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Balance between apoptosis or survival induced by changes in extracellular-matrix composition in human mesangial cells: a key role for ILK- NF*k*B pathway

María del Nogal[1,2,3], Alicia Luengo[1,2,3], Gemma Olmos[1,2,3], Marina Lasa[4], Diego Rodriguez–Puyol[2,3,5,6], Manuel Rodriguez–Puyol[1,2,3] and Laura Calleros[1,2,3,&]

(1) Department of Physiology, Facultad de Medicina, Universidad de Alcalá, Campus Universitario s/n, Alcalá de Henares, 28871 Madrid, Spain

(2) IRSIN, Madrid, Spain

(3) REDinREN (Instituto de Salud Carlos III), Madrid, Spain

(4) Departamento de Bioquímica–Instituto de Investigaciones Biomédicas Alberto Sols, Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, Madrid, Spain

(5) Department of Medicine, Universidad de Alcalá, Madrid, Spain

(6) Nephrology Section and Research Unit Foundation, Hospital Universitario Príncipe de Asturias, Alcalá de Henares, Madrid, Spain

(&) Corresponding autor: e-mail: laura.calleros@uah.es

Abstract

Renal fibrosis is the final outcome of many clinical conditions that lead to chronic renal failure, characterized by a progressive substitution of cellular elements by extracellular-matrix proteins, in particular collagen type I. The aim of this study was to identify the mechanisms responsible for human mesangial cell survival, conditioned by changes in extracellular-matrix composition. Our results indicate that collagen I induces apoptosis in cells but only after inactivation of the pro-survival factor NF*k*B by either the super-repressor I*k*Bα or the PDTC inhibitor. Collagen I activates a death pathway, through ILK/GSK-3*b*-dependent Bim expression. Moreover, collagen I significantly increases NF*k*B-dependent transcription, I*k*Bα degradation and p65/NF*k*B translocation to the nucleus; it activates *b*1 integrin and this is accompanied by increased activity of ILK which leads to AKT activation. Knockdown of ILK or AKT with small interfering RNA suppresses the increase in NF*k*B activity. NF*k*B mediates cell survival through the anti-apoptotic protein Bcl-xL. Our data suggest that human mesangial cells exposed to abnormal collagen I are protected against apoptosis by a complex mechanism involving integrin *b*1/ILK/ AKT-dependent NF*k*B activation with consequent Bcl-xL overexpression, that opposes a simultaneously activated ILK/ GSK-3*b*-dependent Bim expression and this dual mechanism may play a role in the progression of glomerular dysfunction.

Keywords: Apoptosis · NF*k*B · ILK · Cell–matrix- interactions · Collagen · Renal fibrosis

Abbreviations: HMC, Human mesangial cells; ECM, Extracellular matrix; COL I, Collagen type I; COL IV, Collagen type IV; NF*k*B, Nuclear factor *k*B; I*k*B Inhibitor of NF*k*B; ILK, Integrin-linked kinase; GSK-3*b,* Glycogen synthase kinase-3 *b.*

Introduction

Fibrotic diseases are characterized by the presence of a fibrous connective tissue excess in organs such as the lungs, liver, heart or kidneys, as a result of a reparative or reactive process [1]. Renal fibrosis is an organ fibrosis example in which the progressive renal function loss that characterizes primary diseases such as glomerulonephritis, diabetes, urinary tract obstruction or chronic rejection of transplanted kidneys [2–4] is linked to the extracellular matrix (ECM) protein accumulation in different renal structures including mesangium and interstitium. Glomerular mesangial cells seem to play a key role in the genesis of glomerular sclerosis [5].

Progressive cellular elements substitution by ECM proteins, in particular an excessive deposition of abnormal collagens, is the consequence of an imbalance in the rate of cell proliferation, necrosis, and apoptosis [6], as well as the loss of the normal equilibrium between the synthesis and degradation of ECM [7]. Adhesion to the correct type of ECM is essential for the proliferation, differentiation, and survival of most cells [8, 9]. ECM-dependent survival is mediated by integrins, a family of heterotrimeric, trans- membrane receptors, which bind to ECM components and transmit signals through a broad range of signaling protein [10]. One of these signaling proteins is integrin-linked kinase (ILK), which can directly interact with the cytoplasmic domain of the *b*1 and *b*3 integrin subunits [11]. This interaction increases the ILK kinase activity and then phosphorylates and inhibits glycogen synthase kinase-3 (GSK-3) [12]. GSK-3 is not the only substrate for ILK. This kinase also phosphorylates protein kinase B (PKB/ AKT) [13], a mechanism that has been implicated in integrin-mediated survival and gene expression regulation through the activation of transcription factors such as the nuclear factor *k*B (NF*k*B) [14, 15].

NF*k*B is a dimeric complex which is activated by pro- inflammatory extracellular signals and cellular stress, resulting in the transcriptional regulation of several hundreds of cellular genes related to immunity system, inflammation, cell proliferation, differentiation, and apoptosis [16]. In the canonical pathway, NF*k*B function is regulated by a cellular process that involves phosphorylation and degradation of its inhibitory protein I*k*Bα, allowing the translocation of active

NF*k*B complexes to the nucleus and transcriptional activation [17]. The NF*k*B-dependent pro-survival effect has been observed by the ability of this transcription factor to regulate the expression of various anti-apoptotic proteins, like several members of both the *inhibitor of apoptosis (iap)* family and at least three anti-apoptotic members of the bcl-2 gene family: Bcl-2, Bcl-xL, and Mcl-1 [18, 19].

A number of studies link NF*k*B activation to both human and experimental kidney disease [20, 21]. NF*k*B activation has been found in vivo and in vitro in intrinsic glomerular cells such as podocytes and mesangial, tubular and endothelial cells as a result of either renal injury or exposure to inflammatory stimuli. In addition, it was observed that the activation of NF*k*B plays a role in various primary and chronic kidney diseases associated with inflammation and fibrosis [22, 23]. Furthermore the deregulation of NF*k*B-mediated cell survival signals may contribute to renal disease [22, 24, 25]. On the other hand, recent data from both animals and humans have documented the potential reversibility of glomerulosclerosis, and have examined the mechanisms of this glomerular remodeling, considering that NF*k*B could be one of these mechanisms [26].

With these precedents, in the present study we hypothesize that the qualitative changes in the ECM composition observed in glomerulosclerosis, especially those related to collagen type I (COL I) accumulation may play a role in the progression of glomerular dysfunction, through the modulation of the balance between cell apoptosis or survival of human mesangial cells (HMC). We analyzed the possible mechanisms involved in the COL I activated pro-apoptotic pathways and the role of NF*k*B in this process. We demonstrate for the first time that COL I exerts a complex role on apoptosis in HMC, as it simultaneously activates a pro-apoptotic pathway by induction of Bim protein, and an anti-apoptotic mechanism, consisting of the up-regulation of Bcl-xL expression through the ILK/AKT/ NF*k*B signaling pathway.

Materials and methods

Experimental Design

In studies with soluble collagens, cells were grown to 90 % confluence during 3 days and then treated with 12.5 μg/ml COL I or COL IV at several times. For inhibition experiments, cells were preincubated with the corresponding inhibitor for 1 h and continuously exposed to COL I or COL IV plus inhibitor. Studies with COL I- or COL IV- coated plates were performed in cells that were cultured on a thin film of COL I or COL IV. For this, culture plates were incubated for 16 h at 4 ºC in a solution of 12.5 μg/ml COL I or COL IV in bicarbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$ [pH 9]) to allow the formation of a thin film of collagen, as described [27]. Then, the remaining collagen was discarded and Petri dishes were washed with Hanks' balanced salt solution to restore the pH. Cells were grown for the indicated times.

Quantification of sub-G1 DNA content by flow cytometry

Quantification of apoptotic cells was carried out by measurement of the fraction of cells with sub-G1 DNA content by flow cytometry analysis after propidium iodide (PI)- staining. Adherent and floating cells were collected after treatment, washed with ice-cold PBS, and fixed with 70 % ice-cold ethanol (30 min, 4 ºC). Fixed cells were washed twice with PBS and treated with RNAse (1 mg/ml; 30 min, 37 ºC). After staining of cellular DNA with 5 ng/ml PI dissolved in PBS, cells were analyzed on a FACScan flow cytometer (Becton–Dickinson, New Jersey, USA). Percentages of cells in different cell cycle phases were calculated from DNA histograms. Cells with sub-G1 DNA content were considered apoptotic.

Assessment of apoptosis by Annexin V and PI staining

Apoptosis was also detected by Annexin V-FITC Apoptosis Detection Kit (Calbiochem, La Jolla, CA, USA). Briefly, cells were double stained with annexin V-FITC and propidium iodide (PI) following manufacturer's instruction. Staining was measured by flow cytometry on the FACScan and the distribution of cells was analyzed using CellQuest™ software (Becton–Dickinson, New

Jersey, USA). Data from 10,000 cells was collected for each data file. Early apoptosis was defined as Annexin V-FITC-positive and PI-negative cells, and late apoptosis was defined as double positive Annexin V-FITC/PI staining.

NFkB reporter assay

Cells were plated 24 h before transfection. The cells in every well were then incubated 8 h with 1 ml Opti-MEM (Invitrogen, CA, USA) containing complexes of 5 μl LipofectAMINE (Invitrogen, CA, USA), 1.0 μg of the 3x NF*k*B-TK-Luc reporter plasmid and 0.2 μg renilla luciferase reporter as an internal control as previously described [28]. Next, the transfected cells were incubated with complete growth medium for 16 h and then were treated. Finally, firefly luciferase activity was measured with a Lumat LB9506 luminometer (Berthold Technologies, Herts, UK) and normalized against the renilla luciferase activity by using the dual-luciferase reporter assay system (Promega, Madison, WI, USA). Luciferase activity is represented as relative luciferase units of firefly per renilla.

siRNA transfection

To deplete expression of AKT or ILK protein by specific siRNA, oligonucleotides cells were seeded 24 h before transfection. Cells were transfected in 1 ml of OPTIMEM with 100 nM siRNA or Silencer™ negative control (Scrambled RNA) using Lipofectamine. After 8 h incubation with the RNA-complex, 1 ml of medium containing FBS was added. The cells were treated as indicated.

ILK activity measurement

ILK kinase activity in HMC extracts was determined by immunoprecipitation in vitro kinase assay [29]. Briefly 300 μg of extracted cell proteins were immunoprecipitated with an anti-ILK antibody. GSK-3*b* fusion protein was used as the ILK substrate and ATP was the phosphate donor. Proteins were run in 12 % SDS-PAGE gels. Both, the ReliaBLOT® Block and the ReliaBLOT® HRP Conjugate were used for membrane blotting and antibodies incubation following the protocol

provided by the manufacturer. Immunoreactive products were detected as previously described and after autoradiography, the phosphorylated GSK-3*b* protein was quantified by densitometric analysis with Scion Image software.

Data analysis and statistical procedures

All values are presented as mean ± standard error of the mean (SEM). All experiments were repeated a minimum of three times, and the number of experiment is provided in the legends of the figures. Comparisons between groups were performed by using non-parametric statistics (depending on the number of groups and the paired or unpaired nature of data, different test were selected). A *p* value of 0.01 was considered significant.

Results

NFkB pathway protects from COL I-induced apoptosis in HMC cells

In glomerulosclerosis, an abnormal remodeling of the ECM occurs, and normal components, such as collagen type IV (COL IV), are substituted by unusual components, such as COL I [30]. To evaluate the effects of changes in the ECM composition on the apoptosis of HMC, we analyzed the sub-G_1 hypodiploid cell population by flow cytometry. Cells were incubated with COL I or COL IV for 24–96 h. Furthermore, to inhibit NF*k*B activation we used two methods: addition of the inhibitor PDTC for 1 h or cell transfection with a plasmid encoding a phosphorylation- resistant I*k*Bα mutant (I*k*Bα $^{Ser32,36/Ala}$), before 48 h treatment. No changes were observed in the apoptosis of HMC treated with either COL I or COL IV. Interestingly, apoptosis was significantly increased after treatment with COL I in cells overexpressing the super-repressor I*k*Bα $^{Ser32,36/Ala}$ or treated with PDTC with about 30 % of cells becoming apoptotic (Fig. 1a). By contrast, no changes in apoptosis were observed in control cells or in cells treated with COL IV, regardless of the presence of I*k*Bα $^{Ser32,36/Ala}$ or PDTC (Fig. 1a).

These results were verified by both examination of the cleavage of the caspase substrate PARP and determination of the level of phosphatidyl serine exposed outside the cell membrane by labeling with Annexin V-FITC. An increased PARP cleavage (Fig. 1b) and annexin V-labeled cell percentage (36 %) (Fig. 1c) was observed only in cells treated 24 h with COL I in which NF*k*B was inhibited with PDTC, without changes in other cell conditions. These findings suggest that COL I activates simultaneously a death and an NF*k*B-dependent survival pathway in HMC.

COL I induces an NFkB-dependent survival pathway. COL I regulates NF*k*B-*dependent transcription,* I*k*Bα *degradation and p65/* NF*k*B *translocation to the nucleus*

To investigate the effect of COL I on NF*k*B-dependent transcription, cells were transfected with a 3xNF*j* B-TK-Luc reporter plasmid. Cells were then incubated for 24 or 48 h with COL I, COL IV, COL I plus I*k*Bα $^{Ser32,36/\ Ala}$ or COL I plus PDTC, and NF*k*B-dependent transcription was measured. A significant increase in NF*k*B dependent transcription was observed in COL I-treated cells, when compared to either control or COL IV-treated cells. As expected, the addition of PDTC or the cotransfection with I*k*Bα $^{Ser32,36/Ala}$ reversed the activation of NF*k*B-dependent transcription induced by COL I (Fig. 2a). We next studied, by immunoblotting analysis, the kinetics of I*k*Bα degradation in cytosolic extracts from cells incu- bated with either COL I or COL IV. I*k*Bα levels decreased after 2–24 h of incubation with COL I (Fig. 2b). This should be accompanied by an increase in the translocation of the p65/NF*k*B subunit to the nucleus. As expected, we detected by western blotting that the amount of p65/NF*k*B in the nucleus from cells incubated with COL I for 2–24 h was higher than the amount in cells incubated with COL IV (Fig. 2c). These data were confirmed by confocal microscopy experiments, where the images revealed an increase in the nuclear p65/NF*k*B in COL I-incubated cells when compared with either control or COL IV-treated cells (Fig. 2d).

b1 Integrin and ILK play a role in the activation of NF*k*B- *dependent transcription by COL I*

To analyze the mechanism by which COL I induced NF*k*B transcriptional activity, we tested whether COL I activated NF*k*B via binding to specific integrins. Interaction of *b*1 integrin with their ligands (e.g., COL I) induces a conformational switch that is often linked to the initiation of a signaling cascade involving ILK [31]. Our data show that the expression of the activation epitope of *b*1 integrin (Fig. 3a), as well as the in vitro ILK activity measured as GSK-3*b* fusion protein phosphorylation (Fig. 3b), were higher in HMC treated for 2–4 h with COL I than in COL IV-treated cells. At these times, no changes were observed in ILK cellular content (Fig. 3b). Both, a blocking antibody against *b*1 integrin (Fig. 3c) and ILK depletion with a specific siRNA (Fig. 3d) substantially inhibited the NF*k*B activation induced by COL I.

AKT protein is involved in COL I-mediated induction of both NF*k*B-*dependent transcription and protection from apoptosis*

ILK has been shown to directly activate AKT through phosphorylation on Ser473 [32]. To test the involvement of AKT in the effect of COL I, cells were plated with different collagen proportions for 24 h. As shown in Fig. 4a, a significant increase in AKT phosphorylation at Ser473 was observed when COL I proportion was augmented. The implication of AKT was confirmed in AKT-abrogated cells with a specific siRNA, in which the COLI-induced NF*k*B-dependent transcription activation was significantly reduced (Fig. 4b). To demonstrate the importance of ILK as an upstream mediator of this effect, HMC were depleted of ILK with specific siRNA oligonucleotides. ILK depletion completely blocked the increase in AKT phosphorylation induced by COL I (Fig. 4c). Knock-down AKT with specific siRNA promoted an increase of apoptosis in COL I-treated cells (Fig. 4d), supporting the specific involvement of this protein in the protective effect of NF*k*B from the COL I-induced apoptosis. Taken together, these data suggest that the up-regulation of the ILK/AKT/NF*k*B pathway by COL I activate a mechanism that protects from the apoptosis induced by COL I in HMC.

*COL I induces Bcl-xL expression via activation of ILK/AKT/*NF*k*B *pathway*

To determine which anti-apoptotic protein was involved in the NF*k*B mediated protection, we analyzed the expression of some of them in HMC exposed to collagens. There was a potent induction of Bcl-xL expression after 8 h of COL I treatment, when compared to either control or COL IV-treated cells (Fig. 5a). By contrast, the expression of Bcl-2, x-IAP or c-IAP was not modified (Online Resource 2). To test whether Bcl-xL induction was NF*k*B-dependent, we overexpressed the I*k*Bα $^{Ser32,36/Ala}$ and determined Bcl- xL levels after incubation with collagens. The induction of Bcl-xL by COL I was completely inhibited in I*k*Bα $^{Ser32,36/Ala}$ expressing cells (Fig. 5a), as well as in cells pretreated with the NF*k*B inhibitor PDTC (Fig. 5b). ILK/AKT were also involved in the COL I-induced increase of Bcl-xL expression, since depletion of these proteins with specific siRNAs completely reversed COL I-induced Bcl-xL over- expression (Fig. 5c, d).

COL I induces an ILK–GSK-3b pro-apoptotic pathway. COL I increases the cellular content of Bim

To analyze the mechanism by which COL I induces apoptosis, we first analyzed the collagens effect on two well-known pro-apoptotic proteins, Bim and Bax. Bim is a well characterized pro-apoptotic protein induced by GSK-3*b* inhibition [33] and the ILK-dependent GSK-3*b* phosphorylation inhibits the activity of this protein. The treatment of HMC with COL I induced a rapid and sustained increase in Bim expression that was not observed in cells treated with COL IV (Fig. 6a). In these conditions, no changes were observed in Bax expression.

GSK-3b protein is involved in COL I-mediated induction of Bim

To test this hypothesis, the ability of COL I to phosphorylate endogenous GSK-3*b* and the dependence of this effect on ILK were first evaluated. The results indicate that COL I increased

the GSK-3*b* phosphorylation at Ser9 in a dose- and time-dependent manner (Fig. 6b). A marked reduction of COL I-induced GSK-3*b* phosphorylation was observed after depletion of ILK with specific siRNA (Fig. 6c). The effect of COL I on Bim was mimicked by the addition to the cells of LiCl, a GSK-3*b* inhibitor (Fig. 6d) and by the depletion of ILK expression (Fig. 6e).

Taken together, our data suggest that HMC are protected against apoptosis induced by changes in ECM composition, especially COL I, by a mechanism involving both *b*1 integrin and ILK signaling-dependent AKT and NF*k*B activation with consequently augmented expression of Bcl-xL, a compensatory pro-survival mechanism that opposes ILK/ GSK-3*b*/Bim pro-apoptotic pathway.

Discussion

In recent years, our group has demonstrated that HMC cultured on COL I synthesize increased amounts of prof- ibrotic factors and they also produce more collagens and fibronectin [34]. Moreover, we have previously demonstrated that soluble COL I induces the proinflammatory COX-2 protein expression in HMC [35]. Here, we focused in the apoptotic and anti-apoptotic balance, which is important in the resolution of mesangial proliferative glomerulonephritis and in the progression of glomerular sclerosis, and in the abnormal ECM regulation of this balance. This could be relevant in situations in which the progression or the resolution of renal diseases could be dependent on the normal regulation of apoptosis.

COL I did not modify the apoptosis rate of HMC. In fact, the results observed when cells contacted with this protein were comparable to those observed in standard plastic cultures and in cells incubated with COL IV. However, the anti-apoptotic effect of the transcription factor NF*k*B has been widely defined [18, 19] and numerous articles summarized the role of NF*k*B activation in the development of renal fibrosis. Inhibition of NF*k*B signaling effectively attenuates renal injury in experimental animals with unilateral urethral obstruction (UUO), hypertension, subtotal

nephrectomy, protein-overload, angiotensin II infusion or FK506 nephropathy [36–41]. In these studies, agents such as pyrrolidine dithiocarbamate, hepatocyte growth factor, parthenolide or renin-angiotensin system inhibitors were used to inhibit renal NF*k*B activation. Other reports provided similar information with a more specific NF*k*B pathway blockade methodology. Inoue et al. [42] reported that fibroblast expression of an I*k*B dominant-negative transgene attenuates renal fibrosis and Henke et al. [43] generated Cre/lox transgenic mice with endothelial cell-restricted NF*k*B super-repressor I*k*Bα DN overexpression, in which the hypertension- induced renal damage was attenuated. For these reasons, apoptosis was studied in cells incubated with different ECM proteins, in which NF*k*B activity was inhibited. In these conditions, COL I was a potent pro-apoptotic stimulus, suggesting the presence of a dual mechanism of apoptosis regulation in HMC incubated with COL I. The survival pathway seems to be dominant over the apoptotic one, since substantial cell death takes place only after inactivation of NF*k*B. Similar findings were demonstrated in vascular cells incubated with COL I fragments [44] but the present study shows for the first time that the dual effect depends on the nature of the ECM component, in our case COL I but not COL IV. Interestingly, as shown in the ‘‘Results’’ section, even different proportions of both collagens may induce differential effects in the cells.

The mechanism involved in cell survival in the presence of COL I has been studied in detail. We have identified *b*1 integrin, ILK and AKT as being responsible for NF*k*B activation. The implication of these proteins in the COL I-induced NF*k*B activation is supported by two kinds of results: (a) the analysis of protein activation in the presence of COL I, and (b) the consequences of the blockade of a particular protein, with specific antibodies or siRNAs, on the COL I-dependent downstream activation of other pro- teins. According to previous results from our group in HMC [34], *b*1 integrin mediates the COL I-induced ILK activation. In turn, ILK phosphorylates AKT and this activates NF*k*B, through the degradation of I*k*Bα, thereby allowing p65/NF*k*B nuclear translocation. The implication of AKT in the NF*k*B-mediated cellular survival is demonstrated by the increase of the apoptosis in AKT-depleted cells. The role of ILK, AKT and NF*k*B activation in survival pathways has

been extensively described [18, 45, 46]. On the other hand, we have found that the Bcl-xL protein levels are significantly increased in COL I-treated cells, and we have demonstrated the reversion of this increase by using AKT or ILK siRNAs, by overexpressing I*k*Bα $^{Ser32,36/Ala}$ or by using an NF*k*B inhibitor. Therefore, we propose a pathway by which Bcl-xL acts as a downstream effector of NF*k*B, inducing cellular resistance to apoptosis in response to COL I.

The present results also explore the pro-apoptotic pathway induced by COL I. With a similar approach to the one previously described, it has been demonstrated that the inactivation of GSK-3*b* by the ILK-dependent phosphorylation, leads to the increase of the pro-apoptotic protein Bim. Interestingly, LiCl, an inhibitor of GSK-3*b*, also induced Bim expression. The possible functional cross-talk between the pro-apoptotic and anti-apoptotic pathways may take place at the level of the Bcl-2 family proteins. In viable cells, the pro- apoptotic proteins Bax and Bak are restrained by their physical interaction with the pro-survival Bcl-2 proteins, so this is the balance that must be overcome if a cell is to undergo apoptosis. A variety of studies indicate that the BH3 only proteins (e.g., Bim) are primarily responsible for disrupting the balance between Bax/Bak and the pro-survival Bcl-2 proteins in response to cellular stress [47]. However, there has been a debate about precisely how this occurs [48]. For example, in fibroblasts, Bim expression is required for apoptosis following serum withdrawal, by increasing both the abundance of Bim and its binding to Bcl-xL [49]. In our system, the COL I treatment significantly increases both Bcl-xL and Bim expression, while the possibly augmented Bax- expression [50] has been discarded in the present work (Online Resource 3).

Considering the data previously discussed above, a possible putative model of COL I-induced NF*k*B-mediated survival in HMC can be envisaged (Fig. 7). The possibility of alternative mechanisms involved could also exist, and must be explored in other studies. In this regard, von Wnuck et al. [44] demonstrated in smooth muscle cells that the activation of alpha 2 and alphaVbeta3 integrins, with dual stimulation of calpains and NF*k*B, controls IAPs- mediated cellular survival induced by COL I fragments. In our system, the possibility of a IAPs-mediated alternative pathway has also been discarded. On the other hand, it can be proposed that the augmented Bim expression mediated

by GSK-3*b* inhibition may be due to c-MYC transcription factor activation. Previous reports from Kotliarova et al. [33] showed that inhibition of GSK-3*b* activity results in c-MYC activation, leading to the induction of Bim. In contrast to our results, these authors demonstrated that inhibition of GSK-3*b* activity causes a dramatic decrease in intracellular NF*k*B activity.

Here, we demonstrate that COL I activate a dual pathway in HMC, in which NF*k*B is responsible for the protection from the apoptosis induced by pathological ECM composition. This fact could be particularly relevant in glomerular diseases characterized by increased amounts of ECM proteins in basal membranes or the mesangial compartment, and probably by the substitution of normal collagen such as COL IV for interstitial collagens, as COL I. In these conditions, the mesangial cell-restricted blockade or suppression of the pro- survival factor NF*k*B could be used as a possible therapy for early fibrosis progression prevention or reversion.

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Conflict of interest The authors declare that they have no conflict of interest.

References

1. López-Novoa JM, Nieto MA (2009) Inflammation and EMT: an alliance towards organ fibrosis and cancer progression. EMBO Mol Med 1:303–314
2. Liu Y (2006) Renal fibrosis: new insights into the pathogenesis and therapeutics. Kidney Int 69:213–217
3. Okada H, Kalluri R (2005) Cellular and molecular pathways that lead to progression and

regression of renal fibrogenesis. Curr Mol Med 5:467–474

4. Brenner BM, Meyer TW, Hostetter TH (1982) Dietary protein intake and the progressive nature of kidney disease: the role of hemodynamically mediated glomerular injury in the pathogenesis of progressive glomerular sclerosis in aging, renal ablation, and intrinsic renal disease. N Engl J Med 307:652–659
5. Gilbert RD, Turner CL, Gibson J, Bass PS, Haq MR, Cross E, Bunyan DJ, Collins AR, Tapper WJ, Needell JC, Dell B, Morton NE, Temple IK, Robinson DO (2009) Mutations in phospholipase C epsilon 1 are not sufficient to cause diffuse mesangial sclerosis. Kidney Int 75:415–419
6. Intentan HD, Schiffrin EL (2001) Vascular remodeling in hypertension: roles of apoptosis, inflammation, and fibrosis. Hypertension 38:581–587
7. Zeisberg M, Khurana M, Rao VH, Cosgrove D, Rougier JP, Werner MC, Shield CF 3rd, Werb Z, Kalluri R (2006) Stage- specific action of matrix metalloproteinases influences progressive hereditary kidney disease. PLoS Med 3:e100
8. Schwartz MA (1997) Integrins, oncogenes, and anchorage independence. J Cell Biol 139:575–578
9. Koyama H, Raines EW, Bornfeldt KE, Roberts JM, Ross R (1996) Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of Cdk2 inhibitors. Cell 87:1069–1078
10. Giancotti FG, Ruoslahti E (1999) Integrin signaling. Science 285: 1028–1032
11. Hannigan GE, Leung-Hagesteijn C, Fitz-Gibbon L, Coppolino MG, Radeva G, Filmus J, Bell JC, Dedhar S (1996) Regulation of cell adhesion and anchorage-dependent growth by a new beta 1-integrin-linked protein kinase. Nature 379:91–96
12. Legate KR, Montan˜ez E, Kudlacek O, Fassler R (2006) ILK, PINCH and parvin: the tIPP of integrin signalling. Nat Rev Mol Cell Biol 7(1):20–31
13. Troussard AA, Mawji NM, Ong C, Mui A, St-Arnaud R, Dedhar S (2003) Conditional knock-out of integrin-linked kinase demonstrates an essential role in protein kinase B/Akt activation. J Biol Chem 278:22374–22378

14. Sizemore N, Leung S, Stark GR (1999) Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phos- phorylation and activation of the NF-kappaB p65/RelA subunit. Mol Cell Biol 19(7):4798–4805
15. Tan C, Mui A, Dedhar S (2002) Integrin-linked kinase regulates inducible nitric oxide synthase and cyclooxygenase-2 expression in an NF-*k*B-dependent manner. J Biol Chem 277:3109–3116
16. Hayden MS, Ghosh S (2008) Shared principles in NF-kappaB signalling. Cell 132:344–362
17. Perkins ND (2007) Integrating cell-signalling pathways with NF-kappaB and IKK function. Nat Rev Mol Cell Biol 8:49–62
18. Karin M (2006) NF-kappaB in cancer development and progression. Nature 441:431–436
19. Shamas-Din A, Brahmbhatt H, Leber B, Andrews DW (2011) BH3-only proteins: orchestrators of apoptosis. Biochim Biophys Acta 1813(4):508–520
20. Guijarro C, Egido J (2001) Transcription factor-kB (NF-kB) and renal disease. Kidney Int 59:415–424
21. Sanz AB, Santamaría B, Ruiz-Ortega M, Egido J, Ortiz A (2008)
Mechanisms of renal apoptosis in health and disease. J Am Soc Nephrol 19(9):1634–1642
22. Ghosh S, Hayden MS (2008) New regulators of NF-kappaB in inflammation. Nat Rev Immunol 8:837–848
23. Ross MJ, Martinka S, D'Agati VD, Bruggeman LA (2005) NF-kappaB regulates Fas-mediated apoptosis in HIV-associated nephropathy. J Am Soc Nephrol 16:2403–2411
24. Massy ZA, Guijarro C, O'Donnell MP, Kim Y, Kashtan CE, Egido J, Kasiske BL, Keane WF (1999) The central role of nuclear factor-kappa B in mesangial cell activation. Kidney Int 56:S76–S79
25. Sanz AB, Sanchez-Nino MD, Ramos AM, Moreno JA, Santamaria B, Ruiz-Ortega M, Egido J, Ortiz A (2010) NFkB in renal inflammation. J Am Soc Nephrol 21:1254–1262

26. Ma LJ, Fogo AB (2007) Modulation of glomerulosclerosis. Semin Immunopathol 29:385–395
27. Iglesias-De La Cruz MC, Ruiz-Torres MP, De Lucio-Cazana FJ, Rodriguez-Puyol M, Rodriguez-Puyol D (2000) Phenotypic modifications of human mesangial cells by extracellular matrix: the importance of matrix in the contractile response to reactive oxygen species. Exp Nephrol 8:97–103
28. Calleros L, Lasa M, Toro MJ, Chiloeches A (2006) Low cell cholesterol levels increase NFkappaB activity through a p38 MAPK-dependent mechanism. Cell Signal 18(12):2292–2301
29. Ortega-Velazquez R, Diez-Marques ML, Ruiz-Torres MP, Gonzalez-Rubio M, Rodriguez-Puyol M, Rodriguez Puyol D (2003) Arg–Gly–Asp–Ser peptide stimulates transforming growth factor-beta1 transcription and secretion through integrin activation. FASEB J 17(11):1529–1531
30. Stockand JD, Sansom SC (1997) Regulation of filtration rate by glomerular mesangial cells in health and diabetic renal disease. Am J Kidney Dis 29:971–981
31. Wu C, Dedhar S (2001) Integrin-linked kinase (ILK) and its interactors: a new paradigm for the coupling of extracellular matrix to actin cytoskeleton and signaling complexes. J Cell Biol 155:505–510
32. Persad S, Attwell S, Gray V, Mawji N, Deng JT, Leung D, Yan J, Sanghera J, Walsh MP, Dedhar S (2001) Regulation of protein kinase B/Akt-serine-473 phosphorylation by integrin linked kinase (ILK): critical roles for kinase activity and amino acids arginine-211 and serine-343. J Biol Chem 276:27462–27469
33. Kotliarova S, Pastorino S, Kovell L, Kotliarov Y, Song H, Zhang W, Bailey R, Maric D, Zenklusen J, Lee J, Fine H (2008) Glycogen synthase kinase-3 inhibition induces glioma cell death through c-MYC, nuclear factor-KB, and glucose regulation. Cancer Res 68:6643–6651
34. Ortega-Velazquez R, Gonzalez-Rubio M, Ruiz-Torres MP, Diez- Marques ML, Iglesias MC, Rodríguez-Puyol M, Rodríguez-Puyol D (2004) Collagen I upregulates extracellular matrix gene

expression and secretion of TGF-beta 1 by cultured human mesangial cells. Am J Physiol Cell Physiol 286(6):C1335–C1343

35. Alique M, Calleros L, Luengo A, Griera M, Iniguez MA, Punzón C, Fresno M, Rodríguez-Puyol M, Rodríguez-Puyol D (2011) Changes in extracellular matrix composition regulate cyclooxygenase-2 expression in human mesangial cells. Am J Physiol Cell Physiol 300:C907–C918
36. Rodriguez-Iturbe B, Ferrebuz A, Vanegas V, Quiroz Y, Mezzano SA, Vaziri ND (2005) Early and sustained inhibition of nuclear factor-kappaB prevents hypertension in spontaneously hypertensive rats. J Pharmacol Exp Ther 315:51–57
37. Fujihara CK, Antunes GR, Mattar AL, Malheiros DMAC, Vieira JM, Zatz R (2007) Chronic inhibition of nuclear factor-kB attenuates renal injury in the 5/6 renal ablation model. Am J Physiol Renal Physiol 292:F92–F99
38. Esteban V, Lorenzo O, Ruperez M, Suzuki Y, Mezzano S, Blanco J, Kretzler M, Sugaya T, Egido J, Ruiz-Ortega M (2004) Angiotensin II, via AT1 and AT2 receptors and NF-kappaB pathway, regulates the inflammatory response in unilateral ureteral obstruction. J Am Soc Nephrol 15:1514–1529
39. Giannopoulou M, Dai C, Tan X, Wen X, Michalopoulos GK, Liu Y (2008) Hepatocyte growth factor exerts its anti-inflammatory action by disrupting nuclear factor-kB signaling. Am J Pathol 173:30–41
40. Tamada S, Nakatani T, Asai T, Tashiro K, Komiya T, Sumi T, Okamura M, Kim S, Iwao H, Kishimoto T, Yamanaka S, Miura K (2003) Inhibition of nuclear factor-*k*B activation by pyrrolidinedithiocarbamate prevents chronic FK506 nephropathy. Kidney Int 63:306–314
41. Elks CM, Mariappan N, Haque M, Guggilam A, Dewan S, Majid A, Francis J (2009) Chronic NF*k*B blockade reduces cytosolic and mitochondrial oxidative stress and attenuates renal injury and hypertension in SHR. Am J Physiol Renal Physiol 296:F298–F305

42. Inoue T, Takenaka T, Hayashi M, Monkawa T, Yoshino J, Shimoda K, Neilson EG, Suzuki H, Okada H (2010) Fibroblast expression of an I*k*B dominant-negative transgene attenuates renal fibrosis. J Am Soc Nephrol 21:2047–2052

43. Henke N, Schmidt-Ullrich R, Dechend R, Park J, Qadri F, Wellner M, Obst M, Gross V, Dietz R, Luft F, Scheidereit C, Muller D (2007) Vascular endothelial cell-specific NF*k*B suppression attenuates hypertension-induced renal damage. Circ Res 101:268–276

44. von Wnuck Lipinski K, Keul P, Ferri N, Lucke S, Heusch G, Fischer JW, Levkau B (2006) Integrin-mediated transcriptional activation of inhibitor of apoptosis proteins protects smooth muscle cells against apoptosis induced by degraded collagen. Circ Res 98:1490–1497

45. Vivanco I, Sawyers CL (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2(7):489–501

46. Edwards LA, Thiessen B, Dragowska WH, Daynard T, Bally MB, Dedhar S (2005) Inhibition of ILK in PTEN-mutant human glioblastomas inhibits PKB/Akt activation, induces apoptosis, and delays tumor growth. Oncogene 24:3596–3605

47. Willis S, Adams J (2005) Life in the balance: how BH3-only proteins induce apoptosis. Curr Opin Cell Biol 17(6):617–625

48. Ewings K, Wiggins C, Cook S (2007) Bim and the pro-survival Bcl-2 proteins. Cell Cycle 6(18):2236–2240

49. Ewings K, Hadfield-Moorhouse K, Wiggins C, Wickenden J, Balmanno K, Gilley R, Degenhardt K, White E, Cook S (2007) ERK1/2-dependent phosphorylation of BimEL promotes its rapid dissociation from Mcl-1 and Bcl-xL. EMBO J 26:2856–2867

50. Ren D, Tu H, Kim H, Wang G, Bean G, Takeuchi O, Jeffers J, Zambetti G, Hsieh J, Cheng E (2010) BID, BIM, and PUMA are essential for activation of the BAX- and BAK-dependent cell death program. Science 330(6009):1390–1393

Figure legends

Fig. 1 Inhibition of NF*k*B produces collagen I-induced apoptosis in human mesangial cells (HMC). a Cells were incubated with collagen I (COL I, 12.5 lg/ml) or collagen IV (COL IV, 12.5 lg/ml) for 24, 48, 72, or 96 h alone or with PDTC (100 lM) for 48 h. For I*k*Bα $^{Ser32,36/Ala}$ expression, cells were transfected and 24 h after, incubated with COL I or COL IV for 48 h. Apoptosis was determined in PI-stained cells and analyzed by flow cytometry. Representative *graphs* are shown. b Cells were incubated as in (a) for 48 h and PARP proteolysis was analyzed by western blot (a characteristic *blot* is shown). Equal protein loading was confirmed by probing with an actin antibody. The *graphs* present densitometric band analysis normalized to actin. c HMC were treated as in (a) for 24 h and then stained with annexin V-FITC and PI followed by flow cytometry analysis. Etoposide (50 lM, 16 h) was used as a positive control. The results show representatives *dot plots*. The data represent mean ± SEM of five independent experiments. **p* \ 0.01 versus the others groups

Fig. 2 Collagen I induces NF*k*B-dependent transcription, I*k*Bα degradation, and p65/NF*k*B translocation to the nucleus. a HMC were transfected with an NF*k*B driven luciferase reported plasmid (3xNF*k*B-TK-Luc) and pRLTK-Renilla. 24 h after transfection, cells were incubated with COL I, (12.5 lg/ml) or COL IV (12.5 lg/ml) for 24 and 48 h or with COL I plus PDTC (100 lM) for 48 h. For I*k*Bα $^{Ser32,36/Ala}$ expression, cells were transfected and 24 h after, incubated with COL I for 48 h. Cell extracts were assayed for luciferase and renilla activities. Luciferase activity was normalized with renilla activity and expressed as fold increase from control. b and c Cells were incubated as in (a) for 2, 4, 8, and 24 h and I*j*B (b) or p65 (c) protein levels were analyzed in cellular or nuclear extracts by western blot (a characteristic *blot* is shown). Equal protein loading was confirmed by probing with an actin or H3 antibody. The *graphs* present densitometric band analysis normalized to actin or H3. The data represent mean ± SEM of five independent experiments. **p* \ 0.01 versus the others groups. #*p* \ 0.01

versus COL I. d Cells were incubated as in (a) for 24 h. Immunofluorescence staining for p65 (*green*) was performed. Magnifications 940. A representative of four independent experiments is shown. TNF*a* plus LPS (2 and 100 nM, 16 h) was used as a positive control.

Fig. 3 *b*1 integrin and ILK regulates the activation of NF*k*B-dependent transcription induced by COL I. a HMC were incubated with COL I (12.5 lg/ml) or COL IV (12.5 lg/ml) for 4 h and active *b*1 integrin levels were analyzed by western blot (a characteristic *blot* is shown). Equal *b*1 integrin loading was confirmed by probing with an anti-*b*1 integrin antibody. Mn (1 mM) was used as a positive control. Equal protein loading was confirmed by probing with an actin antibody. The *graphs* present densitometric band analysis normalized to actin. b Cells were incubated as in (a) for 2 or 4 h and in vitro kinase activity of ILK was determined in cells lysates, with a GSK-3*b* protein-fusion. GSK-3*b* protein phosphorylation (P-GSK-3*b*) was measured by western blot (a characteristic *blot* is shown). Anti-*b*1 activator antibody was used as a positive control. Equal ILK loading was confirmed by probing with an anti-ILK antibody. The *graphs* present densitometric band analysis normalized to ILK. c HMC were transfected with an NF*k*B driven luciferase reported plasmid (3xNF*k*B-TK-Luc) and pRLTK-Renilla, as indicated. 24 h after transfection, cells were incubated as in (a) for 48 h, alone or with previous incubation with a *b*1 blocking antibody (AC anti *b*1). Cell extracts were assayed for luciferase and renilla activities. Luciferase activity was normalized with renilla activity and expressed as fold increase from control. d Cells were transfected as in (c) and simultaneously were depleted of ILK with a specific siRNA, and a scrambled RNA (Sc) was used as control. After that, cells were treated and NF*k*B transcriptional activity was determined as in (b). Total ILK expression was measured by western blot (ILK). TNF*a* plus LPS (2 and 100 nM, 16 h) was used as a positive control. The data represent mean ± SEM of five independent experiments. **p* \ 0.01 versus the other groups. #*p* \ 0.01 versus COL I.

Fig. 4 Role of PI3K/AKT pathway in the induced NF*k*B transcriptional activity and protection of apoptosis induced by COL I. a HMC were grown on plates coated with different proportions of COLI/COL IV for 24 h. Phosphorylation of AKT protein was analyzed by western blot (a characteristic *blot* is shown). Equal protein loading was confirmed by probing with actin antibody. The graphs present densitometric band analysis normalized to actin. b HMC were transfected with an NF*k*B driven luciferase reported plasmid (3xNF*k*B-TK-Luc) and pRLTK- Renilla, as indicated. 24 h after transfection, cells were incubated with COL I (12.5 lg/ml) or COL IV (12.5 lg/ml) for 48 h, alone or were simultaneously depleted of AKT with a specific siRNA and a scrambled RNA (Sc) was used as control. Cell extracts were assayed for luciferase and renilla activities. Luciferase activity was normalized with renilla activity and expressed as fold increase from control. Total AKT expression was measured by western blot (AKT). TNF*a* plus LPS (2 and 100 nM, 16 h) was used as a positive control. c Cells were depleted of ILK with a specific siRNA, and a scrambled RNA (Sc) was used as control. After that, cells were incubated as in (b) for 24 h. Phosphorylation of AKT protein was analyzed by western blot (a characteristic *blot* is shown). Equal protein loading was confirmed by probing with actin antibody. The graphs present densitometric band analysis normalized to actin. d Cells were depleted of AKT with a specific siRNA or scrambled RNA (Sc) was used as control and then were incubated as in (b). Apoptosis was determined in PI-stained cells and analyzed by flow cytometry. The data represent mean ± SEM of five independent experiments. *p \ 0.01 versus the others groups. #p \ 0.01 versus COL I.

Fig. 5 COL I induces Bcl-xL expression via activation of NF*k*B (a–d). HMC were treated with COL I (12.5 lg/ml) or COL IV (12.5 lg/ml) for 8 h alone or with previous I*k*Bα $^{Ser32,36/Ala}$ transfection (a), preincubation with PDTC (100 lM) (b) or were depleted of AKT (c) or ILK (d) with a specific siRNA and a scrambled RNA (Sc) was used as control. Bcl-xL expression was assessed by western blot (a characteristic *blot* is shown). Equal protein loading was confirmed by probing with actin antibody. The graphs present densitometric band analysis normalized to

actin. The data represent mean ± SEM of five independent experiments. *p \ 0.01 versus the others groups. #p \ 0.01 versus COL I.

Fig. 6 COL I induces the proapoptotic protein Bim expression through a GSK3-dependent mechanism (a and d). Cells were treated with COL I (12.5 lg/ml) or COL IV (12.5 lg/ml) for the indicated time alone or with LiCl (10 mM) and Bim levels was analyzed by western blot (a characteristic *blot* is shown). b HMC were grown on plates coated with different proportions of COLI/COL IV for 24 h or treated as in (a) for 2, 4, 8, and 24 h. Phosphorylation of GSK-3*b* protein (P-GSK-3*b*) was analyzed by western blot (a characteristic *blot* is shown). c and e Cells were depleted of ILK with a specific siRNA, and a scrambled RNA (Sc) was used as control. After that cells were incubated as in (a) for the times indicated and P-GSK-3*b* (c) or Bim (e) levels were analyzed by western blot (a characteristic *blot* is shown). a–e Equal protein loading was confirmed by probing with GSK-3*b* or actin antibody. The graphs present densitometric band analysis normalized to GSK-3*b* or actin. The data represent mean ± SEM of five independent experiments. *p \ 0.01 versus the other groups. #p \ 0.01 versus COL I.

Fig. 7 Schematic diagram of the signaling involved in collagen I-induced apoptotic and survival pathways in human mesangial cells. Putative crosstalk between the Bim-mediated apoptotic pathway and the NF*k*B -mediated survival pathway in HMC treated with COL I. The balance between anti- and proapoptotics Bcl-xL and Bim members of Bcl-2 family may determine the survival or death of the cells. Sites of action of inhibitors are shown.

Figure 1

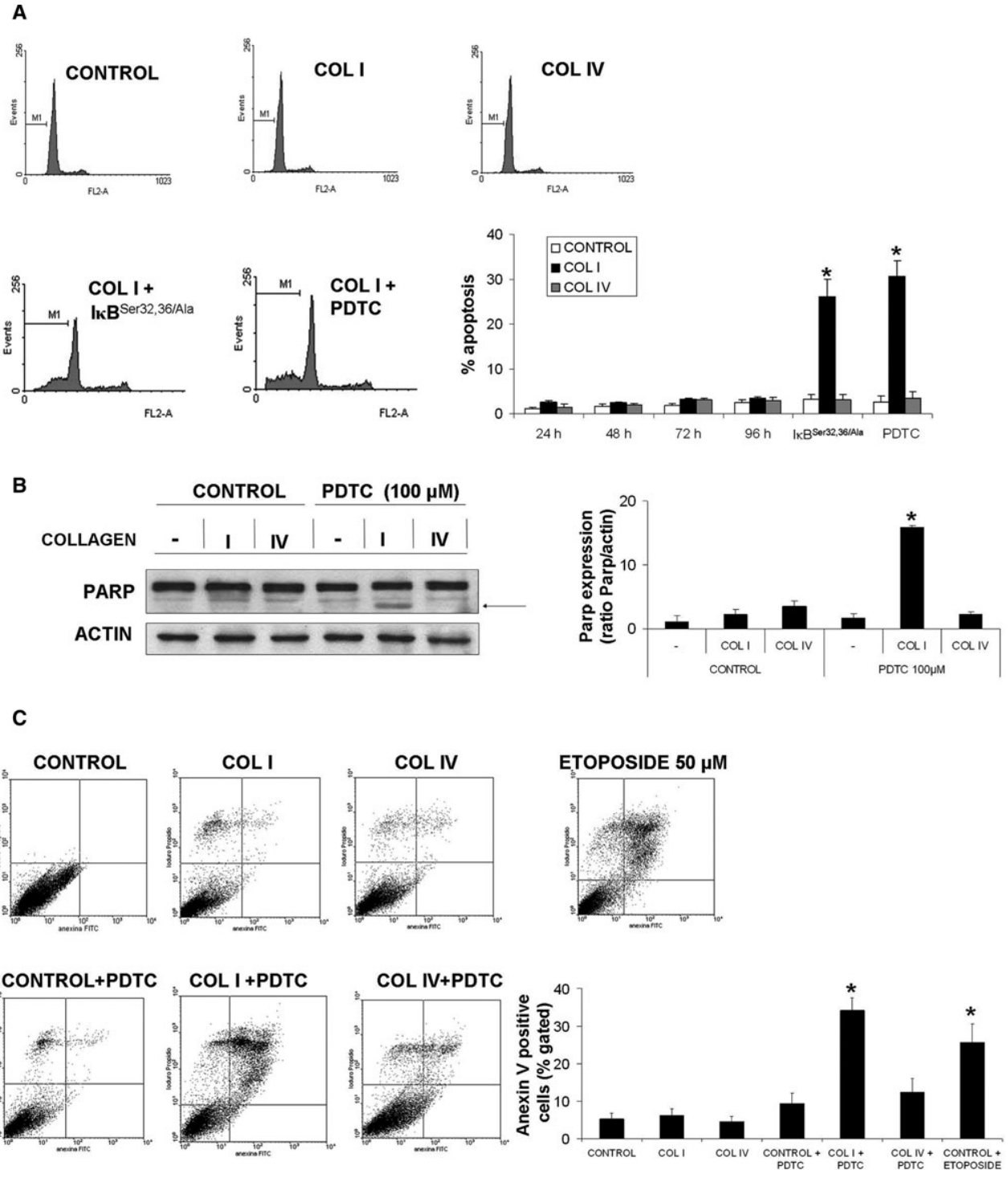

A
CONTROL
COL I
COL IV
COL I + IκBSer32,36/Ala
COL I + PDTC
Events
M1
FL2-A
% apoptosis
24 h
48 h
72 h
96 h
IκBSer32,36/Ala
PDTC
B
CONTROL
PDTC (100 µM)
COLLAGEN
PARP
ACTIN
Parp expression (ratio Parp/actin)
PDTC 100µM
C
CONTROL
COL I
COL IV
ETOPOSIDE 50 µM
CONTROL+PDTC
COL I +PDTC
COL IV+PDTC
anexina FITC
Anexin V positive cells (% gated)
CONTROL + PDTC
COL I + PDTC
COL IV + PDTC
CONTROL + ETOPOSIDE

Figure 2

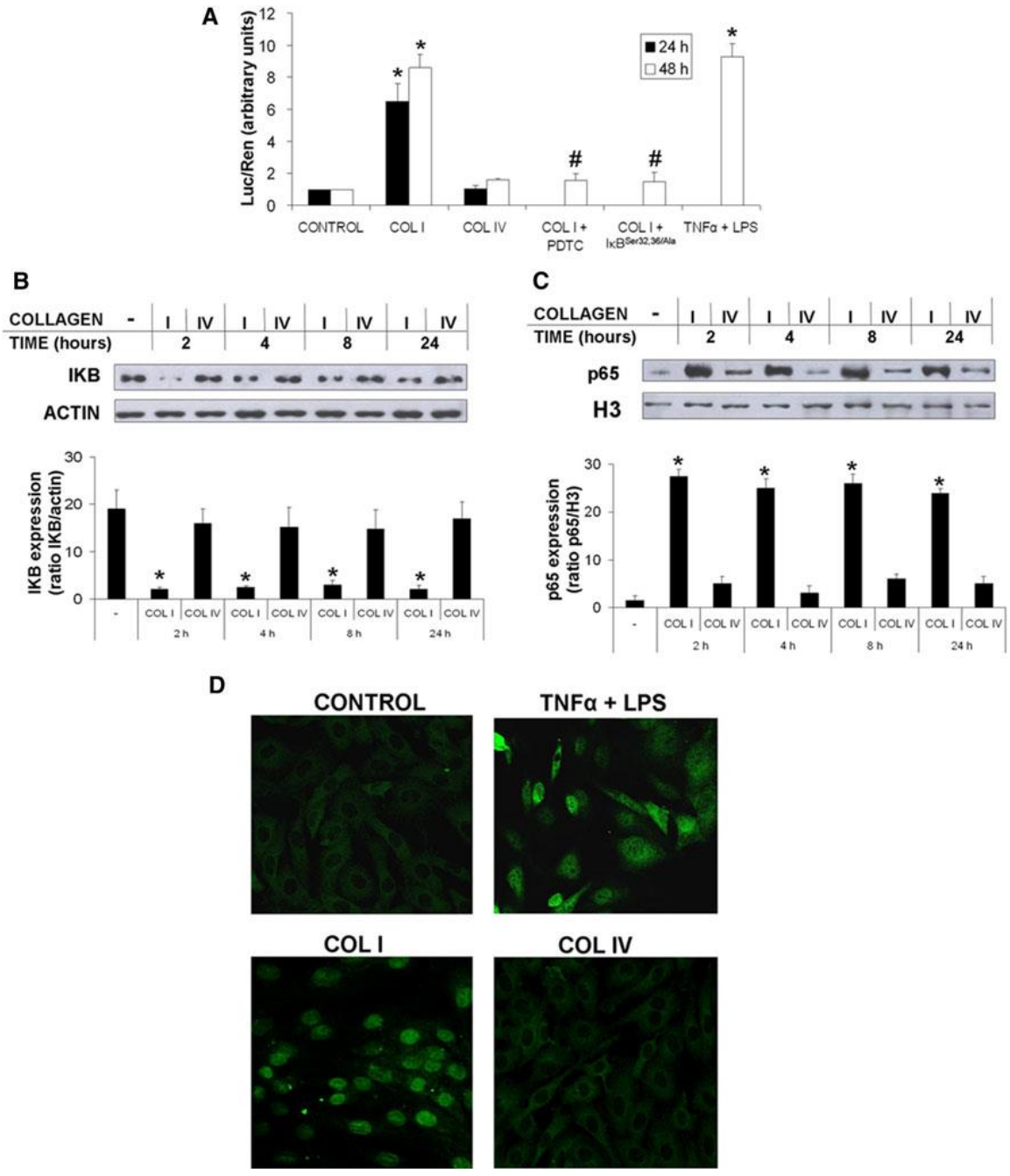

A
Luc/Ren (arbitrary units)
24 h
48 h
CONTROL
COL I
COL IV
COL I + PDTC
COL I + IκB Ser32,36/Ala
TNFα + LPS
B
COLLAGEN
TIME (hours)
IKB
ACTIN
IKB expression (ratio IKB/actin)
C
p65
H3
p65 expression (ratio p65/H3)
D
CONTROL
TNFα + LPS
COL I
COL IV

Figure 3

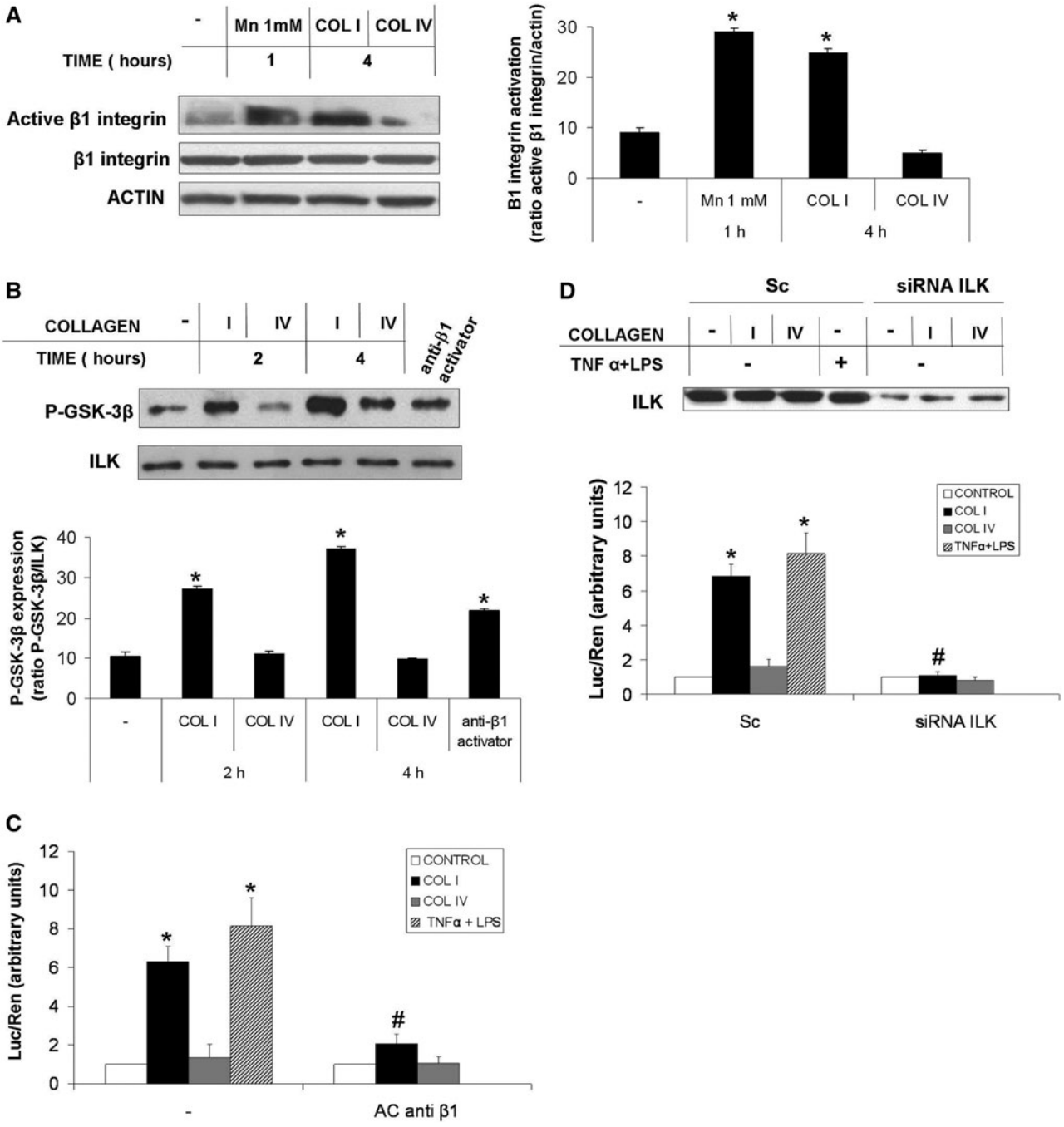

A
-
Mn 1mM
COL I
COL IV
TIME (hours)
1
4
Active β1 integrin
β1 integrin
ACTIN
B1 integrin activation (ratio active β1 integrin/actin)
30
20
10
0
*
*
-
Mn 1 mM
COL I
COL IV
1 h
4 h
B
COLLAGEN
-
I
IV
I
IV
anti-β1 activator
TIME (hours)
2
4
P-GSK-3β
ILK
P-GSK-3β expression (ratio P-GSK-3β/ILK)
40
30
20
10
0
*
*
*
-
COL I
COL IV
COL I
COL IV
anti-β1 activator
2 h
4 h
D
Sc
siRNA ILK
COLLAGEN
-
I
IV
-
-
I
IV
TNF α+LPS
-
+
-
ILK
Luc/Ren (arbitrary units)
12
10
8
6
4
2
0
CONTROL
COL I
COL IV
TNFα+LPS
*
*
#
Sc
siRNA ILK
C
Luc/Ren (arbitrary units)
12
10
8
6
4
2
0
CONTROL
COL I
COL IV
TNFα + LPS
*
*
#
-
AC anti β1

Figure 4

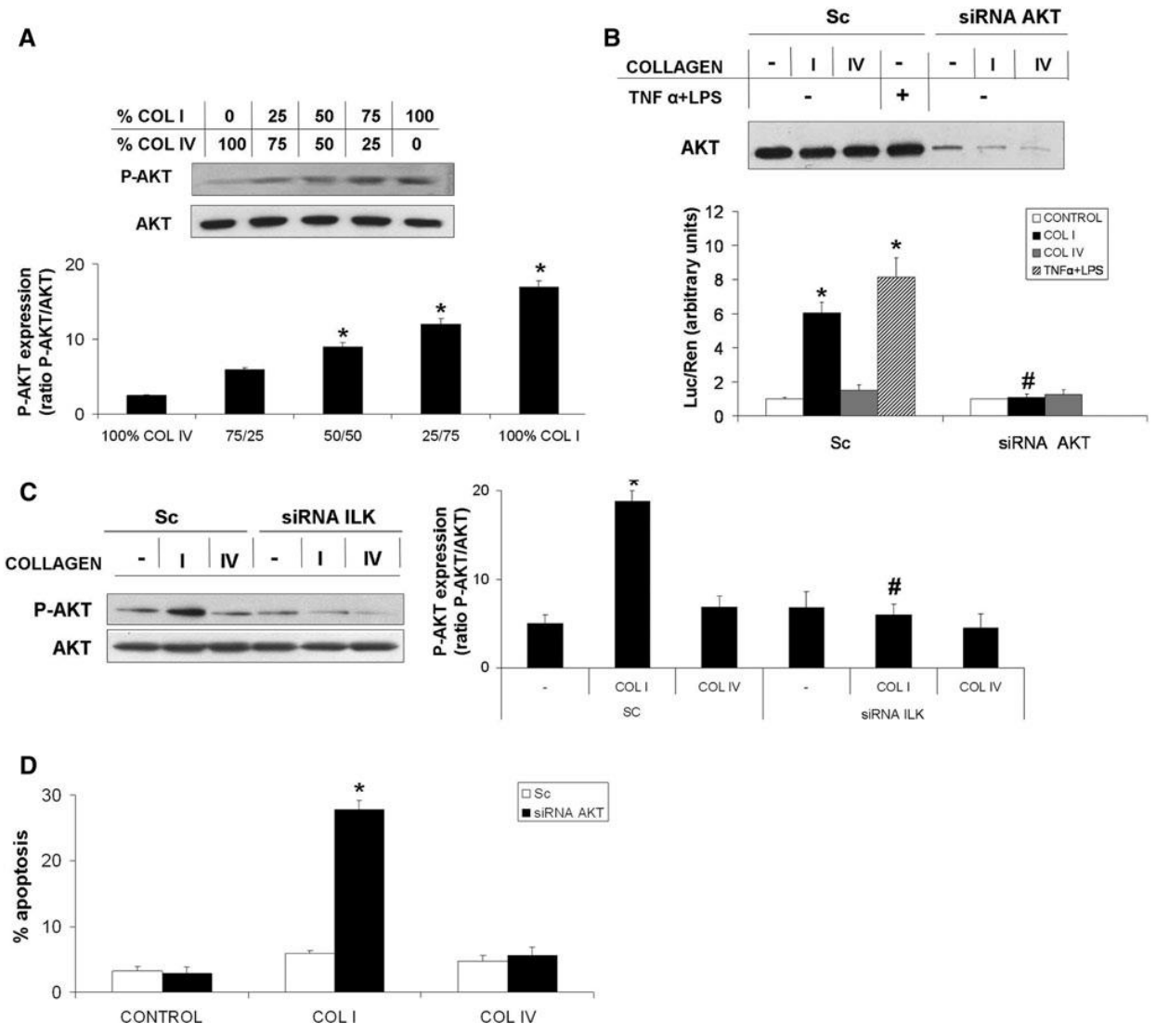

A
% COL I 0 25 50 75 100
% COL IV 100 75 50 25 0
P-AKT
AKT
P-AKT expression (ratio P-AKT/AKT)
100% COL IV
75/25
50/50
25/75
100% COL I
B
Sc
siRNA AKT
COLLAGEN
TNF α+LPS
AKT
Luc/Ren (arbitrary units)
CONTROL
COL I
COL IV
TNFα+LPS
Sc
siRNA AKT
C
Sc
siRNA ILK
COLLAGEN
P-AKT
AKT
P-AKT expression (ratio P-AKT/AKT)
COL I
COL IV
SC
siRNA ILK
D
% apoptosis
Sc
siRNA AKT
CONTROL
COL I
COL IV

Figure 5

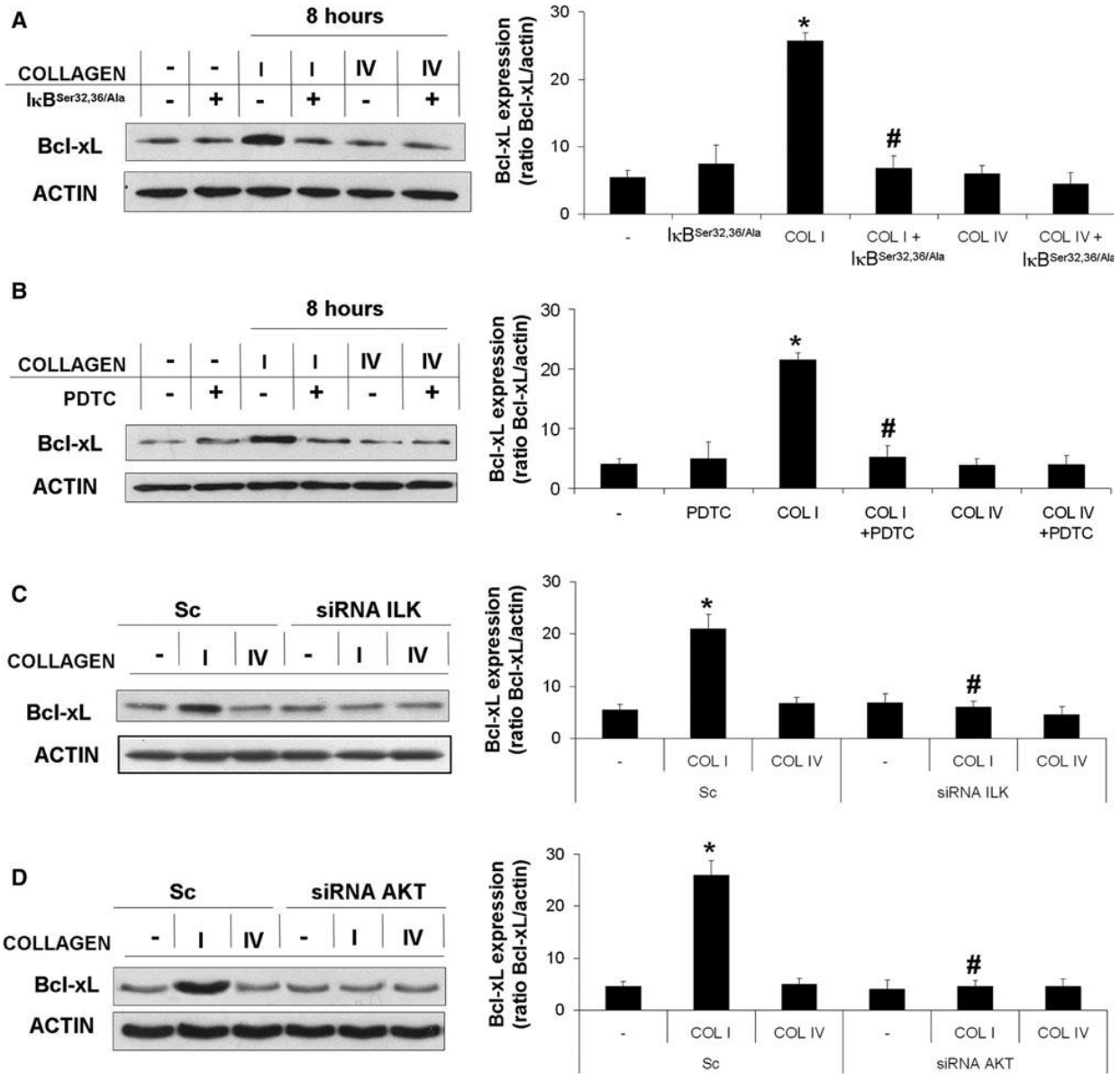

A
8 hours
COLLAGEN
IκB Ser32,36/Ala
Bcl-xL
ACTIN
Bcl-xL expression (ratio Bcl-xL/actin)
COL I
COL I + IκB Ser32,36/Ala
COL IV
COL IV + IκB Ser32,36/Ala
B
PDTC
COL I +PDTC
COL IV +PDTC
C
Sc
siRNA ILK
D
siRNA AKT

Figure 6

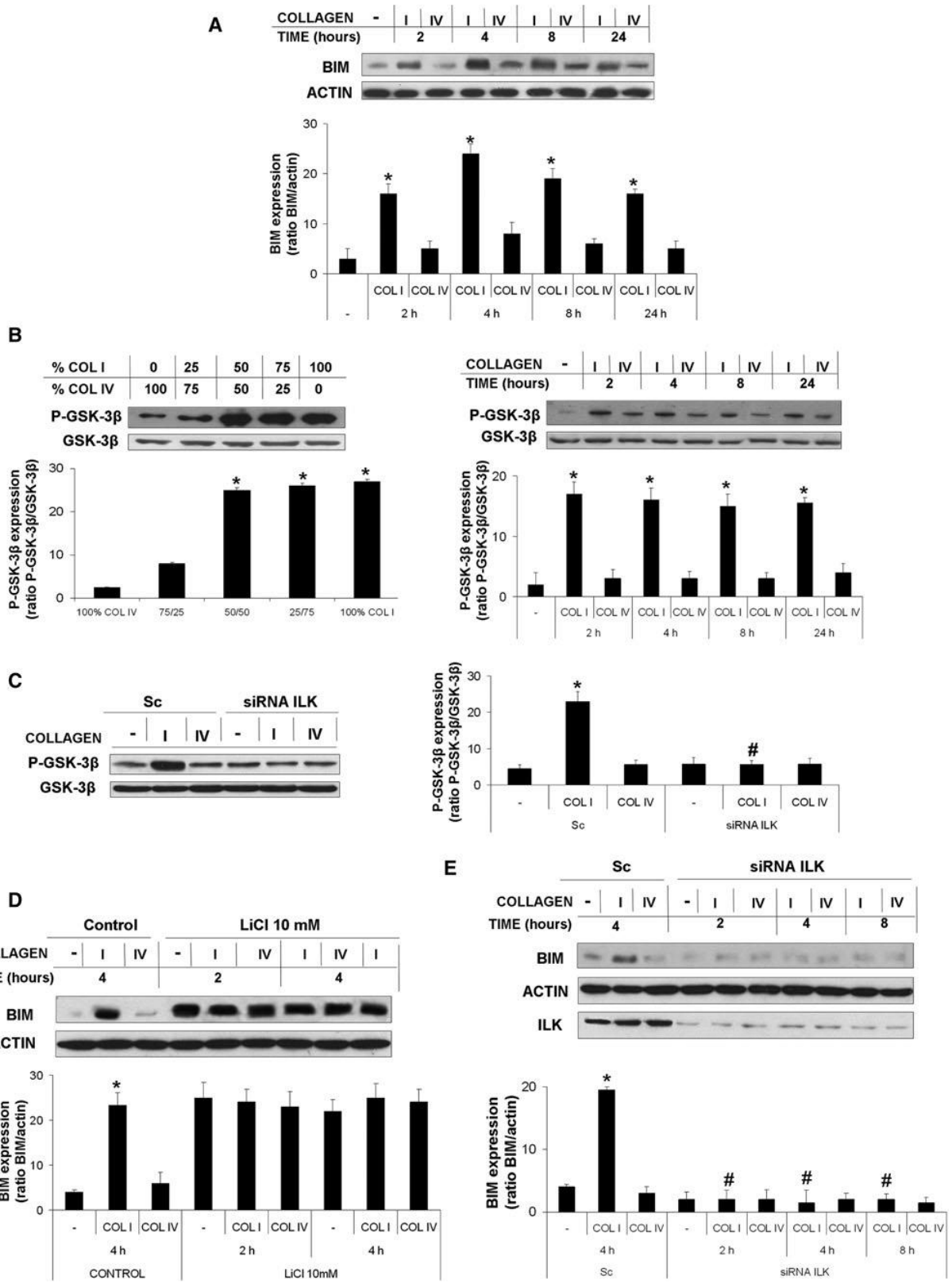
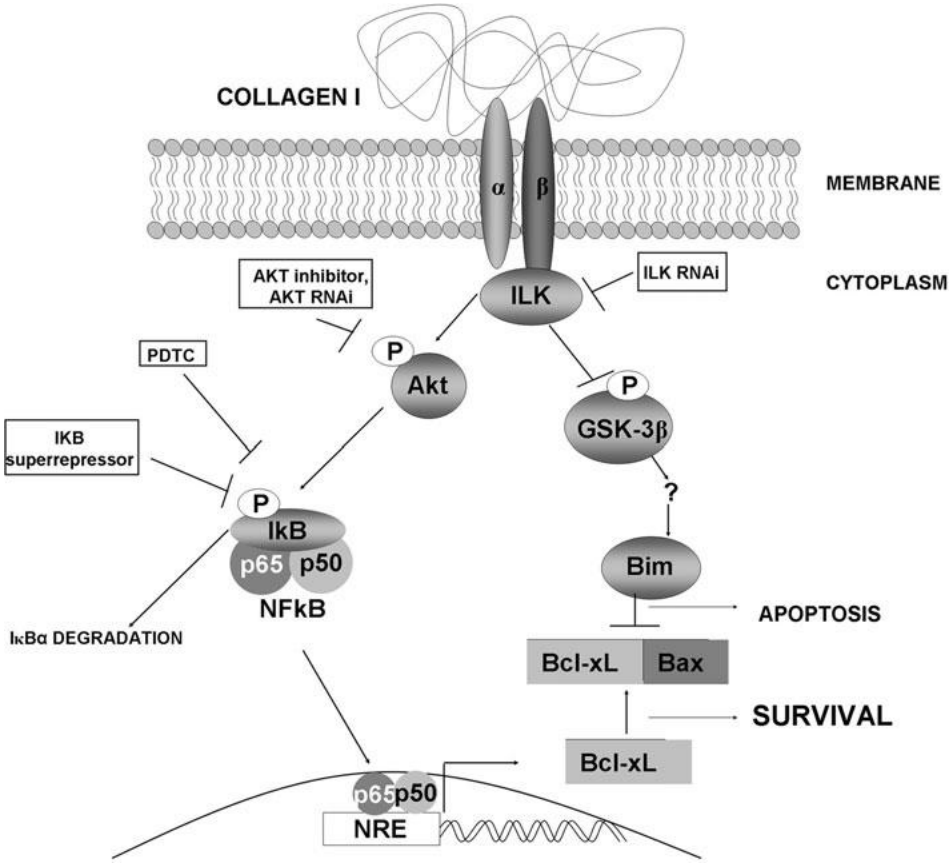

A
COLLAGEN - I IV I IV I IV I IV
TIME (hours) 2 4 8 24
BIM
ACTIN
BIM expression (ratio BIM/actin)
COL I COL IV COL I COL IV COL I COL IV COL I COL IV
2 h 4 h 8 h 24 h
B
% COL I 0 25 50 75 100
% COL IV 100 75 50 25 0
P-GSK-3β
GSK-3β
P-GSK-3β expression (ratio P-GSK-3β/GSK-3β)
100% COL IV 75/25 50/50 25/75 100% COL I
COLLAGEN - I IV I IV I IV I IV
TIME (hours) 2 4 8 24
C
Sc siRNA ILK
COLLAGEN - I IV - I IV
P-GSK-3β
GSK-3β
Sc siRNA ILK
D
Control LiCl 10 mM
COLLAGEN - I IV - I IV I IV I
TIME (hours) 4 2 4
BIM
ACTIN
CONTROL LiCl 10mM
E
Sc siRNA ILK
COLLAGEN - I IV - I IV I IV I IV
TIME (hours) 4 2 4 8
BIM
ACTIN
ILK
Sc siRNA ILK

Figure 7


COLLAGEN I
α
β
MEMBRANE
CYTOPLASM
AKT inhibitor, AKT RNAi
ILK
ILK RNAi
P
Akt
PDTC
P
GSK-3β
IKB superrepressor
?
P
IkB
p65
p50
NFkB
Bim
APOPTOSIS
IκBα DEGRADATION
Bcl-xL
Bax
SURVIVAL
Bcl-xL
p65p50
NRE