REQUIREMENT OF CLUSTERIN EXPRESSION FOR PROSURVIVAL AUTOPHAGY IN HYPOXIC KIDNEY TUBULAR EPITHELIAL CELLS

by

Hatem Alnasser

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Science

in

The Faculty of Graduate and Postdoctoral Studies

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

January 2020

The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the thesis entitled: Requirement of Clusterin Expression for Prosurvival Autophagy in Hypoxic Kidney Tubular Epithelial Cells.

Submitted by Hatem Alnasser in partial fulfilment of the requirements for the degree of Master of Science in Experimental Medicine

Examining Committee:

Dr. Caigan Du, Urologic Sciences

Supervisor

Dr. Michael Cox, Urologic Sciences

Supervisory Committee Member

Dr. Amina Zoubeidi, Urologic Sciences

Supervisory Committee Member

Dr. Vincent Duronio, Medicine

External Examiner

Abstract

Cellular autophagy is a prosurvival mechanism in the kidney against ischemia reperfusion injury (IRI), but the molecular pathways to activating autophagy in ischemic kidneys are not fully understood. Clusterin (CLU) is a chaperone-like protein and its expression is associated with kidney resistance to IRI. This study investigated the role of CLU in the prosurvival autophagy in the kidney. Renal IRI was induced in mice by clamping renal pedicles at 32°C for 45 min. Hypoxia in renal tubular epithelial cell (TEC) cultures was induced by exposure to 1% O_2 atmosphere. Autophagy was determined by either LC3-BII expression in Western blot or LC3-GFP aggregation in confocal microscopy. Cell apoptosis was determined by flow cytometric analysis. Unfolded protein response (UPR) was determined by PCR array. Here, we showed that autophagy was significantly activated by IRI in wild type (WT) but not CLU deficient kidneys. Similarly, the autophagy was activated by hypoxia in human proximal TECs (HKC-8) and WT mouse primary TECs but was impaired in CLU null TECs. Hypoxia activated autophagy was CLU dependent and was positively correlated with cell survival, and inhibition of autophagy significantly promoted cell death both in HKC-8 and mouse WT/CLU expressing TECs, but not in CLU null TECs. Further studies showed that CLU-dependent prosurvival autophagy was associated with UPR activation in hypoxic kidney cells. In conclusion, these data suggest that activation of prosurvival autophagy by hypoxia in kidney cells is required CLU expression, and may be a cytoprotective mechanism of CLU in the protection of the kidney from hypoxia/ischemia-mediated injury.

Lay Summary

Clusterin is a glycoprotein that has been discovered over thirty years ago. It has been studied to have some protective properties in the brain from Alzheimer's and in the cancer field. In our lab, we have established that it has some protective properties in the kidneys. The cells have several defence mechanisms and one of them is called autophagy which translated to self eating. So, when the cells die it leaks some of its materials and proteins out and in order to prevent that from happening the cells self destructs and 'eats itself'. Our study is trying to prove that the defence mechanism of clusterin in the kidney cells is autophagy.

Preface

This thesis has been written in partial fulfilment of the requirements for the degree of Master of Science in Experimental Medicine. I Have written this thesis under the direction and Supervision of Dr. Caigan Du from May 2012 to June 2019.Also my experiments were completed at the Vancouver Prostate Centre with technical skills under the supervision of Lab Manager, Qiunong Guan. I have been involved in the animal experiments All the animals (males, 10-12 weeks old) for the experiments were cared for in accordance with the Canadian Council on Animal Care guideline under the protocols approved by the Animal Use Subcommittee at the University of British Columbia (Ethics approvals:A10-0043, A11-0192, A11-0409). I took care of cell cultures and handling. My supervisory committee were involved in feedback in my regular meetings with them. The thesis has been written based on two papers, the first "Requirement of clusterin expression for prosurvival autophagy in hypoxic kidney tubular epithelial cells" Alnasser HA, Guan Q, Zhang F, Gleave ME, Nguan CY, Du C. Published in the American Journal of Pysiology in 2015. I was the first author and performed all the experiments. The statistical analysis and paper writing were done by myself with the help of my supervisor. The other co-authors were involved in a more supervisory and feedback role. The second paper "From Humans to Experimental Models: The Cytoprotective Role of Cluster in the Kidney" Qiunong Guan, Hatem A Alnasser, Christopher YC Nguan and Caigan Du. Published in the Medical and Surgical Urology-Open Access in 2014. I was involved in the literature review and was helping my lab manager Quinong Guan in performing the experiments. Statistical analysis and writing were done by Dr. Caigan Du. And the rest of the co-authors were involved in a supervisory and feedback role.

Acknowledgements

I offer my enduring gratitude to the faculty, staff and my colleagues at UBC, who have inspired me to continue my work in this field. I owe thanks to my supervisor Dr. Caigan Du, whose guidance has been advocating for the best interest of my learning process encouraging me to take initiatives and explore my potentials in the field of research and experimental medicine. Also, I would like to express my deep appreciation to the lab manager Qiunong Guan, who has been mentoring my lab skills throughout this project and whose continuous enthusiasm in the work place has always been uplifting for all lab members.

I would like to give many thanks to Drs. Michael Cox and Amina Zoubeidi, my supervisory committee, for their interest in this project and my work and for the time and effort they have invested in providing me with enlightening feedback.

Dedication

This degree is dedicated to my family who has been a source of all kinds of support throughout my lifetime academic journey. My overwhelming gratitude is forever owed to my dear parents, Abdulaziz and Salam, my loving wife, Hadel, and my precious little five year old angels Judy & Maya for their providing of constant inspiration, motivation and most valued prayers that are truly the essence of any success and accomplishments of mine.

Table of Contents

1. Introduction.

1.1 Acute Kidney Injury.

The definition of an acute kidney has been a long standing debate and several definitions such as RIFLE and AKIN have come out mostly relying on serum creatinine from baseline associated with a decline in urine output within a certain period of time usually within 48 hours. (93). But for the purpose of this study we will use the common definition being an increase in serum creatinine of > 26.4 unmol/L within 48 hours or a percentage of increase in serum creatinine of 50% or more or a reduction in urine output of < 0.5ml/kg/hour for more than 6 hours. There are several causes of AKIs. The best way to approach it is to divide it into three major categories:

Pre-renal causes, renal causes or intra-renal causes and post renal causes.

Pre -renal AKI is defined as an insult to the kidney(s) that results in a decline of kidney function. The damage in a pre-renal insult happens to the structure supplies the kidneys mainly the blood vessels whether it is decreased perfusion from low volume, obstruction from an external mass or emboli. Renal causes include damage that happens within the kidney structures such as the tubules, interstitium, glomeruli and vessels and post renal causes arises from any obstruction to ureters bladder and urethra such as stones masses and the prostate.There have been several markers as well as prediction scores that have been studied to establish the presence of an acute kidney injury.

Several characteristics have been established in order to choose an ideal biomarker for confirming an AKI and for that creatinine fits most of these characteristics. But so far there are no biomarkers indicating if the kidney has the ability to to protect itself from any injury.

1.2 Ischemic Reperfusion Injury.

1.2.1 Definition.

Ischemia is a hypoxic condition which disrupts blood flow to the tissue, and subsequent reperfusion is the restoration of blood flow to the ischemic tissue, which together result in tissue injury referred to as ischemia-reperfusion injury (IRI). Renal IRI induces acute kidney injury (AKI) and is seen in many clinical situations, including kidney transplantation (1,2) or cardiac surgery-associated AKI (CSA-AKI)(3-5). In kidney transplantation, the severe ischemic injury in donor kidneys from donation after cardiac death (DCD) is a primary reason for their discard (2), and in grafted kidneys it causes delayed graft function (DGF) (6,7), and is a high risk factor for both acute and chronic allograft rejection (8,9), eventually resulting in graft loss(10-13. CSAAKI is characterized by an abrupt reduction in glomerular filtration rate of the kidney after cardiac surgery(5,12), and may contribute to the increased mortality as a small (0.3–0.5 mg/dl) or larger (>0.5 mg/dl) increase in serum creatinine (sCr) after cardiac surgery correlates with a nearly 3- or 18-fold increase in 30-day mortality, respectively(4). Furthermore, CSA-AKI worsens long term complications in patients who have additional risk factors such as advanced age, renal insufficiency, diabetes mellitus, systemic hypertension or systemic lupus erythematosus(5). Unfortunately, there is no effective therapy available to treat renal IRI as of today.

1.2.2 Pathophysiology.

IRI is complicated by the ischemia or hypoxia first, followed by the blood reperfusion or reoxygenation, in which both hypoxia and reoxygenation can induce apoptotic and necrotic cell death(13,14). Evidence in literature suggests that renal IRI is multifaceted including inflammatory and immune responses, oxidative stress, induction of cell death and incomplete renal repair(15-17), but its pathogenesis is still not fully understood. It has been known for a while that in the response to pathological stresses (e.g. nutrient depletion or starvation) cellular autophagy is activated to rebalance the energy source through a catabolic self-degradative process of misfolded or aggregated proteins and/or damaged organelles (i.e. mitochondria, endoplasmic reticulum and proxisomes) (18,19). Recent studies have shown an increase in autophagic activity in the kidneys following IRI or in hypoxic kidney cells(20-23) . Although different biological functions of autophagy have been reported to associate with either cell survival or death under certain circumstances (24-27), it has been clearly demonstrated that the activation of autophagy provides a protective mechanism in the kidney for cell survival during IRI (20,28,29). However, little is known of the molecular pathways that regulate prosurvival autophagy in the kidneys during IRI.

1.3. Clusterin.

1.3.1 Definition.

Clusterin (CLU) is a chaperone-like glycoprotein (30,31), that has been establised by numerous studies that it has cytoprotective effects not only to the kidneys but also to the heart brain and GI tract (32). Clusterin (CLU) protein was first discovered more than thirty years ago (93), and a large volume of research has been dedicated to it since – there are more than two thousand publications in Pubmed/NCBI databases when using 'clusterin' as a keyword search criteria today. Human CLU gene (NCBI Gene ID: 1191) is located at chromosome 8p21-p12, and consists of 10 exons, in which the rest two exons are alternative (designated 1 and 1') (94).

Thus, CLU gene can be transcribed into at least three mRNA variants (NCBI Reference No.: NM_001831.3; NR_038335.1; NR_045494.1) or perhaps even more (95). The mRNA isoform 1 is a major form of CLU mRNA, whereas other forms including mRNA isoform 2 collectively count for less than 1% of total CLU mRNA (95). Two isoforms of CLU proteins have been well characterized; nuclear isoform of CLU (nCLU, isoform 1) containing the nuclear localization signal that is translated due to the splicing at exon 1 and 3 together placing a downstream AUG at exon 3 as the first available translation and lacking of exon 2 (95,96), while pre-secreted isoform of CLU (sCLU) containing the endoplasmic reticulum (ER)-targeting signal encoding in exon 2 (95). The nCLU is translocated into the nucleus after translation and probably without glycosylation (95), whereas the pre-secreted sCLU is targeted to ER and Golgi bodies glycosylation and cleavage between Arg-205 and Ser-206 to produce mature sCLU, a secreted disulfide linked heterodimer of α- and β-chains (97,99). Under certain stress conditions, sCLU however can be retrotranslocalized into the cytosol

instead of secretion (81). However, the cellular localization of all these isoforms and their expression are largely unknown. Murine CLU gene (NCBI Gene ID: 12759; MGI ID: 88423) is found at chromosome 14, and contains nine exons that are only transcribed to a single mRNA (NCBI Reference No.: NM_013492.2, 1808 bp) (100). nCLU isoform has not been found in mice as of yet. The homolog of mouse CLU to human sCLU is 75% at the amino acid level, and both have the same ER-targeting signal peptide and the cleavage site (101). By immunohistological staining using the same anti-CLU α-chain antibody, CLU protein was localized in-human kidney sections in the same pattern as that of mice. Plus, CLU in mice can serve as a counterpart for sCLU both extracellular and intracellular) in humans, particularly in the study of the kidney disorders.

1.3.2 Physiology.

In the human body, sCLU is a major glycoprotein in all the physiological fluids such as plasma, milk, urine, cerebrospinal fluid, and semen (98). It is constitutively produced and secreted by almost all cell types that form the cellular interfaces of fluid compartments (98), and similarly by the liver (101). The serum levels of sCLU in humans are present in a range of 35-353 μg/mL (102-105). In tissue, upregulation of CLU expression (probably including nCLU) is associated with many pathophysiological processes, such as neuropathologies (106,107), heart disease (108), cancer (109-111), kidney transplant rejection, and kidney disease including glomerulonephritis (112,113). In rodents, renal CLU is upregulated following a variety of insults, such as unilateral ureteral obstruction (UUO) and ischemia-reperfusion injury (IRI) (35,114), acute glycerol-induced renal failure, chronic vitamin E and selenium deficiency (115), lupus-like nephritis (116), and in resident glomerular cells exposed

to complement-mediated injury (117). The CLU expression in glomerular mesangial and epithelial cells, as well as renal proximal tubular epithelial cell (TEC) is increased in response to the stimulation of thrombin (118) and hypoxia in our published observations. Further studies indicate that CLU is an apically secreted glycoprotein in renal TECs (119), and is detected in both viable and apoptotic cells following renal injury (113,120). The molecular mechanism(s) for either constitutive or inducible expression of CLU have not been well investigated. It has been reported that CLU gene proximal promoter contains a 'clusterin element' (CLE) that is specifically bound by heat-shock factor (HSF) 1 after heat shock, or by HSF1-HSF2 up on proteasome inhibition (121,122), resulting in the induction of CLU gene transcription. The primary structure of sCLU in both α- and β-chain subunits contains several large molten globule domains, amphipathic regions and coiled-coil αhelices (30,73100,123), which are typical of molecular chaperone - conformational adaptability to allow CLU protein to bind its substrate proteins or lipids with high afinity and low specificity (30,31,90). Furthermore, one study has revealed that the glycosylation of sCLU may not be required for its overall secondary structure content and binding activity to its substrate (124). The biological activities of extracellular sCLU have been studied extensively; it was initially found to induce cell aggregation (125-127), and in the plasma it inhibited the cytolysis of complement membrane attack complex (MAC) by binding to the complement components (128-130), and was associated with both high-density lipoprotein (HDL) and low-density lipoprotein (LDL) complexes (123,131,132). These studies suggest that sCLU in the blood may serve not only as an inhibitor of the lytic terminal complement cascade, but also as a regulator of lipid transport and local lipid redistribution. Furthermore, addition of sCLU prevents cell apoptosis in cultured cells treated with TNF-α, H2O2 or gentamicin probably

by activation of magalin- phosphatidylinositol 3-kinase/Akt pathway (133-135), and mediates clearance of cellular debris into non-professional phagocytes (136).

Based on all of these observations, it has been proposed that sCLU functions as an extracellular chaperone, a previously unknown quality- control system for protein folding that mediates the recognition and disposal of extracellular misfolded proteins via receptor (i.e. megalin)- mediated endocytosis and lysosomal degradation (137). This hypothesis is supported by a recent study showing that sCLU in the blood binds to a panel of proteins, including ceruloplasmin, brinogen, and albumin, in response to physiologically relevant stress (138).

Inside human cells, in addition to nCLU isoform that is mainly localizing in the nucleus and triggers cell death (139), sCLU could redirect to the cytosol under cellular stress (81,140). In mice, CLU in normal tissues (heart and kidney) is present as a single protein band at approximately 40 kDa in Western blot analysis in our studies (38,114,154), while in cultured cells from these tissues two protein bands at approximately 60 kDa and 40 kDa are detected (38,114,154), suggesting that mouse CLU probably is also retrotranslocated to the cytosol following exposure to sub lethal stress in culture conditions. A variety of biological activities of intracellular sCLU or cytoplasmic CLU (cCLU) have been reported; it inhibits apoptosis by the interaction with BAX or GRP78 (80,82,140) or promotes cell survival by the activation of Akt and NF-κB pathway (134,141). It is of much more interest to see that sCLU (~70 kDa) acts as an intracellular chaperone to interact with both ATP7A and ATP7B (Cu-ATPases) and facilitates degradation of misfolded/mislocalized mutant ATP7B (142). Whether or not intracellular sCLU plays a role in ER quality control machinery that facilitates the degradation of mis/ un-folded proteins in ER remains under further investigation.

During rodent embryogenesis, CLU expression is detected in a variety of the tissues in many developing organs, such as the epithelial cells of comma and S-shaped bodies of the primordial kidney (143), developing islet of Langerhans of the primordial pancreas (143), myocardial cells adjacent to developing endocardial cushions of both atrioventricular canal and truncusarteriosus, stromal connective tissue throughout leaflet formation of the developing hearts (144), hypothalamic region, neocortex and hippocampus of the developing brain (145,146). Interestingly however, complete knockout (KO) of CLU expression in CLU KO mice has not been found to cause any phenotypic change in postnatal development as compared to WT mice (42), suggesting that CLU may not be absolutely required for the differentiation and morphogenesis of an organ (i.e. kidney). Although brain weight, neurons, astrocytes and oligodendrocytes are not significantly different between WT and CLU KO mice during postnatal development, it is noted that there is a significant deficit in motor cells (~16%) in the facial nucleus in CLU KO compared with WT mice (147), suggesting that CLU may have a negative impact on neuronal development in certain motor nuclei. Indeed, in cultured progenitor or undifferentiated cells, CLU enhances neuronal differentiation from neural precursor cells (148), and ectopic over expression of CLU significantly up-regulates the expression of morphogenic factor Pdx-1 and Ngn-3 that is correlated with an increase in β-cell transformation from neogenic ductal cells (49), and increases CXCR4 expression and migration of cardiac progenitor cells (150).

We have recently demonstrated that kidney repair or tissue regeneration is impaired after IRI in CLU KO mice (38), suggesting that CLU may play a key role in the differentiation and migration of renal stem/progenitor cells that have been found to contribute to renal repair after injury (151-153) which however remain elusive. Its expression is significantly up-

regulated in the kidneys of animal models of unilateral ureteral obstruction (UUO) and IRI (33,34,35) or in renal biopsies from rejected renal allografts or diseased kidneys(36). Up-regulated CLU may play a protective role in the kidney because a deficiency in CLU expression in mice (CLU knockout) results in the development of age-related glomerulopathy (37), increases the susceptibility to renal IRI or impairs renal repair after IRI (38,39). As a matter of fact, Clusterin has been recently studied that its urinary excretion could be a marker for worsening Diabetic Nephropathy.

Recently, we have found that CLU overexpression enhances cell survival via autophagydependent pathways in prostate tumor cells(40). The objective of this study was to investigate if the cytoprotection of CLU in the kidney was mediated by the activation of prosurvival autophagy during IRI.

2. Methods, Materials, and Animal Models.

2. 1 Animals and cell cultures materials and methods.

Both wild type (WT) C57BL/6 (B6) and CLU knockout ($CLU^{-/-}$, KO) strains of mice in B6 background were received from the breeding colonies in the animal facility at the Jack Bell Research Centre (Vancouver, BC) (39). GFP-LC3 transgenic mice in B6 background were a kind gift from Dr. Noboru Mizushima (Department of Physiology and Cell Biology, Tokyo Medical and Dental University, Tokyo, Japan) (41), and were subsequently backcrossed for ten generations into B6-$CLU^{-/-}$ mice in our facility to generate GFP-LC3 CLU KO ($GFPLC3^{+/+}CLU^{-/-}$) mice as a CLU null control of GFP-LC3 CLU WT (GFP-$LC3^{+/+}CLU^{+/+}$) mice, respectively (40). In these GFP-LC3 transgenic mice, GFP-LC3 was ubiquitously expressed under the control of the CAG promoter, and the accumulation of GFP puncta represented the formation of autophagosomes or autophagy activation(41). Genotypes of both CLU KO and GFP-LC3 knock in strains of mice were confirmed by PCR as described previously(40,42).

Human proximal tubular epithelial cell (TEC) line HKC-8 was kindly provided by Dr. Daniel L. Sparks (Ottawa, ON, Canada) under the permission from Dr. Lorraine Racusen (43).

MKC-1 cells were generated by immortalizing primary TECs from WT B6 mice with origin deficient SV40 DNA (39). Both CLU-expressing TECs and CLU-negative control cells were originated from the same CLU null TECs by stable expression of either human CLU isoform 1 cDNA using pHEX6300 vector (denoted as TEC-CLU^{hCLU}) or empty pHEX6300 vector (denoted as TEC-$CLU^{-/-}$) (38). Primary murine TECs were isolated from the kidney cortex of mice following the protocol as described previously

(44). In brief, renal cortex was collected and minced in HBSS containing penicillin-streptomycin. The tissue fragments were washed twice with HBSS and then digested with 1 mg/ml of collagenase V (Sigma-Aldrich Canada, Oakville, ON, Canada) in HBSS at 37°C for 15 min with intermittent agitation of the tissue. The digested tissue was sieved through a 40 μm Cell Strainer (BD Falcon^{TM}, BD Biosciences, Mississauga, ON, Canada). After washing with HBSS and subsequently with complete K1$^{+/+}$ medium as described previously (18), the sieved cells were seeded and grown in complete K1$^{+/+}$ medium in a collagen-coated flask. Finally, the phenotypes of primary TECs were confirmed by their expression of E-cadherin and CD13 (Alanine aminopeptidase) in fluorescence-activated cell sorter (FACS) analysis. All TEC lines (HKC-8, MKC-1, TEC-CLU^{hCLU} and TEC-CLU$^{-/-}$) and the primary TECs were grown in complete K1$^{+/+}$ medium.

2.2 Reagents and antibodies.

Both Annexin-V conjugated with phycoerythrin (Annexin-V-PE) and 7-aminoactinomycin D (7-AAD) were purchased from BD Biosciences, Bafilomycin A1 (Baf A1) was from Sigma-Aldrich Canada. Spautin-1 was from Cellagen Technology (San Diego, CA, USA) Primary antibodies used in this study were: goat polyclonal anti-CLU-alpha (C-18, Santa Cruz Biotech, Santa Cruz, CA, USA), rabbit polyclonal anti-LC3B ($^{\#}$2775, Cell Signaling Technology, New England Biolabs, Ltd., Whitby, ON, Canada), rabbit polyclonal anti-IRE1 (phospho-ser724) (Biorbyt, Cambridge, UK), rabbit polyclonal anti-IRE1-alpha (NB100-2324, Novus Biologicals, Littleton, CO, USA), and mouse monoclonal anti-β-actin (clone AC-40, Sigma-Aldrich Canada). Secondary antibodies were: horseradish peroxidase (HRP)-conjugated anti-goat IgG (sc-2020), anti-mouse IgG HRP (sc-2314) and anti-rabbit IgG HRP (sc-2313). All were from Santa Cruz Biotech.

2.3 Renal IRI model.

Renal IRI was induced in the left kidney. In brief, mice were anesthetized with the combination of ketamine (100 mg/kg) and xylazine (10 mg/kg), and isoflurane as needed. The left kidneys were exposed through a flank incision, followed by the induction of ischemia in these kidneys through clamping renal pedicles at the body temperature of 32^{o}C for 45 min. After the clamps were released, reperfusion of the kidneys was confirmed visually. In order to examine the increase in autophagic activity (GFP prunta) in the left kidneys of GFP-LC3$^{+/+}$CLU$^{-/-}$ and GFP-LC3$^{+/+}$CLU$^{+/+}$ mice after reperfusion, the non-ischemic right kidneys in the same mice were kept as contralateral controls.

3. Experiments.

3.1 Induction of hypoxia in vitro.

Hypoxia in cultured kidney cells in complete $K1^{+/+}$ medium was induced by incubation in a humidified hypoxic chamber (Coy Laboratory Products, Inc., Grass Lake, MI, USA), pumped with a gas mixture of 1% O_2, 5% CO_2 and 94% N_2 at 37°C, while identical (twin culture) cell cultures were grown a humidified CO_2 incubator in an atmosphere containing 5% CO_2 and 95% air (approximately 20% O_2) at 37°C as normoxia controls.

3.2 Analysis of GFP-LC3 puncta formation.

GFP–LC3 mice are commonly used to monitor the induction of autophagic activity or autophagosome numbers, indicated by the increased number of GFP-LC3 puncta in tissue sections (41,85). For the assessment of IRI-induced autophagy in the kidney, renal tissues after 24 h of ischemia-reperfusion were collected from both injured and contralateral kidneys, and fixed with paraformaldehyde fixation solution (4% paraformaldehyde dissolved in 0.1 M Naphosphate buffer, pH 7.4) for 4 h, incubated overnight with 15% sucrose/PBS, and 30% sucrose/PBS for 4 h. Tissues were then embedded in Tissue-Tek OCT medium and stored at −80°C. Sections (5–7 μm) were prepared with a cryostat and air dried at room temperature for 30 min. To examine hypoxia-induced autophagy in cultured kidney cells, kidney cells on glass coverslips were exposed to hypoxia or normoxia for 24 h, followed by fixing in the paraformaldhyde solution for 15 min and rinsing with PBS. The nuclei in both tissue sections and cultured cells were stained with 4’,6-diamidino-2-phenylindole (DAPI, Cell Signaling Technology). GFP-LC3 puncta formation (green) and DAPI-stained nuclei (blue) in tissue

sections or cultured cells were visualized using a Zeiss LSM 780 confocal microscope (Carl Zeiss, Thornwood, NY, USA).

3.3 Semi-quantitative analysis of renal injury.

Histological assessment of tubular injury in kidney sections was performed in a blinded fashion. Formalin-fixed and paraffin-embedded sections (5 μm thickness, longitudinal) were stained with Periodic acid-Schiff (PAS) reagents. The percentage of damaged tubule (combined both necrosis and vacuolization) in total of tubules was counted in each view, randomly selected in the region of renal cortex under ´400 magnification, and was presented as an average of at least 20 non overlapping fields for each kidney.

3.4 Analysis of cell apoptosis.

Apoptosis in cultured kidney cells was measured by fluorescence-activated cell sorting (FACS) analysis following the manufacturer's protocol (BD Biosciences) as described previously (39), in which Annexin-V-PE stained early apoptosis and 7-AAD late apoptosis or necrosis. In a FACS graph, non-apoptotic (viable) cells were in low left quadrant, necrotic cells in upper left quadrant (7-AAD positive only), late apoptotic cells in upper right quadrant (both Annexin-V and 7-AAD positive) and early apoptotic cells in lower right quadrant (Annexin-V positive only). Briefly, monolayers of kidney cells were released by a brief incubation with Trypsin-EDTA solution (Sigma-Aldrich Canada), and then incubated with Annexin-V-PE in 1 X binding buffer for 15 min, followed by 7-AAD in the dark. The intensity of fluorescence of apoptotic and necrotic cells was measured by flow cytometry and

analyzed as compared to background controls using CELLQUEST software (BD Biosciences).

3.5 Western blot analysis.

The Western blot analysis of protein expression was routinely performed following a standard protocol in the laboratory. In brief, whole cellular protein extract was prepared by a brief sonication of pieces of renal cortex or cell pellets in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitor cocktail (Roche, Mannheim, Germany), and fractioned by 7 to 15% SDSpolyacrylamide gel electrophoresis' (SDS-PAGE) according to the molecular size of the target protein. After transferring to nitrocellulose membranes (Bio-Rad Lab, Hercules, CA, USA), the target proteins on the blot were identified using relevant primary antibodies along with HRPconjugated secondary antibodies as described in 'Reagents and antibodies' section, and visualized by an enhanced chemiluminescence assay (ECL, Thermo Fisher Scientific, Inc., Rockford, IL, USA). The blots were re-probed with anti-β-actin IgG antibody (Sigma-Aldrich Canada) to confirm equal protein loading in each sample. The expression levels of the target proteins were measured using a densitometry and were presented as a ratio unit (RU) of the target protein to beta-actin on the same blots.

3.6 PCR array.

The expression of unfolded protein response (UPR)-associated 84 genes in cultured TECs was quantitatively examined using PCR Arrays kits following manufacturer's instruction (SABiosciences – QIAGEN Inc., Valencia, CA, USA). Each group (CLU-expressing TECCLU^{hCLU} versus CLU null TEC-CLU$^{-/-}$) included four different samples (TEC

cultures grown at four separate times) that were randomly selected for the determination of gene expression profile using PCR arrays. The total RNA from each sample was directly extracted and purified from the monolayer after 24 h of either hypoxia or normoxia using the RNeasy Microarray Tissue Mini kit (QIAGEN) and was converted to cDNA using RT^2 First Strand Kit (QIAGEN). The expression of selected genes was amplified by real-time PCR using RT^2 Profile PCR arrays (QIAGEN). Data were analyzed using Web-based PCR Array Data Analysis Software (www.SABiosciences.com/pcrarraydataanalysis.php.). The functional gene grouping was listed based on the reference provided by the manufacturer's website (http://www.sabiosciences.com/rt_pcr_product/HTML/PAMM-089A.html).

3.7 Statistical Analysis.

Analysis of variance (ANOVA) or t-tests (two-tailed distribution) in Prism GraphPad software (GraphPad Software, Inc., La Jolla, CA, USA) were used as appropriate for comparisons between groups. Data were collected from each individual experiment or each mouse for statistical analysis. A p value of ≤ 0.05 was considered significant.

4 Results.

4.1 CLU is required for activating autophagy and reducing tissue injury in the kidneys with IRI.

Several studies have demonstrated either the up-regulation of CLU expression (25,39) or the activation of autophagy(20,21,28) in tubular cells of the kidneys with IRI. To investigate if the autophagy played a role in the cytoprotectivity of CLU against renal IRI as demonstrated previously(39), the autophagic activity in WT kidneys compared to CLU null kidneys was examined following IRI induction. Like these previous observations, the number of LC3-GFP prunta was markedly increased in the tubules of the kidneys after 24 h of reperfusion as compared to that in contralateral kidneys in WT LC3-GFP transgenic mice (Fig. 1A, left column), indicating the activation of autophagy by IRI in these kidneys. By contrast, in CLU KO LC3-GFP mice, IRI did not induce such significant autophagic activity in the kidneys, as there was not much difference in LC3-GFP prunta between the kidneys with IRI and contralateral controls (Fig. 1A, right column). Similar to our previous study(39), WT kidneys displayed less tubular injury (11.3 ± 2.5%) than CLU KO counterparts (32.6 ± 9.8%) ($p = 0.0057$, two-tailed ttest, $n = 4$) after induction of IRI in LC3-GFP transgenic mice (Fig. 1B), suggesting the association of autophagic activity with the resistance to IRI in WT kidneys. To further confirm the autophagic activity and its role in the kidneys during IRI, LC3 expression was examined by Western blot in the kidney samples from our previous study (39), in which non-transgenic WT mice were shown to be more resistant to renal IRI as compared with CLU KO mice after 48 h of reperfusion. As shown in Fig. 2, the levels of both LC3 I and II in the protein extracts of renal cortex from WT kidneys were significantly higher than those in CLU null kidneys. These data together suggest that the autophagy activated by

IRI in the kidneys is largely dependent on the expression of CLU and is associated with the resistance to tissue injury.

4.2 CLU is required for activating autophagy and promoting cell survival in cultured kidney cells under hypoxia.

IRI is initiated by the insufficient blood flow or reduced oxygen supply to the tissue, resulting in profound tissue hypoxic injury (45), and the autophagy is activated in cultured renal TECs by exposure to hypoxic environment – atmosphere containing 1% of O_2(20,21). As shown in Fig. 3A, LC3B-II expression in human proximal tubular HKC-8 cells was increased after 24 h of incubation under the hypoxic atmosphere (1% of O_2) as compared to that in cultures at 95% of atmospheric O_2 levels (~ 20% of O_2), confirming the induction of autophagic activity in human proximal TECs by hypoxia in vitro. Also, the activation of autophagy in hypoxic tubular cells was positively correlated with the up-regulation of CLU expression (Fig. 3A). The relationship of hypoxia-induced autophagy with CLU expression was further verified in primary renal cortical epithelial cells isolated from WT or CLU KO mice. As shown in Fig. 3B, as compared to the control cells at normoxia LC3B-II expression was markedly up-regulated by hypoxia in WT cells, whereas there was a slightly increase in LC3B-II expression in hypoxic CLU KO cells. The hypoxia-induced autophagy in WT kidney cells was further confirmed in primary renal cortical epithelial cells isolated from LC3-GFP transgenic WT or CLU KO mice (Fig. 3C), in which the number of LC3-GFP prunta was significantly increased only in hypoxic WT kidney cells.

CLU expression increases cell survival in cultured kidney cells exposed to cytokine mixture (IFN-alpha and TNF-alpha) (39). To verify if CLU mediated kidney cell survival under hypoxia, cell viability or death was examined in hypoxic CLU-expressing kidney cells

as compared to CLU null control cells. As shown in Fig. 4, cell viability in cultured primary renal cortical epithelial cells isolated from WT or CLU KO mice was significantly decreased after 24 h of exposure to hypoxia, indicated by in WT cells 86.9 ± 5.95% (n = 5) under normoxia to 67.93 ± 4.17% (n = 9) under hypoxia ($p < 0.0001$, two-tailed t-test), or in CLU KO cells 78.13 ± 5.31% (n = 7) under normoxia to 55.77 ± 8.2% (n = 9) under hypoxia ($p < 0.0001$, two-tailed t-test). Further statistical analyses showed that there were more viable cells in WT cells than in CLU KO cells in both normoxic and hypoxic conditions, indicated by two-tailed t-test (normoxia: $p = 0.0226$; hypoxia: $p = 0.0009$) or two-way ANOVA (WT vs. KO, $p < 0.0001$). These results were further confirmed using human CLU-expressing CLU null kidney cells (TEC-CLU^{hCLU}) compared to CLU null TEC-$CLU^{-/-}$ cells that were established previously (38). As shown Fig. 5, CLU-expressing TEC-CLU^{hCLU} cells were more resistant to hypoxia-induced cell death, indicated by 75.96 ± 3.0% of viable cell population in TEC-CLU^{hCLU} cell cultures compared to 62.68 ± 1.81% in TEC-$CLU^{-/-}$ control ($p = 0.0028$, two-tailed t-test, n = 3). Taken together, these data clearly indicate that CLU expression is required for activating autophagy and increasing cell survival in cultured kidney cells under hypoxia, which are consistent with less tissue injury and higher degree of autophagy activation in WT kidneys as compared to those in CLU null kidneys (Fig. 1).

4.3 CLU-mediated autophagy protects kidney cells from hypoxia-induced cell death.

Autophagic activity links to both types of cell death (apoptosis and necrosis), and it either promotes cell survival or results in cell death in response to adverse environment and stress (46,47). As shown above, a higher level of autophagic activity was found in WT kidneys with less IRI (Fig, 1), and in CLU-expressing kidney cells with less cell death under hypoxia (Figs. 35). To reveal the role of autophagy in kidney cell survival in response to

hypoxia, cell survival was examined in hypoxic kidney cells in the presence of autophagy inhibitors (Baf-A1 and Spautin-1). Both human and mouse kidney cell cultures (HKC-8 and MKC-1) were treated with Baf-A1 (0, 5 or 10 nM) under either hypoxia or normoxia for 24 h. As shown in Fig. 6, hypoxia induced cell death in both HKC-8 and MKC-1 cell cultures as expected, and addition of Baf-A1 further reduced cell survival in hypoxic HKC-8 cells from 88.2 ± 1.5% in the absence of the inhibitor to 79.8 ± 4.2% in the presence of 10 nM Baf-A1 ($p = 0.0245$, one-way ANOVA, $n = 3$) while the inhibitor did not attenuate the viability of cell cultures under normoxia (Fig. 6B). Similar results were seen in cultured MKC-1 cells (Fig. 6C). These data were further confirmed in cultured TEC-CLU^{hCLU} compared to TEC-CLU$^{-/-}$ cells, which were treated with a different autophagy inhibitor Spautin-1. As shown in Fig. 7, Spautin-1 reduced the cell survival in TECCLU^{hCLU} cells under normaxia and more profoundly under hypoxia, which was similar to BafA1-treated MKC-1 cells (Fig. 6). By contrast, the autophagy inhibitor did not show significant impact on the cell death of CLU null TEC-CLU$^{-/-}$ cells under either normoxia or hypoxia (Fig. 7C). These data may suggest that the cytoprotection of CLU in kidney cells is mainly mediated by activating the autophagy, and vice versa.

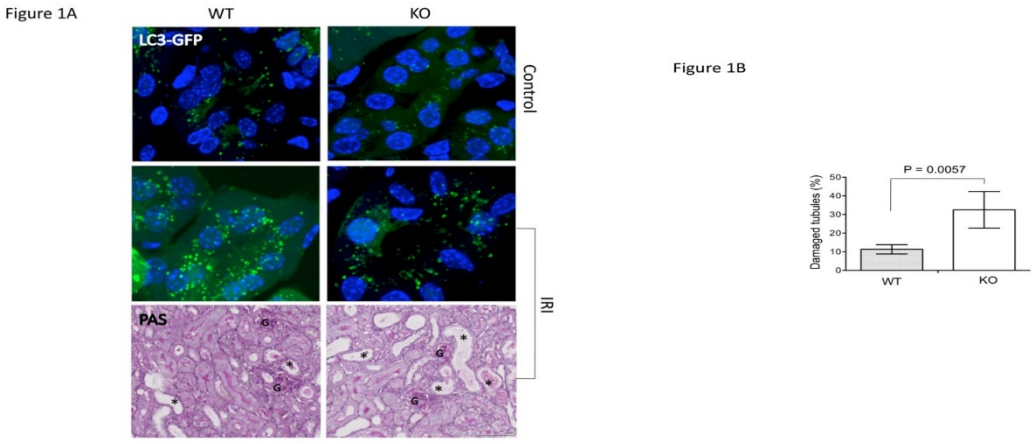
4.4 CLU-dependent prosurvival autophagy correlates with an increase in UPR in hypoxic kidney cells.

Acute ischemia induces endoplasmic reticulum (ER) stress and subsequently activates UPR in the kidney(48,49), and the UPR, autophagy and proteasomal degradation together degrade and recycle the excess or damaged proteins to restore cellular homeostasis and/or to inactivate the cell death (i.e. apoptosis) pathway(50,51). To confirm if CLU-dependent prosurvival autophagy was associated with the UPR, the activation of UPR signaling

pathways was examined in hypoxic TEC-CLU^{hCLU} cells compared to TEC-CLU$^{-/-}$ cells. By statistical comparison of the expression of 84 UPR-related genes in hypoxic TEC-CLU^{hCLU} cells with TEC-CLU$^{-/-}$ cells in PCR array analysis, 57 genes were significantly up-regulated while 8 genes (Ganac, Uggt2, Ero1lb, Hspa2, Htra4, Hspb9, Rrn2 and Creb3l3) down-regulated in hypoxic TEC-CLU^{hCLU} cells (Suppl Table 1). The functional gene grouping showed that the mostly affected functional pathways of UPR by CLU was "regulation of translation", "heart shock protein" and "ER protein folding quality control", followed by "protein folding", "unfold protein binding", "protein disulfide isomerization" and "anti-apoptosis" (Table 1). The CLU-dependent activation of UPR in hypoxic kidney cells was further confirmed by western blot analysis of ER stress, inositolrequiring enzyme 1 (IRE1), one of principal UPR receptors. As shown in Fig. 8, the expression of IRE1 protein was up-regulated by hypoxia in both TEC-CLU^{hCLU} and TEC-CLU$^{-/-}$ cells as compared to those under normoxia. However, only a high level of phospho-IRE1 (p-IRE1) was seen in hypoxic TEC-CLU^{hCLU} cells (not TEC-CLU$^{-/-}$ cells) (Fig. 8), suggesting that this UPR receptor was more active in hypoxic TEC-CLU^{hCLU} cells than those in CLU null TEC-CLU$^{-/-}$ cells. Taken together, these data indicate that CLU-dependent prosurvival autophagy is associated with more profound activation of UPR that may be related to the enhancement of protein translation and heat shock protein expression for protein folding and ER protein folding quality control, resulting in the prevention of cell apoptosis.

5. Figures and Tables.

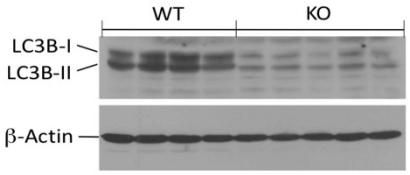
Fig. 1: Association of CLU-mediated IRI resistance with autophagy activation.



Both GFP-LC3$^{+/+}$CLU$^{-/-}$ (CLU KO) and GFP-LC3$^{+/+}$CLU$^{+/+}$(CLU WT) C57BL/6 male mice were subjected to renal ischemia–reperfusion at 32^{o}C of body temperature for 45 min. After 24 h of reperfusion, renal tissues were harvested from both the left (with ischemia-reperfusion) and right (contralateral) kidneys for histological analyses. (A) A typical microscopic view of LC3-GFP puncta in the renal cortex of contralateral kidneys (Control) and kidneys with IRI, and of renal tubular damage in PAS stained sections in each group. Green: LC3-GFP puncta; blue: nucleus; G: glomerulus; *: damaged tubules. (B) The percentage of damaged tubules, including necrotic tubules, tubular dilation, intratubular cast formation and tubular vacuolation, in the section of the renal cortex was counted under ´400 magnification by a semi-quantitative histological analysis with PAS staining in a blinded fashion. At least 20 randomly selected, nonoverlapping views were counted and averaged for each kidney/mouse. Data were presented as mean ± standard derivation
(SD) in each group (KO vs. WT, p = 0.0057, two-tailed t-test, n = 4).

Fig. 2: Increase in LC3 (I and II) expression in the kidneys of WT but not CLU KO mice following renal IRI.

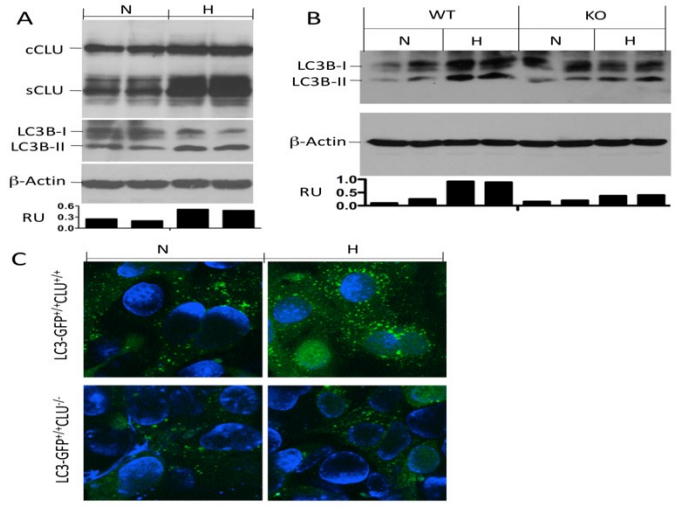
Figure 2



Both WT and CLU KO mice were subjected to renal IRI as described previously (92). After 48 h of reperfusion, the total cellular protein was extracted from the cortex and was fractionated by 12% SDS-PAGE. The levels of both LC3 I and II in the protein sample of WT (n = 4) or KO (n = 5) kidneys with IRI were determined using Western blot. Each lane represented one sample/mouse. Data were a representative of three separate experiments.

Fig 3: Activation of autophagy by hypoxia in CLU-expressing/WT but not in CLU null TECs in vitro.

Figure 3



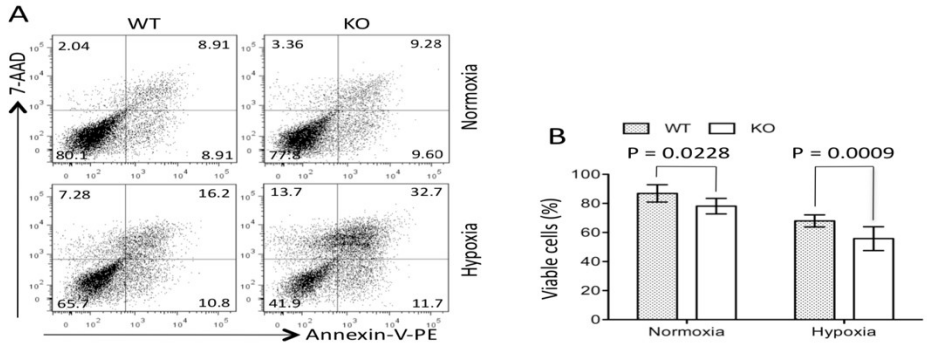
Monolayers of TECs were grown in $K1^{+/+}$ medium overnight, followed by incubation under normoxia (~20% O_2) or hypoxia (1% O_2) for 24 h. The activation of autophagy was determined by either LC3-BII expression in Western Blot or LC3-GFP aggregation in confocal microscopy.

(A) Total protein was extracted from HKC-8 cells, and the levels of CLU (cytoplasmic CLU/cCLU and secreted CLU/sCLU) and LC3B (I and II) were determined using Western blot. Two samples/lanes were collected for each group. Data were a representative of two separate experiments. (B) Primary TECs were isolated from the kidney cortex of WT or CLU KO mice, and were exposed to hypoxia or normoxia for 24 h. Again, the levels of LC3B (I and II) in total protein extracts were determined using Western blot. Two samples/lanes were collected for each group. Data were a representative of two separate experiments. (C) Primary TECs were isolated from the kidney cortex of GFP-$LC3^{+/+}CLU^{+/+}$ or GFP-$LC3^{+/+}CLU^{-/-}$ mice.

The degree of LC3GFP aggregation in these cells after 24 h exposure to hypoxia or normoxia was examined using confocal microscopy. Data were presented as a typical image in each group. Green: LC3-GFP puncta; blue: nucleus; N: normxia; H: hypoxia; RU: ratio units of LC3B-II to Beta-actin.

Fig. 4: More cell survival in WT than CLU null TECs in hypoxia.

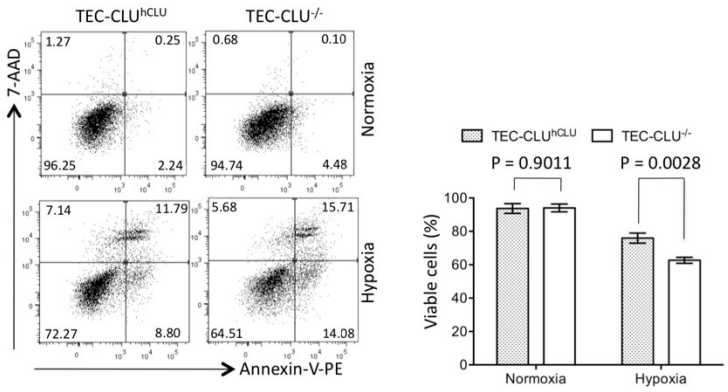
Figure 4



Monolayers of primary TECs from the kidney cortex of WT or CLU KO mice were grown in $K1^{+/+}$ medium overnight, followed by incubation under normoxia (~20% O_2) or hypoxia (1% O_2) for 24 h. Cell death was determined by FACS analysis with 7-AAD (late apoptosis and necrosis) and Annexin-V-PE (apoptosis) staining. (A) A representative FACS graph for each group. (B) Statistical comparison of cell viability indicated by the percentage of doublenegatively staining population in the left lower quadrant in a FACS graph. Data are presented as mean ± SD in each group (WT vs. KO: $p = 0.0028$ in normoxia; $p = 0.0009$ in hypoxia, two-tailed t-test, $n = 9$).

Fig. 5: Increased cell survival in TECs with ectopic expression of human CLU in hypoxia.

Figure 5



Monolayers of TEC-CLU^{hCLU} and TEC-CLU$^{-/-}$ cells were grown in K1$^{+/+}$ medium overnight, followed by incubation under normoxia (~20% O_2) or hypoxia (1% O_2) for 24 h. Cell death was determined by FACS analysis with both 7-AAD and Annexin-V-PE staining. (A) A representative FACS graph for each group. (B) Statistical comparison of cell viability indicated by the percentage of double negatively staining population in the left lower quadrant in a FACS graph. Data are presented as mean ± SD in each group (TEC-CLU^{hCLU} vs. TEC-CLU$^{-/-}$: p = 0.9011 in normoxia; p = 0.0028 in hypoxia, two-tailed t-test, n = 3).

Fig. 6: Decreased cell survival in TECs by autophagy inhibition.

Figure 6A

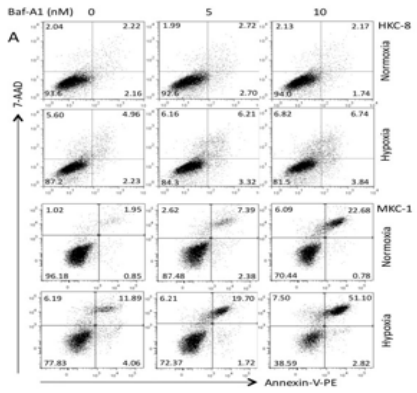
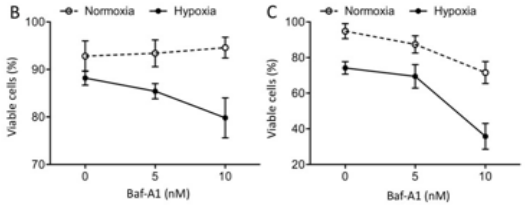
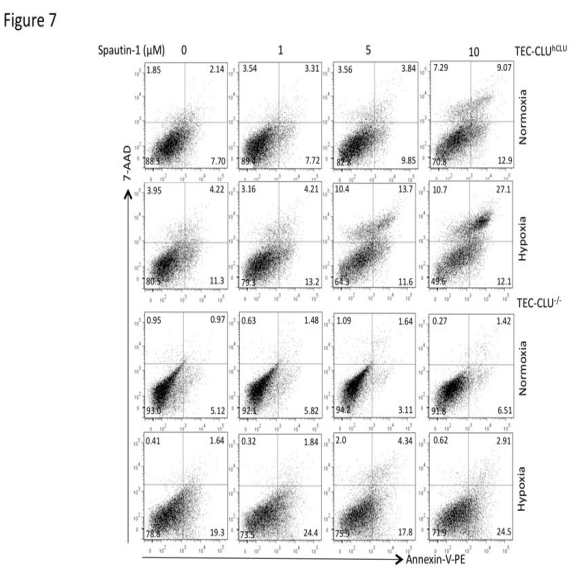


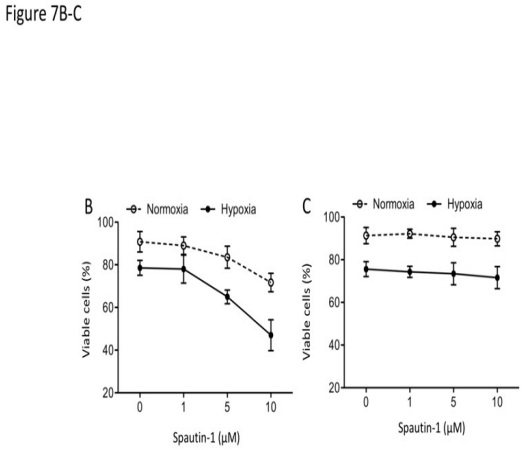
Figure 6B-C



Monolayers of HKC-8 and MKC-1 cells were grown in $K1^{+/+}$ medium overnight, followed by incubation in the absence of presence of Baf-A1 (5 or 10 nM) under normoxia (~20% O_2) or hypoxia (1% O_2) for 24 h. Cell death was determined by FACS analysis with both 7-AAD and Annexin-V-PE staining. (A) A representative FACS graph for each group. (B) Statistical comparison of cell viability of HKC-8 cells treated with different concentrations of Baf-A1 (normoxia: $p = 0.7961$, hypoxia: $p = 0.0245$; one-way ANOVA, n=3).(C) Statistical comparison of cell viability of MKC-1 cells treated with different concentrations of Baf-A1 (normoxia:$p = 0.004$, hypoxia:$p = 0.0004$; one-way ANOVA, n=3).

Fig. 7: Decreased cell survival in CLU-expressing TECs but not in CLU null TECs by autophagy inhibition.

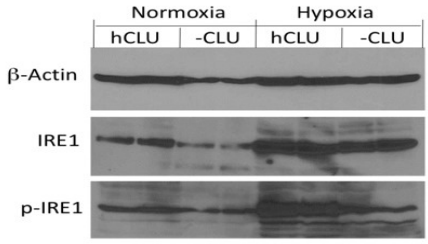




Monolayers of TEC-CLU^{hCLU} and TEC-CLU$^{-/-}$ cells were grown in K1$^{+/+}$ medium overnight, followed by incubation in the absence of presence of Spautin-1 (1, 5 or 10 μM) under normoxia (~20% O_2) or hypoxia (1% O_2) for 24 h. Cell death was determined by FACS analysis with both 7-AAD and Annexin-V-PE staining. (A) A representative FACS graph for each group. B) Statistical comparison of cell viability of TEC-CLU^{hCLU} cells treated with different concentrations of Spautin-1 (normoxia: $p = 0.0039$, hypoxia: $p = 0.0003$; one-way ANOVA, $n = 3$). (C) Statistical comparison of cell viability of TEC-CLU$^{-/-}$ cells treated with different concentrations of spautin-1 (normoxia: $p = 0.8447$, hypoxia: $p = 0.7157$; one-way ANOVA, $n = 3$).

Fig. 8: Activated IRE1 in CLU-expressing TECs but not in CLU null TECs in hypoxia.

Figure 8



Monolayers of TEC-CLU^{hCLU} and TEC-CLU$^{-/-}$ cells were grown in K1$^{+/+}$ medium overnight, followed by incubation under normoxia (~20% O_2) or hypoxia (1% O_2) for 24 h. The total cellular protein was extracted from the cultures using RIPA buffer and was fractionated by 7% SDS-PAGE. The levels of total IRE1, phospho-IRE1 (p-IRE1) and -actin in each sample were determined using Western blot. Each lane represented one sample. hCLU: TEC-CLU^{hCLU} cells, CLU: TEC-CLU$^{-/-}$ cells. Data were a representative of three separate experiments.

Table 1. CLU-dependent UPR pathways in hypoxic kidney cells.

Functional pathways (total number of related genes)	Number of affected genes	Percentage of affected genes/function
Regulation of translation (4)	4 up-regulated	100%
Heat shock proteins (10)	9 up-regulated	90%
ER protein folding quality control (10)	7 up-regulated, 2 down-regulated (Ganc, Uggt2)	90%
Protein folding (19)	16 up-regulated	84.21%
Unfolded protein binding (21)	13 up-regulated; 3 down-regulated (Ero1lb, Hspa2, Hspb9)	76.19%
Protein disulfide isomerization (8)	5 up-regulated, 1 down-regulated (Ero1lb)	75%
Anti-apoptosis (18)	11 up-regulated, 2 down-regulated (Ern2, Hspb9)	72.22%
Regulation of cholesterol metabolism (7)	5 up-regulated	71.43%
Ubiquitination (13)	9 up-regulated	69.23%
ER associated degradation (19)	12 up-regulated, 1 down-regulated (Htra4)	68.42%
Transcription factors (17)	8 up-regulated, 2 down-regulated (Creb3l3, Ern2)	58.82%

Table 2. CLU-dependent Unfolded Protein Response (UPR)-related gene expression in hypoxic TECs.

Functions	Gene (full name)	Fold change	P value
Unfolded Protein Binding	Cct4 (Chaperonin containing Tcp1, subunit 4)	2.2138	0.0069
	Cct7 (Chaperonin containing Tcp1, subunit 7)	1.8599	0.0026
	Htra2 (HtrA serine peptidase 2)	1.722	0.0272
	Tcp1 (T-complex protein 1)		
ER Protein Folding Quality Control	Edem1 (ER degradation enhancer, mannosidase alpha-like 1)	1.7007	0.0075
	Erp44 (ER protein 44)	1.9689	0.0076
	Ganab (Alpha glucosidase 2 alpha neutral subunit)	1.5721	0.0012
	Ganac (Glucosidase, alpha, neutral c)	-0.3576	0.0002
	Prkcsh (Protein kinase C substracte 80K-H)	2.097	0.002
	Serp1 (Stress-associated ER protein 1)	2.1261	0.0005
	Sil1 (ER chaperone SIL1 homolog, S. cerevisiae)	1.9358	<0.0001
	Uggt1 (UDP-glucose glycoprotein glucosyltransferase 1)	1.754	0.0023
Regulation of Cholesterol Metabolism	Insig1 (Insulin induced gene 1)	2.3327	0.0083
	Srebf2 (Sterol regulatory element binding factor 2)	2.1412	0.038
Regulation of	Eif2a (Eukaryotic translation initiation factor 2a)	2.1484	0.0101

Translation	Eif2ak3 (Eukaryotic translation initiation factor 2 alpha kinase 3	1.8803	0.0064
ER-Associated Degradation	Herpud1 (Homocysteine-inducible, ER stress inducible, ubiquitin-like domain member 1	2.2256	0.0075
	Mbtps2 (Membrane-bound transcription factor peptidase, site 2)	1.922	0.0012
	Sel1l (Sel-1 suppressor of lin-12-like, C. elegans)	2.0743	0.0017
	Ubxn4 (UBX domain protein 4)	1.786	0.0035
Ubiquitination	Sec62 (SEC62 homolog, S. cerevisiae)	1.6157	0.0011
	Ube2j2 (Ubiquitin – conjugating enzyme E2, J2 homolog, yeast)	1.9523	0.0365
	Ufd1l (Ubiquitin fusion degradation 1 like)	1.726	0.0195
	Usp14 (Ubiquitin specific peptidase 14)	1.8476	0.0147
	Vcp (Valosin containing protein)	1.7661	0.0158
Transcription Factor	Atf4 (Activating transcription factor 4)	4.2857	0.0038
	Atf6 (Activating transcription factor 6)	2.477	0.0019
	Xbp1 (X-box binding protein 1)	1.7543	0.0001
	Ern2 (ER to nucleus signaling 2)	-0.288	0.0059
Protein Folding	Dnajb2 (DnaJ-Hsp40 homolog, subfamily b, member 2)	1.7447	0.0227

	Dnajc3 (DnaJ-Hsp40 homolog, subfamily c, member 3)	1.6201	0.0058
	Hspa4I (Heat shock protein 4 like)	2.9044	0.0006
	Pfdn2 (Prefoldin 2)	1.95	0.0012
	Ppia (Peptidylprolyl isomerase A)	1.82	0.0433
	Sec63 (SEC63-like – S. cerevisiae)	1.785	0.0049
Protein Disulfide Isomerization	None		
Heat Shock	Haspa4 (Heart shock protein 4)	3.1924	0.0068
Proteins	Haspa5 (Heat shock protein 5)	4.9698	<0.0001
	Hsph1 (Heat shock 105kDa/110kDa protein 1)	2.9439	<0.0001
Apoptosis	H47 (Histocompatibility 47)	1.9258	0.0391
	Hspb9 (Heat shock protein, alpha-crystallinrelated, B9)	-0.464	0.0012
	Manf (Mesencephalic astrocyte-derived neutrophic factor)	2.0582	0.0056
	Mapk10 (Mitogen-activated protein kinase 10)	1.88	0.0083
	Mapk8 (Mitogen-activated protein kinase 8)	2.0566	<0.0001

The levels of UPR-related genes were analyzed using mouse UPR PCR array (Catalog No. PAMM-089Z, QIAGEN, Toronto, ON, Canada).

6. Discussion.

AKI due to IRI is a common pathological factor leading to unacceptably high morbidity and mortality, and to the development of chronic kidney disease (CKD) or the transition from preexisting CKD to end-stage renal disease(87); however, the pathogenesis of renal IRI is not fully understood. Recently, many studies have demonstrated that autophagy is a cytoprotective mechanism in the kidneys against IRI(20,28,29), but the molecular mechanisms regulating this prosurvival autophagy remains largely unknown. This study for the first time demonstrates that CLU expression is required for the activation of the autophagy during IRI in the kidneys or by hypoxia in cultured kidney cells, and the CLU-dependent autophagy is at least one of pathways mediating the cytoprotection of CLU, indicated by less IRI in WT kidneys or more survived cells in cultures of CLU-expressing kidney cells under hypoxia as compared to their CLU null controls. The activation of CLU-dependent, prosurvival autophagy in hypoxic kidney cells also positively correlates with the induction of UPR, but how CLU regulate UPR in kidney cells in response to hypoxia needs further investigation. The kinetics of autophagic activity in the kidneys with IRI has been investigated in a strain of transgenic mice expressing both markers of autolysosome and early autophagic vacuoles, showing that both early autophagic vacuoles and autolysomes reach the maximal at day 1 after surgery, and the early autophagic vacuoles return to the control level at day 3 while the autolysosomes remain unchanged(52). Also, the proliferative activity decreases in cells containing the autolysosomes (52). This study suggests the autophagy initiation at day 1 and autophagosome clearance during renal recovery at day 3, and the autophagic cells are less likely to involve in renal repair after IRI(52).

The present study confirms that hypoxia- or IRI-stimulated autophagy is associated with increased cell survival in the kidneys or cultured kidney cells, and incubation with autophagy inhibitors results in an increase in cell death, suggesting a prosurvival function of autophagy in kidney cells under hypoxia. Many previous studies have shown that autophagic activity in renal tissues is stimulated by ischemia-reperfusion (28,29,44,52) and in cultured TECs by hypoxia (20,21,53); however, the molecular pathways to activating prosurvival autophagy in the kidney in response to hypoxia or IRI are not fully understood. It has been known that AMP-activated kinase (AMPK) is a sensor of cellular energy homeostasis (54), and its activity is required for autophagy activation in response to starvation (55) and hypoxia(56). Indeed, AMPK is strongly activated in the kidneys within 1 min of ischemia and remains so until 30 min(57), and agonist enhancement of AMPK activity improves autophagy and reduces renal IRI(58,59), suggesting that AMPK may be a key regulator of autophagy in the kidneys with IRI. Furthermore, it has been well documented that many biochemical events occur in renal tubular cells during the ischemia or hypoxia, which include a rapid degradation of ATP to ADP and AMP, and an increase in cytosolic Ca^{2+} and reactive oxygen (ROS) species (60). Interestingly, literature reports that hypoxia-activated AMPK in different experimental systems has been found due to elevating mitochondrial ROS and AMP through the activation loop of the upstream kinase LKB1 (61,62). and/or increasing cytosolic Ca^{2+} via Ca^{2+}/calmodulin-dependent kinase kinase beta (62,63). However, a recent study shows that a significant reduction of AMPK activity in the kidneys of AMPK-Beta1 KO mice does not affect renal IRI at all as compared to WT controls(64), indicating that the role of AMPK in the activation of prosurvival autophagy, in particular CLU-dependent, in kidney cells under hypoxia or ischemia needs further investigation.

One of interesting findings in this study is that the prosurvival autophagy is detected in CLU-expressing kidney cells, even in those with ectopic expression of CLU, suggesting a role of CLU in the activation of prosurvival autophagy in the kidneys with IRI or hypoxic kidney cells. If CLU takes part in hypoxia-induced autophagy, autophagy-related proteins together with CLU are likely up-regulated by hypoxia in WT kidney cells. Indeed, hypoxia activates many transcription factors, including hypoxia-inducible factor-1 (HIF-1) (11). Activation of HIF-1 has been seen in the kidneys subjected to 45 min of renal ischemia and in cultured renal epithelial LLC-PK cells under hypoxia within 30 min and persisted for 4 h (20). HIF-1 regulates gene expression through its interaction with a hypoxia response element (HRE) in target gene promoter (65). A recent study identifies three putative HRE sites (A/GCGTG) in CLU gene promoter and demonstrates the binding of HIF-1 to these sites(66), which may result in upregulation of CLU expression in the kidneys in the response to IRI (35,38,39) and in HKC-8 cells (Fig. 3A) or others (67) under hypoxia. On the other hand, HIF-1 activity is also required for hypoxia-induced autophagy, in which HIF-1 induces the expression of Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3) and BNIP3L that play important roles in the induction of autophagy by disrupting the interaction of Beclin 1 with Bcl-2 via their BH3 domain (53,68,69). The second substantial evidence to support the role of CLU in autophagy in hypoxic kidney cells comes from recent studies, showing that CLU is colocalized with autophagy proteins (LC3 II and p62) in renal TECs from patients with nephropathic cystinosis (70) and with LC3 within the autophagosome in prostate cancer cells to enhance cell survival(4). However, how CLU regulates prosurvival autophagy in hypoxic kidney cells is not fully understood as of yet.

Although the biology of autophagy has received increasing interest recently, the understanding of its pathways and functions still remains in its infancy. Beside non-selective macroautophagy, there are several selective autophagies including chaperone-mediated autophagy (CMA), pexophagy and mitophagy – to contribute to degradation of intracellular components in lysosomes in mammalian cells (5); however, the regulatory pathways of each type of these autophagies remain poorly defined. It has been reported that HIF-1-BNIP3 pathway involves autophagy in the kidneys with IRI or hypoxic kidney cells (49), and the HIF-1-BNIP3mediated autophagy selectively targets the mitochondria – mitophagy (49). The prosurvival role of the mitophagy is also found in human umbilical vein endothelial cells and cardiac myocytes under hypoxia (72). Hypoxia or reduced oxygen availability primarily impacts mitochondrial oxidation, and results in mitochondrial damage in renal TECs (73,74,89), and the damaged mitochondria could release inter-membrane proteins or other internal molecules that may trigger autophagy through an unknown mechanism (71). CLU is a chaperone protein containing several large molten globule domains, amphipathic regions and coiled-coil α-helices (1,75), which allow CLU protein to bind various substrate proteins or lipids with high affinity and low specificity (28,64,75). Thus, it is possible that CLU may act as an adaptor protein that facilitates the mitophagy by binding to mitochondrial inter-membrane components, which however needs further investigation. Moreover, there is a low level of autophagic activity in CLU null kidneys with IRI (Fig. 1) as well as in hypoxic CLU deficient kidney cells (Fig. 3), implying that probabaly there is CLU-independent autophagy in kidney cells in response to hypoxia. Indeed, in addition to the HIF-1-BNIP3-mitophagy, p53-sestrin-2 also induces autophagy in hypoxic kidney cells (53), and CMA in hypoxic

neuronal cells for cell survival (74). Whether or not these types of autophagy occur in the absence of CLU remains elusive.

In addition to autophagy, ER stress is also induced by IRI in the kidneys as indicated by the accumulation of aberrant unfolded and misfolded proteins in the ER lumen, and subsequently activates UPR pathway (75). The primary purpose of the UPR to enhance the protein-folding capacity by activating the transcription of UPR target genes, such as Ca^{2+}-dependent molecular chaperones, including glucose-regulated protein-78 (GRP78), GRP94, and calreticulin (60), and to eliminate unfolded or misfolded proteins within the ER (51). In the absence of IRE1, doublestranded RNA-activated protein kinase-like ER kinase (PERK), and the activating transcription factor-6 (ATF6) are normally held in an inactive state in the ER membranes by binding to GRP78, but under the ER stress or as unfolded or misfolded proteins accumulate in the ER lumen, GRP78 releases these transmembrane ER proteins (IRE1, PERK and ATG6), which consequently initiate the UPR signaling (75). By activating the UPR, the unfolded or misfolded proteins are eliminated through an ER-associated degradation pathway (ERAD) (76,77), in which the soluble targeted proteins are retrotranslocated into the cytosol, ubiquitinated, and are degraded by the proteasome in ERAD I (78,79), or the insoluble misfolded protein aggregates are degraded by autolysosomes/lysosome - autophagy in ERAD II (76). The present study shows that the cytoprotection of CLU in hypoxic kidney cells is associated with an increase in the activity of IRE1 (Fig. 8) and the expression of a panel of UPR-targeted genes (Suppl Table 1), suggesting that CLU may play an important role in the elimination of the unfold or misfolded protein within the ER. However, the mechanisms by which CLU facilitates the UPR to restore the functions of the ER for cell survival are not well understood. CLU is associated with

GRP78 in prostate cancer cells under the ER stress (80), and is retrotranslocated into the cytosol by a mechanism similar to ERAD I (80,81) and co-localized with the mitochondria (45). Furthermore, GRP78 stabilizes CLU protein and its hypoglycosylated form, and is required for stress-induced up-regulation of CLU (80) or vice versa (82), resulting in the inhibition of cell apoptosis. It seems that the requirement of CLU for prosurvival UPR in hypoxic kidney cells via the enhancement of GRP67 expression, which may mediate the prosurvival mitophagy via ERAD II pathway.

7. Conclusion.

In conclusion, the incidence of AKI due to transient renal hypoxia is rising but effective treatments and preventative approaches are currently lacking. Hypoxia- or IRI-induced kidney injury significantly contributes to DGF of the kidney transplants as well as to the development of kidney disease after ischemic AKI. Both clinical and experimental studies have demonstrated the cytoprotection of CLU in the kidney (38); however, the protective properties or actions of CLU in the kidney are not fully understood. The finding of this preliminary study for the first time demonstrates that the protection of CLU in hypoxic kidney cells is associated with the prosurvival autophagy and UPR, indicating that CLU-dependent autophagy and/or UPR may be as a potential target for the development of new therapeutic strategies for preventing and/or enhancing recovery following ischemic AKI in patients, but prior to this, an increased mechanistic understanding of the protective nature of CLU is needed, in particular further investigation of how these CLU-dependent pathways are coordinated in the response to hypoxia to increase cell survival.

References.

1. Salahudeen AK. Cold ischemic injury of transplanted kidneys: new insights from experimental studies. Am J Physiol Renal Physiol 287: F181-187, 2004.

2. Salahudeen AK. Cold ischemic injury of transplanted organs: some new strategies against an old problem. Am J Transplant 4: 1, 2004.

3. Hoste EA, Cruz DN, Davenport A, Mehta RL, Piccinni P, Tetta C, Viscovo G, and Ronco C. The epidemiology of cardiac surgery-associated acute kidney injury. Int J Artif Organs 31: 158-165, 2008.

4. Ausania F, White SA, Pocock P, and Manas DM. Kidney damage during organ recovery in donation after circulatory death donors: data from UK National Transplant Database. Am J Transplant 12: 932-936, 2012.

5. Rosner MH and Okusa MD. Acute kidney injury associated with cardiac surgery. Clin J Am Soc Nephrol 1: 19-32, 2006.

6. Cantafio AW, Dick AA, Halldorson JB, Bakthavatsalam R, Reyes JD, and Perkins JD. Risk stratification of kidneys from donation after cardiac death donors and the utility of machine perfusion. Clin Transplant 25: E530-540, 2011.

7. van der Vliet JA, Warle MC, Cheung CL, Teerenstra S, and Hoitsma AJ. Influence of prolonged cold ischemia in renal transplantation. Clin Transplant 25: E612-616, 2011.

8. Requiao-Moura LR, Durao Mde S, Tonato EJ, Matos AC, Ozaki KS, Camara NO, and Pacheco-Silva A. Effects of ischemia and reperfusion injury on long-term graft function.

Transplant Proc 43: 70-73, 2011.

9. Shoskes DA and Cecka JM. Deleterious effects of delayed graft function in cadaveric renal transplant recipients independent of acute rejection. Transplantation 66: 1697-1701, 1998.

10. Kolonko A, Chudek J, Pawlik A, Wilk J, Jalowiecki P, and Wiecek A. Acute kidney injury before organ procurement is associated with worse long-term kidney graft outcome. Transplant Proc 43: 2871-2874, 2011.

11. Yarlagadda SG, Coca SG, Formica RN, Jr., Poggio ED, and Parikh CR. Association between delayed graft function and allograft and patient survival: a systematic review and metaanalysis. Nephrol Dial Transplant 24: 1039-1047, 2009.

12. Bellomo R, Auriemma S, Fabbri A, D'Onofrio A, Katz N, McCullough PA, Ricci Z, Shaw A, and Ronco C. The pathophysiology of cardiac surgery-associated acute kidney injury (CSA-AKI). Int J Artif Organs 31: 166-178, 2008.

13. Saikumar P, Dong Z, Weinberg JM, and Venkatachalam MA. Mechanisms of cell death in hypoxia/reoxygenation injury. Oncogene 17: 3341-3349, 1998.

14. Tsao CC, Nica AF, Kurinna SM, Jiffar T, Mumby M, and Ruvolo PP. Mitochondrial protein phosphatase 2A regulates cell death induced by simulated ischemia in kidney NRK-52E cells. Cell Cycle 6: 2377-2385, 2007.

15. Akcay A, Nguyen Q, and Edelstein CL. Mediators of inflammation in acute kidney injury. Mediators Inflamm 2009: 137072, 2009.

16. Bonventre JV and Yang L. Cellular pathophysiology of ischemic acute kidney injury. J Clin Invest 121: 4210-4221, 2011.

17. Malek M and Nematbakhsh M. Renal ischemia/reperfusion injury; from pathophysiology to treatment. J Renal Inj Prev 4: 20-27, 2015.

18. Glick D, Barth S, and Macleod KF. Autophagy: cellular and molecular mechanisms. J Pathol 221: 3-12, 2010.

19. Murrow L and Debnath J. Autophagy as a stress-response and quality-control mechanism: implications for cell injury and human disease. Annu Rev Pathol 8: 105-137, 2013.

20. Jiang M, Liu K, Luo J, and Dong Z. Autophagy is a renoprotective mechanism during in vitro hypoxia and in vivo ischemia-reperfusion injury. Am J Pathol 176: 1181-1192, 2010.

21. Suzuki C, Isaka Y, Takabatake Y, Tanaka H, Koike M, Shibata M, Uchiyama Y, Takahara S, and Imai E. Participation of autophagy in renal ischemia/reperfusion injury. Biochem Biophys Res Commun 368: 100-106, 2008.

22. Wu HH, Hsiao TY, Chien CT, and Lai MK. Ischemic conditioning by short periods of reperfusion attenuates renal ischemia/reperfusion induced apoptosis and autophagy in the rat.J Biomed Sci 16: 19, 2009.

23. Yeh CH, Hsu SP, Yang CC, Chien CT, and Wang NP. Hypoxic preconditioning reinforces HIF-alpha-dependent HSP70 signaling to reduce ischemic renal failure-induced renal tubular apoptosis and autophagy. Life Sci 86: 115-123, 2010.

24. Kourtis N and Tavernarakis N. Autophagy and cell death in model organisms. Cell Death Differ 16: 21-30, 2009.

25. Kundu M and Thompson CB. Autophagy: basic principles and relevance to disease. Annu Rev Pathol 3: 427-455, 2008.

26. Levine B and Abrams J. p53: The Janus of autophagy? Nat Cell Biol 10: 637-639, 2008.

27. Levine B and Yuan J. Autophagy in cell death: an innocent convict? J Clin Invest 115: 2679-2688, 2005.

28. Isaka Y, Kimura T, and Takabatake Y. The protective role of autophagy against aging and acute ischemic injury in kidney proximal tubular cells. Autophagy 7: 1085-1087, 2011.

29. Kimura T, Takabatake Y, Takahashi A, Kaimori JY, Matsui I, Namba T, Kitamura H, Niimura F, Matsusaka T, Soga T, Rakugi H, and Isaka Y. Autophagy protects the proximal tubule from degeneration and acute ischemic injury. J Am Soc Nephrol 22: 902-913, 2011.

30. Bailey RW, Dunker AK, Brown CJ, Garner EC, and Griswold MD. Clusterin, a binding protein with a molten globule-like region. Biochemistry 40: 11828-11840, 2001.

31. Humphreys DT, Carver JA, Easterbrook-Smith SB, and Wilson MR. Clusterin has chaperone-like activity similar to that of small heat shock proteins. J Biol Chem 274: 6875-6881, 1999.

32. Kim, S., Song, S., Kim, J., Jeon, Y., Kim, B., Kang, M., … Kim, Y. (2017). Urine clusterin/apolipoprotein J is linked to tubular damage and renal outcomes in patients with type 2 diabetes mellitus. Clin. Endocrinol. (Oxf), 87(2), 156–164.

33. Rosenberg ME and Paller MS. Differential gene expression in the recovery from ischemic renal injury. Kidney Int 39: 1156-1161, 1991.

34. Schlegel PN, Matthews GJ, Cichon Z, Aulitzky WK, Cheng CY, Chen CL, Saso L, Goldstein

M, Janne OA, Bardin CW, and et al. Clusterin production in the obstructed rabbit kidney: correlations with loss of renal function. J Am Soc Nephrol 3: 1163-1171, 1992.

35. Witzgall R, Brown D, Schwarz C, and Bonventre JV. Localization of proliferating cell nuclear antigen, vimentin, c-Fos, and clusterin in the postischemic kidney. Evidence for a heterogenous genetic response among nephron segments, and a large pool of mitotically active and dedifferentiated cells. J Clin Invest 93: 2175-2188, 1994.

36. Dvergsten J, Manivel JC, Correa-Rotter R, and Rosenberg ME. Expression of clusterin in human renal diseases. Kidney Int 45: 828-835, 1994.

37. Rosenberg ME, Girton R, Finkel D, Chmielewski D, Barrie A, 3rd, Witte DP, Zhu G, Bissler JJ, Harmony JA, and Aronow BJ. Apolipoprotein J/clusterin prevents a progressive glomerulopathy of aging. Mol Cell Biol 22: 1893-1902, 2002.

38. Nguan CYC, Guan Q, Gleave ME, and Du C. Promotion of cell proliferation by clusterin in the renal tissue repair phase after ischemia-reperfusion injury. Am J Physiol-Renal 306: F724-F733, 2014.

39. Zhou W, Guan Q, Kwan CC, Chen H, Gleave ME, Nguan CY, and Du C. Loss of clusterin expression worsens renal ischemia-reperfusion injury. Am J Physiol Renal Physiol 298: F568-578, 2010.

40. Zhang F, Kumano M, Beraldi E, Fazli L, Du C, Moore S, Sorensen P, Zoubeidi A, and Gleave ME. Clusterin facilitates stress-induced lipidation of LC3 and autophagosome biogenesis to enhance cancer cell survival. Nat Commun 5: 5775, 2014.

41. Lassnigg A, Schmidlin D, Mouhieddine M, Bachmann LM, Druml W, Bauer P, and Hiesmayr M. Minimal changes of serum creatinine predict prognosis in patients after cardiothoracic surgery: a prospective cohort study. J Am Soc Nephrol 15: 1597-1605, 2004.

42. McLaughlin L, Zhu G, Mistry M, Ley-Ebert C, Stuart WD, Florio CJ, Groen PA, Witt SA, Kimball TR, Witte DP, Harmony JA, and Aronow BJ. Apolipoprotein J/clusterin limits the severity of murine autoimmune myocarditis. J Clin Invest 106: 1105-1113, 2000.

43. Racusen LC, Monteil C, Sgrignoli A, Lucskay M, Marouillat S, Rhim JG, and Morin JP. Cell lines with extended in vitro growth potential from human renal proximal tubule: characterization, response to inducers, and comparison with established cell lines. J Lab Clin Med 129: 318-329, 1997.

44. Jevnikar AM, Brennan DC, Singer GG, Heng JE, Maslinski W, Wuthrich RP, Glimcher LH, and Kelley VE. Stimulated kidney tubular epithelial cells express membrane associated and secreted TNF alpha. Kidney Int 40: 203-211, 1991.

45. Eltzschig HK and Eckle T. Ischemia and reperfusion--from mechanism to translation. Nat Med 17: 1391-1401, 2011.

46. Das G, Shravage BV, and Baehrecke EH. Regulation and function of autophagy during cell survival and cell death. Cold Spring Harb Perspect Biol 4, 2012.

47. Nikoletopoulou V, Markaki M, Palikaras K, and Tavernarakis N. Crosstalk between apoptosis, necrosis and autophagy. Biochim Biophys Acta 1833: 3448-3459, 2013.

48. Fougeray S, Bouvier N, Beaune P, Legendre C, Anglicheau D, Thervet E, and Pallet N. Metabolic stress promotes renal tubular inflammation by triggering the unfolded protein response. Cell Death Dis 2: e143, 2011.

49. Montie HL, Kayali F, Haezebrouck AJ, Rossi NF, and Degracia DJ. Renal ischemia and reperfusion activates the eIF 2 alpha kinase PERK. Biochim Biophys Acta 1741: 314-324, 2005.

50. Benbrook DM and Long A. Integration of autophagy, proteasomal degradation, unfolded protein response and apoptosis. Exp Oncol 34: 286-297, 2012.

51. Clarke R, Cook KL, Hu R, Facey CO, Tavassoly I, Schwartz JL, Baumann WT, Tyson JJ, Xuan J, Wang Y, Warri A, and Shajahan AN. Endoplasmic reticulum stress, the unfolded protein response, autophagy, and the integrated regulation of breast cancer cell fate. Cancer Res 72: 1321-1331, 2012.

52. Li L, Wang ZV, Hill JA, and Lin F. New autophagy reporter mice reveal dynamics of proximal tubular autophagy. J Am Soc Nephrol 25: 305-315, 2014. Mizushima N, Yoshimori T, and Levine B. Methods in mammalian autophagy research. Cell 140: 313-326, 2010.

53. Ishihara M, Urushido M, Hamada K, Matsumoto T, Shimamura Y, Ogata K, Inoue K, Taniguchi Y, Horino T, Fujieda M, Fujimoto S, and Terada Y. Sestrin-2 and BNIP3 regulate autophagy and mitophagy in renal tubular cells in acute kidney injury. Am J Physiol Renal Physiol 305: F495-509, 2013.

54. Novikova DS, Garabadzhiu AV, Melino G, Barlev NA, and Tribulovich VG. AMPactivated protein kinase: structure, function, and role in pathological processes. Biochemistry (Mosc) 80: 127-144, 2015.

55. Meley D, Bauvy C, Houben-Weerts JH, Dubbelhuis PF, Helmond MT, Codogno P, and Meijer AJ. AMP-activated protein kinase and the regulation of autophagic proteolysis. J Biol Chem 281: 34870-34879, 2006.

56. Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, Mukherjee C, Shi Y, Gelinas C, Fan Y, Nelson DA, Jin S, and White E. Autophagy promotes tumor

cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell 10: 51-64, 2006.

57. Mount PF, Hill RE, Fraser SA, Levidiotis V, Katsis F, Kemp BE, and Power DA. Acute renal ischemia rapidly activates the energy sensor AMPK but does not increase phosphorylation of eNOS-Ser1177. Am J Physiol Renal Physiol 289: F1103-1115, 2005.

58. Decleves AE, Sharma K, and Satriano J. Beneficial Effects of AMP-Activated Protein

Kinase Agonists in Kidney Ischemia-Reperfusion: Autophagy and Cellular Stress Markers. Nephron Exp Nephrol, 2014.

59. Lempiainen J, Finckenberg P, Levijoki J, and Mervaala E. AMPK activator AICAR ameliorates ischaemia reperfusion injury in the rat kidney. Br J Pharmacol 166: 1905-1915,2012.

60. Devarajan P. Update on mechanisms of ischemic acute kidney injury. J Am Soc Nephrol 17: 1503-1520, 2006. Ni M and Lee AS. ER chaperones in mammalian development and human diseases. FEBS Lett 581: 3641-3651, 2007.

61. Emerling BM, Weinberg F, Snyder C, Burgess Z, Mutlu GM, Viollet B, Budinger GR, and Chandel NS. Hypoxic activation of AMPK is dependent on mitochondrial ROS but independent of an increase in AMP/ATP ratio. Free Radic Biol Med 46: 1386-1391, 2009.

62. Hardie DG, Hawley SA, and Scott JW. AMP-activated protein kinase--development of the energy sensor concept. J Physiol 574: 7-15, 2006.

63. Mungai PT, Waypa GB, Jairaman A, Prakriya M, Dokic D, Ball MK, and Schumacker PT. Hypoxia triggers AMPK activation through reactive oxygen species-

mediated activation of calcium release-activated calcium channels. Mol Cell Biol 31: 3531-3545, 2011.

64. Mount PF, Gleich K, Tam S, Fraser SA, Choy SW, Dwyer KM, Lu B, Denderen BV, Fingerle-Rowson G, Bucala R, Kemp BE, and Power DA. The outcome of renal ischemiareperfusion injury is unchanged in AMPK-beta1 deficient mice. PLoS One 7: e29887, 2012. Nuutinen T, Suuronen T, Kauppinen A, and Salminen A. Clusterin: a forgotten player in Alzheimer's disease. Brain Res Rev 61: 89-104, 2009.

65. Cummins EP and Taylor CT. Hypoxia-responsive transcription factors. Pflugers Arch 450: 363-371, 2005.

66. Park J, Park SY, Shin E, Lee SH, Kim YS, Lee DH, Roh GS, Kim HJ, Kang SS, Cho GJ, Jeong BY, Kim H, and Choi WS. Hypoxia inducible factor-1alpha directly regulates nuclear clusterin transcription by interacting with hypoxia response elements in the clusterin promoter. Mol Cells 37: 178-186, 2014.

67. Poulios E, Trougakos IP, and Gonos ES. Comparative effects of hypoxia on normal and immortalized human diploid fibroblasts. Anticancer Res 26: 2165-2168, 2006.

68. Bellot G, Garcia-Medina R, Gounon P, Chiche J, Roux D, Pouyssegur J, and Mazure NM. Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. Mol Cell Biol 29: 2570-2581, 2009.

69. Zhang H, Bosch-Marce M, Shimoda LA, Tan YS, Baek JH, Wesley JB, Gonzalez FJ, and Semenza GL. Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. J Biol Chem 283: 10892-10903, 2008.

70. Sansanwal P, Li L, and Sarwal MM. Inhibition of intracellular clusterin attenuates cell death in nephropathic cystinosis. J Am Soc Nephrol 26: 612-625, 2015.

71. Yu L, Strandberg L, and Lenardo MJ. The selectivity of autophagy and its role in cell death and survival. Autophagy 4: 567-573, 2008.

72. Du C, Guan Q, Diao H, Yin Z, and Jevnikar AM. Nitric oxide induces apoptosis in renal tubular epithelial cells through activation of caspase-8. Am J Physiol Renal Physiol 290: F1044-1054, 2006.

73. de Silva HV, Stuart WD, Park YB, Mao SJ, Gil CM, Wetterau JR, Busch SJ, and Harmony JA. Purification and characterization of apolipoprotein J. J Biol Chem 265: 1429214297, 1990.

74. Dohi E, Tanaka S, Seki T, Miyagi T, Hide I, Takahashi T, Matsumoto M, and Sakai N. Hypoxic stress activates chaperone-mediated autophagy and modulates neuronal cell survival. Neurochem Int 60: 431-442, 2012.

75. Inagi R. Endoplasmic reticulum stress as a progression factor for kidney injury. Curr Opin Pharmacol 10: 156-165, 2010.

76. Fujita E, Kouroku Y, Isoai A, Kumagai H, Misutani A, Matsuda C, Hayashi YK, and Momoi T. Two endoplasmic reticulum-associated degradation (ERAD) systems for the novel variant of the mutant dysferlin: ubiquitin/proteasome ERAD I and autophagy/lysosome ERAD II. Hum Mol Genet 16: 618-629, 2007.

77. Meusser B, Hirsch C, Jarosch E, and Sommer T. ERAD: the long road to destruction. Nat Cell Biol 7: 766-772, 2005.

78. Buck TM, Wright CM, and Brodsky JL. The activities and function of molecular chaperones in the endoplasmic reticulum. Semin Cell Dev Biol 18: 751-761, 2007.

79. Nakatsukasa K and Brodsky JL. The recognition and retrotranslocation of misfolded proteins from the endoplasmic reticulum. Traffic 9: 861-870, 2008.

80. Li N, Zoubeidi A, Beraldi E, and Gleave ME. GRP78 regulates clusterin stability, retrotranslocation and mitochondrial localization under ER stress in prostate cancer. Oncogene 32: 1933-1942, 2013.

81. Nizard P, Tetley S, Le Drean Y, Watrin T, Le Goff P, Wilson MR, and Michel D. Stressinduced retrotranslocation of clusterin/ApoJ into the cytosol. Traffic 8: 554-565, 2007.

82. Wang C, Jiang K, Gao D, Kang X, Sun C, Zhang Q, Li Y, Sun L, Zhang S, Guo K, and Liu Y. Clusterin protects hepatocellular carcinoma cells from endoplasmic reticulum stress induced apoptosis through GRP78. PLoS One 8: e55981, 2013.

83. Eickelberg O, Seebach F, Riordan M, Thulin G, Mann A, Reidy KH, Van Why SK, Kashgarian M, and Siegel N. Functional activation of heat shock factor and hypoxia-inducible factor in the kidney. J Am Soc Nephrol 13: 2094-2101, 2002.

84. Feldkamp T, Park JS, Pasupulati R, Amora D, Roeser NF, Venkatachalam MA, and Weinberg JM. Regulation of the mitochondrial permeability transition in kidney proximal tubules and its alteration during hypoxia-reoxygenation. Am J Physiol Renal Physiol 297: F1632-1646, 2009.

85. Mizushima N, Yamamoto A, Matsui M, Yoshimori T, and Ohsumi Y. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. Mol Biol Cell 15: 1101-1111, 2004.

86. Semenza GL. HIF-1, O_2, and the 3 PHDs: how animal cells signal hypoxia to the nucleus. Cell 107: 1-3, 2001.

87. Sharfuddin AA and Molitoris BA. Pathophysiology of ischemic acute kidney injury. Nat Rev Nephrol 7: 189-200, 2011.

88. Sun L, Hao Y, An R, Li H, Xi C, and Shen G. Overexpression of Rcan1-1L inhibits hypoxia-induced cell apoptosis through induction of mitophagy. Mol Cells 37: 785-794, 2014.

89. Weinberg JM, Venkatachalam MA, Roeser NF, Saikumar P, Dong Z, Senter RA, and Nissim I. Anaerobic and aerobic pathways for salvage of proximal tubules from hypoxia-induced

90. mitochondrial injury. Am J Physiol Renal Physiol 279: F927-943, 2000. Nuutinen T, Suuronen T, Kauppinen A, and Salminen A. Clusterin: a forgotten player in Alzheimer's disease. Brain Res Rev 61: 89-104, 2009.

91. Mark E Thomas, Caroline Blaine, Anne Dawnay, Mark A J Devonald, Saoussen Ftouh, Chris Laing, . . . Marlies Ostermann. (2014). The definition of acute kidney injury and its use in practice. *Kidney International, 87*(1), 62-73.

92. Mizushima N and Kuma A. Autophagosomes in GFP-LC3 Transgenic Mice. Methods Mol Biol 445: 119-124, 2008.

93. Hogg SD, Embery G (1979) The isolation and partial characterization of a sulphated glycoprotein from human whole saliva which aggregates strains of Streptococcus sanguis but not Streptococcus mutans. Arch Oral Biol 24: 791- 797.

94. Tobe T, Minoshima S, Yamase S, Choi NH, Tomita M, et al. (1991) Assignment of a human serum glycoprotein SP-40,40 gene (CLI) to chromosome 8. Cytogenet Cell Genet 57: 193-195.

95. Prochnow H, Gollan R, Rohne P, Hassemer M, Koch-Brandt C, Baiersdorfer M (2013) Nonsecreted clusterin isoforms are translated in rare amounts from distinct human mRNA variants and do not affect Bax-mediated apoptosis or the NF-kappaB signaling pathway. PLoS One 8:e75303.

96. Leskov KS, Klokov DY, Li J, Kinsella TJ, Boothman DA (2003) Synthesis and functional analyses of nuclear clusterin, a cell death protein. J BiolChem 278: 11590-11600.

97. Burkey BF, deSilva HV, Harmony JA (1991) Intracellular processing of apolipoprotein J precursor to the mature heterodimer. J Lipid Res 32: 1039- 1048.

98. Jones SE, Jomary C (2002) Clusterin. Int J Biochem Cell Biol 34: 427-431.

99. Kapron JT, Hilliard GM, Lakins JN, Tenniswood MP, West KA, et al. (1997) Identification and characterization of glycosylation sites in human serum clusterin. Protein Sci 6: 2120-2133.

100. Jordan-Starck TC, Lund SD, Witte DP, Aronow BJ, Ley CA, et al. (1994) Mouse apolipoprotein J: characterization of a gene implicated in atherosclerosis. J Lipid Res 35: 194-210.

101. Polihronis M, Machet D, Saunders J, O'Bryan M, McRae J, et al. (1993) Immunohistological detection of C5b-9 complement complexes in normal and pathological human livers. Pathology 25: 20-23.

102. Murphy BF, Kirszbaum L, Walker ID, d'Apice AJ (1988) SP-40,40, a newly identified normal human serum protein found in the SC5b-9 complex of complement and in the immune deposits in glomerulonephritis. J Clin Invest 81: 1858-1864.

103. Choi NH, Tobe T, Hara K, Yoshida H, Tomita M (1990) Sandwich ELISA assay for quantitative measurement of SP-40,40 in seminal plasma and serum. J Immunol Methods 131: 159-163.

104. Morrissey C, Lakins J, Moquin A, Hussain M, Tenniswood M (2001) An antigen capture assay for the measurement of serum clusterin concentrations. J BiochemBiophys Methods 48:

13-21.

105. Ståhl AL, Kristoffersson A, Olin AI, Olsson ML, Roodhooft AM, et al. (2009) A novel mutation in the complement regulator clusterin in recurrent hemolytic uremic syndrome. MolImmunol 46: 2236-2243.

106. Polihronis M, Paizis K, Carter G, Sedal L, Murphy B (1993) Elevation of human cerebrospinal fluid clusterin concentration is associated with acute neuropathology. J NeurolSci 115: 230-233.

107. Choi-Miura NH, Oda T (1996) Relationship between multifunctional protein "clusterin" and Alzheimer disease. Neurobiol Aging 17: 717-722.

108. Trougakos IP, Poulakou M, Stathatos M, Chalikia A, Melidonis A, et al. (2002) Serum levels of the senescence biomarker clusterin/apolipoprotein J increase significantly in diabetes type II and during development of coronary heart disease or at myocardial infarction.ExpGerontol 37: 1175-1187.

109. Trougakos IP, Gonos ES (2002) Clusterin/apolipoprotein J in human aging and cancer. Int J Biochem Cell Biol 34: 1430-1448.

110. Zoubeidi A, Chi K, Gleave M (2010) Targeting the cytoprotective chaperone, clusterin, for treatment of advanced cancer. Clin Cancer Res 16: 1088-1093.

111. Zoubeidi A, Gleave M (2012) Small heat shock proteins in cancer therapy and prognosis. Int J Biochem Cell Biol 44: 1646-1656.

112. Murphy BF, Davies DJ, Morrow W, d'Apice AJ (1989) Localization of terminal complement components S-protein and SP-40,40 in renal biopsies. Pathology 21: 275-278.

113. Dvergsten J, Manivel JC, Correa-Rotter R, Rosenberg ME (1994) Expression of clusterin in human renal diseases. Kidney Int 45: 828-835.

114. Zhou W, Guan Q, Kwan CC, Chen H, Gleave ME, et al. (2010) Loss of clusterin expression worsens renal ischemia-reperfusion injury. Am J Physiol Renal Physiol 298: F568578.

115. Nath KA, Dvergsten J, Correa-Rotter R, Hostetter TH, Manivel JC, et al. (1994) Induction of clusterin in acute and chronic oxidative renal disease in the rat and its dissociation from cell injury. Lab Invest 71: 209-218.

116. Moll S, Menoud PA, French L, Sappino AP, Pastore Y, et al. (1998) Tubular up-regulation of clusterin mRNA in murine lupus-like nephritis. Am J Pathol 152: 953-962.

117. Yamada K, Hori Y, Hanafusa N, Okuda T, Nagano N, et al. (2001) Clusterin is up-regulated in glomerular mesangial cells in complement-mediated injury. Kidney Int 59: 137-146.

118. Laping NJ, Olson BA, Short B, Albrightson CR (1997) Thrombin increases clusterin mRNA in glomerular epithelial and mesangial cells. J Am SocNephrol 8: 906-914.

119. Graichen R, Lösch A, Appel D, Koch-Brandt C (1996) Glycolipid-independent sorting of a secretory glycoprotein to the apical surface of polarized epithelial cells. J BiolChem 271: 1585415857.

120. Gobé GC, Buttyan R, Wyburn KR, Etheridge MR, Smith PJ (1995) Clusterin expression and apoptosis in tissue remodeling associated with renal regeneration. Kidney Int 47: 411-420.

121. Michel D, Chatelain G, North S, Brun G (1997) Stress-induced transcription of the clusterin/apoJ gene. Biochem J 328 : 45-50.

122. Loison F, Debure L, Nizard P, le Goff P, Michel D, et al. (2006) Up-regulation of the clusterin gene after proteotoxic stress: implication of HSF1-HSF2 heterocomplexes. Biochem J 395: 223-231.

123. de Silva HV, Harmony JA, Stuart WD, Gil CM, Robbins J (1990) Apolipoprotein J: structure and tissue distribution. Biochemistry 29: 5380-5389.

124. Stewart EM, Aquilina JA, Easterbrook-Smith SB, Murphy-Durland D, Jacobsen C, et al. (2007) Effects of glycosylation on the structure and function of the extracellular chaperone clusterin. Biochemistry 46: 1412-1422.

125. Fritz IB, Burdzy K, Sétchell B, Blaschuk O (1983) Ram rete testis fluid contains a protein (clusterin) which influences cell-cell interactions in vitro. BiolReprod 28: 1173-1188.

126. Cheng CY, Mathur PP, Grima J (1988) Structural analysis of clusterin and its subunits in ram rete testis fluid. Biochemistry 27: 4079-4088.

127. Silkensen JR, Skubitz KM, Skubitz AP, Chmielewski DH, Manivel JC, et al. (1995) Clusterin promotes the aggregation and adhesion of renal porcine epithelial cells. J Clin Invest 96: 2646-2653.

128. Murphy BF, Saunders JR, O'Bryan MK, Kirszbaum L, Walker ID, et al. (1989) SP-40,40 is an inhibitor of C5b-6-initiated haemolysis. IntImmunol 1: 551-554.

129. Choi NH, Nakano Y, Tobe T, Mazda T, Tomita M (1990) Incorporation of SP-40,40 into the soluble membrane attack complex (SMAC, SC5b-9) of complement. IntImmunol 2: 413-417.

130. Tschopp J, Chonn A, Hertig S, French LE (1993) Clusterin, the human apolipoprotein and complement inhibitor, binds to complement C7, C8 beta, and the b domain of C9. J Immunol 151: 2159-2165.

131. Jenne DE, Lowin B, Peitsch MC, Böttcher A, Schmitz G, et al. (1991) Clusterin (complement lysis inhibitor) forms a high density lipoprotein complex with apolipoprotein A-I in human plasma. J BiolChem 266: 11030-11036.

132. Karlsson H, Leanderson P, Tagesson C, Lindahl M (2005) Lipoproteomics I: mapping of proteins in low-density lipoprotein using two-dimensional gel electrophoresis and mass spectrometry. Proteomics 5:551-565.

133. Girton RA, Sundin DP, Rosenberg ME (2002) Clusterin protects renal tubular epithelial cells from gentamicin-mediated cytotoxicity. Am J Physiol Renal Physiol 282: F703-709.

134. Ammar H, Closset JL (2008) Clusterin activates survival through the phosphatidylinositol 3kinase/Akt pathway. J BiolChem 283: 12851-12861.

135. Kim JH, Kim JH, Jun HO, Yu YS, Min BH, et al. (2010) Protective effect of clusterin from oxidative stress-induced apoptosis in human retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 51: 561-566.

136. Bartl MM, Luckenbach T, Bergner O, Ullrich O, Koch-Brandt C (2001) Multiple receptors mediate apoJ-dependent clearance of cellular debris into nonprofessional phagocytes. Exp Cell Res 271: 130-141.

137. Wyatt A, Yerbury J, Poon S, Dabbs R, Wilson M (2009) Chapter 6: The chaperone action of Clusterin and its putative role in quality control of extracellular protein folding. Adv Cancer Res 104: 89-114.

138. Wyatt AR, Wilson MR (2010) Identification of human plasma proteins as major clients for the extracellular chaperone clusterin. J BiolChem 285: 3532-3539.

139. Bettuzzi S, Rizzi F (2009) Chapter 5: Nuclear CLU (nCLU) and the fate of the cell. Adv Cancer Res 104: 59-88.

140. Zhang H, Kim JK, Edwards CA, Xu Z, Taichman R, et al. (2005) Clusterin inhibits apoptosis by interacting with activated Bax. Nat Cell Biol 7: 909-915.

141. Zoubeidi A, Ettinger S, Beraldi E, Hadaschik B, Zardan A, et al. (2010) Clusterin facilitates COMMD1 and I-kappaB degradation to enhance NF-kappaB activity in prostate cancer cells. Mol Cancer Res 8: 119-130.

142. Materia S, Cater MA, Klomp LW, Mercer JF, La Fontaine S (2011) Clusterin (apolipoprotein J), a molecular chaperone that facilitates degradation of the copper-ATPases ATP7A and ATP7B. J BiolChem 286: 10073-10083.

143. French LE, Chonn A, Ducrest D, Baumann B, Belin D, Wohlwend A, et al (1993) Murineclusterin: molecular cloning and mRNA localization of a gene associated with epithelial differentiation processes during embryogenesis. J Cell Biol 122:1119-1130.

144. Min BH, Jeong SY, Kang SW, Crabo BG, Foster DN, et al. (1998) Transient expression of clusterin (sulfated glycoprotein-2) during development of rat pancreas. J Endocrinol 158: 43-52.

145. Witte DP, Aronow BJ, Dry JK, Harmony JA (1994) Temporally and spatially restricted expression of apolipoprotein J in the developing heart defines discrete stages of valve morphogenesis. DevDyn 201: 290-296.

146. Charnay Y, Imhof A, Vallet PG, Hakkoum D, Lathuiliere A, et al. (2008) Clusterin expression during fetal and postnatal CNS development in mouse. Neuroscience 155: 714-724.

147 O'Bryan MK, Cheema SS, Bartlett PF, Murphy BF, Pearse MJ (1993) Clusterin levels increase during neuronal development. J Neurobiol 24: 421-432.

148. Cordero-Llana O, Scott SA, Maslen SL, Anderson JM, Boyle J, et al. (2011) Clusterin secreted by astrocytes enhances neuronal differentiation from human neural precursor cells. Cell Death Differ 18: 907-913.

149. Kim SY, Lee S, Min BH, Park IS (2007) Functional association of the morphogenic factors with the clusterin for the pancreatic beta-cell differentiation. Diabetes Res ClinPract 77 Suppl 1: S122-126.

150. Li Y, Qu J, Shelat H, Gao S, Wassler M, et al. (2010) Clusterin induces CXCR4 expression and migration of cardiac progenitor cells. Exp Cell Res 316: 3435- 3442.

151. Romagnani P, Lasagni L, Remuzzi G (2013) Renal progenitors: an evolutionary conserved strategy for kidney regeneration. Nat Rev Nephrol 9: 137-146.

152. Morigi M, Benigni A (2013) Mesenchymal stem cells and kidney repair. Nephrol Dial Transplant 28: 788-793.

153. Smeets B, Boor P, Dijkman H, Sharma SV, Jirak P, et al. (2013) Proximal tubular cells contain a phenotypically distinct, scattered cell population involved in tubular regeneration. J Pathol 229: 645-659

154. Li S, Guan Q, Chen Z, Gleave ME, Nguan CY, et al. (2011) Reduction of cold ischemiareperfusion injury by graft-expressing clusterin in heart transplantation. J Heart Lung Transplant 30: 819-826