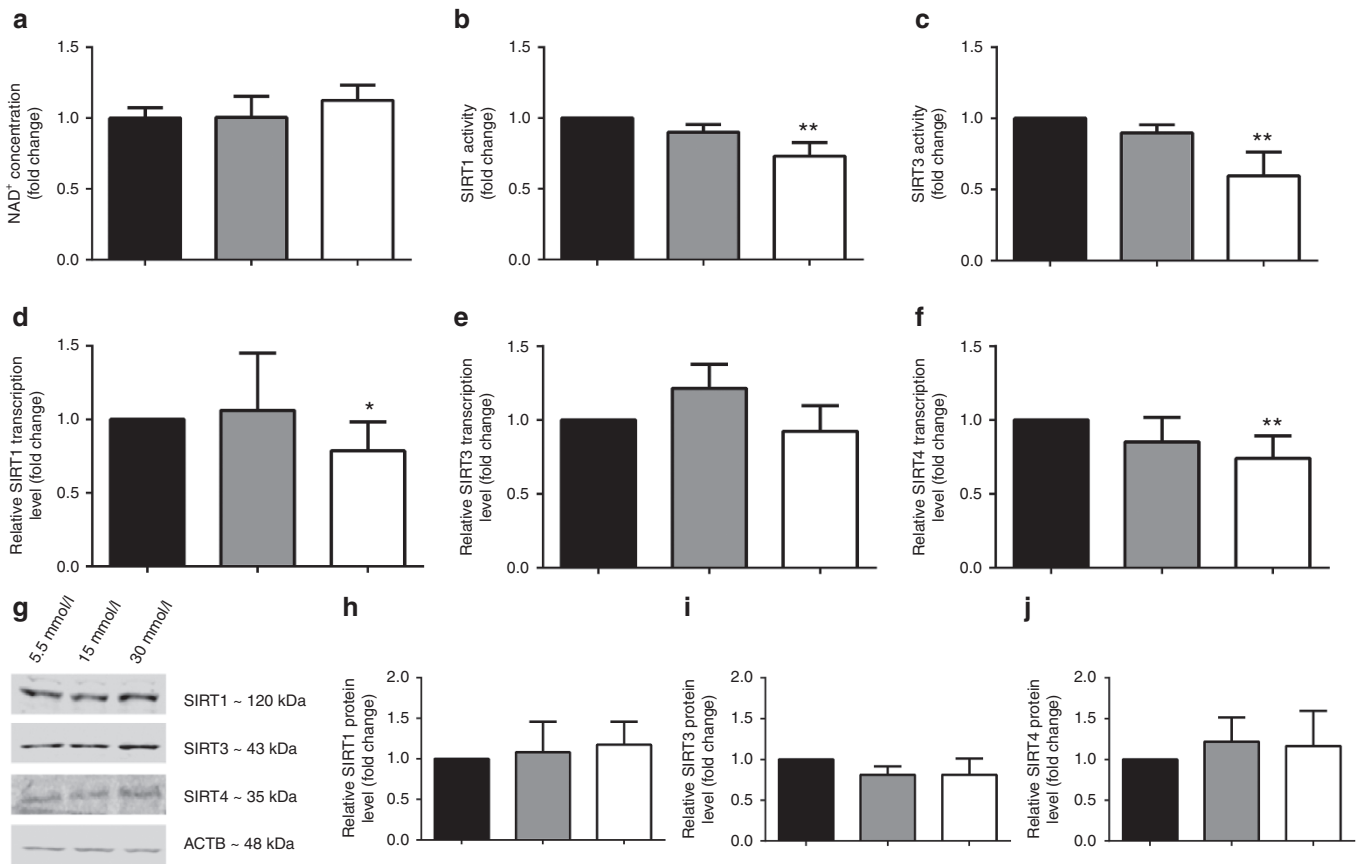
Figure 4. Alterations of NAD$^+$ and sirtuins (SIRTs) in human umbilical vein endothelial cells (HUVECs) from uncomplicated pregnancies (NPs; black) treated with 15-mM (gray) and 30-mM (white) glucose. (**a**) NAD$^+$ concentration, (**b**) SIRT1 activity, (**c**) SIRT3 activity, relative transcription levels of (**d**) *SIRT1*, (**e**) *SIRT3*, and (**f**) *SIRT4* in HUVECs of NPs after incubation with 15-mM or 30-mM glucose compared with standard culture medium (5.5 mM). (**g**) Representative immunoblots and relative protein levels of (**h**) SIRT1, (**i**) SIRT3, and (**j**) SIRT4 of ECFCs from NPs treated with 5.5-, 15-, or 30-mM glucose. No significant alterations in protein levels were detected. (*n* = 5). Data are shown as mean fold changes ± SD of 15- or 30-mM-glucose–treated HUVECs compared with 5.5-mM-glucose–treated HUVECs (control = 1). *$P < 0.05$, **$P < 0.01$ when compared with 5.5-mM-glucose–treated ECFCs.

GDM pregnancies with RSV: 1.20 ± 0.15, $P > 0.05$). SIRT1 activity (NPs with RSV: 1.61 ± 0.25, $P < 0.01$; GDM pregnancies with RSV: 1.66 ± 0.14, $P < 0.01$) and SIRT3 activity (NPs with RSV: 1.52 ± 0.21, $P < 0.01$; GDM pregnancies with RSV: 1.48 ± 0.38, $P < 0.01$) were markedly stimulated in HUVECs from NPs and GDM pregnancies. The relative transcription level of *SIRT1* (NPs with RSV: 1.49 ± 0.97, $P < 0.01$; GDM pregnancies with RSV: 1.40 ± 0.64, $P < 0.05$) was higher in NPs and GDM pregnancies, while the relative transcription levels of *SIRT3* (NPs with RSV: 1.11 ± 0.52, $P > 0.05$; GDM pregnancies with RSV: 0.96 ± 0.28, $P > 0.05$) and *SIRT4* (NPs with RSV: 1.32 ± 0.22, $P > 0.05$; GDM pregnancies with RSV: 1.13 ± 0.33, $P > 0.05$) were not significantly different. Paeonol and RSV both did not significantly change protein levels of SIRT1, SIRT3, and SIRT4 in NPs and GDM pregnancies ($P > 0.05$) (**Figure 6**).

DISCUSSION

In the 1980s, Barker (1) proposed the concept of fetal programming as a possible cause of cardiovascular morbidity and mortality in adulthood. In line with this theory, recent clinical trials have shown that normalization of blood glucose failed to improve cardiovascular morbidity in the diabetic population (20,21).

Furthermore, increased levels of circulating cellular adhesion molecules, putative biomarkers for early subclinical stages of atherosclerosis and diabetes, have been found in offspring exposed to GDM and preexisting maternal diabetes (22). The persistence of hyperglycemic stress despite glucose normalization has been coined "hyperglycemic memory" (23). This emerging concept of a metabolic memory may explain why cardiovascular complications and other sequelae progress in the offspring of GDM pregnancies despite normoglycemia. However, it is not clear how fetal hyperglycemia translates into morbidity and mortality during later life. Therefore, we aimed to study the effect of GDM on SIRTs as potential mediators of altered vascular homeostasis.

In the present study, we show that SIRT levels of ECFCs isolated from cord blood and HUVECs isolated from cord are altered in pregnancies complicated by GDM pregnancies compared with NPs. The activity and expression levels of SIRTs were lower in both GDM-ECFCs and GDM-HUVECs compared with NPs. Comparable effects were also found after exposure of ECFCs and HUVECs from NPs to hyperglycemia *in vitro*. Furthermore, SIRT reduction was more pronounced in GDM-ECFCs compared with GDM-HUVECs. We also found that the SIRT activator RSV could significantly enhance the expression
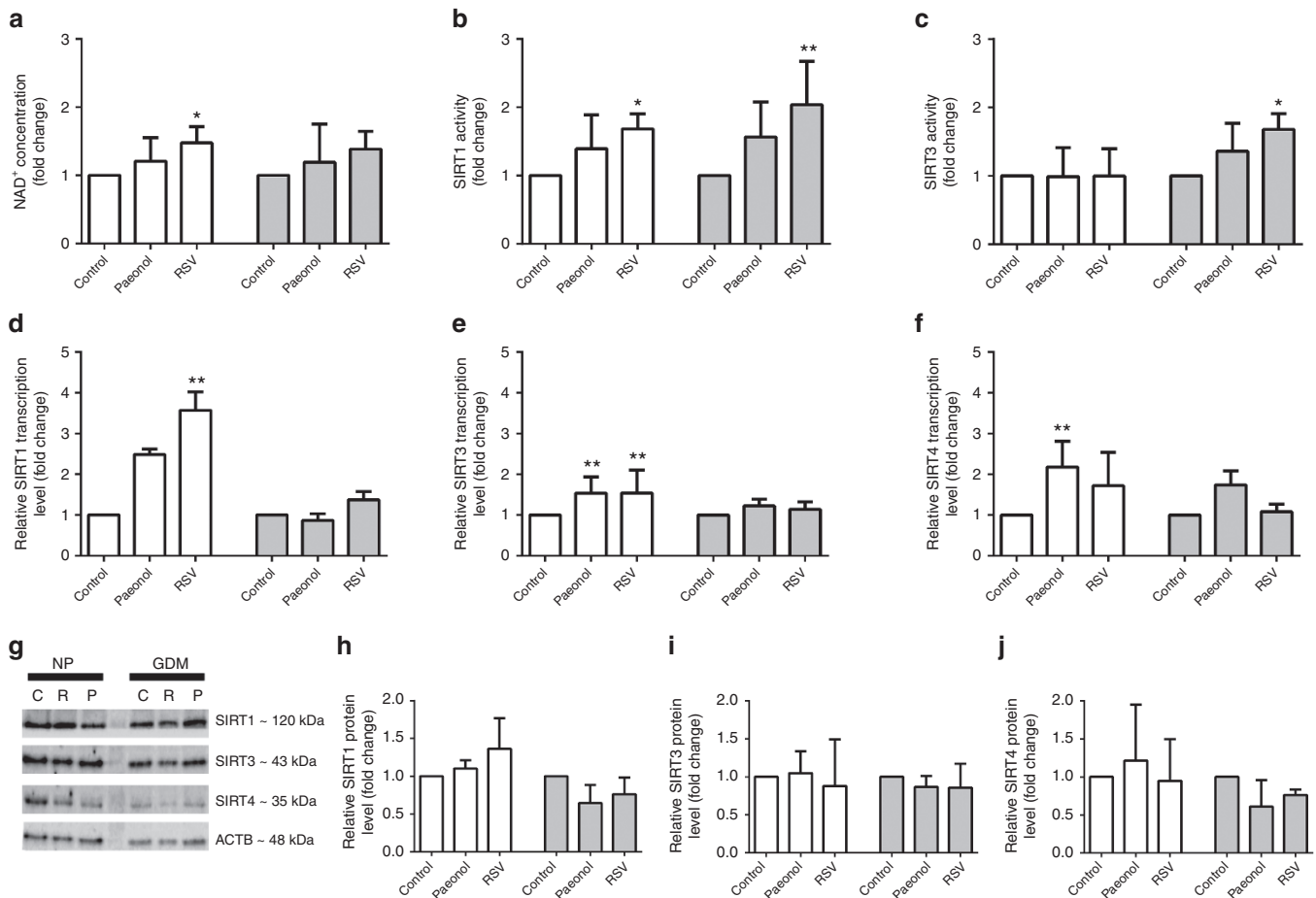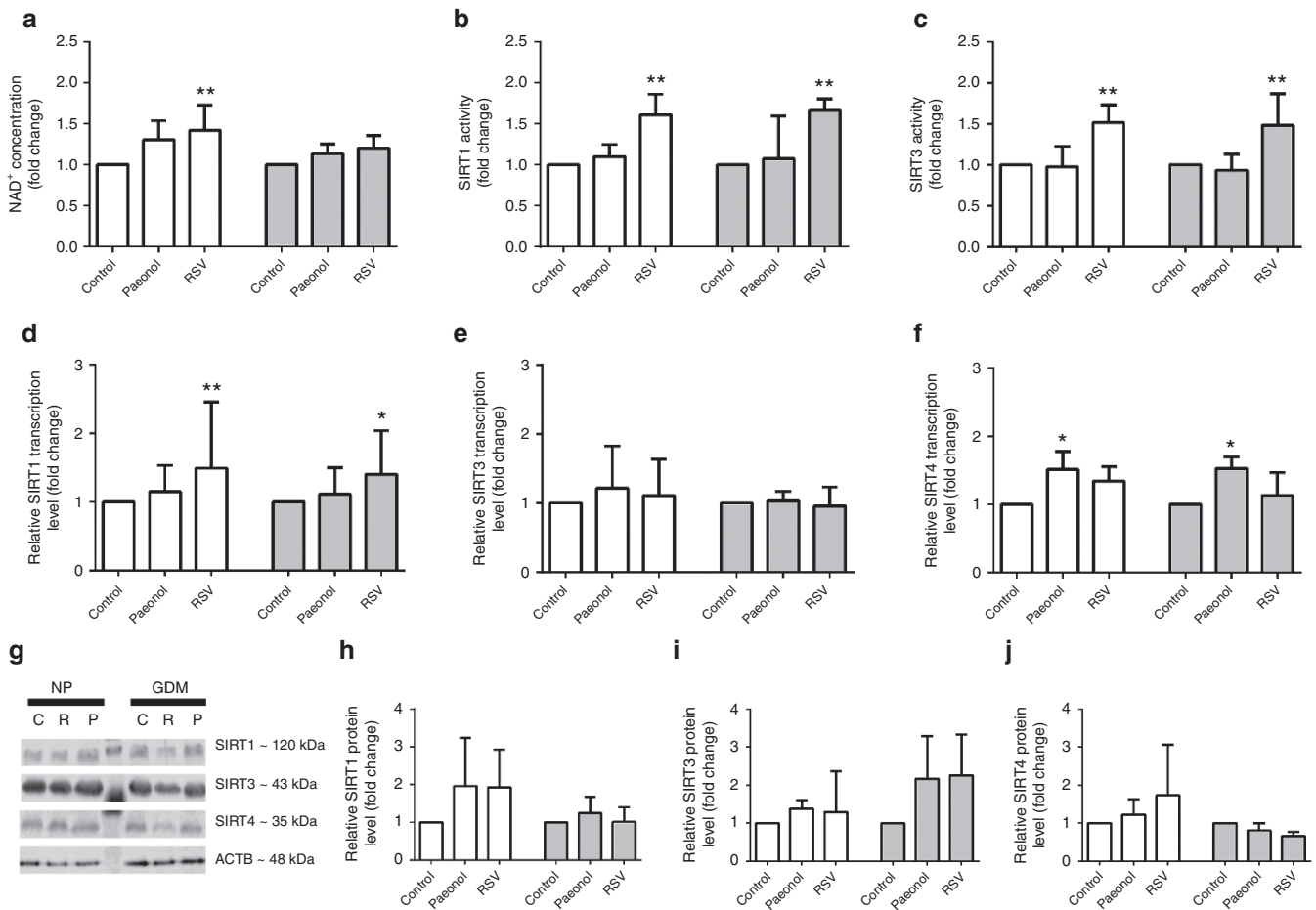
Figure 5. Effect of sirtuin (SIRT) activators on endothelial colony-forming cells (ECFCs) from gestational diabetes (GDM) pregnancies (gray) compared with uncomplicated pregnancies (NPs; white). ECFCs were incubated with 50-μM paeonol resuspended in dimethylsulfoxide or 1-μM resveratrol (RSV) resuspended in ethanol for 24 h before collection. Control cells were mock treated with ethanol. (**a**) NAD+ concentration, (**b**) SIRT1 activity, (**c**) SIRT3 activity and relative transcription levels of (**d**) *SIRT1*, (**e**) *SIRT3*, and (**f**) *SIRT4* in ECFCs of NPs and GDM pregnancies. (**g**) Representative immunoblots and relative protein levels of (**h**) SIRT1, (**i**) SIRT3, and (**j**) SIRT4 of ECFCs from NPs and GDM pregnancies treated with ethanol, RSV, or paeonol. No significant alterations in protein levels were detected. (*n* = 5). Data are presented as mean fold changes ± SD of RSV- or paeonol-treated ECFCs as compared with mock-treated ECFCs (control = 1). *$P < 0.05$ or **$P < 0.01$ when compared with control.

and activity of SIRTs in both ECFCs and HUVECs. However, the SIRT activator paeonol only had an effect in ECFCs.

The downregulation of SIRT1 observed in ECFCs from neonates with pregnancies complicated by GDM suggests that SIRT1 in circulating blood cells may serve as a novel biomarker, confirming results of a study by de Kreutzenberg *et al.* (8) in patients with insulin resistance and metabolic syndrome. Downregulation of SIRT1 has previously been described to be associated with accelerated senescence of EPCs in neonates underlining its function as a key regulator in vascular homeostasis during early life (24). In accordance with our results, Balestrieri *et al.* (10) observed decreased SIRT1 protein levels in EPCs from type 2 diabetic patients, especially those with poor glycemic control. Zheng *et al.* (25) showed that decreased levels of SIRT1 correlated with the cellular "memory" of hyperglycemic stress after glucose levels were normalized. Activation of SIRT1 by RSV or SIRT1 overexpression compromised the memory of hyperglycemic stress, whereas the inhibition of SIRT1 by SIRT1 siRNA increased sensitivity to high-glucose

stress. SIRT1 has been reported to control endothelial angiogenic functions during postnatal vascular growth (26). Our previous findings of impaired functions, e.g., proliferation, migration, and angiogenesis of ECFCs from GDM pregnancies (18) could potentially result from lower SIRT activity. Thus, SIRTs seem to be promising candidate molecules for mediating the feature of "hyperglycemic memory".

In the present study, we found a downregulation of SIRT3 activity and *SIRT3* and *SIRT4* at mRNA level in GDM pregnancies. SIRT activators enhanced *SIRT3* and *SIRT4* mRNA expression. These results may imply metabolic rescue by the induction of SIRT1 activity. As previously described, SIRT1 activates peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) (27), which is a regulator of mitochondrial biogenesis and function. PGC1α integrates different cellular signals and regulates genes of energy metabolism, one of these genes is SIRT3. It is activated by PGC1α binding to the estrogen-related receptor-binding element in the SIRT3 promotor region (28). Feldman *et al.* (29) hypothesized

Figure 6. Effect of sirtuin (SIRT) activators on human umbilical vein endothelial cells (HUVECs) from gestational diabetes (GDM) pregnancies (gray) compared with uncomplicated pregnancies (NPs; white). HUVECs were incubated with 50-μM paeonol resuspended in dimethylsulfoxide or 1-μM resveratrol (RSV) resuspended in ethanol for 24 h before collection. Control cells were mock treated with ethanol. (**a**) NAD$^+$ concentration, (**b**) SIRT1 activity, (**c**) SIRT3 activity and relative transcription levels of (**d**) *SIRT1*, (**e**) *SIRT3*, and (**f**) *SIRT4* in HUVECs of NPs and GDM pregnancies. (**g**) Representative immunoblots and relative protein levels of (**h**) SIRT1, (**i**) SIRT3, and (**j**) SIRT4 of HUVECs from NPs and GDM pregnancies treated with ethanol, RSV, or paeonol. No significant differences in protein levels were detected. (*n* = 5). Data are presented as mean fold changes ± SD of RSV- or paeonol-treated HUVECs as compared with mock-treated HUVECs (control = 1). *$P < 0.05$ or **$P < 0.01$ when compared with control.

that the PGC1α-dependent mitochondrial metabolic reprogramming is SIRT3 mediated. This crosstalk of the nutrient-sensitive SIRT1 and the mitochondrial regulators PGC1α and SIRT3 may be one of the reasons for altered metabolic regulation of GDM cells and later on in children of GDM pregnancies. Furthermore, SIRT3 reduction is associated with altered metabolic conditions like high-fat feeding or fasting. Huynh *et al.* (30) speculated that patients with type 2 diabetes show decreased SIRT3 levels due to an altered metabolic homeostasis. Our results seem to support this theory, even if we are not able to provide a molecular mechanism at this point.

Hyperglycemia has been associated with increased oxygen consumption, which is probably related to an increased production of reactive oxygen species (ROS) (31). SIRT3 plays a key role in regulating mitochondrial ROS homeostasis (32). Increased ROS production in vascular tissue under pathological conditions is the main cause of endothelial dysfunction. The SIRT activator RSV attenuated oxidative injury in endothelial cells through the regulation of mitochondrial ROS

homeostasis, which, in part, was mediated through the activation of SIRT3 (33).

Data on the role of SIRT4 are limited, but inhibition is likely to accelerate the development of type 2 diabetes (34). In general, SIRT4 is associated with the regulation of energy metabolism. Recent data suggest that SIRT4 is a cellular, especially mitochondrial, lipoamidase. Only a few proteins are lipoylated, but they play critical roles in cellular metabolism and are targets of SIRT4 (35). One of those targets is pyruvate dehydrogenase, which is inhibited after SIRT4-dependent hydrolysis of the lipoamide. The reduction of SIRT4 in GDM cells in our study may serve to maintain pyruvate dehydrogenase activity (35) thus allowing for metabolism of glucose. SIRT4 is known to inhibit fatty acid oxidation via increasing levels of malonylCoA. If SIRT4 is reduced in GDM cells, fatty acid oxidation will be stimulated thus enhancing energy production. Furthermore, the efficiency of the adenosine triphosphate production is regulated via the adenosine diphosphate/triphosphate translocator ANT2, which not only acts as an adenosine

diphosphate/triphosphate exchange transporter but as an uncoupler as well (36). Decreased SIRT4 levels would lead to a less efficient energy production. It is not clear if reduced SIRT4 levels are confined to ECFCs and HUVECs or are a general feature in many organs. We do not know which effect is the most important one for the dysregulation of SIRT4 in GDM pregnancies. Further work has to be performed on SIRT4 to get sufficient insight into its regulation.

To the best of our knowledge, this is the first study investigating the effect of the SIRT activators RSV and paeonol on GDM-ECFCs and GDM-HUVECs. The experiments presented here show that RSV could significantly enhance the expression and activity of SIRTs in both ECFCs and HUVECs from NPs and GDM pregnancies. However, the response to paeonol was heterogenous. In fact, paeonol only had a significant effect in ECFC. RSV is a polyphenolic compound from red grapes. It was the first compound described that can mimic the SIRT-mediated effects of caloric restriction. Several different mechanisms for the action of RSV have been proposed (37). Gertz *et al.* (14) show a direct activation via interaction of RSV and the SIRT1 active site using crystal structure analysis. The authors also describe RSV as a strong activator of SIRT5 and an inhibitor of SIRT3. Other studies propose a mechanism via activation of the Akt pathway. Modulating the Akt pathway would explain the effects of RSV on the cell-cycle regulation in neurons (38). Further studies suggest an activation of the 5′ AMP-activated protein kinase (AMPK) pathway in a RSV/SIRT1/SIRT3-dependent manner, but Desquiret-Dumas *et al.* (39) inhibited AMPK and could still measure RSV-induced mitochondrial biogenesis in liver cells. Mechanistic studies in endothelial cells propose an increase in vascular oxidative stress resistance induced by RSV via antioxidant, anti-apoptotic, and anti-inflammatory effects (40,41) and a pathway that involves the activation of SIRT1 and the upregulation of antioxidant defense mechanisms by RSV to attenuate mitochondrial ROS production (42). Paeonol is a phenolic compound derived from peonies. Traditional Chinese medicine uses this compound alleviating various oxidative stress-related diseases. Jamal *et al* (15) suggest a beneficial role of paeonol on premature senescence in a SIRT1-dependent manner. However, the mechanism of action for this compound is still unknown.

SIRT1 activation is a promising new therapeutic approach for treating diseases of aging such as type 2 diabetes (43). SIRT1 activation by RSV improves vascular function by increasing NO availability and attenuates dyslipidemia and obesity-induced metabolic alterations in human subjects (44–46). Paeonol can alleviate the oxidative stress-related cardiovascular diseases predominantly through the modulation of endogenous antioxidants and reduction of free radical formation (47,48).

Two different endothelial cell types—ECFCs and HUVECs—were used in this study. They are the first vascular cells of the fetus that are exposed to aberrant conditions and an adverse metabolic milieu during pregnancy. ECFCs represent progenitor cells, possessing the ability to differentiate into mature endothelial cells and to repair damaged endothelium. In contrast, HUVECs are mature endothelial cells which

may explain an increased vulnerability of the latter, which is reflected in more pronounced changes of SIRT1 (33). At the same time, this finding also supports the hypothesis of "hyperglycemic memory". As ECFCs are responsible for controlling vascular repair and development of the vascular system, they may mediate the "hyperglycemic memory" and altered SIRT expression of ECFCs might be another sign of endothelial cell dysfunction leading to reduced vessel repair capacity.

A few studies compared functional and molecular readouts in these two cell types and showed heterogeneous responses in many cases. Sipos *et al.* (49) describe the ability of human fetal ECFCs, but not human fetal endothelial cells, to transmigrate to the maternal bloodstream of the uterus and then home to locations of maternal uterine vasculogenesis underlining profound functional differences between these cell types. While the response of ECFCs to shear stress was comparable with HUVECs and human umbilical artery endothelial cells (50), a differential response of ECFCs compared with HUVECs was detected on electrospun scaffolds with distinct microfiber diameters (51). In 3D assays, ECFCs induced more robust sprouting vascular structures than HUVECs possibly induced by a stronger phosphorylation of VEGFR2 and its downstream signaling (52). The experiments presented here show that ECFCs but not HUVECs respond to paeonol. Own, unpublished data indicate a strong functional response of ECFCs to vitamin D, while HUVECs did not change their proliferation, migration, and angiogenic behavior. These data are in support of the theory of a differential sensitivity of these cells to the same stimuli.

Our patients were recruited at admission to Labor & Delivery. To avoid the impact of other factors than GDM on our results, we matched the two groups as best as possible according to gestational age, maternal age, and pre-pregnancy BMI. This resulted in an average BMI in the overweight range also in the group of women with NPs and is therefore higher than we would expect it to be in the general population of women with NPs.

Conclusion

In summary, we have shown that GDM and *in vitro* hyperglycemia induced a reduction of SIRTs in fetal ECFCs and HUVECs. These findings might reflect one underlying mechanism resulting in metabolic and vascular diseases in the offspring of GDM pregnancies. SIRT activators, paeonol and RSV, improved the function of SIRTs in ECFCs, which opens new perspectives for the prevention of adverse long-term effects of a diabetic intrauterine environment. Our findings give new insights into understanding and controlling the increased risk of cardiovascular diseases and other long-term sequelae in individuals originating from pregnancies complicated by GDM. However, further research is needed to explore the underlying cellular and molecular mechanisms and the potential of SIRT activators to improve vascular function.

METHODS
Patients
Ten women with NPs and ten women with GDM pregnancies were recruited for this study and matched for gestational age, maternal age,

and pre-pregnancy BMI. The diagnostic standard for GDM was based on the criteria defined by the "International Association of Diabetes and Pregnancy Study Groups". All women had an oral glucose tolerance test at 24–28 wk of gestation, singleton pregnancies, and normal blood pressure during pregnancy. In addition, all women had no evidence of illnesses known to affect glucose metabolism, e.g., no clinical history of preexisting diabetes, renal, hypertensive, or other vascular disease, and none were on medication known to affect glucose metabolism. All patients were nonsmokers by self-report. All participants in the study gave written informed consent. The Ethical Committee at Hannover Medical School approved this study.

Isolation of ECFCs and HUVECs
Umbilical cord and umbilical cord venous blood were collected immediately after delivery. ECFCs from cord blood were isolated as previously described (53). The culture medium (complete endothelial growth medium, EGM-2) was endothelial basal medium 2 (Lonza, Walkersville, MD) with 10% fetal calf serum (Biochrom AG, Berlin, Germany) and 1% penicillin/streptomycin (Biochrom AG) supplemented with EGM-2 SingleQuots Kit (Lonza). The medium was changed daily for 10 d and then every other day until the first passage. Colonies of ECFCs appeared between 5 and 30 d of cultivation and were identified as circumscribed monolayers of more than 50 rapidly proliferating, cobblestone-appearing cells. ECFCs were used at 80–90% confluence at passage 4 or 5.

HUVECs were harvested from fresh human umbilical cord vessels within 1–5 days of delivery. Briefly, umbilical veins were cannulated with blunt needles and rinsed with PBS. Then type I collagenase (180 units/mg, GIBCO, Invitrogen, CA) was infused. Segments were clamped at both ends and placed in an incubator at 37 °C for 30 min. Segments were rinsed with PBS, and the cell suspension was centrifuged. The cell pellet was resuspended and plated in tissue culture flasks in endothelial cell growth medium with supplemental mix (Promocell, Heidelberg, Germany) containing 10% fetal calf serum and 1% penicillin/streptomycin. Passage 5 was used for further experimentation.

Immunofluorescence and flow cytometry were used to confirm the ECFC phenotype as previously described (53). Purity of the HUVEC cultures was determined by flow cytometric analysis (Partec GmbH, Münster, Germany) using the fluorescein-isothiocyanate–conjugated monoclonal anti-human-endothelial cell antibody CD31 and the R-phycoerythrin–conjugated monoclonal anti-human-fibroblast antibody CD90 (clone WM59 and 5E10; Dako Cytomation GmbH, Hamburg, Germany).

Treatment Conditions
Hyperglycemia treatment included a 7-day exposure of NP ECFCs or HUVECs with supplemental D-glucose (Sigma-Aldrich, Seelze, Germany) at 15.0 or 30.0 mM added to complete EGM-2 medium (5.5 mM glucose, standard medium). ECFCs and HUVECs were incubated with 50 μM paeonol (Sigma-Aldrich, St. Louis, MO) resuspended in dimethylsulfoxide (Sigma-Aldrich) or 1 μM RSV (Enzo Life Sciences, NY) resuspended in ethanol for 24 h before collection. The concentrations used of RSV and paeonol were chosen according to published literature, in which a relevant or optimal dose has not been established by human studies and will vary depending on the effect being studied (14,15). Control cells were mock treated with ethanol.

NAD⁺ Measurement
Cells were resuspended in HEPES buffer (Sigma-Aldrich) with a concentration of 500,000 cells/ml. The measurement was performed as previously described (54). The absorption was measured at 340 nm using the Infinite M200pro spectrophotometer (Tecan, Maennedorf, Switzerland).

SIRT Activity
Maximal SIRT activity was determined as per the manufacturer's recommendations using the SIRT1 Fluorometric Drug Discovery Kit BML-AK555 (Enzo Life Sciences, Farmingdale, NY) and the SIRT3 Fluorimetric Drug Discovery Kit BML-AK557 (Enzo Life Sciences). Samples (cells in HEPES buffer at a concentration of 500,000 cells/ml) were diluted at a ratio of 1:3. The signal was measured with the Infinite

M200pro spectrophotometer (Tecan) at an excitation of 365 nm and at an emission of 465 nm.

Western Blot Analysis
After trypsinization, cells were lysed in 3× Laemmli buffer (Tris HCl 2M 4.725 ml, glycerol 20% 7.5 ml, sodium dodecyl sulfate 10% 30 ml, 2-mercaptoethanol 4.5 ml, H₂O 3.275 ml, bromophenolblue 500 μg, pH = 6.8, 20 μl/100,000 cells). Ten microliter of protein were separated on a sodium dodecyl sulfate containing 10% polyacrylamide gel, then transferred to nitrocellulose membranes, and incubated with blocking buffer (Odyssey Li-Cor Biosciences, Lincoln, NE) containing 5% bovine serum albumin (GERBU Biotechnik GmbH, Heidelberg, Germany) for 1 h at room temperature. The membranes were subsequently incubated overnight at 4 °C with primary antibodies (SIRT 1 diluted 1:250, SIRT 3 diluted 1:250, β-actin as internal control diluted 1:2,000, all rabbit polyclonal, Millipore, Temecula, CA), washed in Tris Buffered Saline with 0.1% Tween-20 (TBS-T) 4× for 5 min, and then incubated with secondary antibody (goat anti-rabbit IRDye 800CW, 0.5 mg, 1:20000, Odyssey Li-Cor Biosciences) for 1 h. After washing with TBS-T 3× for 5 min and TBS 1× for 5 min, Odyssey Fc Dual-Mode Imaging System (Li-Cor Biosciences) was used to detect the bands and estimate the density of proteins. For the analysis of β-actin, the membranes were stripped and reprobed with anti-β-actin antibody to account for protein loading variations.

RNA Isolation and Real-Time RT-PCR
Total RNA was isolated using RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. One microgram of RNA was reverse-transcribed using Omniscript reverse transcription kit (Qiagen). SYBR Green PCR Master Mix (Life technologies, Grand Island, NY) was used for real-time RT-PCR according to the manufacturer's instructions in 7900HT Fast Real Time PCR System (Applied Biosystems, Foster City, CA). cDNA templates were diluted 1:30. The program involved initially 15 min at 95 °C, at 60 °C for 30 s, at 72 °C for 30 s for 45 cycles afterwards. Relative quantification of the signals was performed by normalizing the signals of different genes with the average of all internal genes. Signal intensities in the control lanes were arbitrarily assigned a value of 1.0. The primer sequences (forward/reverse, Eurofins, Brussels, Belgium) used were as follows: *SIRT 1*, 5′- CAA CTT GTA CGA CGA AGA C-3′/ 5′-TCA TCA CCG AAC AGA AGG-3′; *SIRT 3*, 5′- CAG TCT GCC AAA GAC CCT TC-3′/ 5′- AAA TCA ACC ACA TGC AGC AA-3′; *SIRT 4*, 5′-GCT GTG AGA GAA TGA AGA TGA GC-3′/5′- CTT GGA AAG GGT GAT GAA GCG-3′; *β-actin*, 5′-TTC CTG GGC ATG GAG TC-3′/5′- CAG GTC TTT GCG GAT GTC-3′; *hHPRT 1*, 5′-GCT GAC CTG CTG GAT TAC-3′/ 5′-TGC GAC CTT GAC CAT CTT-3′; GAPDH, 5′-TGC ACC ACC AAC TGC TTA GC-3′/ 5′-GGC ATG GAC TGT GGT CAT GAG-3′.

Statistical Analysis
Demographic data were analyzed using unpaired *t* test and are expressed as means and SD or percentages. Differences in experimental data between two groups were compared by paired Wilcoxon-signed rank test for repeated measurements. Datasets with three or more groups were statistically analyzed by Kruskal–Wallis test and data are presented as mean fold changes and SD compared with controls. Controls were NPs, 5.5-mM, or mock-treated NPs or GDM samples as appropriate. All calculations were performed using Prism 6 software package (GraphPad Software, La Jolla, CA) with *P* less than 0.05 considered statistically significant.

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