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Inhibition of Endothelial p53 Improves Metabolic Abnormalities Related to Dietary Obesity

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SUMMARY

Accumulating evidence has suggested a role for p53 activation in various age-associated conditions. Here, we identified a crucial role of endothelial p53 activation in the regulation of glucose homeostasis. Endothelial expression of p53 was markedly upregulated when mice were fed a high-calorie diet. Disruption of endothelial p53 activation improved dietary inactivation of endothelial nitric oxide synthase that upregulated the expression of peroxisome proliferator-activated receptor-γ coactivator-1α in skeletal muscle, thereby increasing mitochondrial biogenesis and oxygen consumption. Mice with endothelial cell-specific p53 deficiency fed a high-calorie diet showed improvement of insulin sensitivity and less fat accumulation, compared with control littermates. Conversely, upregulation of endothelial p53 caused metabolic abnormalities. These results indicate that inhibition of endothelial p53 could be a novel therapeutic target to block the vicious cycle of cardiovascular and metabolic abnormalities associated with obesity.

INTRODUCTION

The transcriptional factor p53 is a tumor suppressor protein that is activated by various stresses, such as oncogenic stimuli, hypoxia, and oxidative stress (Serrano and Blasco, 2001; Stewart and Weinberg, 2006). It is thought to be a defensive mechanism against malignant transformation. Accumulating evidence has suggested a role of p53 activation in various age-associated conditions, including vascular senescence (Chen and Goligorsky, 2006; Minamino and Komuro, 2007, 2008), heart failure (McTernan et al., 2003; Plutzky, 2011; Sano et al., 2007), and diabetes (Chang et al., 2004; Minamino et al., 2009). Atherogenic factors like oxidized low density lipoprotein and angiotensin II have been shown to upregulate cell-cycle regulators, including p53, contributing to vascular dysfunction (Minamino and Komuro, 2007, 2008). It has also been reported that vascular p53 is activated in patients with diabetes (Brodsky et al., 2004; Montooth et al., 2003; Orimo et al., 2009) and the development of cardiovascular complications is accelerated by this disease (Beckman et al., 2002; Puigserver et al., 1998). However, it remains unclear whether activation of vascular p53 contributes to metabolic abnormalities associated with obesity.

Central obesity and associated inflammation of visceral fat are thought to play a major role in the development of insulin resistance (Gordon, 2007; Hotamisligil, 2006). There is evidence that microvascular dysfunction also promotes insulin resistance by impairing glucose uptake into skeletal muscle (Clark, 2008; Jonk et al., 2007). The initial human study demonstrated that insulin-stimulated vasodilation contributes significantly to insulin-mediated glucose uptake, which is impaired in patients with type 2 diabetes (Laakso et al., 1990). Subsequent studies have shown that an insulin-stimulated increase in the number of perfused capillaries in the skeletal muscles, a phenomenon known as capillary recruitment, has a more important influence on glucose uptake than the insulin-induced increase of total blood flow (Clark et al., 2003). In individuals with insulin resistance, the influence of insulin on microvascular perfusion is markedly impaired (Czernichow et al., 2010; Muris et al., 2012), suggesting that insulin regulates glucose uptake and insulin sensitivity via a feedforward mechanism. Consistent with this notion, capillary recruitment and glucose tolerance are impaired in mice with endothelial deletion of Irs-2 (Kubota et al., 2011). In contrast, mice with endothelial cell-specific insulin receptor knockout do not exhibit glucose intolerance (Vicent et al., 2003). Moreover, the development of microvascular dysfunction precedes the onset of insulin resistance (Clark, 2008), indicating that additional mechanisms may contribute to vascular dysfunction associated with diabetes.

In the present study, we investigated the role of endothelial p53 in glucose homeostasis. We found that a high-calorie diet

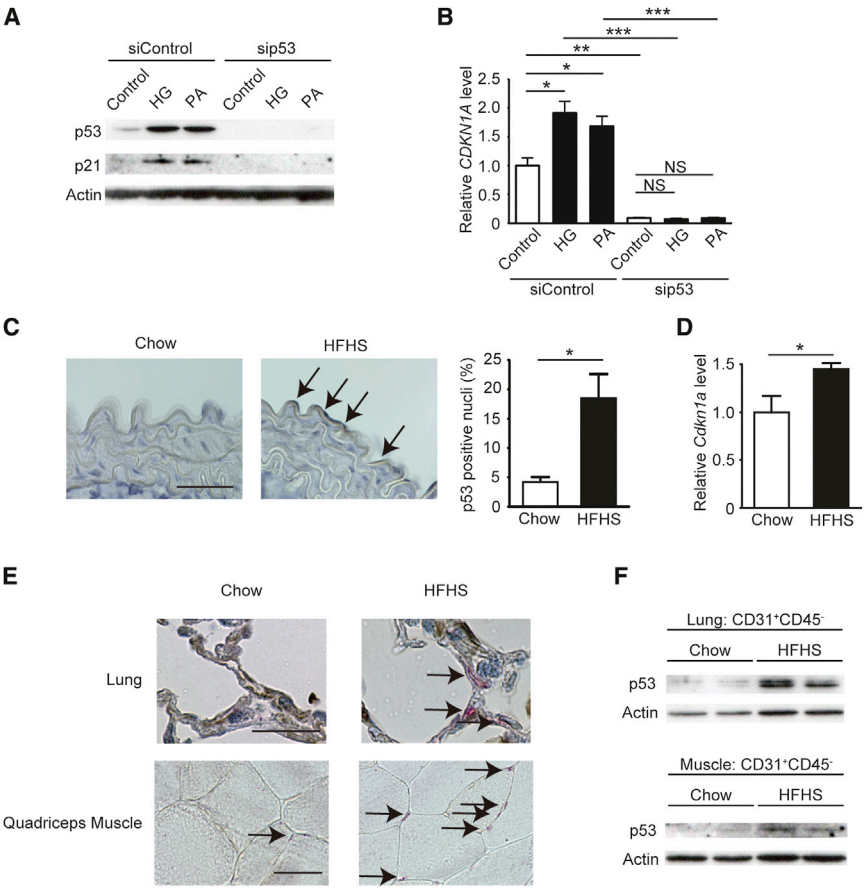


Figure 1. Upregulation of Endothelial p53 under Diabetic Conditions

(A) Human umbilical vein endothelial cells (HUVECs) transfected with siRNA targeting p53 (sip53) or control siRNA (siControl) were treated with high glucose (HG) or with palmitic acid (PA) for 24 hr. Expression of p53 and p21 was examined by western blot analysis.

(B) Real-time PCR assessing the expression of *CDKN1A* (p21) in HUVECs prepared as in Figure 1A (n = 3).

(C and D) Immunohistochemistry for p53 (C) and real-time PCR assessing expression of *Cdkn1a* (p21) (D) in the aorta of mice fed a normal chow (Chow) or a high-fat/high-sucrose (HFHS) diet (n = 5). Arrows indicate p53-positive endothelial cells (Brown).

(E) Immunohistochemistry for isolection B4 (Brown), a marker of endothelial cells, and p53 (Red) in lung and quadriceps muscle tissue of mice prepared as in Figure 1C.

(F) Expression of p53 was examined by western blot analysis in endothelial cells isolated from the lungs and lower limb skeletal muscles of mice fed chow or an HFHS diet. Scale bar, 20 μm. *$p < 0.05$, **$p < 0.01$. Data are shown as the means ± SEM.

increased endothelial expression of p53. Despite no difference of food intake, deletion of endothelial p53 significantly improved fat accumulation and insulin resistance in a dietary obesity model. In addition, endothelial p53 negatively regulated the activation of endothelial nitric oxide synthase (eNOS), which modulates the expression of peroxisome proliferator-activated receptor-γ coactivator-1α (Pgc-1α) in skeletal muscle, so mitochondrial biogenesis was increased by endothelial p53 deletion in this model. Furthermore, we found that endothelial p53 deficiency led to an increase of glucose uptake into skeletal muscle by upregulating glucose transporter (Glut) 1 expression in endothelial cells. Conversely, overexpression of endothelial p53 led to impairment of glucose metabolism. These findings indicate that inhibition of endothelial p53 improves metabolic abnormalities by increasing energy consumption and glucose uptake. They also suggest that dietary activation of endothelial p53 may be a cause of vascular dysfunction that induces insulin resistance in diet-induced obesity.

RESULTS

Metabolic Abnormalities Increase p53 Activity in Endothelial Cells

Features of the diabetic state, such as hyperglycemia and hyperlipidemia, have been shown to cause DNA damage in endothelial cells (Minamino and Komuro, 2007, 2008). In agreement with these reports, we found that exposure of human endothelial cells to high glucose or palmitic acid upregulated the expression of p53 protein (Figure 1A). We also confirmed that these stimuli significantly increased the expression of *cyclin-dependent kinase inhibitor 1a* (*CDKN1A*, known as p21) at both the mRNA and protein levels in a p53-dependent manner (Figures 1A and 1B), leading to cell-cycle arrest in G1 phase (Figure S1A). Likewise, the endothelial expression of p53 protein and *Cdkn1a* mRNA was upregulated in the aorta when the mice were fed a high-fat/high sucrose diet (Figures 1C and 1D). Increased expression of p53 was also observed in microvessels of the lungs and skeletal muscle in mice on a high-calorie diet (Figure 1E). We subsequently examined p53 expression in endothelial cells ($CD31^+$ $CD45^-$) isolated from these tissues and confirmed that expression of endothelial cell-specific genes was significantly increased in the isolated cell fractions compared with whole samples or depleted fractions (Figures S1B and S1C). Moreover, expression of p53 protein was significantly higher in endothelial cells isolated from the tissues of mice fed a high-calorie diet compared with cells from mice on a normal diet (Figure 1F). These findings suggested that a high-calorie state activates p53 in endothelial cells.

Dietary Endothelial p53 Activation Contributes to the Development of Metabolic Abnormalities

To further investigate the role of endothelial p53 in glucose homeostasis, we established endothelial cell-specific p53 knockout (EC-p53 KO) mice (Tie2-Cre; $Trp53^{loxP/loxP}$). Expression of p53 and its target genes (including *Cdkn1a*) were significantly decreased in organs with a high vascularity, including the

lungs (Figure S2A). There were no differences of body weight and glucose metabolisms between EC-p53 KO mice and their control littermates on a normal diet (Figures 2A and 2B). In contrast, EC-p53 KO mice had a significantly lower body weight compared with control mice when fed a high-calorie diet (Figure 2A). Furthermore, compared with control littermates, EC-p53 KO mice showed improvement of insulin sensitivity and glucose tolerance along with a lower plasma insulin level (Figures 2C and 2D) and less accumulation of both visceral and subcutaneous fat, whereas their lean mass was unchanged (Figures 2E and S2B) and there was no difference of dietary intake between the two groups (Figure 2F). Insulin-induced phosphorylation of Akt was restored in the liver, skeletal muscle, and epididymal fat of EC-p53 KO mice (Figures S2C–S2E). Expression of proinflammatory cytokines was also downregulated in fat tissue of EC-p53 KO mice (Figures 2G and S2F). Although we did not find any differences of core body temperature or locomotor activity between EC-p53 KO mice and their control littermates (Figure 2H), oxygen consumption was significantly increased by disruption of endothelial p53 expression (Figure 2I). These results indicated that deletion of endothelial p53 attenuates fat accumulation and weight gain due to a high-calorie diet by increasing energy expenditure and therefore improves metabolic abnormalities related to dietary obesity.

Because *Tie2* can be expressed by hematopoietic cells (Tang et al., 2010), we additionally established Pdgfb-Cre-ER; *Trp53*$^{loxP/loxP}$ mice as a model of endothelial cell-specific p53 deletion. In this model, the efficiency of tamoxifen-induced Cre recombinase activity has previously been tested with ROSA26-lacZ reporter mice, revealing that recombination was achieved in most of the endothelial cells of the capillaries and small arterioles in adult animals (Benedito et al., 2009; Claxton et al., 2008). Although endogenous Pdgfb is also expressed by nonendothelial cells, the previous study demonstrated that the transgene expression was endothelial cell-specific in various tissues, including skeletal muscle (Claxton et al., 2008). We also confirmed the endothelial cell-specific expression of the transgene in the skeletal muscle of Pdgfb-Cre-ER; *Trp53*$^{loxP/loxP}$ mice, by localizing expression of enhanced green fluorescent protein that is encoded by the transgene of these mice (Figures S2G and S2H). In Pdgfb-Cre-ER; *Trp53*$^{loxP/loxP}$ mice, expression of p53 was significantly decreased in isolated endothelial cells, but not in bone marrow cells (Figure S2I). When fed a high-calorie diet, Pdgfb-Cre-ER; *Trp53*$^{loxP/loxP}$ mice showed less weight gain, as well as improved insulin sensitivity and glucose tolerance, compared with their control littermates similar to Tie2-Cre; *Trp53*$^{loxP/loxP}$ mice (Figures S2J and S2K).

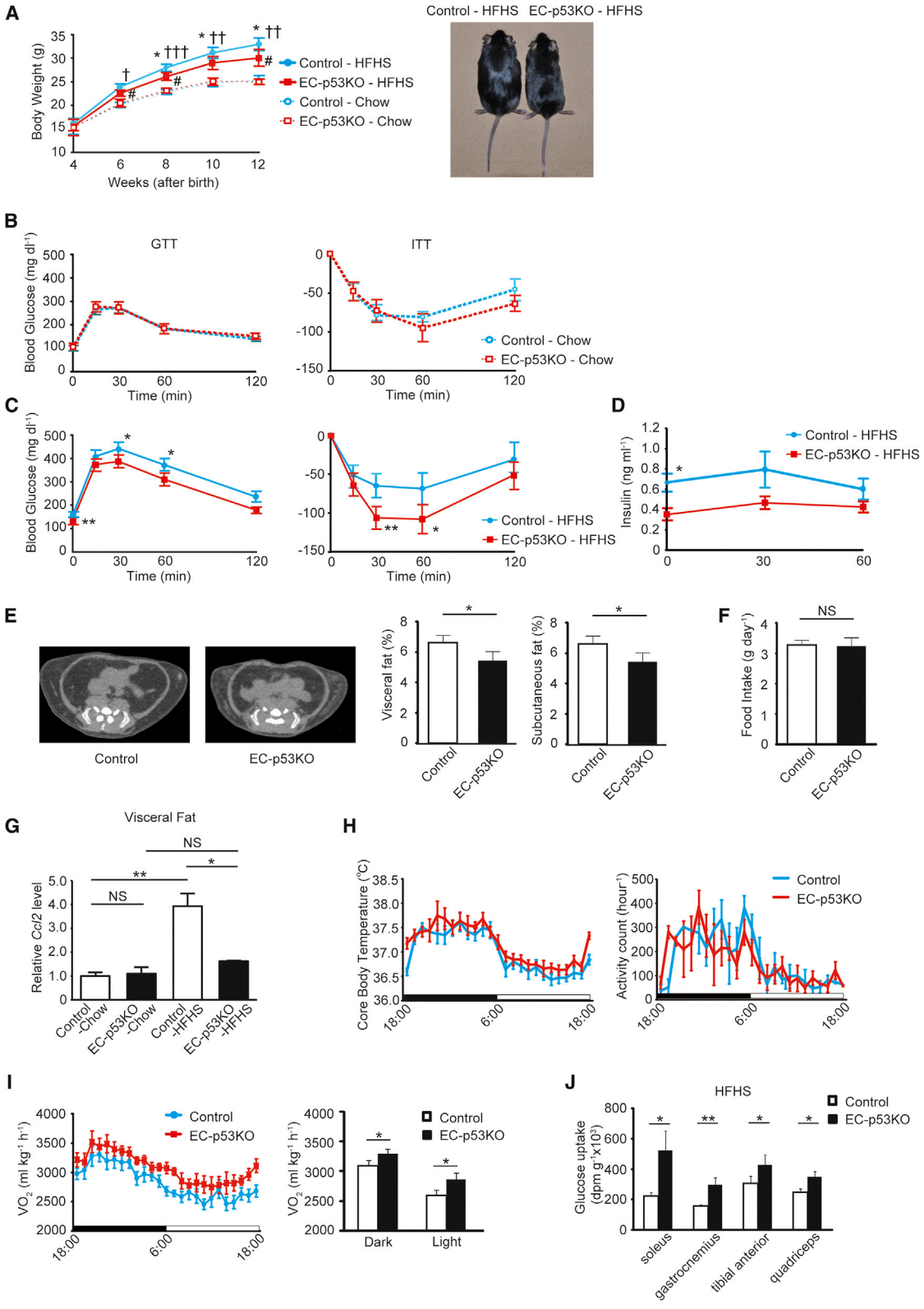
We also found that glucose uptake by skeletal muscle and brown adipose tissue was significantly increased in EC-p53 KO mice on a high-calorie diet, but not when these mice were fed a normal diet (Figures 2J, S2L, and S2M). These results suggest that activation of endothelial p53 reduces the energy consumption and mediates various metabolic abnormalities in diabetes.

Inhibition of p53 Activation in Endothelial Cells Increases Mitochondrial Biogenesis in Skeletal Muscle

We next investigated the mechanisms by which inhibition of endothelial p53 activation increased oxygen consumption in a model of dietary obesity. There were no differences in the weight of brown adipose tissue and the expression of uncoupled protein 1 between the two groups (Figures S3A and S3B). It has been reported that skeletal muscle makes the great contribution to energy expenditure, both at rest and during exercise (Butler and Kozak, 2010). Several studies using genetically modified mice have suggested that an increase of mitochondrial biogenesis in skeletal muscle leads to increased energy expenditure and protection against dietary obesity (Aguilar et al., 2007; Narkar et al., 2008). Accordingly, we next investigated the phenotypic features of skeletal muscle in EC-p53 KO mice.

It has been reported that activation of endothelial p53 leads to various functional abnormalities, including decreased production of nitric oxide (NO), which regulates vascular relaxation, angiogenesis, and mitochondrial biogenesis (Arany, 2008; Chen and Goligorsky, 2006; Leone et al., 2005; Minamino and Komuro, 2007, 2008). We therefore hypothesized that p53-induced inhibition of eNOS might affect oxygen consumption in skeletal muscle. Consistent with this concept, phosphorylation of eNOS was markedly decreased in control mice fed a high-calorie diet, and this decrease was significantly reversed in EC-p53 KO mice (Figure 3A). Likewise, expression of p21 in aortic endothelial cells was markedly increased by a high-calorie diet, and this increase was diminished by deletion of endothelial p53 (Figure S3C). Because NO has been reported to induce mitochondrial biogenesis via upregulation of PGC-1α (Lin et al., 2002), we next examined the effect of endothelial p53 on the expression of Pgc-1α (also known as *Ppargc1a*) and its regulating molecules related to mitochondrial biogenesis, such as *nuclear respiratory factor* (*Nrf*)*-1* and *mitochondrial transcription factor A* (*Tfam*) (Arany, 2008; Arany et al., 2008; Leone et al., 2005), in skeletal muscle cells by using a coculture system. Overexpression of p53 in endothelial cells led to a decrease of phosphorylated eNOS (Figure 3B) that was associated with downregulation of *Ppargc1a*, *Nrf-1*, and *Tfam* in cocultured skeletal muscle cells (Figure 3C). Treatment with an NO donor attenuated the decreased expression of Pgc-1α, Nrf-1, and Tfam (Figure 3C). We also found that Pgc-1α expression and the mitochondrial DNA content were significantly reduced in the skeletal muscle of control mice on a high-calorie diet compared with mice on a normal diet, whereas these abnormalities were improved in EC-p53 KO mice (Figures 3D and 3E). Expression of Pgc-1α-target genes related to the metabolic pathways (in particular the fatty acid oxidation-related pathway) was significantly increased in the skeletal muscle of EC-p53 KO mice (Figure S3D). There was no significant difference of the respiration rate of mitochondria isolated from skeletal muscle between the two groups (Figure S3E). These results suggest that endothelial p53 modulates mitochondrial biogenesis in skeletal muscle by regulating eNOS activity.

In contrast, the vessel density in skeletal muscle and the expression of endothelial cell-specific genes were not altered by inhibition of endothelial p53 (Figures S3F and S3G). Laser Doppler analysis did not detect any difference of blood flow between the two groups (Figure S3H). Moreover, microangiography using fluorescent microspheres showed that microvascular perfusion did not differ between the two groups before and after insulin stimulation (Figure S3I), supporting a crucial role of



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NO-induced mitochondrial biogenesis in improving the metabolism of EC-p53 KO mice. These results raised the possibility that microvascular perfusion was also regulated by NO-independent mechanisms in EC-p53 KO mice.

To further investigate the role of eNOS, we established EC-p53-eNOS double knockout mice (Tie2-Cre; *Trp53*$^{loxP/loxP}$; eNOS $^{+/-}$) and fed these animals a high-calorie diet. Disruption of eNOS inhibited the increase of Pgc-1α expression and the mitochondrial DNA content caused by endothelial p53 deletion (Figures 3F and 3G). Consequently, there was less improvement of metabolic abnormalities acquired by endothelial p53 deletion in EC-p53-eNOS double knockout mice (Figure 3H).

Mechanism of the p53-Induced Decrease of eNOS Phosphorylation in Endothelial Cells

Next, we investigated how p53 induced a decrease of eNOS activity in endothelial cells. Previous studies have demonstrated that the p53-binding element exists in the promoter region of *NOS3* (a gene encoding eNOS) (Mortensen et al., 1999) and that p53 negatively regulates eNOS expression at the transcriptional level (Kim et al., 2008; Kumar et al., 2011). In our models, activation of p53 did not downregulate eNOS protein production, but its level of phosphorylation was decreased (Figures 3A and 3B), suggesting the presence of another regulatory mechanism for eNOS that involves phosphorylation. We therefore examined the expression of various kinases and phosphatases that regulate eNOS phosphorylation (Kukreja and Xi, 2007) and found that p53 suppressed the phosphorylation of Akt, a well-known kinase upstream of eNOS, in endothelial cells (Figure 4A). Conversely, phosphorylation of Akt was increased by knockdown of p53 in human endothelial cells (Figure S4A). We also found that the expression of phosphatase and tensin homolog (PTEN) and pleckstrin homology-like domain, family A, member 3 (PHLDA3) was positively regulated by p53 (Figures 4B, S4A, and S4B), both of which are known to be target genes of p53 that inhibit Akt phosphorylation in cancer cells (Cantley and Neel, 1999; Kawase et al., 2009; Stambolic et al., 2001). Accordingly, we examined the effect of deletion of these genes on eNOS phosphorylation in endothelial cells. The p53-induced decrease of eNOS phosphorylation was not altered by deletion of PHLDA3 (Figure S4C), but knockdown of PTEN reversed the downregulation of phospho-Akt and phospho-eNOS in p53-infected cells (Figures 4A, 4C, 4D, and S4C), suggesting that p53-induced PTEN transactivation negatively regulates the Akt-eNOS pathway.

Endothelial p53 Negatively Regulates Glucose Uptake into Skeletal Muscle by Downregulating GLUT1 Expression

It has been reported that disruption of Glut4 in skeletal muscle impairs glucose uptake and induces insulin resistance (Kim et al., 2005), whereas Pgc-1α upregulates the expression of Glut4 (also known as *Slc2a4*) in skeletal myocytes (Michael et al., 2001). Although EC-p53 KO mice had higher levels of Pgc-1α compared with control littermates, there was no significant difference of Glut4 expression in skeletal muscle between the two groups (Figure 5A). It has also been reported that p53 directly suppresses tumor cell expression of glucose transporters, among which GLUT1 is a major modulator of endothelial glucose transport into skeletal muscle (Russell and Kahn, 2007). We found that overexpression of p53 led to downregulation of GLUT1 (also known as *SLC2A1*) expression in human endothelial cells (Figure 5B). Exposure of these cells to high glucose resulted in the upregulation of p53 expression (Figure 1A) and inhibited glucose transport (Figure 5C). The high glucose-induced decrease of glucose transport in endothelial cells was improved by knockdown of p53 (Figures 5D and S5A), and this effect was dependent on GLUT1 expression (Figures 5D and S5B). Consistent with these in vitro data, the expression of vascular Glut1 (*Slc2a1*) was downregulated in control mice on a high-calorie diet, and this downregulation was reversed by disruption of endothelial p53 (Figures 5E and S5C).

To examine the role of GLUT1 in muscle glucose transport, we treated EC-p53 KO mice with small interfering RNA (siRNA)-targeting *Slc2a1* and analyzed glucose uptake by skeletal muscle. We confirmed that this treatment led to significant downregulation of endothelial gene expression in various tissues, including skeletal muscle, by using a siRNA targeting the endothelial cell-specific gene *Flk-1* (Figure S5D). We found that knockdown of endothelial Glut1 significantly inhibited glucose uptake by skeletal muscle in EC-p53 KO mice (Figure 5F), suggesting that endothelial p53 modulates glucose

Figure 2. Effect of Endothelial p53-Deficiency on Glucose Metabolism

(A) Body weight of EC-p53 KO mice and their littermate controls (Control) on a normal chow (Chow) or a high-fat/high-sucrose (HFHS) diet (n = 5–10). *p < 0.05, Control-HFHS versus EC-p53KO-HFHS; †p < 0.05, ††p < 0.01, †††p < 0.001, Control-Chow versus Control-HFHS; $^{\#}$p < 0.05, EC-p53KO-Chow versus EC-p53KO-HFHS. The right photograph shows the appearances of EC-p53 KO mouse and littermate control after 8 weeks on the HFHS diet.

(B and C) Glucose tolerance test (GTT) and insulin tolerance test (ITT) were examined in 12-week-old mice as prepared in Figure 2A (chow: n = 6, B; HFHS: n = 13, C).

(D) Plasma insulin levels during GTT in mice on the HFHS diet (n = 9).

(E) CT analysis of EC-p53 KO mice and littermate controls (Control) after 8 weeks on the HFHS diet. The graph shows percent of fat tissue/body weight for visceral fat and subcutaneous fat (n = 13).

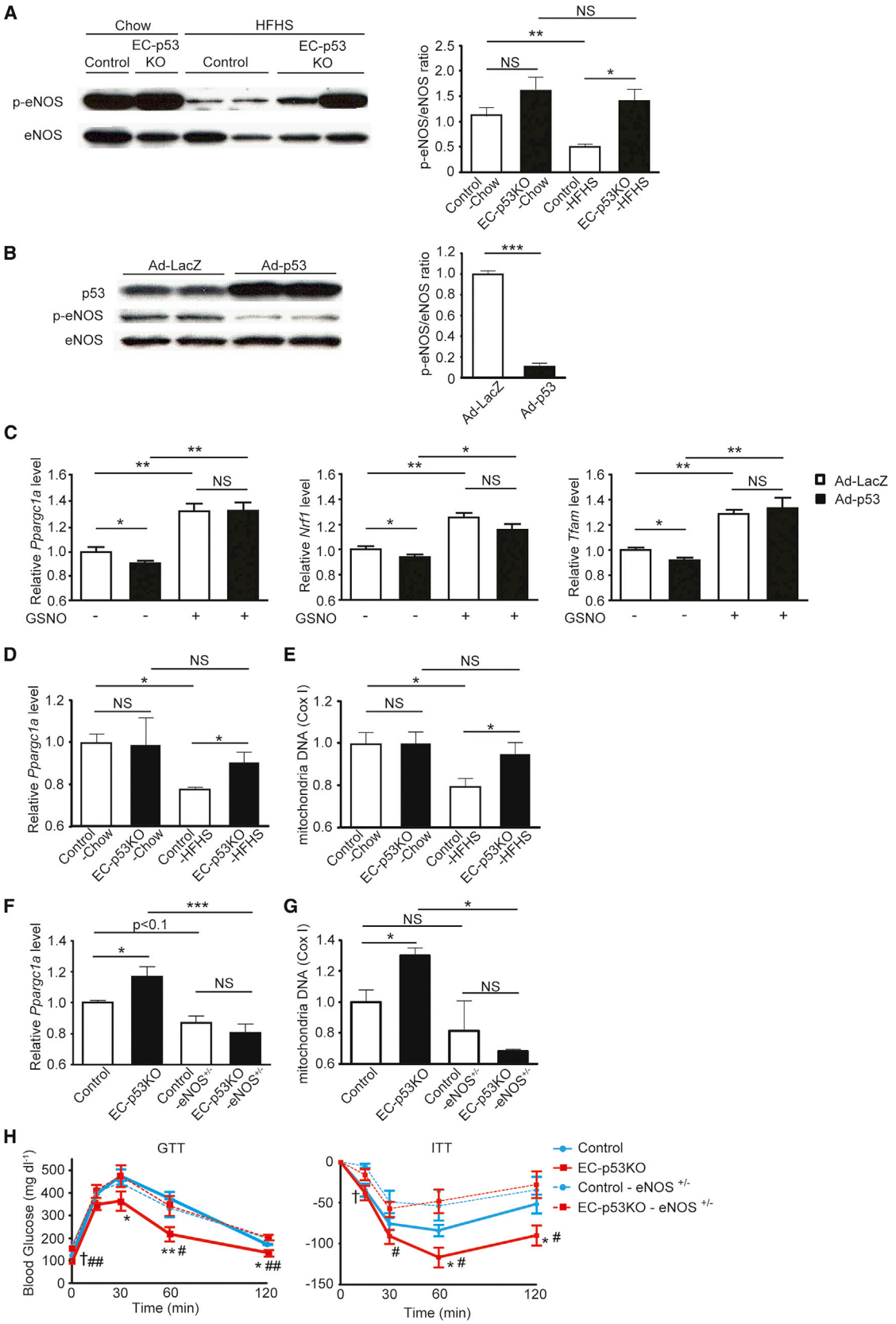
(F) Food intake of EC-p53 KO mice and littermate controls (Control) on the HFHS diet (n = 5).

(G) Real-time PCR analysis for *Ccl2* of the epididymal fat of EC-p53 KO mice and littermate controls (Control) on a normal chow (Chow) or a high-fat/high-sucrose (HFHS) diet (n = 3–4).

(H) Oxygen consumption of EC-p53 KO mice and littermate controls (Control) on the HFHS diet. The graph indicates average oxygen consumption during the light phase (Light) and the dark phase (Dark) (n = 8).

(I) The core body temperature and the activity of EC-p53 KO mice and littermate controls (Control) on the HFHS diet (n = 7).

(J) Glucose uptake by skeletal muscles in lower extremities of EC-p53 KO mice and littermate controls (Control) on the HFHS diet (n = 14). *p < 0.05, **p < 0.01. Data are shown as the means ± SEM.



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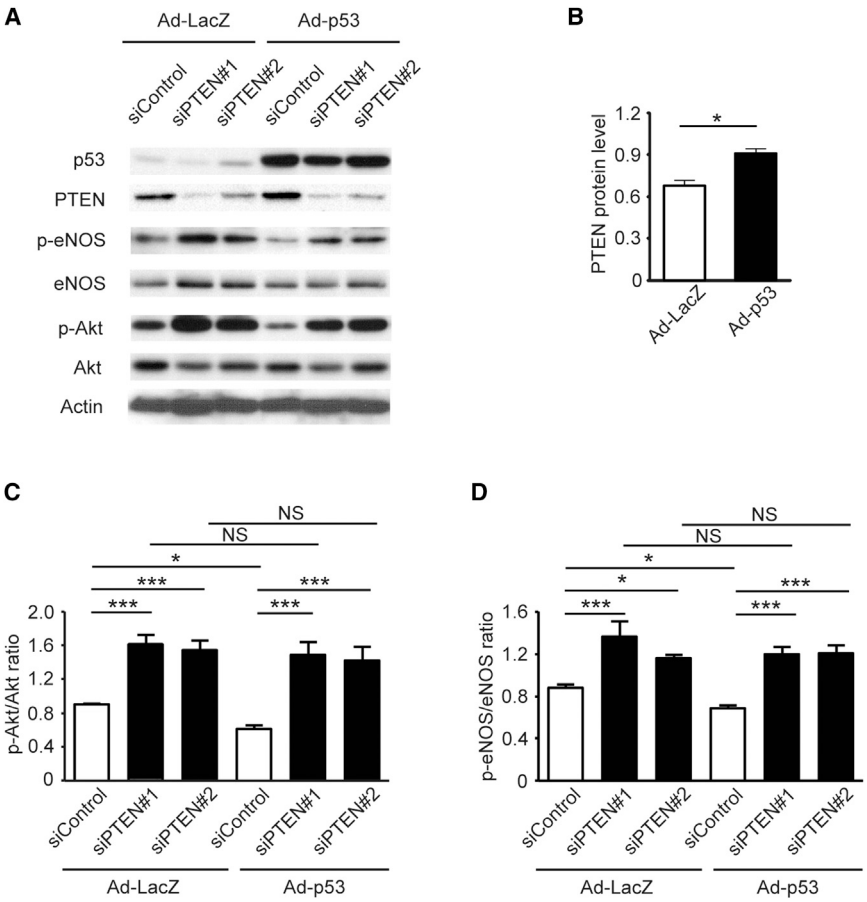

Figure 4. Endothelial p53 Upregulates the Expression of PTEN that Downregulates the Akt-eNOS Pathway

(A) Expression of p53, PTEN, phospho-eNOS, eNOS, phospho-Akt, and Akt were examined by western blot analysis in HUVECs infected with adenovirus encoding LacZ (Ad-LacZ) or p53 (Ad-p53) and transfected with siRNA-targeting PTEN (siPTEN) or control siRNA (siControl). HUVECs were cultured in growth factor-free medium for 24 hr and were harvested after stimulation for 1 hr with growth factors (hEGF, hFGF-B, VEGF, and R3-IGF-1).

(B) Quantitative analysis of expression of PTEN protein levels in HUVECs infected with Ad-LacZ or Ad-p53 (n = 3).

(C and D) Quantitative analyses of phosphorylation levels of Akt (C) and eNOS (D) in HUVECs as prepared in Figure 4A (n = 4). *p < 0.05, **p < 0.01, ***p < 0.001. Data are shown as the means ± SEM.

uptake by skeletal muscle through regulation of Glut1 expression in endothelial cells.

Histological examination showed that Glut1 was predominantly expressed by the endothelium, with its expression being markedly decreased when mice were fed a high-calorie diet (Figure S5C). This decrease of Glut1 expression was attenuated in EC-p53 KO mice (Figure S5C). Although we could not exclude the possibility that deletion of endothelial p53 might regulate Glut1 expression by smooth muscle cells through non-cell-autonomous mechanisms, these results indicated that endothelial p53 mainly influences Glut1 expression in the endothelium.

Presumably, endothelial p53 would be even more important for regulating Glut1 expression in skeletal muscle capillaries, which only have a few pericytes.

Overexpression of p53 by Endothelial Cells Exacerbates Insulin Resistance and Fat Accumulation

The activity of p53 is tightly regulated and immediately diminishes in the absence of critical stress to avoid unwanted consequences. Mdm2 and Mdm4 have been identified as negative regulators of p53, and their essential role in the regulation of p53 expression has been demonstrated in a variety of genetic mouse models (Wade et al., 2010). To test whether upregulation of endothelial p53 causes metabolic abnormalities, we established endothelial cell-specific Mdm4 KO (EC-Mdm4 KO) mice (Pdgfb-Cre-ER; *Mdm4*$^{loxP/loxP}$) on a high-calorie diet. In this model, expression of Mdm4 was deleted in the vascular endothelium by treatment with tamoxifen, leading to upregulation of endothelial p53 expression (Figures 6A and 6B). We initially attempted to establish mice with endothelial cell-specific

Figure 3. Influence of Endothelial p53 Activity on Mitochondrial Biogenesis in Skeletal Muscle

(A) Expression of phosphoendothelial NO synthase (eNOS) and eNOS were examined by western blot analyses in the aortas of EC-p53 KO mice and littermate controls (Control) after 8 weeks on a normal chow (Chow) or a high-fat/high-sucrose (HFHS) diet (n = 3).

(B) Expression of p53, phospho-eNOS, and eNOS were examined by western blot analysis in HUVECs infected with adenovirus encoding LacZ (Ad-LacZ) or p53 (Ad-p53) (n = 3).

(C) HUVECs as prepared in Figure 3B were treated with S-Nitrosoglutathione (GSNO), an NO donor, or vehicle. Expression of *Ppargc1a* (Pgc-1α), *Nrf1*, and *Tfam* was examined by real-time PCR (n = 3).

(D) Real-time PCR analysis assessing expression of *Ppargc1a* (Pgc-1α) in the skeletal muscle of mice as prepared in Figure 3A (n = 5).

(E) Mitochondria DNA contents in the skeletal muscle of mice as prepared in Figure 3A (n = 5).

(F and G) Real-time PCR assessing expression of *Ppargc1a* (*Pgc-1α*) (F) and mitochondria DNA contents (G) in the skeletal muscle of littermate controls (Control), EC-p53 KO mice, eNOS hetero knockout mice (Control-eNOS$^{+/-}$), and EC-p53-eNOS double knockout mice (EC-p53KO-eNOS$^{+/-}$) on the HFHS diet. *p < 0.05, **p < 0.01, ***p < 0.001.

(H) Glucose tolerance test (GTT) and insulin tolerance test (ITT) were examined in 12-week-old mice as prepared in Figure 3F (n = 4–6). *p < 0.05, **p < 0.01, Control versus EC-p53KO; †p < 0.05, Control versus Control-eNOS$^{+/-}$; $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, EC-p53KO versus EC-p53KO-eNOS$^{+/-}$. Data are shown as the means ± SEM.

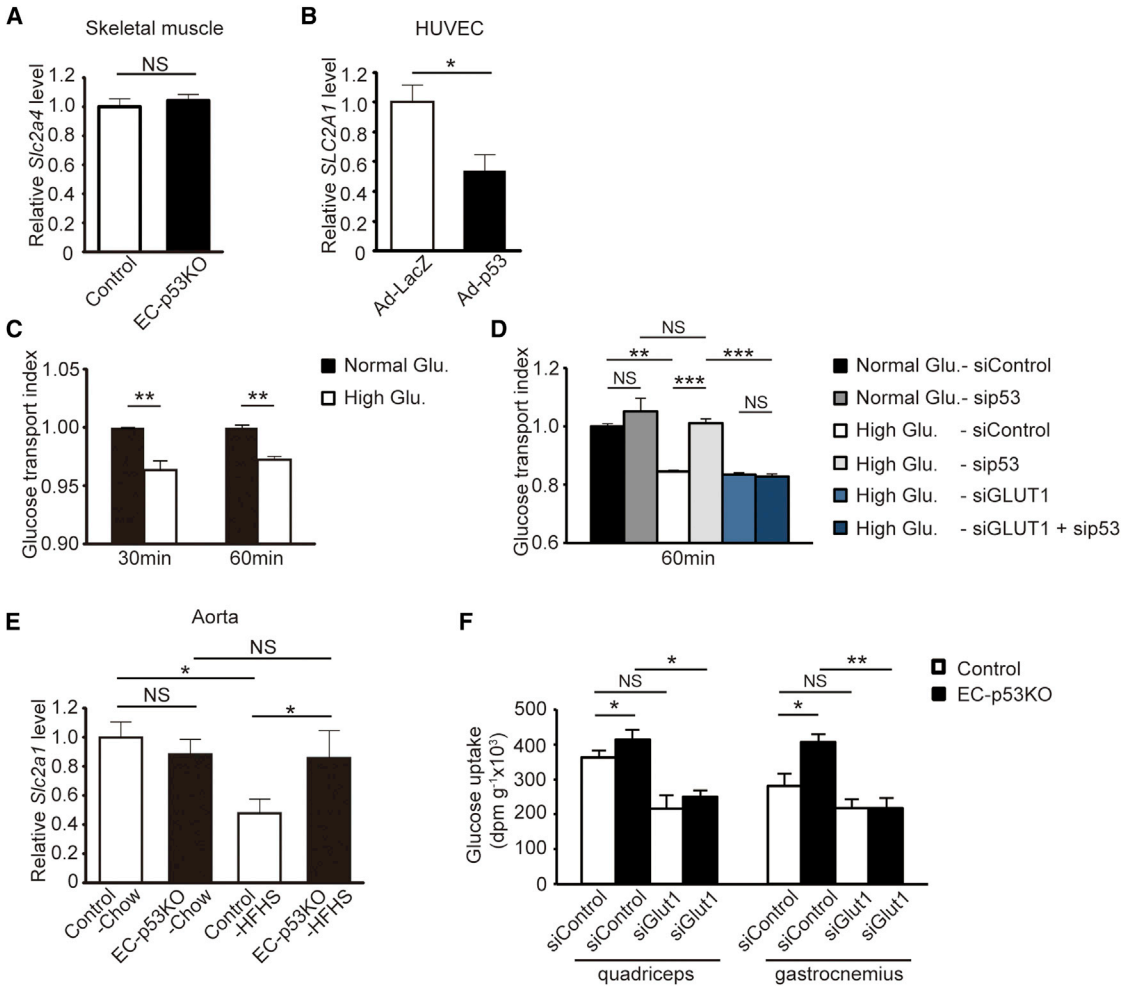

Figure 5. Effect of Endothelial p53 Activity on Glucose Transport into Skeletal Muscle

(A) Real-time PCR assessing expression of *Slc2a4* (Glut4) in the skeletal muscle of EC-p53 KO mice and littermate controls (Control) on the HFHS diet (n = 8).

(B) Real-time PCR analysis assessing expression of *SLC2A1* (*GLUT1*) in HUVECs infected with adenovirus encoding LacZ (Ad-LacZ) or p53 (Ad-p53) (n = 3).

(C) Relative glucose transport index was measured in HUVECs cultured under the normal or high-glucose condition (n = 3) as described in the Experimental Procedures.

(D) Relative glucose transport index in HUVECs transfected with siRNA-targeting p53 or GLUT1 (n = 3).

(E) Real-time PCR analysis for *Slc2a1* (*Glut1*) of the aorta of EC-p53 KO mice and littermate controls (Control) on a normal chow (Chow) or a high-fat/high-sucrose (HFHS) diet (n = 5).

(F) Glucose uptake by skeletal muscle in lower extremities of EC-p53 KO mice and littermate controls (Control) on the HFHS diet treated with siRNA-targeting Glut1 or control siRNA (n = 5–10). *$p < 0.05$, **$p < 0.01$. Data are shown as the means ± SEM.

deletion of Mdm4 by using noninducible Tie2-Cre mice, but we failed to obtain adult Tie2-Cre; $Mdm4^{loxP/loxP}$ mice, presumably due to lethality of this deletion during embryonic development. In contrast to EC-p53 KO mice, phosphorylation of eNOS in the aorta and *Pgc-1α* expression in the skeletal muscle were significantly reduced in EC-Mdm4 KO mice compared with their control littermates (Figures 6B and 6C). Likewise, endothelial *Glut1* expression was markedly downregulated in EC-Mdm4 KO mice (Figure 6D). Consequently, insulin sensitivity and glucose tolerance were significantly impaired by endothelial p53 activation (Figure 6E). We also found that dietary obesity and fat accumulation were exacerbated in EC-Mdm4 KO mice compared with their control littermates (Figures 6F and 6G), even though there was no difference in food intake between the two groups (Figure 6H). We noted that expression of *Ppargc1a* in skeletal muscle was significantly decreased as early as 5 weeks after p53 activation in the endothelium, followed by upregulation of proinflammatory cytokine expression in fat tissue of EC-Mdm4 KO mice (Figures S6A and S6B). These results suggest that endothelial p53 activation decreases mitochondrial biogenesis in skeletal muscle, leading to fat accumulation and inflammation, and exacerbates metabolic abnormalities associated with obesity.

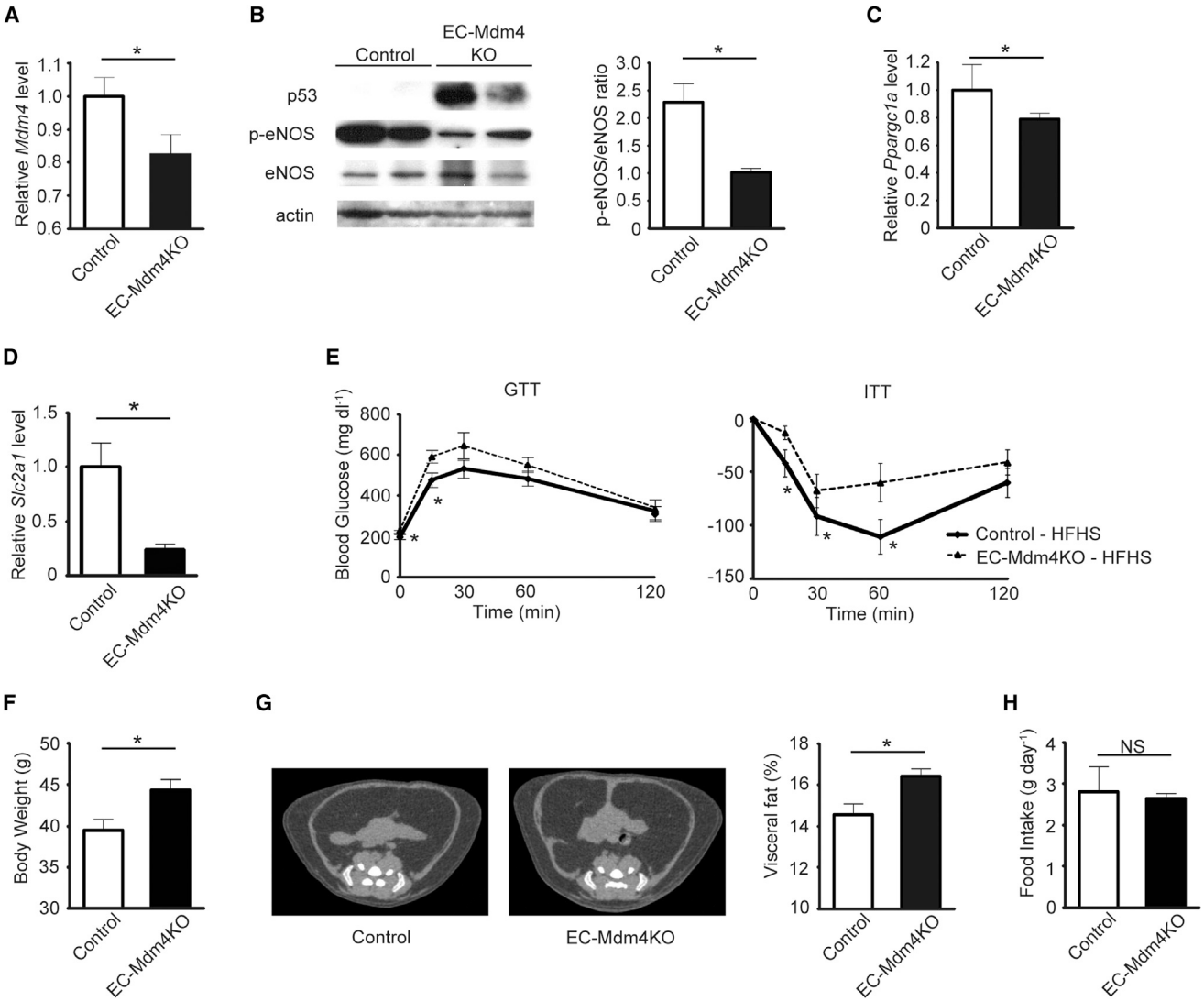

Figure 6. Upregulation of Endothelial p53 Exacerbates Metabolic Abnormalities

(A) Real-time PCR assessing expression of *Mdm4* in the lungs of EC-Mdm4 KO mice and littermate controls (Control) on a high-fat/high-sucrose (HFHS) (n = 5).

(B) Expression of p53, phospho-eNOS, and eNOS was examined by western blot analyses in the aortas of mice as prepared in Figure 6A (n = 3).

(C and D) Real-time PCR assessing expression of *Ppargc1a* (Pgc-1α) in the skeletal muscle (C) and *Slc2a1* (Glut1) in the aorta of mice (D) in 16-week-old mice (8 weeks after gene deletion) prepared in Figure 6A (n = 4–6).

(E) Glucose tolerance test (GTT) and insulin tolerance test (ITT) were examined in 16-week-old mice prepared in Figure 6A (n = 7).

(F–H) Body weight (F), CT analysis (G), and food intake (H) of 16-week-old mice as prepared in Figure 6A (n = 5–7). *p < 0.05. Data are shown as the means ± SEM.

DISCUSSION

Alterations of the metabolic state could directly affect the function of vascular cells via the bloodstream. Endothelial cells exist throughout the vascular system from the great vessels to the capillaries and regulate the tissue microenvironment by producing various molecules, such as nitric oxide and inflammatory cytokines. We observed upregulation of p53 protein expression in various organs/tissues (particularly highly vascular organs) of mice fed a high-calorie diet. We found that the increased expression of p53 was localized to the endothelium and that this change accelerated the onset of metabolic abnormalities, such as obesity and insulin resistance, by reducing energy expenditure and glucose transport. Inhibition of endothelial p53 attenuated fat accumulation and inflammation, leading to improvement of insulin resistance in mice fed a high-calorie diet. Our results do not exclude the possibility that endothelial p53 regulates microvascular perfusion but rather provide an additional insight into how excessive calorie intake promotes vascular dysfunction and thereby induces metabolic abnormalities.

The transcriptional coactivator PGC-1α was first identified through its interaction with nuclear receptor peroxisome proliferator-activated receptor-γ in brown adipose tissue and was found to regulate adaptive thermogenesis (Puigserver et al.,

1998). Recent evidence suggests that PGC-1α may have a critical role in skeletal muscle regulation by modulating a number of gene expression programs (Arany, 2008), including those for mitochondrial biogenesis, angiogenesis, and fiber composition. It has been reported that PGC-1α modulates skeletal adaptations by interacting with various transcription factors, such as NRF-1, NRF-2, the estrogen-related receptors, and myocyte enhancer factor-2 (Arany, 2008). Consistent with our results, forced expression of Pgc-1α in skeletal muscle was reported to increase oxidative type I fibers along with increased expression of mitochondrial markers in mice (Lin et al., 2002). Conversely, Pgc-1α-deficient mice have fewer mitochondria and a lower respiratory capacity in oxidative skeletal muscle (Leone et al., 2005). Furthermore, Pgc-1α-deficient mice show striking failure to restore limb blood flow after an ischemic insult, whereas transgenic expression of Pgc-1α in skeletal muscle has a protective effect (Arany et al., 2008). In contrast, we did not find any changes of vessel density or expression of proangiogenic factors in the skeletal muscle of EC-p53 KO mice, presumably because NO-induced upregulation of Pgc-1α has different transcriptional effects.

Although expression of PGC-1α and mitochondrial genes is coordinately repressed in the skeletal muscles of diabetic patients (Mootha et al., 2003; Patti et al., 2003), the role of skeletal muscle PGC-1α in insulin resistance remains equivocal. For example, mice with Pgc-1α overexpression in skeletal muscle are not sensitized to insulin signaling (Calvo et al., 2008; Miura et al., 2003), although upregulation of Pgc-1α in skeletal muscle is associated with improvement of insulin sensitivity in various genetic models (Iwabu et al., 2010; Oike et al., 2005). It is noteworthy that the expression of Glut4 was significantly downregulated in mice with overexpression of Pgc-1α in skeletal muscle. Thus, increasing Pgc-1α expression by transgenic intervention could have unfavorable effects and may not improve glucose homeostasis in diabetic models. In our study, we found that disruption of endothelial p53 increased glucose uptake by upregulating endothelial Glut1 expression, whereas skeletal muscle expression of Glut4 was unchanged. Hence, endothelial p53 activity may affect various pathways involved in glucose homeostasis rather than simply increasing PGC-1α expression.

We found that p53 plays a critical role in glucose homeostasis by regulating the expression of genes, such as *PTEN* and *SLC2A1*, presumably through its action as a transcription factor. It is possible that p53 also regulates the expression of various molecules secreted by the vascular endothelium and media, which could influence the metabolism of neighboring cells via a paracrine mechanism. Angiogenesis and vascular functions have been shown to modulate obesity and adipose metabolism (Cao, 2013), which could be involved in endothelial p53-induced insulin resistance. Because overexpression of p21 has been reported to alter the expression of various genes, including proinflammatory cytokines (Kunieda et al., 2006; Miyauchi et al., 2004), it is also conceivable that upregulation of p21 expression by a high-calorie diet had an additional influence on glucose homeostasis in our model.

It is generally accepted that the endothelium does not play a rate-limiting role in glucose metabolism. However, there is evidence suggesting that Glut1 has a rate-limiting role in determining endothelial glucose transport in an insulin-independent manner (Alpert et al., 2005; Huang et al., 2012; Rajah et al., 2001). For example, Huang et al. (2012) reported that a decrease of Glut1 expression by endothelial cells in mice reduces glucose uptake into various tissues, including the heart, leading to impaired glucose clearance and glucose intolerance. In the present study, we found that a high-calorie diet downregulated Glut1 expression in endothelial cells in association with the upregulation of p53 expression, whereas the deletion of endothelial p53 inhibited diet-induced downregulation of Glut1 expression and improved glucose uptake by skeletal muscle. For analysis of glucose uptake in mice, we used a minimal dose of 2-deoxyglucose that did not stimulate insulin secretion, so our results demonstrate a role of endothelial Glut1 in insulin-independent glucose transport.

In conclusion, our results indicate that endothelial p53 regulates glucose metabolism by modulating mitochondrial biogenesis and glucose uptake into skeletal muscle. There is evidence that features of the diabetic state, such as hyperglycemia and hyperinsulinemia, lead to upregulation of endothelial p53 expression through mechanisms involving an increase of oxidative stress and activation of stress-response kinases, thus promoting the development of cardiovascular complications (Chen and Goligorsky, 2006; Minamino and Komuro, 2007, 2008). The present findings demonstrate the mechanism of a vicious cycle in which upregulation of endothelial p53 induces metabolic abnormalities that in turn promote cardiovascular dysfunction. Inhibition of endothelial p53 could be a new therapeutic strategy for blocking this vicious circle in obese patients.

EXPERIMENTAL PROCEDURES

Animal Models

All animal study protocols were approved by the Chiba University review board. C57BL/6 mice were purchased from the SLC Japan. Mice were fed a high-fat/high sucrose (HF/HS) diet (Maeda et al., 2002) or normal chow from 4 to 12 weeks of age before metabolic analyses were performed. Mice that expressed Cre recombinase in endothelial (Tie2-Cre) were purchased from Jackson Laboratories. We then crossed Tie2-Cre mice (with a C57BL/6 background) with mice that carried floxed *Trp53* alleles (with a C57BL/6 background) (Marino et al., 2000) to generate endothelial cell-specific p53 knockout mice. These mice used in this study were 12–14 weeks old. To inhibit NOS activity, we crossed endothelial cell-specific p53 knockout mice with eNOS knockout mice that were purchased from Jackson Laboratories to establish endothelial cell-specific p53 and eNOS double knockout mice. We also crossed Pdgfb-Cre-ER mice (with a C57BL/6 background) (Claxton et al., 2008) with mice that carried floxed *Trp53* alleles or floxed *Mdm4* alleles (with a C57BL/6 background) (Grier et al., 2006) to generate other endothelial cell-specific p53 knockout mice or endothelial cell-specific Mdm4 knockout mice. These mutant mice received 10 mg kg^{-1} of tamoxifen (Sigma-Aldrich) intraperitoneally once a day for 5 consecutive days at 8 weeks of age to induce Cre-mediated recombination and were fed the HF/HS diet afterward. After 5–10 weeks, they were analyzed for metabolic parameters.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were grown in EBM-2 medium (Lonza) with 2% FBS, growth factors (hEGF, hFGF-B, VEGF, and R3-IGF-1), heparin, hydrocortisone, ascorbic acid, and antibiotics (50mg ml^{-1} gentamicin and 50 μg/ml amphotericin B). To examine the effects of exposure to high glucose or palmitic acid, HUVECs were cultured in the medium with 30 mM glucose or 500 μM palmitic acid for 24 hr before harvesting. Palmitic acid stock solution (5 mM) was prepared by conjugation with BSA, as described

previously (Cousin et al., 2001). For the control, 5 mM glucose was added to the basal medium, and D-mannitol and BSA solution were also added to adjust the osmotic pressure. Growth factors were not added during exposure to high glucose or palmitic acid. C2C12 mouse myoblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% serum. To induce differentiation into myotubes, C2C12 myoblasts were cultured in DMEM with 2% serum. The coculture assay system was established by using 24-well cell culture inserts (0.4 μm pore size; BD Falcon). HUVECs and C2C12 were seeded on outer well and insert membrane, respectively. S-Nitrosoglutathione (GSNO, 5 μM; Sigma) was used as an NO donor to evaluate the effect of NO.

Physiological Analyses

We housed mice individually and monitored their body weight and food intake. The adiposity of mice was examined using CT (LaTheta, ALOCA) in accordance with the manufacturer's protocol. We performed computed tomography scanning at 2 mm intervals from the diaphragm to the floor of the abdominal cavity. Oxygen consumption was measured by using an O_2/CO_2 metabolic measurement system (model MK-5000; Muromachikikai) as described previously (Oike et al., 2005). The core body temperature and activity of mice were measured by using implantable intra-abdominal radiofrequency probes and receivers (TA10TA-F20 and RPC-1; Data Sciences International) (Barber et al., 2004). The data were sampled in a continuous mode and analyzed by Dataquest ART2.1. Blood flow in skeletal muscle was measured with a laser Doppler perfusion analyzer (moorLDI2-IR; Moor Instruments) in lower limbs denuded of skin.

Laboratory Tests

For the intraperitoneal glucose tolerance test, mice were fasted overnight and were given glucose at a dose of 1 g kg^{-1} (body weight). For the insulin tolerance test, mice were given human insulin intraperitoneally (1 U kg^{-1} body weight). Tail vein blood was collected at 0, 15, 30, 60, and 120 min after administration, and blood glucose levels were measured with Antisense III (HORIBA), whereas plasma insulin levels were measured by insulin immunoassay (Morinaga).

Viral Infection and Transfection

Small interfering RNA (siRNA)-targeting p53 or Glut1 was purchased from Invitrogen (designed by BLOCK-iT RNAi Designer) and introduced into human endothelial cells by using Lipofectamine RNAiMax (Invitrogen) in accordance with the manufacturer's instructions. High-titer adenoviral stocks (10^9 plaque-forming units) were generated with the Adeno-X Expression System (Clontech) in accordance with the manufacturer's instructions.

Statistical Analysis

Data are shown as the mean ± SEM. Differences between groups were examined by Student's t test or ANOVA followed by Bonferroni's correction for comparison of means. For all analyses, $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.04.046.

AUTHOR CONTRIBUTIONS

M.Y. and T.M. designed and conducted experiments and wrote manuscripts. S. Okada, A. Nakagomi, J.M., I.S, A. Nojima, and Y.Y. performed animal experiments. M.F. and G.L. helped with knockout mouse experiments. H.I., N.K., and S. Ohta measured mitochondrial function. Y.K. analyzed the data. T.M. directed and supervised all aspects of the study.

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