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Original Article

Immunolocalization of membrane-type 1 MMP in human rheumatoid synovium tissues

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Abstract: Membrane-type 1 matrix metalloproteinase (MT1-MMP, also known as MMP14), the best characterized membrane-anchored MMP, is an important matrix-degrading proteinase that could digest a broad spectrum of extracellular matrix proteins and accelerate angiogenesis. We have previously reported that some MMPs involved in the angiogenesis and the pannus formation within the joint, leading to the erosion of articular cartilage and bone in the pathological process of rheumatoid arthritis (RA). In the present study, we used immunohistochemistry assay and con-focal scanning technique to study the detailed immunolocalization of MT1-MMP in human RA synovium tissues as well as the infiltrating immune cell subsets. Our results showed that the positive MT1-MMP immunostaining could be found in synoviocytes, vascular endothelial cells, infiltrating macrophages and monocytes in RA synovium tissues, while weak or negative immunostaining could be found in infiltrating T cells, B cells and NK cells, respectively. Moreover, the Ki-67$^+$ highly proliferating synoviocytes also showed higher MT1-MMP expression in RA synoviocytes. Thus, the aberrant expression of MT1-MMP in RA synoviocytes as well as infiltrating immune cells may contribute to the proliferation of the synoviocytes, and the angiogenesis and the pannus formation in RA pathological progression.

Keywords: MT1-MMP, immunostaining, con-focal scanning, pannus, rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA), a chronic autoimmune inflammatory disease of unknown aetiology, reveals progressive destruction of bone and articular cartilage with functional impairment and disability, which was characterized by synovial inflammation, hyperplasia and pannus formation [1]. As we know, the invading synovial pannus tissues could usually produce proteinases that degrade components of the joint extracellular matrix, and followed by a vascular phase with high increase in vessel growth [2]. Thus, due to the angiogenesis has been considered as an essential event in perpetuating inflammatory and immune responses, as well as supporting pannus growth and development of RA, inhibition of angiogenesis has been proposed as a novel therapeutic strategy for RA. We have previously reported that some MMPs, such as MMP-2 and MMP-9, were involved in the angiogenesis and the pannus formation in RA synovium tissues, suggesting that these two MMPs expressed by synoviocytes as well as certain infiltrating immune cells role importantly in RA progression [3].

The membrane-type MMPs (MT-MMPs) are important sub-group members of the matrix metalloproteinases [4]. As of now, it has been demonstrated that there are six major members of the MT-MMP subgroup, namely MT1-, MT2-, MT3-, MT4-, MT5- and MT6-MMPs. Moreover, these MT-MMPs could also be further sub-classified into type I transmembrane-type (MT1-, MT2-, MT3- and MT5-MMPs) and glycosylphosphatidylinositol (GPI)-anchored type (MT4- and MT6-MMPs) [5]. The MT-MMPs have also been showed important role in vascular system stabilization, maturation, and leakage, and were thus closely associated with angiogenesis in many physio-pathological process [6]. For example, we have previously demonstrated that MT2-MMP expression level in

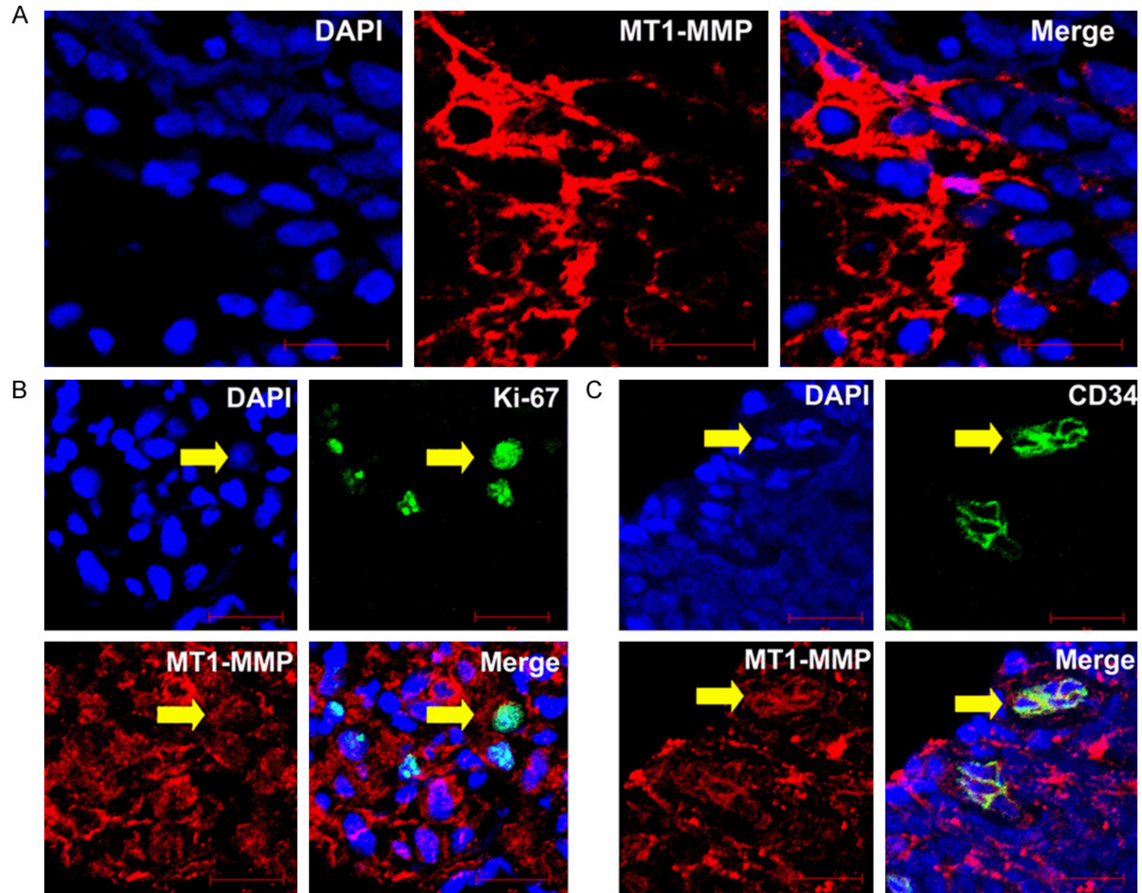

Figure 1. Immunolocalization of MT1-MMP in synoviocytes and vascular endothelial cells. A: Positive staining of MT1-MMP in synoviocytes of rheumatoid synovium. B: Positive staining of MT1-MMP in Ki-67 positive synoviocytes. C: Positive staining of MT1-MMP in CD34$^+$ vascular epithelial cells. Panel A to C, scale bar = 20 μm.

human esophageal cancer tissues was significantly associated with intra-tumoral micro-vessel densities [7]. The MT1-MMP was the one of the most important member of MT-MMPs, and was well characterized in angiogenesis [8].

In the present study, we focused on the immunolocalization of MT1-MMP in the angiogenesis and the pannus formation in RA synovium tissues. The immunohistochemistry and the confocal scanning were performed to characterize the MT1-MMP in synoviocytes, vascular endothelial cells as well as infiltrating immune cells in the pannus of RA synovium. Our results showed that MT1-MMP could be expressed on proliferating synoviocytes, CD34$^+$ vascular endothelial cells and CD68$^+$ infiltrating macrophages and CD14$^+$ monocytes, but weakly expressed on CD3$^+$ T cells, CD20$^+$ B cells and CD57$^+$ NK cells, and thus our data represented a detailed immunolocalization of MT1-MMP in the angiogenesis and the pannus formation in RA synovium tissues.

Methods and materials

Patient and tissue samples

The synovial tissue samples were obtained from one patient with RA at active stage by using arthroscopic biopsy in the Department of Orthopedics of our hospital, which has been described in our previous study [3]. The patient met the American College of Rheumatology criteria for RA, and the diagnosis of pathological changes was confirmed by using H & E staining. Laboratory assessment including the measurement of serum levels of C reactive protein (CRP), erythrocyte sedimentation rate (ESR), anti-CCP antibody, rheumatoid factors (RF), and blood routine examination were performed.

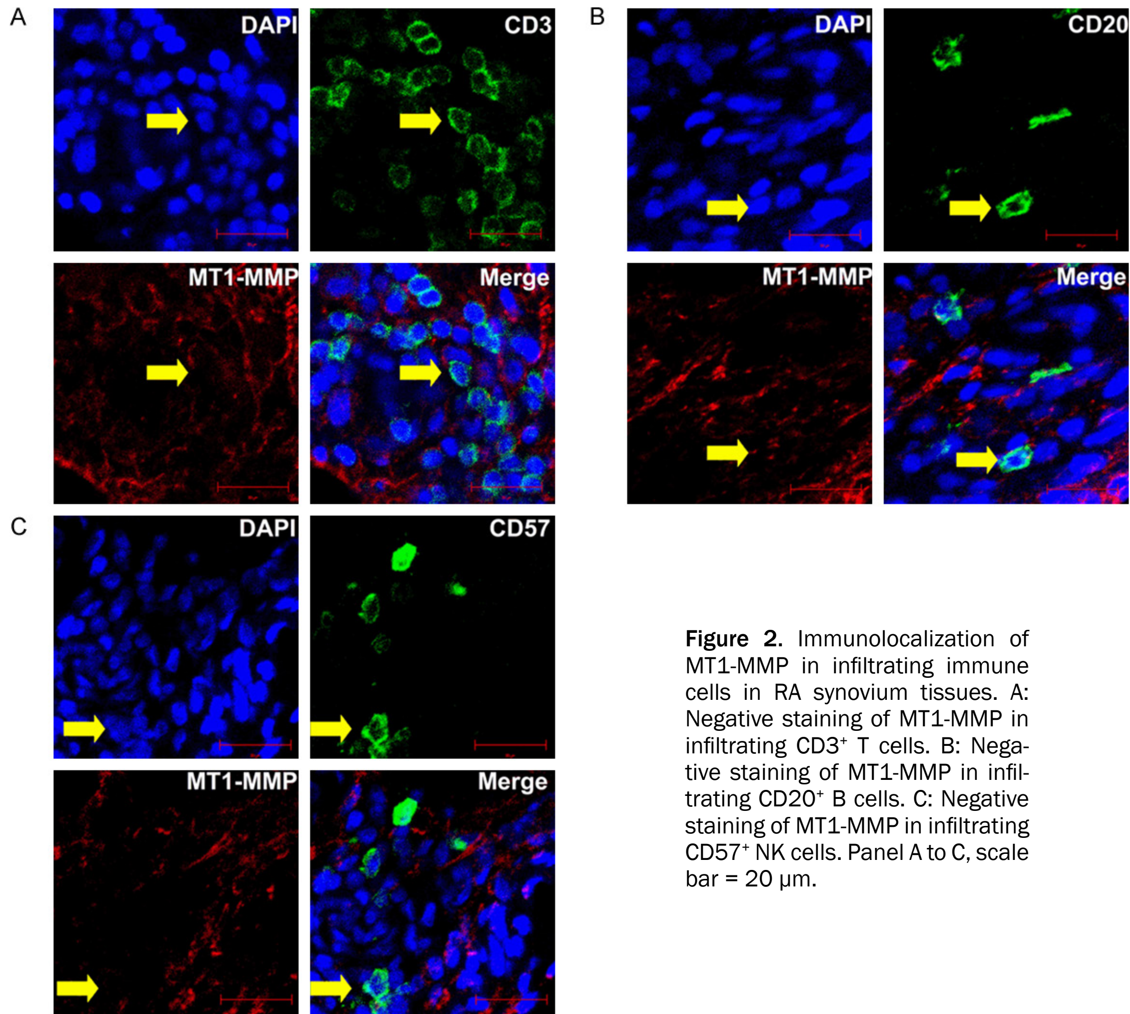

Figure 2. Immunolocalization of MT1-MMP in infiltrating immune cells in RA synovium tissues. A: Negative staining of MT1-MMP in infiltrating $CD3^{+}$ T cells. B: Negative staining of MT1-MMP in infiltrating $CD20^{+}$ B cells. C: Negative staining of MT1-MMP in infiltrating $CD57^{+}$ NK cells. Panel A to C, scale bar = 20 μm.

The protocol for the present study was approved by the Ethics Committee of our hospital.

Antibodies and reagents

Rabbit anti-human MT1-MMP monoclonal antibody (AB6004, diluted in 1:300) was purchased from Millipore (Billerica, MA, USA), mouse anti-human CD3 monoclonal antibody (ready to use) was purchased from Beijing Zhongshan Golden Bridge Biology (Beijing, China). Mouse anti-human CD20 monoclonal antibody (ready to use), mouse anti-human CD68 monoclonal antibody (ready to use), mouse anti-human CD34 monoclonal antibody (ready to use), mouse anti-CD57 monoclonal antibody (ready to use), mouse anti-Ki-67 monoclonal antibody (ready to use) and goat serum used in blockade were purchased from Fuzhou Maxin Biotechnology (Fuzhou, China). Mouse anti-human CD14 monoclonal antibody was purchased from Novus Biologicals (Littleton, CO, USA). Alexa Fluor® 488 goat anti-mouse IgG (H+L) and Alexa Fluor® 555 goat anti-rabbit IgG (H+L) were purchased from Invitrogen (Grand Island, NY, USA). Rabbit IgG was purchased from Southern Biotech (Birmingham, AL, USA), and mouse IgG1 was purchased from eBioscience (San Diego, CA, USA). 4, 6-Diamino-2-phenyl indole (DAPI) used in fluorescent counterstaining of cell nuclei was purchasedfrom Beyotime (Shanghai, China).

Immunofluorescence staining and con-focal laser scanning

The immunofluorescence staining and the confocal laser scanning were performed as de-

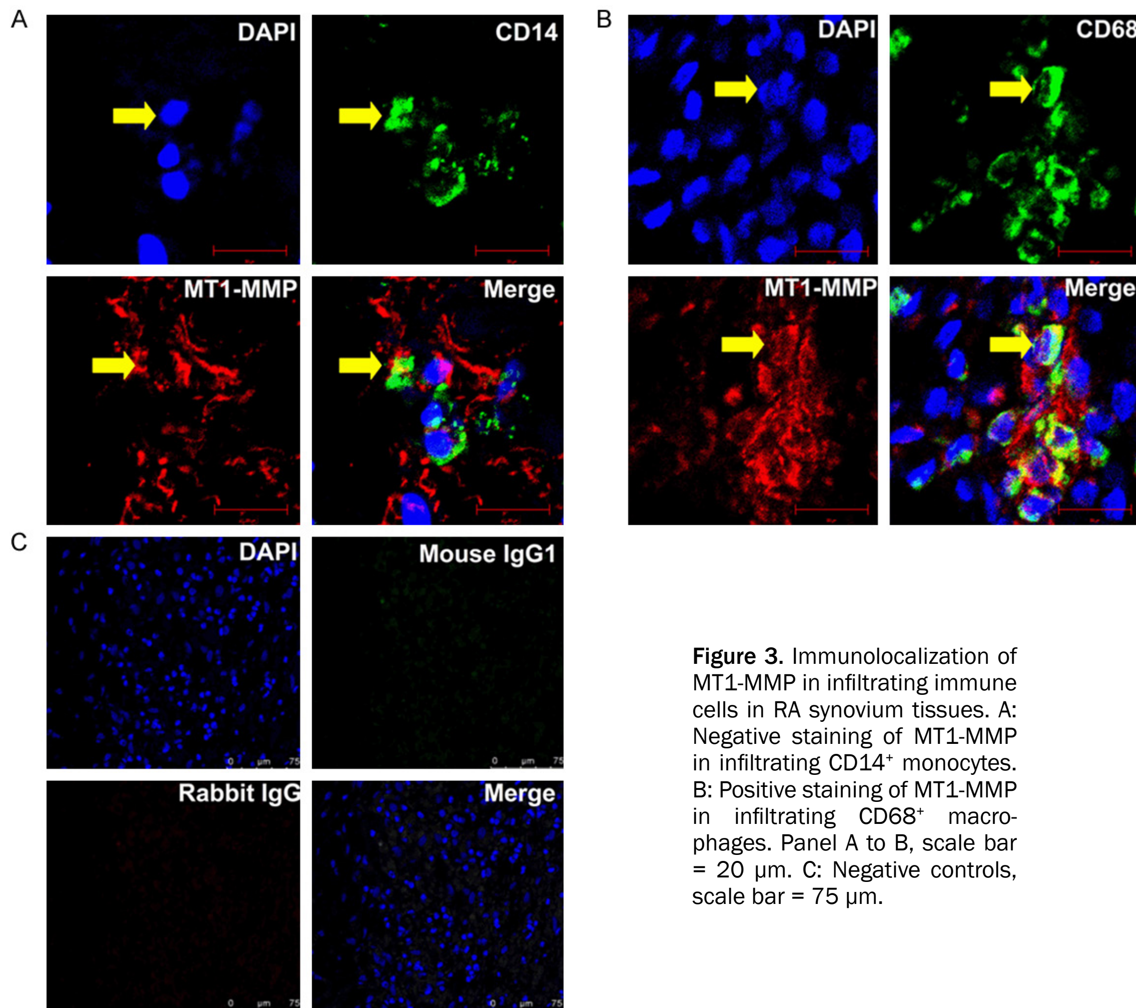

Figure 3. Immunolocalization of MT1-MMP in infiltrating immune cells in RA synovium tissues. A: Negative staining of MT1-MMP in infiltrating CD14+ monocytes. B: Positive staining of MT1-MMP in infiltrating CD68+ macrophages. Panel A to B, scale bar = 20 μm. C: Negative controls, scale bar = 75 μm.

scribed in our previous study [3, 9]. In brief, the formalin-fixed, paraffin-embedded rheumatoid synovium tissues were cut into 3-μm-thick sections. After the sections were dewaxed in xylene, rehydrated via graded ethanol solutions, antigen retrieval was performed by heating the sections for 30 min at 100°C in EDTA solution. Then the sections were cooled and rinsed in PBS for 5 min, and subsequently blocked using 3% bovine serum albumin. The sections were then incubated with primary antibody against MT1-MMP (diluted in 1:300) in combination with anti-CD3, anti-CD20, anti-CD34, anti-CD68, anti-Ki67, anti-CD57 and anti-CD14 respectively, at 4°C overnight. The section incubated with rabbit IgG in combination with mouse IgG1 instead of the primary antibodies was performed as a negative control. After washing in PBS solution for three times (5 min per wash), the sections were then incubated with mixed Alexa Fluor® 488 goat anti-rabbit IgG (diluted in 1:100) and Alexa Fluor® 555 goat anti-mouse IgG (diluted in 1:100) for one hour at 37°C. Then the sections were rinsed in PBS for 5 min, and incubated with DAPI for 10 min to stain the nuclei and mounted by using anti-fade mounting medium. Finally, the sections were examined by using a LSM 710 laser scanning con-focal microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany).

Results

Immunolocalization of MT1- MMP in synoviocytes and vascular endothelial cells in RA synovium tissue

Our immunofluorescence staining and confocal scanning results showed that the immunolocalization of MT1-MMP could be found in

synoviocytes in RA synovium tissues (**Figure 1A**), and the Ki-67 positive synoviocytes, namely highly proliferating synoviocytes also showed positive MT1-MMP staining (**Figure 1B**), which suggested that MT1-MMP was positively involved in the synovial hyperplasia in the RA progression. Moreover, when we stained the micro-vessels in the RA synovium by using CD34 endothelial marker, we also found the positive staining of MT1-MMP in the neo-vessels in the pannus of RA synovium (**Figure 1C**), suggesting the potential role of MT1-MMP in promoting angiogenesis in the pannus formation and the synovial hyperplasia of RA.

Immunolocalization of MT1-MMP in infiltrating immune cells in RA synovium tissue

We have previously demonstrated that the MMPs members, such as MMP-2 and MMP-9 could be found in infiltrating immune cells in RA synovium, suggesting that these metalloproteinases were involved in the angiogenesis and the pannus formation in RA progression [3]. Therefore, in the present study, we also aimed to characterize the immunolocalization of MT1-MMP in infiltrating immune cell subsets in RA synovium, and we found that the MT1-MMP could not be expressed by infiltrating $CD3^{+}$T cells (**Figure 2A**), $CD20^{+}$ B cells (**Figure 2B**) and $CD57^{+}$ NK cells (**Figure 2C**), but the positive staining of MT1-MMP could be found in filtrating $CD14^{+}$ monocytes (**Figure 3A**) and $CD68^{+}$ macrophages (**Figure 3B**). Thus, the molecular and cellular mechanism of MT1-MMP expressed on infiltrating monocytes and macrophages and its physio-pathological contribution need further investigation.

Discussion

It has been demonstrated that the matrix metalloproteinase family is one of the most important proteinases that degrade the extracellular matrix, and play an essential role in many physio-pathological process, such as extracellular matrix remodeling, inflammation, fibrosis, angiogenesis and *etc* [10]. As we know, the matrix metalloproteinase family members are zinc-containing, calcium-dependent proteases, which could be divided into six major subgroups, namely the collagenases, gelatinases, stromelysines, matrilysins, MT-MMPs and other MMPs, on the basis of substrate specificity, sequence similarity, and domain organization [11-13]. MT1-MMP, is one of the most important members of MT-MMPs, firstly characterized as a cell surface proMMP-2 activator expressed in invasive cancer cells, but many other cell types also expressed this enzyme has been implicated in various physio-pathological processes for its extracellular matrix degrading and accelerating angiogenesis [5, 14]. The current studies in experimental cancer models showed that the tumor cell expressed MT1-MMP was essential for the remodeling and transmigration through the base membrane and for the invasive migration ability [15]. As of know, the detailed expression pattern of MT1-MMP in RA synovium tissues especially those infiltrating immune cells still remains elusive.

We have previous showed that the positive staining MMP-2 and MMP-9 could be found in RA synoviocytes as well as CD34 positive vascular endothelial cells, infiltrating CD14 positive monocytes and CD68 positive macrophages, suggesting that these MMPs were involved in the angiogenesis and in the synovium hyperplasia, and finally contributed to the pannus formation of RA progression [3]. In the present study also found that MT1-MMP was highly expressed in RA synovium tissues, we identified the membranous staining of MT1-MMP in synoviocytes, CD34 positive vascular endothelial cells, CD14 positive monocytes and CD68 positive macrophages, therefore, as the most important member of the MT-MMPs subgroup, MT1-MMP expression in the RA synovium tissues could potentially promote the synovium hyperplasia, angiogenesis and pannus formation in RA progression. In our present study, the proliferating synoviocytes characterized by Ki-67 positive staining also showed that positive staining of MT1-MMP, suggesting MT1-MMP expressed by synoviocytes could regulate the cellular biological function of synoviocytes themselves. It has been suggested that in human cancers, MT1-MMP is a cellular migration/invasion promoter and involved in cancer cell invasion, metastasis and tumor growth [16]. MT1-MMP could regulate the cell invasion and migration behavior via several means, for example, it can directly degrade the extracellular matrix, especially fibrillar collagens, and also can regulate epithelial-to-mesenchymal transition of the cancer cells [5, 16]. Thus, it's important for us to uncover the molecular

mechanism of MT1-MMP regulating cellular behavior changes of synoviocytes in RA progression.

We have previously demonstrated that the angiogenesis and the pannus formation could not only provide oxygen and nutrients to the hypertrophic granulation tissue, and also could provide the means for recruitment of inflammatory cells forward to the articular synovium, and finally leads to the irreversible destruction of joint structure and function [1, 3, 17, 18]. The immune cell subsets are important components of the RA pannus, such as macrophages, T cells, B cells, monocytes and dendritic cells, *etc.* [1]. Although some studies demonstrated that MT1-MMP could be expressed by T cells and B cells, but we found negative MT1-MMP staining in infiltrating T cells and B cells in RA synovium tissues [5]. Macrophages have been indentified to contribute to the angiogenesis, especially in human caner progression [19]. The infiltrating macrophages in hyperplastic rheumatoid synovium tissue also could contributed to the angiogenesis and proteolytic degradation via expressing MMPs, such as MMP-2 and MMP-9 [3]. Our present study also showed the infiltrating macrophages could also highly expressed MT1-MMP. *In vivo* studies showed that, the blockade of MT1-MMP by using the inhibitory antibody could lead to the decreased the secretion of immunosuppressive TGF-β, polarized macrophages to an antitumor phenotype, increased iNOS, and improved tumor perfusion, resulting in reduced primary tumor growth and enhanced response to radiation therapy, especially in high MMP14-expressing tumors [20]. Thus, the macrophage expressed MT1-MMP might not only contribute the angiogenesis, but also could inflect the immune modulation in the microenvironment of RA synovium.

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Disclosure of conflict of interest

None.

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