

**Kallikrein gene ‘knock-down’ by siRNA transfection induces a pro-fibrotic phenotype in rat mesangial cells**

Izabella Z.A Pawluczyk<sup>a,c</sup>, Eddie K.C. Tan<sup>c</sup>, David Lodwick<sup>b</sup>, Kevin P.G. Harris<sup>a,c</sup>  
Department of Infection, Immunity and Inflammation<sup>a</sup>, and Department of Cardiovascular  
Sciences<sup>b</sup>, University of Leicester  
John Walls Renal Unit<sup>3</sup>, Leicester General Hospital<sup>c</sup>, Leicester, UK

Running title: kallikrein suppression reduces mesangial cell scarring

Correspondence:

Dr I.Z.A. Pawluczyk  
John Walls Renal Unit  
Leicester General Hospital  
Gwendolen Road  
Leicester LE5 4PW  
Tel 0116 2584111  
FAX 0116 2584617  
e-mail [izap1@le.ac.uk](mailto:izap1@le.ac.uk)

## **Abstract**

Emerging evidence suggests that kallikrein exerts reno-protective effects independent of its haemodynamic actions. The aim of the current investigation was to delineate the role of kallikrein in the regulation of fibrosis, by 'knocking down' its expression using specific small interfering RNAs (siRNA). Rat mesangial cells were treated with 12, 60, 120 nmol kallikrein-specific siRNAs. The consequent cellular genotypes and phenotypes were analysed. Western blotting demonstrated that mesangial cells produced a kallikrein protein, which was of a different molecular weight to urinary kallikrein from rats of the same species. Treatment of cells with siRNA resulted in a dose dependent decrease in kallikrein mRNA levels, which impacted on other components of the kallikrein-kinin system, dose dependently reducing bradykinin B2 receptor mRNA expression. Kallikrein suppression resulted in significant increases in fibronectin and TGF $\beta$  protein levels in culture supernatants over control levels. Gelatin zymography demonstrated a siRNA dose dependent decrease in active MMP2 enzyme levels. Bradykinin, an effector molecule of the kallikrein system is known to stimulate tPA production. However, paradoxically tPA protein levels were augmented with increasing kallikrein mRNA silencing. This was accompanied by a dose dependent decrease in low density lipoprotein receptor related protein (LRP) mRNA levels indicating that increased tPA levels were due to an attenuation of receptor mediated protease clearance. These data lend strong support to the hypothesis that kallikrein exerts anti-fibrotic, reno-protective effects that are independent of its classical haemodynamic actions.

## **Key words**

Kallikrein, fibrosis, mesangial cells,

## **Introduction**

Tissue kallikrein is a serum protease largely present in tissues, which are critical for blood pressure regulation and vascular function such as the kidney, heart, lungs, adrenals, and vasculature [1-3]. Kallikrein enzymatically cleaves kininogen to release kinins, peptide hormones that mediate a

number of biological responses including vasodilation, increased smooth muscle relaxation and increased vascular permeability [4] via bradykinin B2 receptor binding [5,6]. To date most of the reported beneficial effects of kallikrein have been associated with amelioration of hypertension. Epidemiological studies have shown that urinary kallikrein levels are inversely correlated with blood pressure in essentially hypertensive patients [7,8]. Consistent with these observations is the fact that an allele expressing high urinary kallikrein excretion may be associated with reduced risk of essential hypertension [8]. Genetic manipulation and pharmacological administration of kallikrein into experimental animals has not only been shown to reduce blood pressure but also to attenuate glomerulosclerotic and tubular injury [5, 9-11]. This is not entirely unexpected as improvement in hypertension is known to preserve kidney function. However, it has been shown that long-term infusion of tissue kallikrein can attenuate glomerulosclerosis in Dahl sensitive rats without affecting their blood pressure [12]. Furthermore, infusion of a bradykinin B2 receptor antagonist was able to abrogate kallikreins reno-protective effect whilst leaving the rise in blood pressure unaffected [5] These studies would suggest that the protective actions of kallikrein manipulation may occur independently of kallikrein's ability to lower blood pressure. We have previously shown in *in vitro* studies, and therefore independently of any confounding effects of haemodynamic control, that drugs, which are able to enhance the kallikrein-kinin system concomitantly, reduce fibronectin protein levels secreted by injured human mesangial cells [13-15]. This has also led us to speculate that the kallikrein-kinin system functions as a regulator of fibrosis [15].

A number of studies in experimental animals have investigated the effects of kallikrein gene transfer in conditions of renal disease to observe its potential protective effects [9-11]. In the current study we aim to investigate the role of kallikrein in the regulation of fibrosis, *in vitro*, independently of hemodynamic effects, by 'knocking down' its expression, using kallikrein specific small interfering RNAs (siRNA) and observing potentially detrimental effects.

## **Methods**

Unless otherwise stated all chemicals were obtained from Sigma Chemical Company, Poole, Dorset, UK.

### **Cell culture**

Glomerular mesangial cells were cultured from the glomerular explants of age matched (8 week) male and female Wistar rat kidneys (University of Leicester colony) using standard techniques. The cells were cultured in RPMI 1640 (Invitrogen, Paisley, UK) supplemented with 20% heat inactivated foetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin and 2mM glutamine. Cultured cells were characterized as mesangial by their typical stellate, fusiform morphology, their positive staining for the Thy-1 antigen and their resistance to the toxic effects of D-valine and PAN. Mesangial cells were used in passage 2-10. In all experiments they were cultured in 24 well plates (Costar-Corning), allowed to grow to 50-70% confluence for kallikrein siRNA transfection.

### **Transfection of cells with kallikrein siRNA**

Two different siRNA preparations to renal kallikrein (kallikrein 1) were used:

siRNA 1: 5'-AACCAACAUGCCAGCCAUC99-3' annealed with 5'-GAUGGCUGGCAU GUUGGUU99-3', siRNA 2: 5'-UUCCCUGAUGAUCUCCAGU99-3' annealed with 5'-ACUGGA GAUCAUCAGGGAA99-3'. Scrambled control siRNA 5'-UCGUCCUAUUCGCAGAU U99-3' annealed with 5'-AAUCUGCGGAAUAGGACGA99-3' (99 refers to dTdT over hang) (RNA oligonucleotides were designed and custom-made by Eurogentec, Liege, Belgium))

Transfections were carried out broadly according to Eurogentec's protocol using the company's recommended transfection agent. Briefly, rat mesangial cells were grown in 24 well plates until they were approximately 50-70% confluent. 0.42µl jetSI®-ENDO transfection agent was added per 10pmol siRNA (and to medium containing no siRNA as zero control). Therefore 0.42, 2.1 or 4.2µl of transfection agent was added to 50µl of serum free medium, vortexed strongly and incubated for 10min. 0.14µg (10pmol), 0.70µg (50pmol), 1.4µg (100pmol) of siRNA duplex was added to 50µl of

serum free medium and vortexed gently. The solution containing the transfection agent was added to the solution containing the siRNA duplex. The mixture was vortexed immediately for 10s and incubated at room temperature for 30 min to allow the complexes to form. During complex formation growth medium from the plates was removed and 500µl of RPMI medium containing 1% FCS was added. 100µl of the jetSI®ENDO/siRNA duplex mixture was added to the medium in each well with swirling. The plate was then incubated for 4hr after which 250µl of 1% FCS RPMI medium was added to each well, thus making the final concentration of siRNA in each well 12, 60 and 120nM. After 24hr the supernatants were removed and saved for analysis. RNA was extracted from the cells using TRIzol.

### **RT-PCR**

0.5µg aliquots of total RNA were reverse transcribed using AMV reverse transcription system (Promega, Southampton, UK) according to the manufacturer's instructions. The resulting cDNA was amplified using ReddyMix™ PCR Mastermix (ABgene, Surrey, UK) and 50pmol of specific sense and anti-sense primers. Thermocycling conditions were optimised for each primer pair.

Oligonucleotide primers used: kallikrein sense 5'-ATGTGGTTCCTGATCCTATTCCTCGACCTG-3' antisense 5'-TCAGGGGTTTTCTTCATAACTTCTTT-3', bradykinin B2 receptor sense 5'GGACCATGAAGGACTACAGG-3', antisense 5'TAGGCCACGTAGGAACTGAT-3' [16], MMP2 sense 5'CCAGCGACCTCAGGGTGACA-3' antisense 5'-TAGTGGAGCACCAGAGGAA-3' [17], MMP9 sense 5'-CTCACCAGCGCCAGC CGACTTAT-3', antisense 5'-GGCACTGAGGAATGATCTAA-3' [17], LRP sense 5'-GCAGGC TACTGTGCTAACAAACAG-3' antisense 5'-TGCTAAGGGATCCCCTATCTCATC-3'. All primers were custom synthesised by Invitrogen. 18S ribosomal primers were from TaqMan™ (PE Applied Biosystems, CA, USA). 18s ribosomal RNA was used as the 'house keeping gene to normalise for loading.

### **Custom antibody production**

Anti-kallikrein antibodies were designed and custom manufactured by Eurogentech (Liege, Belgium) using the following peptide as an immunogen H<sub>2</sub>N-KVIDLPTEEPKVGST-CPNH<sub>2</sub>

### **Western blotting**

Culture supernatants from mesangial cells were mixed 1:1 with non-reducing sample buffer. The samples were boiled for 5min and resolved on 12% SDS-polyacrylamide gels. The gels were blotted onto nitrocellulose membranes and immunostained. Briefly, the membranes were blocked with a 2% solution of BSA in TTBS (TBS containing 0.5% Tween 20) for 1hr. The membranes were then washed once in TTBS prior to incubation with polyclonal rabbit anti-rat kallikrein (1:1000) (Eurogentec), for at least 2hr at room temperature. After 3 more washes the membranes were incubated with horseradish peroxidase-labelled goat anti rabbit immunoglobulins for at least 2hr at room temperature. The membranes were washed a further 3 times before addition of chromogenic substrate (SigmaFast™ DAB (diamino benzidine) tablets).

### **Kallikrein deglycosylation**

N-deglycosylation of kallikrein was carried out using GlycoProfileII Enzymatic In-solution Deglycosylation kit according to the manufacturer's instructions.

### **Preparation of cell lysates**

After removal of tissue culture supernatants, cell monolayers were washed with PBS, scraped into 1.0% IGEPAL (BDH, Merck,UK.) in wash buffer (PBS containing 0.3 M NaCl, 0.1% Tween 20) and then incubated at 37°C for 2 hours. The cell scrapings were then transferred into 2ml tubes and sonicated for 5s using a Jencons 50 watt sonicator. The cell lysates were then centrifuged for 30s at 13000 rpm before determination of protein concentration.

### **Measurement of fibronectin production**

Culture supernatants or cell lysates were assayed for fibronectin using an inhibition ELISA as previously described [18].

### **Protein assay**

Detergent-lysed cell monolayer protein was assayed using a commercial BioRad DC protein assay kit using BSA standards (BioRad, UK).

### **Preparation of macrophage-conditioned medium (mpcm)**

Macrophage conditioned medium was prepared from LPS stimulated peritoneal macrophages as previously described [18].

### **Cloning of the kallikrein gene into a pTarget expression vector.**

The full coding region of the renal kallikrein-1 gene was amplified by RT-PCR using the following forward and reverse primers: 5'-ATG TGG TTC CTG ATC CTA TTC CTC GAC CTG-3', 5'-TCA GGG GTT TTC CTT CAT AAC TTC TTT-3'. The PCR product was purified by electrophoresis on a 1% agarose gel and extracted from the gel using Sephglas Band Prep (Amersham-Pharmacia) according to the manufacturer's instructions. The purified PCR product was then ligated into the pTarget mammalian expression vector (Promega) according to the manufacturer's instructions. The kallikrein-vector construct was transformed into E coli (JM109 strain), and plated onto selective agar (containing ampicillin, IPTG and X-gal). White colonies were picked off and expanded in culture. The plasmids were then extracted using Wizard Minipreps (Promega) and digested using EcoR1. The digests were resolved by electrophoresis on agarose gels to see which of the plasmids contained the kallikrein insert. The chosen vector construct was expanded in culture and extracted using the Wizard Maxiprep (Promega). The purified vector construct was sequenced across the insertion site to confirm correct orientation using *ABPRISM*<sup>™</sup> dRhodamine Terminator Cycle Sequencing Ready Reaction

System. The plasmid vector construct was then used to transiently transfect rat mesangial cells with the kallikrein gene.

### **Transfection of cells with kallikrein gene**

Transfection reagent Transfast (Promega) was resuspended in water and frozen and thawed as directed by the manufacturer. The plasmid containing the kallikrein gene was diluted in serum free RPMI to give 0.75µg DNA per 200µl/well of a 24 well plate. 16µl Transfast was added to the DNA and vortexed briefly. The DNA transfection agent mixture was incubated for 10-15 min at room temperature. Growth medium was removed from the cells and 200µl of the mixture was added per well. The plate was returned to 37°C. After 1hr 800µl of RPMI containing 5% FCS or MPCM (containing 5% FCS) was added per well. The plate was returned to the incubator for a further 48hr. The supernatants were subsequently removed and saved for analysis. RNA was extracted from the cell monolayers using TRIzol.

### **Statistics**

In the siRNA experiments mesangial cell fibronectin, TGFβ and tPA levels were corrected for cell protein. Representative agarose gels, Western blots and zymograms are shown. Results are expressed as means ± SEM. For comparison of means between two groups, an unpaired *t*-test was employed. To compare values between multiple groups, an analysis of variance (ANOVA) with Bonferroni correction was applied. Statistical significance was defined as  $P < 0.05$ .

### **Results**

#### **Mesangial cell kallikrein**

Western blotting of mesangial cell supernatants for renal kallikrein protein demonstrated expression of a major protein band of the order of 40kD. On occasion, bands of the order of 65kD and 92kD were also observed (Fig1).

When mesangial cell supernatant kallikrein protein was compared to the urinary form from rats of the same species it was noted that the major kallikrein band in the urine was of the order of 20kD with only a faint band around 40kD. A band at 92kD was also observed, which probably represents a protein-bound form of kallikrein (Fig 1). The differences in molecular weight between the major bands in culture supernatants and urine can probably be explained by differences in glycosylation.

When mesangial cell and urinary kallikrein were N-deglycosylated, the molecular weight of mesangial cell kallikrein was reduced to around 25kD (Fig 1) (not quite to level of native urinary kallikrein at 20kD). When urinary kallikrein was N-deglycosylated a band appeared approximately of the same order of size as deglycosylated mesangial kallikrein (around 25kD) but the majority of the immunoreactive material was of a lower molecular weight (Fig 1). These data support previous reports of differentially glycosylated isoforms of kallikrein but more importantly suggest that mesangial cell-derived kallikrein does not contribute significantly to the major form of kallikrein found in the urine, which has come to be regarded as a marker of blood pressure control.

#### **‘Knock-down’ of mesangial cell kallikrein gene by siRNA**

In order to suppress kallikrein mRNA expression, mesangial cells were treated with 0, 12, 60, 120nM kallikrein specific siRNA for 24hr. RT-PCR of RNA extracted from the cells demonstrated that expression of the kallikrein mRNA was reduced with increasing concentrations of siRNA (Fig 2A). This effect was specific since 60nM scrambled siRNA had no effect on kallikrein gene expression (Fig 2A). Similar effects were observed with both siRNA preparations.

Reduction in secreted kallikrein protein by western blotting was not observed at this time point (24hr)

#### **Effect of kallikrein mRNA suppression on other components of the kallikrein-kinin system.**

In order to ascertain whether other components of the kallikrein-kinin system were also affected by kallikrein mRNA silencing RT-PCR for bradykinin B2 receptor The results demonstrated that

bradykinin B2 receptor mRNA expression was dose dependently down regulated with siRNA treatment (Fig 3a).

Western blotting of mesangial cell lysates confirmed that bradykinin B2 receptor suppression was also down regulated at the protein level (Fig 3b).

Since bradykinin B2 receptor signalling involves nitric oxide (NO), PCR for inducible NO synthase (iNOS) was also carried out. The RT-PCR demonstrated that kallikrein mRNA suppression resulted in an upregulation of iNOS mRNA levels (Fig 3c). Similar effects were observed with both siRNAs

### **Effect of suppressed kallikrein mRNA expression on fibronectin and modulators of matrix turnover.**

In order to observe the direct effects of kallikrein mRNA silencing on mesangial cell scarring supernatants from kallikrein siRNA transfected mesangial cells were analysed for fibronectin protein. We found that supernatant fibronectin levels were maximally increased by 60nM kallikrein siRNA ( $p < 0.05$  vs control and all other siRNA concentrations,  $n=7$ )(Fig 4).

The pro-fibrotic growth factor TGF $\beta$  is frequently up regulated in conditions of injury and particularly during matrix deposition. As kallikrein gene transfer has already been shown to reduce expression of TGF $\beta$  in DOCA salt hypertensive rats [19] we wished to determine whether 'knocking down' kallikrein could have the reverse effect. The ELISA demonstrated that immunoreactive TGF $\beta$  levels were indeed increased with kallikrein gene suppression. 60nM kallikrein siRNA treatment resulted in significantly higher TGF  $\beta$  levels than control and other siRNA concentrations ( $p < 0.05$ ,  $n=7$ ) (Fig 5).

We have previously shown that the anti-fibrotic responses that result from enhancement of the kallikrein-kinin system in human mesangial cells have involved the modulation of the expression of proteases [13-15]. RT-PCR demonstrated that both matrix metalloproteinase (MMP)-9 and MMP2 mRNA levels were up regulated with increased kallikrein silencing (Fig 6A). When mesangial cell culture supernatants were analysed by gelatin zymography only faint bands of MMP9 lytic activity

were detected. However, MMP2 activity was observed in mesangial cell supernatants, and was reduced dose-dependently with increasing siRNA concentration (Fig 6B).

Bradykinin, an effector molecule of the kallikrein-kinin system, is known to be a potent inducer of tissue plasminogen activator (tPA) [20,21], a protease also known to be involved in extra-cellular matrix degradation. We therefore analysed the effects on culture supernatant tPA levels of kallikrein 'knock-down'. The results unexpectedly demonstrated that tPA levels were significantly increased with 60nM and 120nM siRNA ( $p < 0.001$  vs control and 12nM siRNA, no significant difference was observed between 60nM and 120nM siRNA treatment) (Fig 7a). These data would suggest that either more tPA was being synthesised or that the protease was accumulating in the culture supernatants as a result of its being prevented from being taken up or scavenged by the cell.

RT-PCR demonstrated that siRNA treatment had no effect on tPA mRNA levels indicating that the observed increase in supernatant tPA levels was not a result of increased synthesis.

Among its many functions low-density lipoprotein receptor-related protein (LRP) is known to be a scavenger receptor for tPA [22]. RT-PCR on RNA extracted from siRNA treated cells was therefore carried out to determine whether LRP expression was affected by kallikrein mRNA suppression. RT-PCR demonstrated that LRP expression was down regulated on treatment with increasing concentrations of siRNAs (Fig 7b), thereby indicating that at least some of the increased tPA levels could be accounted for by an accumulation of tPA in the supernatant as a result of a reduced ability of kallikrein gene suppressed cells to endocytose tPA. This observation lends further support to our previous preliminary observations, which suggested that altered LRP activity was involved in the anti-fibrotic effects of anti-hypertensive drugs [23].

### **Transfection of the kallikrein gene**

In order to observe the effects of over expression of the kallikrein gene the whole coding region of rat renal kallikrein 1 was transfected into rat mesangial cells. Plasmid without insert was used as a negative control. RT-PCR of RNA extracted from mesangial cells treated with kallikrein or control

plasmid demonstrated that the kallikrein gene had been successfully transfected into and was being expressed by the mesangial cells (Fig 8a).

To observe whether over expression of kallikrein mRNA had any effect on mesangial cell fibronectin production particularly during injury, kallikrein-transfected mesangial cells were cultured in the presence or absence of macrophage conditioned medium (mpcm), which we have previously shown induces profibrotic responses in mesangial cells [18]. The results demonstrated that fibronectin levels in the supernatants of control (non-mpcm treated) kallikrein transfected mesangial cells were significantly reduced compared to control-transfected cells ( $p < 0.05$ ). However, kallikrein transfection had no beneficial effect on fibronectin levels in mpcm treated mesangial cells (Fig 8b). We were unable to demonstrate any change in protein expression by Western blotting.

## **Discussion**

It has been known for many years that the concentration of kallikrein detected in the urine is a predictor of blood pressure status [24]. Urinary kallikrein is known to come from the kidney [25,26] and more specifically from the distal tubular cells [27]. Whether renal and urinary kallikreins are actually the same molecule has been a matter for debate. Kidney, salivary gland and pancreatic kallikreins are known to be immunologically identical to urinary kallikrein when analysed by radioimmunoassay [28]. However, kallikrein is known to be differentially glycosylated [29] and as such is found in various isoforms and therefore different molecular weights. Although renal tissue kallikrein has previously been described as being histologically localised in the renal cortex (and only in response to physiological or pathological stimuli [30]) no expression has been reported in the glomeruli. In this study we have shown that mesangial cells constitutively express the kallikrein gene. Moreover, mesangial cell-derived kallikrein is a different size from urinary kallikrein of the same species. Since it is thought that kallikrein is excreted in urine without any further modification [31] our data would suggest that mesangial cell-derived kallikrein is not the major urinary factor

associated with hypotension. However, we do have strong evidence to indicate that kallikrein plays an important protective role in controlling the cell's fibrotic phenotype.

Recent *in vivo* gene transfer studies have highlighted the important role that kallikrein plays in reno-protection [21,32]. These studies suggested that the beneficial effects of kallikrein in terms of reducing fibrosis scores were mediated via activation of NO/cGMP signalling pathways, which ultimately resulted in a reduction in oxidative stress and TGF  $\beta$  expression [32]. The current *in vitro* study was in accord with these observations in that it showed that fibronectin and TGF $\beta$  levels were increased when kallikrein mRNA was suppressed. The fibrotic status of a cells environment is however, dependent on a fine balance between extracellular matrix synthesis and degradation. We were able to demonstrate a down regulation of active MMP2 enzyme activity in the culture media of kallikrein siRNA-transfected cells providing additional evidence for kallikrein favouring the anti-fibrotic side of the fibrosis equation. Paradoxically however, MMP2 mRNA expression was upregulated.

Kallikrein mRNA suppression also resulted in an increase in supernatant tPA protein levels. Tissue PA is generally thought to be beneficial in the pathogenesis of fibrosis because of its ability to increase matrix degradation and decrease matrix accumulation [33], it is also known to be up regulated in tissues and cells following stimulation with bradykinin [20,21]. The raised tPA levels induced by kallikrein suppression observed in this study would intuitively be contrary to expectations. However, increased tPA levels were most probably the result of a combination of down regulation of LRP, the receptor known to scavenge tPA, and through which tPA can signal [34] and a pathophysiological increase in synthesis. As the decrease in LRP expression occurs as an additional consequence of kallikrein mRNA suppression and since LRP is also known to be a receptor involved in the catabolism of fibronectin [34], this may provide another explanation as to why fibronectin accumulates following kallikrein mRNA suppression and further underlines kallikrein's direct protective role in reducing fibrosis.

Kallikrein gene silencing exerted down stream effects on other components of the kallikrein-kinin system. Bradykinin B2 receptor mRNA expression was seen to be dose dependently suppressed suggesting a common controlling mechanism or the involvement of an auto regulatory negative feed back loop.

Interestingly iNOS mRNA expression was upregulated. NO produced by iNOS is known to be a signalling molecule involved in bradykinin B2 receptor activity. It is also a ubiquitous molecule involved in many physiological reactions. NO has been shown to have protective effects in several models of renal disease [35,36]. Over expression of iNOS in a model of unilateral ureteral obstruction was shown to improve renal function [36]. However, during excessive NO production initiated by excessive production of iNOS derived from infiltrating immune cells it behaves as a pro-inflammatory mediator initiating nitrate damage and oxidative stress [37]. Elevated levels of iNOS have also been associated with an augmentation of oxidative stress in spontaneously hypertensive rats [32].

One of the most interesting observations of this study is the fact that specific silencing of the kallikrein gene also has profound effects on the expression of other related genes and proteins. This suggests that either there is a common controlling mechanism between the related genes or that the observations are due to non specific 'off target' effects. The fact that kallikrein siRNAs targeted to different parts of the kallikrein message exhibit comparable effects on other related genes and proteins, suggests that the observed 'lateral effects' are not likely to be due to 'off target' phenomena. The fact that the 60nM dose of siRNA most often resulted in optimal protein effects when the strongest knockdown effect on mRNA expression was seen with the 120nM siRNA suggests that the latter higher dose was possibly inducing a degree of toxicity.

In the inverse experiments where the kallikrein gene was over expressed in mesangial cells, fibronectin levels were reduced but only in the non mpcm-injured transfected cells. This may be due to the fact that not enough kallikrein is produced to counter the effects of injury in this system. Unfortunately during the course of this study it has only been possible to observe the consequences

of suppressing or over-expressing kallikrein message without concomitant measurable effects in protein levels. This may be due to the fact that the anti-kallikrein antibody is reactive against several other kallikrein isoforms. The reduction in targeted isoform may be masked by expression of an isoform, which is not suppressed. Further modulation of kallikrein protein expression to a measurable level would significantly assist in delineating the effects of kallikrein expression particularly in the injured state.

Several important *in vivo* studies have alluded to an important role for kallikrein in protection from fibrosis during hypertension-induced injury [19,32]. Although, blood pressure independent events *in vivo* can to a certain extent be teased out and studied, the influence of blood pressure itself can never be totally discounted. This is the first *in vitro* study, which presents strong evidence for the kallikrein-kinin system being directly involved in regulating the mesangial cell's fibrotic status in an injury free setting, which is totally independent of the confounding effects of haemodynamic control and provides a valuable platform for further research extending the observations to an *in vivo* model of renal injury.

## **Acknowledgments**

Dr Eddie Tan was supported by a Baxter Fellowship.

## **References**

1. Clements JA. The glandular kallikrein family of enzymes:tissue specific expression and hormonal regulation. *Endocr Rev* 1989; 10:393-419.
2. Wang DZ, Song Q, Chen LM, Chao L, Chao J. Expression and cellular localisation of tissue kallikrein-kinin systems in human adrenal gland. *Am J Physiol* 1996; 272:F709-F716.
3. Wolf WC, Harley RA, Sleuce D, Chao L, Chao J. Localisation and expression of tissue kallikrein-kinin and kallistatin in human blood vessels. *J Histochem Cytochem* 1999; 47:221-228.

4. Bhoola KD, Figueroa CD, Worthy K. Bioregulation of kinins, kallikreins, kininogens, and kinases. *Pharmacol Rev* 1992; 44:1-80.
5. Hirawa N, Uehara Y, Suzuki T, Kawabata Y, Numabe A, Gomi T et al. Regression of glomerular injury by kallikrein infusion in Dahl salt-sensitive rats is a bradykinin B2 receptor-mediated event. *Nephron* 1999; 81:183-193.
6. Wang DZ, Chao L, Chao J. Hypotension in transgenic mice over-expressing human bradykinin B2 receptor. *Hypertension* 1997; 29:488-493.
7. Margolius HS, Horowitz DZ, Pisano JJ, Keiser HR. Urinary kallikrein excretion in hypertensive man: Relationships to sodium intake and sodium retaining steroids. *Circ Res* 1974; 35:820-825.
8. Berry TD, Hasstedt SJ, Hunt SC, Wu LL, Smith JB, Ash KO et al. A gene for high urinary kallikrein may protect against hypertension in Utah kindreds. *Hypertension* 1989;123: 3-8.
9. Lin L, Zhang JJ, Chao L, Chao J. Gene therapy in hypertension: Adenovirus-mediated kallikrein gene delivery in hypertensive rats. *Hum Gene Ther* 1997; 8:1753-1761.
10. Chao J, Zhang JJ, Lin KF, Chao L. Human kallikrein gene delivery attenuates hypertension, cardiac hypertrophy and renal injury in Dahl salt-sensitive rats, *Hum Gene Ther* 1998; 9: 21-31.
11. Yamaha K, Wang C, Chao L, Chao J. Kallikrein gene delivery attenuates hypertension and cardiac hypertrophy and enhances renal function in Goldblatt hypertensive rats. *Hypertension* 1998; 31:1104-1110.
12. Uehara Y, Hirawa N, Kawabata Y, Suzuki T, Ohshima N, Oka K et al. Long term infusion of kallikrein attenuates renal injury in Dahl salt-sensitive rats. *Hypertension* 1994; 24:770-877.
13. Pawluczyk IZA, Patel SR, Harris KPG. The role of bradykinin in the anti-fibrotic effects of perindoprilat on human mesangial cells. *Kidney Int* 2004; 65:1240-1251.

14. Pawluczyk IZA, Patel SR, Harris KPG. The role of the alpha-1 Adrenoceptor in modulating human mesangial cell matrix production. *Nephrology Dial Transpl* 2006; 21:2417-2424.
15. Pawluczyk IZA, Patel SR, Harris KPG. Pharmacological enhancement of the kallikrein-kinin system promotes anti-fibrotic responses in human mesangial cells. *Cell Physiol Biochem* 2006; 18:327-336.
16. Yayama K, Nagaoka M, Takano M, Okamoto H. Expression of kininogen, kallikrein, and kinin receptor genes by rat cardiomyocytes. *Biochim Biophys Acta* 2000; 1495:69-77.
17. Martin J, Knowlden J, Davies M, Williams JD. Identification and independent regulation of human mesangial cell metalloproteinases. *Kidney Int* 1994; 46: 877-885.
18. Pawluczyk IZA, Harris KPG. Macrophages promote pro-sclerotic responses in cultured rat mesangial cells. A mechanism for the initiation of glomerulosclerosis. *J Am Soc Nephrol* 1997; 8: 1525-1536.
19. Xia CF, Bledsoe G, Chao L, Chao J. Kallikrein gene transfer reduces renal fibrosis, hypertrophy and proliferation in DOCA-salt hypertensive rats. *Am J Renal Physiol* 2005; 289: F622-F631.
20. Markwardt F, Klocking HP. Studies on the release of plasminogen activator. *Throm Res* 1976; 8:217-223.
21. Emeis JJ, Tranquille N. The role of bradykinin in secretion from vascular endothelial cells. *Agents Actions* 1992; 38 (suppl), 285-291.
22. Herz J. and Strickland DK. LRP: a multifunctional scavenger and signalling receptor. *J Clin Invest* 2001;108:779-784.
23. Pawluczyk IZA, Patel SR, Harris KPG. The ACE inhibitor perindoprilat modulates the activity of the low density lipoprotein receptor-related protein (LRP) in human mesangial cells [Abstract]. *J Am Soc Nephrol* 2006; 17: 251A.
24. Frey EK. Kreislaufhormon und innere secretion. *Munch. Med. Wschr.* 1929;76: 1951-1952.
25. WP Raab. Enzymes and isoenzymes in urine. *Curr probl Clin Biochem* 1968; 2: 17-83.

26. Frey EK, Kraut H, Werle E, Vogel R, Zickgraf-Rudel G, Trautschold I. In: Das kallikrein-kinin system und seine inhibitoem. Edited by Enke F, Stuttgart, 1968.
27. Goud HD, Oza NB, Mitsialis SA, Levinsky NG. Regulation of renal rK1 kallikrein in spontaneously hypertensive rats *Ren Physiol Biochem* 1993; 16: 125-130.
28. Van Leewen BH, Grinblat SM, Johnson CI. Tissue specific control of glandular kallikreins. *Am J Physiol (Renal Fluid Electrolyte Physiol)* 1984; 247: F760-F764.
29. Tomiya N, Yamaguchi T, Awaya J, Kurono M, Endo S, Arata Y, Takahashi N, Ishihara H, Mori M, Tejima S. Structural analysis of asparagines-linked oligosaccharides of porcine pancreatic kallikrein. *Biochem* 1988; 27:7146-7154.
30. Cumming AD, Walsh T, Wojtacha D, Fleming S, Thomson D, Jenjins DA. Expression of tissue kallikrein in human kidney *Clin Sci* 1994; 87: 5-11.
31. Takahashi S, Irie A, Miyake Y. Primary structure of human urinary prokallikrein. *J Biochem* 1988; 104: 22-29.
32. Zhang JJ, Bledsoe G, Kato K, Chao L, Chao J. Tissue kallikrein attenuates salt-induced renal fibrosis by inhibition of oxidative stress. *Kidney Int* 2004; 66:722-732.
33. Kitching AR, Holdsworth SR, Ploplis VA, Plow EF, Collen D, Carmeliet P, Tipping PG. Plasminogen and plasminogen activators protect against renal injury in crescentic glomerulonephritis. *J Exp Med* 1997; 185: 963-968.
34. Salicioni AM, Mizelle KS, Loukinova E, Mikhailenko I, Strickland DK, Gonias SL. The low density lipoprotein receptor-related protein mediates fibronectin catabolism and inhibits fibronectin accumulation on cell surfaces. *J Biol Chem* 2002; 277:16160-16166.
35. Ito K, Chen J, Khodadadian JJ, Seshan SV, Eaton C, Zhao X et al. Liposome-mediated transfer of nitric oxide synthase gene improves renal function in ureteral obstruction in rats. *Kidney Int* 2004; 66:1365-1375.
36. Datta PK, Sharma M, Duann P, Lianos EA. Effect of nitric oxide synthase inhibition on proteinuria in glomerular immune injury. *Exp Biol Med* 2006; 231:576-584.

37. Kumar U, Chen J, Sapoznikhov V, Canteros G, White BH, Sidhu A. Over expression of inducible nitric oxide synthase in the kidney of the spontaneously hypertensive rat. Clin Exp Hypertens 2005; 27: 17-31.

### **Figure Legends**

**Fig 1.** Mesangial cell kallikrein. Western blot shows pattern of kallikrein protein expression in native rat urine and mesangial cell culture supernatants and after N-deglycosylation (DG).

**Fig 2.** ‘Knock-down’ of mesangial cell kallikrein gene by siRNA.

RNA was extracted from mesangial cells following transfection with specific kallikrein siRNA or scrambled (control) siRNA. RNA was reverse transcribed and amplified using kallikrein specific primers.

**A.** Agarose gel shows kallikrein amplicons following transfection of mesangial cells with 12, 60, 120 nM specific kallikrein siRNA.

**B.** Agarose gel shows kallikrein amplicons following treatment with 60nM kallikrein specific siRNA vs 60nM scrambled siRNA.

**Fig 3** Effect of kallikrein siRNA treatment on other components of the kallikrein-kinin system.

RNA extracted from mesangial cells treated with kallikrein siRNAs was extracted, reverse transcribed and amplified using bradykinin B2 receptor (BkB2R) specific primers.

Agarose gels show BkB2R amplicons in response to 12, 60, 120 nM siRNA transfection.

**Fig 4.** Effect of suppressed kallikrein mRNA expression on fibronectin protein levels.

Histogram shows fibronectin protein levels in mesangial cell supernatants following treatment with kallikrein siRNAs. Data is presented as means $\pm$ sem of fibronectin levels (ng fibronectin/ $\mu$ g cell protein) expressed as a fold increase over control (zero siRNA) levels. Supernatant fibronectin

levels were maximally increased by 60nM kallikrein siRNA ( $p < 0.05$  vs control and all other siRNA concentrations (ANOVA),  $n=7$ )

**Fig 5** Effect of suppressed kallikrein mRNA expression on TGF $\beta$  protein levels.

Histogram shows TGF $\beta$  levels in mesangial cell supernatants following treatment with kallikrein siRNAs. Data is presented as means $\pm$ sem of TGF $\beta$  levels (pg TGF $\beta$  / $\mu$ g cell protein) expressed as fold increase over control (zero siRNA) levels. 60nM kallikrein siRNA treatment resulted in significantly higher TGF  $\beta$  levels than control and other siRNA concentrations ( $p < 0.05$  (ANOVA),  $n=7$ )

**Fig 6** Effect of siRNA treatment on MMP2 and MMP9 expression.

RNA extracted from mesangial cells treated with kallikrein siRNAs was extracted; reverse transcribed and amplified using MMP2 and MMP9 specific primers.

A. Agarose gel shows expression MMP2 and MMP9 amplicons in response to treatment with 12, 60, 120 nM kallikrein siRNA.

B. Gelatin zymogram shows bands of lysis corresponding to pro and active MMP2 activity and very faint bands of pro and active MMP9 activity following kallikrein siRNA treatment.

**Fig 7.** Effect of siRNA treatment on culture supernatant tPA levels.

A Histogram shows tPA protein levels in mesangial cell culture supernatants after transfection with kallikrein siRNA. Data are presented as means $\pm$  sem of tPA levels (ng tPA/ $\mu$ g cell protein) expressed as fold increase over control (zero siRNA) levels. tPA levels were significantly increased with 60nM and 120nM siRNA ( $p < 0.001$  vs control and 12nM siRNA, no significant difference was observed between 60nM and 120nM siRNA treatment (ANOVA),  $n=5$ ).

RNA extracted from mesangial cells treated with kallikrein siRNAs was extracted, reverse transcribed and amplified using specific LRP primers.

B. Agarose gel shows LRP amplicons following transfection with 12, 60, 120nM kallikrein siRNA.

**Fig 8.** Effect of kallikrein gene over expression on mesangial cell culture supernatant fibronectin levels in normal and injured mesangial cells.

A. Agarose gel showing kallikrein amplicons from mesangial cells transfected with kallikrein or control plasmid and then exposed to medium alone or mpcm.

B. Histogram showing mesangial cell fibronectin levels expressed as mg/ml in the culture supernatants of cells transfected with kallikrein or plasmid control and then exposed to medium alone or mpcm. Results are means  $\pm$  sem, (\* $p < 0.05$  vs control plasmid,  $n=3$ ).

Fig 1

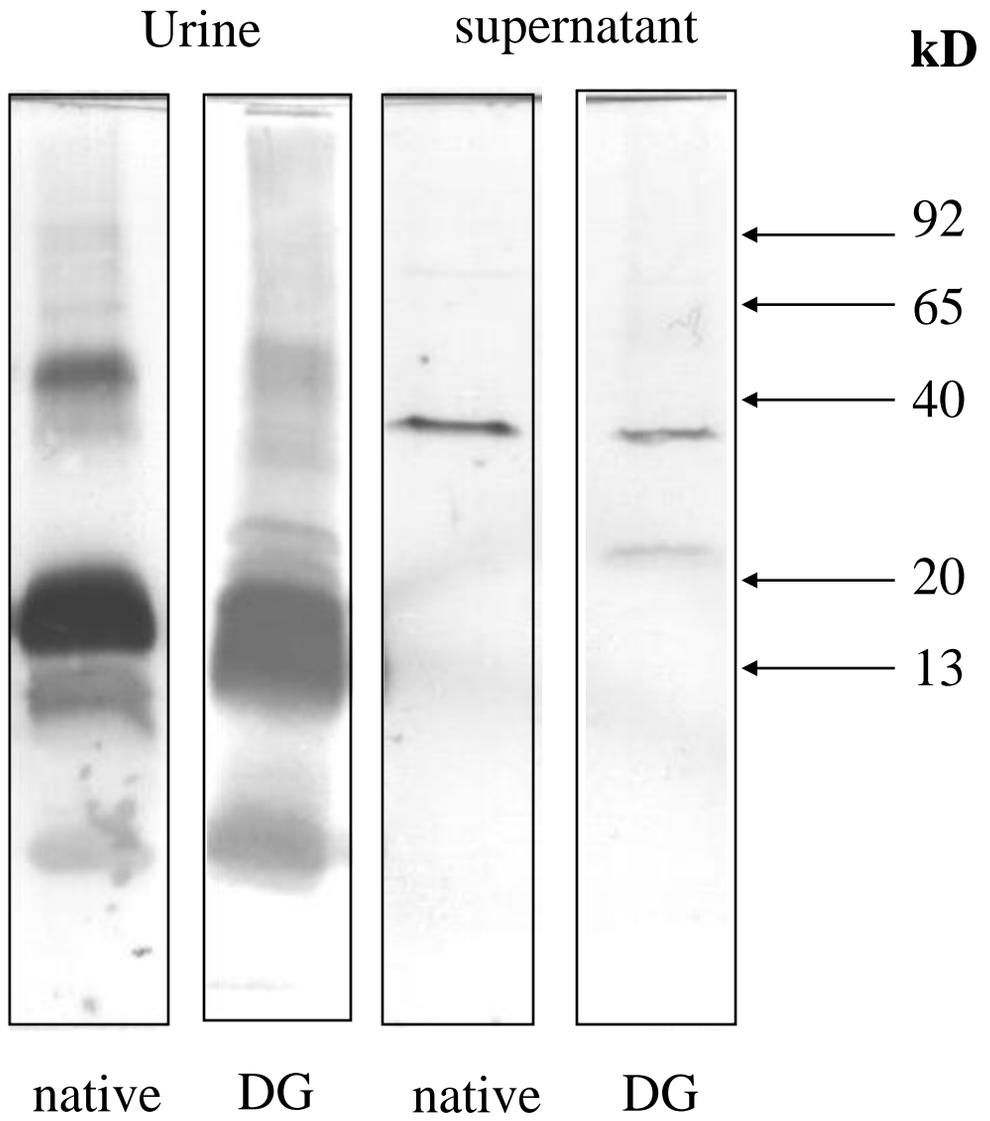
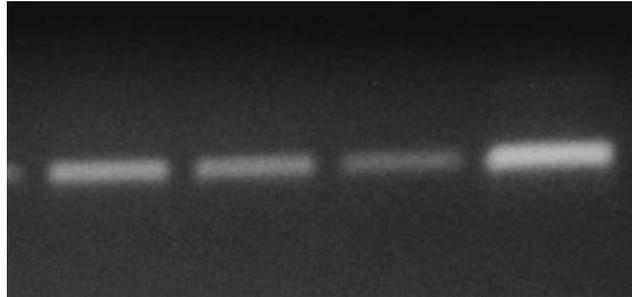


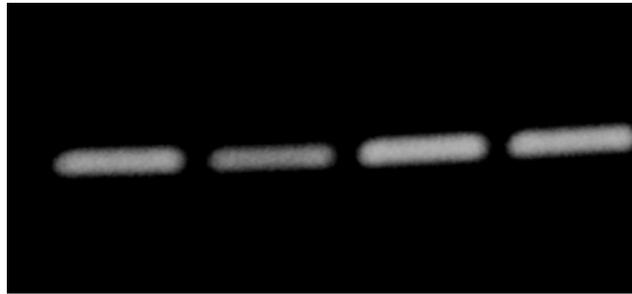
Fig 2

**A**



12    60    120    0  
Kallikrein siRNA nM

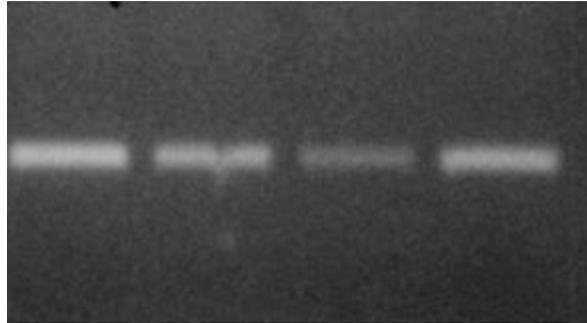
**B**



0    60    0    60  
Kallikrein    Scrambled  
siRNA        siRNA

Fig 3

Bk B2R



12 60 120 0

Kallikrein siRNA nM

Fig 4

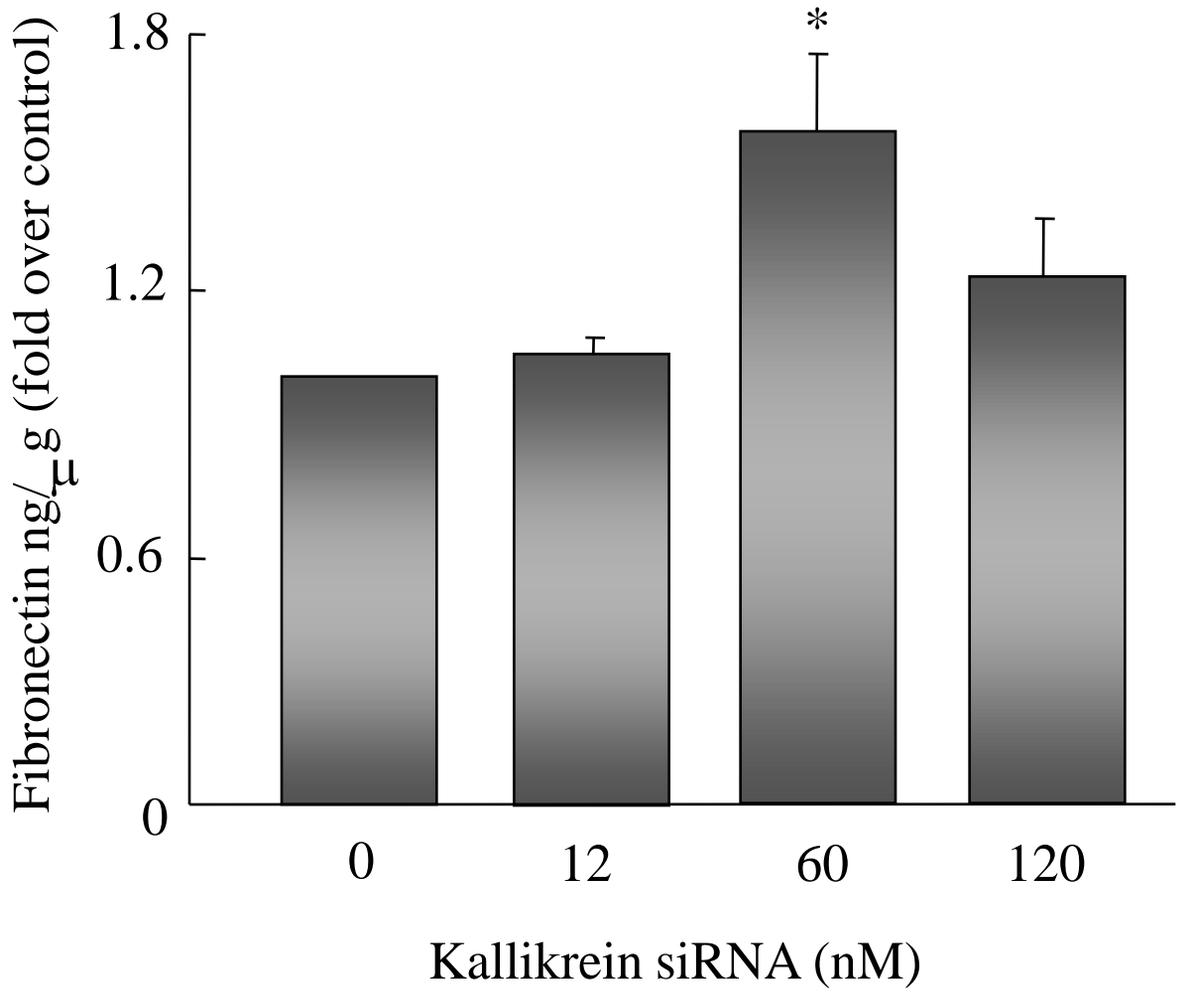


Fig 5

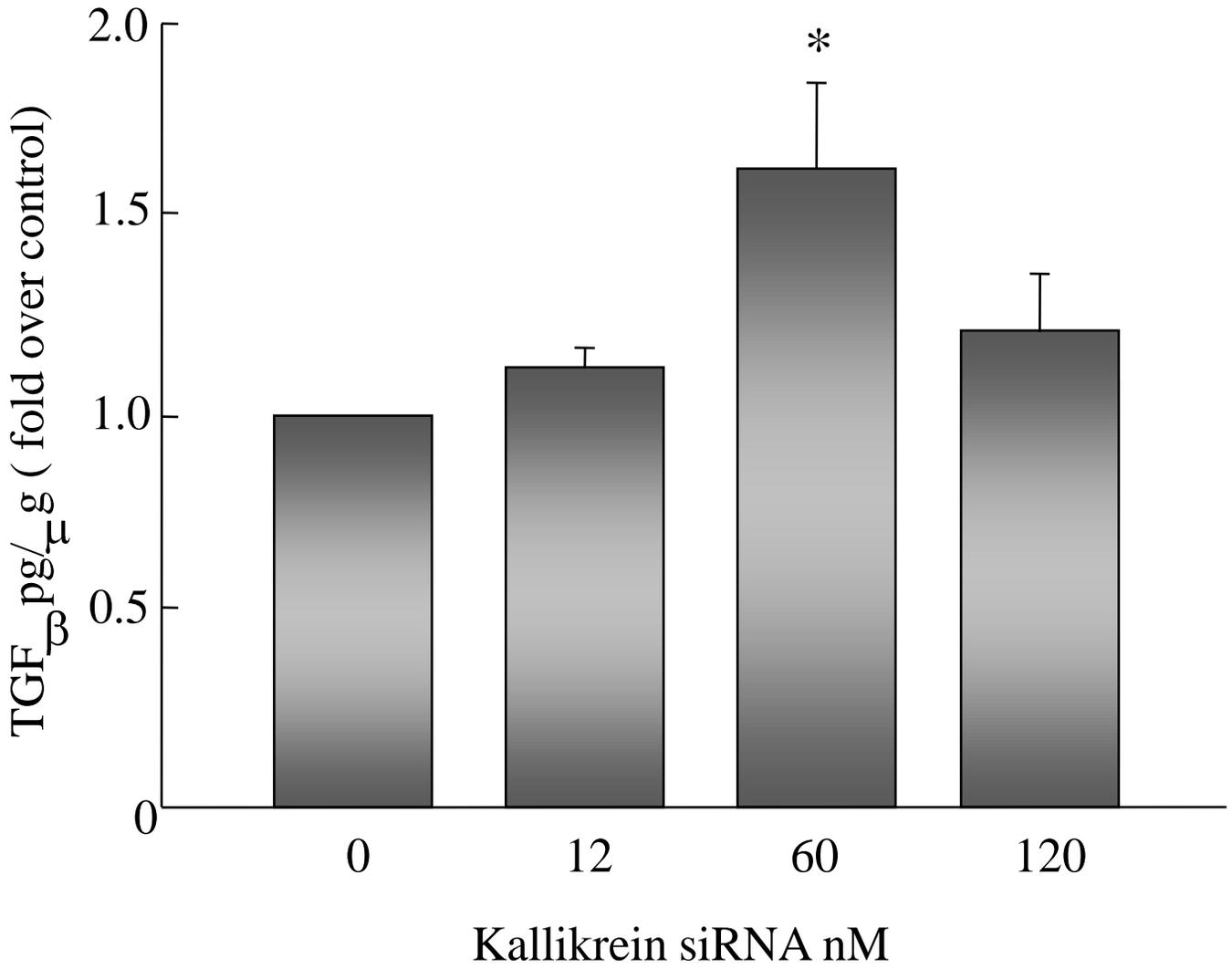
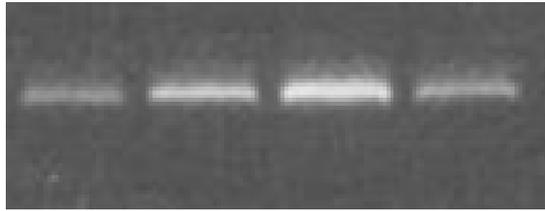


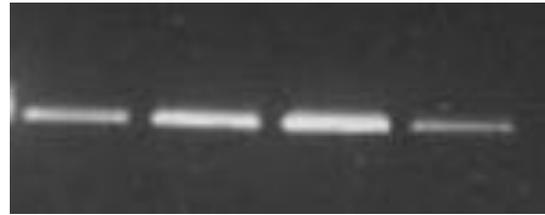
Fig 6

**A**

MMP2



MMP9

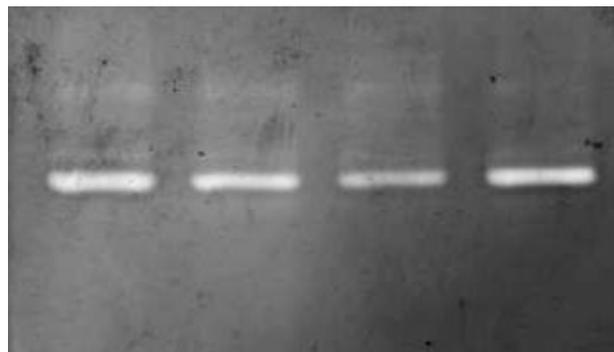


12 60 120 0

Kallikrein siRNA nM

**B**

MMP9 →  
proMMP2 →  
Active MMP2 →



12 60 120 0

Kallikrein siRNA nM

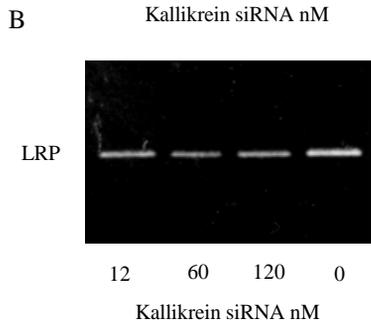
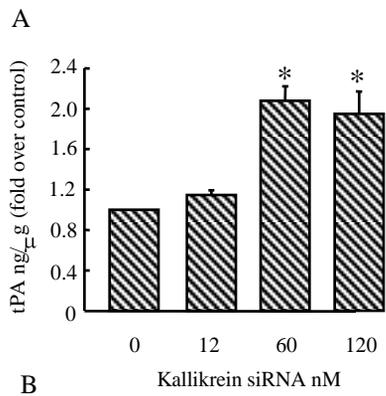
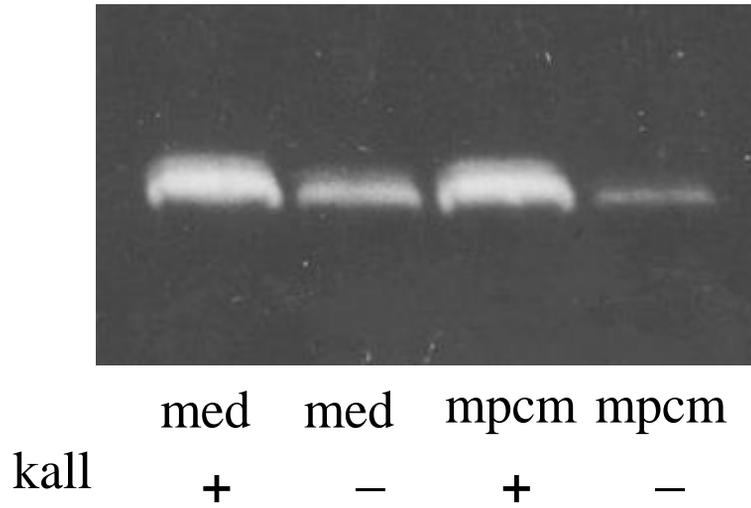


Fig 8

A



B

