Matrix metalloproteinase-2 (MMP-2) and membrane-type 1 MMP (MT1-MMP) affect the remodeling of glomerulosclerosis in diabetic OLETF rats

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Abstract

Background. We reported previously that diabetic glomerular nodular-like lesions were formed during the reconstruction process of mesangiolysis. However, the precise mechanism has yet to be elucidated. Here, we investigated the roles of matrix metalloproteinase (MMP)-2, which is activated from proMMP-2 by membrane-type (MT)-MMP in the sclerotic and endothelial cell injury process of a type II diabetic model, Otsuka Long–Evans Tokushima Fatty (OLETF) rats.

Methods. Monocrotaline (MCT) or saline only was injected three times every 4 weeks in 36-week-old OLETF rats and control Long–Evans Tokushima Otsuka rats. Glomerular expression and enzymatic activity of MMP-2 and MT1-MMP were assessed by immunohistochemistry, gelatin zymography of cultured glomerular supernatants, in situ enzymatic detection and reverse transcription–polymerase chain reaction.

Results. Mesangial matrix increased in OLETF rats. In addition, mesangiolysis and nodular-like mesangial expansion were observed only in MCT-injected endothelial injured OLETF rats. MMP-2 and MT1-MMP proteins increased in the expanded mesangial lesions in OLETF rats. Gelatin zymography revealed an increase in 62-kDa activated MMP-2 in the culture supernatants of isolated glomeruli from OLETF rats. In situ enzymatic activity of MMP in the mesangial areas was also detected in 50-week-old MCT-injected OLETF rats.

Conclusion. These results suggest that MMP-2 and MT1-MMP are produced and activated in glomeruli through the progression of diabetic nephropathy and may have some effect on the remodeling of the glomerular matrix in diabetic nephropathy.

Keywords: diabetic nephropathy; glomerulosclerosis; matrix metalloproteinase-2; membrane-type 1 matrix metalloproteinase; diabetic nodular-like lesion

Introduction

Diabetic nephropathy is one of the most important causes of end-stage renal disease. Characteristic pathological changes of diabetic nephropathy include accumulation of extracellular matrix (ECM) in glomerular and tubulointerstitial tissues. These pathological abnormalities are speculated to be induced by alterations in ECM production or degradation. Fioretto et al. [1] reported that 10 years of normoglycemia after pancreas transplantation ameliorated the lesions of diabetic nephropathy, suggesting the importance of degradation of accumulated ECM in diabetic nephropathy.

The glomerular ECM, which composes the glomerular basement membrane and mesangial matrix, has highly specialized mechanical and biological functions in the renal glomeruli [2, 3]. These functions are maintained by tight regulation of the balance between the rates of synthesis and degradation of ECM. Alterations of this balance may lead to pathological changes in glomerular structure and function. Therefore, studies on the regulatory mechanisms of ECM are very important to understand the pathogenesis of glomerular injury associated with qualitative and quantitative changes of ECM [4].

The degradation of ECM, including type IV and V collagens, laminin, fibronectin and proteoglycans, is mediated by proteinases, such as aspartic, cysteine, serine proteinases and matrix metalloproteinases (MMPs) [5]. In fact, the glomerular expression of MMPs, including MMP-2, 3, 9, 13, 14, 24, 25, 27 and 28, and membrane-type MMP (MT1, 2-MMP) has been reported in various glomerular diseases, including diabetic nephropathy [6–15]. Among these MMPs, MMP-2 has a specific activation mechanism. MMP-2 is activated on the cell membrane by MT-MMP [16]. Among these MMPs, MMP-2 has a specific activation mechanism. MMP-2 is activated on the cell membrane by MT-MMP.
MMP-2 and MT1-NMP in diabetic nephropathy

Here, we investigated the glomerular expression of MMP-2, MT1-MMP and in situ activity of MMP-2 in diabetic nephropathy using a diabetic model, Otsuka Long–Evans Tokushima Fatty (OLETF) rats, which resembles human type 2 diabetes mellitus [17–19]. In addition, we also attempted to induce nodular-like lesions resembling those seen in human patients through vascular endothelial injury and mesangiosis by administration of monocrotaline.

Materials and methods

Animals and experimental design

OLETF rats and Long–Evans Tokushima Otsuka (LETO) rats used as controls were donated from Tokushima Laboratory of Otsuka Pharmaceutical Co. Ltd. We administered monocrotaline (2% fluid; Sigma, St Louis, MO) subcutaneously at a dose of 30 mg/kg three times every 4 weeks to 36-week-old male OLETF and LETO rats. Control rats were injected with the same amounts of saline three times. The rats were sacrificed at 40, 44, 46 and 50 weeks old for histological examination of kidney tissues and isolation of glomeruli for culture and RNA purification. There were no pathological changes in the lungs or livers of monocrotaline-injected control LETO rats. There were also no differences in body features between monocrotaline-injected or non-injected control LETO rats.

All procedures used in the animal experiments complied with the standards set out in the ‘Guideline for the Care and Use of Laboratory Animals in Takara-machi Campus of Kanazawa University’.

Measurement of urinary albumin excretion

At 36, 46 and 50 weeks old, we examined urinary albumin excretion using 24-h urine collection. Measurements were performed by enzyme-linked immunosorbent assay (Nephrat; Exocell, Inc., Philadelphia, PA).

Histological examinations

Renal tissues were fixed in 10% buffered formalin, followed by embedding in paraffin and staining with hematoxylin and eosin, periodic acid–Schiff (PAS) reagent and periodic acid silver methenamine (PAM). Two sections were cut from each paraffin block and stained with hematoxylin and eosin, periodic acid–Schiff (PAS) reagent and periodic acid silver methenamine (PAM). Two sections were cut from each paraffin block and stained with hematoxylin and eosin, periodic acid–Schiff (PAS) reagent and periodic acid silver methenamine (PAM). The cross-reactivity of these antibodies to rat molecules was confirmed

Immunohistochemical detection of MMP-2 and MT1-MMP

Fresh specimens were prepared in optimal cutting temperature compound, snap frozen in n-hexane cooled with a mixture of dry ice and acetone and cut into 6-μm sections on a cryostat (Tissue-Tek II systems; Miles, Naperville, IL). Anti-human MMP-2 (clone 42-SD11) and anti-human MT1-MMP (clone 113-5B7) mouse monoclonal antibodies (Fuji Pharma Co., Ltd., Takaoka, Japan) were used as the primary antibodies. The cross-reactivity of these antibodies to rat molecules was confirmed by western blot analysis (data not shown). Immunohistochemistry was performed by the indirect avidin–biotin–alkaline phosphatase method (Vectastain ABC-AP kit; Vector Labs, Burlingame, CA). Normal mouse IgG was used as a negative control. Human synovial tissues resected from patients with rheumatoid arthritis were used as positive controls for MMP-2 and MT1-MMP staining. The percentage MMP-2- or MT1-MMP-positive area in each glomerulus was evaluated using NIH Image.

Messenger RNA (mRNA) was extracted from formalin-fixed paraffin-embedded tissue using an IsoGen PB Kit (Nippon Gene, Tokyo, Japan). MMP-2 mRNA expression was evaluated by real-time polymerase chain reaction (PCR) and normalized relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Glomerular isolation and culture

Rats were anesthetized with diethyl ether and the kidneys were perfused with saline via the aorta and then excised. The capsules were removed and the cortex was separated from the medulla and minced with a razor blade. Glomeruli were isolated by the differential sieving technique. The tissue was passed sequentially through 250-, 150- and 100-μm sieves. intact glomeruli retained on the 106-μm sieve were washed with saline and resuspended in serum-free RPMI 1640 medium (Gibco, New York, NY) containing 100 μM of penicillin (Gibco), 100 μM of streptomycin (Gibco) and 0.2% lactalbumin (Sigma) in 12-well multi-well plates. The purity of the final suspension was determined by light microscopy. On average, there were fewer than five tubular fragments per 100 glomeruli. After 24 h of incubation at 37°C in 5% CO2 in air, the culture media were removed.

In vitro and in vivo detection of MMP activity by zymography

The in vitro activities of MMPs in glomerular culture medium from LETO and OLETF rats was detected by zymography. Briefly, media were diluted appropriately to normalize for glomerular number. Gel loading buffer [0.5 M Tris–HCl, pH 6.8, 10% sodium dodecyl sulfate (SDS), 5% glycerol and 0.1% bromphenol blue] was added to the culture supernatant, and electrophoresis was performed on SDS–polyacrylamide gels (8%) containing 0.8 mg/mL gelatin (Sigma) under non-reducing conditions [20]. After electrophoresis, the gels were incubated in 2.5% Triton X-100 solution for 1 h at room temperature and then in a buffer containing 50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 10 mM CaCl2 and 0.02% NaN3 for 24 h at 37°C. The gels were stained with Coomassie brilliant blue, de-stained and photographed [21]. The zones of lysis were visualized and analyzed by densitometry. SDS–polyacrylamide gel electrophoresis (PAGE) molecular weight standards low and high (Bio-Rad, Richmond, VA) were used as molecular weight markers.

In situ detection of MMP activity on glomeruli was examined as follows. Renal frozen specimens were incubated with 1 μm synthetic peptides NFF-N1-N2-N3-N4-C176 (MOC-ac-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys(Dnp)-NH2; Pep-2; Peptide Institute, Osaka, Japan) resolved in dimethylsulfoxide (Sigma) and incubated with 0.02% NaN3 and 0.05% Brij35 with 2 mM aminophenyl mercuric acetate as a substrate of MMP-2 for 48 h at 37°C and examined then with an immunofluorescence microscope using a U-MWU filter (AX-80; Olympus, Tokyo, Japan) [22].

Detection of MMP-2, MT1-MMP, TIMP-2 and fibronectin transcripts in isolated glomeruli

We performed semiquantitative analysis of the glomerular MMP-2, MT1-MMP, TIMP-2 and fibronectin gene expression by reverse transcription–polymerase chain reaction (RT-PCR). Total RNA was purified from isolated glomeruli by acid guanidinium thiocyanate–phenol–chloroform extraction [23]. Sense and antisense primers, designed using the previously published complementary DNA sequence for rat MMP-2, rat GAPDH, rat MT1-MMP, TIMP-2 and fibronectin, were synthesized. Primers for MMP-2, MT1-MMP and TIMP-2 were designed using the primer program GENETYX-MAC (GENETYX, Tokyo, Japan). Primer pairs were chosen to yield an expected product of 248 bp for rat MMP-2, 363 bp for rat GAPDH [24], 398 bp for rat MT1-MMP, 403 bp for rat TIMP-2 and 294 bp for rat fibronectin [25] (Table 1). Aliquots of 1 μg of total RNA were subjected to reverse transcription. PCR was then performed using an RNA PCR kit (AMV; Takara, Kyoto, Japan). The thermal cycler was programmed with an initial incubation of 94°C for 1 min, followed by 35 – 40 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The entire reaction mixture was subjected to 2% agarose gel electrophoresis, and PCR products were visualized with ethidium bromide staining and ultraviolet transillumination and were analyzed semiquantitatively by densitometry. RT–PCR data were normalized relative to the housekeeping gene GAPDH as a control.

Statistical analysis

Data are presented as means ± SEMs. Statistical significance of differences was assessed by analysis of variance (ANOVA) and the Kruskal–Wallis test. In all analyses, P < 0.05 was taken to indicate statistical significance.
Results

Urinary albumin excretion was increased in diabetic OLETF rats but was not influenced by monocrotaline

The urinary albumin excretion in control LETO rats was 1.5 ± 0.5, 1.2 ± 0.1 and 1.0 ± 0.3 mg/day at 36, 46 and 50 weeks old, respectively. However, the urinary albumin excretion in diabetic LETO rats was significantly elevated as compared to that of LETO rats (86.6 ± 30.6, 155.0 ± 22.7, 112.0 ± 16.4 mg/day at 36, 46 and 50 weeks old, respectively; P < 0.01 by ANOVA). After monocrotaline injection, there were no significant changes in urinary albumin excretion in either LETO or OLETF rats (Figure 1).

Monocrotaline-accelerated diabetic glomerulopathy in diabetic OLETF rats

There was no significant glomerular changes in LETO rats (13.3 ± 3.3 at 40 weeks, 2.9 ± 1.8 at 50 weeks) (Figure 2). After monocrotaline injection, mild mesangial matrix expansion was seen in LETO rats (27.5 ± 11.1 at 40 weeks, 8.6 ± 4.6 at 50 weeks) (Figure 2). In contrast to LETO rats, marked mesangial expansion was observed in OLETF rats (166.7 ± 23.3 at 40 weeks, 154.0 ± 28.4 at 50 weeks) (Figure 2). Moreover, monocrotaline treatment in OLETF rats significantly increased mesangial matrix scores at 50 weeks (140.0 ± 17.3 at 40 weeks, 160.0 ± 4.1 at 44 weeks, 205.0 ± 18.5 at 46 weeks, 219.3 ± 6.0 at 50 weeks) (Figure 4A). In addition, some glomeruli showed typical mesangiolysis lesions and nodular-like lesions (Figure 2) in monocrotaline-treated OLETF rats at 50 weeks, concomitant with glomerular capillary endothelial cell swelling (Figure 3). Similar to mesangial matrix expansion, quantitative analysis indicated that nodular-like lesions and mesangiolysis gradually increased in monocrotaline-treated OLETF rats (Figure 4B and C).

MMP-2 and MT1-MMP proteins were upregulated in monocrotaline-treated diabetic LETO rats

MMP-2 and MT1-MMP proteins were only faintly detected on the mesangial area of the glomeruli of LETO and monocrotaline-treated LETO rats by immunohistochemical study (data not shown). In OLETF rats, both proteins were observed mainly in the mesangial area and were enhanced in the area of severely expanded mesangial matrix. In monocrotaline-treated OLETF rats, the expression levels of both proteins were upregulated in the expanded mesangial areas and the segmental sclerotic lesions of glomeruli (Figure 5A and B).

Monocrotaline-treated diabetic OLETF rats showed in vivo activity of MMP-2 in glomeruli

We examined the gelatinolytic activity of glomerular culture media from 50-week-old LETO and OLETF rats by zymography using gelatin-substrate SDS–PAGE (Figure 6A and B). In the culture media from 50-week-old LETO rats, there were gelatinolytic activities at 62 and 68 kDa. The 62-kDa band was thought to indicate the activated form of MMP-2. The activities of 62 and 68 kDa were enhanced in monocrotaline-treated LETO (2.3- and 2.3-fold, respectively), saline-injected OLETF (4.8- and 2.7-fold, respectively) and monocrotaline-treated OLETF rats (5.5- and 3.1-fold, respectively). In addition, in situ MMP activity was detected in the mesangial areas of the glomeruli from the monocrotaline-treated OLETF rats at 50 weeks (Figure 6C) but not in the other groups.

Transcription of MMP-2 mRNA was enhanced more than those of TIMP-2 and fibronectin by monocrotaline treatment, especially in diabetic OLETF rats

MMP-2, MT1-MMP, TIMP-2 and fibronectin mRNAs were detected in isolated glomeruli by RT–PCR (Figure 7A). Optical densities of MMP-2, MT1-MMP, TIMP-2 and fibronectin RT–PCR products were corrected relative to those of GAPDH as an internal control in the same lane. Compared with the saline-injected LETO rats, MMP-2 gene expression was enhanced in monocrotaline-treated LETO, saline-injected OLETF and monocrotaline-treated OLETF rats by 15.8-, 2.8- and 18.1-fold, respectively. MT1-MMP mRNA was also increased by 1.9-, 1.1- and 2.8-fold in these rats, respectively. The levels of TIMP-2 and fibronectin mRNA were also increased by 1.4- and 2.2-fold, respectively, in monocrotaline-treated LETO rats, by 1.1- and 2.2-fold, respectively, in saline-injected OLETF rats and by 2.2- and 3.0-fold, respectively, in monocrotaline-treated OLETF rats in comparison with control saline-injected LETO rats (Figure 7B).

Table 1. Primer sequences and expected PCR product sizes

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<th>Target</th>
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<td>MMP-2</td>
<td>Sense 5'-GTCTTCCCCCTCCTTTTCTG-3</td>
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<tr>
<td></td>
<td>Antisense 5'-CGGAATTCTTGGTGTTAGGTG-3</td>
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<td>Sense 5'-GACTGAGATCAAGGCCAATG-3'</td>
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<td>Antisense 5'-TGTCATTCCCATATTAGATCC-3'</td>
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<tr>
<td></td>
<td>398 bp</td>
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<tr>
<td>TIMP-2</td>
<td>Sense 5'-CAAAAGCAAGTGAGCAGAAGGA-3'</td>
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<td>Antisense 5'-CAAGGAAGGAGTCAAAAGCCTG-3'</td>
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Discussion

In this study, we produced nodular-like lesions of diabetic glomerulopathy in a diabetic model with administration of monocrotaline. Diabetic OLETF rats, which present symptoms similar to those of human type 2 diabetes mellitus, showed vascular endothelial injury and mesangiolysis following administration of monocrotaline. Our results also indicated glomerular expression of MMP-2, MT1-MMP and in situ activity of MMP-2 in the diabetic nephropathy model. These data suggested that glomerular MMP-2 and MT1-MMP may play some role in the process of glomerular matrix remodeling in the progression of diabetic nephropathy.

The renal histology of OLETF rats mimics that of human diabetic nephropathy [17–19]. However, typical nodules in human diabetic nephropathy, which have a laminar structure and show weak positive PAM staining, mainly composed of type VI collagen were faintly seen in the kidneys of this rat model. Previously, we hypothesized that these human diabetic nodules were formed during the reconstruction of mesangiolytic lesions [26]. In this study, we attempted to produce human diabetic nodule-like lesions by administration of monocrotaline, which can induce mesangiolysis by glomerular capillary endothelial injury. Although the lamination of nodules in this model was incomplete, the nodule-like lesions resembling human diabetic nodules were seen in some glomeruli of monocrotaline-treated OLETF rats as well as diffuse glomerulosclerosis. An experimental model for nodular-like lesions was reported previously by Inagi et al. [27] utilizing megsin overexpression in RAGE and inducible nitric oxide synthase transgenic mice. However, these nodule-like lesions in monocrotaline-treated OLETF rats may be formed due to the influence of hyperglycemia.
or metabolic factors during the process of reconstruction from mesangiolysis because monocrotaline-induced glomerulopathy in Sprague–Dawley rats presents with mesangiolysis, following repair or remodeling process, but not nodular-like lesions. Therefore, both diabetic conditions and some toxic events associated with monocrotaline injection would be necessary for the formation of nodular-like lesions in this rat model.

MMP-2 is the principal MMP involved in ECM degradation in the glomeruli. MMP activity is regulated through the following three steps: (i) transcription, (ii) activation of the latent form and (iii) inhibition by TIMPs. The first step is MMP-2 transcription. In the present study, we demonstrated that MMP-2 was mainly localized to the expanded mesangial area and some proportion of visceral epithelial cells in monocrotaline-treated OLETF rats. In addition, isolated glomeruli clearly showed that MMP-2 gene expression, protein production and activation were locally upregulated. Previous in vitro studies indicated that visceral epithelial cells and glomerular endothelial cells could produce MMP-2 as well as mesangial cells [28, 29]. Moreover, mesangial cells were reported to be positive for MMP-2 in Schoenlein–Henoch nephritis and IgA nephropathy [30].

MMP-2 gene was regulated by cell-specific transcriptional systems [31–33]. MMP-2 gene expression in mesangial cells was speculated to be regulated by activation of extracellular signal-regulated kinases 1 and 2, plasminogen activator inhibitor -1, transforming growth factor (TGF)-β and renin/prorenin [34, 35]. Among these regulatory systems, TGF-β is well known as an inducer of MMP-2 in mesangial cells in vitro [11]. Moreover, TGF-β was suggested to play a central role in animal models of diabetic nephropathy as well as human diabetic nephropathy [36–39]. Furthermore, it has been reported that glomerular TGF-β and type IV collagen expression were increased in the kidneys of OLETF rats at 30 weeks old [40, 41]. In addition, MMP-2 mRNA was upregulated coincident with the enhancement of TGF-β mRNA in anti-Thy1.1 glomerulonephritis model [11]. These data indicate that MMP-2 may have some role in the pathogenesis of diabetic nephropathy under conditions of TGF-β
stimulation. In future, blocking studies of TGF-β may be helpful to confirm the in vivo inducers of MMP-2 in this OLETF rat model.

The second regulatory step of MMP is activation. We detected MT1-MMP, which is a proMMP-2 activator, in the expanded mesangial area and glomerular visceral epithelial cells, and the distribution of this protein was coincident with that of MMP-2. Cultured mesangial cell can produce MT1-MMP [42]. Moreover, MT1-MMP mRNA was detected in the mesangial area of anti-Thy1.1 glomerulonephritis, using in situ hybridization [21]. Although McLennan et al. [43] reported that cultured mesangial cell-derived MT1-MMP gene expression was reduced by high glucose concentration, the glomerular expression levels of this gene in OLETF and LETO rats were not different in our study. In addition, glomerular MT1-MMP mRNA levels of both OLETF and LETO rats were increased by monocrotaline injection, coinciding with the increase of activated MMP-2 protein. Thus, MT1-MMP may play a role in glomerular MMP-2 activation. In addition, MT1-MMP itself can degrade type I and III collagens which accumulates in sclerotic glomeruli [44]. These observations suggest that MT1-MMP may play a dual role in digestion of ECM through direct cleavage of the substrates and the activation of proMMP-2 in diabetic glomeruli.

The third regulatory step of MMP involves inhibition by TIMPs. Among the TIMP family, TIMP-2 plays a major role in inhibiting MMP-2 activity. We demonstrated that both MMP-2 and TIMP-2 gene and protein expression levels were increased in monocrotaline-treated OLETF rats, and the upregulation of MMP-2 mRNA expression was much higher than those of TIMP-2. We also detected MMP-2 enzyme activation in glomeruli of monocrotaline-treated OLETF rats. It was reported that TIMP-2 shows different roles for MMP-2 activation depending on its concentration; high concentrations of TIMP-2 inhibit proMMP-2 and MMP-2 activation, while low concentrations activate...
proMMP-2 concomitant with MT-MMP [45]. Moreover, TIMP-2 and MMP-2 gene expression in mesangial cells were regulated by glucose concentration in vivo and in vitro [9, 43, 46]. Under these complicated control systems, MMP-2 activity overcame TIMP inhibition, and MMP-2 enzyme activity may play some role in ECM degradation associated with diabetic nephropathy.

Finally, the balance between ECM production and degradation is important for progression of diabetic nephropathy. Although the MMP-2 activity was enhanced in the glomeruli, glomerulosclerosis (ECM accumulation) progressed in monocrotaline-treated OLETF rats. These results suggested that ECM production may overcome ECM degradation in monocrotaline-treated OLETF rats. It has been reported that type III, IV and VI collagen, fibronectin and laminin expression are increased at the transcript and protein levels in the presence of high glucose concentrations [19, 47]. Moreover, hemodynamic abnormalities such as glomerular hypertension and hyperglycemia-induced metabolic disorders, such as advanced glycation end product C activation, will stimulate ECM production. Furthermore, normalization of hyperglycemia-ameliorated glomerular diffuse lesions, suggesting that the degradation system of accumulated ECM was dominant under normoglycemic conditions [1]. These observations suggest that overproduction of ECM induced by hyperglycemia may play an important role in the progression of diabetic nephropathy.

In summary, MMP-2 and MT1-MMP are produced and activated in glomeruli through the progression of diabetic glomerulosclerosis and may play important roles in the remodeling process of glomerular matrix in the diabetic nephropathy model of monocrotaline-treated OLETF rats. The altered synthesis of MMP, TIMP and ECM components will determine the outcome of ECM metabolism.

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Conflict of interest statement. None declared.

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