PGC-1α Protects from Notch-Induced Kidney Fibrosis Development

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Directed by Professor Shin-Wook Kang

The Doctoral Dissertation
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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December 2017

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December 2017

ACKNOWLEDGEMENTS

I would like to thank everyone who helped me to finish this thesis.

First of all, I want to express my warmest thanks to Professor Shin-Wook Kang for his careful and kind guidance not only in my study but also in my life in Korea. He did his best to give me warm support and shared his astonishing knowledge with me to finish my thesis. I will always try to keep his words of enthusiasm and kindness in my heart.

In addition, I especially feel thankful to Professor Seung Hyeok Han for supporting and guiding me in many ways. His valuable advices, encouragement, and belief in me have made me possible to complete many research works including various laboratory techniques.

I would also like to express my appreciation to Professor Jeon-Soo Shin, Professor Heon Yung Gee, Professor Dong-Ryeol Ryu, and Professor Tae-Hyun Yoo, who always helped and supported me, and to thank Professor Jung Tak Park for his valuable advice regarding my research.

I would like to extend my thanks to Bo Young Nam, the postdoctoral researcher in our lab, who taught me many skills and laboratory technologies. Moreover, I have to say thanks

to Seonghun Kim, Jimin Park, Sukyung Kang and Arum Choi for their help during my graduate school study.

Needless to say, I cannot express my warm gratitude enough to my husband who has always brought me confidence and support. I was absolutely able to finish this work because of his encouragement and understanding.

Finally, I really appreciate my parent's hard work and sacrifice that enabled me to achieve this result. I would also like to thank my parents-in law for their unconditional support and love.

Thanks everyone.

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ABSTRACT

PGC-1α Protects from Notch-Induced Kidney Fibrosis Development

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Kidney fibrosis is the histological manifestation of chronic kidney disease (CKD) and interstitial fibrosis shows the strongest correlation with future functional decline. Sustained activation of developmental pathways, such as Notch, in tubular epithelial cells has been shown to have a key role in fibrosis development. However, the mechanism of Notch-induced fibrosis is poorly understood. Several recent studies indicate that metabolic alterations and insufficient energy supply in tubule cells play a role in fibrosis development. Therefore, I hypothesized that metabolic alteration mediates the Notch-induced

kidney fibrosis development, and particularly tested the role of peroxisomal proliferator-gamma coactivator-1 alpha (PGC-1α), a key regulator of energy metabolism and mitochondrial biogenesis.

Using human kidney samples, I analyzed expression pattern of PGC-1α in CKD. For animal study, mice expressing *Pax8-rtTA/tetO-ICN1* were crossed with mice harboring *Pax8-rtTA/tetO-Ppargc1a* to create *Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a* mice. The littermate *tetO-ICN1* or *tetO-Ppargc1a* mice were considered controls. Using kidney tissues from these mice, I examined fibrosis, PGC-1α expression, fatty acid oxidation pathway, and apoptosis. To delineate the mechanism of Notch1-induced fibrosis, chromatin immunoprecipitation (ChIP) assay and luciferase assay were performed with primary cultured renal tubular epithelial cells.

Compared with kidneys from control counterparts, kidneys from mice overexpressing Notch1 in tubular epithelial cells (*Pax8-rtTA/ICN1* transgenic mice), mouse models of fibrosis, and patients with renal fibrosis have decreased expression of PGC-1α. ChIP assay revealed that the Notch1 target gene, Hes1, directly binds to the regulatory region of *Ppargc1a*, which encodes PGC-1α. Compared with *Pax8-rtTA/ICN1* transgenic animals, *Pax8-rtTA/ICN1/Ppargc1a* transgenic mice showed rescue of renal structural alteration and molecular defect of profibrotic genes. Overexpression of PGC-1α

restored mitochondrial structure and content and reversed the fatty acid oxidation defect induced by Notch1 overexpression in vitro in tubule cells. Furthermore, compared with *Pax8-rtTA/ICN1* mice, *Pax8-rtTA/ICN/Ppargc1a* mice exhibited improvement in renal fatty acid oxidation gene expression and apoptosis.

This study suggests that metabolic pathway plays key roles in Notch1-induced fibrosis development. PGC-1α is a direct Notch target gene and restoration of PGC-1α activity could be a promising treatment strategy against CKD.

Key words: PGC-1α, Notch1, kidney fibrosis, fatty acid oxidation, mitochondria

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I. INTRODUCTION

Chronic kidney disease (CKD) has become an important public health problem worldwide. It is diagnosed by reduction of the estimated glomerular filtration rate (eGFR) < 60 ml/min/1.73m^2 or abnormal leakiness of the glomerulus to albumin as urine albumin-to-creatinine ratio >30 mg/g.[1] Patients with CKD have at least three- to five-fold greater mortality rate when compared to matched subjects without CKD.[2-4]

Interstitial fibrosis shows the strongest correlation with future renal functional decline. Kidney fibrosis is the final common pathway that is observed in all forms of CKD. Fibrosis represents a complex architectural change characterized by glomerulosclerosis, tubular atrophy, accumulation of myofibroblast, collagen, and inflammatory cells, and peritubular capillary

loss.[5]

Patients and animal models of CKD are characterized by sustained expression of developmental genes such as Wnt, Notch and Hedgehog in renal tubular epithelial cells (RTECs). Studies have shown the critical role and contribution of these pathways to kidney fibrosis development.[8-11] Notch is a well-known master regulator of cell specification, differentiation, and tissue patterning. In mammals, there are four Notch receptors (Notch 1-4) and two classes of canonical ligands, Jagged 1 and 2, and Delta-like ligand 1, 3, and 4. The canonical Notch signaling pathway is initiated when the ligand binding to Notch receptors, thus causing proteolytic cleavage on the extracellular face by an ADAM/TACE protease and the intracellular side of the plasma membrane by γ-secretase. After the cleavage, the Notch intracellular domain translocates to the nucleus and forms a complex with RBPj and Mastermind-like proteins, leading to transcription of Notch target genes, such as helix-loop-helix proteins of the Hes and Hey families. Notch signaling is a crucial regulator of kidney development, although it is expressed at low level in normal healthy adults.[12]

It is hypothesized that, in CKD kidneys, Notch is activated in tubule cells in response to injury and cell death, likely as part of an injury repair mechanism.[9,10,13,14] Sustained and high Notch expression seems to be harmful. Tubule-specific expression of Notch induces severe epithelial dedifferentiation, interstitial fibrosis, and death of the animals. Notch is not only sufficient but also necessary for fibrosis development, because inhibition of Notch signaling attenuates tubulointersitial fibrosis (TIF) in a mouse model

of folic acid- and ureteral obstruction-induced fibrosis.[10]

The mechanism of Notch-induced fibrosis is poorly understood. Several pathways have been proposed to contribute to Notch-induced development of fibrosis, including dedifferentiation, partial epithelial-to-mesenchymal transition (EMT), and enhanced proliferation.[12,15] The role of these pathways has not been substantiated by *in vivo* studies. Several recent studies have indicated that metabolic alterations and insufficient energy supply in tubule cells play a role in fibrosis development.[16,17] In fact, in absence of sufficient energy supply, RTECs die or dedifferentiate, creating a nidus for fibrosis development.[18] I hypothesized that metabolic alteration mediated the Notch-induced kidney fibrosis development and particularly tested the role of PGC-1α.

II. MATERIALS AND METHODS

1.Generation of mice overexpressing *ICN1* and/or *Ppargc1a* in the RTECs

Transgenic mice harboring the *tetO-ICN1* (Intracellular Notch1) transgene have been described previously.[19] FVB-Tg (*tetO-Ppargc1a)1Dpk/J* mice were purchased from the Jackson Laboratory. To generate mice with conditional inducible expression of Notch1 and PGC-1α in RTECs, *Pax8-rtTA* mice were crossed with *tetO-ICN1* and *tetO-Ppargc1a* mice, respectively. I identified transgenic mice by genomic PCR analysis using transgene-specific primers. The primer sequences for *tetO-ICN1* and *tetO-Ppargc1a* are presented in Table 1. Mice were put on a doxycycline-containing chow diet (Bioserv S3888) starting at 4 weeks of age. Animal care and experiments were performed in accordance with the National Institutes of Health guidelines.

2. Quantitative real-time polymerase chain reaction (qPCR) analysis

I compared transcript levels of genes related to Notch signaling, β-oxidation, apoptosis, and dedifferentiation (*Procol1a1, Procol3a1, Fn,* and *smooth muscle a-actin*) by qPCR. To this end, total RNA samples from mouse kidney were prepared using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). I examined the RNA quality on agarose gels, and determined the quantity using Nano Drop. Next, 1 μg of total RNA was reverse-transcribed using the cDNA Archival Kit (Applied Biosystems). I performed qPCR analysis on a ViiA™ 7 Real-Time PCR System (Life Technologies), with the SYBR Green Master Mix, using three-step standard cycling conditions, with sequence-specific primers. The sequences of primers are presented in Table 1. I examined the

melting curve to confirm that a single PCR product was amplified. For quantitative analysis, samples were normalized to *Ubiquitin C* gene expression by the $2^{-\Delta\Delta CT}$ value method.

3. Mitochondrial DNA analysis

Genomic DNA was extracted from kidney and cells using a commercially available kit (Wizard Genomic DNA Purification Kit, Promega, Madison, WI, USA) according to the manufacturer's instructions. qPCR was then conducted with primers specific for the mtDNA-encoded *Cox1* gene and the nuclear-encoded *Ndufv1* gene; the relative mtDNA copy number was presented as the mtDNA/nuclear DNA ratio.

4. Western blot analysis

Protein expression levels of key enzymes of the fatty acid oxidation pathway and apoptosis markers were examined by Western blot analyses. I prepared cell lysates in RIPA lysis buffer containing a protease inhibitor cocktail (Complete Mini, Roche) and phosphatase inhibitor (PhosSTOP, Roche). Proteins were resolved by electrophoresis on 5–15% gradient gels, transferred onto polyvinylidenedifluoride membranes and probed with the antibodies against the following proteins; phospho-acetyl-coenzyme A carboxylase (ACC) (1:500; 3661; Cell Signaling Technology), carnitine palmitoyltransferase 1 (CPT1) (1:500; ab176320; Abcam, Cambridge, UK), Hes-1 (1:1000; ab108937; Abcam), activated notch1 (1:2000; ab8925; Abcam), PGC-1α (1:1000; ab54481; Abcam), BCL2 (1:500; sc-509; Santa

Cruz Biotechnology, Santa Cruz, CA), cleaved-caspase 3 (1:500; 9664; Cell Signaling Technology), and β-actin (1:5000; A5316; Sigma-Aldrich). A horseradish peroxidase (HRP)-conjugated anti-rabbit (1:2000; 7074; Cell Signaling Technology) or anti-mouse IgG antibody (1:2000; 7076; Cell Signaling Technology) was served as a secondary antibody. After repeated washes, the membrane was developed by chemiluminescence (Western Lightning-ECL; Thermo Scientific). To quantify the band densities, I used ImageJ v1.49 software (National Institutes of Health, Bethesda, MD; online at http://rsbweb.nih.gov/ij).

5. Histology, immunohistochemistry and immunofluorescence staining

To evaluate histologic features, formalin-fixed, paraffin-embedded kidney sections were stained with periodic acid Schiff (PAS). I also performed Masson's trichrome staining and immunohistochemical analysis. All slide pictures were captured using an Olympus DP73 microscope.

For Masson's trichrome staining, 5 μm-thick sections of paraffin-embedded tissues were deparaffinized, hydrated in ethyl alcohol, washed in tap water, and re-fixed in Bouin solution at 56°C for 1 hr. After washing in running tap water for 10 min and staining with Weigert iron hematoxylin working solution for 10 min, I stained the slices with the Biebrich scarlet-acid fuchsin solution for 15 min, followed by a 10-min wash. The slides were then differentiated in phosphomolybdic-phosphotungstic acid solution for 15 min, transferred to an aniline blue solution and stained for 10 min, and were reacted with 1% acetic acid solution for 5 min.

For immunohistochemical staining, the tissue slices were deparaffinized, hydrated in ethyl alcohol, and washed in tap water. Antigen retrieval was carried out in 1 mM EDTA, pH 8.0, or sodium citrate buffer, pH 6.0, by microwaving for 15 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min. The slices were blocked in 0.2% fish skin gelatin for 60 min or 5% normal serum for 30 min at room temperature and incubated overnight at 4 °C with a rabbit anti–cleaved-caspase 3 (1:100; Cell Signaling) antibody, rabbit anti-PGC-1α (1:250; ab54418; Abcam) antibody, a rat anti-mouse F4/80 (1:100; MCA497GA; Bio-rad) antibody, a mouse anti-mouse CD68 (1:100; ab31630; Abcam) antibody, a rabbit anti-mouse Ki67 (1:100; ab15580; Abcam) and a rabbit anti-PCNA (1:200; PA5-16797; Thermo Scientific). Staining was visualized using HRP-conjugated antibodies against rabbit IgG and 3, 3-diaminobenzidine bymeans of the Dako Envision Kit as per manufacturer's protocol (Vector Labs).

6. Primary cell cultures and transfection

RTECs were isolated from wild type mouse kidneys. The cells were cultured in the RPMI 1640 medium (Gibco) containing 10% of fetal bovine serum (FBS, Gibco), 100 U/mL penicillin G (Sigma-Aldrich), 2.5 μg/mL amphotericin B (Sigma-Aldrich), and 20 ng/mL epidermal growth factor (Sigma-Aldrich). In short, kidneys were dissected, placed in 1 mL of ice-cold DPBS (Cellgro), and minced into pieces of approximately 1 mm^3. These pieces were transferred and digested for 60 min at 37°C, and the supernatants were sieved through a 100-μm nylon mesh. After centrifugation for 10 min at

3000 rpm, the pellet was resuspended in sterile red blood cell lysis buffer (8.26 g NH_4Cl, 1 g $KHCO_3$, 0.037 g EDTA per L ddH_2O) and seeded in 10-cm culture dishes. At confluence of ~ 70 %, the cells were transfected with *pCDNA3*, *ICN1* plasmid, *Ppargc1a* plasmid (Addgene, Cambridge, MA, USA), and Renilla luciferase plasmid, *pGL-basic*, *pGL3– Ppargc1a* promoter luciferase plasmid (Promega, Wisconsin, USA), Hes1 expression plasmid (Origene, Rockville, USA), *Hes-1* siRNA and *ICN1* siRNA (Dharmacon, Lafayette, CO, USA) using Lipofectamine 2000 and Plus reagents (Invitrogen). Next, 14 hours after transfection, media were changed to 1% complete media and the cells were incubated for additional 36-48 h.

7. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was conducted as previously described.[20] Briefly, 2x 10^7 primary culture cells, which were transfected with *pCDNA3*, *ICN1* plasmid, or 100 nM of *ICN1* siRNA respectively, were cross-linked, washed, and sonicated. The resulting lysates were subjected to immunoprecipitation with antibodies against Hes1 (Abcam) or control IgG (Santa Cruz Biotechnology). Protein Aagarose/Salmon sperm DNA (Temecula, CA 92590) were used to capture the immunoprecipitates. After washing, bound proteins were eluted and ChIP-enriched DNA was isolated by phenol:chloroform extraction. The eluted ChIP DNA and input control samples were analyzed by qPCR using the following primer pair within the *Ppargc1a* enhancer promoter. The primer sequences were sense 5'-TTTCAGTGTTTTTCCTTCATT-3' and anti-sense 5'-CCCAGAAAACAAA

TGCTAGA-3'. Data were analyzed by the two methods and normalized to the input samples as described elsewhere.[17]

8. Luciferase assay

Primary RTECs were obtained as described above and seeded in 6-well plates. The next day, 100 ng of a Renilla luciferase-encoding plasmid, 400 ng of the *pGL-basic* or *pGL3– Ppargc1a* promoter luciferase plasmid, and 600 ng of the *Hes1* expression plasmid, and 100 nM of *Hes-1* siRNA were cotransfected using Lipofectamine 2000 according to the manufacturer's instructions. The cells were harvested 36 hours after transfection and analyzed with the Dual-Luciferase Reporter Assay System (Promega). Luminescence was measured on a CentroXS3 LB9601 luminometer. Luciferase activity of each group was normalized to Renilla luciferase activity and between-group differences were expressed as relative fold changes.

9. Electron microscopic examination

I next examined mitochondrial structure by standard transmission electron microscopy. Primary RTECs were fixed with a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde overnight, washed, dehydrated, and embedded in a resin according to standard procedures. Mitochondria were examined under a JEOL 1011 microscope (Tokyo Japan).

10. Statistics

Statistical analysis was performed using the statistical package SPSS software ver. 20.0 for Windows (SPSS, Chicago, IL, USA). All the results are presented as mean ± SD. To analyze a difference between two groups, Student's t-test was used. One-way ANOVA with a *post hoc* Bonferroni's test was applied when more than two groups were present. Difference with a *P*-values < 0.05 were considered statistically significant.

Table 1. Sequences of oligonucleotide primers used for qPCR test

	Forward	Reverse
Ubc	GCCCAGTGTTACCACCAAGAAG	GCTCTTTTTAGATACTGTGGTGAGGAA
Ppargc1a	AGTCCCATACACAACCGCAG	CCCTTGGGGTCATTTGGTGA
Hes1	CCCCAGCCAGTGTCAACAC	TGTGCTCAGAGGCCGTCTT
Hey1	GCCTCCGACCCGCTTCT	TGGGATGCGTAGTTGTTGAGAT
Procol1a1	GCCAAGAAGACATCCCTGAA	GTTTCCACGTCTCACCATTG
Procol3a1	ACAGCTGGTGAACCTGGAAG	ACCAGGAGATCCATCTCGAC
Fn	ACAAGGTTCGGGAAGAGGTT	CCGTGTAAGGGTCAAAGCAT
Acta2	CTGACAGAGGCACCACTGAA	AGAGGCATAGAGGGACAGCA
Cpt1	GGTCTTCTCGGGTCGAAAGC	TCCTCCCACCAGTCACTCAC
Acox1	CTTGGATGGTAGTCCGGAGA	TGGCTTCGAGTGAGGAATT
Bcl-2	TGGGATGCCTTTGTGGAACT	CAGCCAGGAGAAATCAAACAGA
Tfam	GGAATGTGGAGCGTGCTAAAA	TGCTGGAAAAACACTTCGGAATA

III. RESULTS

1. Decreased PGC-1α expression in Notch1-induced kidney injury

Conditional inducible expression of Notch1 in tubule cells (in the *Pax8-rtTA/tetO-ICN1* transgenic mice) resulted in the development of severe fibrosis (**Figure 1A**). Transcript levels of *Ppargc1a* were significantly reduced in whole kidney samples of these transgenic animals compared to controls (**Figure 1B**). Similarly, *in vitro* expression of Notch1 in cultured tubule cells resulted in reduced PGC-1α expression, indicating an inverse association between Notch and PGC-1α (**Figure 1C**). These findings were further confirmed at protein levels by Western blot analyses (**Figure 1D and 1E**) and immunohistochemistry (**Figure 1F**).

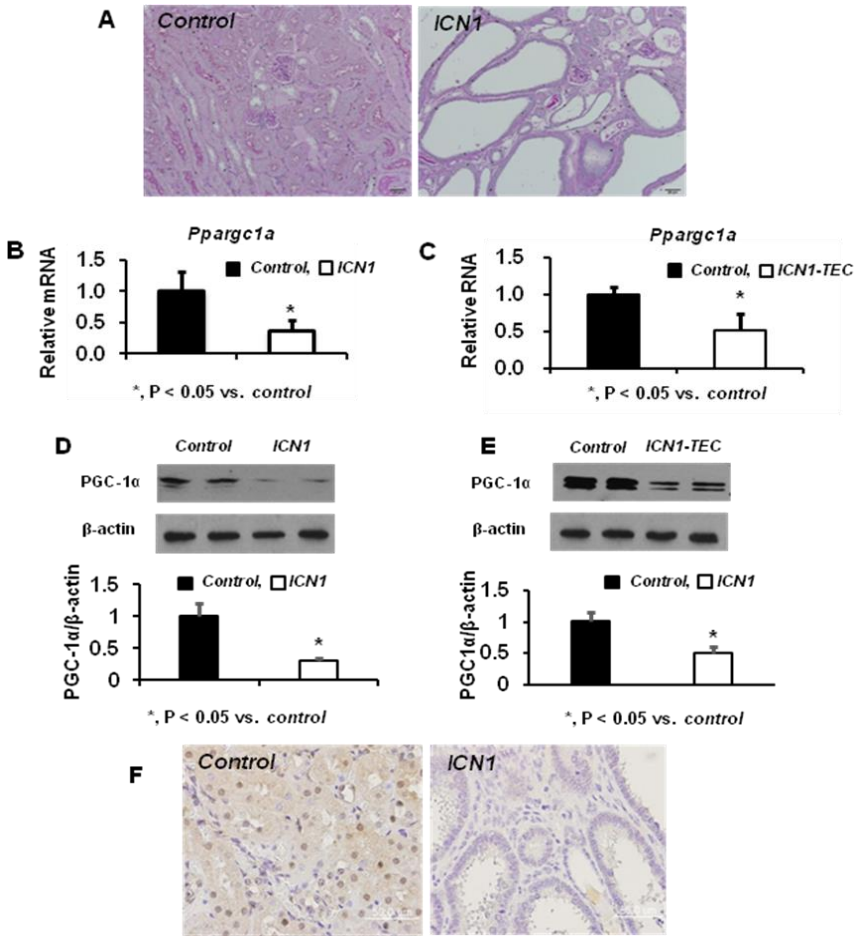


Figure 1. Decreased PGC-1α expression in Notch1-induced kidney injury. (A) Representative PAS stained kidney sections of *Pax8-rtTA/tetO-ICN1* transgenic and control mice. (B-C) *Ppargc1a* transcript levels in *Pax8-rtTA/tetO-ICN1* transgenic animals (B) and in cultured renal tubule cells expressing Notch1 (C). (D-E) Western blot images of PGC-1α protein expression levels in *Pax8-rtTA/tetO-ICN1* transgenic animals (D) and in cultured renal tubular cells expressing Notch1 (E). (F) Immunohistochemical staining of PGC-1α in *Pax8-rtTA/tetO-ICN1* transgenic animals.

2. Decreased PGC-1α expression in human kidneys with fibrosis

Next, I examined the expression of PGC-1α in patients with kidney disease. I quantified transcript levels of *PPARGC1A* in 95 microdissected human kidney tubule samples by Affymetrix microarrays.[21,22] CKD was defined by reduced eGFR (< 60 mL/min/1.73 m^2), as per NKF guidelines.[1] In the dataset, 39 (41.1%) samples met the criteria for CKD.[9] As expected, CKD samples also showed significantly higher glomerulosclerosis and interstitial fibrosis. Demographics and clinical characteristics of these samples are shown in Table 2. Patients were classified into five groups; healthy control, hypertension without kidney disease (HTN), diabetes without kidney disease (DM), hypertensive nephrosclerosis (HTN-CKD), and diabetic nephropathy (DN-CKD). There were no differences in age, gender, race, and serum albumin among five groups. However, eGFR was significantly lower and blood pressure levels were significantly higher in the HTN-CKD and DN-CKD groups than in control groups.

In patient samples, *PPARGC1A* transcript levels significantly and positively correlated with eGFR (**Figure 2A**). Immunohistochemical analysis confirmed the transcript level data. PGC-1α was mainly expressed in the nuclei of the whole TECs. The nuclear staining of PGC-1α was evident in normal kidney, but it was significantly decreased in CKD **(Figure 2B)**. Notably, in advanced stages of CKD, relatively less damaged tubules showed PGC-1α activity. Immunofluorescence study showed that PGC-1α was expressed in both proximal and distal tubules, and the intensity of PGC-1α was weak in CKD **(Figure 2C)**. In aggregates, the analysis indicated that PGC-1α expression

correlates with kidney function in patient samples.

Finally, I further performed double immunofluorescence staining with antibodies against Notch1 and PGC-1α using human samples of CKD. Interestingly, in tubules with high expression of Notch1, PGC-1α expression was low. In contrast, Notch1 staining was weak in tubules with high PGC-1α activity **(Figure 2D)**. This finding suggests that Notch signaling negatively regulates PGC-1α.

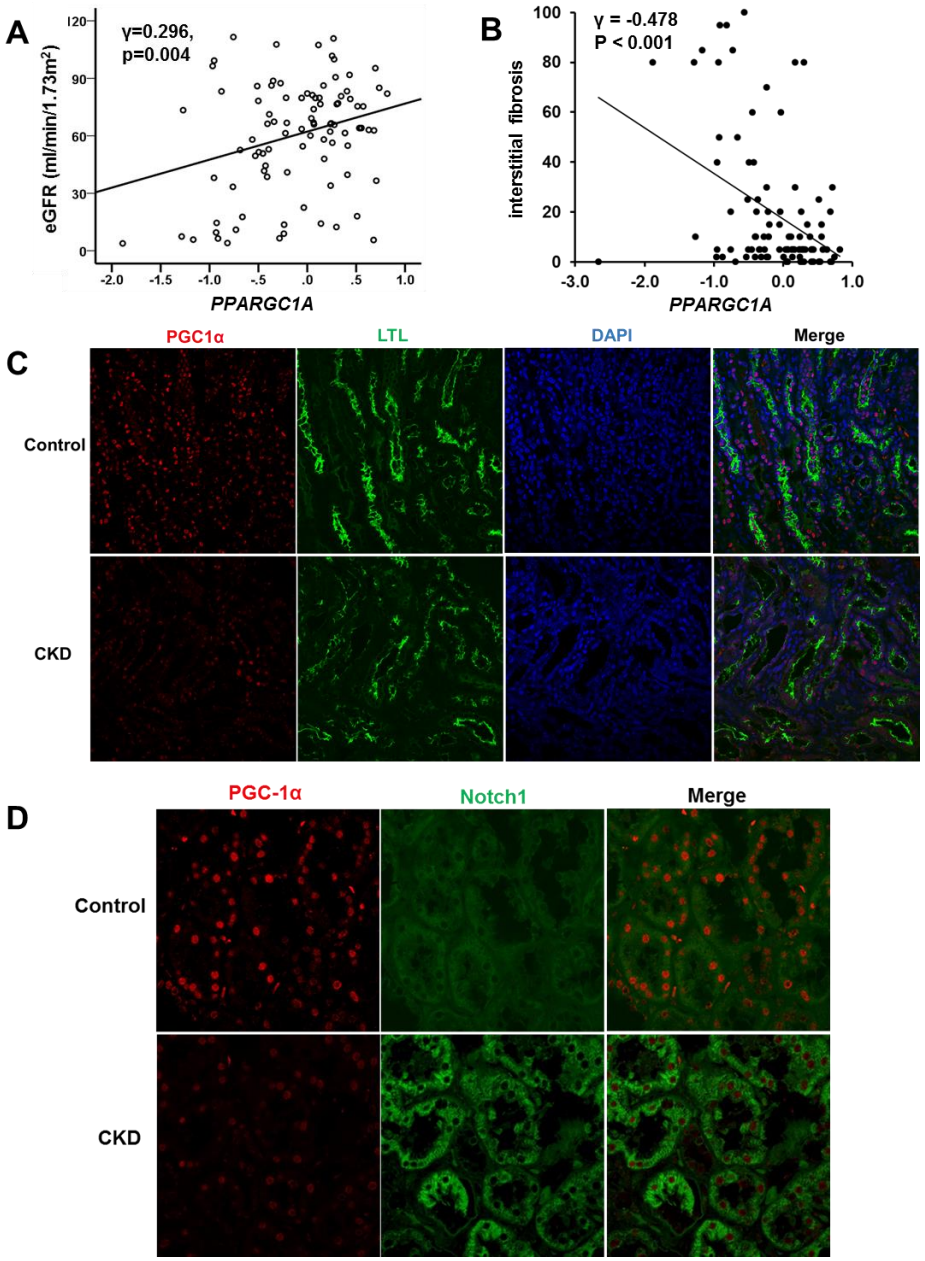


Figure 2. Decreased PGC-1α expression in human fibrotic kidney samples.

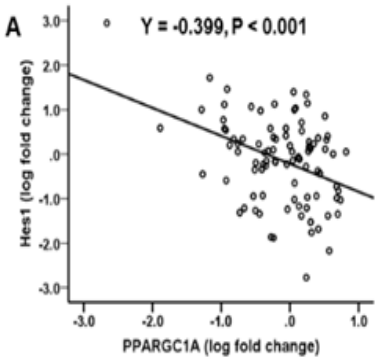
(A) Correlation between estimated glomerular filtration rate and *PPARGC1A* transcript level. (B) Representative images of PGC-1α immunohistochemical staining in control and diseased human kidney samples. (C) Double immunofluorescence staining with antibodies against PGC-1α and Lotus tetragonolobus lectin (LTL) in human samples of CKD. (D) Double immunofluorescence staining with antibodies against Notch1 and PGC-1α in human samples of CKD.

Table 2. Patients characteristics

	All	Control	HTN	DM	HTN-CKD	DKD	p-value
N (%)	95	19 (20%)	20 (21.1%)	17 (17.9%)	19 (20.0%)	20 (21.1%)	-
Age (years)	63.6 ±13.5	62.0 ± 9.7	64.1 ± 10.8	64.5 ± 12.7	59.3 ± 20.5	67.9 ± 10.8	0.363
Gender (Male, N, %)	55 (57.9%)	11 (57.9%)	12 (60.0%)	10 (58.8%)	12 (63.2%)	10 (50.0%)	0.942
Race (N, %)							0.825
African American	36 (37.9%)	6 (35.3%)	7 (43.8%)	5 (41.7%)	11 (51.1%)	7 (36.8%)	
Caucasian	19 (20.0%)	5 (29.4%)	5 (31.2%)	2 (16.7%)	2 (11.1%)	5 (26.3%)	
Hispanic	6 (6.3%)	1 (5.9%)	1 (6.2%)	2 (16.7%)	1 (5.6%)	1 (5.3%)	
Asian	5 (5.3%)	1 (5.9%)	1 (6.2%)	2 (16.7%)	0 (0.0%)	1 (5.3%)	
BMI (kg/m^2)	29.8 ± 9.3	28.6 ± 6.3	35.0 ± 15.2	28.7 ± 4.7	25.0 ± 5.8	31.6 ± 7.9	0.014
Systolic BP (mmHg)	138.7 ± 24.8	128.7 ± 19.3	142.8 ± 35.7	134.0 ± 16.8	152.4 ± 24.3	134.5 ± 27.5	0.061
Diastolic BP (mmHg)	78.1 ± 13.7	74.5 ± 12.8	79.2 ± 7.2	74.0 ± 11.4	87.4 ± 13.8	71.2 ± 12.7	0.010
Serum albumin (g/dL)	4.0 ± 0.7	4.3 ± 0.4	4.1 ± 0.5	3.9 ± 0.6	4.1 ± 0.5	3.7 ± 0.9	0.051
eGFR (mL/min/1.73 m^2)	60.3 ± 29.8	85.5 ± 18.6	77.1 ± 15.4	78.4 ± 10.7	29.6 ± 21.7	33.3 ± 18.4	<0.001

3. Notch1 directly regulates PGC-1α

I then explored the cellular and molecular mechanisms by which Notch1 signaling regulates PGC-1α in RTECs. Using microarray data from human kidney tubule samples, I examined the correlation between *PPARGC1A* and Notch pathway genes. I found that among several Notch receptors, ligands, and target gene *Hes1* shows the strongest correlation with *PPARGC1A* **(Figure 3A)**. Therefore, I hypothesized that Hes1 might directly regulate the expression of PGC-1α. To test this hypothesis, I performed ChIP assay using primers spanning the putative *Hes1*-responsive element region within the N-box flanking region of *Ppargc1a* promoter. This region has been reported to have regulatory function in mouse RTECs.[20] The ChIP assay indicated the direct binding of Hes1 to the *Ppargc1a* promoter region **(Figure 3B)**. The enrichment of *Hes1* binding region in the *Ppargc1a* promoter was significantly decreased by *Hes1* knock-down, whereas it was increased by *ICN1* plasmid transfection **(Figure 3C)**. To substantiate this finding, I also carried out a luciferase reporter assays. *Ppargc1a* promoter–driven luciferase reporter activity was significantly lower upon *Hes1* overexpression. In contrast, *Hes1* knock-down significantly increased this activity **(Figure 3D)**. These findings together suggest that the transcriptional repressor Hes1 (a downstream target of Notch1 signaling) can directly and negatively regulate PGC-1α in RTECs.



	Pearson's coefficient (γ)	p-value
NOTCH1	-0.096	0.353
NOTCH2	-0.301	0.003
NOTCH3	-0.382	< 0.001
NOTCH4	-0.102	0.323
HES1	-0.399	< 0.001
HES2	0.029	0.778

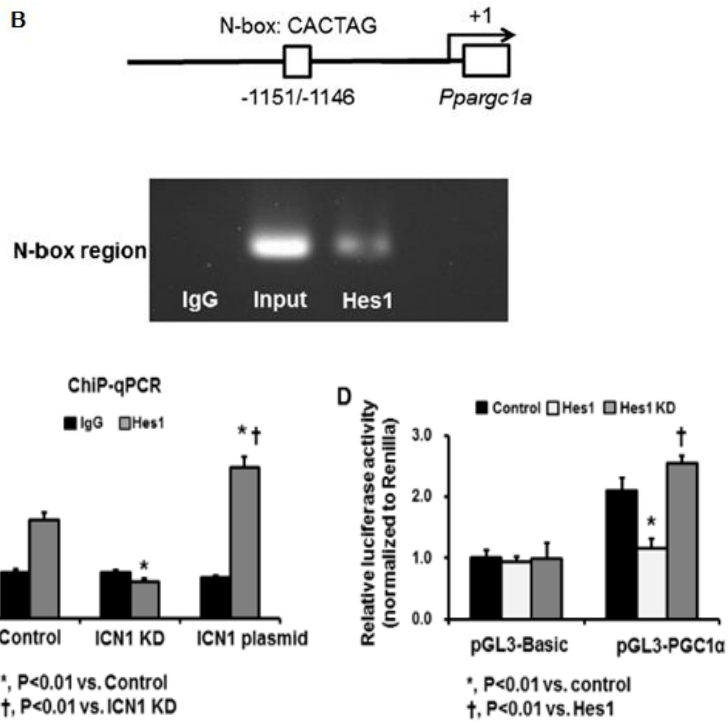


Figure 3. Hes1 directly regulates PGC-1α. (A) Correlation between *PPARGC1A* and *Hes1* transcript levels in 95 microdissected human kidney

tubule samples. (B) ChIP assay using a primer spanning the putative *Hes1*-responsive element region within the N-box flanking region of *Ppargc1a*. (C) ChIP assay after knock-down (KD) of *ICN1* and *ICN1* plasmid transfection. (D) Luciferase reporter activity assay using TECs obtained from primary culture after co-transfection of *pGL3– Ppargc1a* and *Hes1* plasmid or *Hes1* siRNA.

4. Tubule-specific overexpression of *Ppargc1a* ameliorates Notch1-induced kidney injury

Next, I hypothesized that decreased PGC-1α expression and a downstream metabolic defect are involved in Notch1-induced fibrosis development *in vivo*. To investigate the functional role of PGC-1α, I generated mice with conditional inducible overexpression of *Ppargc1a* in RTECs and I crossed mice expressing *Pax8-rtTA/tetO-Ppargc1a* with mice harboring *Pax8-rtTA/tetO-ICN1* to create *Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a* mice (**Figure 4A**). Littermates of *tetO-ICN1* or *tetO-Ppargc1a* mice (without *Pax8-rtTA*) were served as controls. As expected, transcript levels of *ICN1* in whole kidney tissue of *Pax8-rtTA/tetO-ICN1* and *Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a* mice were significantly higher than in control animals. In addition, mRNA expression levels of Notch target genes, *Hes1* and *Hey1*, were also higher in mice overexpressing Notch1 (**Figure 4B**). The mRNA expression of *Ppargc1a* was statistically and significantly increased in mice with tubule-specific PGC-1α expression (**Figure 4B**). Western blot analyses also confirmed the three results **(Figure 4C)**. At baseline, *Pax8-rtTA/tetO-Ppargc1a* mice did not show gross or histological abnormalities and were born at the expected Mendelian ratio. As described above, the *Pax8-rtTA/tetO-ICN1* mice became very sick and died at around 6 weeks after the initiation of doxycycline-containing chow.

I did not observe gross phenotypic abnormalities or increased mortality in triple transgenic (*Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a*) animals. Animals were sacrificed 6 weeks after the initiation of doxycycline-containing food. As

in a previous study,[10] kidneys were enlarged in *Pax8-rtTA/tetO-ICN1* mice compared to controls (**Figure 5A**). Normal renal architecture was lost, and kidneys showed severe tubular degeneration, dilatation, and interstitial fibrosis (**Figure 5B**). The interstitium was widened with accumulation of matrix and activated myofibroblasts. In contrast, kidney size was close to normal in *Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a* mice. Tubule dilatation was significantly attenuated, and fibrosis was markedly ameliorated in triple-transgenic mice compared to *Pax8-rtTA/tetO-ICN1* mice (**Figure 5B**).

In line with the morphological alterations, qPCR analysis showed that transcript levels of profibrotic genes such as *Procol1a1, Procol3a1*, *Fn* and *smooth muscle a-actin* were significantly increased in *Pax8-rtTA/tetO-ICN1* mice compared to control and *Pax8-rtTA/tetO-Ppargc1a* mice (**Figure 6A**). In addition, expression levels of inflammatory markers such as CD68 and F4/80 were significantly higher in mice with ICN1 overexpression (**Figure 6B and 6C**). These changes were significantly reduced in *Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a* mice. Masson trichrome staining, which showed severe fibrosis in mice expressing Notch1, indicated significant attenuation in mice expressing PGC-1α (**Figure 6D**). In summary, mice with tubule-specific expression of PGC-1α were protected from Notch1-induced kidney fibrosis.

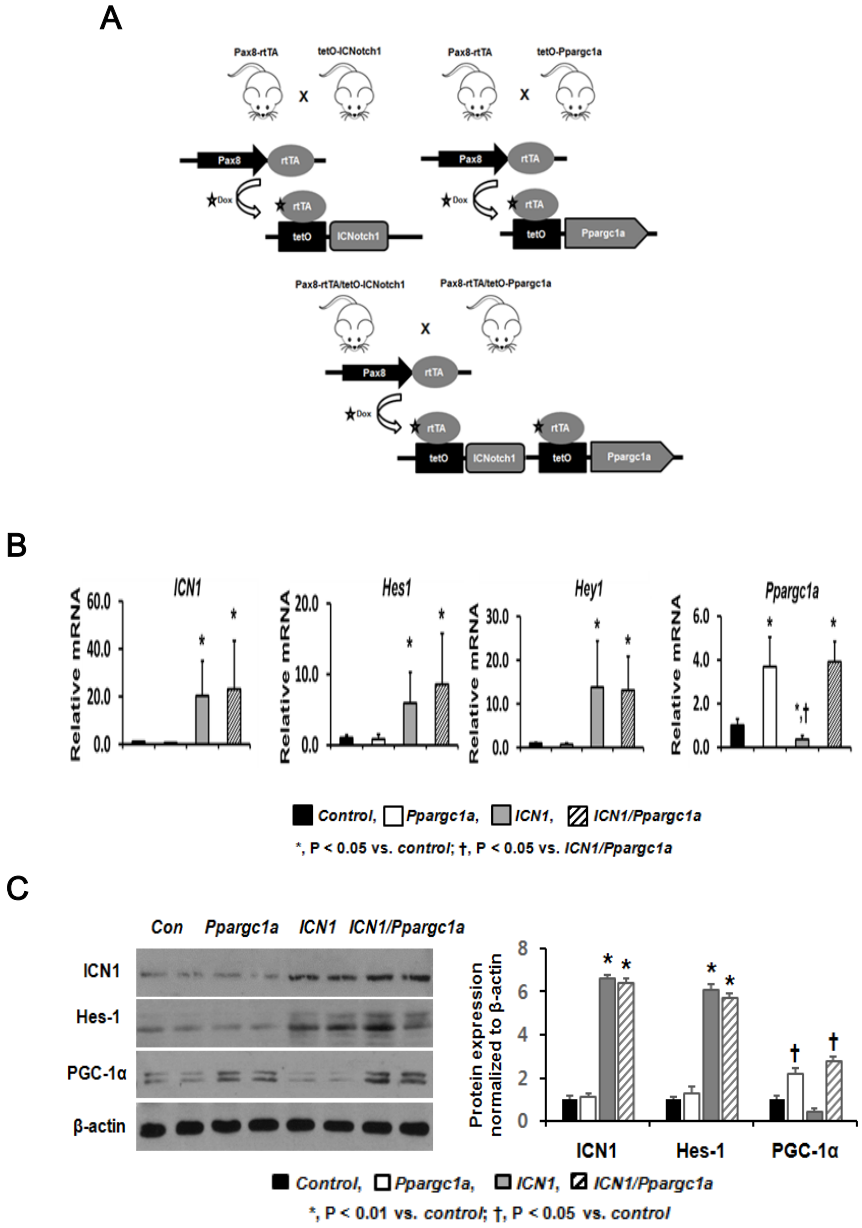


Figure 4. Generation of mice with conditional inducible overexpression of *ICN-1* and *Ppargc1a*. (A) Schematic figure depicting generation of mice with tubule-specific overexpression of *ICN-1* and *Ppargc1a*. (B) Quantitative real

time PCR analysis of *ICN1*, *Hes1*, *Hey1*, and *Ppargc1a* transcript levels in kidneys of control, *Pax8-rtTA/tetO-Ppargc1a*, *Pax8-rtTA/tetO-ICN1*, and *Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a* mice. (C) Western blot images of ICN1, Hes-1, and PGC-1α protein levels in the same mice.

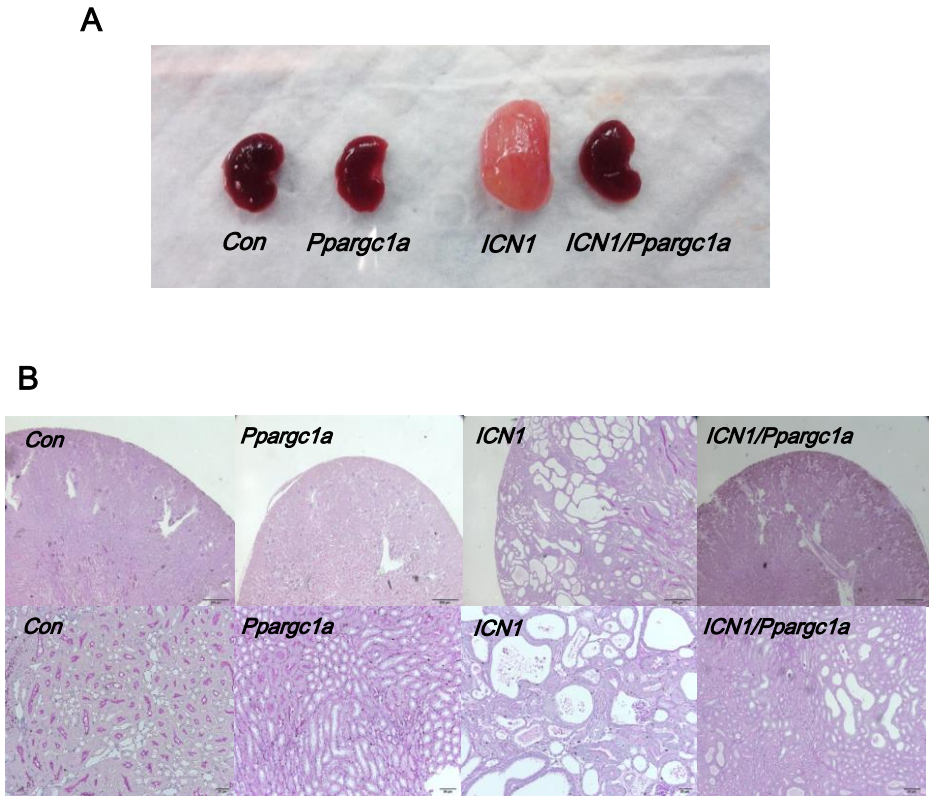
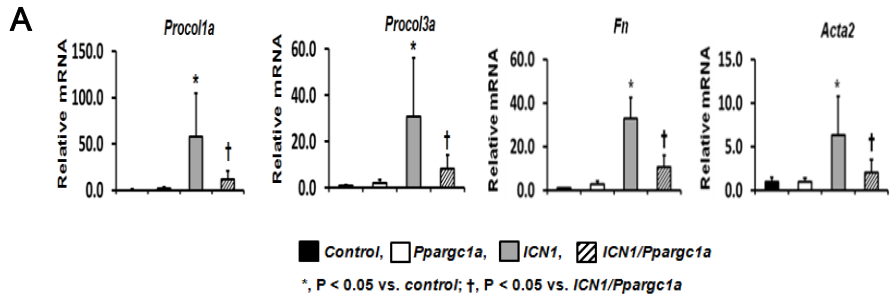
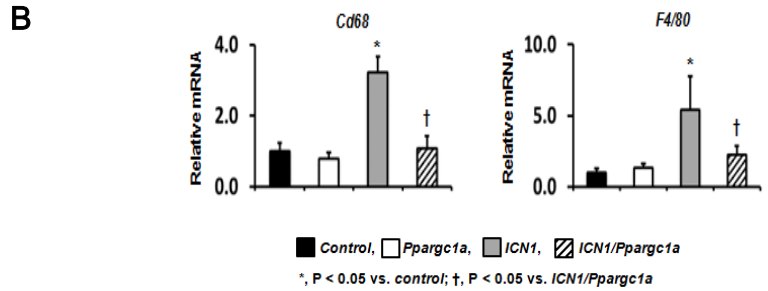
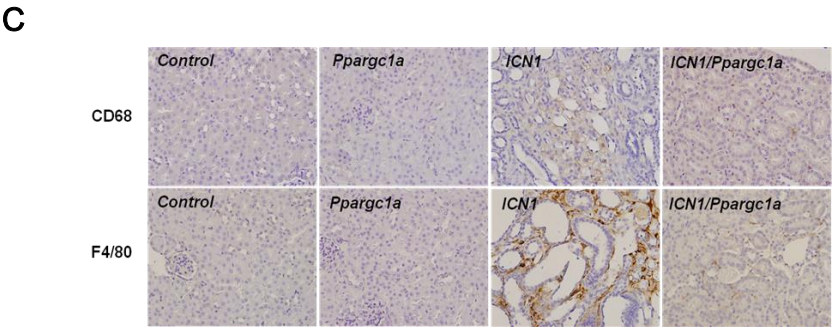


Figure 5. Phenotypic characterization of kidneys from *Pax8-rtTA/tetO-Ppargc1a, Pax8-rtTA/tetO-ICN1, and Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a* mice. (A) Gross morphology. (B) Representative images of PAS-stained kidney sections from control, *Pax8-rtTA/tetO-Ppargc1a*, *Pax8-rtTA/tetO-ICN1, and Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a* mice.







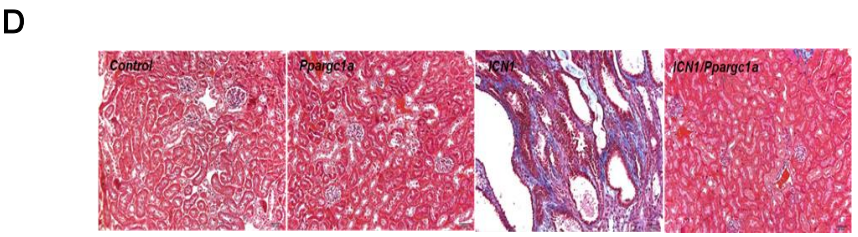


Figure 6. Tubule-specific overexpression of *Ppargc1a* ameliorates Notch1-induced kidney injury. Transcript levels of pro-collagen 1al (*Procol1a1*), pro-collagen 3a1 (*Procol3a1*), fibronectin (*Fn*) and smooth muscle α-actin

(*Acta2*) in whole kidneys lysates of *Pax8-rtTA/tetO-Ppargc1a*, *Pax8-rtTA/tetO-ICN1*, and *Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a* mice. (B) Relative mRNA levels of Cd68 and F4/80 in whole kidneys lysates of control, *Pax8-rtTA/tetO-Ppargc1a*, *Pax8-rtTA/tetO-ICN1*, and *Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a* mice. (C) Representative images of immunohistochemial staining for CD68 and F4/80 in the same mice. (D) Representative images of Masson's trichrome staining of each group.

5. PGC-1α restores impaired mitochondrial morphology and fatty acid oxidation (FAO) defect induced by Notch1 overexpression *in vitro*

PGC-1α is a key regulator of mitochondria biogenesis. Thus, I then evaluated morphological changes of mitochondria in TECs overexpressing ICN1 and/or PGC-1α. Primary tubular cells were transfected with *Notch1*- and *Ppargc1a*-containing plasmids. Electron microscopic analysis showed normally looking and elongated mitochondria with intact cristae in TECs transfected with control (*pcDNA3*) or *Ppargc1a* plasmids. By contrast, mitochondrial structure was lost and mitochondria appeared fragmented, small, and round in RTECs expressing *ICN1*. Morphological alterations in mitochondria were almost abrogated in *ICN1* expressing cells on *Ppargc1a* expression **(Figure 7A)**.

In parallel, PGC-1α expression also attenuated the Notch-induced reduction of mitochondrial transcripts such as *Tfam* and *mtDNA* in mice and cells expressing *ICN1* (**Figure 7B and 7C**).

I then examined transcript levels of FAO-related genes induced by Notch expression (**Figure 7D**). Cells expressing ICN1 had significantly reduced transcript levels of *Ppargc1a, Cpt1,* and *acyl-coenzyme A oxidase 1(Acox1)*. These alterations were attenuated by PGC-1α co-transfection. Notch1 expression in TECs resulted in increased expression of profibrotic genes. The increased expression of profibrotic genes, such as *Procol1a* and *FN,* were attenuated by PGC-1α. In summary, in cultured cells, Notch1 induced changes of mitochondrial transcript level and structural mitochondrial defects. PGC-1α reversed mitochondrial defects induced by Notch1 expression.

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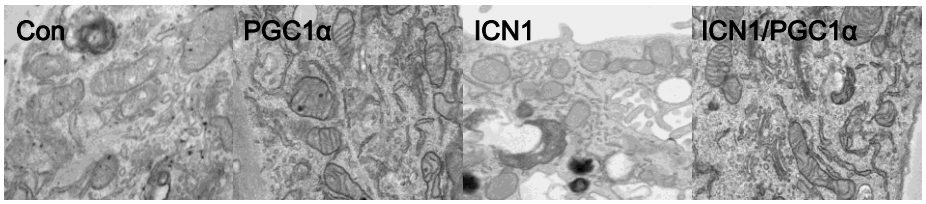

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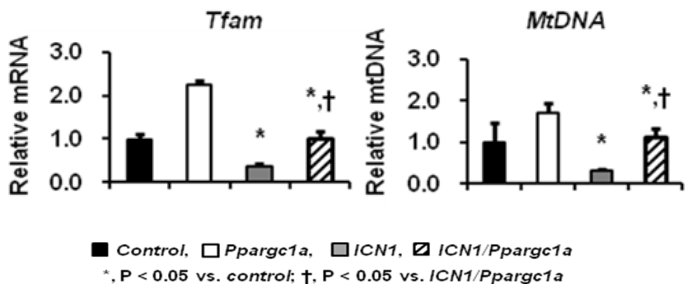

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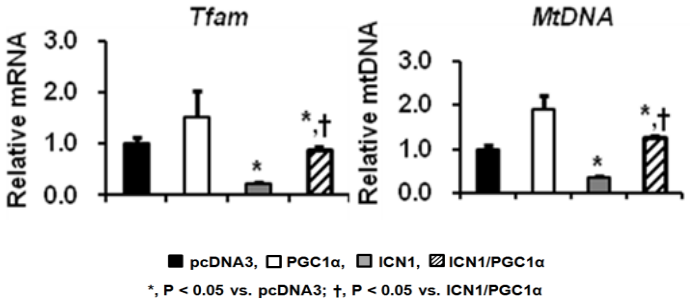

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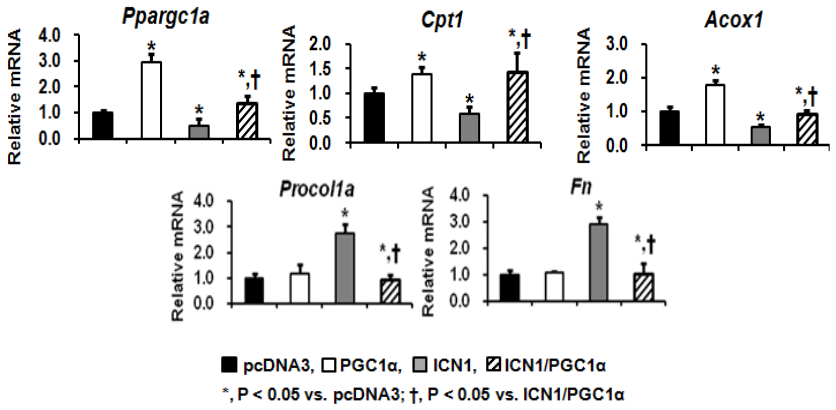

Figure 7. PGC-1α restores the impaired mitochondrial morphology and fatty acid oxidation pathway induced by Notch1 overexpression. (A) Representative transmission electron microscopy images of TECs transfected with *pcDNA3*, *Ppargc1a*, *ICN1*, and *ICN1/Ppargc1a* plasmids. (B) mRNA expression of *Tfam and MtDNA* in control, *Pax8-rtTA/tetO-Ppargc1a*, *Pax8-rtTA/tetO-ICN1*, *and Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a* mice and (C) in cultured TECs transfected with *pcDNA3*, *Ppargc1a*, *ICN1*, and *ICN1/Ppargc1a* plasmids. (D) qPCR assay for fatty acid oxidation-related profibrotic markers in control and TECs transfected with *Ppargc1a*, *ICN1*, and *ICN1/Ppargc1a* plasmids.

6. PGC-1α attenuates alteration of fatty acid oxidation and Notch1-induced kidney injury *in vivo*

Next, I examined the effects of PGC-1α on Notch1-induced kidney injury *in vivo*. I quantified transcript levels of genes related to FAO. In *Pax8-rtTA/tetO-ICN1* mice, mRNA expression levels of *Cpt1* and *Acox1* were significantly decreased when compared to control mice. PGC-1α expression almost completely normalized *Cpt1* and *Acox1* levels *in vivo* (**Figure 8A**). Western blot analysis revealed that protein levels of phospho-ACC and CPT1 were significantly decreased in *Pax8-rtTA/tetO-ICN1* mice but were restored in *Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a* mice (**Figure 8B**).

Next, I tested whether Notch1-induced apoptotic cell death was also attenuated by PGC-1α. qPCR analysis indicated that mRNA expression levels of *B cell CLL/lymphoma 2* (Bcl2), an anti-apoptotic gene, were significantly lower in *Pax8-rtTA/tetO-ICN1* mice (**Figure 9A**) and in RTECs transfected with the *ICN1* plasmid (**Figure 9B**). Western blot analysis confirmed the decreased BCL2 levels in mice with tubule-specific Notch1 overexpression (**Figure 9C**). Notch transgenic mice also showed increased cleaved-caspase3 levels, likely consistent with increased apoptosis. *Ppargc1a* expression protected tubule cells from the Notch-induced increased apoptosis as examined by cleaved caspase 3 immunostaning (**Figure 9D**). I also examined ICN1-induced cell proliferation activity in vivo. Immunohistochemical staining study showed that the number of PCNA and Ki67 positive cells was significantly decreased in *Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a* mice compared to *Pax8-rtTA/tetO-ICN1* mice (**Figure 9E**).

In summary, in vivo expression of PGC-1α improved fatty acid oxidation, reduced apoptosis and cell proliferation, and ameliorated kidney fibrosis.

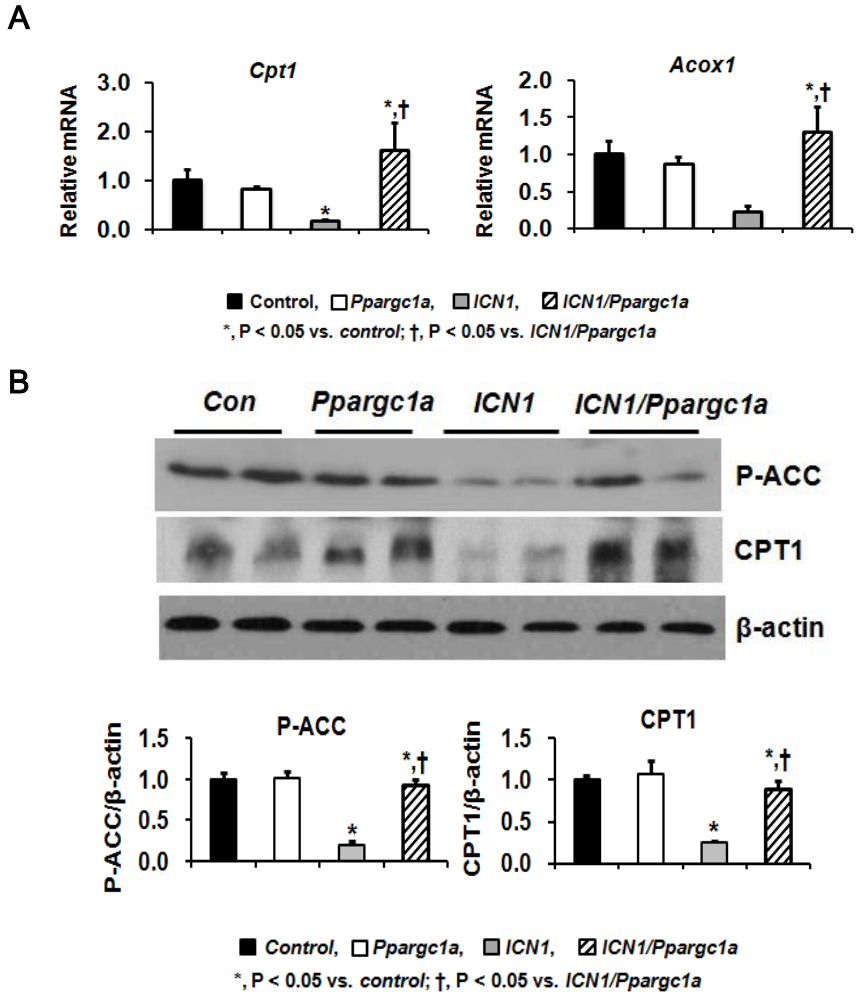

Figure 8. PGC-1α improves altered fatty acid oxidation and Notch1-induced kidney injury. (A) qPCR assay for fatty acid oxidation-related genes in kidneys of control, *Pax8-rtTA/tetO-Ppargc1a*, *Pax8-rtTA/tetO-ICN1*, and *Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a* mice. (B) Representative images and quantification of Western blot analyses for phospho-ACC, and CPT1 in these mice groups.

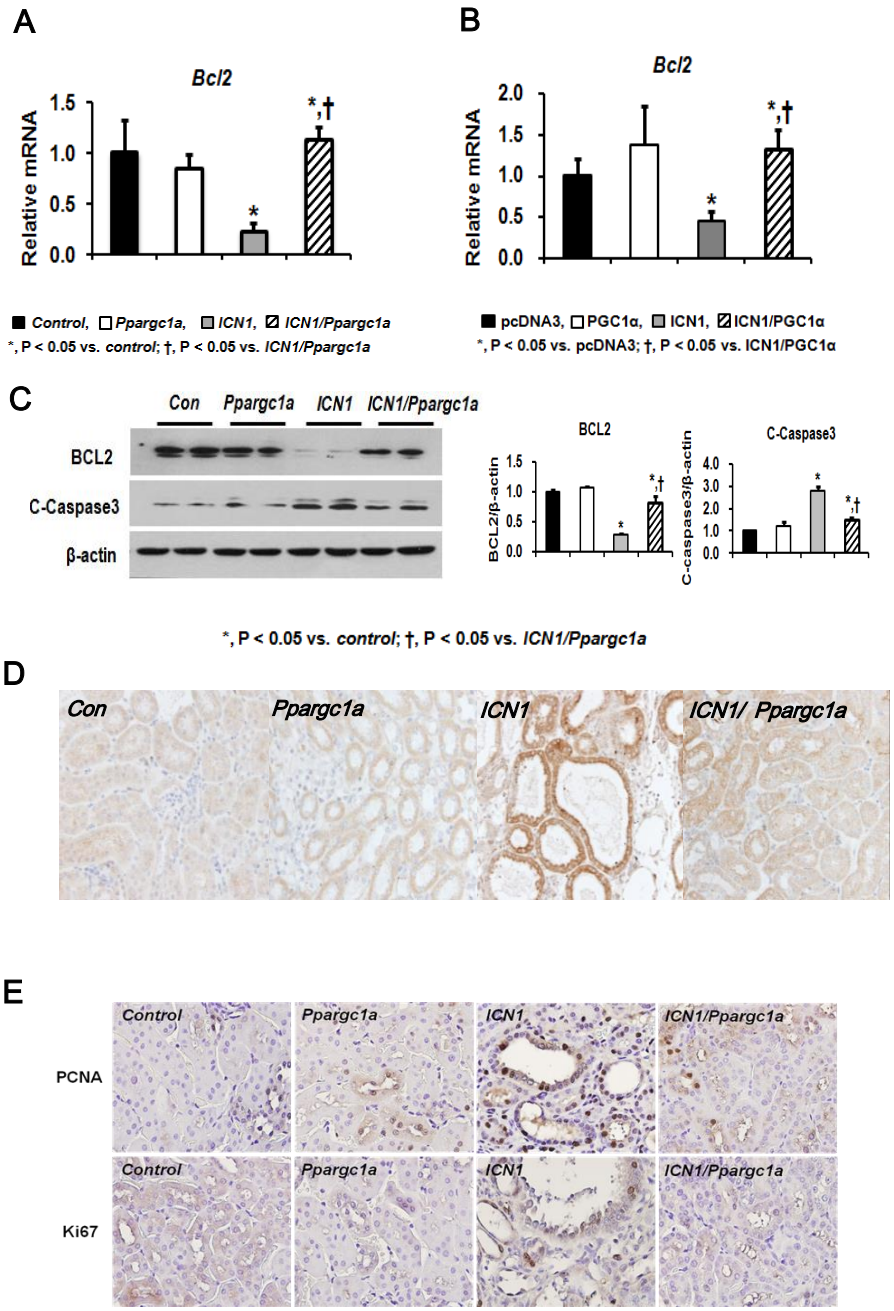

Figure 9. PGC-1α overexpression in TECs attenuates Notch1-induced apoptotic cell death. (A) Relative mRNA level of *Bcl2* in control, *Pax8-*

rtTA/tetO-Ppargc1a, Pax8-rtTA/tetO-ICN1, and *Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a* mice. (B) Relative mRNA level of *Bcl2* in control and TECs transfected with *Ppargc1a*, *ICN1*, and *ICN1/Ppargc1a* plasmids. (C) Representative images and quantification of Western blot analyses for BCL2 and cleaved-caspase 3 in 4 mice groups. (D) Representative immunohistochemial staining with cleaved-caspase 3 in control *Pax8-rtTA/tetO-Ppargc1a, Pax8-rtTA/tetO-ICN1, and Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a* mice. (E) Representative images of immunohistochemical staining for PCNA and Ki67 in *control, Pax8-rtTA/tetO-Ppargc1a, Pax8-rtTA/tetO-ICN1,* and *Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a* mice.

IV. DISCUSSION

In this study, I showed that Notch1 overexpression resulted in mitochondrial defects, FAO dysregulation, cell death, and severe kidney fibrosis. These alterations were restored by PGC-1α. I also found that Notch1 signaling directly regulates PGC-1α. These findings unravel novel mechanisms for Notch1-induced kidney injury and underscore the potential role of PGC-1α to improve kidney dysfunction.

Notch signaling has dual roles. Notch signaling molecules are expressed throughout kidney development and contribute as key factors to the nephrogenesis. Receptors such as Notch1 and Notch2, and the ligands Delta1 and Jagged1 are expressed in developing glomerular and tubular epithelial cells.[23,24] However, the expression of these molecules is dramatically decreased in the mature kidneys.[12] Notch activation is likely secondary to kidney injury and a part of a regenerative response. Increased Notch activity is associated with higher tubular proliferation. Although some researchers believe that cell proliferation may be associated with a less-differentiated state and contribute to matrix proteins for fibrosis,[25] Notch-induced proliferation is more likely to play an important role in epithelial repair and regeneration after kidney injury, not increasing epithelial cell mass.[12,26] During development and *in vitro* settings, Notch is a strong inducer of EMT,[15,22] but the role of EMT in fibrosis development is a highly controversial issue.[27]

Nevertheless, sustained Notch activation has been described in patients and in mouse models of CKD.[8-10] Furthermore, genetic activation of Notch receptors and ligands results in severe kidney injury. Conditional re-

expression of ICN1 in vivo in podocytes increased urinary protein excretion and prominent glomerulosclerosis.[9,14] In addition, RTEC-specific expression of ICN1 induced rapid and severe TIF, which was ameliorated by a γ-secretase inhibitor, and pharmacologic inhibition of Notch pathway also aggravated the TIF in the folic acid-induced and the ureteral obstruction-induced mouse models.[10] In the histologic analysis of different human kidney disease samples, Notch1 expression was significantly correlated with severity of glomerlulosclerosis, eGFR and TIF.[8] These findings together suggest that activated Notch signaling is indeed a major contributor to the development of kidney fibrosis. In line with these studies, I recapitulated kidney fibrosis by tubule-specific Notch1 overexpression.

PGC-1α is a master regulator of mitochondrial biogenesis.[28] Mitochondria are the powerhouse of the cell and play an important role in cellular homeostasis. Dysfunctional mitochondria eventually jeopardize cell viability,[18,29] leading to cell death and dedifferentiation. PGC-1α orchestrates mitochondrial biogenesis by interacting with estrogen-related receptor-α, peroxisome proliferator-activated receptors, and nuclear respiratory factors 1 and 2, and thereby increases mitochondrial mass and overall mitochondrial function.[6,30,31] One of the main targets of PGC-1α is FAO, which is the preferred energy source for highly metabolic cells. In particular, kidney exclusively utilizes FAO as an energy source, which was uncovered through the effect on the kidney injury by genetic or phamarcologic alteration in FAO pathway, even without restoring the defect in the glucose utilization, in different TIF mouse models.[16] Notably, PGC-1α plays the key co-transcription

factor to regulate the expression of genes involved in fatty acid uptake and oxidation such as *Cpt1* and *Acox1*, the rate-limiting and key enzymes in FAO.[16,32] In this context, it can be presumed that deficiency or inactivation of PGC-1α can impair mitochondrial function, FAO, and energy supply. This consequently can lead to cell death and tissue damage. In fact, transcript levels of *PPARGC1A* and genes related to FAO were notably decreased in patients with CKD as compared to controls.[8] In different animal models of acute kidney injury, PGC-1α was suppressed and mitochondria were damaged, and these defects were significantly attenuated by excess PGC-1α.[18,33] Also, PGC-1α reduction was observed in aldosterone-induced podocyte injury, and activation of it ameliorated mitochondrial dysfunction and protected podocyte.[34] In line with these findings, in the present study, the expression of PGC-1α and enzymes involved in FAO was significantly decreased and mitochondrial structure was distorted in Notch1-induced kidney fibrosis model. Here, I specifically asked the question whether metabolic alterations through PGC-1α are involved in Notch-induced fibrotic process. The results showed that PGC-1α is not only regulated by Notch but is involved in Notch-induced fibrosis development. Transgenic expression of PGC-1α was able to ameliorate Notch-induced FAO protein reductions and fibrosis development. These results indicate that metabolic dysregulation plays a key role in cellular dedifferentiation and providing sufficient metabolic input can prevent RTECs from Notch-induced dedifferentiation, death, and fibrosis development.

Hes1 belongs to the highly conserved family of Hairy-related proteins and it is a key Notch1 target gene, and mainly mediates the harmful effects of

Notch signaling.[35] It has a domain which can bind a DNA motif, called the N-box.[36] Similarly to many Hairy-related proteins, *Hes-1* can recruit histone deacetylases and Groucho/TLE family, transcriptional co-repressors, thus leading to deacetylation of the promoter region of target genes, making them transcriptionally inactive.[37] The expression of *Hes1* was increased significantly in different mouse models of TIF.[10] In addition, the results of the present microarray data from human kidneys showed that transcript level of *Hes1* was also significantly increased in diseased kidney. Based on these findings, I was particularly interested in *Hes1* as a main target of Notch1 signaling and used this gene for further study. I evaluated the direct interaction between Notch1 and PGC-1α by ChIP and luciferase reporter assays, and found that Notch signaling directly modulates transcriptional activity of PGC-1α in RTECs, and it is mediated by Hes1. Several recent reports suggest that Notch is a key regulator of metabolism.[21,38,39] A study by Bi et al.[21] showed that constitutive activation of Notch signaling inhibits *Ppargc1a* transcription in white adipocytes, demonstrating the binding of Hes1 to the *Ppargc1a* regulatory region. Taken together, these findings suggest that PGC-1α appears to be a direct target in RTECs of Notch1-mediated kidney fibrosis.

V. CONCLUSION

In this study, I showed that Notch1 induces downregulation of PGC-1α, impairment of fatty acid oxidation, and mitochondrial dysfunction. *Ppargc1a* is a direct Notch1 target gene and genetic re-expression of PGC-1α in RTECs abrogates Notch1-induced kidney fibrosis. These findings suggest that metabolic pathways play key roles in Notch1 (a developmental pathway) induced fibrosis development, and restoration of PGC-1α activity could be a promising treatment strategy against CKD.

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ABSTRACT (IN KOREAN)

Notch에 의하여 유도된 신섬유화에 대한 PGC-1α의 억제효과

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신섬유화는 만성 신장질환의 특징적인 조직학적 변화이며, 특히 세뇨관-간질 섬유화는 신기능의 저하와 밀접한 연관이 있는 것으로 알려져 있다. 신세뇨관 상피세포에서 Notch 신호전달체계의 지속적인 활성화는 신섬유화의 발생에 중요한 역할을 하는 것으로 보고되고 있지만, Notch에 의하여 유도되는 신섬유화의 근원적 기전에 대해서는 아직까지 명확하게 규명되지 않은 실정이다. 한편, 최근의 일부 연구에서는 에너지대사의 변화 및 불충분한 에너지 공급이 신섬유화의 발생과 관련이 있는 것으로 밝혀졌다. 이에 본 연구에서는 Notch에 의하여 유도된 신섬유화의 과정에 에너지대사의 변화가 관

여할 것이라는 가정 하에 에너지 및 미토콘드리아 대사에 관여하는 것으로 알려져 있는 peroxisomal proliferator-gamma coactivator -1α (PGC-1α)의 역할에 대하여 알아보고자 하였다.

실험으로는 만성 신장질환 환자의 신장 조직에서 PGC-1α의 발현 변화를 확인하였을 뿐만 아니라, PGC-1α의 역할을 규명하기 위하여 *Pax8-rtTA/tetO-ICN1* 마우스, *Pax8-rtTA/tetO-Ppargc1a* 마우스, 그리고 *Pax8-rtTA/tetO-ICN1* 마우스와 *Pax8-rtTA/tetO-Ppargc1a* 마우스를 교배하여 얻은 *Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a* 마우스를 이용한 동물실험도 시행하였다. 각각의 실험 동물군의 신장 조직을 이용하여 신섬유화 정도, PGC-1α의 발현 및 지방산 산화 경로의 변화, 그리고 세포사멸 등을 확인하였다. 생체외 실험으로는 Notch에 의하여 유도된 신섬유화의 근원적 기전을 규명하기 위하여, 1차 배양 신세뇨관 상피세포를 이용하여 chromatin immunoprecipitation (ChIP) assay와 luciferase assay를 수행하였다.

실험 결과를 보면, Notch1이 신세뇨관에서 과발현된 *Pax8-rtTA/tetO-ICN1* 마우스의 신장과 신섬유화가 동반된 환자의 신장에서 PGC-1α의 발현이 의미있게 감소되었다. 또한, ChIP assay를 통하여 Notch1의 표적 유전자인 Hes1이 PGC-1α를 코딩하는

Ppargc1a 유전자의 전사조절 부위에 직접적으로 결합함을 확인하였다. 동물실험상 *Pax8-rtTA/tetO-ICN1* 마우스에 비하여 *Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a* 마우스의 신장에서 신섬유화와 관련된 유전자들의 발현 감소뿐만 아니라 신장의 조직학적 호전이 관찰되었다. 1차 배양 신세뇨관 상피세포에서 PGC-1α를 과발현시켰을 때, Notch1의 과발현으로 인한 미토콘드리아의 구조 변화와 지방산 산화 장애가 개선되었다. *Pax8-rtTA/tetO-ICN1* 마우스에 비하여 *Pax8-rtTA/tetO-ICN1/tetO-Ppargc1a* 마우스의 신장에서도 지방산 산화와 관련 유전자의 발현 변화와 세포사멸이 유의하게 완화되었다.

이상의 결과로, Notch1에 의하여 유도된 신섬유화 과정에 에너지 대사 경로가 중요한 역할을 하며, Notch1의 직접적 조절 하에 있는 PGC-1α의 활성을 유지 또는 증가시키는 것이 만성 신장질환의 치료에 유용할 것으로 사료된다.

핵심되는 말: PGC-1α, Notch1, 신섬유화, 지방산 산화, 미토콘드리아

PUBLICATION LIST

Han SH, **Wu MY**, Nam BY, Park JT, Yoo TH, Kang SW, et al. PGC-1α Protects from Notch-Induced Kidney Fibrosis Development. *J Am Soc Nephrol* 2017;28:3312-22.