Co-operative interactions between NFAT (nuclear factor of activated T cells) c1 and the zinc finger transcription factors Sp1/Sp3 and Egr-1 regulate MT1-MMP (membrane type 1 matrix metalloproteinase) transcription by glomerular mesangial cells

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The transition of normally quiescent glomerular MCs (mesangial cells) to a highly proliferative phenotype with characteristics of myofibroblasts is a process commonly observed in inflammatory diseases affecting the renal glomerulus, the ultimate result of which is glomerulosclerosis. Generation of proteolytically active MMP (matrix metalloproteinase)-2 by the membrane-associated membrane type 1 (MT1)-MMP is responsible for the transition of mesangial cells to the myofibroblast phenotype [Turck, Pollock, Lee, Marti and Lovett (1996) J. Biol. Chem. **271**, 15074–15083]. In the present study, we show that the expression of MT1-MMP within the context of MCs is mediated by three discrete *cis*-acting elements: a proximal non-canonical Sp1 site that preferentially binds Sp1; an overlapping Sp1/Egr-1-binding site that preferentially binds Egr-1; and a more distal binding site for the NFAT (nuclear factor of activated T cells) that binds the NFAT c1 isoform present in MC nuclear extracts. Transfection with an NFAT c1 expression plasmid, or activation of calcineurin with a calcium ionophore, yielded major increases in NFAT c1 nuclear DNA-binding activity, MT1-MMP transcription and protein synthesis, which were additive with the lower levels of transactivation provided by the proximal Sp1 and the overlapping Sp1/Egr-1 sites. Specific binding of NFAT c1 to the MT1-MMP promoter was confirmed by chromatin immunoprecipitation studies, while MT1-MMP expression was suppressed by treatment with the calcineurin inhibitor, cyclosporin A. These studies are the first demonstration that a specific NFAT isoform enhances transcription of an MMP (MT1-MMP) that plays a major role in the proteolytic events that are a dominant feature of acute glomerular inflammation. Suppression of MT1-MMP by commonly used calcineurin inhibitors may play a role in the development of renal fibrosis following renal transplantation.

Key words: Egr-1, glomerular mesangial cell, membrane type 1 matrix metalloproteinase (MT1-MMP), nuclear factor of activated T cells (NFAT), Sp1, Sp3.

INTRODUCTION

The glomerular basement membrane and the mesangial matrix constitute the primary physical components involved in the regulation of plasma ultrafiltration, disposition of circulating macromolecules and maintenance of structure within a high-capillary-pressure environment [1]. Our studies and those of others over the past decade have elucidated critical roles for specific members of the large matrix metalloproteinase gene family in the basal turnover of glomerular extracellular matrix components [2,3], as well as enhanced synthesis during acute inflammatory processes [4,5]. In particular, the role of MMP (matrix metalloproteinase)-2 and its transcriptional regulation within the context of glomerular MCs (mesangial cells) have been the focus of extensive analysis [6–10]. MMP-2 is secreted as a latent proenzyme, and is activated by several physiological processes, including interactions as an MMP-2–TIMP-2 (tissue inhibitor of MMP-2) complex with the membrane-associated matrix metalloproteinase, membrane type 1 (MT1)-MMP [11,12]. In addition, MT1-MMP is a *bona fide* extracellular-matrix-degrading enzyme capable of cleaving multiple substrates [13]. Enhanced expression of MT1-MMP has been reported in several experimental models of glomerulonephritis, including the mesangial proliferative model induced by anti-Thy1.1 injection [4,14], and in experimental crescentic glomerulonephritis [15]. Increased MT1-MMP expression has also been reported in fibrotic renal cortex of glomeruli of dogs with the X-chromosome-linked Alport syndrome, a syndrome characterized by progressive glomerulonephritis, hearing loss, retinopathy and lens abnormalities [16]. High glucose concentrations have been associated with an increase in mesangial matrix quantity, and this has been attributed to a suppressive effect of high glucose on MT1-MMP synthesis by cultured MCs [17].

In spite of its importance for the generation of the active proteolytic complex of MT1-MMP–MMP-2–TIMP-2, there has been only limited investigation of the mechanisms that regulate MT1-MMP transcription. Lohi et al. [18] provided an initial characterization of the human MT1-MMP promoter region and observed, within the context of HT-1080 fibrosarcoma cells, that a proximal Sp1 site was responsible for nearly 90 % of measured luciferase reporter activity. Haas et al. [19] defined a proximal overlapping Egr-1/Sp1-binding site in the murine MT1-MMP promoter within the context of cultured microvascular endothelial

Abbreviations used: BCA, bicinchoninic acid; C/EBP CCAAT/enhancer-binding protein; ChiP, chromatin immunoprecipitation; EMSA, electrophoretic mobility-shift assay; IL, interleukin; MC, mesangial cell; (MT1)-MMP, (membrane type 1) matrix metalloproteinase; NFAT, nuclear factor of activated T cells; SV40, simian virus 40; TIMP, tissue inhibitor of MMP; TNF-α, tumour necrosis factor α.

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cells. Induction of angiogenesis by culture in three dimensions was dependent upon increased Egr-1 occupancy of this site and enhanced MT1-MMP transcription. This proximal overlapping Egr-1/Sp1 site also regulates enhanced MT1-MMP transcription in response to cyclic strain [20].

Owing to the importance of MT1-MMP expression by glomerular MCs for the generation of locally active MMP-2, we initiated a comprehensive evaluation of the murine MT1-MMP promoter within the context of this cell type. These studies confirmed the significance of the previously defined overlapping Egr-1/Sp1 site for basal promoter activity in MCs. This site was found to act co-operatively with a more proximal non-canonical Sp-1-binding site. Notably, the sequence of the non-canonical Sp1 site is conserved in the human sequence, and it is located further downstream (3′) than the Sp1 site investigated by Lohi et al. [18]. Perhaps most significant for acute inflammatory processes affecting the glomerulus, we found that a third, considerably more potent enhancer element located between − 360 and − 392 bp relative to the translational start site represented a specific binding site for the transcription factor NFAT (nuclear factor of activated T cells). The specificity of NFAT isoform NFAT c1, binding to the enhancer element, was confirmed by EMSA (electrophoretic mobility-shift assay), co-transfection experiments and ChiP (chromatin immunoprecipitation). The total levels of MT1-MMP transcription in MCs represent a summation of the transactivating function provided by the proximal non-canonical Sp1 and overlapping Egr-1/Sp1 sites, with the most significant amount of activity generated by the NFAT-binding enhancer. The present study is the first demonstration that a member of the large MMP gene family is regulated by a specific NFAT isoform, a finding of considerable significance for MT1-MMP regulation during acute glomerular inflammatory processes.

MATERIALS AND METHODS

Constructs

A murine MT1-MMP genomic clone extending from − 3364 bp to the first intron was originally isolated by Dr Suneel S. Apte, (Cleveland Research Foundation, Cleveland, OH, U.S.A.), and was generously given by Dr Tara L. Haas and Dr Joseph A. Madri (Department of Pathology, Yale University School of Medicine, New Haven, CT, U.S.A.). PCR-generated deletion constructs of the original MT1-MMP genomic clone were subcloned into the promoterless luciferase expression vector pGL3-Basic (Promega) for use in transient transfection assays. All fragments extended from − 50 bp relative to the translational start site to − 3364 bp, − 2681 bp, − 2256 bp, − 1685 bp, − 1143 bp and − 519 bp respectively. Seven additional deletion constructs of the fragment − 519, which showed the strongest activity, were constructed in the same fashion. All constructs were denominated pMT1-MMP-Luc followed by the number that corresponded to the last base included in the construct.

In order to analyse a third enhancer region, located between − 312 and − 392 bp, three constructs were made by cloning the fragment extending from − 392 to − 312 bp, and two subfragments of this sequence, extending from − 392 to − 360 bp and from − 359 to − 312 bp, into pGL3-Promoter, which includes the heterologous SV40 (simian virus 40) promoter. These constructs were denoted pMT1MMP-Luc 392/312p, pMT1MMP-Luc 392/360p and pMT1MMP-Luc 359/312p respectively. The constructs carrying GG-to-TA mutations (which are shown subsequently in boldface, lower-case letters) of the Egr-1-binding site (5′-G^{-285}CGGGGGCGG-3′), pMT1-MMP-Luc 311mut 1 (G^{-285}C**ta**GGGCGG) and pMT1-MMP-Luc 311mut 2 (G^{-285}CGGG**ta**CGG), were made to construct pMT1-MMP-Luc 311 using site-directed mutagenesis (Stratagene), and confirmed by sequencing. The constructs carrying three G-to-T mutations of the non-canonical Sp1-binding site (5′-G^{-259}AAGGGAGGGA-3′ to 5′-G^{-259}AAG**tt**A**t**GGA-3′) were made to construct pMT1-MMP-Luc 275 (denoted pMT1-MMP-Luc 275 mutant), pMT1-MMP-Luc 311 (denoted pMT1-MMP-Luc 311 non-canonical Sp mutant; 'pMT1-MMP-Luc 311 ncSpm' in the Figures/legends) and pMT1-MMP-Luc 311mut 1 (denoted pMT1-MMP-Luc 311 double mutant; 'pMT1-MMP-Luc 311double m' in the Figures/legends). The mutations of the NFAT site (5′-TTTTTTCCCCTT-3′ to 5′-TTTTT**ctt**CCTT-3′) at − 362 bp were made to construct pMT1-MMP-Luc 392, which was denoted pMT1-MMP-Luc 392 mutant. The Egr-1-pcDNA3 and Sp1-pcDNA3 expression vectors encoding the full-length human Egr-1 and Sp1 coding sequences were provided by Dr Joseph A. Madri. The *Drosophila* pPacSp1 and pPacSp3 expression vectors were kindly provided by Dr Jonathan M. Horowitz (College of Veterinary Medicine, North Carolina State University, Raleigh, NC, U.S.A). The pPac-β-galactosidase expression vector was provided by Dr Robert Tjian (Department of Molecular and Cell Biology, Howard Hughes Medical Institute, University of California, Berkeley, CA, U.S.A.). The plasmid expressing the cDNA of the human NCAT 2(pSH160c-hNFAT c1) was kindly provided by Dr Gerald R. Crabtree (Department of Microbiology and Immunology, Beckman Center, Stanford, CA, U.S.A.). An Egr-1-pPac expression vector was generated by PCR amplification of the fragment encoding the human Egr-1 coding sequence cloned in pCDNA3-Egr-1 using forward and reverse primers containing the *Bam*HI site. The PCR product was then inserted into the pPac vector digested with *Bam*HI.

Cells and culture conditions

The isolation and maintenance of rat glomerular MCs have been described in detail [7]. MCs were maintained in RPMI 1640 medium supplemented with 1 % non-essential amino acids, 2 mM glutamine, 100 μg of streptomycin, 100 units/ml penicillin and 10 % fetal bovine serum. *Drosophila* SL-2 cells were maintained in Schneider's medium supplemented with 10 % fetal bovine serum at 25 °C.

Transient transfections

Transient transfections of the various MT1-MMP-Luc promoter constructs and the normalizing pSV-β-galactosidase expression plasmid were performed using Fugene-6 in cultured rat MCs according to the manufacturer's instructions (Roche). On day 3, cells were lysed in reporter lysis buffer (Promega) and assayed for luciferase (Luciferase Assay System Promega) and β-galactosidase activity (Luminescent β-galactosidase Reporter System; Clontech) according to the manufacturer's instructions. In a second series of experiments, pcDNA3-Egr-1, pcDNA3-Sp1, or both were co-transfected into MCs with pSV-β-galactosidase and either pGL3-Basic or the respective MT1-MMP-Luc promoter constructs. In a third series of experiments, Fugene 6-based transient transfections of the MT1-MMP-Luc promoter constructs and pPac-β-galactosidase were performed on *Drosophila* SL-2 cells. Co-transfection experiments in SL-2 cells were performed adding the same amount of pPac-Sp1 expression vector and/or pPac-Egr-1 expression vector and/or pPac-Sp3 expression vector, together with the MT-MMP-Luc promoter constructs.

Results from three independent experiments, each with quadruplicate wells, were normalized for β-galactosidase activity, and averaged. Data are expressed as means $\pm$ 1 S.D.

Nuclear cell extracts

MCs were grown to 90 % confluence in 150 mm tissue-culture dishes in the presence or absence of ionomycin (1 μM) for 30 min, washed twice with ice-cold PBS without calcium and magnesium, and scraped into 10 ml of cold PBS. Nuclear cell extracts were prepared according to Dignam et al. [21]. Proteins were extracted in 20 mM Hepes, pH 7.9, 1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF and 20 % (v/v) glycerol, and dialysed overnight in a similar buffer containing 100 mM NaCl instead of KCl. Protein concentrations were determined by the BCA (bicinchoninic acid) protein assay (Pierce). Nuclear cell extracts were stored at − 80 °C prior to EMSA.

EMSA

Complementary nucleotides (Operon Technologies) corresponding to bp − 262 to − 243 (5′-GGAGAAGGGAGGGACCAAAG-3′), − 292 to − 273 bp (5′-CATTGGGGCGGGGGCGGAGG-3′) and − 383 to − 342 bp (5′-GCACCACAGAAAAGACAAATTTTTTTTCCCCTTCTCTCCTGC-3′) of the MT1-MMP promoter were annealed, end-labelled using [γ-^{32}P]ATP and purified on acrylamide gels. Gel-shift reactions consisted of labelled probe (2×10^4 c.p.m.), 20 μl of binding buffer [20 mM Hepes (pH 7.9)/0.1 M NaCl/0.2 mM EDTA/0.5 mM dithiothreitol/0.2 mM PMSF/10 % glycerol], 1 μg of poly(dI-dC), 300 μg/ml of acetylated BSA and 10 μg of nuclear extracts incubated for 30 min at room temperature. For antibody supershift assays, 2 μl of the appropriate antibody [Egr-1 (588), Sp1 (1C6), Sp3 (D-20) or NFAT c1 (7A6), all from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.] were added to the reactions, and incubated for 45 min at room temperature ($\approx$ 17 °C) before gel loading. The reactions were electrophoresed on a 5 % (w/v) non-denaturing polyacrylamide gel, followed by autoradiography.

Cell lysates

MCs were grown to 90 % confluence in 100 mm tissue-culture dishes in the presence or absence of ionomycin (1 μM) for 6 h. For calcineurin inhibition studies, cells were pre-treated with cyclosporin A (1 μM) for 30 min, followed by incubation in the presence or absence of ionomycin. A final set of cells was transiently transfected 48 h before harvesting with the NFAT c1 expression plasmid pSH160c-hNFAT (5 μg/dish) using FuGENE™-6. The cells were washed with PBS, scraped and pelleted in 400 μl of PBS. Pellets from two dishes were resuspended in 200 μl of lysis buffer [0.1 M Tris/HCl (pH 8.1)/1 % (v/v) Triton X-114/10 mM EDTA/1 mM PMSF/1 × protease inhibitor cocktail (Boehringer Mannheim)]. The cell suspension was left on ice for 10 min and clarified by centrifugation (3000 ***g*** for 15 min at 4 °C). The lysate was subjected to temperature-induced detergent phase separation [22] by incubation at 37 °C for 10 min, followed by centrifugation at 20 °C at 1200 ***g*** for 15 min. The upper aqueous phase was removed and replaced by an equal volume (200 μl) of cold lysis buffer to wash the lower detergent-rich phase. Phase separation was then repeated as above. The new upper phase was removed, and the same volume of cold lysis buffer was added to the lower detergent phase. To prevent a renewed phase separation, CHAPS was added to the detergent phase to a final concentration of 0.25 %. Protein concentrations were determined by the BCA protein assay.

Western blotting

The detergent phase from Triton X-114 protein extracts was reduced, subjected to SDS/PAGE and electroblotted on to nitrocellulose membranes. The membranes were blocked with 5 % milk-blocking solution (PBS/5 % fat-free dry milk/0.05 % Tween 20), and probed with the specific monoclonal antibody for MT1-MMP (Ab-4; Oncogene Research Products, San Diego, CA, U.S.A.). After washing, the membranes were incubated with secondary antibody [horseradish-peroxidase-conjugated sheep anti-mouse Ig antibody (Amersham Biosciences UK Ltd)]. Immunoreactive bands were detected with a chemiluminescence detection system (ECL Plus Western Blotting Detection System; Amersham Biosciences UK Ltd).

ChiP experiments

Rat MCs were grown on 15 cm^2 dishes to near-confluence, washed in PBS and cross-linked by the addition of 1 % formaldehyde/PBS for 30 min. Cross-linking was terminated by addition of 100 mM glycine/PBS, and the cells were harvested into PBS (Buffer A) containing 10 % glycerol, 0.5 % Nonidet P40, 0.25 % Triton X-100 and Complete™ protease inhibitor cocktail (Roche). After incubation at 4 °C for 10 min, the cells were disrupted in a Dounce homogenizer and centrifuged at 50 ***g*** to remove debris. The supernatant was centrifuged at 500 ***g*** for 20 min over Buffer A containing 500 mM sucrose. The nuclear pellet was washed twice in PBS, suspended in 250 μl of TE buffer [10 mM Tris/HCl (pH 8.0)/1 mM EDTA] containing 10 % glycerol and sonicated on ice to an average genomic length of 800 bp (Branson microtip, maximum power, three pulses of 5 s duration). Nuclei from 2×10^7 cells were used for each ChiP reaction.

Protein-G–Sepharose beads were blocked in Buffer B (TE buffer containing 0.5 % Triton X-100, 0.5 % CHAPS, 0.1 % SDS, 150 mM NaCl, 1 mg/ml salmon sperm, 1 mg/ml BSA and protease inhibitors) for 4 h at 4 °C and used to pre-clear the chromatin for 4 h at 4 °C in Buffer B. Thereafter, the Protein-G–Sepharose beads were pelleted by centrifugation, and the cleared chromatin solution was incubated with 4 μg of murine monoclonal anti-NFAT c1 IgG (7A6; Santa Cruz Biotechnology) in a total volume of 250 μl of Buffer B at 4 °C overnight. An aliquot (50 μl) of a 50 % slurry of blocked Protein-G–Sepharose beads was added, followed by incubation for 6 h at 4 °C and four subsequent washes in 500 μl of Buffer B. The beads were resuspended in 150 μl of TE buffer and incubated at 37 °C for 3 h with 10 μg of RNAse A, followed by incubation with 10 μg of Proteinase K at 65 °C overnight. The samples were extracted with phenol/chloroform/3-methylbutan-1-ol (49/49/1, by vol.) and the organic layer was back-extracted with 150 μl of TE buffer. Glycogen (20 μg) was added to the aqueous phase, and DNA was precipitated with ethanol. The DNA pellets were suspended in 20 μl of TE buffer and analysed by PCR.

The homologous rat MT1-MMP promoter was localized by the BLAST algorithm to the *Rattus norvegicus* contig NW_047454.1 using the murine MT1-MMP promoter as the query sequence, and had an overall identity of 87 %, including complete conservation of the NFAT-binding site in the same relative position to the transcriptional start site observed with the murine MT1-MMP promoter. PCR (35 cycles) was performed in aliquots of 10 μl vol. with 2 μl of the immunoprecipitated DNA solution using a forward primer (5′-GCATGGAAGACTTCATGTCTCCAAC-3′) and a reverse primer (5′-GAATGTGATTCCTAGAGCGCAGTCG-3′) to generate a 475 bp product centred on the NFAT-binding site. Under these conditions, amplification was in the linear range and each experiment was performed at least three times.

RESULTS

To identify the transcriptional elements that regulate MT1-MMP expression by glomerular MCs, transient transfections were

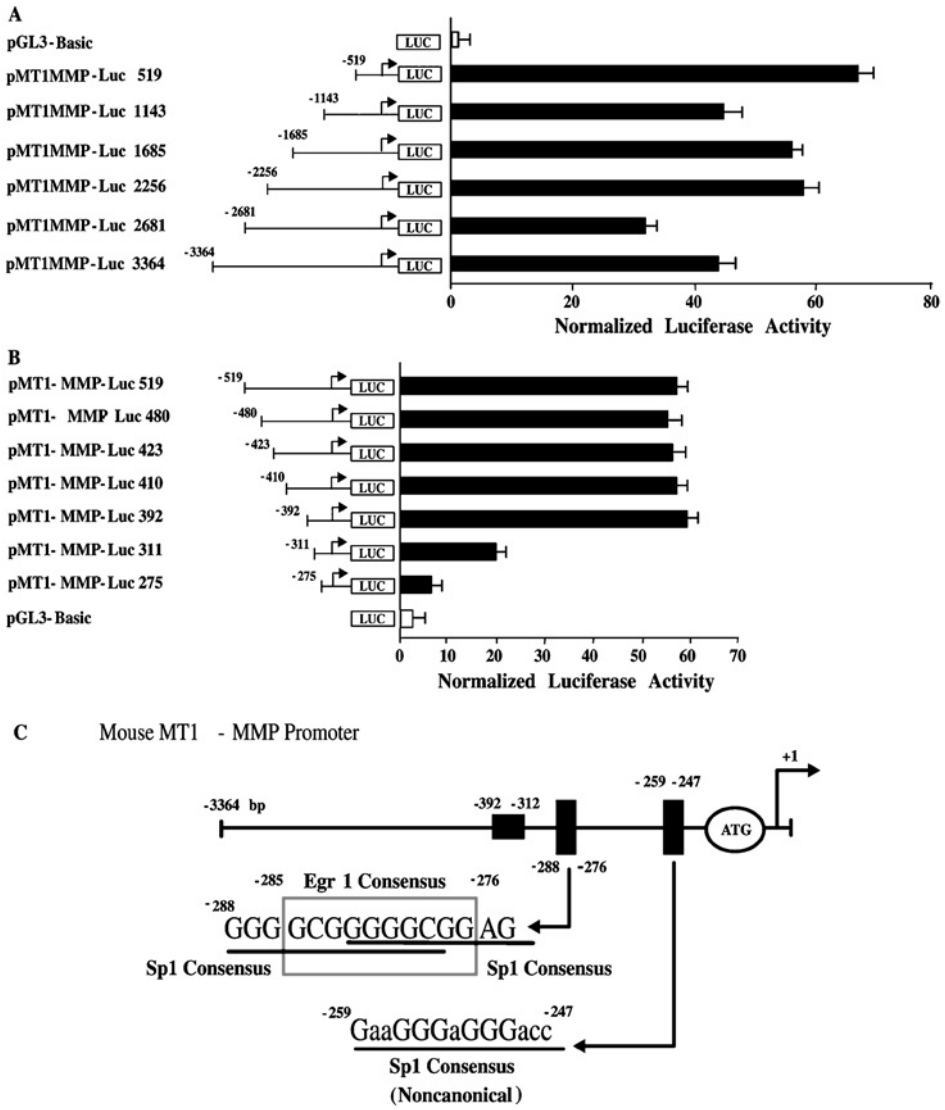

Figure 1 Transcriptional activity of the MT1-MMP promoter in MCs (A), transcriptional activity of the proximal murine MT1-MMP-promoter in MCs (B) and consensus elements for transcription factors present in the murine MT1-MMP promoter (C)

(**A**) Constructs containing deletions of murine MT1-MMP promoter sequences are shown schematically relative to construct pMT1-MMP-Luc 3364, which contains −3364 bp of MT1-MMP 5′ flanking sequence. All constructs start at −50 bp relative to the ATG codon. The 5′ end of each construct is shown at the left. Luciferase activities of each sample were normalized to β-galactosidase activity, averaged, and then expressed as the fold increase above the promoterless luciferase activity of construct pGL3Basic (results are shown as means ± S.D.). The construct closest to the ATG, pMT1-MMP-Luc 519, presents the strongest activity. (**B**) Constructs containing deletions of the proximal MT1-MMP promoter sequences are shown schematically relative to construct pMT1-MMP-Luc 519, which contains −519 bp of MT1-MMP 5′ flanking sequence. Basal promoter activity was localized to a region −275 bp relative to the ATG codon. The promoter activity of construct pMT1-MMP-Luc 311 was 3-fold higher than the activity of the construct pMT1-MMP-Luc 275. The promoter activity of the construct pMT1-MMP-Luc 392 was 9-fold higher than the activity of the basal promoter and 3-fold higher than the activity of the construct pMT1-MMP-Luc 311. These results are consistent with the existence of three physically discrete enhancer elements located between −50 to −275 bp, −276 to −311 bp and −312 to −392 bp. (**C**) The region between the translational start site and −275 bp contains a potential Sp1-binding site with a matrix identity score of 0.74 (bp G^{-259}aAGGgAGGGA^{-249} compared with consensus GRGGCRGGGW). Within the region between −288 and −276 bp is a GC-rich sequence that contains two putative Sp1 sites (underlined) and an overlapping Egr-1 site (boxed). A third enhancer region extends from −312 to −392 bp.

performed with a set of 5′-deletion constructs subcloned into a promoterless luciferase expression vector (Figure 1A). Co-transfection with a β-galactosidase-expressing plasmid was performed to normalize for differences in transfection efficiency. The most proximal construct, pMT1-MMP-Luc 519, was responsible for the highest transcriptional activity observed, suggesting that the important *cis*-regulatory elements were present in this region. A second set of deletion constructs based on pMT1-MMP-Luc 519 was assembled, and the results of transient transfections of MCs are summarized in Figure 1(B). Basal promoter activity was detected within the construct extending to −275 bp relative to the translational start site (pMT1-MMP-Luc 275). Basal promoter activity increased 3-fold with the addition of an additional 36 bp fragment (construct pMT1-MMP-Luc 311). A third region located between −312 and −392 bp increased luciferase reporter activity by an additional 3-fold over that observed with the pMT1-MMP-Luc 311 construct. These initial mapping studies are consistent with a distribution of three discrete enhancer sites located between the translational start site and −275 bp, between −275 and −311 bp and between −312 and −392 bp. Furthermore, the inclusion of all three elements results in an additive 9-fold increase in transcriptional activity. As shown in Figure 1(C),

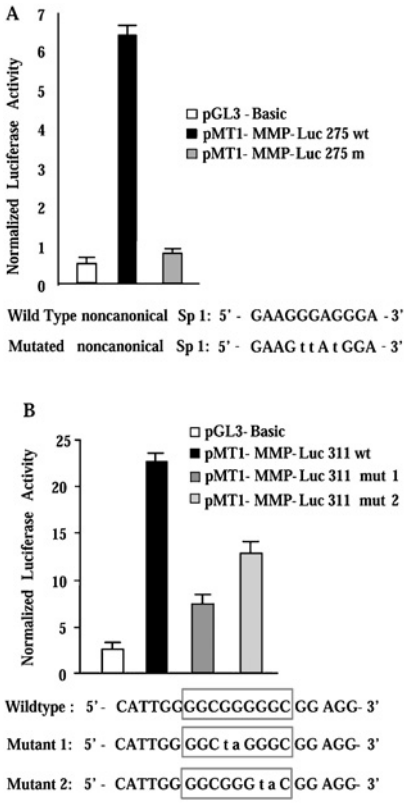

Figure 2 Transcriptional activity of the non-canonical Sp1 site included in the proximal MT1-MMP promoter (A), and transcriptional activity of the MT1-MMP promoter overlapping the Sp1/Egr-1-binding site in MCs (B)

MCs were transfected with the constructs wild-type pMT1-MMP-Luc 275 (pMT1-MMP-Luc 275 wt) or pMT1-MMP-Luc-275 mutant (pMT1-MMP-Luc-275 m). The construct pMT1 MMP-Luc-275 m contains three G-to-T mutations at the core sequence of the non-canonical Sp1 site. The sequences of the wild-type and mutated non-canonical Sp1 sites are described at the bottom of the panel. Lower-case letters denote sites of mutagenesis. The mutation of the non-canonical Sp1 site almost eliminated the activity expressed by the wild-type pMT1-MMP-Luc-275 construct, underscoring its importance in the expression of the MT1-MMP promoter region up to 275 bp in MCs. (**B**) MC were transfected with the wild-type construct pMT1-MMP-Luc 311 (pMT1-MMP-Luc-275 wt), or with two variants containing functional mutations of the overlapping Sp1/Egr-1 site (pMT1-MMP-Luc-275 mut 1 and pMT1-MMP-Luc-275 mut 2). Construct pMT1-MMP-Luc 311 mut 1 contains a G^{-283}G-to-T^{-283}A mutation that blocks both Egr-1 and Sp-1 binding. Construct pMT1-MMP-Luc 311 mut 2 contains a G^{-270}G-to-T^{-270}A mutation that blocks only Egr-1 binding, leaving Sp-1 interactions intact. The luciferase reporter activity generated by the pMT1-MMP311 mut 1 construct is only 25 % of that generated by the wild-type control, whereas reporter activity generated by the pMT1-MMP311 mut 2 construct is approx. 50 % of the wild-type control. These results are consistent with the ability of either Egr-1 or Sp1 to interact with this site within the context of MCs.

the region between −276 and −288 bp includes an overlapping Egr-1/Sp1 consensus binding site previously shown to regulate MT1-MMP transcription by endothelial cells [19]. The region between the translational start site and −275 bp was notable for a potential Sp1-binding site that had a matrix identity score of 0.74 {bp G^{-259}aAGGgAGGGA^{-249} compared with the consensus of GRGGCRGGGW [where the bp that differ from the consensus are shown in lower-case letters; TESS (Transcription Element Search Software), URL: http://www.cbil.upenn.edu/tess]}.

Several approaches were used to determine the functional significance of the overlapping Egr-1/Sp1 and non-canonical Sp1-binding sites for MT1-MMP transcription within the context of glomerular MCs. As shown in Figure 2(A), mutagenesis of the proximal non-canonical Sp1-binding sequence 5′-G^{-259}AAGGG-AGGGA-3′ to 5′-G^{-259}AAG**tt**A**t**GGA-3′ (where boldface, lower-case letters denote sites of mutagenesis) virtually eliminated the luciferase activity expressed by the wild-type pMT1-MMP-Luc275 construct. To investigate the functional significance of the overlapping Egr-1/Sp1 site within this cellular context, we used the wild-type pMT1-MMP-Luc311 construct or two variants containing functional mutations. pMT1-MMP-Luc 311mut 1 contains a G^{-283}G-to-T^{-283}A mutation that blocks both Egr-1 and Sp1 binding [21]. Construct pMT1-MMP-Luc 311mut 2 contains a G^{-270}G-to-T^{-270}A mutation that blocks only Egr-1 binding, leaving Sp1 interactions intact [21]. As shown in Figure 2(B), the luciferase reporter activity generated by the pMT1-MMP-Luc 311mut 1 construct is only 25 % of that generated by the wild-type control, whereas reporter activity generated by the pMT1-MMP 311mut 2 construct is approx. 50 % that of the wild-type control. These results are consistent with the ability of either Egr-1 or Sp1 to interact with this site within the context of MCs, and that reporter activity generated by the wild-type constructs represents the summation of either Egr-1 or Sp1 binding to this region.

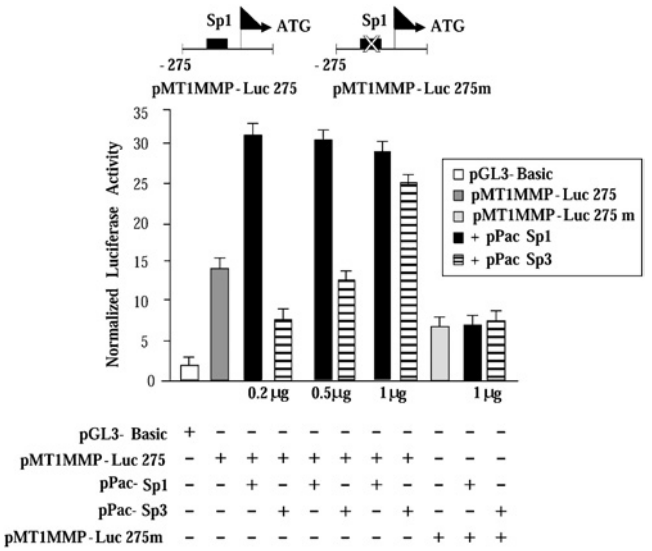

Figure 3 Functional profiles of Sp1 and Sp3 in the regulation of the non-canonical Sp1 site of the proximal MT1-MMP promoter in *Drosophila* SL-2 cells

Drosophila SL-2 cells were co-transfected with the constructs pMT1-MMP-Luc 275 or pMT1-MMP-Luc 275 mutant (pMT1-MMP-Luc-275 m), and with increasing concentrations of pPac-Sp1 or pPac-Sp3 expression plasmids. The construct pMT1-MMP-Luc 275 contains the wild-type non-canonical Sp1 site, whereas the construct pMT1-MMP-Luc 275 m contains three G-to-T mutations of the non-canonical Sp1-binding site (5′-G^{-259}AAGGGAGGGA-3′ to 5′-G^{-259}AAG**tt**A**t**GGA-3′). It required nearly 5 times as much Sp3 expression plasmid as the Sp1 plasmid to achieve equivalent levels of luciferase activity. The enhanced transcription activity with Sp1 or Sp3 co-transfection was completely blocked when the construct pMT-MMP-Luc275 m was used, indicating the specificity of the binding.

To evaluate the potential interaction of Sp proteins with the proximal non-canonical binding site in more detail, we performed a series of transfections, detailed in Figure 3, with the pMT1MMP-Luc 275 construct within the context of Schneider SL-2 cells, a *Drosophila melanogaster* cell line that lacks Sp and Egr protein expression [23]. Transient transfection of the pMT1-MMP-Luc 275 construct alone yielded moderate levels of luciferase activity within this cellular context. Co-transfection with a *Drosophila*-optimized expression plasmid pPac-Sp1 resulted in a 2.5–3-fold increase in luciferase reporter activity, even at the lowest plasmid concentration of 0.2 μg/dish. Co-transfection with pPac-Sp3,

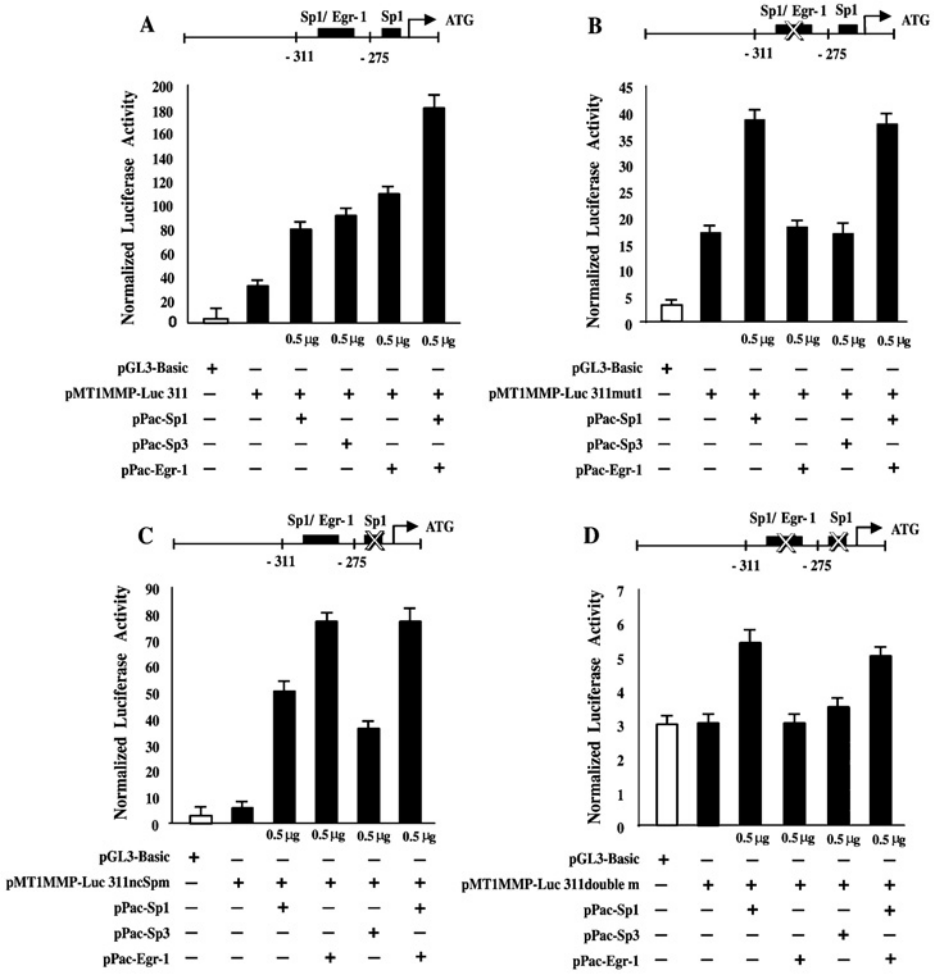

Figure 4 Functional profiles of Sp1, Sp3 and Egr-1 in the regulation of the non-canonical Sp1 and overlapping Sp1/Egr-1-binding sites in *Drosophila* SL-2 cells (A), functional profiles of Sp1, Sp3 and Egr-1 in the regulation of the non-canonical Sp1 independent of the Sp1/Egr-1 enhancer in *Drosophila* SL-2 cells (B), functional profiles of Sp1, Sp3 and Egr-1 in the regulation of the overlapping Sp1/Egr-1 enhancer contained in the MT1-MMP promoter in *Drosophila* SL-2 cells (C), and functional profiles of Sp1, Sp3 and Egr-1 in the regulation of the MT1-MMP promoter when both the non-canonical Sp1 and the overlapping Sp1/Egr-1 site are mutated (D)

(**A**) *Drosophila* SL-2 cells were co-transfected with the construct pMT1-MMP-Luc 311 and with the same concentrations (0.5 μg) of the expression plasmids pPac-Sp1, pPac-Sp3, pPac-Egr-1, or both pPac-Sp1 and pPac-Egr-1. The construct pMT1-MMP-Luc 311 contains the wild-type non-canonical Sp1 and wild-type overlapping Sp1/Egr-1 sites. Sp1, Sp3 and Egr-1 induced 3–4-fold increases in luciferase activity. Co-transfection with equal concentrations of pPac-Sp1 and pPac-Egr-1 yielded an additive increase in transcriptional activity. (**B**) *Drosophila* SL-2 cells were co-transfected with the construct pMT1-MMP-Luc 311 mut 1 plus the addition of the same concentration (0.5 μg) of the expression plasmids pPac-Sp1, pPac-Sp3, pPac-Egr-1, or both pPac-Sp1 and pPac-Egr-1. The construct pMT1-MMP-Luc 311 mut 1 carries a G^{-283}G-to-T^{-283}A mutation of the overlapping Sp1/Egr-1-binding site that blocks both Egr-1 and Sp1 binding. The wild-type Egr-1 site is 5′-G^{-285}CGGGGGCGG-3′; the mutated Egr-1 site present in the construct pMT1-MMP-Luc 311 mut 1 is 5′-G^{-285}C**ta**GGGCGG. Co-transfection with pPac-Sp1 significantly increased luciferase activity, whereas equivalent concentrations (0.5 μg) of pPac-Sp3 or pPac-Egr-1 did not, consistent with a specific interaction of Sp1 with the proximal non-canonical Sp1 site. (**C**) *Drosophila* SL-2 cells were co-transfected with the construct pMT1-MMP-Luc 311 non-canonical Sp mutant (pMT1-MMP-Luc 311 ncSpm) plus the addition of the same concentration (0.5 μg) of the expression plasmids pPac-Sp1, pPac-Sp3, pPac-Egr-1, or both pPac-Sp1 and pPac-Egr-1. The construct pMT1-MMP-Luc 311 ncSpm carries three G-to-T mutations of the non-canonical Sp1-binding site and an intact overlapping Sp1/Egr-1 site. Co-transfection with either of the three expression plasmids yielded significant increases in transcriptional activity. Co-transfection with equal amounts of pPacSp1 and pPac-Egr-1 reflected only the activity of pPac-Egr-1, instead of the additive effect observed with both proteins, when the non-canonical Sp1 site was also intact, as described in the legend to Figure 6(A). (**D**) *Drosophila* SL-2 cells were co-transfected with the construct pMT1-MMP-Luc 311 double mutant (pMT1-MMP-Luc 311double m) plus the addition of the same concentrations (0.5 μg) of the expression plasmids pPac-Sp1, pPac-Sp3, pPac-Egr-1, or both pPac-Sp1 and pPac-Egr-1. The construct pMT1-MMP-Luc 311 double m carries three G^{-255}-to-T^{-255} mutations of the non-canonical Sp1-binding site, and a G^{-283}G-to-T^{-283}A mutation of the overlapping Sp1/Egr-1-binding site. Mutation of both the non-canonical Sp1 and the overlapping Egr-1/Sp1 sites resulted in basal levels of reporter activity that were the same as those found with the control pGL-3-Basic plasmid. Co-transfection with pPac-Sp1, pPac-Sp3 or pPac-Egr-1 did not increase the activity of the promoter.

which expresses the Sp3 protein, also increased pMT1-MMP-Luc 275-generated reporter activity. The dose–response curves for co-transfection with pPac-Sp3 indicated that it required nearly five times as much Sp3 expression plasmid as the Sp1 plasmid to achieve equivalent levels of luciferase activity. The enhanced transcriptional activity with Sp1 or Sp3 co-transfection was completely blocked when the pMT-MMP-Luc 275m plasmid was used. These experiments confirm a specific interaction of Sp1 and Sp3 proteins with the non-canonical binding site in the pMT1-MMP-Luc 275 construct, and suggest a preferential interaction or response with the Sp1, as opposed to the Sp3, protein. Sequence specificity of binding was confirmed by the mutagenesis experiments in which the core Sp1-binding site was mutated.

The next series of studies summarized in Figure 4 addressed the issue of potential additive or competitive interactions between the proximal non-canonical Sp1-binding site and the

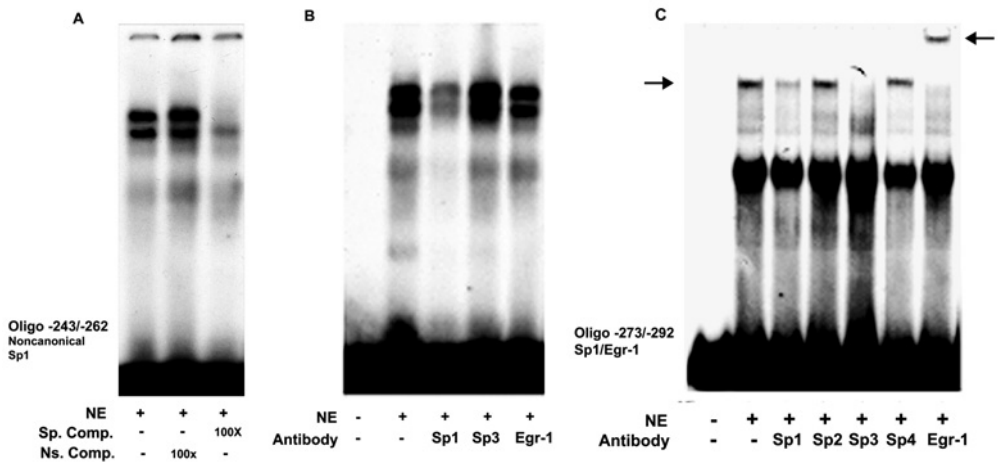

Figure 5 Binding profiles of MC nuclear extracts to the non-canonical Sp1-binding site (A), antibody supershift studies (B), and binding profiles of MC nuclear extracts to the overlapping Sp1/Egr-1-binding site (C)

(**A**) Gel mobility-shift assays were performed by incubating nuclear extracts of MCs with a 20 bp ^{32}P-labelled DNA probe extending from −262 to −243 bp, containing the non-canonical Sp1-binding site extending from −259 to −247 bp. Incubation of the labelled oligonucleotide yielded two closely migrating bands, which were competed by addition of 100-fold excess of unlabelled oligonucleotide, but not by addition of a non-specific oligonucleotide generated by scrambling the −262 to −243 sequence. (**B**) Antibody supershift assays are shown: lane 1, probe alone; lane 2, MC nuclear extracts (NE); lanes 3, 4 and 5, MC nuclear extracts incubated with antibodies against Sp1, Sp3 and Egr-1 respectively. Two gel-shifted complexes (lane 2) were depleted by the antibody against Sp1 (lane 3). Inclusion of antibodies against Sp3 and Egr-1 did not modify the primary gel-shifted complex (lanes 4 and 5). (**C**) Gel mobility-shift assays were performed by incubating nuclear extracts of MCs with a 20 bp ^{32}P-labelled DNA probe extending from −292 to −273 bp, containing the overlapping Sp1/Egr-1 consensus binding site extending from −288 to −276 bp. Lane 1, probe alone; lane 2, MC nuclear extracts (NE); lane 3, antibody against Sp1; lane 4, antibody against Sp2; lane 5, antibody against Sp3; lane 6, antibody against Sp4; lane 7, antibody against Egr-1. The protein–DNA complex with the lowest electrophoretic mobility was supershifted by an antibody against Egr-1 (lane 7, arrow), and was depleted by an antibody against Sp3 (lane 5, arrow). The antibody against Sp1 partially depleted the same protein–DNA complex.

previously defined overlapping Egr-1/Sp1 site located at −288 bp. Transfection of construct pMT1-MMP-Luc 311, which includes both of these sites, into Schneider SL-2 cells yielded normalized luciferase activity of approx. 30 arbitrary units, which is nearly twice that observed with the proximal pMT1-MMP-Luc 275 construct shown in Figure 3 (Figure 4A). In contrast with what was observed with construct pMT1-MMP-Luc 275 (Figure 3), co-transfection of pMT1-MMP-Luc 311 with both pPac-Sp1 and pPac-Sp3 expression plasmids induced an approx. 3-fold increase in luciferase reporter activity, whereas co-transfection with an equivalent concentration of pPac-Egr-1 yielded a slightly greater, approx. 4-fold increase in reporter activity. Co-transfection with equal concentrations of the Sp1 and Egr-1 expression plasmids yielded additive increases in luciferase reporter activity.

To determine the relative contributions of the overlapping Egr-1/Sp1 and non-canonical Sp1 sites to total observed luciferase activities, we sequentially mutated these sites and determined basal and induced luciferase activities. The consequences of mutation of the overlapping Egr-1/Sp1 site are summarized in Figure 4(B). Basal activity of the Egr-1/Sp1 mutant was approx. 50 % of that observed with the intact site (Figure 4A). As expected, co-transfection with the pPac-Egr-1 expression plasmid did not increase luciferase reporter activities. Co-transfection with the pPac-Sp1 expression plasmid did increase reporter activity by 2–3-fold, whereas co-transfection with an equal concentration of the pPac-Sp3 expression plasmid had no effect on luciferase reporter activities. These experiments again indicate a preferential effect of Sp1, as opposed to Sp3, for interaction with the proximal non-canonical Sp1-binding site, an observation supported by EMSA, as detailed below. Mutation of the proximal non-canonical Sp1 site with an intact overlapping Egr-1/Sp1 site resulted in major decreases in basal luciferase activity that was only minimally greater than that observed with the control pGL3-Basic vector (Figure 4C). Whereas co-transfection with pPac-Sp1 or pPac-Sp3 yielded significant increases in luciferase activity, a significantly greater level of reporter activity was observed with an equivalent concentration of the pPac-Egr-1 expression plasmid. These findings are consistent with a generally recognized model of overlapping Egr-1/Sp1 sites, in which basal levels of transcription due to occupancy of the site of Sp1/Sp3 are followed by increased levels of transcription due to displacement with the transcriptionally more potent Egr-1 protein [24]. The model is consistent with the finding that co-transfection with equal concentrations of Sp1 and Egr-1 did not enhance further the activity of the pMT1-MMP-Luc 311 non-canonical Sp mutant construct. As anticipated, mutation of both the non-canonical Sp-1 and the overlapping Egr-1/Sp1 sites resulted in basal levels of reporter activity that were virtually the same as those found with the control pGL-3-Basic plasmid, and no significant transcriptional response was observed with co-transfection of pPac-Sp1, pPac-Sp3 or pPac-Egr-1 (Figure 4D).

EMSA provided confirmation of the specific interactions of the Sp1, Sp3 and Egr-1 proteins with the proximal non-canonical Sp1 and the overlapping Egr-1/Sp1 sites. As summarized in Figure 5(A), the non-canonical Sp1 oligonucleotide spanning −243 to −262 bp demonstrated specific binding to MC nuclear proteins, based on competition with specific and non-specific unlabelled oligonucleotides. Addition of antibody against Sp1 depleted the specifically shifted bands, whereas addition of antibody against Sp3 or Egr-1 had no effect on nuclear protein or oligonucleotide mobilities (Figure 5B). These studies are consistent with a specific interaction of the non-canonical Sp1 site with the Sp1 protein, and not the closely related Sp3 protein present in nuclear extracts. The specificity of Sp1 binding to this site determined by EMSA is consistent with the preferential enhancement in luciferase reporter activity observed with Sp1, as opposed to Sp3 (see Figure 4B).

The next set of mobility studies examined the potential interactions of Sp1 proteins and Egr-1 with the overlapping Egr-1/Sp1

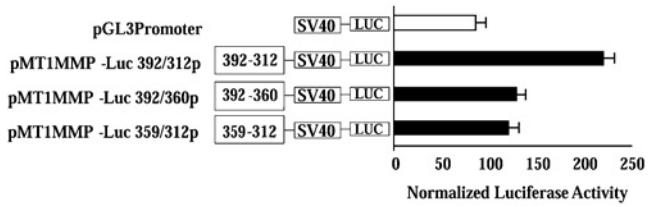

Figure 6 Transcriptional activity of the murine MT1-MMP −392/−312 enhancer fragment in the context of the SV40 promoter

To confirm the activity of the putative enhancer region extending from −392 to −312 bp, three constructs were made by cloning the whole fragment and two subfragments into the luciferase vector pGL3-Promoter upstream of a heterologous SV40 promoter. The constructs were denoted pMT1-MMP-Luc 392/312p, pMT1-MMP-Luc 392/360p and pMT1-MMP-Luc 359/312p respectively. Transient transfections with the pMT1-MMP-Luc 392/312p construct increased the activity of the SV40 promoter nearly 3-fold, whereas the constructs pMT1-MMP-Luc 392/360p and pMT1-MMP-Luc 359/312p had reporter activities in the same range of the control pGL3-Promoter vector.

site located between −273 and −292 bp (Figure 5C). Addition of antibody against Sp1 or Sp3 depleted the shifted oligonucleotide, consistent with a common and expected interaction of both these transcription factors with this element. Addition of antibody against Egr-1 resulted in a clear supershifted band. Taken together, these studies demonstrate specific interactions of the overlapping Egr-1/Sp1 site in the MT1-MMP promoter with Sp1/3 and Egr-1, and are consistent with the luciferase reporter activities shown in Figure 4(C), in which both Sp1 and Sp3 stimulated reporter activity, although to a lesser degree than Egr-1.

The functional analysis of the MT1-MMP-Luc reporter constructs shown in Figure 1(B) indicated that a probable enhancer element was located between −312 and −392 bp, with a resultant 3-fold increase in the transcriptional activity over that generated by the proximal non-canonical Sp1 and overlapping Egr-1/Sp1 sites detailed above. To assess this potential enhancer activity independently from the proximal Sp1 and Egr-1/Sp1 sites, the fragment extending from −312 to −392 bp was subcloned into the vector pGL3-Promoter, which includes the heterologous SV40 promoter. This construct was denoted pMT1-MMP-Luc 392/312p. Two subfragments of this sequence were also subcloned into pGL3-Promoter, and denoted pMT1-MMP-Luc 392/360p and pMT1-MMP-Luc 359/312p respectively. Transient transfection of MCs with the pMT1-MMP-Luc 392/312p construct yielded a nearly 3-fold increase in luciferase reporter activity as compared with the control pGL3-Promoter vector alone (Figure 6). Notably, this activity was completely eliminated by cleavage of the original −312 to −392 bp fragment into two equal segments centred on bp −360, suggesting that the cleavage process had disrupted the actual enhancer site. Examination of the sequence surrounding bp −360 revealed a potential single binding site for the transcription factor NFAT. The consensus binding sequence for NFAT from the mouse IL (interleukin)-2 promoter [25] is 5′-CAAAT**TTTCC**TC-3′, where the bold nucleotides denote the core binding site; the sequence centred on the −360 bp of the murine MT1-MMP genomic sequence is 5′-TTTTT**TTTCC**CC-3′, and has a matrix identity score of 0.96.

To determine the significance of the potential NFAT-binding site for the enhancer activity centred around the −360 bp site, EMSA and co-transfection experiments were performed. EMSA used MC nuclear extracts and a radiolabelled oligonucleotide extending from −383 to −342 bp, which includes the putative NFAT-binding sequence located at −362 to −351 bp. Sugimoto et al. [26] previously reported that cultured rat MCs express only one of the five known isoforms of NFAT, NFAT c1. MC nuclear

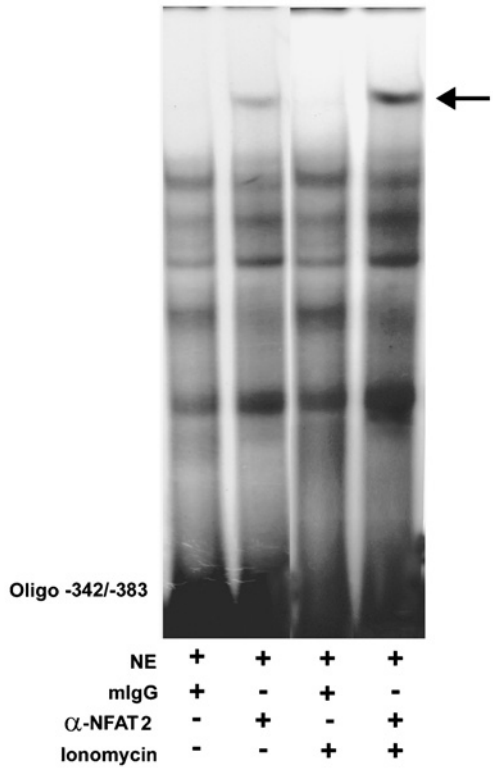

Figure 7 Binding profile of MC nuclear extracts to the NFAT site

Gel mobility-shift assays were performed by incubating nuclear extracts of MCs with a 42 bp ^{32}P-labelled DNA probe extending from −383 to −342 bp, containing the NFAT consensus binding site extending from −362 bp to −351 bp. Lane 1, MC nuclear extracts incubated with murine IgG (mIgC); lane 2, MC nuclear extracts incubated with a specific antibody against NFAT c1 (7A6); lane 3, nuclear extracts obtained from MC exposed to ionomycin (1 μM) for 30 min and incubated with murine IgG; lane 4, nuclear extracts obtained from MC exposed to ionomycin, and incubated with a specific antibody against NFAT c1 (7A6). The antibody against NFAT c1 induced a supershifted protein–DNA complex. Exposure of MCs to ionomycin significantly increased NFAT c1/DNA-binding activity (cf. lanes 2 and 4).

extracts demonstrated specific binding to the −383 to −342 bp oligonucleotide that could be competed with 100× excess of unlabelled oligonucleotide, but not with a similar concentration of scrambled, unlabelled oligonucleotide (results not shown). As shown in Figure 7, inclusion of a monoclonal antibody against NFAT c1 in the incubation mixture resulted in a supershifted band not obtained with control murine IgG. The activity of NFAT c1 is regulated by calcineurin, which dephosphorylates the NFAT c1 protein and permits nuclear translocation [27]. Calcineurin activity is stimulated by the calcium influx caused by ionophores, including ionomycin. We therefore incubated MCs with 1 μM ionomycin for 30 min before harvesting and preparation of nuclear extracts. As shown in Figure 7, nuclear extracts from ionomycin-treated cells had significantly more shifted NFAT c1 oligonucleotide, functionally confirming the specific interaction of NFAT c1 with the −383/342 bp oligonucleotide.

Transient transfection experiments with an NFAT c1 expression plasmid confirmed the transactivating ability of NFAT c1 for MT1-MMP transcription. As shown in Figure 8, the NFAT c1 expression plasmid induced a concentration-dependent increase in luciferase reporter activity from the pMT1-MMP-Luc 392 construct, which includes the NFAT-binding site at −360 bp and three more proximal, putative sites located at −162, −187 and −203 bp. At the highest concentration of NFAT c1 expression plasmid used

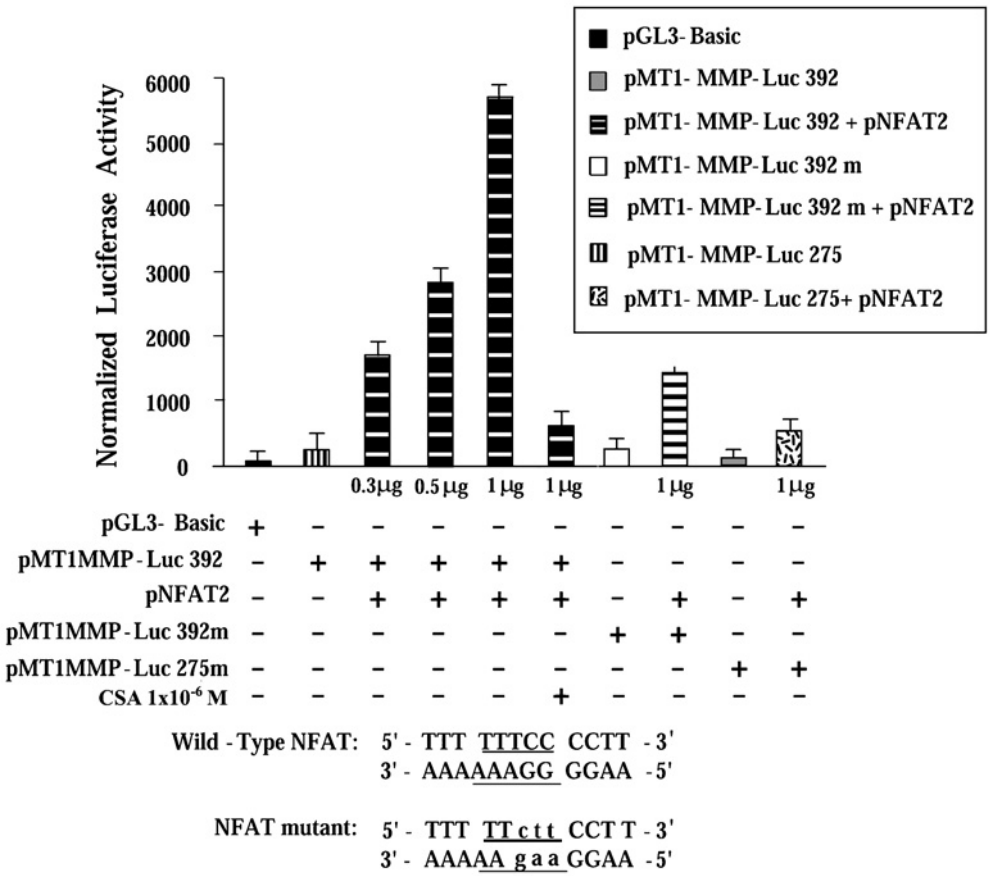

Figure 8 Comparison of NFAT c1 transactivation efficacy: proximal compared with distal NFAT-binding sites

MCs were co-transfected with the construct pMT1-MMP-Luc 392, which includes one distal and three proximal NFAT-binding sites, and increasing concentrations of a NFAT c1 expression plasmid. MCs were also transfected with the construct pMT1-MMP-Luc 392 m, which has 3 bp mutations at the core of the distal NFAT site. To assess the action of NFAT c1 on the three proximal NFAT sites, MCs were transfected with the construct pMT1-MMP-Luc 275, which does not include the distal NFAT and the Sp1/Egr-1 sites, and with the NFAT c1 expression plasmid. Co-transfection of the pMT1-MMP-Luc 392 construct with increasing concentrations of the NFAT c1 expression plasmid yielded a nearly 100-fold increase in luciferase activity, which was reduced, but not completely eliminated, by mutation of the distal NFAT-binding site. Co-transfection with pNFAT c1 of the pMT1-MMP-Luc 275 construct, which includes only the proximal three NFAT sites and lacks the Egr-1/Sp1 site, yielded small increases in luciferase activity.

(1 μg/well), a nearly 100-fold increase in luciferase activity was observed. Considerably less promoter activation by the NFAT c1 expression plasmid was seen with construct pMT1-MMP-Luc-392m, in which the core of the distal NFAT binding site was mutated. The transactivation activity of the NFAT c1 expression plasmid was almost completely inhibited by the inclusion of the calcineurin inhibitor, cyclosporin A, at a concentration of 1 μM.

The three proximal, putative NFAT-binding sites are located more 3′ than the non-canonical Sp1 and the overlapping Sp1/Egr-1 sites. In order to assess the possible response of these proximal NFAT sites to NFAT c1, transient transfections of the pMT1-MMP-Luc-275 construct, which lacks the overlapping Sp1/Egr-1 site at -288 bp and the distal NFAT site at -362 bp, were performed with or without the NFAT c1 expression plasmid. The NFAT c1 expression plasmid increased only modestly the activity of the construct pMT1-MMP-Luc-275; however, the activity generated was 13-fold less than the activity generated by the same amount of NFAT c1 expression plasmid when the distal NFAT-binding site was included in the construct (compare the NFAT c1-induced activation of construct pMT1-MMP-Luc-392 with the NFAT c1-induced activation of construct pMT1-MMP-Luc-275; Figure 8). These results indicate that the distal NFAT site is responsible for the bulk of the NFAT c1-mediated transactivation of MT1-MMP.

ChiP assays were employed to demonstrate occupancy of the NFAT c1-binding site on the intrinsic (rat genomic) MT1-MMP promoter. The homologous rat MT1-MMP promoter was localized by the BLAST algorithm to the *R. norvegicus* contig NW_047454.1 using the murine MT1-MMP promoter as the query sequence, and had an overall identity of 87 %, including complete conservation of the NFAT-binding site in the same relative position to the transcriptional start site observed with the murine MT1-MMP promoter. As shown in Figure 9, incubation of isolated MC chromatin with a monoclonal antibody against NFAT c1, followed by PCR, yielded the anticipated 475 bp amplicon product centred on the NFAT-binding site. Pre-incubation of the cells for 30 min with 1 μM ionomycin, followed by ChiP, yielded an approx. 4-fold increase in the abundance of the 475 bp amplicon product, consistent with increased occupancy by NFAT c1 of the intrinsic MT1-MMP-promoter-binding site following activation of calcineurin.

The stimulatory effect of NFAT c1 on MT1-MMP transcription was associated with an increase in actual MT1-MMP protein synthesis. Cultured MCs were exposed to 1 μM ionomycin or

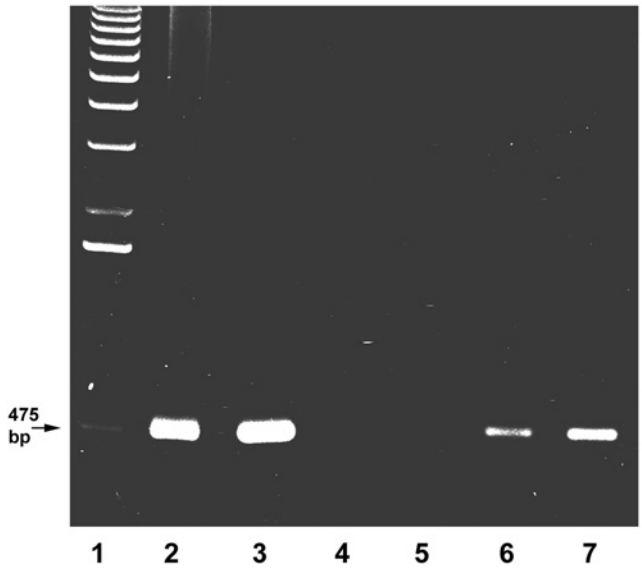

Figure 9 ChiP of NFAT c1 binding to the intrinsic (rat genomic) MT1-MMP promoter

ChiP was performed as detailed in the Materials and methods section. Lane 1, DNA ladder; lane 2, rat genomic DNA PCR positive control; lane 3, isolated chromatin PCR positive control; lane 4, antibody against NFAT c1 without chromatin, negative control; lane 5, chromatin without antibody against NFAT c1, negative control; lane 6, antibody against NFAT c1 plus chromatin from control MCs; lane 7, antibody against NFAT c1 plus chromatin from MC treated for 30 min with 1 μM ionomycin. The arrow denotes the predicted PCR product of 475 bp, which is centred on the NFAT c1-binding site at -360 bp. Treatment with ionomycin increased the abundance of the 475 bp amplicon by approx. 4-fold, consistent with increased occupancy of the NFAT c1-binding site following calcineurin activation.

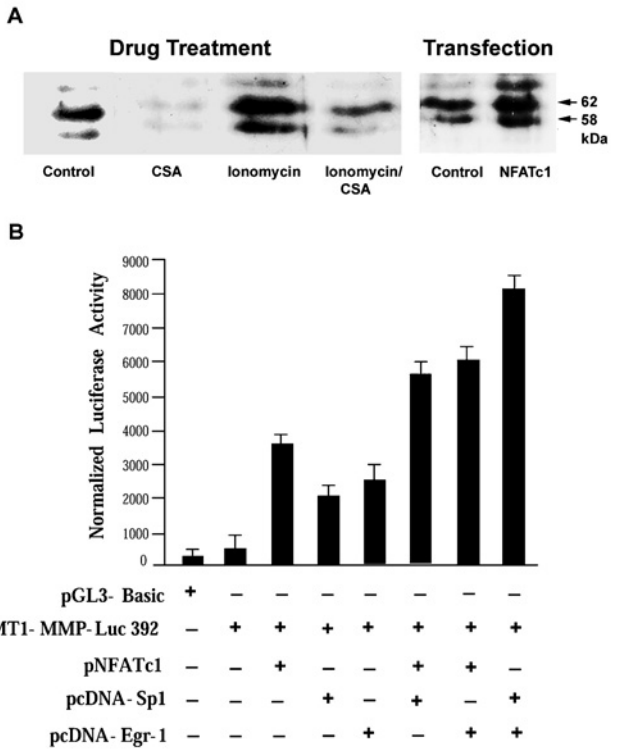

Figure 10 Western blot analysis of MT1-MMP in MCs treated with calcineurin-activity modifiers or by transient transfection with a NFAT c1 expression plasmid (A), and functional profile of the transcription factors NFAT c1, Egr-1 and Sp1, alone or in combination, in the regulation of MT1-MMP in MC (B)

The four lanes of the left panel represent controls treated with: vehicle (Control); 1 μM cyclosporin A (CSA); 1 μM ionomycin (Ionomycin); or cyclosporin A pre-treatment for 30 min, followed by addition of ionomycin (Ionomycin/CSA). For the transient transfection studies, MC were transfected with the empty plasmid pSH160c or the pSH160c-NFAT c1 expression plasmid for 48 h, followed by extraction of membrane-enriched fractions, as detailed in the Materials and methods section. For both sets of experiments, a total of 20 μg of protein was loaded/lane. The arrows denote the 62 kDa (latent) and 58 kDa (active) forms of the MT1-MMP protein. (**B**) MCs were co-transfected with the construct PMT1-MMP-Luc 392 and with equivalent, submaximal amounts (0.5 μg) of NFAT c1, Egr-1 and Sp1 expression plasmids. Used alone, NAFT2, Egr-1 and Sp1 all induced significant increases in luciferase activity. NFAT c1 plus Egr-1 or NFAT c1 plus Sp1 yielded additive increases in reporter activity. Inclusion of all three expression plasmids yielded levels of reporter activity that are an arithmetic sum of the three individual activities.

vehicle for 6 h, followed by preparation of a plasma-membrane-enriched fraction and Western blot analysis. To confirm the requirement for calcineurin activation for increased MT1-MMP synthesis, cells were also pre-treated for 30 min with 1 μM cyclosporin A, followed by addition of ionomycin or vehicle. A final set of cells was transiently transfected 48 h before harvesting with the NFAT c1 expression plasmid (5 μg/dish). As shown in Figure 10(A), treatment of cells with cyclosporin A almost completely suppressed basal levels of MT1-MMP protein synthesis, whereas incubation with ionomycin yielded a nearly 5-fold increase in MT1-MMP protein synthesis. Pre-treatment of the cells with cyclosporin A for 30 min before the addition of ionomycin significantly suppressed MT1-MMP protein synthesis. Finally, NFAT c1 transfection also increased MT1-MMP protein synthesis 3–4-fold. These studies complement the findings obtained with ChiP, and clearly demonstrate that the intrinsic MT1-MMP promoter is regulated by NFAT c1 binding.

A final series of studies examined the potential additive effects of Sp1, Egr-1 and NFAT c1 on MT1-MMP transcription. The results of these experiments are shown in Figure 10(B). When equivalent plasmid concentrations of the respective expression plasmids were used, NFAT c1 induced a 63-fold increase in luciferase activity, whereas Sp1 and Egr-1 induced increases of 35-fold and 40-fold respectively. The combination of NFAT c1 with either Sp1 or Egr-1 induced approx. 100-fold increases in luciferase reporter activity, and inclusion of the three expression plasmids increased MT1-MMP-Luc 392 reporter activity by nearly 140-fold. Thus NFAT c1, Sp1 and Egr-1 act in an additive fashion, via their respective and physically discrete binding sites, to enhance MT1-MMP transcription by MCs.

DISCUSSION

Proteinases, specifically MMP2 and its activator MT1-MMP, are among the many regulatory molecules secreted by MCs with the potential to influence local structure and function of the glomerular capillary and mesangial extracellular matrices [1]. Regulation of MT1-MMP production is of critical importance in determining the overall impact of MMP2 on glomerular function in health and disease [28,29]. In the present study, we have investigated the transcriptional mechanisms underlying the expression of MT1-MMP by cultured MCs. A series of 5′-deletion constructs defined three proximal regions of the MT1-MMP promoter that mediate expression of the gene in cultured MCs: the non-canonical Sp1 site, the overlapping Sp1/Egr-1 site and the distal NFAT-binding site.

EMSA using extracts from MC nuclei revealed that Sp1 preferentially bound the non-canonical Sp1 site, whereas endogenous Egr-1 and Sp3 did not. Supershift analysis showed that Egr-1, and both Sp1 and Sp3, bound the overlapping Sp1/Egr-1 site. Mutation

of the proximal non-canonical Sp1-binding site abolished the activity of the basal promoter. Mutations of the overlapping Sp1/Egr-1 site that affected exclusively the binding of Egr-1 diminished significantly the enhancer activity of the overlapping Sp1/Egr-1 site. Hence we have demonstrated a co-operative interaction between Sp1 and Egr-1 via binding to physically separated sites in the promoter that enhances transcription of MT1-MMP by MCs. While Sp1 and Sp3 have equivalent binding affinities and specificities within the context of the canonical Sp1-binding site [30], it is possible that nucleotide differences present in the MT1-MMP non-canonical site confer differential Sp1 compared with Sp3 binding.

Our finding of the involvement of Egr-1 in the regulation of MT1-MMP in MCs, which in culture present a pro-inflammatory phenotype characterized by augmented proliferation and synthesis of extracellular matrix proteins [28], correlates with the findings described by Hofer et al. [31]. In the Hofer study, it was demonstrated by antisense-oligonucleotide strategies that Egr-1 is critical for induction of MC proliferation. Furthermore, it has been shown *in vivo* that specific inhibition of Egr-1 prevents MC hypercellularity in the anti-Thy 1.1 model of mesangioproliferative glomerulonephritis [32]. In the latter study, changes in the expression of MT1-MMP were not investigated, but a previous report from different investigators found enhanced expression of MT1-MMP in the same model of mesangioproliferative glomerulonephritis [14]. Egr-1-induced expression of MT1-MMP has also been found in a model of cell proliferation using endothelial cells undergoing angiogenesis in a three-dimensional extracellular matrix environment [19]. Co-operative activation by Egr-1 and Sp1 has been found in the regulation of the IL-2Rβ gene, in which co-operation of Sp1 with Egr-1, by binding to contiguous sites, mediates maximal IL-2Rβ gene expression [33].

We have also found that high-level transcription of MT1-MMP is mediated by NFAT c1. NFAT c1 increased 100-fold the promoter activity of construct pMT1-MMP-Luc 392. Construct pMT1-MMP-Luc 392 included three proximal and one distal NFAT-binding sites, with the distal site being the main contributor to the enhancer activity observed. We also found an additive effect of NFAT c1 with Egr-1 and Sp1 on MT1-MMP gene transcription.

The NFAT proteins are a family of transcription factors whose activation is controlled by calcineurin, a Ca^{2+}-dependent phosphatase [27]. Five different members of the NFAT family have been identified [34]. NFAT proteins were originally identified in T cells as inducers of cytokine gene expression [35]. More recent data have implicated NFAT in the control of gene expression influencing the development and adaptation to environmental changes of numerous mammalian cell types [36]. NFAT does not usually function alone, but in co-operation with other transcription factors, and there are several different patterns of combinatorial interactions [37].

NFAT-mediated gene transcription is orchestrated, in part, by formation of composite regulatory elements [34]. Composite regulatory elements involve not only functional synergy, but the existence of co-operative physical interactions between two or more different transcription factors that bind to adjacent binding sites [34]. It has been shown that adjacent Sp1/Egr-1 and NFAT elements are conserved in the IL-2 and TNF-α (tumour necrosis factor α) gene promoters [38]. These studies found that, upon binding to adjacent promoter sites, members of the Egr and NFAT protein families form stable heterodimeric complexes and co-operate in tissue-specific expression of the genes IL-2 and TNF-α. In those two promoters, the Sp1/Egr-1 (ZIP)-binding site is located further 5′ than the NFAT site, and both sites are in close proximity, being separated by 12 bp in the IL-2 promoter and by 6 bp in the TNF-α promoter [38]. Even though NFAT c1 and Egr-1 functionally co-operate in the regulation of the MT1-MMP gene, the overlapping Sp1/Egr-1 site and the distal NFAT elements present in the MT1-MMP promoter have a different position, orientation and distance than the conserved sequence. In the MT1-MMP promoter, the distal NFAT site is located more 5′ than the ZIP site, has an opposite orientation, and both sites are separated by 69 bp. The distance between the ZIP site and the closest proximal NFAT site is 77 bp, making any physical interaction also unlikely. These findings suggest that NFAT co-operates with Egr-1 in the regulation of the MT1-MMP promoter by binding independently and without physical interaction.

Co-operative interaction between NFAT and Sp proteins has been also reported [39]. In the MT1-MMP promoter, the Sp1-binding sites present in the Sp1/Egr-1 overlapping site are 66 and 71 bp removed from the distal NFAT site, again suggesting that NFAT and Sp1 co-operate in the regulation of MT1-MMP transcription without physical interaction.

Careful analysis of the sequences surrounding the distal and proximal NFAT-binding sites suggests putative binding sites for the transcription factors C/EBP (CCAAT/enhancer-binding protein) and Oct-1. These proteins have been shown to form composites with NFAT to regulate several genes [40,41]. Analysis of the sequence of the MT1-MMP promoter up to -392 bp revealed two putative binding sites for C/EBP, but which do not conform to the consensus NFAT:C/EBP composite [40].

Composite NFAT:Oct elements have been found to regulate the T-cell-specific IL-3 enhancer [41]. Two putative binding sites for the transcription factor Oct-1 are present in the MT1-MMP promoter. The distal Oct-1-binding site is 14 nt removed from the NFAT core and has a different orientation. The proximal Oct-1 site is in close proximity to two of the proximal NFAT sites, but the orientation of both binding sites differs from the NFAT:Oct composite. Future studies will examine the potential interactions of NFAT with C/EBP and Oct-1 to determine whether functional interactions occur in the absence of heteromeric complex formation.

As opposed to Egr-1, which has been implicated in kidney cell gene regulation under normal conditions and during pathologic processes [31,42], there is only a very limited amount of information concerning renal expression of NFAT isoforms and their potential roles in kidney development or disease processes. Sugimoto et al. [26] previously reported that endothelin-1-mediated induction of cyclo-oxygenase-2 in cultured glomerular MCs is mediated by NFAT c1, which was also shown to be the only NFAT isoform expressed by this cell type. Treatment of the cells with the calcineurin inhibitor cyclosporin A completely blocked NFAT c1 nuclear translocation and endothelin-1-mediated cyclo-oxygenase-2 expression. The authors suggest that chronic inhibition of cyclo-oxygenase-2 expression by use of cyclosporin for transplant treatment may contribute to the development of the glomerulosclerosis commonly seen in patients with cyclosporin-induced nephrotoxicity [43]. The development of glomerulosclerosis has also been postulated to be a consequence of diminished MMP synthesis [44], and our finding that the critical component for regulation of MMP-2 activation, MT1-MMP, is regulated by a calcineurin–NFAT c1 pathway is consistent with this concept. Indeed, inclusion of cyclosporin A in the culture medium significantly suppressed both basal and ionomycin-mediated MT1-MMP protein synthesis.

Our finding that MT1-MMP is transcriptionally regulated by NFAT c1 has implications beyond the kidney. NFAT isoforms play critical roles in cell differentiation and development, including embryonic cardiac and vascular development [36]. Significantly, we have recently found, using MMP-2 promoter/lacZ transgenic mice in conjunction with *in situ* hybridization for MT1-MMP,

that these tissues are intensely positive for these products (M. A. Alfonso-Jaume and D. H. Lovett, unpublished work).

Evidence for a critical role of NFAT transcription factors in carcinogenesis was provided recently by Jauliac et al. [45]. In that study, NFAT1 and NFAT5 were shown to be expressed by invasive breast carcinoma cells and to facilitate tumour cell invasion in response to integrin $\alpha_6\beta_4$ signalling. Although not examined in that study [45], the critical role of MT1-MMP, in conjunction with MMP-2, for tumour invasion and metastasis is well documented [46–49]. Since the MDA-MB-435 breast carcinoma cells used in the study of Jauliac et al. [45] are capable of MT1-MMP expression [50], it is highly probable that NFAT-mediated enhancement of MT1-MMP transcription is a critical component of the enhanced invasive activity expressed by these cells. This issue is currently under investigation.

In conclusion, this study has defined several key regulatory elements that work in concert to control MT1-MMP transcription and synthesis within the context of glomerular MCs. Demonstration that the most potent enhancer effect is mediated by a specific NFAT isoform could have important implications for our understanding of the role of these transcription factors in numerous biological processes involving extracellular matrix remodelling and carcinogenesis.

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