Research Repository UCD

Title	High-mobility group box protein 1: a novel mediator of inflammatory-induced renal epithelial-mesenchymal transition
Authors(s)	Lynch, Julie, Nolan, Stephen, Slattery, Craig, Feighery, Ronan, Ryan, Michael P., McMorro, Tara
Publication date	2010-11-24
Publication information	Lynch, Julie, Stephen Nolan, Craig Slattery, Ronan Feighery, Michael P. Ryan, and Tara McMorro. "High-Mobility Group Box Protein 1: A Novel Mediator of Inflammatory-Induced Renal Epithelial-Mesenchymal Transition" 32, no. 6 (November 24, 2010).
Publisher	Karger
Item record/more information	http://hdl.handle.net/10197/3631
Publisher's version (DOI)	10.1159/000320485

Downloaded 2023-04-07T10:52:29Z

The UCD community has made this article openly available. Please share how this access benefits you. Your story matters! (@ucd_oa)

© Some rights reserved. For more information

HMGB-1 - A novel mediator of inflammatory-induced renal epithelial mesenchymal transition

**Julie Lynch, *Stephen Nolan, Craig Slattery, Ronan Feighery, Michael P. Ryan and #Tara McMorrow*

UCD School of Biomolecular and Biomedical Science, UCD Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland.

* These authors contributed equally to the work.

Corresponding Author:

Dr. Tara McMorrow,
UCD School of Biomolecular & Biomedical Science,
UCD Conway Institute,
University College Dublin,
Belfield, Dublin 4. Ireland.
Telephone: +353-1-7166819;
Fax: +353-1-7166456;
Email: tara.mcmorrow@ucd.ie

Keywords: Renal Fibrosis, PBMC, HMGB-1, epithelial-mesenchymal transition

Short Summary: The aim of this study was to investigate the interactions of proximal tubular epithelial cells with infiltrating immune cells, in the context of chronic kidney disease progression. We have identified HMGB-1 as a potentially important mediator of immune-mediated injury eliciting its effect through the receptor for AGEs (RAGE) and TGF- β 1.

Abstract

Background. High mobility group box protein 1 (HMGB-1) is a chromatin binding protein that bends DNA thereby facilitating gene transcription. HMGB-1 has also been observed as an extracellular secreted protein in serum of patients with sepsis and has putative intracellular signalling effects regulating the production of interleukin-1 and tumour necrosis factor in a number of inflammatory conditions.

Methods. We established a model of immune-mediated epithelial-mesenchymal transition (EMT) in human proximal tubular epithelial cells (PTECs). PTECs were cultured with conditioned medium containing supernatant from activated peripheral blood mononuclear cells (aPBMC). The model was characterised using phenotypic and transcriptomic approaches and suppression subtractive hybridisation was performed to identify differentially regulated genes.

Results. Activation of PBMCs resulted in increased secretion of HMGB-1. In addition, treatment of PTECs with aPBMC-conditioned medium resulted in significant upregulation of HMGB-1 in PTECs. Direct treatment of PTECs with recombinant human HMGB-1 induced alterations in epithelial morphology consistent with EMT including reduced E-cadherin expression, increased α -SMA expression and enhanced cell migration. HMGB-1 effects were mediated at least in part by the receptor for advanced glycation end products (RAGE) and through induction of TGF- β 1 secretion from PTECs.

Conclusions. These results suggest that HMGB-1 is a key mediator of immune-mediated EMT of PTECs and a potentially important signalling molecule in the development of renal fibrosis.

Introduction

Tubulointerstitial fibrosis is characterised by loss of renal tubules, an increased myofibroblast population, and accumulation of extracellular matrix proteins (ECM) such as collagen, fibronectin and laminin [19]. Myofibroblasts, identified by expression of mesenchymal marker α -smooth muscle actin (α -SMA), are the primary source of ECM and are the best prognostic indicator of disease progression in both human and experimental glomerulonephritis [11]. These cells are virtually absent in the normal kidney but have been shown to be critical in renal fibrosis progression. The origin of myofibroblasts within the diseased kidney remains a point of debate [5].

Evidence suggests that one source of these myofibroblasts may be proximal tubular epithelial cells (PTECs) by a process termed epithelial-mesenchymal transition (EMT) [12,22,37]. While there is currently debate regarding the role of PTEC EMT in disease progression *in vivo*, EMT has been extensively observed *in vitro* in response to a variety of stimuli including TGF- β , epidermal growth factor (EGF) and interleukin-1 (IL-1) [6]. Mononuclear cell infiltration, predominantly consisting of lymphocytes and macrophages [25] is another widely observed characteristic of tubulointerstitial fibrosis. It has been suggested that these infiltrating immune cells play a role in promoting myofibroblast activation/accumulation through their ability to secrete cytokines [29]. The aim of this study was to further investigate the interaction of the infiltrating mononuclear cells with PTECs in the context of tubulointerstitial fibrosis development.

High mobility group box protein-1 (HMGB-1), also known as amphoterin, is best known as an architectural transcription factor because of its ability to regulate gene activity through DNA bending [28]. However additional roles for HMGB-1 have been identified including the ability to bind to and modulate NF- κ B [2], and as a secreted factor capable of regulating the expression of pro-inflammatory cytokines tumour necrosis factor-beta (TNF- β) and interferon-beta (IFN- β) [8]. As an extracellular signalling molecule, HMGB-1 elicits its effects through the receptor for advanced glycation end products (RAGE) which has been shown to interact with HMGB-1 with high affinity [15]. A member of the immunoglobulin superfamily of cell surface molecules, RAGE is a multi-ligand receptor that also interacts with amyloid- β {Yan, 1996 #20} and advanced glycation end products (AGEs) [20,31]. RAGE is expressed in vascular smooth

muscle cells, endothelial cells, mononuclear phagocytes, mesangial cells, neurons and lung alveolar epithelial cells [17]. Binding of HMGB-1 to RAGE has been shown to induce cell migration, cell invasion, tumour growth and metastasis [4]. The role of RAGE-HMGB-1 interactions in disease have been widely suggested [26], however the context of the interaction and mechanisms underlying the involvement in disease remain poorly understood.

Previously we reported an *in vitro* model of immune-mediated PTEC damage using conditioned medium containing supernatant from activated peripheral blood mononuclear cells (aPBMC-CM) and human PTECs [14]. Using similar techniques, Nightingale *et al* [24] suggested that aPBMC-CM induced EMT in primary renal epithelial cells. In the current study we have used the PTEC line, HK-2 to investigate the effects of aPBMC-CM on PTEC phenotype and identify key mediators of the effects observed.

Materials and Methods

Reagents. Anti-HMGB-1 and anti-E-cadherin antibodies were obtained from BD Transduction Laboratories (Lexington, KY). Anti-RAGE antibody was obtained from Chemicon Ltd. Anti-TGF- β 1 antibody was obtained from R&D systems, (UK). Recombinant HMGB-1 protein was obtained from Sigma. FITC secondary antibodies were obtained from DAKO. Cell culture dishes and slides were purchased from Falcon, (UK).

Cell Culture and Treatments. HK-2 proximal tubular epithelial cells were maintained as previously described [23]. For the indicated treatments: 10 μ g/ml of HMGB-1 (Sigma-Aldrich) was prepared according to manufacturer's instructions and added in the indicated concentrations. aPBMC-CM was prepared as previously described [14]. Primary human renal proximal tubular cells (RPTECs) were purchased from Clonetics and maintained as per the manufacturer's instructions.

PCR Analysis. Total RNA was extracted from cells using TRIzol reagent (Life Technologies-BRL) as per manufacturer's instructions. mRNA abundance was assessed by real-time PCR or by RT-PCR. Briefly, real-time PCR was performed on a ABI 7900HT real-time PCR system (Applied biosystems, Foster City, USA). Relative gene expression levels of E-cadherin, ZO-1, vimentin genes were assayed using pre-optimised single tube primer/probe Taqman Gene Expression Assays (Applied Biosystems), Ribosomal 18S RNA levels were used as an endogenous normalising control throughout. Reaction cycling conditions were: 2 min at 50°C, 10 min at 95°C for enzyme activation and 40 cycles of 15 sec at 95°C for denaturation followed by 1 min at 60°C for annealing and extension. A negative-RT control was run alongside all assays to control for genomic DNA contamination. Results were analysed using the Ct method. For RT-PCR, the number of PCR cycles used, were determined to be within the linear range of the reactions. PCR primers for E-cadherin, HMGB-1 and α -SMA were supplied by Sigma-Genosys (UK). The sequences of the primers were as previously described [23,34]. Primer sequences for human HMGB-1 were as follows, forward: 5'-GGAGAGATGTGGAATA-3', reverse: 5'-GGGAGTGAGTTGTGTA-3'.

Immunofluorescence and Western Blot analysis. Immunofluorescent staining was performed as previously described [23,34]. Cells were visualised using a Zeiss Axioplan 2 imaging fluorescent microscope. Western blot analysis was also performed as previously described [23,34].

Migration Assay. The migratory behaviour of PTECs was analysed using an *in vitro* wound healing assay. Briefly, monolayers of confluent cultures were experimentally wounded and subsequently cultured under different conditions as indicated. Cultures were observed at 24 hour intervals for up to 72 hours post wounding.

Suppression subtractive hybridisation (SSH). An SSH was performed with the PCR-SELECT cDNA subtraction kit (CLONTECH) as directed by the manufacturer.

Cloning and sequencing of cDNAs. PCR products generated by the SSH procedure were subcloned into PCR2.1 vector using the original TA cloning kit (Invitrogen). Subcloned cDNAs were sequenced using the ABI prism dye terminator cycle sequencing ready reaction kit (Perkin-Elmer). The sequences obtained were compared against GenBankTM/EBI and expressed sequence tag databases using BLAST searches.

Statistical analysis. Data was analysed by one-way ANOVA (analysis of variance) and multiple comparisons between treatment groups were made using the Student T test using GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA, USA). Results were expressed as the mean +/- S.E.M. A probability of 0.05 or less was deemed statistically significant. The following scheme was used throughout * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

aPBMC-CM induced EMT in proximal tubular epithelial cells.

To model the interactions between immune cells and renal tubular epithelial cells, PTECs were exposed to 30% (v/v) of control or aPBMC conditioned medium (aPBMC-CM). Cell morphology was assessed after 48 hours treatment with aPBMC-CM by phase contrast microscopy (Fig 1A). Control cells exhibited typical epithelial, cuboidal morphology with extensive cell-cell contacts. Following treatment with aPBMC-CM, PTECs morphology was significantly affected. PTECs were more elongated, formed less cell-cell attachments and produced filopodia. Similar effects were observed in primary proximal tubular cells (RPTECs), whereby treatment with aPBMC-CM induced a more severe morphological change. The expression and distribution of key epithelial proteins was examined by immunofluorescent microscopy. The cytoplasmic F-actin network regulates cell shape and modulates many aspects of cell phenotype. In control PTECs, F-actin filaments were observed in concentrated peripheral bundles, which is indicative of polarised epithelial cells with established cell-cell junctions. aPBMC-CM treatment disrupted this distribution with F-actin filaments appearing as stress fibres which are associated with a more mesenchymal phenotype (Fig 1B). Junctional integrity is critical to epithelial monolayer function. We examined the distribution and expression of E-cadherin (adherens junctions) and ZO-1 (tight junctions). E-cadherin mRNA was significantly reduced after 48 hours aPBMC-CM treatment (Fig 2A). This reduction was also reflected at the protein level as assessed by immunofluorescent microscopy and by Western blot (Figs 1B and 2B). ZO-1 mRNA was also significantly downregulated by aPBMC-CM treatment in our model (Fig 2A). Again, this was also reflected at the protein level (Fig 1B and 2B). Taken together these results suggest significant disruption of the adherens and tight junctions. The phenotypic changes associated with EMT include increased expression of myofibroblast-associated proteins. Therefore effects of aPBMC-CM on α -SMA and vimentin expression were assessed (Fig 1B, 2A and 2B). Increased staining for α -SMA was observed after 48 hours aPBMC-CM treatment. In addition, vimentin, a type III intermediate filament protein highly expressed in myofibroblasts significantly increased both at the transcript and whole cell protein levels. Taken together, the effects on cell morphology, epithelial junctions and myofibroblast-associated proteins suggest

that PTECs exposed to aPBMC-CM undergo significant phenotypic changes consistent with those observed during EMT [22].

HMGB-1 is upregulated in proximal tubular epithelial cells exposed to aPBMC-CM and is secreted by activated PBMCs.

In the current study, a suppression subtractive hybridisation (SSH) was performed to identify genes that were differentially regulated in PTECs by exposure to aPBMC-CM. After amplification and sequencing HMGB-1 was identified as being significantly upregulated after aPBMC-CM treatment. This observation was confirmed by RT-PCR demonstrating increased HMGB-1 mRNA (Fig 3A). This effect was then examined at protein level. In whole cell lysates, equal amounts of HMGB-1 were observed in control and aPBMC-CM treated PTECs across all time points suggesting that intracellular levels of HMGB-1 were unchanged (Fig 3B). In contrast, HMGB-1 protein levels in cell supernatants were significantly increased in response to aPBMC-CM treatment compared to controls, at all timepoints (Fig 3C). Also of note was a modest increase in HMGB-1 protein in the supernatant of PBMCs after activation compared to inactivated controls (Fig 3D). Together, these results demonstrate that exposure to aPBMC-CM resulted in significant induction and secretion of HMGB-1 in PTECs.

Recombinant HMGB-1 protein induces EMT in proximal tubular epithelial cells.

To investigate the potential role of secreted HMGB-1 in this model of immune-mediated PTEC damage, the effects of direct application of recombinant human HMGB-1 to PTECs was examined. Firstly, the effects of increasing concentrations of HMGB-1 on PTEC morphology was investigated (Fig 4A). PTECs were treated with 1µg/ml, 5µg/ml and 10µg/ml recombinant HMGB-1 protein for 72 hours. HMGB-1 induced dose-dependent alterations in PTEC morphology, inducing EMT-like effects as cells became scattered, elongated and formed filopodia. The effects of HMGB-1 on PTEC phenotype were further examined by RT-PCR assessment of E-cadherin and α -SMA mRNA levels (Fig 4B & 4C). After 72 hours, exposure to 5µg/ml HMGB-1 resulted in significant downregulation of E-cadherin mRNA in PTECs. After the same treatment, α -SMA mRNA was significantly upregulated and this effect was also

observed at the protein level by indirect immunofluorescent microscopy (Fig 4D). Aberrant expression of HMGB-1 has been observed in some invasive cancers [7,35]. Since our studies demonstrated loss of cell-cell contacts following treatment with HMGB-1 we investigated the effect of HMGB-1 on cell motility using an *in vitro* wound healing assay. Migrating cells were quantified at 24, 48 and 72 hours post-wounding. At each timepoint, significantly higher numbers of PTECs cultured in the presence of HMGB-1 had entered the wound area compared to control cells (Fig 4E).

HMGB-1 induced EMT in proximal tubular epithelial cells is mediated by RAGE and TGF- β 1

HMGB-1 binds to the RAGE receptor with high affinity and ligand binding to RAGE has been shown to activate positive auto-regulatory signals to cause RAGE upregulation in cells to enhance RAGE signalling [32]. This potentially important mechanism was investigated in this study. PTECs were treated with 5 μ g/ml HMGB-1 over 72 hours. By 48 hours, treatment with HMGB-1 had increased RAGE compared to time-matched controls (Fig 5A). By 72 hours levels of RAGE were strongly increased by HMGB-1 treatment. The role of RAGE in HMGB-1 induced EMT was examined using a RAGE neutralising antibody (nAb). PTECs were pre-treated with increasing doses of RAGE nAb for 1 hour prior to treatment with 5 μ g/ml HMGB-1. RAGE nAb caused a dose-dependent protection against the alterations in epithelial morphology induced by HMGB-1 with the greatest degree of protection observed using 2 μ g/ml RAGE nAb. PTECs pre-treated with RAGE nAb retained a more epithelial morphology compared to HMGB-1 alone (Fig 5B). This protective effect on PTEC morphology coincided with a significant inhibition of HMGB-1-induced α -SMA mRNA upregulation (Fig 5C). A number of studies have suggested links between RAGE activation and TGF- β 1 signalling. TGF- β 1 plays a significant role in renal fibrosis development through the regulation of ECM production and the induction of EMT in PTECs. We investigated the role for TGF- β 1 in mediating the effects of HMGB-1 in this model. In PTECs exposed to increasing concentrations of HMGB-1, dose and time-dependent increases in TGF- β 1 secretion were detected (Fig 6A). In PTECs pre-treated with 100 μ g/ml of a TGF- β 1 nAb, significant inhibition of HMGB-1-induced α -SMA mRNA upregulation was observed (Fig 6B). This finding would suggest that HMGB-1 is indeed inducing TGF- β 1 expression and that TGF- β 1 could be a key mediator in HMGB-1 induced

EMT. Taken together, these results suggest the involvement of both RAGE and TGF- β 1 in mediating the pro-fibrotic effects of HMGB-1 on PTEC phenotype in this model.

Discussion

Activated immune cells secrete significant quantities of cytokines and growth factors at the site of renal injury [10]. Many of these factors are capable of activating fibroblasts and have so been implicated in renal disease progression [18]. However, the role of the tubular epithelial cell and in particular the interaction between the immune cell and tubular epithelial cell in renal fibrosis development is less well studied. Tubular EMT is a process by which renal tubular epithelial cells lose features of their epithelial phenotype and acquire new characteristic features of myofibroblasts [22]. The process of EMT is essential for morphogenetic movements within the developing embryo. The capacity for this phenotypic conversion suggests an incredible plasticity of the differentiated tubular epithelial cell and has been associated with generation of the matrix-producing fibroblasts under pathological settings such as fibrosis and in tumour invasion [37]. A number of features associated with EMT have been characterised including loss of epithelial cell adhesion, F-actin reorganisation, *de novo* expression of fibroblast proteins (such as α -SMA and vimentin), and altered cell migration [21]. While the importance of the later feature is currently under debate, the likely contribution of PTECs to renal fibrosis development is widely accepted. Despite advances in our understanding of the EMT process, mechanistic links between the *in vitro* observations and the *in vivo* disease environment are lacking. In the current work we have utilised an *ex-vivo* preparation to model immune cell activation in a disease context and investigate the interaction of the tubular epithelium with the immune system.

Previously, we demonstrated significant effects on primary tubular epithelial cells following treatment with the secretome of activated peripheral blood mononuclear cells [14]. The current study confirms and extends these observations using a well established proximal tubular epithelial cell line. The current study investigated the effects of aPBMC-CM on PTEC phenotype, examining a number of the previously outlined features associated with EMT. aPBMC-CM caused significant morphological changes consistent with those reported previously in primary tubular epithelial cells [14]. This was likely underpinned by the observed alterations in F-actin distribution. aPBMC-CM also induced strong upregulation of fibroblast associated proteins α -SMA and vimentin at mRNA and protein level. α -SMA was distributed in a stretched and elongated pattern indicative of stress fibre formation, suggesting a dynamic alteration of PTEC cellular architecture likely contributing to the more mesenchymal appearance [13].

aPBMC-CM treatment also significantly impacted cell-cell adhesions in PTECs. Both E-cadherin and ZO-1 were significantly downregulated at mRNA and protein level following treatment with aPBMC-CM. Together these results strongly suggest that exposure of PTECs to aPBMC-CM resulted in significant restriction of epithelial phenotype suggesting an EMT-like effect.

EMT can be initiated by a number of extracellular signals [30]. ECM components such as collagen, as well as soluble factors including epidermal growth factor (EGF), hepatocyte growth factor/scatter factor (HGF/SF), interleukin 1 (IL-1), tumour necrosis factor α (TNF- α), members of the fibroblast growth factor (FGF) and transforming growth factor β 1 (TGF- β 1) have all been shown to contribute to EMT induction *in vitro* and *in vivo* [13]. In an effort to identify novel factors of importance in this *ex-vivo* model of EMT a suppression subtractive hybridisation approach was employed. Sequence analysis of the cDNA library generated in our laboratory revealed the upregulation of HMGB-1 in cells exposed to aPBMC-CM. This observation was confirmed by RT-PCR and was also observed at protein level. Importantly however, the increases in HMGB-1 production appeared to be restricted to the secreted protein fraction. As a result of this finding we next investigated the effect of recombinant human HMGB-1 protein on PTEC morphology and phenotype. We observed alterations consistent with EMT demonstrating, for the first time, a possible link between HMGB-1 and EMT in PTECs.

Several characteristics of HMGB-1 suggest associations with mesenchymal phenotypes. HMGB-1 has been shown to localise at infiltrating filopodial edges, and HMGB-1 is associated with enhanced outgrowth and migration in embryonic neurons, lung cells and tumour cells [16]. HMGB-1 may associate with α -SMA at filopodial membranes. Another member of the HMGB family, HMG-Y, has been shown to induce EMT in the MCF-7 breast epithelial cell line [27]. Our results suggest that the effects of HMGB-1 in PTECs are mediated, at least in part by the RAGE receptor and by TGF- β 1. In addition to the effects observed on cell phenotype, HMGB-1 treatment also resulted in upregulation of RAGE expression. This is similar to the effects observed by Arumugam *et al.* [1], demonstrating that ligand binding to RAGE upregulated RAGE expression thereby amplifying the pro-inflammatory effects of RAGE and its ligands. In PTECs pre-treated with a neutralising antibody against RAGE, significant protection of epithelial phenotype was observed. It has also been observed that the downstream effects of RAGE

activation can be mediated by TGF- β 1 [3,35]. Evidence suggests that positive crosstalk links RAGE activation and TGF- β 1 signalling leading to enhancement of both signals [33]. TGF- β 1 induced a time dependent increase in *de novo* synthesis of RAGE protein in hepatic stellate cells and RAGE agonist AGE-BSA caused up-regulated TGF- β 1 mRNA levels in tubular epithelial cells [9,36]. In addition, upregulation of TGF- β 1 in sub-mesothelial fibrosis was prevented using anti-RAGE antibodies [3]. In the current study, HMGB-1 induced TGF- β 1 secretion from PTECs in a dose dependent manner. Inhibition of TGF- β 1 with a neutralising antibody prevented induction of α -SMA in HMGB-1 treated cells, thus suggesting that it is also involved in the subsequent signalling from the HMGB-1/RAGE interaction. TGF- β 1 has been shown to be a key signalling molecule in the induction of EMT in renal epithelial cells, therefore this would suggest that it may also play an important role in HMGB-1 induced EMT [23,34].

In summary, we have demonstrated that the supernatant from aPBMCs induced EMT in proximal tubular epithelial cells. Differential gene expression screening identified HMGB-1 as being significantly upregulated by aPBMC-CM treatment. HMGB-1 alone was capable of inducing EMT in PTECs. Inhibition of RAGE and TGF- β 1 conferred a protective effect against the alterations induced by HMGB-1 suggesting their involvement in mediating HMGB-1 actions. Our results strongly implicate HMGB-1 in the induction of immune-mediated EMT. A schematic representation of the key signalling events involved is outlined in Figure 7. These results contribute to our better understanding of the molecular mechanisms of EMT indicating the importance of the immune-cell secretome in renal EMT induction and subsequent fibrosis development.

Acknowledgements

This project was supported by the Conway Institute of Biomolecular and Biomedical Research and the Dublin Molecular Medicine Centre (DMMC); under the Programme for Research in Third Level Institutions (PRTL) administered by the Higher Education Authority (HEA). This project was also supported by Science Foundation Ireland, the Health Research Board of Ireland, Enterprise Ireland, and by a research bursary from the Irish Nephrological Society and Amgen Ltd.

Transparency Declarations

Authors have no conflicts of interest to declare.

The results presented in this paper have not been published previously in whole or part, except in abstract format.

References

1. Arumugam T, Simeone DM, Schmidt AM, *et al.* S100P stimulates cell proliferation and survival via receptor for activated glycation end products (RAGE). *J Biol Chem* 2004; 279:5059-65.
2. Currie RA. Functional interaction between the DNA binding subunit trimerization domain of NF-Y and the high mobility group protein HMG-I(Y). *J Biol Chem* 1997; 272:30880-8.
3. De Vriese AS, Flyvbjerg A, Mortier S, *et al.* Inhibition of the interaction of AGE-RAGE prevents hyperglycemia-induced fibrosis of the peritoneal membrane. *J Am Soc Nephrol* 2003; 14:2109-18.
4. Degryse B, de Virgilio M. The nuclear protein HMGB1, a new kind of chemokine? *FEBS Lett* 2003; 553:11-7.
5. Eddy AA. Progression in chronic kidney disease. *Adv Chronic Kidney Dis* 2005; 12:353-65.
6. El-Nahas AM. Plasticity of kidney cells: role in kidney remodeling and scarring. *Kidney Int* 2003; 64:1553-63.
7. Fages C, Nolo R, Huttunen HJ, *et al.* Regulation of cell migration by amphotericin. *J Cell Sci* 2000; 113 (Pt 4):611-20.
8. Fashena SJ, Reeves R, Ruddle NH. A poly(dA-dT) upstream activating sequence binds high-mobility group I protein and contributes to lymphotoxin (tumor necrosis factor-beta) gene regulation. *Mol Cell Biol* 1992; 12:894-903.
9. Fehrenbach H, Weiskirchen R, Kasper M, *et al.* Up-regulated expression of the receptor for advanced glycation end products in cultured rat hepatic stellate cells during transdifferentiation to myofibroblasts. *Hepatology* 2001; 34:943-52.
10. Ferenbach D, Kluth DC, Hughes J. Inflammatory cells in renal injury and repair. *Semin Nephrol* 2007; 27:250-9.

11. Goumenos DS, Brown CB, Shortland J, *et al.* Myofibroblasts, predictors of progression of mesangial IgA nephropathy? *Nephrol Dial Transplant* 1994; 9:1418-25.
12. Guarino M, Tosoni A, Nebuloni M. Direct contribution of epithelium to organ fibrosis: epithelial-mesenchymal transition. *Hum Pathol* 2009; 40:1365-76.
13. Hay ED, Zuk A. Transformations between epithelium and mesenchyme: normal, pathological, and experimentally induced. *Am J Kidney Dis* 1995; 26:678-90.
14. Healy E, Leonard M, Madrigal-Estebas L, *et al.* Factors produced by activated leukocytes alter renal epithelial cell differentiation. *Kidney Int* 1999; 56:1266-9.
15. Hori O, Brett J, Slattery T, *et al.* The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. *J Biol Chem* 1995; 270:25752-61.
16. Huttunen HJ, Kuja-Panula J, Sorci G, *et al.* Coregulation of neurite outgrowth and cell survival by amphoterin and S100 proteins through receptor for advanced glycation end products (RAGE) activation. *J Biol Chem* 2000; 275:40096-105.
17. Ishihara Y, Nishikawa T, Iijima H, *et al.* Expression of matrix metalloproteinase, tissue inhibitors of metalloproteinase and adhesion molecules in silicotic mice with lung tumor metastasis. *Toxicol Lett* 2003; 142:71-5.
18. Iwano M, Neilson EG. Mechanisms of tubulointerstitial fibrosis. *Curr Opin Nephrol Hypertens* 2004; 13:279-84.
19. Lan HY. Tubular epithelial-myofibroblast transdifferentiation mechanisms in proximal tubule cells. *Curr Opin Nephrol Hypertens* 2003; 12:25-9.
20. Li JH, Wang W, Huang XR, *et al.* Advanced glycation end products induce tubular epithelial-myofibroblast transition through the RAGE-ERK1/2 MAP kinase signaling pathway. *Am J Pathol* 2004; 164:1389-97.

21. Liu Y. Epithelial to mesenchymal transition in renal fibrogenesis: pathologic significance, molecular mechanism, and therapeutic intervention. *J Am Soc Nephrol* 2004; 15:1-12.
22. Liu Y. New insights into epithelial-mesenchymal transition in kidney fibrosis. *J Am Soc Nephrol* 2009; 21:212-22.
23. McMorro T, Gaffney MM, Slattery C, *et al.* Cyclosporine A induced epithelial-mesenchymal transition in human renal proximal tubular epithelial cells. *Nephrol Dial Transplant* 2005; 20:2215-25.
24. Nightingale J, Patel S, Suzuki N, *et al.* Oncostatin M, a cytokine released by activated mononuclear cells, induces epithelial cell-myofibroblast transdifferentiation via Jak/Stat pathway activation. *J Am Soc Nephrol* 2004; 15:21-32.
25. Penny MJ, Boyd RA, Hall BM. Role of T cells in the mediation of Heymann nephritis. ii. Identification of Th1 and cytotoxic cells in glomeruli. *Kidney Int* 1997; 51:1059-68.
26. Rauvala H, Rouhiainen A. Physiological and pathophysiological outcomes of the interactions of HMGB1 with cell surface receptors. *Biochim Biophys Acta*; 1799:164-70.
27. Reeves R, Edberg DD, Li Y. Architectural transcription factor HMGI(Y) promotes tumor progression and mesenchymal transition of human epithelial cells. *Mol Cell Biol* 2001; 21:575-94.
28. Reeves R, Wolffe AP. Substrate structure influences binding of the non-histone protein HMG-I(Y) to free nucleosomal DNA. *Biochemistry* 1996; 35:5063-74.
29. Rodgers KD, Rao V, Meehan DT, *et al.* Monocytes may promote myofibroblast accumulation and apoptosis in Alport renal fibrosis. *Kidney Int* 2003; 63:1338-55.
30. Savagner P. Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. *Bioessays* 2001; 23:912-23.

31. Schmidt AM, Vianna M, Gerlach M, *et al.* Isolation and characterization of two binding proteins for advanced glycosylation end products from bovine lung which are present on the endothelial cell surface. *J Biol Chem* 1992; 267:14987-97.
32. Schmidt AM, Yan SD, Yan SF, *et al.* The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. *J Clin Invest* 2001; 108:949-55.
33. Simm A, Bartling B, Silber RE. RAGE: a new pleiotropic antagonistic gene? *Ann N Y Acad Sci* 2004; 1019:228-31.
34. Slaterry C, Campbell E, McMorrow T, *et al.* Cyclosporine A-induced renal fibrosis: a role for epithelial-mesenchymal transition. *Am J Pathol* 2005; 167:395-407.
35. Taguchi A, Blood DC, del Toro G, *et al.* Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases. *Nature* 2000; 405:354-60.
36. Yamagishi S, Inagaki Y, Okamoto T, *et al.* Advanced glycation end products inhibit de novo protein synthesis and induce TGF-beta overexpression in proximal tubular cells. *Kidney Int* 2003; 63:464-73.
37. Zeisberg M, Kalluri R. Fibroblasts emerge via epithelial-mesenchymal transition in chronic kidney fibrosis. *Front Biosci* 2008; 13:6991-8.

FIGURE LEGENDS

FIGURE 1 - *aPBMC-CM induced phenotypic changes in renal proximal tubular cells.* HK-2 cells or primary RPTECs were grown on 60mm tissue culture dishes or Falcon 8-chamber glass slides. Cells were cultured in the presence of 30% (v/v) control or active peripheral blood mononuclear cell conditioned medium (aPBMC-CM) for 48 hours. **(A)** Morphological changes were examined by phase-contrast microscopy (Magnification 100X). **(B)** The effects of aPBMC-CM on F-Actin arrangement, ZO-1, E-cadherin and α -SMA expression was assessed by immunofluorescent microscopy (Magnification 400X). Images shown are representative of at least 3 independent experiments.

FIGURE 2 - *aPBMC-CM induced gene expression changes in renal proximal tubular cells.* HK-2 cells were grown on 60mm tissue culture dishes and cultured in the presence of 30% (v/v) control or active peripheral blood mononuclear cell conditioned medium (aPBMC-CM) for 24, 48 or 72 hours. **(A)** Real-Time-PCR was performed at 48 hours using primers designed against E-cadherin, ZO-1 and vimentin as described in Experimental Procedures. Data shown represents the mean relative fold change compared to time matched controls $\pm$ S.E.M. (* $P < 0.05$, ** $P < 0.01$; n=4). **(B)** E-cadherin, ZO-1 and vimentin protein levels in whole cell lysates were examined by Western blot. β -actin levels were used as a loading control. Band intensities were quantified densitometrically. Data shown represents the mean relative fold change compared to time matched controls $\pm$ S.E.M. (* $P < 0.05$, ** $P < 0.01$; n=3).

FIGURE 3 – *aPBMCs induce expression and secretion of HMGB-1 from renal proximal tubular cells.* HK-2 cells were grown on 60mm tissue culture dishes and cultured in the presence of 30% (v/v) control or active peripheral blood mononuclear cell conditioned medium (aPBMC-CM) for 24, 48 or 72 hours. **(A)** RT-PCR was carried out using primers designed against HMGB-1 and GAPDH as described in Experimental Procedures. Band intensity was quantified densitometrically. Results are expressed as mean relative optical density units $\pm$ S.E.M. of 3 independent experiments performed in duplicate. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). **(B)** Equal amounts of HK-2 whole cell lysates or concentrated cell supernatants **(C)** were analysed by Western blot using an anti-HMGB-1 antibody. β -actin levels were used as a loading control. **(D)** Equal concentrations of activated and un-activated PBMC-CM were analysed by Western blot

using an anti-HMGB-1 antibody. Images shown are representative of at least 3 independent experiments performed in duplicate.

FIGURE 4 - HMGB-1 induces phenotypic and transcriptomic changes in renal proximal tubular cells. HK-2 cells were grown on 60mm tissue culture dishes or Falcon 8-chamber glass slides. Cells were cultured in the absence or presence of increasing concentrations of HMGB-1 for 24 ,48 or 72 hours. **(A)** Cell morphology was examined by phase-contrast microscopy (Magnification 100X). Images are representative of at least three independent experiments performed in duplicate. **(B + C)** RT-PCR was carried out using primers designed against E-cadherin, α -SMA and GAPDH as described in Experimental Procedures. Band intensity was quantified densitometrically. Results are expressed as mean relative optical density units $\pm$ S.E.M. of 3 independent experiments performed in duplicate. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). **(D)** The effect of HMGB-1 on α -SMA expression was assessed by immunofluorescent microscopy (Magnification 200X). Images shown are representative of at least three independent experiments performed in duplicate. **(E)** HK-2 cells were grown on 60 mm tissue culture dishes and experimentally wounded. Cells were then cultured in the absence and presence of 5 μ g/ml HMGB-1 for indicated timepoints. Migrating cells were quantified by manual cell counts and are shown as mean $\pm$ S.E.M. of 3 independent experiments performed in duplicate (* $p < 0.05$, ** $p < 0.01$).

FIGURE 5 - The effects of HMGB-1 are mediated by RAGE in renal proximal tubular cells . HK-2 cells were grown on 60mm tissue culture dishes and were cultured in the absence or presence of 5 μ g/ml HMGB-1, with and without a RAGE neutralising antibody (2 μ g/ml) for 24 ,48 or 72 hours. **(A)** Equal amounts of HK-2 whole cell lysates were analysed by Western blot using an anti-RAGE antibody. Images shown are representative of at least 3 independent experiments performed in duplicate. β -actin levels were used as a loading control. **(B)** Cell morphology was assessed by phase contrast microscopy. **(C)** RT-PCR was carried out using primers designed against α -SMA and GAPDH as described in Experimental Procedures. Band intensity was quantified densitometrically. Results are expressed as mean relative optical density units $\pm$ S.E.M. of 3 independent experiments performed in duplicate. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

FIGURES 6 - *The effects of HMGB-1 are dependent on TGF- β 1 secretion in renal proximal tubular cells.* HK-2 cells were grown on 60mm tissue culture dishes and were cultured in the absence or presence of increasing concentrations of HMGB-1, with and without a RAGE neutralising antibody (100 μ g/ml) for 24 ,48 or 72 hours. **(A)** Equal amounts of HK-2 whole cell lysates were analysed by Western blot using an anti-TGF- β 1 antibody. β -actin levels were used as a loading control. Images shown are representative of at least 3 independent experiments performed in duplicate. **(B)** RT-PCR was carried out using primers designed against α -SMA and GAPDH as described in Experimental Procedures. Band intensity was quantified densitometrically. Results are expressed as mean relative optical density units $\pm$ S.E.M. of 3 independent experiments performed in duplicate. (*p<0.05, **p<0.01, ***p<0.001).

FIGURE 7 - *Proposed role of HMGB-1 in immune-mediated epithelial-mesenchymal transition in renal proximal tubular cells.*

A

B

A

B

C