Overexpression of p53 due to excess protein *O*-GlcNAcylation is associated with coronary microvascular disease in type 2 diabetes

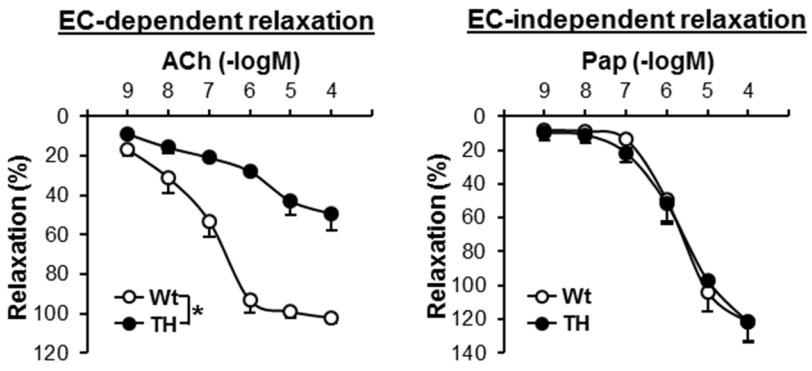
[1, 5]Rui Si, [1,3,4]Qian Zhang, [1]Atsumi Tsuji-Hosokawa, [1]Makiko Watanabe, [1]Conor Willson, [3,4]Ning Lai, [2,4]Jian Wang, [3]Anzhi Dai, [3]Brian T Scott, [3]Wolfgang H. Dillmann, [2,3]Jason X.-J. Yuan, and [1,2,3]Ayako Makino

[1]Department of Physiology and [2]Department of Medicine, The University of Arizona, Tucson, Arizona;
[3]Department of Medicine, University of California, San Diego, La Jolla, California;
[4]State Key Laboratory of Respiratory Disease, Guangzhou Institute of Respiratory Disease, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou, China;
[5]Department of Cardiology, Xijing Hospital, Fourth Military University, Shaanxi, China

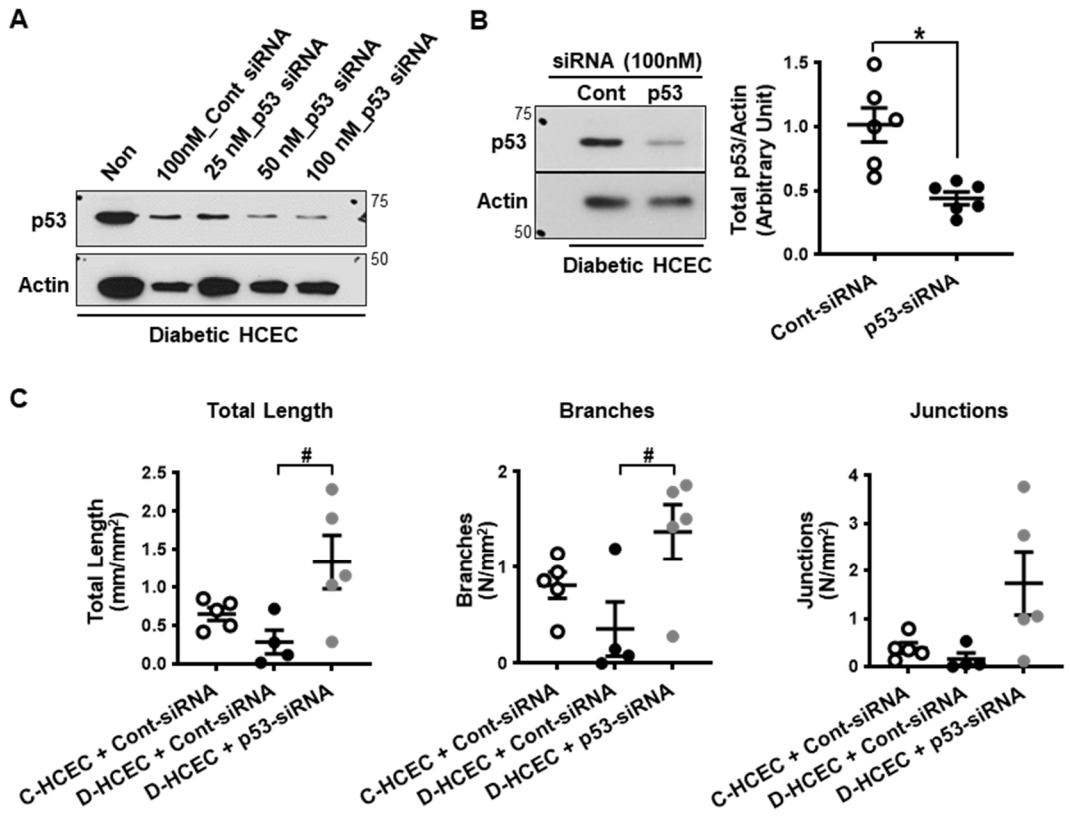
Short Title: p53 and endothelial function in Diabetes

Corresponding Author:
Ayako Makino, Ph.D.
Associate Professor of Medicine
Division of Endocrinology and Metabolism
Department of Medicine
University of California, San Diego
9500 Gilman Dr., MC-0856
La Jolla, CA 92093-0856
Email: amakino@ucsd.edu
Tel: 858-246-5790

Category of the Manuscript: Original Article



Supplemental Figure 1. Endothelium-dependent relaxation in coronary artery is attenuated in TH mice compared to Wt mice. Isometric tension experiments were conducted using 3rd order coronary arteries to assess EC-dependent relaxation using acetylcholine (ACh) or EC-independent relaxation using papaverine (Pap). N_{mice}=4 per group. Data are presented as mean ± S.E. *P<0.05 vs. Wt. Statistical comparison between dose-response curves was made by two-way ANOVA with Bonferroni post hoc test.



Supplemental Figure 2. Inhibition of p53 by p53 siRNA increases tube formation in diabetic HCECs. Control siRNA or p53 siRNA was transfected into diabetic HCECs using lipofectamine RNAiMAX reagent. Forty-eight hours later, cells were used for the experiment. **A**: Western blot analysis showing p53 protein level in ECs after siRNA transfection in a dose dependent manner. **B**: Western blot image (left) showing p53 protein levels after transfection of control and p53 siRNA in ECs at 100 nM. Summarized data (right) showing protein level of p53 ($n_{experiments}$=6 per group) normalized by the protein level of actin. Data are expressed as mean ± SE. **P*<0.05 vs. control siRNA (Cont-siRNA). Unpaired Student's *t*-test was used for comparing two experimental groups. **C**: Summarized data of tube formation in control HCECs (C-HCEC) treated with Cont-siRNA (100 nmol/L, $n_{experiments}$=5), diabetic HCECs (D-HCEC) treated with Cont-siRNA ($n_{experiments}$=4), and D-HCEC treated with p53-siRNA (100 nmol/L, $n_{experiments}$=5). $^{\#}P$<0.05 vs. D-HCEC + Cont-siRNA. Data are mean ± SE. Statistical comparison among three groups was made by one-way ANOVA with Bonferroni *post hoc* test.

Supplement Table 1. Antibody list.

Antibody	Catalog Number / Company	Dilution Rate
Anti *O*-GlcNAc (RL2)	ab2739 / Abcam (Cambridge, MA)	1:2000
Anti OGA	SAB4200267 / Sigma-Aldrich Corp. (St. Louis, MO)	1:2000
Anti p53	sc-393031 / Santa Cruz Biotechnology, Inc. (Dallas, TX)	1:2000
Anti Actin	sc-376421 / Santa Cruz Biotechnology, Inc. (Dallas, TX)	1:4000

Supplement Table 2. Human coronary endothelial cell.

Group	Company	Catalog Number	Lot Number
Control	Lonza (Basel, Switzerland)	cc-2585	305907 419238 547320 7F4281
Control	Cell Applications, Inc. (San Diego, CA)	300-05a	2991
Type 2 diabetic	Lonza (Basel, Switzerland)	cc-2922	233691 234247 239103
Type 2 diabetic	Cell Applications, Inc. (San Diego, CA)	300T2D-05a	3105

Note: All commercially-available T2D cells were purchased at the time the studies were performed.

Supplement Table 3. Mouse p53 signaling pathway PCR array result. Gene names of which mRNA levels were altered in TH mice and restored by chronic PFT administration are listed.

RefSeq Number	Symbol	Description	$2^{-\Delta\Delta CT}$			
			Wt + Vehicle	TH + Vehicle	TH + PFT	*p*-value
NM_013693	*TNF*	Tumor necrosis factor	0.95 ± 0.01	1.60 ± 0.27	1.06 ± 0.10	3.92E-02
NM_007527	*BAX*	Bcl2-associated X protein	0.78 ± 0.14	1.62 ± 0.12	0.86 ± 0.06	4.67E-04
NM_133234	*BBC3*	BCL2 binding component 3	1.02 ± 0.10	1.47 ± 0.08	1.03 ± 0.10	1.21E-02
NM_007610	*CASP2*	Caspase 2	1.07 ± 0.02	1.32 ± 0.08	1.12 ± 0.06	4.03E-02

Wt + Vehicle, n_{mice}=5; TH + Vehicle, n_{mice}=4, TH + PFT, n_{mice}=5. Data are presented as mean ± S.E. One-way ANOVA was carried out to determine the significance between groups.

Supplement Table 4. Primer information used for real-time PCR

	Forward	Reverse
Mouse TRP53	GCGTAAACGCTTCGAGATGTT	TTTTTATGGCGGGAAGTAGACTG
Mouse TNF	CCTGTAGCCCACGTCGTAG	GGGAGTAGACAAGGTACAACCC
Mouse BAX	AGACAGGGGCCTTTTTGCTAC	AATTCGCCGGAGACACTCG-3
Mouse BBC3	ATGGCGGACGACCTCAAC	AGTCCCATGAAGAGATTGTACATGAC
Mouse CASP2	GGAGCAGGATTTTGGCAGTGT	GCCTGGGGTCCTCTCTTTG
Mouse 18S ribosomal RNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

1. Supplementary methods for supplemental figures/tables

Isometric tension measurement in coronary arterial ring (Supplemental Figure 1)

Isometric tension measurement in isolated coronary arteries (CAs) from mice was performed as described previously[1-6]. Briefly, third-order small CAs were dissected from the hearts and then cut into 1-mm segments. The CA rings were mounted on a myograph (DMT-USA, Inc. Ann Arbor, MI, USA) using thin stainless wires (20 μm in diameter) and the resting tension was set at 0.1 g. CAs were allowed to equilibrate for 45 min with intermittent washes every 15 min. After equilibration, each CA ring was contracted by superfusion of $PGF_{2\alpha}$ to generate similar contraction level in all groups. Acetylcholine (ACh) or papaverine (Pap, a phosphodiesterase inhibitor) was administrated in a dose-dependent manner (1 nmol/l to 100 μmol/l).

p53-siRNA transfection in diabetic HCECs (Supplemental Figure 2)

Downregulation of p53 in diabetic HCECs was achieved using p53 siRNA (siRNA ID# s606, Thermo Fisher Scientific) and lipofectamine RNAiMAX reagent (Thermo Fisher Scientific). Specific protein knockdown was verified with Western blotting 48 hours after transfection.

RT2 profile PCR array (Supplemental Table 3)

mRNAs from MCECs were isolated using the miRNeasy Mini Kit (QIAGEN, Chatsworth, CA). First-strand cDNA was synthesized using a RT2 first-strand kit (QIAGEN). Pathway-focused gene expression analyses were performed using RT2 Profile PCR Arrays (Mouse p53 Signaling Pathway PCR Array, PAMM-027ZA, QIAGEN). The results from the PCR array were analyzed done based on manufacturer's instructions. Each array contained five housekeeping genes and we used beta actin to normalize other gene expression levels. The transcript levels of Gene of Interest (GOI) were quantified according to the cycle threshold (ΔCt) method. Ct values > 35 were not included in the analysis and were considered as negative.

Statistical analysis

Statistical analysis was performed using SigmaPlot 14 (Systat Software, Inc.). Data were presented as mean ± S.E. One-way ANOVA was used for multiple comparisons. Statistical comparison between dose-response curves was made by two-way ANOVA with the Bonferroni correction performed post hoc to correct for multiple comparisons. Differences were considered to be statistically significant when $*P<0.05$.

References

1. Makino A, Platoshyn O, Suarez J, Yuan JX, Dillmann WH. Downregulation of connexin40 is associated with coronary endothelial cell dysfunction in streptozotocin-induced diabetic mice. *Am J Physiol Cell Physiol* 2008;**295**:C221-230.
2. Makino A, Wang H, Scott BT, Yuan JX, Dillmann WH. Thyroid hormone receptor-α and vascular function. *Am J Physiol Cell Physiol* 2012;**302**:C1346-1352.
3. Makino A, Dai A, Han Y, Youssef KD, Wang W, Donthamsetty R, Scott BT, Wang H, Dillmann WH. *O*-GlcNAcase overexpression reverses coronary endothelial cell dysfunction in type 1 diabetic mice. *Am J Physiol Cell Physiol* 2015;**309**:C593-599.
4. Han Y, Cho YE, Ayon R, Guo R, Youssef KD, Pan M, Dai A, Yuan JX, Makino A. SGLT inhibitors attenuate NO-dependent vascular relaxation in the pulmonary artery but not in the coronary artery. *Am J Physiol Lung Cell Mol Physiol* 2015;**309**:L1027-1036.
5. Estrada IA, Donthamsetty R, Debski P, Zhou MH, Zhang SL, Yuan JX, Han W, Makino A. STIM1 restores coronary endothelial function in type 1 diabetic mice. *Circ Res* 2012;**111**:1166-1175.
6. Cho YE, Basu A, Dai A, Heldak M, Makino A. Coronary endothelial dysfunction and mitochondrial reactive oxygen species in type 2 diabetic mice. *Am J Physiol Cell Physiol* 2013;**305**:C1033-1040.