

Article

Global Gene Expression Profiling Reveals Functional Importance of Sirt2 in Endothelial Cells under Oxidative Stress

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Abstract: The NAD⁺-dependent deacetylases Sirt1 and Sirt2 mediate cellular stress responses and are highly expressed in vascular endothelial cells. In contrast to the well-documented protective actions of Sirt1, the role of endothelial Sirt2 remains unknown. Using cDNA microarray and PCR validation, we examined global gene expression changes in response to Sirt2 knock down in primary human umbilical vein endothelial cells under oxidative stress. We found that Sirt2 knock down changed expression of 340 genes, which are mainly involved in cellular processes including actin binding, cellular amino acid metabolic process, transmembrane receptor protein serine/threonine kinase signaling, ferrous iron transport, protein transport and localization, cell morphogenesis, and functions associated with endosome membrane and the trans-Golgi network. These genes and associated functions were largely non-overlapping with those altered by Sirt1 knock down. Moreover, we showed that pharmacological inhibition of Sirt2 attenuated oxidant-induced cell toxicity in endothelial cells. These suggest that Sirt2 is functionally important in endothelial cells under oxidative stress, and may have a primarily distinct role as compared to Sirt1. Our results may provide a basis for future studies aiming to dissect the specific signaling pathway(s) that mediates specific Sirt2 functions in endothelial cells.

Keywords: Sirt1; Sirt2; endothelial cell; oxidative stress; functional genomics; microarray

Abbreviations: eNOS, nitric oxide synthase; NF- κ B, nuclear factor- κ B; HUVEC, human umbilical vein endothelial cell; siRNA, small interfering RNA; qPCR, quantitative polymerase chain reaction; GO, Gene Ontology; GPx, glutathione peroxidase; SOD, superoxide dismutase.

1. Introduction

Mammalian Sirt proteins (Sirt1 to Sirt7) are orthologues of the yeast *SIR2* gene product, an NAD⁺-dependent class III histone deacetylase [1–4]. All Sirt proteins contain a conserved NAD⁺-dependent catalytic core domain of ~275 amino acids [3,4]. Among the seven Sirt proteins identified, Sirt1, 2, and 3 have the highest homology to yeast Sir2 and all exhibit specific protein deacetylase activity [5,6]. In addition to their important roles in aging and metabolic regulation, different studies have suggested that Sirt is also involved in modulating cardiovascular physiology and disease [1,7]. In the heart, for example, Sirt1 and Sirt3 have been implicated in promoting cardiomyocyte survival and preventing cardiac remodeling in response to different stress stimuli [8]. In blood vessels, activation of Sirt functions, especially those of Sirt1, is associated with multiple beneficial effects such as preventing vascular cell senescence, suppressing inflammation, decreasing cellular oxidative stress, and promoting vascular regeneration [1,7]. Moreover, Sirt may also exert cardiovascular protective actions by improving global glucose and lipid metabolism [8].

Endothelial cells have a pivotal role in maintaining the homeostasis of blood vessels. Endothelial dysfunction is recognized as a major cellular basis of the development of many cardiovascular diseases such as hypertension, atherosclerosis and heart failure [9]. Several lines of evidence have indicated that Sirt1 has an important role in modulating endothelial cell functions. In particular, Sirt1 physically interacts with and deacetylates endothelial nitric oxide synthase (eNOS), leading to enhanced eNOS activation [10]. Both *in vitro* and *in vivo* experiments revealed that activation of Sirt1 function led to increases in nitric oxide production and endothelium-dependent vasorelaxation, decreased inflammatory reactions in endothelial cells, and suppressed endothelial cell apoptosis and senescence [1,11–14].

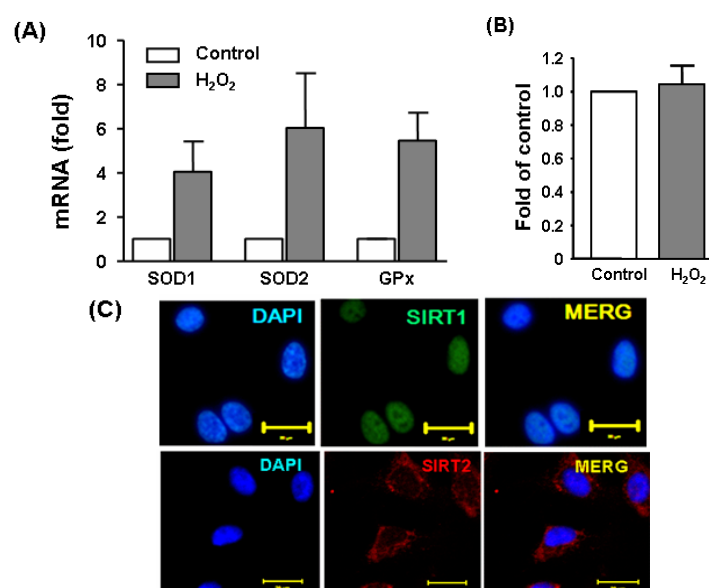
Several Sirt members have pivotal roles in modulating cellular stress responses [2,6]. Under oxidative stress, Sirt1 exhibited broad cytoprotective effects in endothelial cells [7,15–19]. The molecular mechanisms by which Sirt1 produces these cytoprotective actions are not totally understood, while current evidence indicates that activation of the FoxO family members by Sirt1 is likely to be a major signaling route [2,6]. In contrast to Sirt1, the biological functions of Sirt2 in endothelial cells remain unknown [1]. Results from previous studies in non-endothelial cells indicate that the effects of Sirt2 on cell viability under stress are variable and appear to be cell type- and context-dependent [20–27]. Currently, research efforts have been made in the development of selective Sirt2 inhibitors, which may be used as novel chemotherapy agents [28]. Hence, it is important to determine whether and how Sirt2 is involved in modulating endothelial cell homeostasis under stress conditions. Like Sirt1, Sirt2 expression is also responsive to oxidative stress [24]. Moreover, Sirt2 and Sirt1 share a number of common substrates, including FoxO1, FoxO3, nuclear factor (NF)- κ B, histone H3 and p300 [4,8,24,29–32]. These results prompted us to hypothesize that Sirt2 may also have critical

functions in endothelial cells under oxidative stress. Therefore, in the present study we aim to examine the importance of endothelial Sirt2 on a systematic biology basis, by characterizing global gene expression changes after Sirt2 knock down in primary human umbilical vein endothelial cells (HUVECs) using mRNA microarray, an approach that has been used to delineate Sirt1 functions at the whole genome level [33,34].

2. Results and Discussion

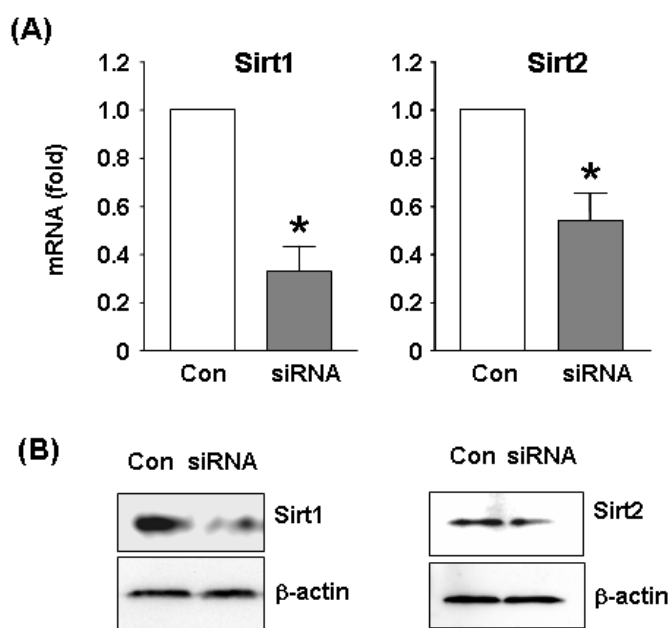
To induce oxidative stress in cultured endothelial cells, we treated the cells with H_2O_2 at 300 μM for 6 h. Induction of a cellular stress response under this condition was demonstrated by the upregulation of detoxification enzymes glutathione peroxidase (GPx) and superoxide dismutases (SOD) as measured by qPCR (Figure 1A). We also confirmed that this treatment protocol did not induce obvious cytotoxic effects as assessed by a MTS cell viability assay (Figure 1B). A previous study demonstrated that Sirt2 expression was upregulated upon oxidant stimulation in adipocytes [24]. To clarify whether this is also the case in endothelial cells, we measured Sirt2 expression with qPCR in H_2O_2 challenged cells. We found that as compared to untreated cells, H_2O_2 increased Sirt2 expression by ~ 2 fold (data not shown). To demonstrate the intracellular localization of Sirt2 and in endothelial cells, we performed immunofluorescence staining. As shown in Figure 1C, the majority of Sirt2 showed a cytosolic localization, which was in contrast to Sirt1, which was mainly nuclear.

Figure 1. (A) An oxidative stress response induced by H_2O_2 (300 μM for 6 h) in cultured human umbilical vein endothelial cells (HUVECs), as revealed by the upregulation of glutathione peroxidase (GPx) and superoxide dismutases (SOD) as measured by qPCR; (B) H_2O_2 treatment at 300 μM did not result in obvious cytotoxicity in the present experimental system. Cell viability was assessed with a MTS-based assay. Data are mean $\pm$ SEM, $n = 3$ –4; (C) pseudo-colored immunofluorescence images showing the intracellular localization of Sirt1 and Sirt2 in untreated HUVEC. Nuclei were counter stained with DAPI (blue). Bar = 20 μm . MERG, merged image.



To clarify the functions of Sirt2 in endothelial cells at the genome level, we performed microarray experiments comparing the global gene expression profiles between control and Sirt2i cells. The efficiency of siRNA-mediated gene knock down was confirmed by qPCR and Western blot (Figure 2). We showed that under oxidative stress conditions, knock down of Sirt2 significantly changed the expression level of 340 genes, with 152 being upregulated and 188 downregulated (Figure 3A and Table 1). GO analysis of the Sirt2-sensitive genes showed that these genes were mainly involved in cellular processes related to actin binding, cellular amino acid metabolic process, transmembrane receptor protein serine/threonine kinase signaling pathways, ferrous iron transport, protein transport and localization, cell morphogenesis involved in differentiation, and functions associated with endosome membrane and the trans-Golgi network.

Figure 2. (A) qPCR and (B) Western blot results showing gene silencing efficiency of siRNA sequences targeting Sirt1 or Sirt2 in H₂O₂-treated HUVECs. A non-specific siRNA was used as control. Data are mean $\pm$ SEM. * $p < 0.05$, student's t -test, $n = 4$ –5. Western blots were representative images from two independent experiments.



To confirm that the altered gene expression after Sirt2 knock down was not caused by non-specific off target effects, we run a parallel experiment by knocking down Sirt1 using the same protocol. Sirt1 gene knock down induced significant alterations of expression of 162 genes (87 upregulated and 75 downregulated) (Figure 3B and Table 1). Among the upregulated genes with Sirt1i, only 31 (36%) overlapped with those changed in Sirt2i cells (20% of those changed in Sirt2i cells). Similarly, among the downregulated genes, only 4 (5%) overlapped with those changed in Sirt2i cells (2% of those changed in Sirt2i cells) (Figure 3C). GO analysis of the Sirt1-sensitive genes showed that these genes were mainly involved in cellular processes related to actin binding, ion binding, endoplasmic reticulum, cellular macromolecule biosynthetic process, cytoskeletal protein binding, and Golgi apparatus, of which the majority was distinct from those related to Sirt2. Further analysis of the differentially expressed genes in relation to disease processes with IPA software revealed that Sirt2-sensitive genes were enriched in categories including infectious disease, connective tissue

disorders, developmental disorder, skeletal and muscular disorders, and cardiovascular disease. In comparison, Sirt1-sensitive genes were mainly enriched in categories including cardiovascular disease, inflammatory response, cancer, organismal injury and abnormalities, and connective tissue disorders. We also compared the two sets of cellular pathways significantly over-represented by Sirt1- or Sirt2-sensitive genes respectively, and found that the pathways affected by Sirt1 were primarily distinct from those affected by Sirt2 (Figure 4A). Moreover, IPA-Tox analysis revealed that Sirt1i and Sirt2i exhibited a discrete pattern of gene enrichment in categories of biological mechanisms that were related to toxicity responses (Figure 4B).

Figure 3. Heat map diagrams illustrating the significantly changed ($p < 0.05$ with a fold change value >1.5 as compared to control cells) genes in HUVECs with gene silencing of (A) Sirt2 and (B) Sirt1, determined by Affymetrix Human Genome U219 Array ($n = 3$ biological replicates each); (C) Venn graphs showing the number of genes up- and downregulated by Sirt1 or Sirt2 gene silencing. A high-resolution version for Figure 3A,B is available online.

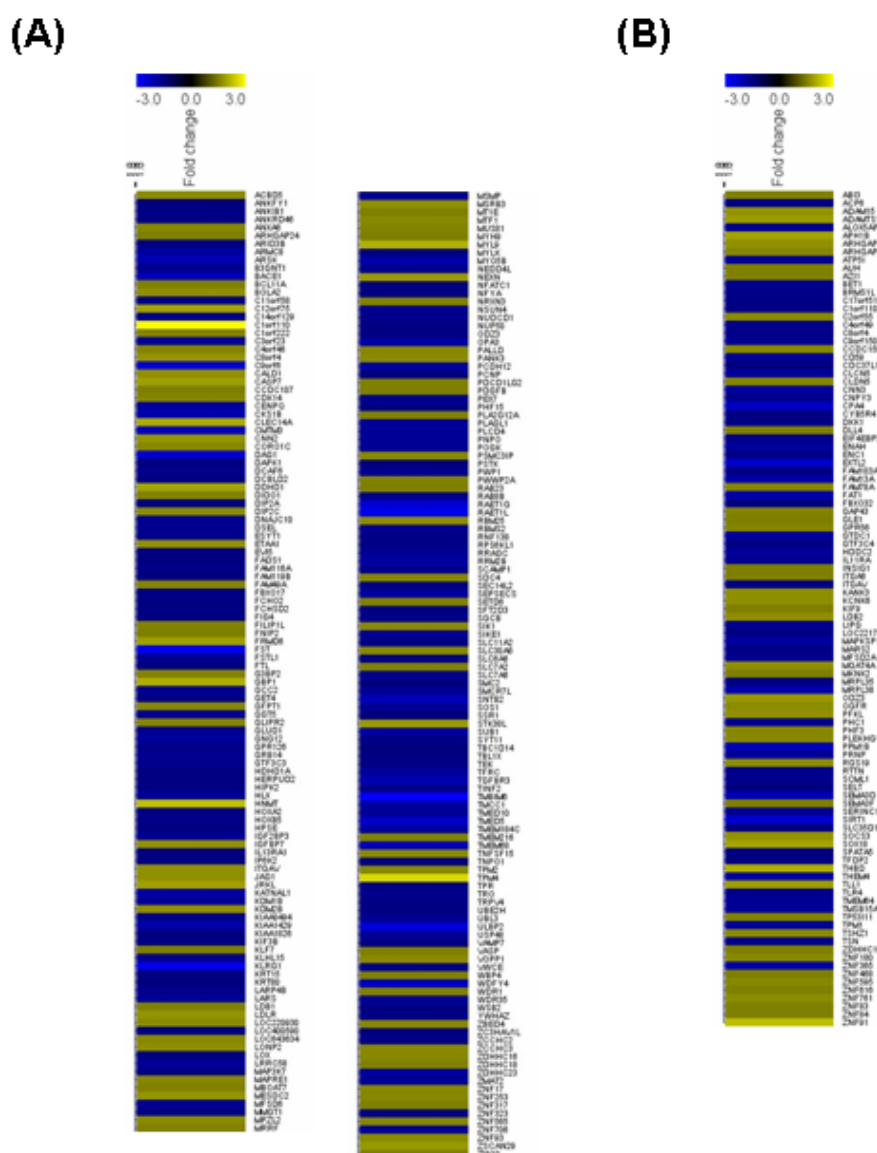


Figure 3. Cont.

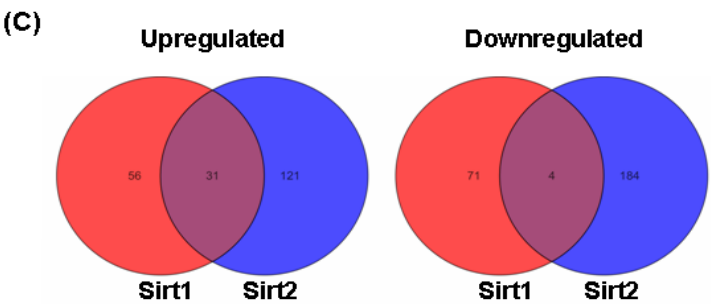
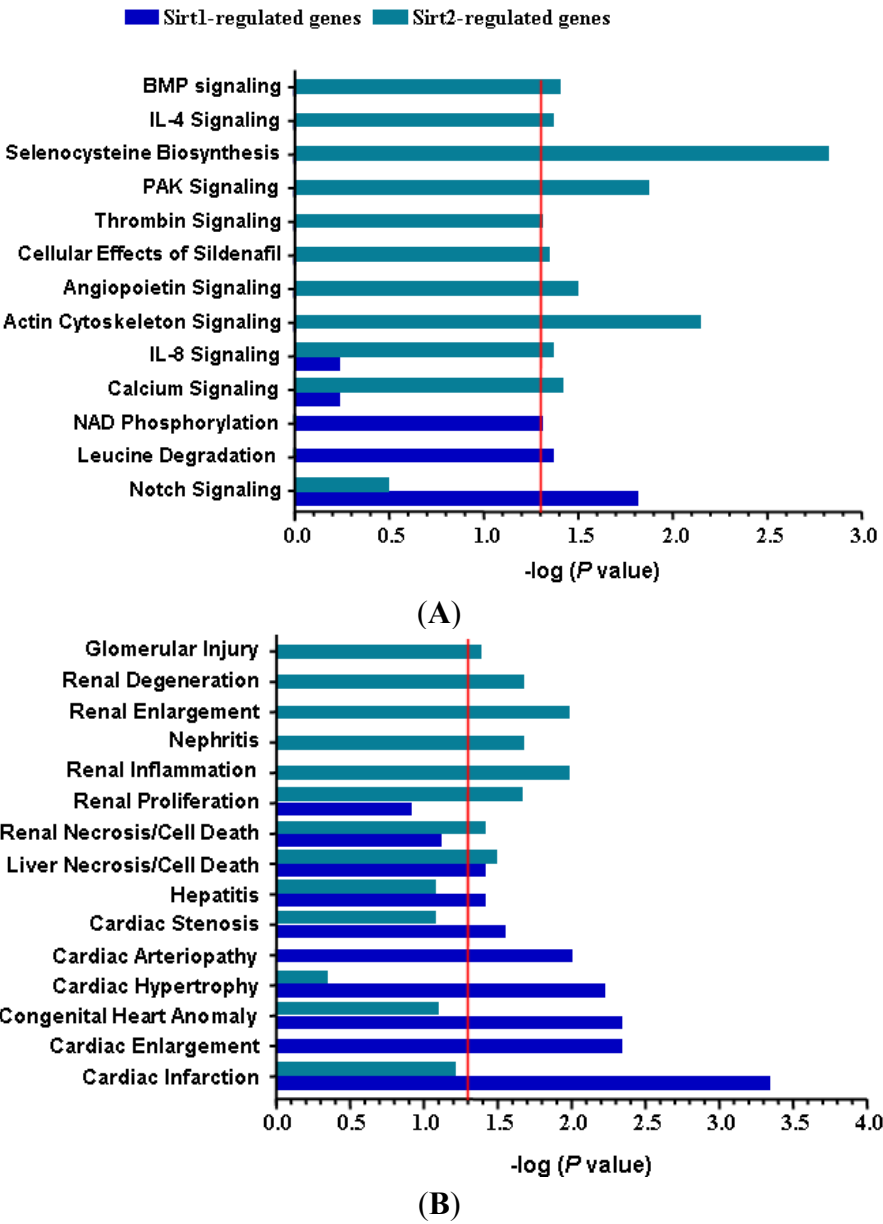


Figure 4. Comparison of the potential categories of (A) intracellular pathways and (B) biological mechanisms related to toxicity responses that were significantly over-represented by Sirt1- or Sirt2-regulated genes respectively in stressed endothelial cells. The red line indicates the threshold of statistical significance. Functional gene enrichment analysis was performed with IPA software.



To validate our microarray data of Sirt2 effects on global gene expression, we carried out qPCR assays on selected genes including *CALD1*, *CASP7*, *CNN2*, *RRAGC*, *ULBP2*. We showed that Sirt2i induced upregulation *CALD1*, *CASP7*, *CNN2* and downregulation of *RRAGC*, *ULBP2* (Figure 5A). These changes were in accordance with the trend as detected by microarray (see Table 1). In contrast, expressions of these genes were not altered in Sirt1i cells (Figure 5B). To further clarify whether Sirt2 was functionally important in endothelial cells under stress, we treated HUVEC cells with a higher concentration (600 μ M) of H_2O_2 for 2 h in the absence and presence of a selective Sirt2 inhibitor AGK2 (from Merck, Darmstadt, Germany) [35]. We found that pre-treatment with AGK2 (10 μ M) attenuated H_2O_2 -induced cell toxicity (Figure 6A), suggesting that under oxidative stress, activation of the Sirt2 pathway might have a detrimental effect on cell viability. In contrast, we showed that pre-treatment with the selective Sirt1 inhibitor EX-527 (10 μ M) (from Merck) increased H_2O_2 -induced cell toxicity (Figure 6B).

Figure 5. Validation of microarray results with qPCR. The expression levels of *CALD1*, *CASP7*, *CNN2*, *RRAGC*, *ULBP2* were measured in (A) Sirt2i cells and (B) Sirt1i cells in the presence of oxidant stress (H_2O_2 300 μ M for 6 h). Gene expression levels were expressed as fold of control. Data are mean $\pm$ SEM. * $p < 0.05$ vs. Con, Student's t -test, $n = 3$ –4.

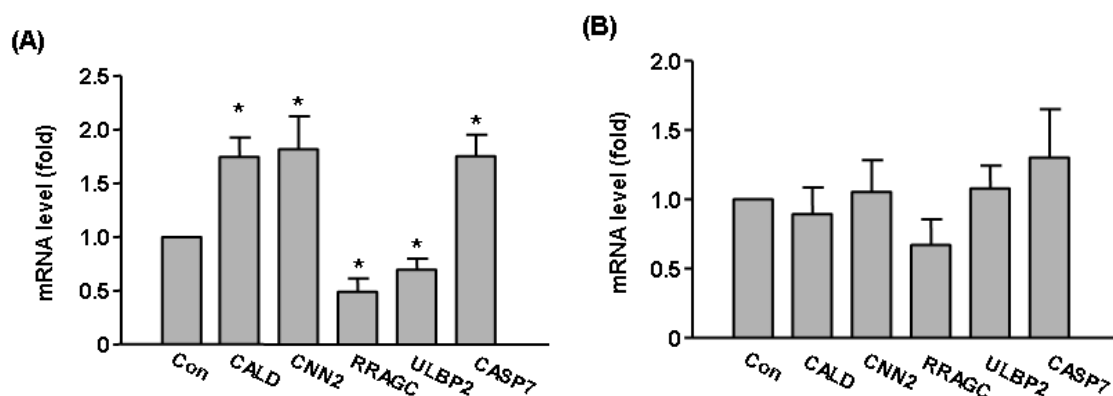
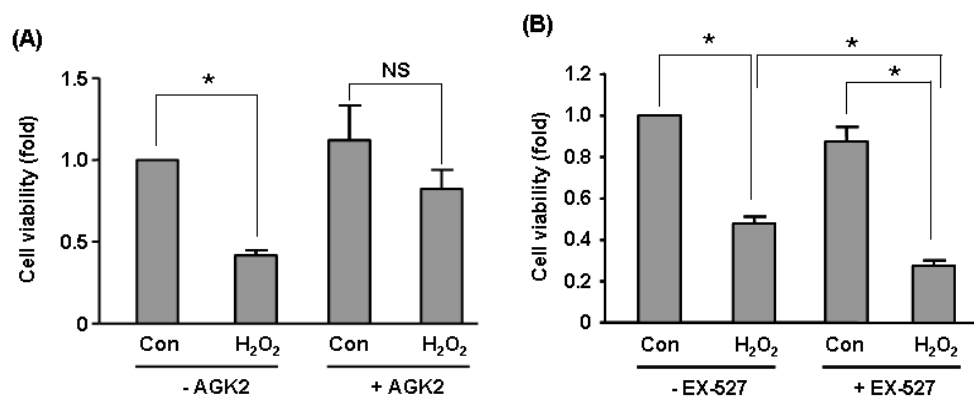


Figure 6. Effects of (A) selective Sirt2 inhibitor AGK2 (10 μ M) and (B) selective Sirt1 inhibitor EX-527 (10 μ M) on H_2O_2 -induced cell toxicity in HUVECs measured with MTS assay. Cells were treated with H_2O_2 (600 μ M) for 2 h in the presence and absence of AGK2 or EX-527 pre-treatment. Data are mean $\pm$ SEM. * $p < 0.05$, one-way ANOVA, $n = 4$ –6. NS: non-significant.



In the present study, we explored the functional importance of Sirt2 in endothelial cells under oxidative stress by measuring global gene expression changes in cells in which Sirt2 was knocked down. We found that Sirt2 gene knock down significantly altered the expression profile of 340 genes, which were involved in different cellular processes (see Table 1). We also confirmed the microarray data with qPCR for selected genes. Gene clustering analysis suggests that Sirt2-sensitive genes in endothelial cells may be involved in regulation of protein transport and localization, cellular amino acid metabolic process, and functions associated with endosome membrane and the trans-Golgi network. These functional annotations are in agreement with findings from cellular function studies showing that Sirt2 may have a pivotal role in modulating cell autophagy, an intracellular mechanism responsible for clearance of damaged proteins and organelles involving activation and mobilization of the endogenous membranous system [36]. Interestingly, a recent study demonstrated that overexpression of Sirt2 inhibited lysosome-mediated autophagic turnover and increased the sensitivity of cells to proteasomal stress-induced cytotoxicity [37]. Conversely, accumulation of ubiquitinated proteins and cytotoxicity in stressed cells were attenuated by Sirt2 knock down. These results indicate that a complex interaction between Sirt2 and autophagic process may be present. In line with these findings, we observed that pharmacological inhibition of autophagy in endothelial cells augmented H₂O₂-induced cell death [38]. Moreover, we found that inhibition of Sirt2 decreased H₂O₂-induced endothelial cytotoxicity (see below). Taken together, we propose that regulation of cellular autophagic processes might be a mechanistic link between Sirt2 and oxidative stress-induced injury in endothelial cells. In addition to the above-mentioned pathways, results from our gene function clustering analysis indicate that Sirt2-regulated genes may also be involved in actin binding, transmembrane receptor protein serine/threonine kinase signaling pathways, ferrous iron transport, and cell morphogenesis involved in differentiation.

Similar to Sirt1, Sirt2 has strong deacetylase activity, and may affect gene expression by modulating functions of multiple transcription factors and co-activators such as FoxO, NF- κ B, p300, and histone [4–6,23,24,29–32]. However, the present gene profiling study showed that the potential intracellular pathways regulated by Sirt2 in stressed endothelial cells were primarily different from those regulated by Sirt1. Consistently, Sirt1- and Sirt2-sensitive genes were involved in distinct categories of diseases, for example inflammatory response, cancer, and organismal injury for Sirt2, and infectious disease, developmental disorder, skeletal and muscular disorders for Sirt1. As observed in neural cells [39], our data suggest that Sirt2 is likely to have a distinct functional role from Sirt1 in endothelial cells under stress conditions. Moreover, these data support that the observed gene expression changes in response to Sirt2 knock down are unlikely to be a result of non-specific off target effects of RNA interference.

The precise cellular functions of Sirt2 in endothelial cells remain largely unknown. A previous study has shown that Sirt2 may be implicated in mediating angiotensin II-induced endothelial cell migration via modulating α -tubulin acetylation and microtubule reorganization [40]. Consistent with this observation, we identified (and confirmed with qPCR) that Sirt2 knock down altered the expression of several genes involved in cytoskeletal organization, cell contraction and migration, such as *CALD1* (caldesmon) and *CNN2* (calponin) [41,42]. Moreover, we demonstrated that Sirt2 also affected expression of genes involved in modulating cell viability. This is exemplified by

CASP7 (caspase 7), which is a master regulator of cell apoptosis, and *RRAGC* (Ras-related GTP binding C), which is involved in activation of the mTOR pathway [43].

To clarify the general role of Sirt2 in endothelial cells under oxidative stress, we challenged the cells with a high concentration of H₂O₂ and demonstrated that pharmacological inhibition of Sirt2 activity attenuated H₂O₂-induced cytotoxicity. This result is consistent with previous experiments in neural and cardiac cells showing that activation of Sirt2 promotes cell death, whereas knock down or inhibition of Sirt2 enhances cellular stress-tolerance [25,35]. Moreover, we confirmed that inhibitions of Sirt2 and Sirt1 had divergent effects on endothelial cell viability under H₂O₂-induced oxidative stress, an observation that was consistent with our microarray data revealing that there was only a small intersection between Sirt2- and Sirt1-sensitive genes in H₂O₂-challenged endothelial cells. Our experiments supported previous findings that Sirt1 exhibited profound cytoprotective effects in vascular endothelial cells in response to oxidative stress [7,15,16].

Table 1. List of differentially expressed genes after Sirt1 or Sirt2 gene silencing (read the entire table column-wise).

Sirt1								
			11722277_s_at	U	ZBED4	11724786_s_at	D	C14orf129
11716338_a_at	U	INSIG1	11722388_at	U	MTF1	11725510_a_at	D	SLC7A6
11716395_a_at	U	GPR56	11722391_at	U	MTF1	11725569_at	D	KLHL15
11718915_a_at	U	RGS19	11722531_a_at	U	CALD1	11725596_a_at	D	ZDHHC23
11719218_at	U	SOC3	11722532_s_at	U	CALD1	11725733_a_at	D	NFYA
11719513_a_at	U	ADAM15	11722533_x_at	U	CALD1	11725850_at	D	USP48
11719745_s_at	U	ARHGAP27	11723092_at	U	FNIP2	11726455_x_at	D	MYO5B
11720832_x_at	U	SOX18	11723414_a_at	U	CNN2	11726551_s_at	D	RAET1L
11722324_a_at	U	ZNF84	11723416_x_at	U	CNN2	11726552_x_at	D	ULBP2
11722353_s_at	U	LDB2	11724389_a_at	U	ZDHHC16	11727222_at	D	EVI5
11724390_x_at	U	ZDHHC16	11725054_a_at	U	HNMT	11727286_a_at	D	ZNF323
11724394_at	U	C2orf55	11726328_x_at	U	GBP1	11727361_a_at	D	MYLK
11724395_a_at	U	ZNF83	11726796_a_at	U	NEXN	11727406_a_at	D	TEK
11724396_x_at	U	ZNF83	11726797_x_at	U	NEXN	11727485_at	D	TPR
11727984_at	U	ODZ3	11726824_a_at	U	ZNF565	11727854_s_at	D	NUP50
11728347_at	U	ABI3	11727545_at	U	PANK3	11727905_a_at	D	IL13RA1
11729918_at	U	ADAMTS18	11727784_x_at	U	TPM4	11727984_at	D	ODZ3
11729919_a_at	U	ADAMTS18	11728028_a_at	U	PWWP2A	11728195_s_at	D	TRO
11730211_x_at	U	PFKL	11728226_a_at	U	CASP7	11728288_a_at	D	KRT15
11731622_x_at	U	ZNF91	11728276_s_at	U	PLA2G12A	11728353_at	D	MSMP
11733299_a_at	U	CLDN5	11728488_a_at	U	NRXN3	11728960_a_at	D	KRT80
11736013_at	U	GLE1	11728744_at	U	C4orf46	11729449_s_at	D	TINF2
11736029_a_at	U	ITGA6	11729665_a_at	U	STK38L	11729550_a_at	D	GPR126
11736458_x_at	U	KCNK6	11730613_at	U	MBOAT7	11729840_s_at	D	ZCCHC2
11737089_a_at	U	TLL1	11731665_a_at	U	PDGFB	11730033_a_at	D	RPS6KL1
11737870_s_at	U	FAM78A	11732339_at	U	BCL11A	11730195_at	D	SEC14L2
11739146_a_at	U	MKNK2	11733043_a_at	U	SLC7A2	11730615_a_at	D	PLCD4
11739491_a_at	U	MGAT4A	11733084_a_at	U	PALLD	11730616_at	D	PLCD4
11739492_a_at	U	MGAT4A	11734281_a_at	U	ZNF17	11730623_at	D	DSEL
11739493_at	U	MGAT4A	11734865_a_at	U	PSMC3IP	11732160_a_at	D	NUDCD1
11739650_at	U	DLL4	11736812_at	U	PDCD1LG2	11732713_at	D	FST
11740601_a_at	U	APH1B	11736959_a_at	U	TNFSF15	11733051_a_at	D	SCAMP1

Table 1. Cont.

11740602_s_at	U	APH1B	11739010_a_at	U	MYH9	11733054_a_at	D	SCAMP1
11740624_a_at	U	AZI1	11739086_x_at	U	MESDC2	11733929_a_at	D	ARMC8
11742830_a_at	U	PHF3	11739088_at	U	MESDC2	11734059_a_at	D	PSTK
11744239_a_at	U	CCDC159	11739451_a_at	U	FRMD6	11734150_x_at	D	PLAGL1
11744430_a_at	U	KIF9	11739672_x_at	U	ZNF253	11735224_a_at	D	KLRG1
11744505_x_at	U	TP53I11	11740133_a_at	U	CALD1	11735991_at	D	LARS
11744948_x_at	U	SEMA3F	11740743_a_at	U	TPM2	11736192_at	D	RRM2B
11745450_a_at	U	AUH	11740744_x_at	U	TPM2	11736343_x_at	D	OPA3
11745927_x_at	U	ADAM15	11741168_a_at	U	MSRB3	11736345_x_at	D	OPA3
11746516_a_at	U	ADAM15	11741188_a_at	U	SLC30A6	11736528_a_at	D	SMC2
11747060_a_at	U	MGAT4A	11742483_a_at	U	C1orf110	11736785_at	D	HOXA2
11747977_a_at	U	ZNF180	11743015_a_at	U	DIP2C	11737052_x_at	D	PLAGL1
11748731_a_at	U	ZNF616	11743020_at	U	ZSCAN29	11737816_x_at	D	FAM119B
11749511_a_at	U	ZNF595	11743253_x_at	U	CALD1	11739064_s_at	D	GNG12
11751946_a_at	U	ARHGAP21	11743458_a_at	U	FAM49A	11739245_a_at	D	ANKFY1
11752002_a_at	U	PLEKHG1	11743696_at	U	CLEC14A	11739596_a_at	D	KIAA1429
11752675_a_at	U	OGFR	11743705_at	U	ETAA1	11739640_at	D	DIP2A
11754022_s_at	U	GAP43	11744034_a_at	U	VASP	11739942_s_at	D	SEPSECS
11754446_x_at	U	ZNF761	11744323_s_at	U	PWWP2A	11740096_a_at	D	TMCC1
11754754_s_at	U	ADAMTS18	11745608_a_at	U	WDR1	11740176_at	D	ARSK
11755219_a_at	U	THBD	11745924_at	U	LOC220930	11740213_a_at	D	TBL1X
11755232_s_at	U	ZNF468	11746173_a_at	U	DDHD1	11741152_x_at	D	PLAGL1
11755474_a_at	U	ADAM15	11746476_x_at	U	CALD1	11742720_at	D	LRRCS58
11757861_a_at	U	TSHZ1	11746548_s_at	U	CNN2	11742722_at	D	LRRCS58
11760814_x_at	U	KANK3	11747300_a_at	U	CDK14	11742962_a_at	D	IP6K2
11715679_s_at	D	PRNP	11747469_x_at	U	KDM2B	11743404_at	D	ZMAT2
11715889_a_at	D	ATP5I	11747711_a_at	U	LDB1	11743573_at	D	TMEM184C
11715890_x_at	D	ATP5I	11748208_a_at	U	ZNF317	11743574_x_at	D	TMEM184C
11716027_at	D	SELT	11748391_x_at	U	ZDHHC16	11743648_a_at	D	DCAF6
11716028_x_at	D	SELT	11748400_s_at	U	LOC643634	11743649_a_at	D	DCAF6
11716404_s_at	D	EIF4EBP2	11748401_x_at	U	TPM4	11743763_at	D	GTF3C3
11718102_at	D	CD59	11748403_x_at	U	CNN2	11744083_at	D	ANKIB1
11718141_at	D	DKK1	11748527_a_at	U	ARHGAP24	11744415_s_at	D	MFSD6
11718769_a_at	D	MAPKSP1	11749172_x_at	U	NEXN	11744788_x_at	D	TMEM68
11719164_a_at	D	CLCN5	11749732_a_at	U	LONP2	11745010_a_at	D	DCBLD2
11719267_s_at	D	SERINC1	11749734_s_at	U	JRKL	11745230_a_at	D	C3orf23
11719394_a_at	D	FBXO32	11749921_a_at	U	SDC4	11745231_a_at	D	C3orf23
11719395_at	D	EXTL2	11750198_a_at	U	CASP7	11745700_s_at	D	ULBP2
11719396_a_at	D	EXTL2	11750623_a_at	U	FILIP1L	11746163_a_at	D	LARP4B
11719479_at	D	ALOX5AP	11751244_s_at	U	CNN2	11746536_a_at	D	WSB2
11719712_at	D	PPM1B	11751245_x_at	U	CNN2	11747146_s_at	D	TMBIM6
11719816_s_at	D	BET1	11751993_a_at	U	JAG1	11748416_a_at	D	DCAF6
11720240_at	D	TMSB15A	11752164_x_at	U	KDM2B	11749027_x_at	D	HERPUD2
11720514_at	D	C9orf150	11752276_a_at	U	DIDO1	11750354_a_at	D	TMEM184C
11721024_a_at	D	IL11RA	11752361_s_at	U	NEXN	11750993_x_at	D	MAP3K7
11721112_a_at	D	ACP6	11752499_a_at	U	CALD1	11751165_a_at	D	RBMS2

Table 1. Cont.

11722843_a_at	D	ENAH	11752930_a_at	U	GBP1	11751297_s_at	D	SUB1
11723533_x_at	D	BRMS1L	11754084_x_at	U	MYL9	11751305_a_at	D	B3GNT1
11723534_at	D	BRMS1L	11754644_x_at	U	CNN2	11751353_a_at	D	RRAGC
11723580_at	D	LOC221710	11754887_a_at	U	MSRB3	11751354_a_at	D	HDHD1A
11724238_at	D	CYB5R4	11754911_x_at	U	NEXN	11753308_s_at	D	ULBP2
11726140_s_at	D	SIRT1	11755122_a_at	U	PALLD	11753549_a_at	D	CMTM3
11726750_a_at	D	GTF3C4	11755734_x_at	U	CCDC107	11754031_s_at	D	CKS1B
11727022_at	D	TMEM64	11757637_a_at	U	MUS81	11754827_x_at	D	FBXO17
11727370_at	D	TSN	11758013_s_at	U	C8orf4	11755251_x_at	D	FADS1
11727935_at	D	C4orf49	11759169_a_at	U	C1orf222	11756152_s_at	D	PCNP
11729128_at	D	CPA4	11759711_a_at	U	RBM25	11756156_s_at	D	TFRC
11729710_a_at	D	MARS2	11760202_at	U	IGFBP7	11756181_x_at	D	YWHAZ
11731195_at	D	SEMA3D	11760611_x_at	U	SETD6	11756205_x_at	D	DCAF6
11731263_a_at	D	ZNF365	11760918_a_at	U	MRRF	11756254_a_at	D	GGT5
11734371_a_at	D	SCML1	11763500_a_at	U	ZNF93	11756259_s_at	D	NFATC1
11734955_a_at	D	SCML1	11715207_at	D	WDFY4	11756285_s_at	D	IGF2BP3
11736470_at	D	SLC35D1	11715265_at	D	FIG4	11756497_a_at	D	VWCE
11738893_s_at	D	TPM1	11715477_at	D	TFRC	11756603_a_at	D	C9orf6
11739119_s_at	D	CNPY3	11715545_at	D	TMED10	11756861_s_at	D	ULBP2
11742308_s_at	D	TPM1	11715550_at	D	DAG1	11757430_s_at	D	TMED5
11742483_a_at	D	C1orf110	11715651_s_at	D	FSTL1	11757523_s_at	D	WDR35
11742743_a_at	D	CNN3	11715761_a_at	D	TBC1D14	11757542_s_at	D	SSR1
11743092_at	D	THEM4	11716015_a_at	D	CMTM3	11757787_x_at	D	FTL
11743197_at	D	TLR4	11716208_s_at	D	GLUD1	11757799_s_at	D	VAMP7
11743334_a_at	D	MRPL35	11716288_s_at	D	ESYT1	11757810_s_at	D	TMED10
11743973_a_at	D	MRPL38	11716391_a_at	D	BACE1	11757880_s_at	D	DAG1
11743974_at	D	MRPL38	11716620_a_at	D	TNPO1	11757989_s_at	D	ANKRD46
11745482_s_at	D	PRNP	11716626_at	D	KIF3B	11758200_x_at	D	CKS1B
11746622_a_at	D	PHC1	11716787_a_at	D	B3GNT1	11758212_s_at	D	KIAA0494
11746928_a_at	D	ENC1	11716788_at	D	B3GNT1	11758452_s_at	D	CENPQ
11747834_a_at	D	SPATA5	11718184_a_at	D	FCHSD2	11758454_s_at	D	FAM116A
11748315_s_at	D	PRNP	11718287_at	D	UBL3	11758750_x_at	D	YWHAZ
11750985_a_at	D	EXTL2	11718288_at	D	UBL3	11758751_at	D	YWHAZ
11751191_a_at	D	LIPG	11718406_s_at	D	TMBIM6	11758809_at	D	RRAGC
11752333_a_at	D	ITGAV	11718439_at	D	NSUN4	11758820_at	D	DNAJC10
11754940_s_at	D	TSN	11718734_a_at	D	POGK	11758873_a_at	D	HPSE
11754976_x_at	D	CNPY3	11718900_a_at	D	TGFBR3	11758995_at	D	LOX
11755458_a_at	D	HDHC2	11718901_at	D	TGFBR3	11758999_s_at	D	UBE2H
11755848_a_at	D	C17orf51	11719088_at	D	MMGT1	11759720_x_at	D	LOC400590
11756471_a_at	D	MFS2A	11719353_s_at	D	GCC2	11763276_a_at	D	PWP1
11756882_a_at	D	RTTN	11719397_a_at	D	RRAGC	11763395_a_at	D	ZC3HAV1L
11757738_s_at	D	FAT1	11719398_s_at	D	RRAGC	11764275_s_at	D	SLC11A2
11758013_s_at	D	C8orf4	11719409_a_at	D	HIPK2	Co-regulated		
11758101_s_at	D	EIF4EBP2	11719628_a_at	D	HDHD1A	11715698_a_at	U	NOLC1
11758326_s_at	D	THEM4	11719786_at	D	SMCR7L	11717961_at	U	MED11
11758872_at	D	CDC37L1	11719912_a_at	D	KDM1B	11718908_s_at	U	CHST2

Table 1. Cont.

11758929_at	D	TFDP2	11720046_x_at	D	DNAJC10	11718909_x_at	U	CHST2
11759503_at	D	FAM103A1	11720111_at	D	SNTB2	11721860_s_at	U	STX12
11759776_at	D	GTDC1	11720112_at	D	SNTB2	11721862_a_at	U	STX12
11759943_at	D	FAM13A	11720146_a_at	D	DAPK1	11722853_a_at	U	HABP4
		Sirt2	11720273_at	D	SFT2D3	11724388_at	U	ZNF721
11715270_s_at	U	KLF7	11720570_a_at	D	PHF15	11724959_s_at	U	CDK14
11715586_at	U	MAPRE1	11720599_s_at	D	SUB1	11727522_a_at	U	ZNF267
11715713_a_at	U	VOPPI	11720602_at	D	SYT11	11727523_x_at	U	ZNF267
11715803_a_at	U	ANXA6	11720798_at	D	RAB8B	11727782_a_at	U	TPM4
11716299_a_at	U	ITGAV	11720799_s_at	D	RAB8B	11727783_s_at	U	TPM4
11716344_a_at	U	ZCCHC3	11720800_a_at	D	RAB8B	11728155_s_at	U	FUT4
11716413_x_at	U	MT1E	11720893_s_at	D	SOS1	11731868_a_at	U	BICD2
11716582_a_at	U	G3BP2	11721524_s_at	D	ZNF706	11734554_a_at	U	BCL7B
11716771_s_at	U	SIK1	11721585_a_at	D	TMCC1	11738988_a_at	U	GANAB
11717055_a_at	U	CORO1C	11721622_a_at	D	KATNAL1	11738989_a_at	U	GANAB
11717056_a_at	U	CORO1C	11721834_a_at	D	GET4	11738990_x_at	U	GANAB
11717952_at	U	ZDHHC18	11721993_at	D	SLC6A6	11743449_a_at	U	BICD2
11717968_at	U	TMEM216	11722111_at	D	HLX	11744786_x_at	U	OGFR
11719647_a_at	U	CASP7	11722273_s_at	D	KIAA1826	11748926_a_at	U	GANAB
11719648_a_at	U	CASP7	11722338_at	D	PEX7	11752163_a_at	U	KDM2B
11719833_at	U	MPZL2	11722377_at	D	PNPO	11752676_x_at	U	OGFR
11720028_x_at	U	LDLR	11722425_s_at	D	NEDD4L	11753089_a_at	U	OGFR
11720063_a_at	U	GLIPR2	11722460_at	D	PCDH12	11753090_x_at	U	OGFR
11720223_at	U	GFPT1	11722475_a_at	D	ARID3B	11754869_s_at	U	ZNF267
11720823_at	U	JAG1	11722662_a_at	D	HPSE	11755270_a_at	U	GANAB
11720849_a_at	U	RAB23	11722969_s_at	D	TRPV4	11757525_s_at	U	BICD2
11721218_a_at	U	MSRB3	11722977_at	D	HOXB5	11759004_at	U	SLC33A1
11721525_s_at	U	LOC440354	11723228_s_at	D	SGCB	11759757_a_at	U	SLC33A1
11721684_a_at	U	ZW10	11723230_a_at	D	RNF138	11741758_x_at	D	TRPV4
11721778_a_at	U	ACBD5	11723586_a_at	D	GRB14	11754398_at	D	LOC644538
11722193_a_at	U	C12orf75	11723639_s_at	D	C11orf58	11758147_s_at	D	MAPK13
11722218_a_at	U	WBP4	11724011_at	D	SIKE1	11758902_at	D	ZNF641
11722220_a_at	U	WBP4	11724171_a_at	D	FCHO2			

3. Experimental Section

3.1. Cell Culture

HUVECs were purchased from the American Type Culture Collection and maintained in ECM (from ScienCell Research Laboratories, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum, 1% ECG (endothelial cell growth supplement, ScienCell), and antibiotics (penicillin 100 U/mL, streptomycin 100 µg/mL). Cells were cultured at 37 °C with 5% CO₂. Confluent cells were subcultured with 0.25% trypsin-EDTA, and cells of passage 3 to 5 were used for experimentation. Cell viability was assessed with the tetrazolium-based (MTS) assay using CellTiter 96 Aqueous kit (from Promega, Madison, WI, USA) according to the manufacturer's direction.

3.2. RNA Interference

Small interfering RNA (siRNA) molecules targeting Sirt1 and Sirt2 were synthesized by GenePharma (Shanghai, China). For each target, 3 different siRNA sequences were tested with quantitative polymerase chain reaction (qPCR), and the one with highest efficacy was selected for following experiments. For siRNA transfection, cells were subcultured 24 h before treatment. Cells were incubated with siRNA (final concentration 30 nM) mixed with Lipofectamin RNAiMAX Reagent (Life Technologies, Carlsbad, CA, USA) for 6 h in antibiotic-free medium, and then changed to normal medium for additional 18 h.

3.3. Microarray Experiments and Data Processing

Cells were transfected with a control siRNA, Sirt2-specific siRNA (Sirt2i) or Sirt1-specific siRNA (Sirt1i). Three biological replicates were included for each group (hence a total of 9 arrays were analyzed). To induce oxidative stress, all transfected cells were treated with H₂O₂ at 300 µM for 6 h. Total RNA was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quality was tested with Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA) and further purified with RNeasy Micro kit (Qiagen, Hilden, Germany). Microarray analysis was performed using Affymetrix Human Genome U219 Array, using standard labeling, hybridization and scanning protocols (ShanghaiBio Corporation, China). The raw data were processed and analyzed with GeneSpring GX software. Genes with a fold change of >1.5 and with a *p* value of <0.05 as compared to control were selected as differentially expressed genes. Gene Ontology (GO) functional annotation of the differentially regulated genes was carried out using DAVID Bioinformatics Resources 6.7 [44]. Further gene function clustering analysis was performed with IPA software (Ingenuity Systems, Redwood City, CA, USA).

3.4. Real-Time qPCR

Total RNA (500 ng) was reverse transcribed to cDNA using Prime Script RT reagent Kit (TaKaRa Biotechnology, Dalian, China). Real-time qPCR was performed with TaqMan gene expression assays primer-probe sets (Applied Biosystems, Carlsbad, CA, USA) or using a Sybr green-based master mix kit (SsoFast EvaGreen from Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. *GAPDH* or *18S* was used as the housekeeping gene.

3.5. Fluorescent Immunocytochemistry

Cells grown on Lab-Tek II chamber slides (Nunc, Roskilde, Denmark) were fixed with cold methanol for 30 min, washed in PBS and blocked with 5% bovine serum albumin. Cells were incubated overnight with polyclonal anti-Sirt1 (1:200) (from Abcam, Cambridge, UK) or anti-Sirt2 (1:100) (from Millipore, Billerica, MA, USA). Immunofluorescent labeling was performed with DyLight594-conjugated donkey anti-rabbit IgG (1:400) (Jackson ImmunoResearch, West Grove, PA, USA). Cell nuclei were counter stained with DAPI. Images were captured using a Zeiss laser scanning confocal microscope (Zeiss LSM710, Oberkochen, Germany). Negative control experiments were performed using corresponding non-immune IgGs.

3.6. Western Blot

Total protein was resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membrane was blocked with 5% non-fat milk at room temperature for 1 h and then incubated with primary antibodies at 4 °C overnight. The blots were developed with ECL Prime reagents from GE Life Sciences (Piscataway, NJ, USA).

3.7. Data and Statistics

Microarray data were tested with Benjamini and Hochberg False Discovery Rate multiple testing correction. Other data were presented as mean $\pm$ SEM and tested with unpaired Student's *t*-test or one-way ANOVA as appropriate, with a value of $p < 0.05$ being regarded as statistically significant. SPSS18.0 was used for statistical analysis.

4. Conclusions

In conclusion, to our knowledge this is the first genome-wide characterization of the gene expression profile in response to Sirt2 knockdown in endothelial cells. Sirt2-sensitive genes are involved in multiple cellular functions. Pharmacological inhibition of Sirt2 attenuated oxidant-induced endothelial cell death. These data suggest that Sirt2 is functionally important in endothelial cells under oxidative stress. Our results may provide a basis for future studies aiming to dissect the specific signaling pathway(s) that mediates specific Sirt2 functions in endothelial cells. Nevertheless, a limitation of the present study was that the microarray data did not provide direct evidence about the specific gene products that were involved in mediating the observed effects of Sirt2. Given the number of genes that are responsive to the changed Sirt2 level, it is likely that multiple mechanisms may be implicated in each specific biological function of Sirt2.

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Conflict of Interest

The authors declare no conflict of interest.

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