석 사 학 위 논 문

GLDC Protects Cisplatin-Induced Damage of Renal Proximal Tubular Cell by Activating UCP1

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이 논문을 석사학위 논문으로 제출함

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Bui Anh Phuc

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1. Introduction

Glycine decarboxylase (GLDC) is one of four individual components of the glycine cleavage system (GCS) (1). Glycine is one of the major inputs for one-carbon metabolism, and excess glycine is converted into aminoacetone and methylglyoxal, which impair cell growth (2). GLDC drives nucleotide biosynthesis and cellular methylation reaction during cell proliferation. Additionally, a study reported that GLDC induces non-small cell lung cancer and glioma (2). Although its expression in the kidney is second highest next to the liver, very little is known as to the role of GLDC in the kidney.

Cisplatin is a chemotherapy medication used to treat several cancers such as head and neck, lung, testis, ovarian, and bladder cancers and causes acute kidney injury (AKI) (3). AKI is a potential cause of chronic kidney disease (CKD) (4). Proximal tubular kidney cells in AKI show premature renal senescence and excess production of reactive oxygen species (ROS). ROS reacts with proteins, nucleic acids, carbohydrates, and lipid, contributing to cell apoptosis and necrosis (5). Cisplatin induces AKI via accelerating cellular senescence and stimulating ROS production in renal proximal tubular cells. Therefore, reducing cellular senescence and ROS production would reduce nephrotoxicity.

ROS is mainly generated in mitochondria of cells (6). Electrons form the electron transport chain of the inner membrane directly contribute oxygen to ROS. Uncoupling protein 1 (UCP1), a member in mitochondria, transports protons to matrix from inner membrane, which cause a limited decrease in membrane's potential, inhibits ROS

emission the electron transport chain, uncoupling respiration from ATP production (7-9).

In the present study, I addressed these issues as follows: (i) I evaluated the effects of GLDC in reactive oxygen species generation cisplatin-induced, (ii) GLDC reduced cisplatin-induced premature senescence, (iii) GLDC regulated apoptosis via UCP1-mediated. My results indicated that GLDC may protect against kidney injury cisplatin-induced through inhibiting oxidative stress.

2. Materials and Methods

2.1. Cell culture:

HK2 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS) and 1:100 penicillin/streptomycin in a humidified incubator (37 °C, 5% CO_2). Cells were subcultured twice a week by harvesting with trypsin/EDTA. To knock down GLDC, HK2 was transfected with 40 nM of small interfering RNA (siRNA) and lipofectamine 2000 (Invitrogen, Carlsbad, USA) was employed to transfect the cells with siRNA following the manufacturer's instructions. GLDC siRNA and negative control siRNA were prepared by Bioneer, Daejeon, Korea.

2.2. Generation of expression vector:

HK2 cells were seeded in 24-well plates. Mixture containing polybrene, lentivirus and media was prepared. Cells were incubated with the prepared mixture overnight at 37 °C, 5% CO_2. Cells were replaced with new media without polybrene. Next, cells were cultured in media with puromycin at 1-10 μg/mL for a month.

2.3. RNA isolation and real-time polymerase chain reaction (RT-PCR) analysis:

The TRizol reagent (Thermo, Massachusetts, USA) was used to

extract total RNA of cells according to the manufacturer's instructions. The NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) was used to measure the purity and concentration of the RNA solution. RT-PCR analysis was conducted with the SYBR green mix (Thermo, Massachusetts, USA) on LightCycler 480 II (Roche, Basel, Switzerland). Samples were normalized against β-actin (10).

2.4. Immunobloting:

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Boston, USA) with protease/phosphatase inhibitor cocktail. Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes (Amersham Bioscience, Buckinghamshire, UK). The protein-specific signals were detected using LAS-3000 (Fujifilm, Tokyo, Japan).

Antibodies: p21 (#2947T, Cell Signaling, 1:3000), p27 (#3688T, Cell Signaling) UCP1 (ab10983, abcam), lamin B1 (sc 30264, Santa Cuz), GLDC (VL3143388C, Invitrogen), β-actin (sc 47778, Santa Cruz), cleaved caspase 3 (9664S, Cell Signaling), cleaved parp (5625S, Cell Signaling).

2.5. Colony formation assay:

Cells were seeded in 6-well plates. After 6 days, cells were evaluated colony formation. Cells were washed with phosphate buffered saline (PBS), then fixed in 4% paraformaldehyde (Solarbio,

Beijing, China) for 15 min. Next, cells were stained with 0.5% crystal violet and surviving colonies were visualized. For quantification of colonies, cells were washed with PBS. Then, cells were incubated with methanol for 10 min. Intensity was measured at 590 nm by a microplate reader.

2.6. Mitochondrial ROS generation assay:

Mitochondrial ROS generation was detected using MitoSOXTM red mitochondrial superoxide indicator (Thermo Fisher Scientific, Boston, USA). In immunofluorescence assay, cells were seeded in 24-well plates containing sterile coverslips. They were treated with 2 μM cisplatin for 24 hr. Cells were washed with PBS and then fixed in 4% paraformaldehyde (Solarbio, Beijing, China) for 15 min at room temperature (RT). After washing three times with PBS, cells were incubated with MitoSOXTM reagent working solution 5 μM for 10 min at 37 ℃. Then, cells were washed three times with PBS and stained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min. Coverslips were mounted and visualized with confocal laser scanning microscopy (Zeiss LSM 5 exciter, Overkochen, Germany). To quantitate mitochondrial ROS, cells were treated with 2 μM cisplatin. Then, cells were harvested and washed with PBS. Next, cells were incubated with MitoSOXTM reagent working solution 5 μM for 10 min at 37 ℃. Mitochondrial ROS level was analysed by flow cytometry BD FACS Canto II system (BD, New Jersey, USA).

2.7. Apoptosis:

Cells were seeded into 6-well plates and treated with cisplatin 10 μM. Cells were harvested and washed with PBS. Cells were stained with FITC Annexin V Apoptosis Detection Kit 7-AAD (Biolegend, California, USA) according to the manufacturer's instructions. Apoptosis was detected by flow cytometry, BD FACS Canto II (BD, New Jersey, USA).

2.8. Cell cycle:

Cells were seeded into 6-well plates. Cells were collected, washed with PBS, and fixed in 70% cold ethanol for at least 30 min. After fixation, cells were incubated with 200 μg/mL ribonuclease A (Thermo Fisher Scientific, Boston, USA) and propidium iodide for 15 min at 37 °C. Data was acquired by BD FACS Canto II system (BD, New Jersey, USA).

2.9. Cytotoxicity assay:

Cells were seeded in 96-well plates (100 μL media per well). The next day, cells were treated with different concentrations of cisplatin. After 1, 2, and 3 days, cell counting kit 8 (CCK-8) solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added 10 μL for each well and incubated for 1 hr. Intensity was measured at 450 nm absorbance.

2.10. Cell growth curve assay:

Cell proliferation was evaluated by CCK-8. Cells were seeded in 96-well plates in 100 μL media. CCK-8 solution was added 10 μL for each well and incubated for 1 hr. Intensity was measured the absorbance at 450 nm.

2.11. β-galactosidase staining:

The senescence associated β-galactosidase (SA-β-Gal) activity was widely used for cell senescence test which was determined using senescence associated β-galactosidase assay kit (Cell Signaling Technology, Boston, USA). Positive signals were calculated by Image J.

2.12. Statistical analysis:

All experiments were performed with three independent experiments and all data represented as mean ± standard deviation (SD). Statistical analyses were conducted using the t-test. Statistical significance was determined with p value < 0.05.

3. Results

3.1. GLDC protects cells against cisplatin and reduces in proximal tubular cell:

To investigate the relevance of GLDC expression in HK2 cell and cisplatin treatment, I examined cell viability of GLDC overexpression (GLDC OE) and GLDC knockdown (GLDC KD) cells treated with cisplatin. My analysis revealed that GLDC OE significantly increased the percentage of live cell (Figure 1A). Moreover, cell viability in GLDC KD was lower than control samples with cisplatin treatment. This finding provides evidence for a potential protective role of high GLDC expression in HK2 cell.

To identify the role of GLDC in apoptosis by cisplatin treatment, flow cytometry analysis and western blot were performed. Interestingly, flow cytometry assay showed that GLDC OE significantly reduced percentage of apoptosis with and without cisplatin treatment, compared to control (Figure 1B&1C). However, knockdown of GLDC markedly increased apoptosis, which was consistent with the results of GLDC OE. Western blot results showed that cleaved caspase 3 and cleaved parp expression in GLDC OE were downregulated, compared to empty vector in the presence of cisplatin (Figure 1B). In contrast, the level of apoptotic biomarkers in GLDC KD increased significantly with cisplatin treatment. These results indicate that GLDC could inhibit apoptosis and conferred protective effects against cisplatin.

3.2. GLDC overexpression reduces cisplatin-induced ROS generation:

To explore the effect of GLDC in proximal tubular cell, I analyzed ROS generation by flow cytometry assay and immunofluorescence. The results showed that deletion of GLDC promoted ROS generation with and without cisplatin. However, the level of positive signals in GLDC OE were less than control samples in presence of cisplatin (Figure 2A). Additionally, RT-PCR data revealed overexpression of GLDC markedly elevated mRNA expressions of superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), and glutathione peroxidase 1 (GPX1) (Figure 2B). Furthermore, knockdown of GLDC decreased mRNA levels of these antioxidative genes (Figure 2B). Together, these data demonstrate that expression of GLDC could reduce cisplatin-induced ROS in proximal tubular cell.

3.3. GLDC regulates cisplatin-induced senescence:

Cisplatin treatment induces premature senescence in renal tubular cells in vitro and in vivo (11). SA-β-Gal staining was performed to detect cellular senescence in HK2 cell with and without cisplatin (12). Positive signals for SA-β-Gal were observed after 24 hr treatment and increased significantly in empty vector but not in GLDC OE. Meanwhile, GLDC KD induced higher level of senescence than control (Figure 3A). Next, western blot data showed that expression of lamin B1, well-known senescence protein maker, is reduced significantly in GLDC KD in presence of cisplatin, compared to control (Figure 3B). Additionally, GLDC OE increased lamin B1

expression as compared with empty vector with or without cisplatin. The data indicate that GLDC could regulate cellular senescence induced by cisplatin.

3.4. GLDC promtoes cell proliferation:

In HK2 cell, GLDC OE could reduce the effect of cisplatin, so GLDC might support cell proliferation rate. To test this hypothesis, I performed colony formation assay and growth curve. HK2 cells transduced with GLDC exhibited significantly colonies formation compared to control. Conversely, suppression of GLDC markedly inhibited the number of colonies (Figure 4A). Likewise, the growth curve graphs also supported my hypothesis (Figure 4B). Flow cytometry analysis was performed to assess cell cycle and indicated similar results. GLDC affected S phase of cell cycle, so GLDC supported to produce material for DNA replication (Figure 4C). Next, I observed p21 and p27 expression, the regulators of cell cycle progression by western blot and RT-PCR analysis. The results showed that level of p21, p27 protein and mRNA expression decreased in GLDC OE compared to empty vector. Meanwhile, GLDC KD increased p21 and p27 expression in western blot and RT-PCR assay (Figure 4D). Taken together, GLDC is essential to induce cell proliferation in HK2.

3.5. Uncoupling protein 1 (UCP1) knockdown aggravates ROS generation and supresses cell proliferation:

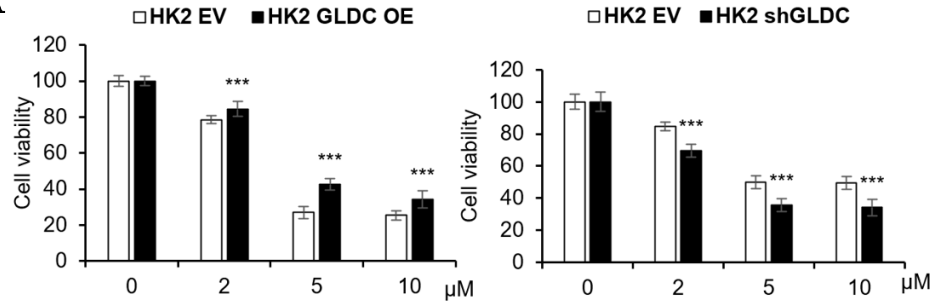
Previous studies supported that UCP1 may reduce mitochondrial

ROS generation in brown adipose tissue (BAT) (7,9). I hypothesized that UCP1 could inhibit ROS generation in HK2 cell in presence of cisplatin. To support this hypothesis, flow cytometry and immunofluorescence assay were analyzed. Based on GLDC OE cells, I constructed UCP1 knockdown by transfecting UCP1 small interfering (siRNA). Immunofluorescence data showed that UCP1 knockdown induced an increase in ROS with cisplatin treatment compared with control (Figure 5A). Consistent with this results, loss UCP1 exhibited a significant decrease in lamin B1 expression by western blot (Figure 5B). Next, I examined the proliferation rate of UCP1 knockdown by flow cytometry, growth curve, and colony formation assays. The results supported that the proliferation rate of cell was repressed in deletion of UCP1 (Figure 5C&5D). Especially, UCP1 knockdown downregulated S phase compared with control. Both GLDC knockdown and UCP1 knockdown affected S phase in cell cycle.

3.6. GLDC regulates UCP1 expression:

To further illustrate the regulation of GLDC-UCP1 in HK2, I analyzed western blot and RT-PCR assay which revealed that an increase in UCP1 expression in GLDC overexpression. In contrast, GLDC knockdown downregulated UCP1 expression (Figure 6A, 6B). However, UCP1 was not required for GLDC expression (Figure 6C). Taken together, GLDC is a effector of UCP1 expression and UCP1 does not affect GLDC expression. Interestingly, ATP measurement assay revealed increased ATP level in GLDC OE compared to empty vector (Figure 6D). UCP1 expression in GLDC OE was higher than empty vector.

A



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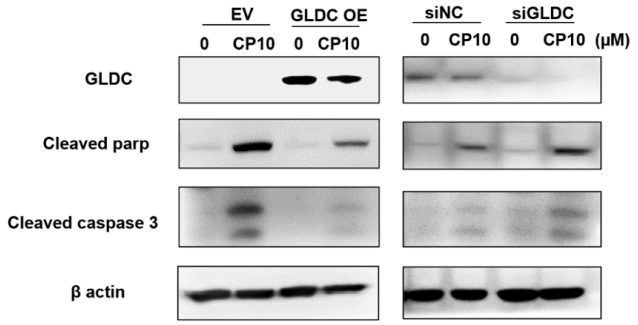

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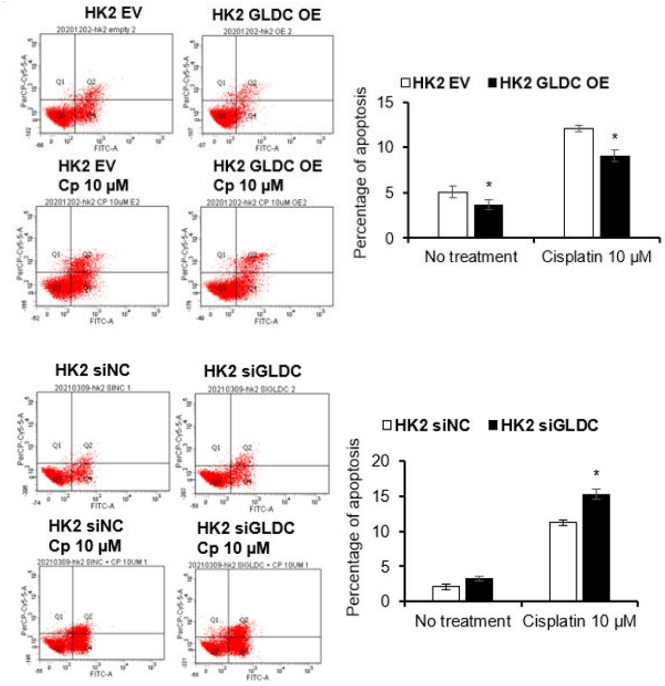
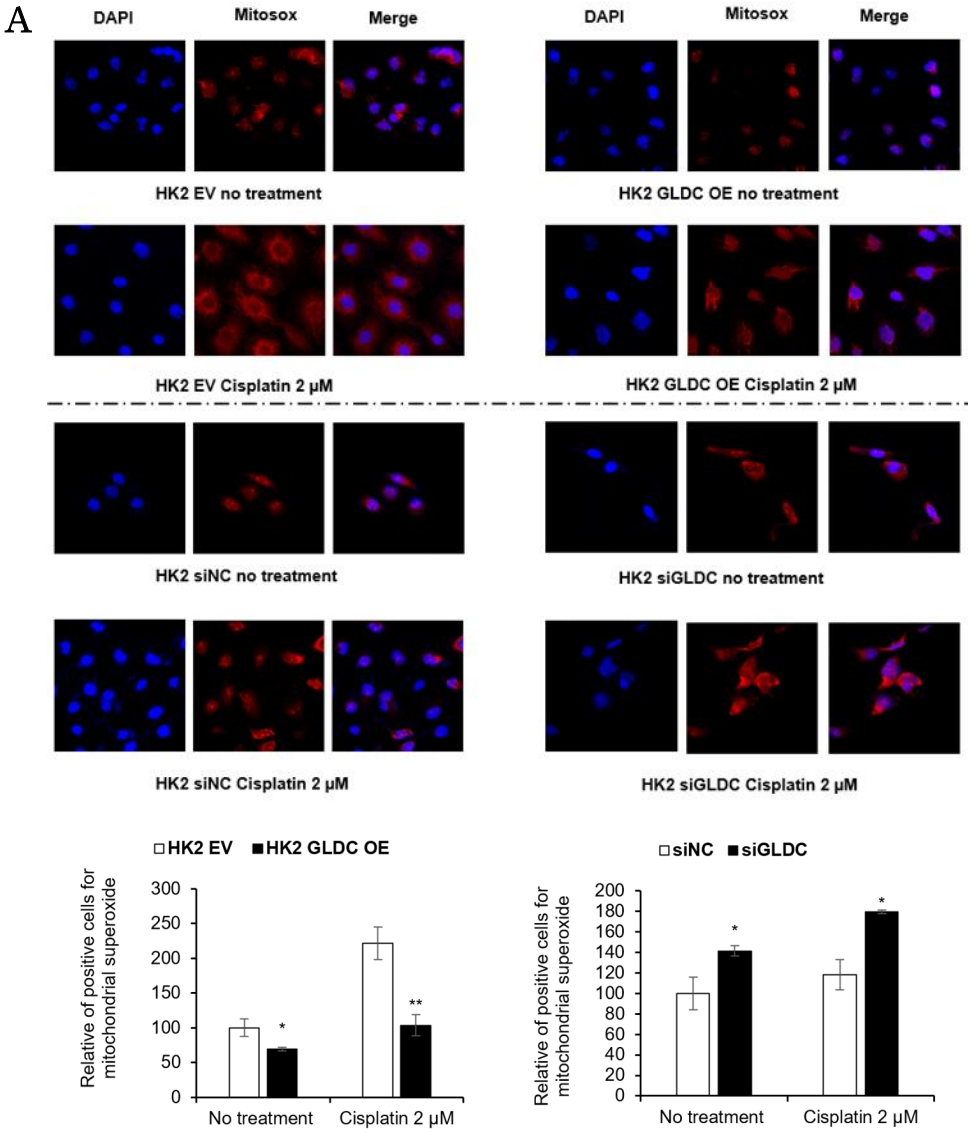


Figure 1. GLDC protects cells against cisplatin and reduces apoptosis. (A) HK2 cells were treated cisplatin with 2, 5, and 10 μM for 2 days in 2 groups: GLDC overexpression & control and GLDC knockdown & control. (B) Cleaved parp and cleaved caspase 3 were detected by western blot with and without cisplatin treatment. (C) Apoptosis were analyzed by Annexin V, 7ADD analysis kit. Data was represented as mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.



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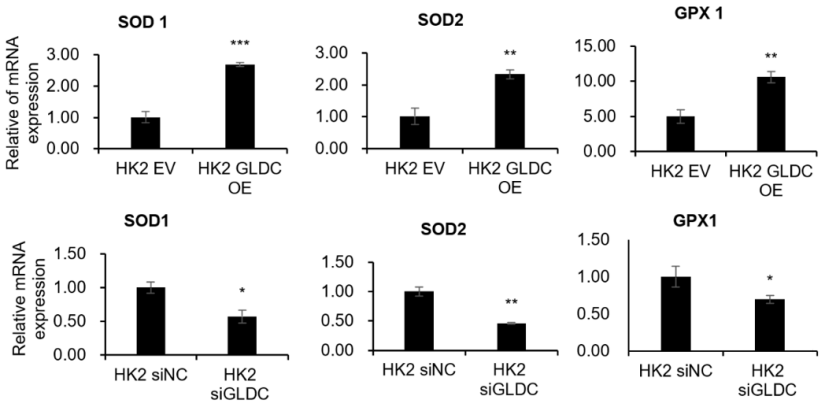

Figure 2. GLDC overexpression suppresses mitochondrial reactive oxygen species generation. (A) Images of mitochondrial reactive oxygen species (ROS) generation were analyzed under confocal microscopy. Scale bar, 20 μm. Quantification of ROS was indicated by flow cytometry. (B) RT-PCR analysis of the antioxidant genes were evaluated in GLDC overexpression & empty vector and GLDC knockdown & control. Data was represented as mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

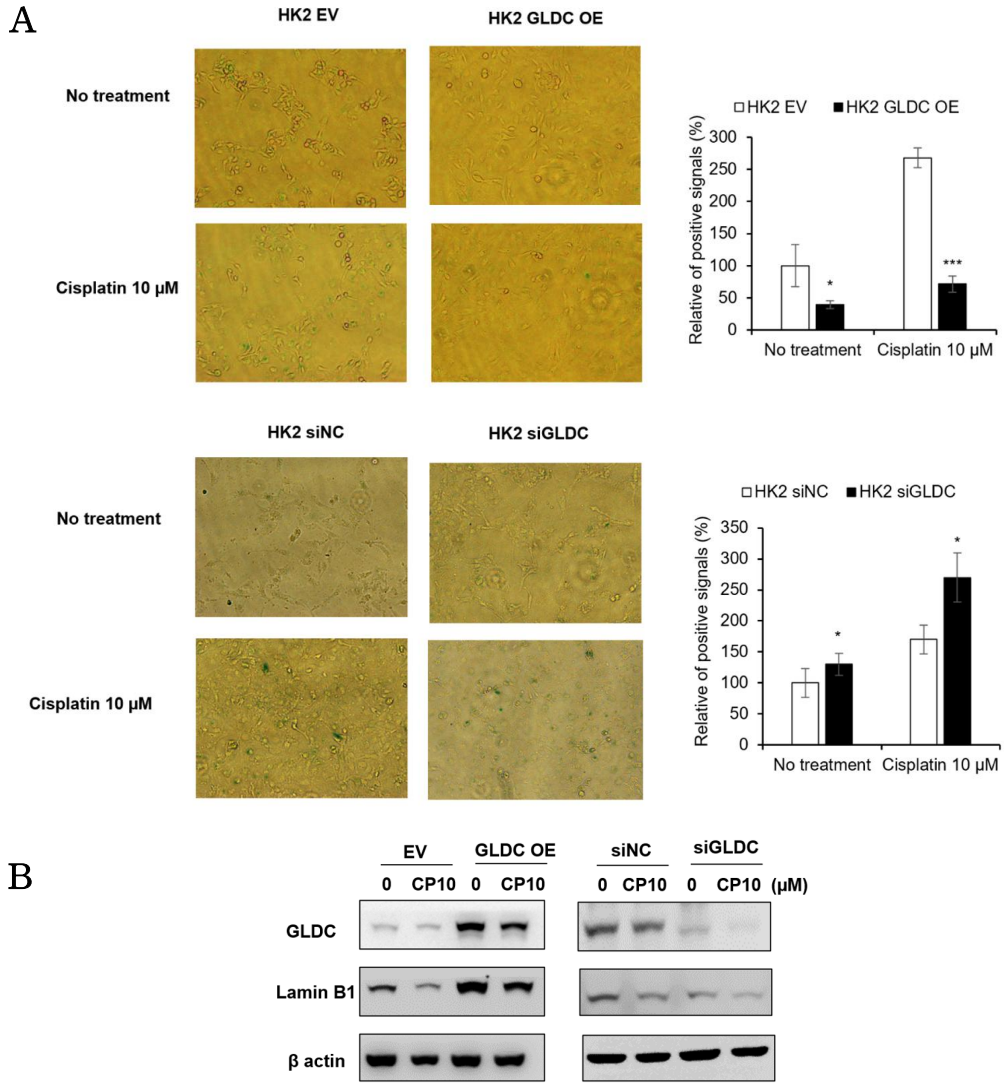

Figure 3. Deletion of GLDC aggravates cisplatin-induced cellular senescence. (A) Images and quantification of β-galactosidase staining described cellular senescence in HK2 (magnification x100). (B) Lamin B1 expression was detected with and without cisplatin treatment by western blot. Data was represented as mean ± SD. $*p < 0.05$, $***p < 0.001$.

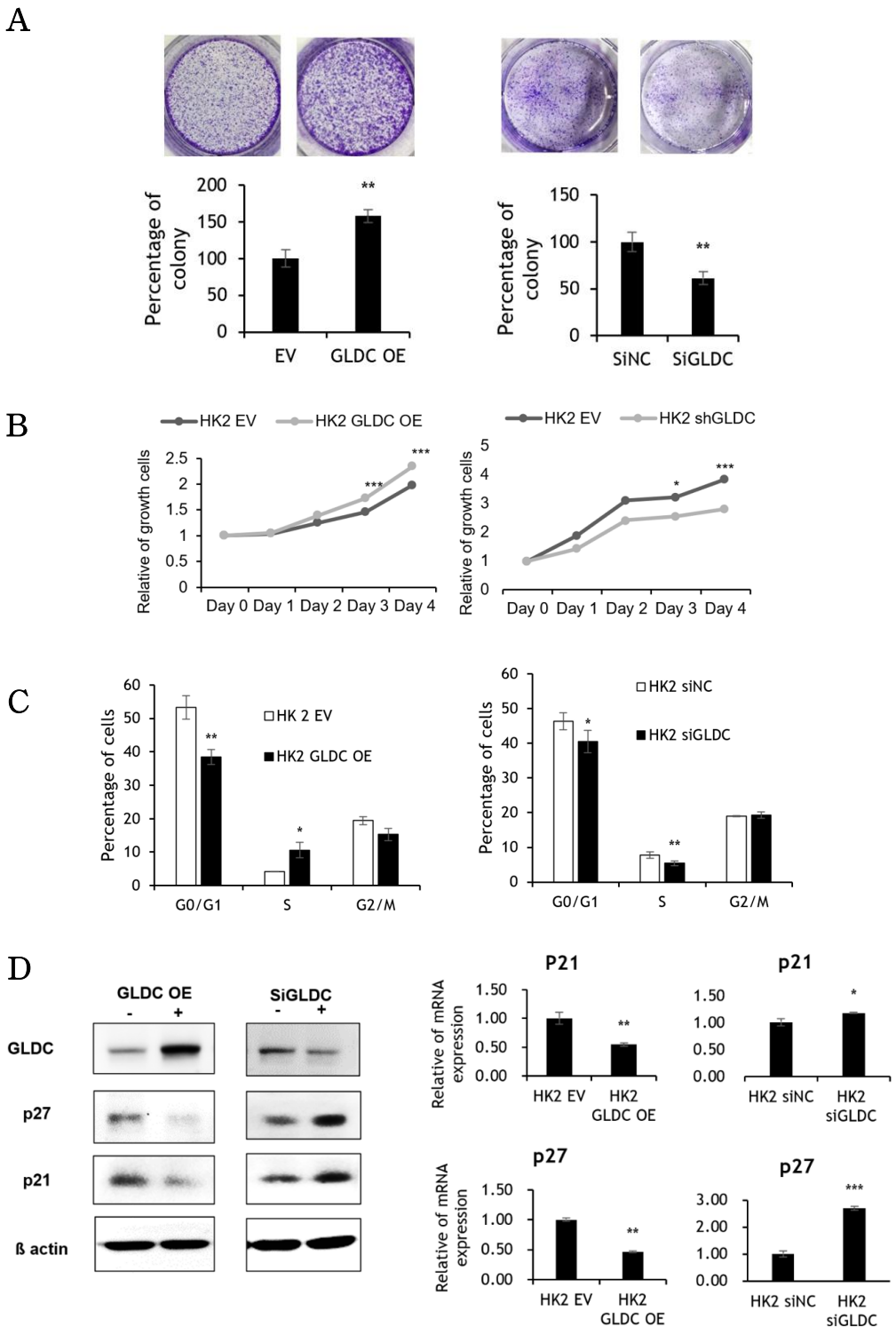

Figure 4. GLDC promotes cell proliferation. (A-C) Colony formation, growth curve and flow cytometry analysis were indicated in GLDC overexpression & empty vector and GLDC knockdown & control. (D) p21 and p27 expression were analyzed by western blot and RT-PCR. Data was represented as mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

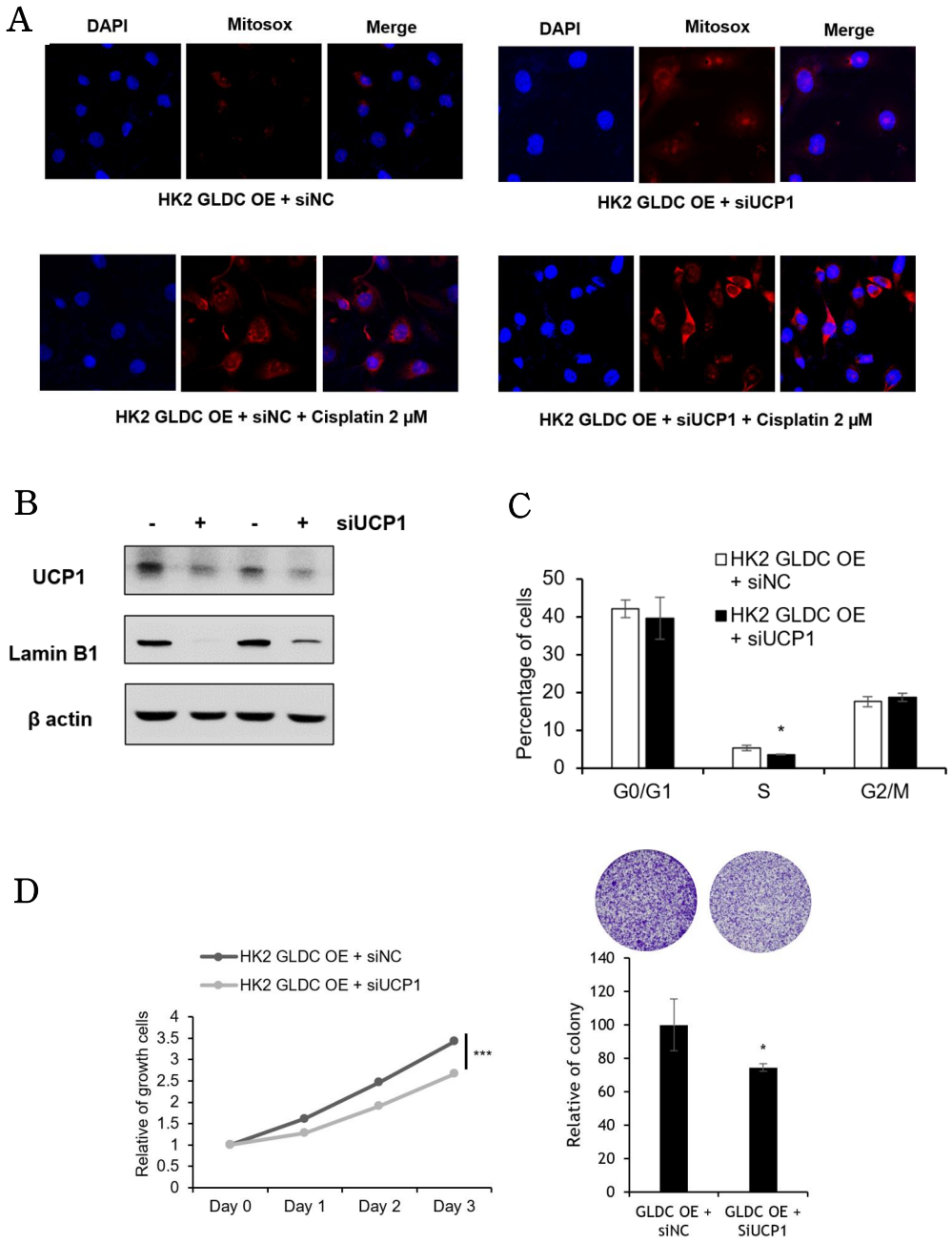

Figure 5. Uncoupling protein 1 (UCP1) knockdown aggravates reactive oxygen species generation (ROS) and suppresses cell proliferation. (A) Fluorescence images of

ROS generation in UCP1 knockdown were visualized using a confocal microscope. Scale bar, 20 μm. (B) Western blot data showed expression of lamin B1. (C) Cell cycle result was indicated UCP1 knockdown by flow cytometry assay. (D) Colony formation & growth curve determined that UCP1 silencing reduced cell proliferation. Data was represented as mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

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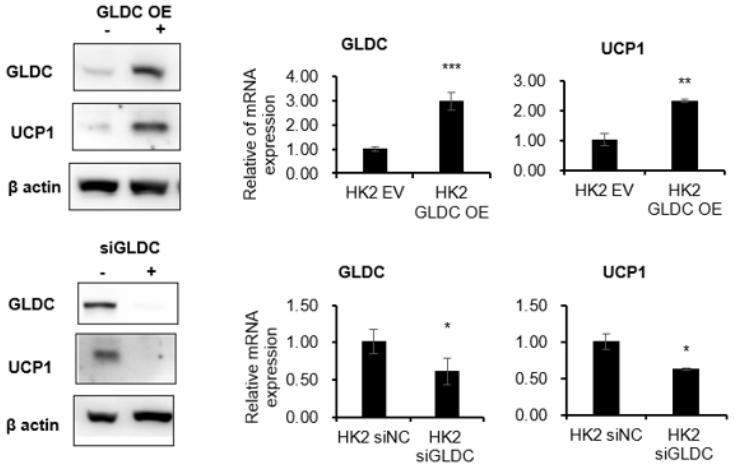

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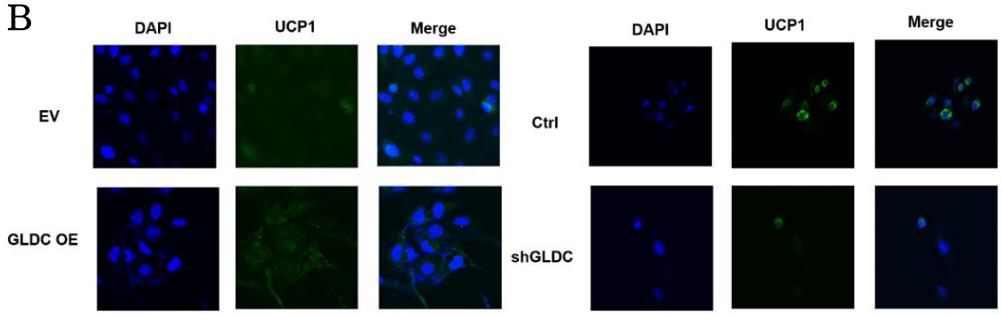

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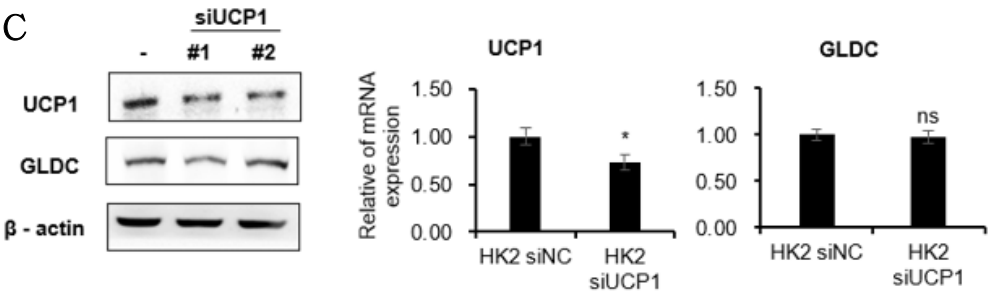

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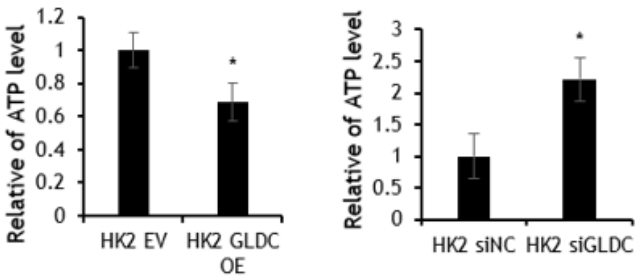

Figure 6. GLDC regulates UCP1 expression. (A) Western blot & RT-PCR data showed that UCP1 expression was affected by GLDC. (B) Immunofluorescence images of UCP1 expression in GLDC OE & empty vector and GLDC KD & control are visualized by confocal microscope. Scale bar, 20 μm. (C) UCP1 expression did not regulate GLDC (D) ATP level was measured in GLDC OE and GLDC KD. Data was represented as mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

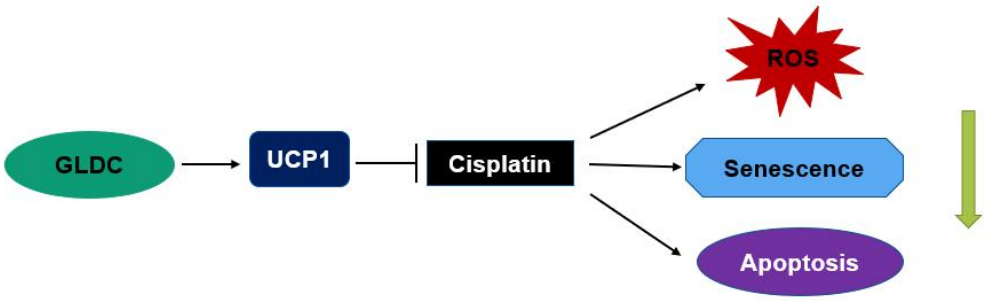

Figure 7. Proposed schema of the pathway underlying protective effect of GLDC in renal proximal tubular cells. GLDC suppresses mitochondrial reactive oxygen species generation, senescence, and apoptosis by activating UCP1.

4. Discussion

In this study, I identified that GLDC acts as a regulator of kidney injury via UCP1-mediated, in particularly regulation of ROS generation and inhibition of apoptosis. (Figure 7). Overexpression of GLDC upregulated UCP1 expression and protected cell viability in cisplatin treatment. In contrast, GLDC knockdown was not able to protect against cisplatin-induced cellular senescence, apoptosis, and ROS generation.

My data showed that GLDC expression is required for cell proliferation. GLDC is an enzyme that catalyzes glycine breakdown generating CO2, NH3, NADH and one-carbon unit 5,10-MTHF (13,14). GLDC knockdown leads to the accumulation of glycine and decreases supply of one-carbon units, and lead to reduced percentage of S phase in cell cycle. GLDC OE inhibited p21, p27 expression which supported cell proliferation. Additionally, the results indicated that GLDC activation could protect HK2 from cisplatin-induced cellular senescence. Senescence, irreversible arrest of the cell cycle, normally occurs in response to stresses such as DNA damage, oxidative, and toxic metabolites (15,16). Cisplatin induces senescence through DNA damage response and the p53/p21 pathway (17). Deletion of GLDC not only elevated senescence levels and p21, p27 expression but also decreased lamin B1 expression, a marker of senescence. Taken together, these findings suggest that GLDC regulated the phenotype in proximal tubular cells.

UCPs are transmembrane proteins that decrease the proton gradient generated in oxidative phosphorylation. UCPs plays an important role in suppressing oxidative stress, and reduces

mitochondrial ROS generation (18,19). UCP1 expressions are limited to fat tissue, most abundant in brown adipose tissue (20,21). Previous studies demonstrated that UCP1 decreases ROS generation and oxidative stress (22). However, other studies supported that UCP1 could reduce mitochondrial ROS generation in brown adipose tissue, thymus, beige adipose tissue (9,19). My data also indicated that UCP1 could inhibit ROS generation with cisplatin treatment. UCP1 knockdown increased ROS level dramatically compared to control. Additionally, UCP1-mediated heat generation in brown fat uncouples the respiratory chain, allowing for fast substrate oxidation with a low rate of ATP production which is important to maintain cellular function (23). That is the reason why ATP production in GLDC OE decreased. Further experiments would be required to improve my knowledge of cellular proliferation and ATP generation.

5. Summary

This study investigated the response of HK2 cell in cisplatin treatment with glycine decarboxylase (GLDC) overexpression and GLDC knockdown. Firstly, cytotoxicity assay and flow cytometry were performed to indicate that GLDC could protect cells against cisplatin and reduce apoptosis in HK2 cell. Additionally, western blot data showed that apoptosis protein expression increased in GLDC knockdown significant compared to control sample. Secondly, immunofluorescence analysis confirmed that GLDC overexpression reduces mitochondrial reactive oxygen species (ROS) generation in cisplatin treatment. Opposite, deletion of GLDC induced higher ROS level than control in presence of cisplatin. Thirdly, GLDC regulated cisplatin-induced senescence. Positive signals in β-galactosidase staining in GLDC overexpression were less than empty vector. GLDC knockdown had higher level of senescence than control sample. Fourthly, GLDC promoted cell proliferation using cell viability, cell cycle, western blot, and RT-PCR assay. Fifthly, western blot, RT-PCR assay indicated that GLDC regulated UCP1 expression. These data suggested that GLDC protects cisplatin-induced damage of renal proximal tubular cell by activating UCP1.

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GLDC Protects Cisplatin-induced Damage of Renal Proximal Tubular Cell by Activating UCP1

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(Abstract)

Glycine decarboxylase (GLDC) is an enzyme that is involved in one carbon metabolism via mediating the breakdown of glycine. I found that GLDC attenuated cisplatin (CP)-induced damages in HK2 renal proximal tubular cells. GLDC overexpression decreased CP-induced cellular senescence, mitochondrial production of reactive oxygen species (ROS) as well as apoptosis. Additionally, deletion of GLDC increased CP-induced cellular senescence, mitochondrial ROS production, and apoptosis. Mechanistically, GLDC downregulated ROS generation and apoptosis through activating uncoupling protein 1 (UCP1). Knockdown of UCP1 resembled GLDC knockdown phenotype in vitro in HK2 proximal tubular cells. In summary, the data indicated that GLDC protects CP-induced damages in proximal

tubular cells via UCP1 mediated pathway and present a scientific foundation for the potential therapeutic usage of GLDC for the treatment of acute kidney injury.

신근위세뇨관세포에서 UCP1 활성화 매개에 의한 GLDC의 시스플라틴 유도 손상 보호기전 연구

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(초록)

Glycine decarboxylase (GLDC)는 글라이신 분해를 통한 1 탄소 대사에 관여하는 효소이다. 본 연구에서 GLDC 단백질이 신장의 근위세뇨관세포인 HK2 세포에서 시스플라틴으로 유도된 손상을 지연시키는 것을 확인하였다. GLDC 단백질의 과발현은 시스플라틴 처리에 따른 세포 노화, 마이토콘드리아의 활성산소 생성뿐만 아니라 세포사멸을 감소시켰다. 더 나아가, GLDC 단백질 발현의 감소는 시스플라틴에 의해 발생하는 세포 노화, 마이토콘드리아의 활성산소 생성과 세포사멸을 증가시켰다. 분자 기전으로, GLDC는 활성산소의 생성을 억제하고 uncoupling protein 1 (UCP1) 단백질을 활성화시켜 세포사멸을 감소시켰다. HK2 세포에서 UCP1 단백질을 감소시켰을 때 GLDC 단백질의 감소와 유사한 표현형을 보였다. 따라서, GLDC 단백질은 근위세뇨관세포에서 UCP1 매개 경로를 통해 시스플라틴에 의한 손상으로부터 신장세포를 보호하고, 급성 신장손상 치료에 유용하게 사용할 수 있다