รายงานวิจัยฉบับสมบูรณ์

โครงการ
การศึกษาหน้าที่ของเอนไซม์ต้านอนุมูลอิสระในเซลล์ท่อไตที่ถูกเหนี่ยวนำโดยการยึดเกาะของผลึกแคลเซียมอ๊อกซาเลทในโรคนิ่วไต

โดย

นางสาวจุฑาทิพย์ มานิสสรณ์

มีนาคม 2558

สัญญาเลขที่ TRG5580013

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โดย
นางสาวจุฑาทิพย์ มานิสสรณ์
หน่วยโปรตีโอมิกส์ทางการแพทย์ สถานส่งเสริมการวิจัย
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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
และมหาวิทยาลัยมหิดล

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Abstract

Project Code: TRG5580013

Project Title: Functional characterizations of antioxidant enzymes in renal tubular cells induced by calcium oxalate crystal adhesion in kidney stone disease

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Adhesion of calcium oxalate crystals on renal tubular epithelial cells is a critical event that triggers many cascades of cellular responses such as the alteration in protein expressions. The aim of this study was to clarify protein function based on proteome data. From our previous study, calcium oxalate dihydrate (COD) crystals (1000 μg/mL) induced down-regulation of various intracellular proteins in renal distal tubular epithelial cells (MDCK) such as anti-oxidant enzymes, proteasomes, and cytoskeletal proteins. Western blot analysis was performed to validate proteome changed. The result showed that anti-oxidant enzyme (SOD1) was not changed whereas cytoskeletal protein (alpha-tubulin) was dramatically decreased. Therefore, in this study, we further clarify the protective roles of alpha-tubulin during COD crystal adhesion in MDCK cells using overexpression technique. Cell death and cell proliferation were observed by trypan blue exclusion assay. The results indicated that alpha-tubulin overexpression could inhibit cell death and promote cell proliferation in MDCK cells. Wound healing assay which was generated by scratch method revealed that cell migration and tissue repair were increased in alpha-tubulin overexpressing cells. Moreover, crystal adhesion assay showed the reduction of crystal adhesion in alpha-tubulin overexpressing cells. Apical membrane isolation using peeling method confirmed that alpha-tubulin overexpression could attenuate crystal adhesion by decreasing some potential crystal-binding proteins including HSP90, HSP70, and alpha-enolase. This finding implied that alpha-tubulin has important role for protecting renal tubular epithelial cells during COD crystal adhesion.

Keywords: Alpha-tubulin, overexpression, calcium oxalate dihydrate (COD), MDCK cells, kidney stone disease

บทคัดย่อ

รหัสโครงการ: TRG5580013

ชื่อโครงการ: การศึกษาหน้าที่ของเอนไซม์ต้านอนุมูลอิสระในเซลล์ท่อไตที่ถูกเหนี่ยวนำโดยการยึดเกาะของผลึกแคลเซียมอ๊อกซาเลทในโรคนิ่วไต

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ระยะเวลาโครงการ: 2 ปี

การยึดเกาะของผลึกแคลเซียมอ๊อกซาเลทในเซลล์ท่อไตส่งผลต่อกระบวนการตอบสนองของเซลล์ในหลายด้าน เช่น การเปลี่ยนแปลงการแสดงออกของปริมาณโปรตีนภายในเซลล์ วัตถุประสงค์ของงานวิจัยนี้เพื่อศึกษาโปรตีนในเชิงหน้าที่โดยอาศัยข้อมูลที่ได้มาจากการศึกษาการแสดงออกของโปรตีนต่างๆ ภายในเซลล์ จากงานวิจัยก่อนหน้านี้พบว่าแคลเซียมอ๊อกซาเลทชนิดไดไฮเดรตปริมาณ 1,000 ไมโครกรัม/มิลลิลิตร ชักนำให้โปรตีนหลายชนิดในเซลล์ท่อไตมีปริมาณลดลง เช่น กลุ่มเอนไซม์ต้านอนุมูลอิสระ โปรตีเอโซม และโปรตีนกลุ่มที่เป็นโครงสร้างของเซลล์ การตรวจสอบผลการเปลี่ยนแปลงของปริมาณโปรตีนภายในเซลล์โดยเทคนิค Western blot พบว่ากลุ่มเอนไซม์ต้านอนุมูลอิสระ เช่น SOD1 ไม่มีการเปลี่ยนแปลง แต่พบว่าโปรตีนกลุ่มที่เป็นโครงสร้างของเซลล์ เช่น โปรตีนอัลฟา-ทูบูลิน มีการเปลี่ยนแปลงที่ลดลงอย่างชัดเจน ดังนั้นในงานวิจัยนี้ จึงมุ่งเน้นไปที่การศึกษาหน้าที่ของโปรตีนอัลฟา-ทูบูลินต่อการยึดเกาะของผลึกแคลเซียมอ๊อกซาเลทชนิดไดไฮเดรตในเซลล์ท่อไต โดยอาศัยเทคนิคทางชีวโมเลกุลมาใช้เพิ่มการแสดงออกของโปรตีน งานวิจัยนี้ได้ทำการศึกษาการตายของเซลล์และการเพิ่มจำนวนของเซลล์ โดยใช้เทคนิคการติดสีของ trypan blue ผลการทดลองแสดงว่าโปรตีนอัลฟา-ทูบูลินที่มีการเพิ่มการแสดงออก สามารถป้องกันการตายของเซลล์ และกระตุ้นให้เกิดการเพิ่มจำนวนของเซลล์ท่อไต การศึกษาผลต่อการสมานแผลของเซลล์โดยเทคนิคการทำเซลล์ให้เกิดรอยแผลบ่งชี้ว่าโปรตีนอัลฟา-ทูบูลินช่วยในการสมานแผลของเซลล์ได้ดีขึ้น นอกจากนี้โปรตีนอัลฟา-ทูบูลินยังช่วยลดการยึดเกาะของผลึกแคลเซียมอ๊อกซาเลทชนิดไดไฮเดรตในเซลล์ท่อไตได้ การศึกษาโปรตีนบนผิวเซลล์แสดงให้เห็นว่าการเพิ่มขึ้นของโปรตีนอัลฟา-ทูบูลินช่วยลดโปรตีนที่สามารถจับกับผลึกแคลเซียมอ๊อกซาเลทบนผิวเซลล์ได้ เช่น HSP90, HSP70, และ alpha-enolase งานวิจัยนี้แสดงให้เห็นว่าโปรตีนอัลฟา-ทูบูลิน มีความสำคัญต่อเซลล์ท่อไตในการป้องกันการยึดเกาะของผลึกแคลเซียมอ๊อกซาเลทชนิดไดไฮเดรต

คำหลัก: อัลฟา-ทูบูลิน, การเพิ่มการแสดงออก, แคลเซียมอ๊อกซาเลทชนิดไดไฮเดรต, เซลล์ท่อไต, โรคนิ่วไต

Chapter 1

Introduction

Kidney stone disease remains a public health problem around the world. The disease causes substantial suffering and ultimately renal failure but the disease mechanism is poorly understood. The supersaturated of calcium and oxalate concentration caused to stone formation (1). Calcium oxalate (CaOx) is the major crystalline component of kidney stone (23). The most common forms of calcium oxalate crystals found in stone matrices include calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD). Both COM and COD crystal can nucleate and adhere directly onto the apical surface of renal tubular epithelial cells (25, 34, 42). The adhesion of crystals to renal tubular epithelial cells is a critical event which triggers many cascades of responses such as cell injury, cell cytotoxicity, cell proliferation, and cell apoptosis leads to the development of kidney stone formation (31, 37). Crystals also evoke an inflammatory response leading to fibrosis, loss of nephrons and eventually to chronic renal failure (22, 28). Moreover, when renal epithelial cells are exposed to CaOx crystals, this event induces downstream cellular responses. However, molecular mechanisms of the downstream cellular responses remain largely unknown. From our previous studies, we have identified the altered proteins in MDCK cells in response to 1000 μg/mL COD crystals by 2-DE analysis. Those altered proteins involved with various biological processes e.g., signal transduction, RNA metabolism, antioxidant enzyme, proteasome, calcium-binding protein, chaperone, metabolic enzyme, and cellular structural protein. The altered proteins obtained from 2-DE have provided an important data for further functional study.

Overexpression method is one of a classical genetic approach for exploring biological functions. It is a versatile tool that can be applied in several purposes (17, 32). In this study, overexpression method was performed for functional characterization of decreased proteins including antioxidant enzyme (SOD1) and cellular structural protein (alpha-tubulin).

The antioxidative system plays an important role in protecting many tissues from oxidative stress. Superoxide dismutases (SODs) are the antioxidant defense systems against reactive oxygen species (ROS) which consist of three isoforms in mammals including the cytoplasmic Cu/ZnSOD (SOD1), the mitochondrial MnSOD (SOD2), and the extracellular Cu/ZnSOD (SOD3). SODs catalyze the conversion of ROS to hydrogen peroxide (14). Various studies reported that

overexpression of SOD1 attenuated oxidative stress and protected cell injury and apoptosis (3, 27, 41). Thamilselvan *et al.* reported that antioxidant enzymes such as catalase and SOD can protect proximal renal tubular epithelial cells from free radical induced by CaOX (36).

Alpha-tubulin was one of the identified proteins which highly decreased when exposed with COD crystals. Alpha-tubulin is a cytoskeletal protein which is a major component of microtubules and plays a crucial role in regulation of cell shape, intracellular transport, cell motility, cell migration and cell division (9, 44). Additionally, microtubule is important for tight junction preservation and restoration (15). Oxidative stress can induce microtubule depolymerization (18). Depolymerization of microtubule even disrupted tight junction but also reduce cellular adhesion and spreading (8, 10). Moreover, emerging evidence suggests that tubulins and microtubule-associated proteins may play a role in cellular stress response thus conferring survival advantage to cancer cells (29).

In this study, we firstly confirmed the proteome data and then the functional roles of altered proteins which responses to COD crystals were deeply characterized in distal renal tubular epithelial (MDCK) cells using overexpression technique. The obtained data will lead to better understanding of molecular mechanisms involved in crystal-cell interaction and may lead to new therapeutic approaches for kidney stone disease.

Chapter 2

Material and methods

Preparation of COD crystals

COD crystals were prepared as previously described by our group (33). Briefly, 125 mL of 25.08 mM calcium chloride dihydrate ($CaCl_2$•$2H_2O$) was added into 250 mL of a buffer containing 19.26 mM trisodium citrate dihydrate ($C_6H_5Na_3O_7$•$2H_2O$), 23.1 mM magnesium sulfate heptahydrate ($MgSO_4$•$7H_2O$), and 127.4 mM potassium chloride (KCl) and the pH was adjusted to 6.5. The solution was incubated at 25°C for 15 min. Then, 125 mL of 6.4 mM sodium oxalate ($Na_2C_2O_2$) was added and incubated for 15 min with continuous stirring. The COD crystals were collected using centrifugation at 2000 x*g* for 5 min and the supernatant was discarded. Thereafter, the crystals were resuspended in methanol and centrifugation at 2000 x*g* for 5 min. The methanol was discarded and the crystals were air-dried. The morphology of COD crystal was observed under phase-contrast microscope.

Cell culture and COD-treated MDCK cells

Mardin-Darby Canine kidney (MDCK) cells were inoculated in 6-well plate containing a complete Eagle's minimum essential medium (MEM) (GIBCO, Life Technologies; Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). Then, the cultured cells were maintained in a humidified incubator at 37°C with 5% CO_2 for 24 h. In this experiment, COD-treated cells were maintained in medium containing 1000 µg/mL COD crystals whereas control cells were maintained in medium without COD crystals and both cells were grown for a further 48 h.

Confirmation of the altered proteins by Western blot analysis

To confirm the results of proteome data in our previous study, Western blot analysis was performed. At 48 h after treatment MDCK cells with COD crystals, cells were harvested and extracted with 1X Laemmli's buffer and separated by SDS-PAGE. The down-regulated proteins were confirmed by specific antibodies including SOD-1 and alpha-tubulin. Equal protein loading was controlled by detection of GAPDH expression.

RNA extraction and RT-PCR amplification of alpha-tubulin gene (*TUBA1A*)

To overexpression of *TUBA1A* gene, the cDNA was prepared from MDCK cells. Brifely, MDCK cells were grown in 60-mm dishes and then harvested for total RNA using Trizol reagent (Invitrogen, Life Technologies; Carlsbad, CA). Then, the cDNA was prepared using Super Script™

III (Invitrogen) and reverse transcription-PCR (RT-PCR) was performed using specific primers. PCR primers were designed for *TUBA1A* gene based on human sequence retrieved from CCDS database (accession no. CCDS8781) and the forward primer (5' -GCAACAACCTCTCCTCTTCG- 3' and the reverse primer (5' -TCCCTGTAAAAGCAGCACCT- 3') were used to amplified the entire *TUBA1A* gene. The PCR product was amplified using Phusion® high fidelity DNA polymerase (New England BioLabs; Beverly, MA) and the amplification was carried out under the following conditions: a preliminary denaturation at 98°C for 3 min, 34 cycles of denaturation at 98°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min, with a final extension at 72°C for 10 min. PCR product was separated by 1.2% agarose gel electrophoresis and detected by staining with ethidium bromide. The DNA bands were visualized using ChemiDoc™ MP Imaging System (Bio-Rad; Berkeley, CA).

Cloning of the TUBA1A gene into expression vector and mammalian cell transfection

The 1.5 kb of *TUBA1A* gene was cloned into an expression vector using Gateway® Technology (Invitrogen). The entry clone was generated using pCR®8/GW/TOPO vector (Invitrogen). Then, the plasmid DNA was extracted before cloned into destination vector using Vivid colorsTM pcDNA™ 6.2/EmGFP-Bsd/V5-DEST and LR clonase II enzyme (Invitrogen). Plasmid DNA was extracted and confirmed by DNA sequencing. The DNA sequence of *TUBA1A* was submitted to GenBank/EMBL/DDBJ (accession no. AB853091). Thereafter, both control (no vector) and alpha-tubulin overexpressing cells (contained pcDNA6.2-TUBA1A vector) were transfected using Lipofectamin™ 2000 (Invitrogen). Briefly, a 24-well plate of MDCK cells was cultured in MEM medium supplemented with 10% fetal bovine serum (FBS) with 90-95% confluent before transfection. Plasmid DNA was diluted with Opti-MEM® I reduced serum medium (GIBCO) and mixed gently with diluted Lipofectamin™ 2000 and incubated for 20 min at RT. Then, the complexes was added into MDCK cells and incubated at 37°C with 5% CO_2 for 24 h. After 48 h post-transfection, 8 µg/mL of blasticidin was added to generate the stable cell line. The selective medium was refreshed every 2-3 day for 2 weeks until blasticidin-resistant colonies could be identified. Overexpression of the alpha-tubulin was confirmed by Western blot analysis.

Western blot analysis

To validate protein levels, Western blot analysis was performed. Cell lysate and apical membrane from MDCK cells were prepared in Leammli's buffer and resolved by 12% SDS-PAGE under reducing condition. The resolved proteins were transferred onto a nitrocellulose membrane using a semi-dry transfer apparatus (GE Healthcare; Uppsala, Sweden) at 85 V for 1:30 hours.

Non-specific bindings were blocked by 5% skim milk in PBS. Then, the membrane was incubated with the following primary antibodies; mouse-monoclonal anti-alpha-tubulin (1:1000 in 1% skim milk/PBS) (Santa Cruz Biotechnology Inc.; Santa Cruz, CA), mouse-monoclonal anti-vimentin (1:1000 in 1% skim milk/PBS) (Santa Cruz), goat-polyclonal anti-ANXA2 (1:1000 in 1% skim milk/PBS) (Santa Cruz), mouse-monoclonal anti-HSP70 (1:1000 in 1% skim milk/PBS) (Santa Cruz), mouse-monocloanl anti-HSP90 (1:1000 in 1% skim milk/PBS) (Santa Cruz), rabbit-polyclonal anti-alpha-enolase (1:1000 in 1% skim milk/PBS) (Santa Cruz), and mouse-monoclonal anti-GAPDH (1:1000 in 1% skim milk/PBS) (Santa Cruz). Thereafter, the membrane was washed with PBS for 3 times and further incubated with the specific secondary antibodies conjugated with horseradish peroxidase (1:2000 in 1% skim milk/PBS). The reactive protein bands were visualized with SuperSignal West Pico chemiluminescence substrate (Pierce Biotechnology, Inc.; Rockford, IL) using autoradiogram.

Cell death and cell proliferation assay

Control and alpha-tubulin overexpressing cells were determined of cell death and cell proliferation after COD-treatment for 48 h using trypan blue exclusion assay (n=3). Briefly, The cells were detached from the culture well using 0.1% trypsin in 2.5 mM EDTA and immediately resuspended in MEM medium with 10% FBS to terminate trypsin activity. Aliquots of cell suspension were mixed with 0.4% trypan blue solution (GIBCO) and then the cells were counted using hemocytometer.

Wound healing assay

Wound healing was studied by scratch method. Briefly, both control and alpha-tubulin overexpressing cells were inoculated in 6-well plate (n=3). After 24 h incubation, the cells were treated with 1000 μg/mL COD crystals for 48 h. Then, the confluent monolayers of normal and alpha-tubulin overexpressing cells were horizontally scratched along the culture well diameter using a 200-μl plastic pipette tip to create a cell-free area. After gently washing with PBS to remove the debris and detached cells, the cultures were further maintained at 37°C with 5% CO2. At the indicated time-points (0, 3, 6, 9, 12 and 15 h after the scratch), each scratch wound was observed using BioStation CT (Nikon Corp.; Tokyo, Japan). This image was submitted to Tarosoft® Image framework v.0.9.6 (Nikon) which adopted to accurately measure the cell-free width.

Crystal adhesion assay

Control and alpha-tubulin overexpressing cells were cultured in a 6-well plate for 48 h (n=6). Thereafter, the culture medium was removed and the cells were washed with PBS twice.

COD crystal-cell adhesion was initiated by the addition of 10% FBS-supplemented MEM containing 100 µg/mL COD crystals into each culture well. The cells were further incubated in a humidified incubator at 37°C with 5% CO_2 for 30 min. Then, the cells were vigorously washed using PBS five times to remove unbound COD crystals. Finally, numbers of the adhered crystals were quantitatively analyzed by counting in 20 randomized high power fields (HPF) per culture well.

Isolation of apical membrane by peeling method

Control and alpha-tubulin overexpressing cells were inoculated in 100 mm disc (approximately $2x10^6$ cells/disc) and maintained in complete MEM medium supplemented with 10% fetal bovine serum (FBS) for 24 h. For COD-treated cells, COD crystals were added into the culture medium and incubated for 48 h until the cells were semi-polarized. The apical membrane of semi-polarized MDCK cells were isolated by peeling method (11). Briefly, the culture medium was removed from semi-polarized cells and then rinsed twice with ice-cold membrane-preserving buffer containing 1mM $MgCl_2$, and 0.1 mM $CaCl_2$ in PBS. Thereafter, Whatman filter paper (0.18-mm-thick, Whatman International Ltd.; Maidstone, U.K.) prewetted with DI water was placed onto the monolayer cells and incubated for 5 min. Then, the filter paper was peeled out and the apical membranes retained at the filter paper were harvested by gentle scrapping with DI water. The apical membrane was enriched using lyophilization and then resuspended in 1X Laemmli's buffer. The isolated apical membrane was performed using Western blot analysis for detection of crystal-binding proteins.

Protein network analysis

The protein-protein interactions from proteome data in our previous study were predicted using STRING 9.1 (http://string-db.org/)(13). This tool provides an integration data on protein-protein associations, both physical and functional interactions from numerous sources. Known and predicted associations are scored and integrated, resulting in comprehensive protein networks covering >1100 organisms. From our previous study, the significantly altered proteins which induced by COD crystals (1000 µg/mL) in MDCK cells were inputted into STRING 9.1 to retrieve the protein function and protein interaction.

Statistical analysis

All quantitative data are presented as mean ± SEM. Comparisons between the two groups of samples were performed using unpaired Student's *t*-test whereas multiple comparisons of more than two groups of samples were performed using one-way analysis of variance (ANOVA) with

Tukey's post-hoc test (SPSS; version 16.0). *P*-values less than 0.05 were considered as statistical significant.

Chapter 3

Results

Proteome data and protein network analysis of significantly altered proteins in MDCK cells induced by COD crystals.

MDCK cells were treated with 1000 μg/mL COD crystals and the proteome data by 2-DE was done in our previous study. We found that thirteen proteins were increased and twenty-four proteins were decreased. Those altered proteins involved in ubiquitination pathway, signal transduction, cellular structure, purine biosynthesis, metabolic enzyme, retinol biosynthesis, cellular transportation, protein degradation, RNA metabolism, RNA binding protein, cell surface antigen, nucleic acid metabolism, antioxidant enzyme, chaperone, carrier protein, and protein biosynthesis. All the significantly altered proteins were submitted to STRING 9.1 software to analyze protein-protein interaction. The interaction between these altered proteins induced by COD crystals are shown in Fig. 1.

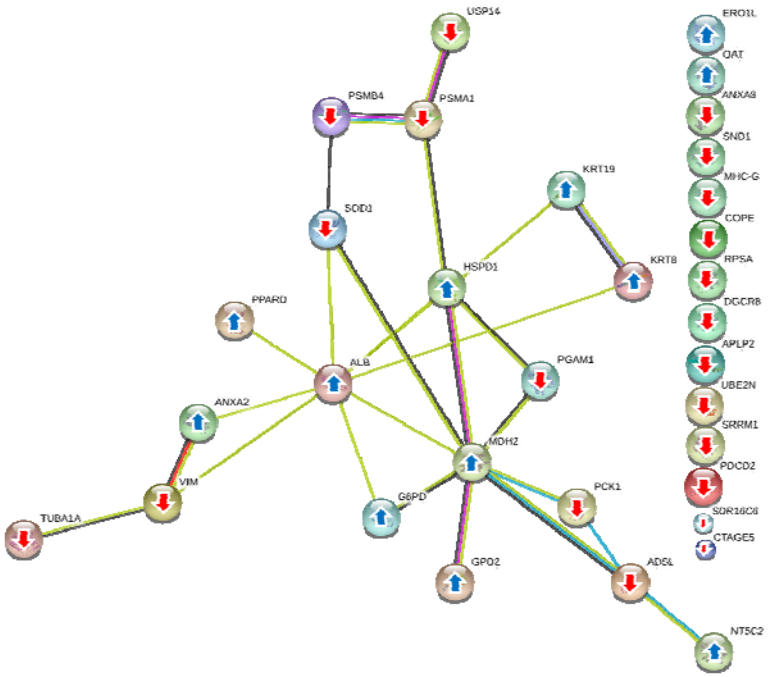


Figure 1. Protein network analysis of altered proteins induced by COD crystals in MDCK cells. STRING 9.1 was used to analyze the protein-protein interaction of altered proteins from our previous study which induced by 1000 μg/mL COD crystals. All altered proteins are shown with

upward and downward arrows (increased protein is labeled with upward arrows and decreased proteins labeled with downward arrows). The connecting line between protein nodes demonstrated the specific type of association. Proteins that were identified but had no interaction are lists in the right side.

Confirmation of the altered proteins by Western blot analysis

To confirm the results of proteome data in our previous study, Western blot analysis was performed with specific antibodies. The results showed antioxidant enzyme (SOD-1) did not change whereas a cytoskeleton protein (alpha-tubulin) was decreased in COD-treated cells. Therefore, alpha-tubulin was focus in the further study to characterize functional role. (Fig. 2)

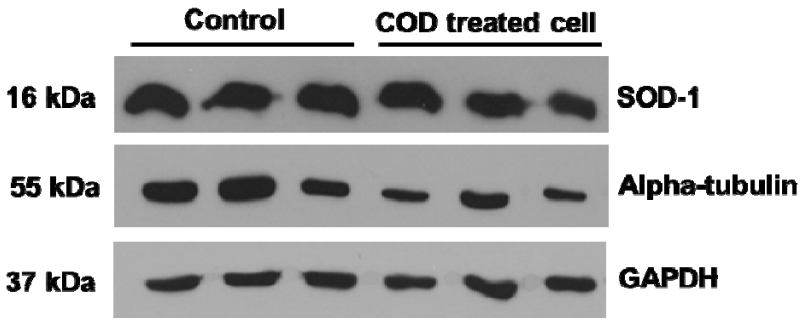
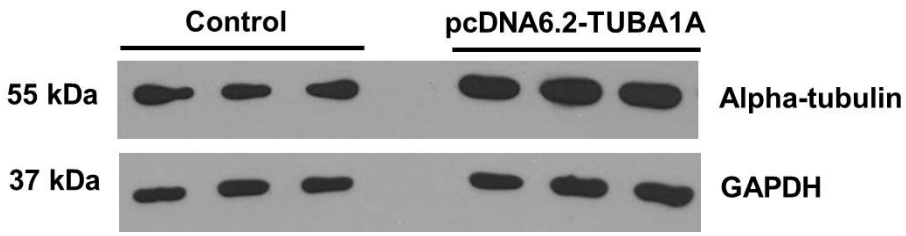


Figure 2. Confirmation of proteomic data by Western blot analysis. SOD-1 was not decreased while alpha-tubulin was decreased in COD treated cells. GAPDH was used to control equal protein loading.

Alpha-tubulin overexpression in MDCK cells

Western blot analysis revealed that alpha-tubulin overexpressing cells (contained pcDNA6.2-TUBA1A) showed higher expression of alpha-tubulin than control (no vector) approximately 1.5-fold as shown in Fig. 3.



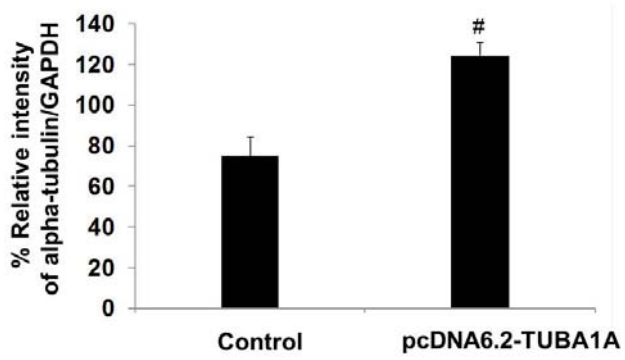


Figure 3. Confirmation of alpha-tubulin overexpression in MDCK cells. Alpha-tubulin overexpression was analyzed by Western blot. A total 10 µg proteins derived from whole cell lysates were resolved by 12% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated with mouse monoclonal anti-alpha-tubulin as a primary antibody and rabbit anti-mouse IgG conjugated with HRP as a secondary antibody. The immunoreactive bands were visualized by chemiluminescence and autoradiography. Alpha-tubulin (55 kDa) could be overexpressed compared to control cells. GAPDH was used as loading control. Percent relative intensity of alpha-tubulin to GAPDH is presented as mean ± SEM of three independent experiments. $^{\#}$ = $P < 0.05$ compared to normal cell.

Effect of alpha-tubulin overexpression in MDCK cells induced by COD crystals

Madin-Darby canine kidney (MDCK) is a distal renal tubular epithelial cell that was wildly used as a model for study kidney stone disease. Cell morphology of control and alpha-tubulin overexpressing cells was observed under phase-contrast microscope. Without COD crystal, cell morphology between control and alpha-tubulin overexpressing cells was not difference. However, in COD-treated condition, control cells showed the disruption of cell border whereas alpha-tuublin overexpressing cells was still maintained the cell border and cell integrity as shown in Fig. 4.

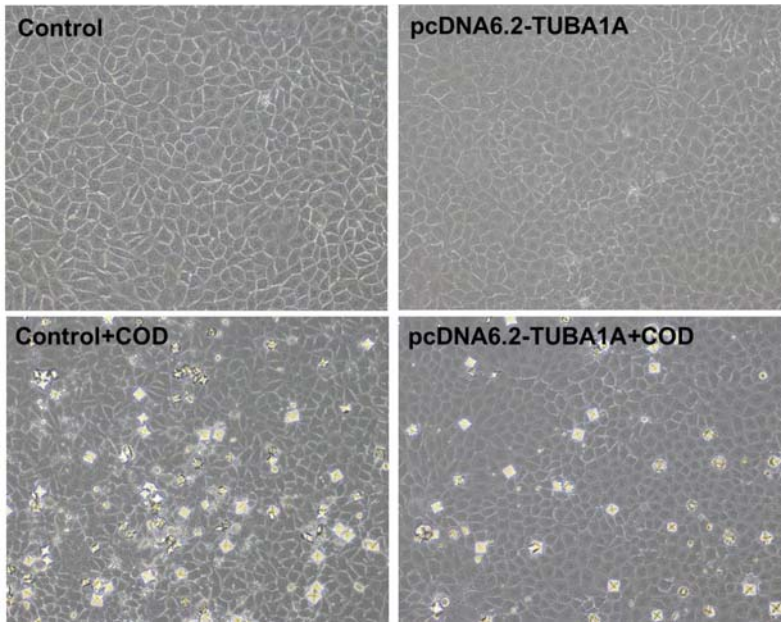
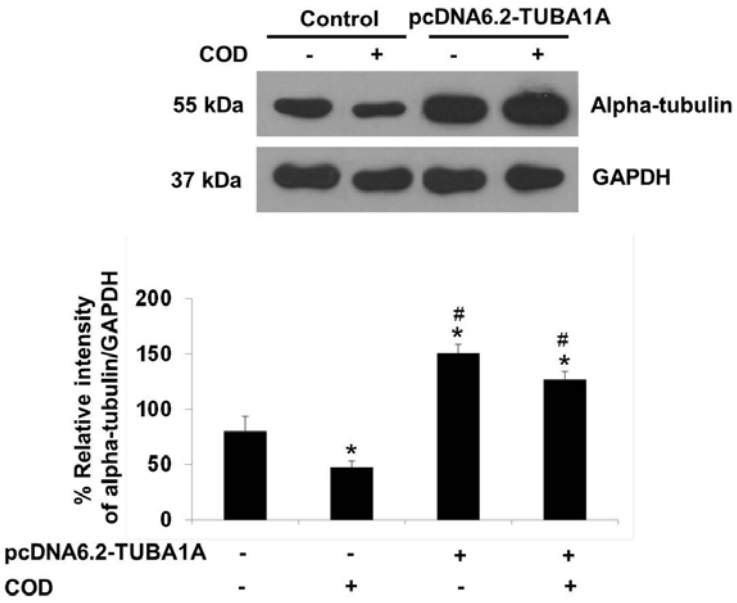


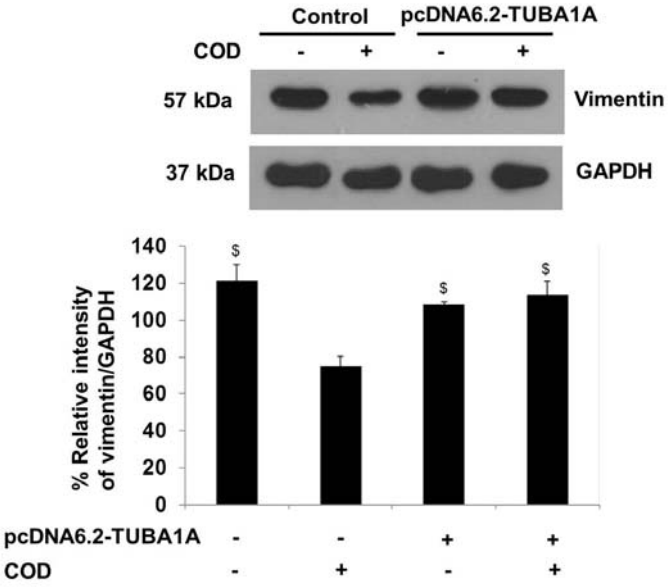
Figure 4. Effect of COD crystal on cell morphology of MDCK cells. Phase-contrast microscopy of control cells (upper left), alpha-tubulin overexpressing cells (upper right), COD-treated control cells (lower left) and COD-treated alpha-tubulin overexpressing cells (lower right) demonstrated that COD crystals affected to cell morphology of control cells whereas alpha-tubulin overexpressing cells could preserve cell morphology compared to control cells. In all panels, the magnification power was 200X.

To study effect of COD crystals on protein expression, whole cell lysate of control and alpha-tubulin overexpressing cells were scrapped after treated with 1000 μg/mL COD crystals for 48 h and resuspended in Laemmli's buffer and analyzed by Western blot as shown in Fig. 5. The results showed alpha-tubulin was decreased in COD-treated control cells whereas increased in alpha-tubulin overexpressing cells and COD-treated alpha-tubulin overexpressing cells (Fig. 5A). Vimentin which is another altered cytoskeletal protein identified by 2-DE and interacted with alpha-tubulin (Fig. 1) was decreased in COD-treated control cell but could be preserved at basal level in alpha-tubulin overexpressing cells and COD-treated alpha-tubulin overexpressing cells (Fig.5B). Moreover, ANXA2, a multi-functional and calcium-binding protein, was increased in all conditions including COD-treated control cells, alpha-tubulin overexpressing cells and COD-treated alpha-tubulin overexpressing cells (Fig. 5C). This indicated that alpha-tubulin overexpression could induce the up-regulation of ANXA2.

A


Control
pcDNA6.2-TUBA1A
COD
- + - +
55 kDa
Alpha-tubulin
37 kDa
GAPDH
% Relative intensity of alpha-tubulin/GAPDH
200
150
100
50
0
#
*
pcDNA6.2-TUBA1A - - + +
COD - + - +

B


Control
pcDNA6.2-TUBA1A
COD
- + - +
57 kDa
Vimentin
37 kDa
GAPDH
% Relative intensity of vimentin/GAPDH
140
120
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0
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pcDNA6.2-TUBA1A - - + +
COD - + - +

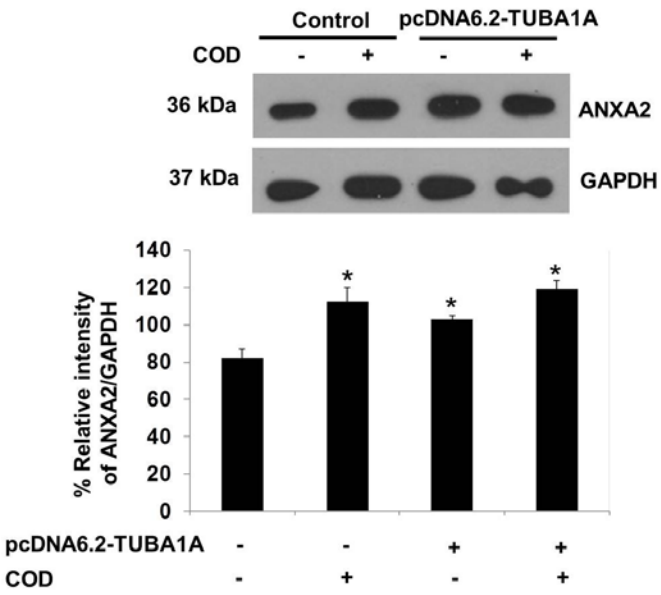

Figure 5. Effect of COD crystals on protein expression in MDCK cells. Whole cell lysate of MDCK cells which exposed with or without 1000 µg/mL COD crystals were analyzed by Western blot. **(A)** alpha-tubulin was decreased in COD-treated control cells whereas alpha-tubulin was increased in alpha-tubulin overexpressing cells and COD-treated alpha-tubulin overexpressing cells. **(B)** Vimentin was decreased in COD-treated control cells whereas could be preserved at basal level in alpha-tubulin overexpressing cells with or without COD crystals. **(C)** ANXA2 was increased in both COD-treated control cells and alpha-tubulin overexpressing cells with or without COD crystals. The quantitative of band intensity was normalized by GAPDH which used as loading control. The data are reported as mean ± SEM (n = 3 independent experiments). * = $P < 0.05$ compared to control cell, $^{\#}$ = $P < 0.001$ compared to control+COD, and $^{\$}$ = $P < 0.01$ compared to control+COD.

Cell death and cell proliferation assay

To observed cell death and cell proliferation, MDCK cells were maintained with or without 1000 µg/mL COD for 48 h. From trypan blue exclusion assay, COD-treated control cells showed the highest cell death with approximately 19.12% whereas alpha-tubulin overexpressing cells and COD-treated alpha-tubulin overexpressing cells showed only 7.98% and 8.99%, respectively and normal cells had 6.25% of cell death (Fig. 6).

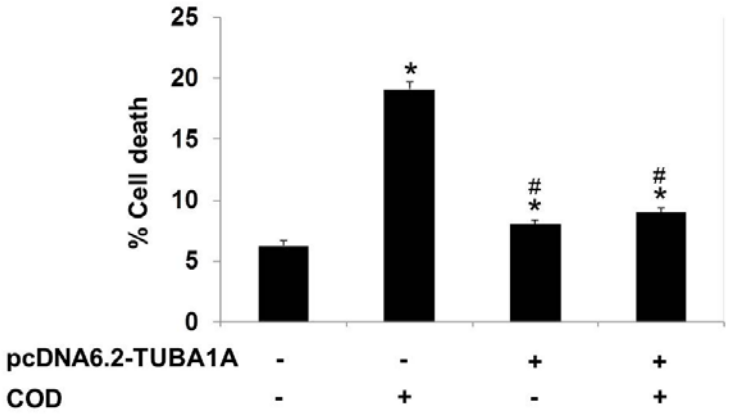

Figure 6. Effect of alpha-tubulin overexpression on cell death. The effect of COD crystals on cell death was examined by trypan blue exclusion assay and shown in percent of cell death. The data showed that percent cell death was highest in COD-treated control cells whereas alpha-tubulin overexpressing cells and COD-treated overexpressing cells showed no significant difference. The data are reported as mean ± SEM (n = 3 independent experiments). * = $P < 0.01$ compared to control cell, $^{\#}$ = $P < 0.01$ compared to control+COD.

However, both alpha-tubulin overexpressing and COD-treated alpha-tubulin overexpressing cells revealed no significant difference of cell death. These results indicated that alpha-tubulin overexpression could reduce cell death. We hypothesized that if alpha-tubulin overexpression could decrease of cell death then it may be promote cell proliferation. To address this hypothesis, the total cell number was determined. Both alpha-tubulin overexpressing and COD-treated alpha-tubulin overexpressing cells showed the total cell number more than control and COD-treated control cells (Fig. 7).

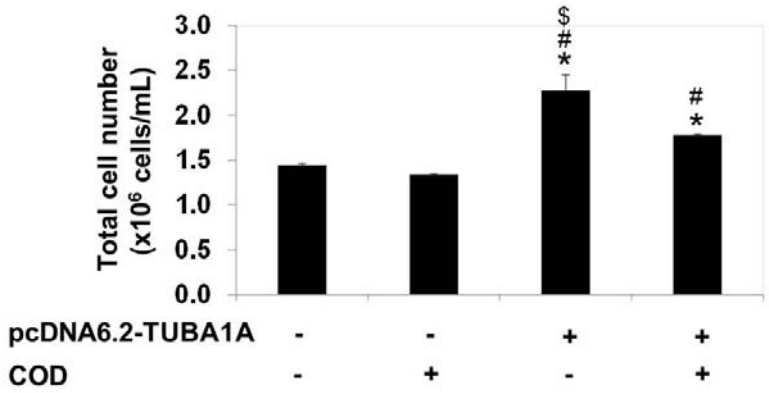

Figure 7. Effect of alpha-tubulin overexpression on cell proliferation. The effect of COD crystals on cell proliferation was examined by trypan blue exclusion assay and shown as total cell number. Alpha-tubulin overexpressing cells showed the highest total cell number followed by COD-treated overexpressing cells and the total cell numbers of alpha-tubulin overexpressing cells were significant higher than control cells. * = $P < 0.05$ compared to control cell, $^{\#}$ = $P < 0.01$ compared to control+COD, and $^{\$}$ = $P < 0.01$ compared to pcDNA6.2-TUBA1A+COD.

Effect of alpha-tubulin overexpression on tissue repair

To evaluate tissue repair and cell migration, wound healing was analyzed using scratch method. Both control and alpha-tubulin overexpressing cells were treated with 1000 µg/mL COD crystal for 48 h and then the artificial wounds were created on the confluent monolayer cells to observe the repair of injurious cells. The scratched wound was measured for cell-free width at the indicated time-points (0, 3, 6, 9, 12 and 15 h after the scratch) and three independent experiments were carried out in triplicate. We initiated the scratch-wound width with approximately 748 µm in all conditions. The morphological changes and cell migration could be observed after 3 h postscratching (Fig. 8A). At time-point 3, 6, 9, and 12 h, cell migration was highest in alpha-tubulin overexpressing cells followed by COD-treated overexpressing cell, control cell and COD-treated control cell, respectively. Moreover, the wounds in all conditions were closed at 15 h after scratching whereas the wounds in COD-treated control cell remained unclosed (Fig. 8B). This result indicated that the delay of tissue repair in control cells compared with alpha-tubulin overexpressing cells.

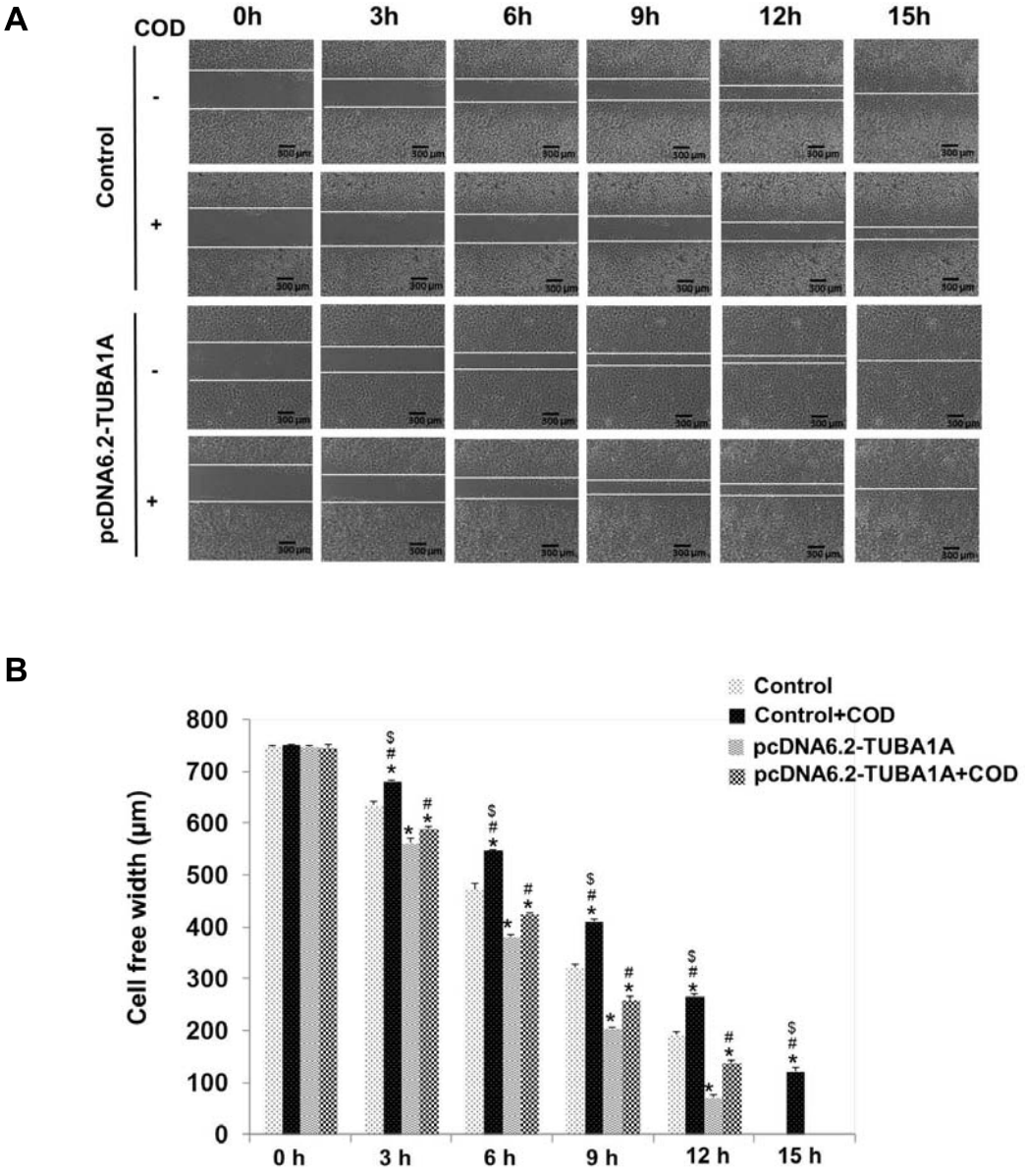

Figure 8. Effect of alpha-tubulin overexpression on tissue repair. Evaluation of tissue repair was generated by scratch assay. Both control and alpha-tubulin overexpressing cells were treated with 1000 µg/mL COD crystal for 48 h and the artificial wounds were created on the confluent monolayer cells. The scratched wound was measured for cell-free width at 0, 3, 6, 9, 12 and 15 h after scratching and observed using BioStation CT with magnification 40X **(A)** and the cell-free widths were measured and analyzed by Tarasoft Image framework v.0.9.6 software **(B)**. All the quantitative data are reported as mean ± SEM (n = 3 independent experiments). * = $P < 0.05$

compared to normal cell, # = $P < 0.05$ compared to pcDNA6.2-TUBA1A, and $ = $P < 0.05$ compared to pcDNA6.2-TUBA1A+COD.

Effect of alpha-tubulin overexpression on crystal adhesion and evaluation of crystal-binding proteins in apical membranes

Crystal adhesion was observed in control and alpha-tubulin overexpressing cells using light microscope after added COD crystals for 30 min. The result showed crystal adhesion in alpha-tubulin overexpressing cells was less than control cells as shown in Fig. 9.

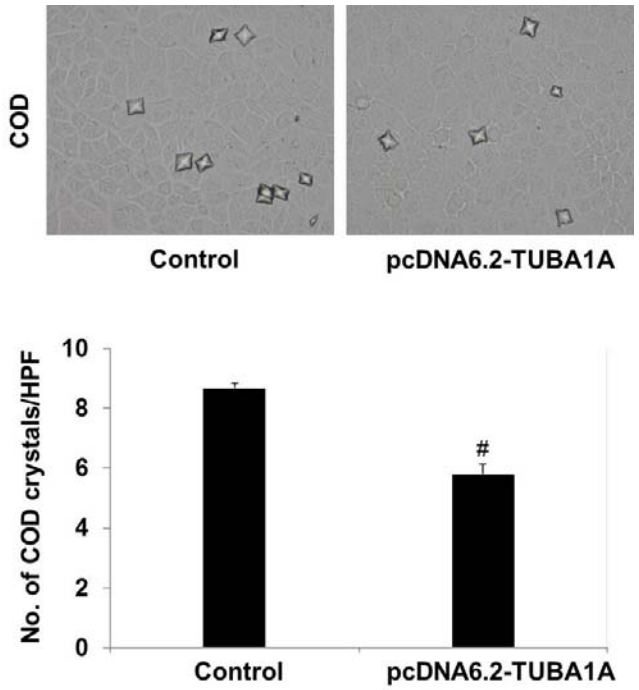

Figure 9. Effect of alpha-tubulin overexpression on COD crystal adhesion. Phase-contrast microscopic showed COD-crystal adhesion in normal cells (left) and alpha-tubulin overexpressing cells (right) with magnification 400X. The quantitative analysis of the number of COD crystals/HPF was significantly decreased in alpha-tubulin overexpressing cells compared to control cells (n = 6 per group). # = $P < 0.001$ compared to control cell.

In addition, Western blot analysis was used to evaluate the expression level of some potential crystal-binding proteins in apical membranes. The results showed that crystal-binding proteins including HSP90, HSP70, and alpha-enolase were increased when exposed with COD

crystals as shown in Fig. 9 (lane 2) and significantly decreased in tubulin overexpression. (Fig. 10, lane 4).

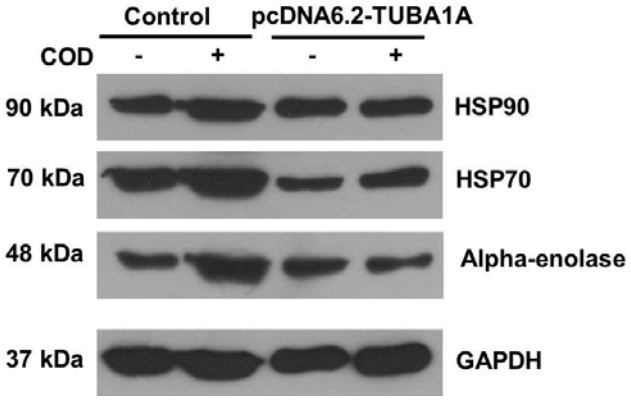

Figure 10. Validation of crystal-binding proteins in MDCK cells induced by COD crystals and protein expression levels were analyzed by Western blot.

Chapter 4

Discussion

From our previous studies, we have identified the altered proteins which response to COD crystal adhesion in MDCK cells by 2-DE analysis. In this study, we further explored those altered proteins to clarify their functions. Based on global protein network analysis, the protein-protein interaction was analyzed using STRING 9.1 and we performed Western blot analysis to examine protein expression levels. The confirmation of protein expression form 2-DE showed that alpha-tubulin was decreased as expected but SOD1 was not decreased in COD-treated cells compared to control. Although we could not confirm decrease of SOD1, we could exhibit that alpha-tubulin was dramatically reduced in COD exposure. Therefore, in this study, alpha-tubulin was overexpressed to determine its function on COD crystals adhesion in MDCK cells.

Microtubules, one of a main cytoskeletal protein in eukaryotic cells, perform essential functions such as regulation of cell shape, intracellular transport and cell motility (44). Alpha-tubulin is a major component of microtubules which form a heterodimer with beta-tubulin (40). With cytotoxicity dose of COD (1000 μg/mL) could induce cell injury and decrease some cytoskeletal proteins such as alpha-tubulin and vimentin and increase ANXA2 which has been identified as a multifunctional and CaOx-binding molecules (24, 26).

Under supersaturated concentrations of calcium and oxalate ion, crystals are formed occasionally and they are either removed in the urine or they are adhered and internalized by the epithelial cells and the endocytosed crystals appear to change cellular functions (6). COD crystals displayed mild cellular damage and mild cytotoxicity under hypercalciuric and hyperoxaluric conditions in HK-2 cells and some adhered crystals were internalized via endocytosis (49). Yap *et al.* demonstrated that alterations in microtubule composition affected the integrity of epithelial cell sheets (46). In our present study, down-regulation of alpha-tubulin affected cell integrity of MDCK cells which observed by inverted phase contrast microscope. Interestingly, alpha-tubulin overexpression could induce vimentin at basal level. Vimentin has been reported to maintain cell and tissue integrity and found interacting with several organelles such as plasma membrane, lysosomes, golgi, nucleus, and microtubules (38).

MDCK cells which were exposed with 1000 μg/mL COD crystals showed high cytotoxicity and cell death whereas alpha-tubulin overexpression could protect MDCK cells from cell damage and cell death. In addition, alpha-tubulin overexpression was found clearly promoted cell

proliferation and wound healing. The scratch wound healing assay facilitates a study of cell migration, tissue reorganization, and cell division (47). When MDCK cells were disrupted with cell-cell contact by artificial wounds, they tend to increase wound healing through a combination of proliferation and migration (5, 45, 47). In addition, microtubules play a role in cell division and cell migration (9, 20, 44). This implied that alpha-tubulin overexpression protected cell injury by increasing cell proliferation and promoted cell migration. Moreover, in our present study found alpha-tubulin overexpression increased expression of vimentin and ANXA2 and the increasing levels of these proteins could promote cell proliferation, cell migration and wound healing. Corresponding to previous evidence, vimentin has been report that involves in wound repair in mice and down-regulation of ANXA2 not only disrupts cell proliferation and cell migration of gastric cancer cells but also induces microtubules disassembly (7, 35).

Many reports showed that calcium oxalate induced renal tubular cell injury and its enhanced crystal attachment on renal cell surface (21, 39, 43). COD crystals are injurious to cell at high dose and renal injury results in cell damage. When crystals adhere on the epical cell surface, it can damage cell membrane (31, 48). In addition, several studies reported that high calcium and high oxalate could enhance crystal-binding proteins and these proteins have been identified as COM-binding proteins in apical membrane of MDCK cells such as HSP90, HSP70, and alpha-enolase (4, 12, 19). To prove the hypothesis of COD crystals evoked cell damage and promoted crystal adhesion by increasing crystal-binding proteins, we analyzed crystal adhesion and evaluated crystal-binding proteins expression levels. The results showed that crystal adhesion and some potential crystal-binding proteins including HSP90, HSP70, and alpha-enolase were reduced in tubulin overexpression. This implied that alpha-tubulin is an important protein for protecting renal tubular epithelial cell injury and crystal adhesion. In addition, several reports also described that heat shock proteins are expressed at low levels in unstress cells and they are induced in various type of cell stress such as oxidative stress, osmotic stress, and thermal stress (2, 16, 30). HSP70 hardly detects under normal condition but it highly evaluate after exposure to physiological stresses (30). It suggested that overexpression of alpha-tubulin could preserve heat shock proteins at basal levels and protected MDCK cells from stress condition which induced by COD crystals as well as reduced crystal adhesion. In previous study of Kanlaya *et al.*, showed that high oxalate ion induced up-regulation of alpha-enolase on apical surface of renal tubular epithelial cells (19). In this present study, we have shown that overexpression of alpha-tubulin could decrease alpha-enolase. The

reduction of those crystal-binding proteins indicated that alpha-tubulin is important for protecting COD crystal adhesion in renal tubular epithelial cells.

Chapter 5

Summary

In conclusion, we carried out the cellular response during COD crystal adhesion in MDCK cells. Our study demonstrated that overexpression of alpha-tubulin could protect renal tubular epithelial cells injury by inhibit cell death and crystal adhesion. Alpha-tubulin overexpression promoted cell proliferation, cell migration and tissue repair in MDCK cells. Moreover, alpha-tubulin overexpression could decrease some potential crystal-binding proteins including HSP90, HSP70, and alpha-enolase. This finding implied that alpha-tubulin is an important protein for protecting renal tubular epithelial cells from COD crystal adhesion.

Chapter 6

Output

Manuscript is being prepared for submission.

Chapter 7

Appendix

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