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Carboxymethyl lysine induces EMT in podocytes through transcription factor ZEB2: Implications for podocyte depletion and proteinuria in diabetes mellitus

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Short Title: Induction of EMT in podocytes by CML

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Highlights:

- Carboxymethyl lysine (CML) induces NF-kB activation which in turn induces ZEB2 in glomerular podocytes.
- CML elicit epithelial mesenchymal transition of podocytes and induce migratory properties.
- CML suppresses podocyte slit-diaphragm proteins and alter their permselectivity.
- Elevated levels of CML in kidney from diabetic rats associated with reduced podocyte count and proteinuria.

Abbreviations used: AGEs, advanced glycation end-products; RAGE, receptor for AGEs; CML, N-carboxymethyl-lysine; KLH, keyhole limpet hemocyanin; DM, diabetic mellitus; DN, diabetic nephropathy; EMT, epithelial-mesenchymal transition; GFB, glomerular filtration barrier; GBM, glomerular basement membrane; SD, slit-diaphragm; STZ, streptozotocin; Snail1, Snail family zinc finger 1; Slug; Snail family zinc finger 2; ZEB1, zinc finger E-box-binding homeobox 1; ZEB2, zinc finger E-box-binding homeobox 2.

ABSTRACT:

Diabetes mellitus, characterized by hyperglycemia due to either absolute or relative deficiency of insulin, is implicated in the etiology of diabetic nephropathy (DN). Diabetic nephropathy is a predominant cause of end-stage renal disease and is morphologically characterized by reduced glomerular podocyte density and clinically evidenced by proteinuria. Among several biochemical events that manifest during diabetes, advanced glycation end-products (AGEs) are implicated in the pathogenesis of DN. N-carboxymethyl-lysine (CML) is one of the predominant AGEs that accumulate in all renal compartments of diabetic patients. Nevertheless, the direct effect of CML on podocyte biology has not been explored. In this study, we demonstrate the induction of zinc-finger E-box binding homeobox2 (ZEB2) protein in podocytes upon exposure to CML through activation of NF-kB signaling cascade. ZEB2 orchestrates epithelial-mesenchymal transformation, during which cell-cell and cell-extracellular matrix interactions are feeble and enable epithelial cells to become invasive. CML treatment induced both NF-kB and ZEB2 promoter activity and suppressed E-cadherin promoter activity. Inhibition of NF-kB activity prevented CML dependent induction of ZEB2 and loss of E-cadherin. While the exposure of podocytes to CML results in increased podocyte permeability, shRNA-mediated knockdown of ZEB2 expression abrogated CML-mediated podocyte permeability. Further, *in vivo* findings of elevated CML levels concurrent with increased expression of ZEB2 in glomeruli and proteinuria in diabetic rats confirm that CML-mediated manifestations in the kidney under chronic diabetes conditions. These *in vitro* and *in vivo* results envisage the novel role of NF-kB-ZEB2 axis in podocytes playing a significant role in pathogenesis of DN.

Keywords: Podocytes, carboxymethyl lysine, NF-kB, ZEB2, E-cadherin, diabetic nephropathy

1. Introduction

The vertebrate kidneys are indispensable for maintaining body homeostasis by regulating electrolyte, water and acid-base balance. These functions are carried out by the collective effort of approximately one million nephrons in each kidney. Each nephron consists of a glomerulus and a renal tubule. Under normal conditions, kidneys ensure ultra-filtrated urine with tightly regulated protein concentration. Appearance of protein in urine indicates damage to the glomerular filtration barrier (GFB), which is a size selective molecular sieve that firmly regulates the filtration of large macromolecules while allowing the passage of only small molecules and water [1]. The three components that constitute GFB are the fenestrated glomerular endothelium, the glomerular basement membrane (GBM) and the podocytes. While all the three components of GFB are essential for normal renal filtration, a wealth of work over the past decade has highlighted the crucial role of podocytes in this filtering process [2].

Podocytes are terminally differentiated visceral epithelial cells that are made up of numerous lamellipodia that branch into primary and secondary processes which further ramify into smaller foot processes. Foot processes from neighbouring podocytes interdigitate and connected by a modified adherent junction called slit diaphragm (SD) that provides intercellular space for the passage of glomerular filtrate [3]. The ability of podocytes to act as filtration barrier depends on the integrity of SD [4]. Alterations in the morphology of podocyte lead to the disruption of foot process architecture, which results in the loss of entire podocytes, ultimately leading to proteinuric state [5-7]. Studies in patients and animal models of diabetes mellitus (DM) revealed that the onset of proteinuria is associated with decreased density and altered podocyte morphology [8, 9]. Reduction in podocyte number has been shown to predict a progressive decline in renal function and proteinuria in Pima Indians [10]. Podocyte number is markedly reduced in diabetic nephropathy (DN) where the foot process is significantly widened and the SD becomes narrower resulting in a declined glomerular filtration rate [7, 10-12]. Studies from diabetic rodents suggested that injured podocytes undergo apoptosis and also detach from GBM into the urinary space [13]. Since podocytes are considered as terminally differentiated cells, loss or injury to the podocytes will impede the glomerular function as the remaining healthy podocytes are unable to compensate for glomerular filtration, thus resulting in proteinuria. Podocyte injury is the leading cause of chronic kidney disease in patients requiring renal replacement therapy [14]. It was reported that podocytes undergo either apoptosis or epithelial-mesenchymal transition (EMT) that accounts for decreased podocyte count and proteinuria [1].

Among various risk factors prevalent in DM, chronic hyperglycemia plays a critical role in abetting several complications including DN. Prolonged hyperglycemia is an exacerbating factor leading to elevated advanced glycation end-products (AGEs) in circulation and tissues. AGEs are a heterogeneous group of compounds derived non-enzymatically from the reaction of reducing sugars including glucose with free amino groups in proteins through a series of oxidative and non-oxidative reactions. AGEs accumulate in DM patients and tend to rise further with the progression of DN [15]. The involvement of AGEs in the pathogenesis of renal damage has been supported by a study in non-diabetic rats wherein administration of AGEs induced proteinuria and histological changes that were observed in diabetic kidney disease [16, 17]. On the other hand preventing AGEs formation attenuates diabetic complications [18, 19]. In renal biopsies from patients with DN, AGE-accumulation is primarily found in GBM and its accumulation involves upregulation of receptor for AGE (RAGE) on podocytes [20]. N-carboxymethyl-lysine (CML) is one of the well-characterized AGE that accumulates predominantly in all renal compartments of diabetics [21]. While extracellular AGEs interfere with cellular signalling via their interaction with RAGE, intracellular AGEs influence several signalling events involving protein kinase C, mitogen-activated protein (MAP) kinase and transcription factors such as NF-kB [22]. In an earlier study, it was shown that AGEs induced tubulo epithelial-myofibroblast transdifferentiation through interaction with RAGE [23, 24]. Both AGE-breaker (ALT711) and neutralizing antibodies to RAGE reduced AGE mediated transdifferentiation of epithelial cells [23]. Although, CML is the predominant AGE present in renal compartments and podocytes from diabetic subjects, it is not entirely established how CML contributes to podocyte damage. In this study, we demonstrated that CML activates ZEB2, a canonical

transcription factor that mediates EMT. Thus CML mediated NF-kB dependent up-regulation of ZEB2 could be linked to albuminuria in diabetic conditions.

2. Materials and Methods:

2.1 Materials: Acrylamide, bis-acrylamide, ammonium persulfate, 2-mercaptoethanol, TEMED, Tris, Tri-reagent, Streptozotocin, keyhole limpet haemocyanin (KLH), glyoxylic acid, sodium cyanoborohydride, were purchased from Sigma (St. Louis, MO). BCA protein assay kit and immobilon nitrocellulose membrane were from Bio-Rad Laboratories (Hercules, CA). Antibodies for NF-kB signaling and EMT were purchased from Cell-Signaling Technologies (Beverly, MA). All other antibodies used in this study were procured from Santa-Cruz Biotechnology (Dallas, Tx). The high capacity cDNA reverse transcription kit and Power SYBR Green Master Mix were obtained from Applied Biosystems (Warrington, UK). Fetal bovine serum, RPMI and cell-culture reagents were procured from Life Technologies (Bangalore, India). Anti-nephrin antibody is a gift from Dr. Rakesh Verma (University of Michigan). E-cadherin wild type construct was a gift from Dr. Eric R Fearon (University of Michigan). ZEB2-luciferase reporter construct was provided by Dr. Antonio García de Herreros (Institut Municipal d'Investigacio´ Me`dica-Hospital del Mar, Universitat Pompeu Fabra, Barcelona, Spain).

2.2 Preparation of CML-KLH and production of CML antibodies: CML-KLH was prepared as described in our earlier study [19]. Briefly, 50 mg/ml KLH was incubated in 0.2 M sodium phosphate buffer (pH 7.8) containing 45mM glyoxylic acid and 150mM sodium cyanoborohydride at 37°C for 24 h. After incubation, KLH preparation was dialyzed extensively to remove any unbound glyoxylic acid. Dialyzed CML-KLH was stored at -80^{o}C until further use. A working solution of CML-KLH was prepared in plain RPMI-1640 medium before treating podocytes. Antibodies for CML-KLH were produced in New Zealand white rabbits as described earlier [19].

2.3 Podocyte culture: Human immortalized podocytes were cultured as described earlier [25]. Briefly, conditionally immortalized human podocytes cells (LY813) from University of Bristol were maintained under growth-permissive conditions at 33°C under 5% CO_2 in RPMI-1640 medium containing 10% FBS, 100 units/ ml penicillin, 100 µg/ml streptomycin, and 1% insulin-transferrin-selenium solution. To induce differentiation, podocytes were shifted to non-permissive conditions at 37°C and maintained for 10 days. Differentiated podocytes were maintained for 4h in serum-free medium before treating with CML-KLH.

2.4 Transient transfection and luciferase assay: Podocytes are resistant to tranfection; therefore we employed HepG2 cells to investigate effect of CML on promoter activities of down-stream targets. HepG2 cells ($1x10^5$ cells/well) were transfected with promoter-reporter construct (pNifty/ZEB2/E-cadherin) and internal control expressing the Renilla luciferase, pRL-TK (Promega). HepG2 cells were transfected using FuGENE (Roche Applied Sciences). Forty-eight hours after transfection, cells were washed twice with PBS and harvested with 100 µl passive lysis buffer (Promega). After a brief freeze-thaw cycle, the insoluble debris was removed by centrifugation at 4^{o}C for 5 min at 12,000xg, and 20 µl aliquots of the supernatant was used for sequential quantification of firefly and Renilla luciferase activity (Dual Luciferase Assay System; Promega). The activity of the cotransfected Renilla reporter plasmid was used to normalize transfection efficiency.

2.5 ZEB2 Knockdown using shRNA lentiviral particles: A panel of five lentiviruses expressing unique ZEB2 shRNA sequences was purchased from Sigma-Aldrich. Proliferating human podocytes were plated in a 6-well plate at 40% confluence ($2x10^5$ cells/well) and cultured overnight for 12–16 h. The cells were then pre-treated with polybrene (6µg/ml for 20 h), infected with lentiviral particles, and incubated at 33°C. The following day the medium was replaced with fresh medium containing puromycin (1 µg/ml) as a selection marker. The puromycin-resistant cells were induced to differentiate using the protocol detailed

above, and ZEB2 expression was measured in the differentiated cells using RT-qPCR and Western blot analysis. ZEB2 knockdown cells were employed for further studies.

2.6 Real-time quantitative PCR: Total RNA was extracted using TRI-reagent (Invitrogen). qRT-PCR using the ABI Prism 7500 sequence detection system (Applied Biosystems) was performed in triplicate using QuantiTect SYBR Green RT-PCR kit (QIAGEN). Primers were designed using Primer Express 2.0 software. mRNA expression of each gene was normalized using the expression of β-actin as a housekeeping gene and data analyzed via the comparative threshold cycle method [26]. The sequences of primers used in the study are as follows: ZEB1, forward: 5-GCACCTGAAGAGGACCAGAG-3, ZEB1 reverse: 5-TGCATCTGGTGTTCCATTTT-3; ZEB2, forward: 5-CAAGGAGCAGGTAATCGCAAGT-3; ZEB2, reverse: 5-GAA CGT CAA ACC CGT GAG CAT-3; Snail, forward: 5-CCACTATGCCGCGCTCTT-3; Snail, reverse: 5-GGTCGTAGGGCTGCTGGAA-3; Slug, forward: 5-TGTTGCAGTGAGGGCAAGAA-3; Slug, reverse: 5′-GACCCTGGTTGCTTCAAGGA-3.

2.7 Sub-cellular fractionation: Nuclear and cytoplasmic sub-cellular fractionation was carried out using the NE-PER kit (Pierce, Rockland, IL) as per the manufacturer's instructions. Fractionated cytoplasmic and nuclear aliquots were stored at -80^{o}C until used for Western blot analysis, wherein β-actin and histone-H3 were used as markers for cytoplasmic and nuclear fractions, respectively.

2.8 Western blotting: Lysate from podocytes treated with or without CML was processed for western blotting. Aliquot of total cell lysate (routinely 50 µg) were heated for 5 min at 100^{o}C using 2x Laemmli buffer. The protein samples were then electrophoresed through a 4% stacking, 8-12% resolving SDS-polyacrylamide gel. BLUeye Prestained Protein Ladder (Geneflow) was also concurrently electrophoresed. After electrophoresis, the proteins were transferred to nitrocellulose membrane by electroblotting. The nitrocellulose membrane was then soaked in 5% non-fat dry milk for 1h and subsequently probed with the respective primary (1:1000 dilution) and secondary antibodies (1:10000 dilution). Blots were developed using the ECL Western blotting substrate (Bio-Rad Laboratories).

2.9 Wound healing assay: Human podocytes were seeded in 6 well plates and grown under normal growth conditions to reach 95% confluence. When the cells are confluent, media was aspirated and a scratch was made in the shape of "+" in a smooth sweeping motion using a sterile pipette tip. Cells were washed twice with pre-warmed media in order to remove any debris from damaged cells. Images of cells were taken before and after treating with CML-KLH at regular time points to monitor the rate of migration of cells into the wound field. The area covered by cells is quantified using Image J software (NIH, USA) and represented as the percent area coverage (Cell Area/ROI Area x 100).

2.10 Albumin influx assay: Human podocytes were placed on a 12-well plate with collagen-coated Transwell filters (Corning) and differentiated at non-permissive conditions as described above by culturing for 10 days. The cells were then treated with CML-KLH (1 µg/ml) for 48 h. Subsequently, the medium was removed, and cells were washed twice with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ mixture to preserve cadherin junctions. 2 ml of RPMI 1640 medium with 40 mg/ml BSA was then placed in the bottom chamber, and 0.3 ml of RPMI 1640 medium (without albumin) was placed in the top chamber. The cells were incubated at 37°C, aliquots of medium was collected from the top chamber at various (1, 2, and 4 h) time points, and albumin concentration was measured in these aliquots using the BCA protein assay kit (Sigma-Aldrich). Absorbance was measured at 562 nm using a Spectra max plate reader (Molecular Devices).

2.11 Studies with diabetic rodents: Two-month-old male Wistar-NIN rats with an average body weight of 205g (obtained from the National Center for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India) were used for this study. All the animals were fed ad libitum with a semisynthetic AIN-93 diet. The control rats (n=6) received 0.1 M citrate buffer (pH 4.5) as vehicle,

whereas the experimental rats received a single intraperitoneal injection of streptozotocin (STZ; 35 mg/kg) in the same buffer. After 72 h of STZ injection, rats with fasting blood glucose levels >150 mg/dL were considered as the diabetic animals (n=6) and were maintained for 10 weeks. Urine was collected at the end of 10 weeks and was analyzed for albuminuria, if any. At the end of the experiment, under general anesthesia, kidneys were perfused with PBS containing 50 U/ml sodium heparin through a cannula placed in the abdominal aorta. At constant hydrostatic pressure of 100 mm Hg perfusion was carried out till the kidneys were blanched. Kidneys from these rats were harvested and cortical regions were dissected and used for immunohistochemistry and western blotting as described above. All animal care protocols were approved by the Institutional Animal Ethics Committee of the National Institute of Nutrition.

2.12 Immunohistochemistry: Kidneys that were collected at the end of animal experiment were fixed in 4% paraformaldehyde. Paraffin embedded 4μM thick sections were obtained and immunolocalization of CML, ZEB2 and E-cadherin was performed by employing standard protocols. Briefly, kidney sections were deparaffinized at 60°C for 45 minutes followed by incubating the slides in xylene for 10 min and same was repeated three times. The sections were then dehydrated in decreasing grades of isopropyl alcohol (90%, 70% and 50%). Antigen retrieval was done by heating the slides in 10 mM sodium citrate buffer, pH 6.0 for 10 min at 60°C in microwave oven. The endogenous peroxidase activity was quenched by incubating the slides in 3% H_2O_2 for 30 min. To prevent non-specific binding of the antibody, blocking was done by incubating the slides in 3% normal goat serum in PBS at room temperature for 1 hour. Later the slides were incubated overnight at 4°C with primary antibody in 1.5% normal goat serum in PBS. After overnight incubation with primary antibody, slides were washed with PBS and incubated with corresponding secondary antibody solution for 45 min and followed by incubation of slides with Vectastain elite ABC reagent (Vector Laboratories). The protein was localized in the kidney sections by addition of DAB solution containing H_2O_2. Slides were examined under an epifluorescence microscope (LMD 6000, Leica microsystems, Germany) and images were captured using appropriate filters.

2.13 Statistical analysis: Majority of the experiments were performed in triplicate and were repeated four to six times. The values represent the mean ± SE of the values obtained. Statistical analysis was carried out with the non-parametric Mann-Whitney U test, considering $p < 0.05$ as significant.

3. Results:

3.1 CML induces ZEB2 expression in human podocytes: Human immortalized podocytes treated with varying concentrations of CML-KLH (0.1-1μg/ml) showed induction of ZEB2 in a dose dependent manner (Fig.1A&B). Further, we have noticed a significant loss of E-cadherin in podocytes treated with CML-KLH, which was more evident at a concentration of 1μg/ml (Fig.1A). E-cadherin is a predominant epithelial marker and its expression is regulated transcriptionally by ZEB2. We then employed a concentration of 1μg/ml CML-KLH to investigate the time dependent effect of CML on expression of a panel of EMT markers. While ZEB2 expression was elevated till 48 h of exposure to CML-KLH, E-cadherin expression was depleted in a time dependent manner (Fig.1C&D). Also, the expression of other epithelial marker (EpCAM) was decreased and mesenchymal marker (β-catenin) was increased with CML-KLH treatment. However, we did not notice significant induction of other transcriptional factors that regulate EMT such as Snail1, Slug and ZEB1 (Fig.1E). Further, wound healing (scratch) assay demonstrated that podocytes exposed to CML-KLH exhibited enhanced motility (Fig.2A). We have also assessed whether CML induced migration is specific to podocytes or it can induce migration of any other cells. MDA-MB-231, mammary carcinoma cells treated with CML-KLH also displayed enhanced motility compared with cells naïve to CML-KLH (Fig.2B). We did not notice induction of ZEB2 in podocytes treated with KLH alone (data not shown).

3.2 CML activates NF-kB signalling in human podocytes: AGEs including CML elicit their effect on cells by binding to the receptor for AGE (RAGE). RAGE belongs to the family of Toll-like receptor-4 and transduces the intracellular signalling via activation of NF-kB. We investigated the effect of CML on

NF-kB activation in podocytes by treating cells with CML-KLH for varying periods of time. We found that exposure of podocytes to CML resulted in activation of NF-kB signalling as evidenced by phosphorylation of IKKα/β and IKBα with eventual phosphorylation of p65NF-kB (Fig.3A). Further, we investigated the translocation of phosphorylated p65NF-kB into nucleus upon exposure of podocytes to CML-KLH. In podocytes exposed to CML, we found that both enhanced phosphorylation and accumulation of p65NF-kB in the nucleus (Fig.3B). Activation of p65NF-kB correlated with increased expression of ZEB2 and decreased expression of E-cadherin in podocytes (Fig.3C&D). Our data also revealed that MG132 (NF-kB inhibitor) attenuated CML induced expression of ZEB2 in podocytes (Fig.3C&D). This suggests, at least partly, that CML dependent activation of EMT phenomenon may be mediated via RAGE-NF-kB axis. Furthermore, we found that exposure of HepG2 cells that were transiently transfected with pNifty (NF-kB promoter-report construct) to CML-KLH showed enhanced pNifty promoter activity (Fig.3E).

3.3 CML-dependent activation of ZEB2 promoter activity: HepG2 cells that were transiently transfected with ZEB2 promoter-reporter construct showed increased luciferase activity upon treatment with CML-KLH (Fig.4A). It was known from previous studies that ZEB2 promoter region has a NF-kB binding site [27]. In this study upon inhibition of NF-kB with MG-132 (1μM), we noticed attenuation of CML dependent expression of ZEB2. Based on the temporal relationship between these two effects of CML on NF-KB and ZEB2 in podocytes, we hypothesized that the effect of CML on ZEB2 expression is mediated via activation of NF-kB. Thus, we tested the effect of CML-KLH on expression of E-cadherin, a putative target of ZEB2. E-cadherin promoter has conserved regulatory elements including two E2 boxes (CACCTG) that bind ZEB2 and elicit the suppressive action of ZEB2 on E-cadherin expression. We tested this hypothesis by exposing HepG2 cells that were transiently transfected with wild-type E-cadherin promoter or E-cadherin promoter mutated in ZEB2 binding E-box region (CATCTG) to CML-KLH. While the promoter activity of wild-type E-cadherin was decreased with CML treatment (Fig.4B), mutation in ZEB2 binding region abolished the inhibitory effect of CML on E-cadherin promoter (Fig.4C). Taken together, these results suggest that CML induces loss of E-cadherin via ZEB2, whereas ZEB2 is induced by NF-kB.

3.4 ZEB2 knockdown abolishes CML-dependent decrease in P-cadherin in human podocytes: Previous studies reported that ZEB2 suppresses the expression of P-cadherin, which is a component of podocyte SD [28]. Therefore, we next ascertained whether ZEB2 played an essential role in CML-dependent decrease in P-cadherin expression in human podocytes. CML treated podocytes showed decreased P-cadherin expression compared to cells that were naïve to CML treatment (Fig.5A&B). In $ZEB2^{-/-}$ podocytes, CML-KLH failed to repress in P-cadherin expression (Fig.5A&B). Hence, these results indicate that ZEB2 is essential for transducing the effect of CML on P-cadherin expression in podocytes.

3.5 Essential role of ZEB2 in transducing effect of CML on podocyte permselectivity: The data described above indicated that CML induced ZEB2 in NF-kB dependent manner and a concomitant loss of E- and P-cadherin expression in podocytes. P-cadherins are the key components of SD, and SD plays an instrumental role in the permselectivity of podocytes. Hence, we hypothesized that the effect of CML on ZEB2 and in turn on P-cadherin expression would manifest as changes in the functioning of the filtration barrier of podocytes. To assess the functional consequence of podocyte exposure to CML, we examined the filtration barrier function of podocytes by a paracellular permeability assay that measures the rate of albumin flux across the monolayer of differentiated podocytes. For this, human podocytes were grown and differentiated on a transwell filter and exposed to CML-KLH (1μg/ml) for 48 h, and the influx of albumin across the transwell membrane was measured. These results revealed a CML-dependent increase in albumin influx across the podocyte monolayer (Fig.5C). To delineate the role of ZEB2 in transducing the effect of CML on the permeability of the podocytes, we next performed paracellular albumin influx assay in ZEB2-/- podocytes. These results indicate that knockdown of ZEB2 expression

resulted in blunting the ability of CML to increase podocyte permeability to albumin (Fig.5C). Hence, these results indicate that CML alters podocytes permeability via ZEB2 and ZEB2 could be a key factor in eliciting both EMT and altered permeability of podocytes by CML.

3.6 CML formation and increased expression of RAGE in diabetic rat kidney linked to EMT and proteinurea: We next investigated the accumulation of CML and expression of RAGE in cortical region of kidney from control and STZ-induced diabetic rats. Both immunoblotting and immunohistochemical data demonstrated that there was an accumulation of CML and increased expression of RAGE in STZ-induced diabetic rat kidneys when compared to control rats (Fig.6A,B&E). Moreover, diabetic rat kidney showed increased expression of mesenchymal markers such as ZEB2 and vimentin and decreased expression of epithelial markers; E-cadherin and nephrin (Fig.6C&D). Further, immunohistochemsitry analysis of diabetic rat kidneys revealed elevated expression of ZEB2 and attenuation of E-cadherin expression in glomerular regions (Fig.6E). The STZ-diabetic rats showed impaired renal function as evidenced by elevated urinary albumin/creatinine ratio (Fig.7A). Immunohistochemical data reveal a decreased podocyte count in STZ-diabetic rat glomeruli (Fig.7B). The data obtained with experimental diabetic rat model imply that CML could lead to proteinurea through induction of EMT.

3.7 Renal genome-wide gene expression reveals an inverse association of RAGE and E-cadherin: To study any association between the expression of RAGE (*AGER*) and E-cadherin (*CDH1*) under normo- and hyperglycemic conditions, we have performed meta-analysis using Nephromine (http://www.nephromine.org). Nephromine is an online search tool of renal genome-wide gene expression data sets and it provides information about both gene expression and specific diseases conditions. We have analysed expression of *AGER* and *CDH1* genes in data set from Hodgin diabetic mouse and it revealed that *AGER* expression increased by 2.02 fold in mice with hyperglycemia (fasting blood glucose 301-600 mg/dL) compared to mice with fasting glucose <300 mg/dL (Fig.8A). On the other hand, *CDH1* expression was decreased by 2.17 fold in hyperglycemic mouse compared with normoglycemic mouse (Fig.8A). Renal genome-wide gene expression revealed an inverse association of *AGER* and *CDH1* in Hodgin Diabetic Mouse.

4. Discussion:

Post-translational modification of both intra- and extra-cellular proteins by non-enzymatic glycation to form AGEs is not only an age-related process, but is also shown to correlate with various complications associated with diabetes and metabolic syndrome [29-32]. Chronic hyperglycemia is an exacerbating factor for enhanced AGE formation as increased levels of AGEs were found in serum samples from diabetic patients and implicated in the pathogenesis of an array of diabetic complications including DN [33-36]. Administration of AGEs into non-diabetic rats induced histological changes and albuminuria that were observed in DN [16, 17]. On the other hand, preventing AGEs formation improved diabetic complications in animal models [18, 19, 37-39]. These studies argue that AGEs are deleterious and are implicated in the etiology of DN. DN is one of the major causes of end-stage renal disease and it is morphologically characterized by reduced podocyte density. However, the specific effect of AGEs on the podocyte biology is largely unknown, and in this study we attempted to investigate the effect of AGEs on the EMT of podocytes. In our study, we found that CML treatment induced promoter activity of both NF-kB and Zeb2 and suppressed the E-cadherin promoter activity. Inhibition of NF-kB activity by MG-132 prevented CML dependent induction of Zeb2 and loss of E-cadherin, suggesting that CML dependent elevated Zeb2 expression is mediated via NF-kB. CML dependent induction of Zeb2 and loss of E-cadherin altered podocyte permeability to albumin whereas shRNA-mediated knockdown of ZEB2 expression abrogated the specific effect of CML to suppress E-cadherin expression in podocytes. We speculate that these actions of CML on EMT of podocytes play a critical role in the pathogenesis of albuminuria of DN (Fig.8B) and preventing AGEs formation could be a desirable therapeutic approach for treating diabetic renal disease.

Podocytes account for about 30% of all glomerular cells. The SD dictates the glomerular permselectivity and is freely permeable to water and small solutes but is a size selective barrier to the passage of large molecules including proteins. Several proteins (nephrin, CD2-associated protein, ZO-1, podocin, P-cadherin) orchestrate the SD structure and enable it to act as a size, charge and shape selective filtration barrier. The podocyte surface is divided into two parts: the apical membrane and the basal membrane, which are above and below the SD, respectively. The apical membrane of podocyte is strongly negatively charged due to the presence of the glycoprotein glycocalyxin. Glycocalyxin repels negatively charged serum albumin and keeps adjacent foot-processes separated from each other [40]. Owing to the significance of podocytes in the glomerular filtration, it is argued that podocytes play a crucial role in the regulation of glomerular function and adequate podocyte count is essential for normal renal function. Thus podocytes exit the cell-cycle to remain terminally differentiated with a quiescent phenotype. It is thus hypothesized that with loss of a critical proportion of podocyte population from the glomerulus, the remaining cells are unable to compensate for glomerular filtration and this results in glomerulosclerosis.

Two mechanisms are proposed to explain the loss of podocytes: apoptosis and detachment. Apoptosis was proposed as a mechanism of podocyte loss and glomerulosclerosis in TGF-β1 transgenic mice, $CD2AP^{-/-}$ mice and puromycin aminonucleoside treated rats [41-43] and it has been argued that ~90% of podocytes detected in urine are apoptotic [8]. However, if this is the case, it is not clear how podocytes from urine could be viable and can be cultured [44]. **Alternatively, a decreased podocyte count could be explained by impaired podocyte adhesion to the GBM**. Elevated expression of anti-adhesive proteins and integrin receptors in DN argues that podocyte detachment could be a mechanism for decreased podocytes count [13, 45-47]. Also, the data from current study provides further evidence for the possibility of podocyte detachment thus reduced podocyte count in diabetic glomeruli. Although, several observations identify podocyte depletion as one of the earliest cellular features of DN [11, 12, 48], the molecular pathways that manifest in decreased podocyte count in DM have only been partially characterized.

Although, AGE mediated generation of reactive oxygen species has been implicated in apoptosis, the direct effect of CML on decreased podocyte density has not been explored. In this study, we demonstrated that podocytes upon exposure to CML showed activation of NF-kB and induced expression of ZEB2. ZEB2, a zinc-finger transcription factor that suppresses E-cadherin expression, orchestrates a series of events in which cell-cell and cell-ECM interactions are altered to release epithelial cells. This phenomenon of EMT is considered as one of the mechanism of podocyte depletion in DN. In this study we showed that CML treatment resulted in decreased E-cadherin expression in dose and time dependent manner and CML induced podocyte EMT as demonstrated by both microscopic methods and biochemical analysis. AGEs were also implicated in pathogenesis of non-diabetic nephropathy such as glomerulosclerosis and secondary focal segmental glomerulosclerosis, and lupus nephritis [49]. In uremia, in the absence of diabetes, a 2-3 fold increase in AGEs has been reported, which indicates that the kidney plays an important role in the accumulation of these compounds [50].

We conclude that CML increases expression of ZEB2 by activation of NF-kB. CML-dependent increase in ZEB2 expression results in EMT via down-regulation of E-cadherin expression thus results in increased podocyte permeability to albumin. We speculate that these actions of CML on EMT of podocytes plays a vital role in podocyte depletion and the pathogenesis of albuminuria during DN. Whereas lessening the load of AGE could be a desirable therapeutic approach for treating diabetic renal disease.

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Author Contribution: The results in the paper were obtained by PAK, GIW and GR. RKM provided ZEB2-/- cells and E-cadherin mutant promoter constructs. PAK, GIW, MAS and GBR planned the experiments. PAK and GBR analysed the data and wrote the paper.

Declarations of interest: The author declares no potential conflict of interests.

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Figure legends:

Figure 1: CML induces ZEB2 expression in podocytes. A- Immortalized differentiated human podocytes were exposed to CML-KLH (0.1- 1.0 μg/ml) for 48 h and cell lysates were prepared and equal amounts of protein fractionated by SDS-PAGE. Immunoblotting was performed for ZEB2 and E-cadherin. Results shown are representative of three independent experiments. **B-** Densitometric quantification of immunoblot (A) analysis and data are mean ± SE (n=4, *denotes p<0.05 *vs* control group). **C-** Immortalized differentiated human podocytes were exposed to 1.0 μg/ml of CML-KLH for varying time periods (0-48 h) and cell lysates were subjected to immunoblottingfor ZEB2, E-cadherin, β-catenin and EpCAM. **D**: Densitometric quantification of immunoblot (C) analysis and data are mean ±SE (n=4, *p<0.05 *vs* control group). **E:** Expression of Snail, Slug, ZEB1 and ZEB2 was measured by qRT-PCR in podocytes treated with or without CML (1 ug/ml) for varying periods (up to 12 h); β-actin expression was used for normalization. Data are mean ± SE; *n*=4. *, *p*<0.05 compared with expression prior to exposure to CML.

Figure 2: CML induces migration of cells. Immortalized differentiated human podocytes were subjected to wound healing assay as detailed in "Experimental Procedures" and exposed to CML-KLH (1.0 μg/ml) for varying periods of time (0-12 h). **A-** Representative images of podocytes treated with or without CML-KLH for 12 h**. B-** Migration of cells was presented as % area covered by cells. Results represent the mean ± SE of four independent experiments carried out in triplicates. **p*<0.05 *vs* % area covered by cells naïve to CML-KLH. **C-** MDA-MB-231 cells were subjected to wound healing assay as detailed in "Experimental Procedures" and exposed to CML-KLH (1.0 μg/ml) for varying periods of time (0-12h). Results represent the mean ± SE of four independent experiments carried out in triplicates. **p*<0.05 *vs* % area covered by cells devoid of CML-KLH.

Figure 3: CML-dependent NF-kB activation in podocytes. A- Immortalized differentiated human podocytes were exposed to CML-KLH (1 μg/ml) for the indicated time periods. Whole-cell protein extracts were prepared and equal amounts of protein was analyzed by SDS-PAGE. Immunoblotting was performed with anti-phospho-IKKα/β, IKBα, p65 NF-kB and corresponding total antibodies. **B-** Cytoplasmic and nuclear fractions of CML-KLH (1.0 μg/ml for 10 min) treated immortalized human podocytes was obtained as detailed in materials and methods. Aliquots of cytoplasmic (Cyto), and nuclear fractions (Nu) were analyzed by immunoblotting using antibodies against phospho p65 NF-kB, total NF-kB, β-actin and, histone H3. **C-** Immortalized human podocytes were treated with CML-KLH (1.0 μg/ml for 10 min) in the absence and presence of NF-kB inhibitor MG-132. Whole-cell protein extracts were prepared and immunoblotting was performed with anti-ZEB2, E-cadherin and β-actin antibodies. **D-** Densitometric quantification of ZEB2 and E-cadherin normalized to β-actin. Expression of these markers in untreated cells considered as one fold. The results are shown as mean ± SE. (n=4, *, P <0.05). **E-** HepG2 cells were transiently transfected with NF-kB promoter luciferase reporter plasmid (pNifty), and an internal control, pRL-TK, expressing the Renilla luciferase. Transfected HepG2 cells were treated with or without CML (1.0 μg/ml) for indicated time periods. Normalized luciferase activity of the HepG2 cells treated with CML is depicted relative to activity of cells treated without CML. Data are mean ± SE (n=4); P < 0.05 *vs* control.

Figure 4: CML-dependent decrease in E-cadherin promoter activity is mediated via ZEB2. A- HepG2 cells were transiently transfected with ZEB2 promoter-reporter luciferase construct and then exposed to CML-KLH (1.0 μg/ml) for 0-24 h. Co-transfection of Renilla luciferase was used to normalize transfection efficiency. The normalized luciferase activity of the cells exposed to CML-KLH is depicted relative to activity of the cells naïve to CML-KLH. Results represent the mean ± SE (n=6); * p<0.05 *vs* cells naïve to CML. **B-** HepG2 cells were transiently transfected with E-cadherin promoter-luciferase

construct and then exposed to the indicated concentration of CML-KLH for 48 h. Co-transfection of Renilla luciferase was used to normalize transfection efficiency. The normalized luciferase activity of the cell exposed to CML-KLH is depicted relative to activity of the cells naïve to CML-KLH. Results represent mean ± SE (n=6); * p<0.05 *vs* untreated cells. **C**-, HepG2 cells were transiently transfected with E-cadherin promoter-luciferase construct with point mutations in the two E2 boxes and exposed to CML-KLH (1.0 μg/ml) for 48 h. Co-transfection of Renilla luciferase was used to normalize transfection efficiency. The normalized luciferase activity for each individual construct exposed to CML-KLH is depicted relative to activity of the respective construct naïve to CML-KLH treatment. Data are mean ± SE (n=6); * p<0.05 *vs* untreated cells.

Figure 5: CML-dependent decrease in P-cadherin expression and increase in permeability of podocytes. A- Immortalized differentiated human podocytes (control and ZEB2 shRNA) were exposed to CML-KLH (500 ng/ml) for 12 h. Whole-cell protein extracts were prepared and analysed by immunoblotting. **B-** Densitometric quantification of P-cadherin normalized to β-actin. Expression of these markers in untreated cells (-CML) considered as one fold. The results are shown as mean ± SE (n=4, *, P <0.05). **C**- Immortalized human podocytes transduced with ZEB2 shRNA and untransduced were grown as a monolayer on collagen-coated Transwell filters and induced to differentiate for 10 days prior to treatment with CML-KLH or vehicle for 24 h, and albumin permeability across the podocyte monolayer was determined at 2, 4 and 6 h. Data are mean ± SE (n=4); *, p<0.05 *vs* cells treated without CML. CML did not alter permeability of podocytes that were transduced with scrambled shRNA (data not shown).

Figure 6: CML accumulation in diabetic rat kidney correlate with increase in ZEB2 expression. A- Renal cortex lysate from vehicle treated rats (Con) or STZ-induced diabetic (Dia) rats were analyzed by immunoblotting using antibodies against CML and RAGE. **B**- Densitometric quantification of CML and RAGE expression normalized to β-actin. Expression CML and RAGE from control rat kidneys considered as one fold. The results are shown as mean ± SE (n=4, *, P <0.05). **C**- Renal cortex lysate from control and STZ-induced diabetic rats was subjected to immunoblotting using antibodies against ZEB2, E-cadherin, β-catenin and nephrin. **D**- Densitometric quantification of ZEB2, E-cadherin, β-catenin and nephrin normalized to β-actin. Expression of these markers from control rat kidneys considered as one fold. The results are shown as mean ± SE (n=4,*,P <0.05). **E**- Immunohistochemical analysis of glomerular expression of ZEB2 and E-cadherin in control or diabetic rats.

Figure 7: Proteinuria is associated with decreased podocyte count: A- Urinary albumin/creatinine ratio was measured from urine collected for 24 h from control (Con) and STZ- induced diabetic (Dia) rats. Data are mean ± SE (n=6), * p<0.05 *vs* control. **B**- Number of podocytes per glomeruli from control (con) and STZ- induced diabetic (Dia) rats were counted from WT-1 stained paraffin sections from control and diabetic rats. Data are mean ± SE (n=6), * p<0.05 *vs* control.

Figure 8: An inverse association of RAGE and E-cadherin expression in diabetic mouse: A- Analysis of RAGE (*AGER*) and E-cadherin (*CDH1*) mRNA expression between control and diabetic Hodgin mouse kidney using Nephromine™. Group 1 with fasting blood glucose 1.0 – 300 mg/dL (n=5) and group 2 with fasting blood glucose 301-600 mg/dL (n=16). *AGER* in group 2 showed 2.02 fold expression over group 1 at p value: 3.88E-7; and *CDH1* in group 2 showed decreased expression (-2.17 fold) over group1. Nephromine (Life Technologies, Ann Arbor, MI) was used for analysis and visualization. **B**-Proposed model for the role of CML in inducing both podocytopathy and proteinuria. The results from our study demonstrated activation of RAGE and NF-kB signaling cascade in podocytes exposed to CML. Expression of ZEB2, the possible target of NF-kB is elevated in cells treated with CML; concurrently the expression of E- and P-cadherin is reduced.

Fig. 1

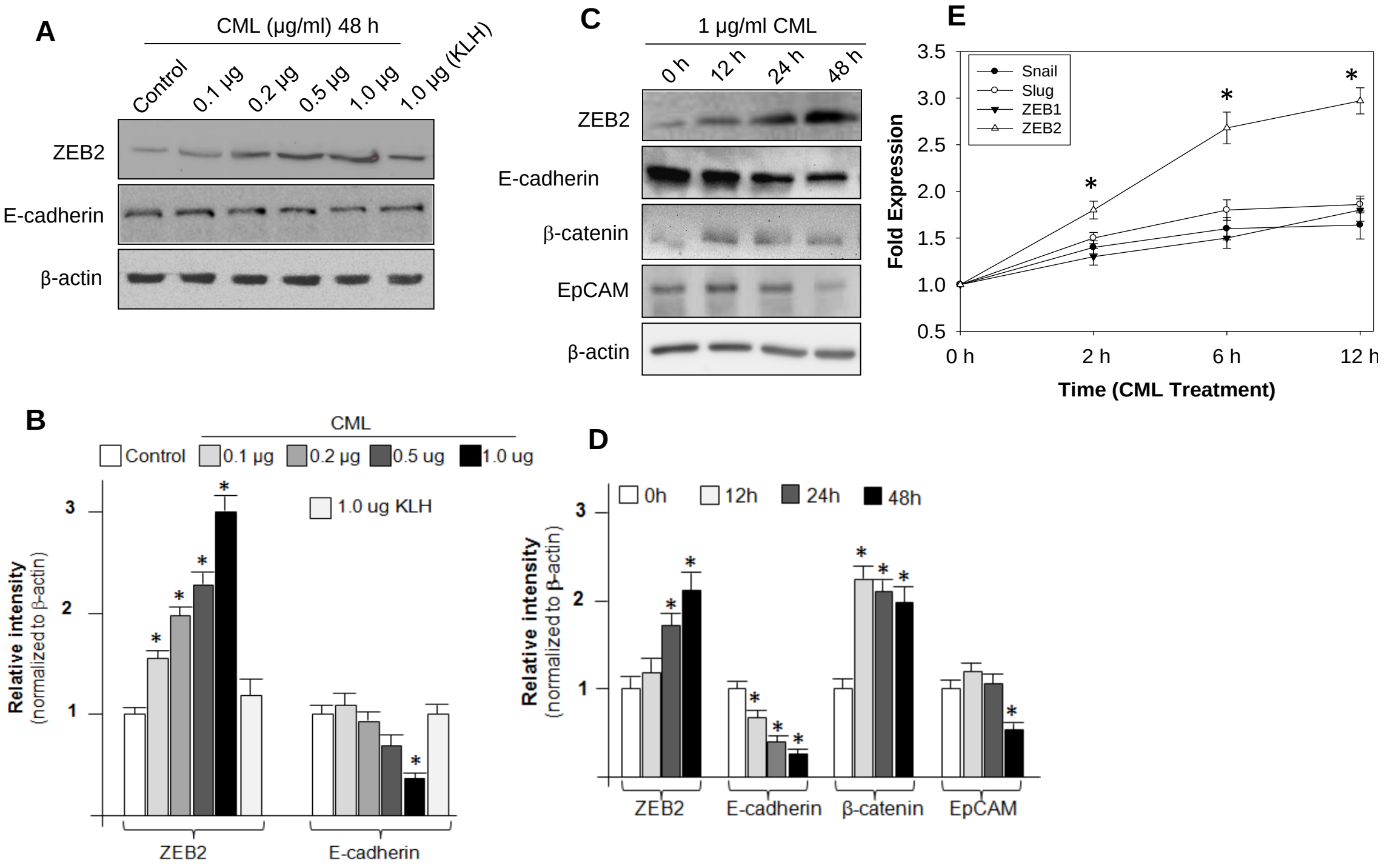

A
CML (μg/ml) 48 h
Control
0.1 μg
0.2 μg
0.5 μg
1.0 μg
1.0 μg (KLH)
ZEB2
E-cadherin
β-actin
C
1 μg/ml CML
0 h
12 h
24 h
48 h
ZEB2
E-cadherin
β-catenin
EpCAM
β-actin
E
Snail
Slug
ZEB1
ZEB2
Fold Expression
Time (CML Treatment)
0 h
2 h
6 h
12 h
B
CML
Control
0.1 μg
0.2 μg
0.5 ug
1.0 ug
1.0 ug KLH
Relative intensity
(normalized to β-actin)
ZEB2
E-cadherin
D
0h
12h
24h
48h
Relative intensity
(normalized to β-actin)
ZEB2
E-cadherin
β-catenin
EpCAM

Fig. 2

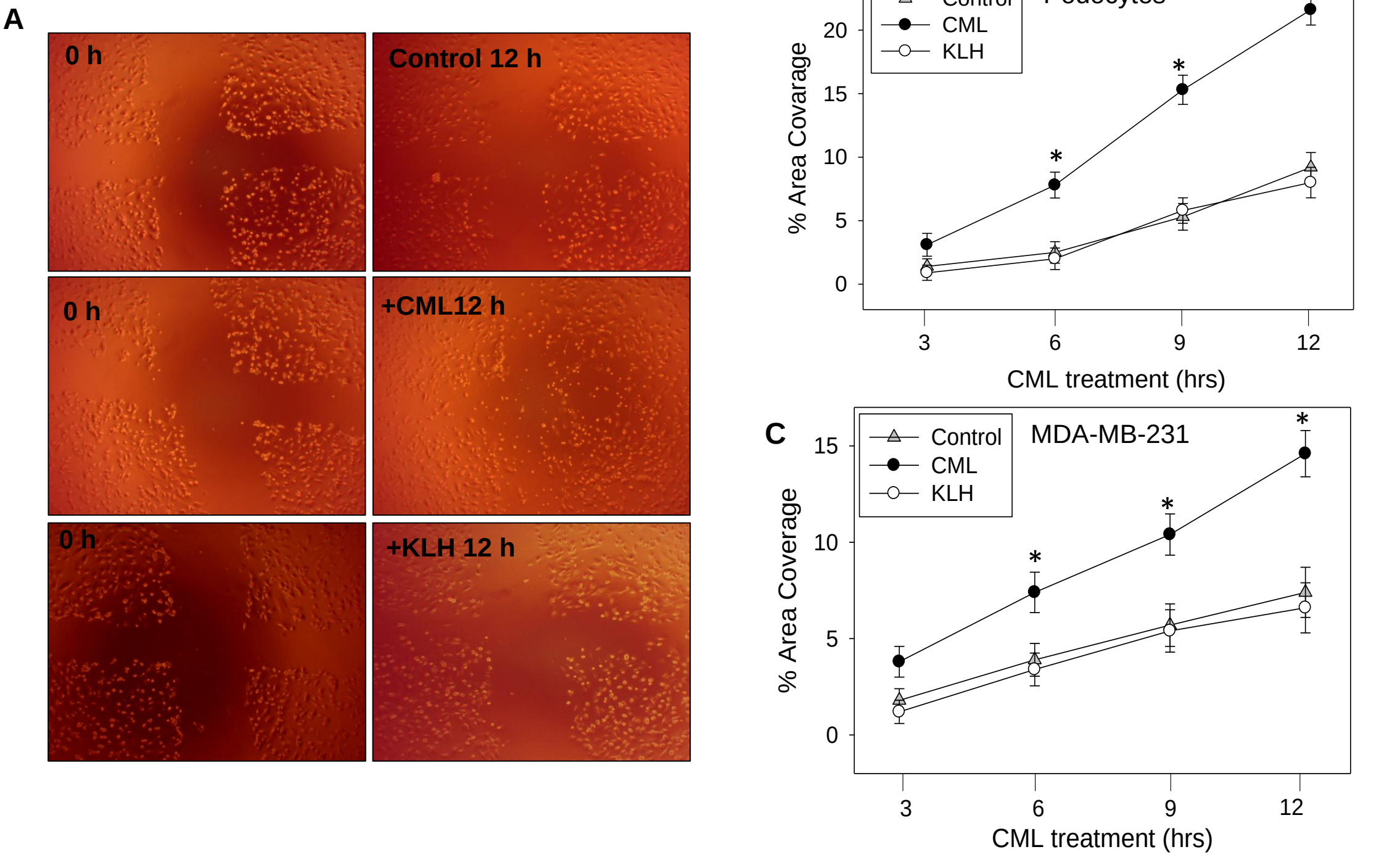

A
0 h
Control 12 h
0 h
+CML12 h
0 h
+KLH 12 h
B
Podocytes
Control
CML
KLH
% Area Covarage
CML treatment (hrs)
C
MDA-MB-231
Control
CML
KLH
% Area Coverage
CML treatment (hrs)

Fig. 3

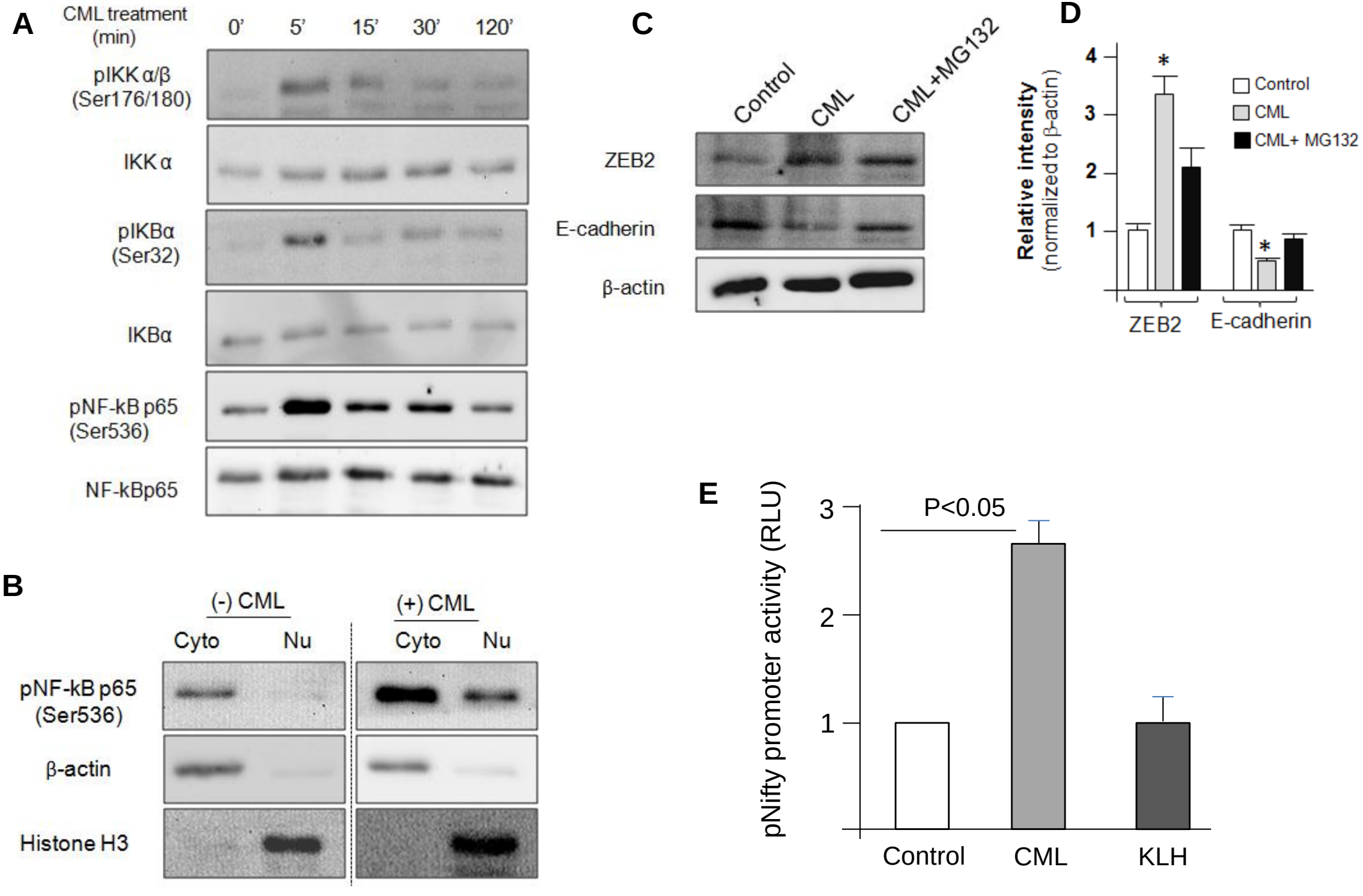

A
CML treatment (min)
0'
5'
15'
30'
120'
pIKK α/β (Ser176/180)
IKK α
pIKBα (Ser32)
IKBα
pNF-kB p65 (Ser536)
NF-kBp65
B
(-) CML
(+) CML
Cyto
Nu
Cyto
Nu
pNF-kB p65 (Ser536)
β-actin
Histone H3
C
Control
CML
CML+MG132
ZEB2
E-cadherin
β-actin
D
Relative intensity (normalized to β-actin)
4
3
2
1
*
*
Control
CML
CML+ MG132
ZEB2
E-cadherin
E
pNifty promoter activity (RLU)
3
2
1
P<0.05
Control
CML
KLH

Fig. 4

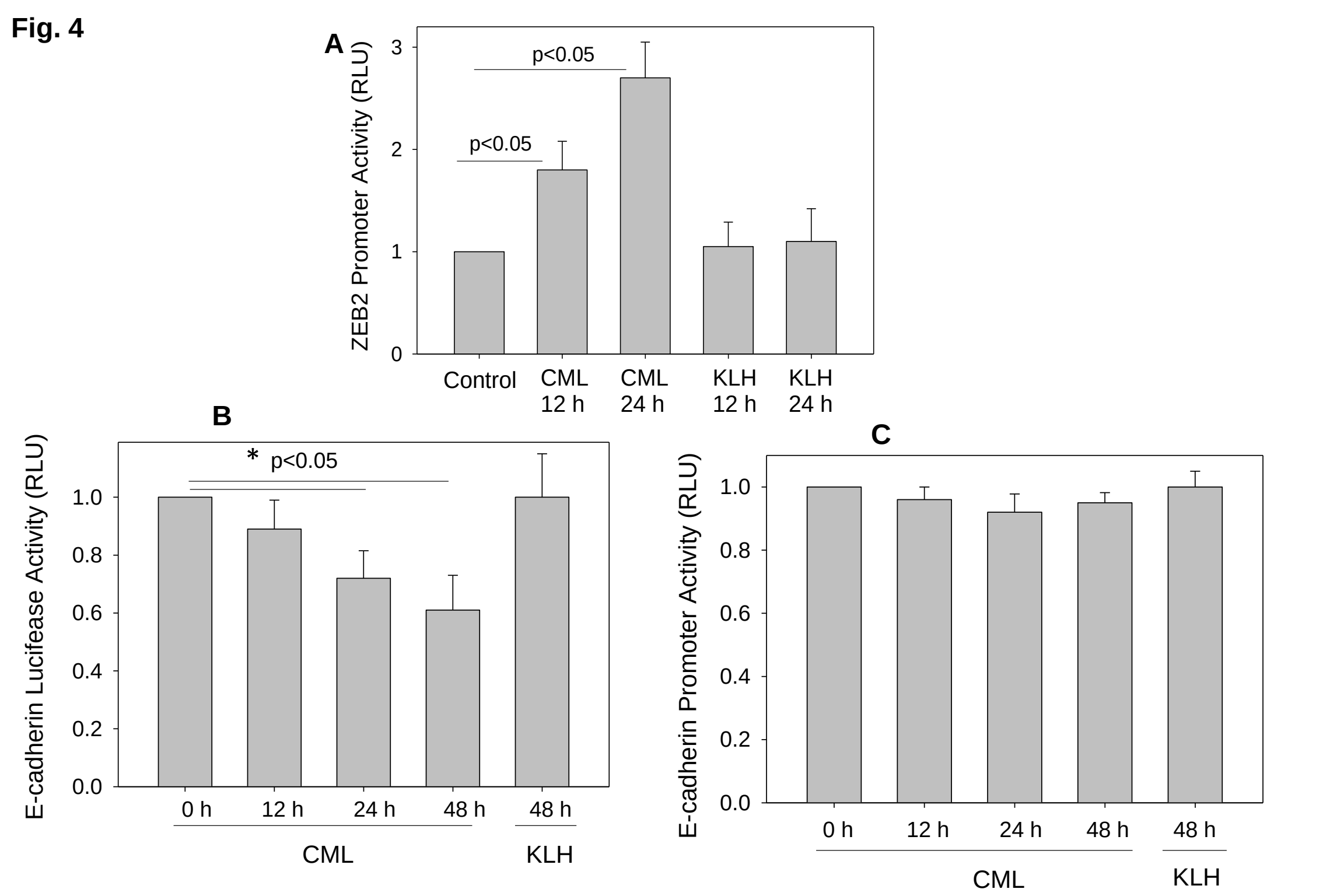

A
ZEB2 Promoter Activity (RLU)
p<0.05
p<0.05
0
1
2
3
Control
CML 12 h
CML 24 h
KLH 12 h
KLH 24 h
B
E-cadherin Lucifease Activity (RLU)
* p<0.05
0.0
0.2
0.4
0.6
0.8
1.0
0 h
12 h
24 h
48 h
48 h
CML
KLH
C
E-cadherin Promoter Activity (RLU)
0.0
0.2
0.4
0.6
0.8
1.0
0 h
12 h
24 h
48 h
48 h
CML
KLH

Fig. 5

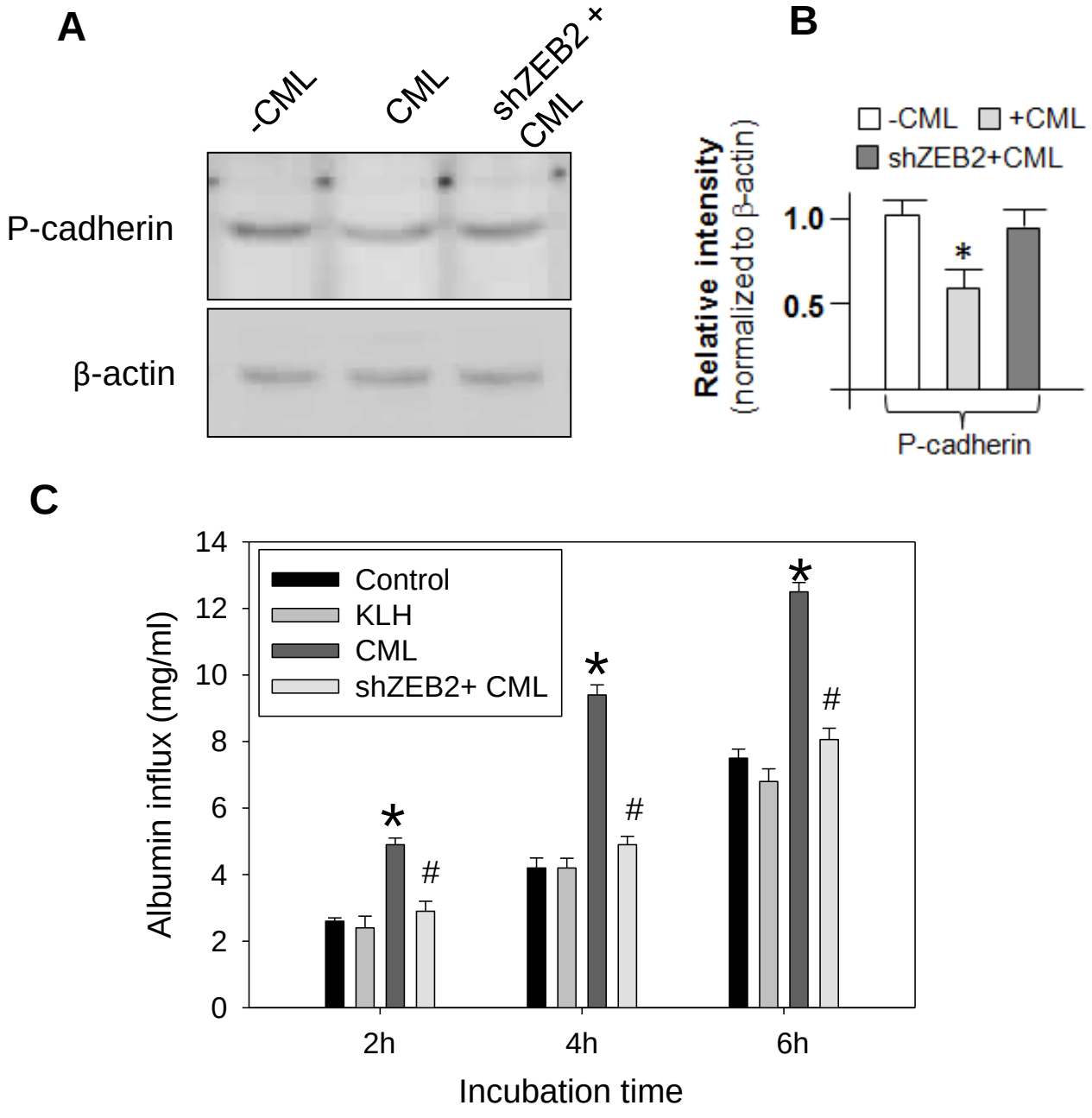
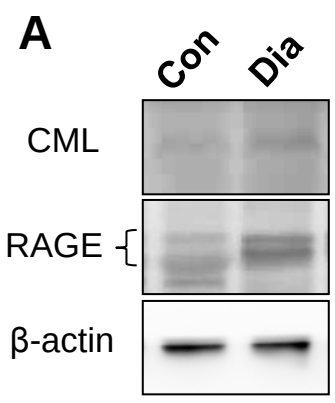
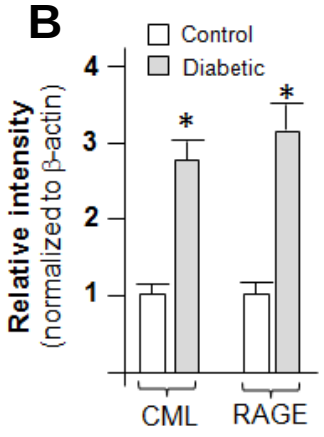
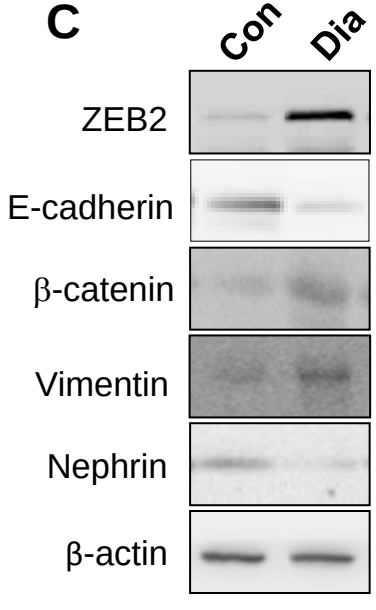
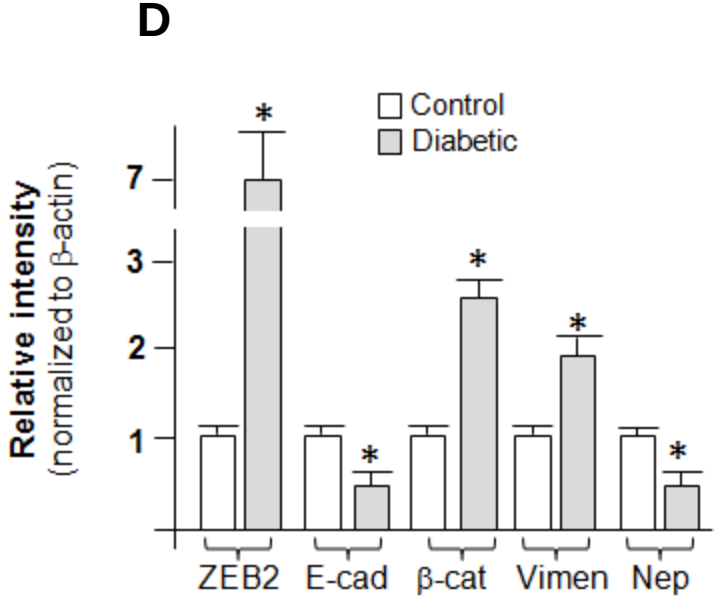

A
-CML
CML
shZEB2 + CML
P-cadherin
β-actin
B
-CML
+CML
shZEB2+CML
Relative intensity
(normalized to β-actin)
1.0
0.5
*
P-cadherin
C
Control
KLH
CML
shZEB2+ CML
Albumin influx (mg/ml)
14
12
10
8
6
4
2
0
*
#
2h
4h
6h
Incubation time

Fig. 6


A
Con
Dia
CML
RAGE
β-actin


B
Control
Diabetic
Relative intensity
(normalized to β-actin)
4
3
2
1
*
*
CML
RAGE


C
Con
Dia
ZEB2
E-cadherin
β-catenin
Vimentin
Nephrin
β-actin


D
Control
Diabetic
Relative intensity
(normalized to β-actin)
7
3
2
1
*
*
*
*
*
ZEB2
E-cad
β-cat
Vimen
Nep

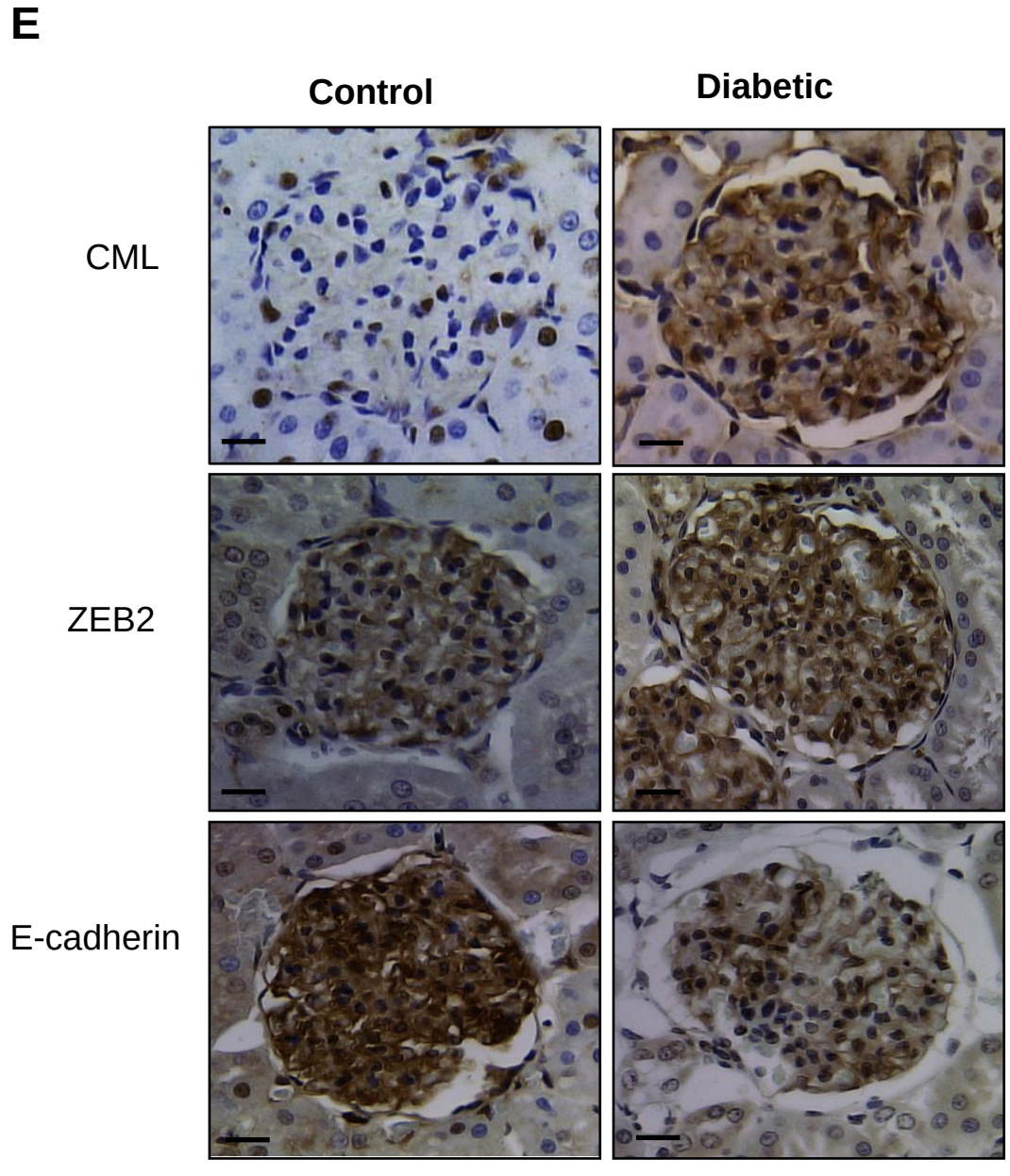

E
Control
Diabetic
CML
ZEB2
E-cadherin

Fig. 7

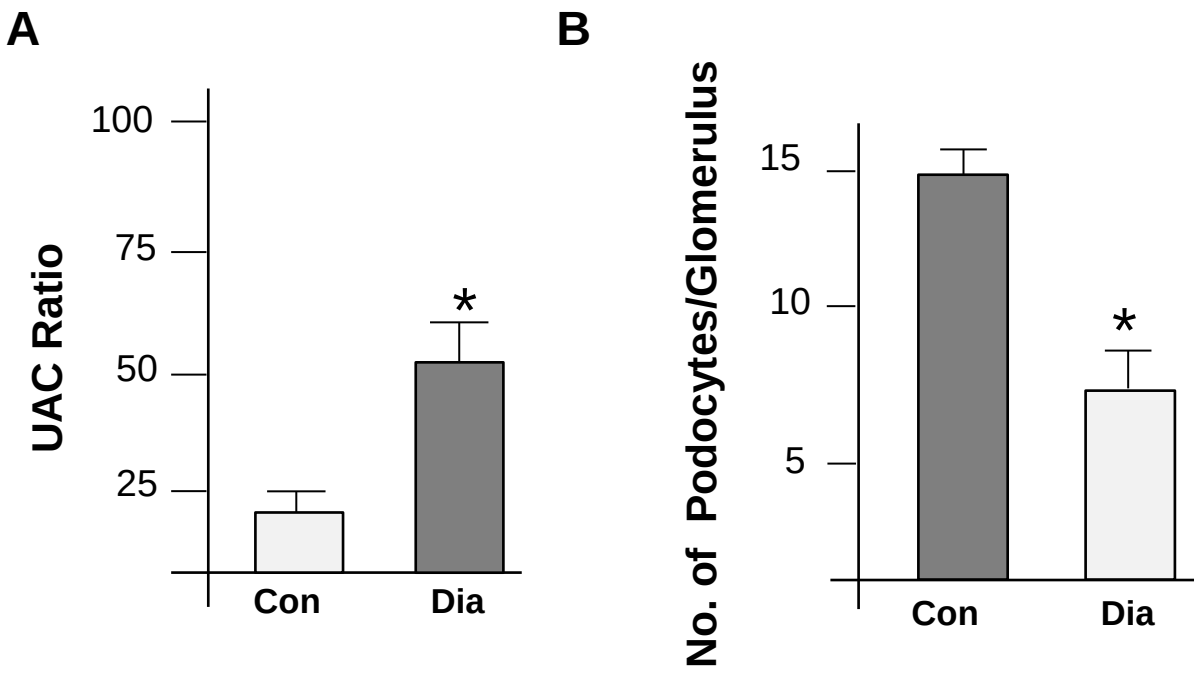
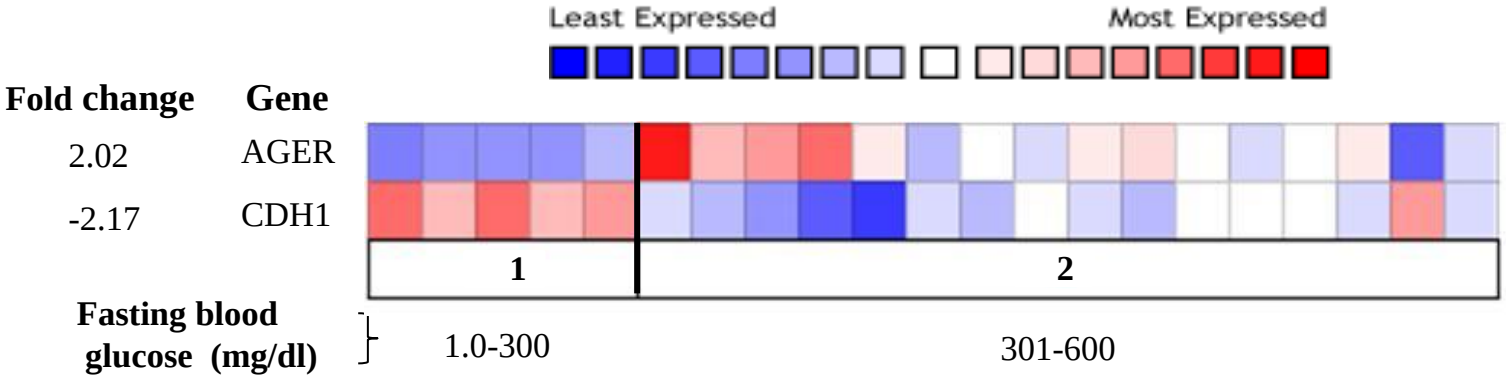
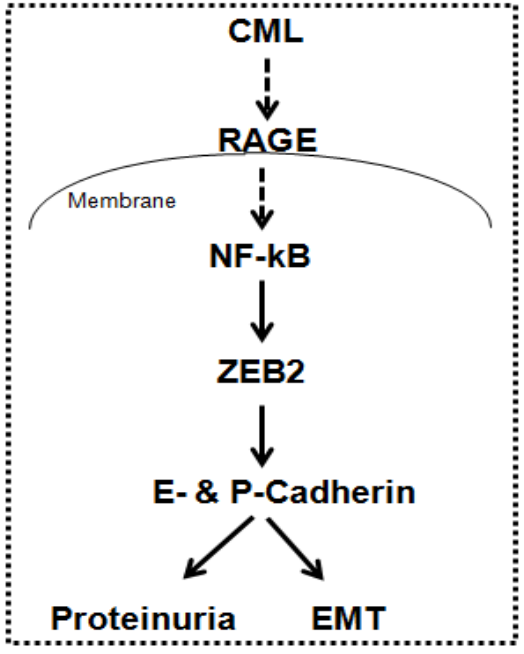

A
UAC Ratio
100
75
50
25
*
Con
Dia
B
No. of Podocytes/Glomerulus
15
10
5
*
Con
Dia

Fig. 8

A


Least Expressed
Most Expressed
Fold change
Gene
2.02
AGER
-2.17
CDH1
1
2
Fasting blood glucose (mg/dl)
1.0-300
301-600

B


CML
RAGE
Membrane
NF-kB
ZEB2
E- & P-Cadherin
Proteinuria
EMT