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Fibroblastic Synoviocytes Secrete Plasma Proteins Via α_2-Macroglobulins Serving as Intracellular and Extracellular Chaperones

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ABSTRACT

Changes in plasma protein levels in synovial fluid (SF) have been implicated in osteoarthritis and rheumatoid arthritis. It was previously thought that the presence of plasma proteins in SF reflected ultrafiltration or extravasation from the vasculature, possibly due to retraction of inflamed endothelial cells. Recent proteomic analyses have confirmed the abundant presence of plasma proteins in SF from control and arthritic patients. Systematic depletion of high-abundance plasma proteins from SF and conditioned media from synoviocytes cultured in serum, and protein analysis under denaturing/reducing conditions have limited our understanding of sources and the native structures of "plasma protein" complexes in SF. Using Western blotting, qPCR, and mass spectrometry, we found that Hig-82 lapine fibroblastic synovicytes cultured under serum-free conditions expressed and secreted plasma proteins, including the cytokine-binding protein secreted phosphoprotein 24 kDa (Spp24) and many of the proteases and protease inhibitors found in SF. Treating synoviocytes with TGF-β1 or BMP-2 for 24 h upregulated the expression of plasma proteins, including Spp24, α_2-HS-glycoprotein, α_1-antitrypsin, IGF-1, and C-reactive protein. Furthermore, many of the plasma proteins of mass <151 kDa were secreted as disulfide-bound complexes with members of the α_2-macroglobulin (A2M) family, which serve as intracellular and extracellular chaperones, not protease inhibitors. Using brefeldin A to block vesicular traffic and protease inhibitors to inhibit endogenous activation of naïve A2M, we demonstrated that the complexes were formed in the endoplasmic reticulum lumen and that Ca^{2+}cysteine protease-dependent processes are involved. J. Cell. Biochem. 116: 2563–2576, 2015.

KEY WORDS: α_2-MACROGLOBULIN; α_1-ANTITRYPSIN (A1AT); BLOOD: SYNOVIUM BARRIER; BONE MORPHOGENETIC PROTEIN-2; C-REACTIVE PROTEIN; CHAPERONE; INSULIN-LIKE GROWTH FACTOR-1; INTER-α-TRYPSIN INHIBITOR HEAVY CHAIN H4; MATRIX METALLOPROTEINASE; TRANSFORMING GROWTH FACTOR-β1; HIG-82 CELLS; Spp24

The synovial joints are among the most important anatomical structures in the human body for accommodating movement and changes in load bearing, as well as reducing friction between bones [Hui et al., 2012]. Synovial joints consist of articular cartilage covering the subchondral bone of opposing surfaces, a fibrous capsule containing an inner lining or synovium, and a cavity filled with synovial fluid (SF) [Smith, 2011]. The cells of the inner lining are designated synoviocytes, and can be derived from precursors of the monocytic (type A) or fibroblastic (type B) lineages [Ritchlin, 2000; Smith, 2011]. Synoviocytes may form gap junctions with other synoviocytes or chondrocytes, but there are no basement membranes or tight junctions. SF contains the lubricating molecules hyaluronan (HA) and lubricin (encoded by the PRG4 gene and also known as proteoglycan 4, megakaryocyte stimulating factor, and

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superficial zone protein) [Hui et al., 2012]. In addition, proteins normally associated with plasma, including α_2-HS-glycoprotein (AHSG), serum amyloid A4 (SAA4), apolipoproteins, complement proteins, fibrinogens (FN), haptoglobin, pregnancy-zone protein (PZP), and transferrin (TF), are abundant in SF [Gobezie et al., 2007]. Many plasma proteins such as secreted phosphoprotein-24 kDa (Spp24), albumin (ALB), α_2-macroglobulin (A2M), and complement C3 (C3), are more abundant in the SF of patients with osteoarthritis (OA) or animal models of joint injury than in controls. These proteins (e.g., inter-α-trypsin inhibitor H4 or ITIH4 and A2M) are often differentially expressed in early vs. late OA [Levick, 1981; Meng et al., 2005; Gobezie et al., 2007].

At one time, plasma proteins found in the SF were assumed to be derived from interstitial fluid filtered from blood plasma because: (1) the concentrations of SF plasma proteins are about one third of those of the circulation; (2) the concentrations of relatively small proteins, such as ALB (67 kDa) and TF (90 kDa), are higher than those of larger proteins, such as FN (340 kDa) [Hui et al., 2012]; and (3) vascular permeability is higher in pathological conditions marked by inflammation, such as rheumatoid arthritis (RA), than in control conditions [Levick, 1981]. It was demonstrated that the concentration of plasma proteins in the SF of normal joints is inversely related to the radius of the protein [Pejovic et al., 1995]. For instance, the small protein α_1-antitrypsin (A1AT or serpinA1, 44 kDa) is more abundant than the large protein A2M (720 kDa). However, it has become apparent that while the synovium is permeable to water, gases, nutrients, small molecules, and small peptides, it is not freely permeable to proteins and proteoglycans larger than 10 kDa [Yamazaki et al., 2014].

Spp24 is a BMP/TGF-β cytokine-binding protein secreted primarily by the liver [Zhao et al., 2013b; Murray et al., 2015]. Induction of experimental arthritis increases the expression of Spp24 in joint cartilage by more than 30-fold, an increase that is greater than that of any other protein [Meng et al., 2005]. We reported that Spp24 is present in serum in a high molecular weight (>500 kDa) disulfide-bonded complex with A2Ms (A2M itself, PZP, and C3) and A1AT [Zhao et al., 2013b]. In studies aimed at characterizing the SF proteome of patients with RA and OA, Spp24 was found to be present if A2M, its abundant carrier protein, had not first been depleted to enhance the detection of less abundant proteins [Balakrishnan et al., 2014b]. In contrast, Spp24 was undetectable when A2M was selectively removed prior to analysis [Balakrishnan et al., 2014a]. The cellular origin of Spp24 in SF has not been determined. Since the analytical methods used to detect Spp24 in SF were dissociative and reductive, it could not be determined whether Spp24 was present as a high-molecular weight complex with A2M, as previously reported [Zhao et al., 2013b].

Over 30 years ago, Hamanishi [1978] and Knight and Levick [1984] suggested that the ultrastructure of the synovium, which consists of dispersed synoviocytes overlying a fenestrated capillary network, acts as a semipermeable blood: synovial barrier (BSB) that effectively blocks free migration of high-molecular solutes. Thus, the large Spp24∼A2M∼A1AT complex would be excluded from the SF. We hypothesized that Spp24 is present in SF as a high molecular weight complex with A2M, similar to that secreted by the liver and found in the plasma [Zhao et al., 2013b]. We also hypothesized that the large size of the complex would prevent it from simply diffusing from the interstitial fluid into the synovium. Furthermore, we hypothesized that the complex was synthesized and secreted by cells within the synovium. These hypotheses were tested in the rabbit intra-articular synoviocyte cell line Hig-82 cultured under defined, serum-free conditions. We used non-reducing SDS–PAGE and Western blotting to determine if Hig-82 synoviocytes secrete a high-molecular weight complex containing A2M and Spp24. We investigated the expression of genes for various other "plasma proteins" under basal conditions and after treatment with TGFβ1 or BMP2, and used mass spectrometric analysis of proteins partially purified from serum-free condition medium of Hig-82 cells.

With respect to the general mechanism of plasma protein secretion, currently accepted concepts hold that plasma proteins produced by the liver (hepatocytes) are mostly glycosylated and secreted individually by the vesicular secretory pathway [Bowman, 1993; Donohue et al., 1996]. The addition of N-linked oligosaccharide ($Glc_3Man_9GlcNAc_2$) chain en bloc to nascent proteins, a common feature of glycoproteins [Elbein, 1987], takes place in the endoplasmic reticulum (ER). This is followed by trimming and modification into complex glycoproteins in the ER, vectoral transition from the ER to the cis- and trans-Golgi networks, and fusion with the plasma membrane during active secretion. We provided evidence that the non-glycosylated Spp24 depends on glycosylation as the Spp24∼A2M complex moves from the ER through the Golgi and is actively secreted.

The mechanism of A2M complex formation with cytokine ligands in vitro has been known for more than two decades [Feinman, 1994], and the concept of A2M serving as an extracellular chaperone for other proteins is beginning to emerge [Misra et al., 2011; Wyatt et al., 2013]. However, details of A2M activation and ligand binding in the extracellular environment are not fully understood, and there is no report of A2M serving as an intracellular chaperone in the scientific literature. Using various protease inhibitors and activators, we explored the molecular mechanism of A2M in serving as both an intracellular and an extracellular chaperone in the biosynthesis and processing of various other plasma proteins, including Spp24 by synoviocytes.

MATERIALS AND METHODS

CELL CULTURE

The Hig-82 rabbit synoviocyte was obtained from the American Type Culture Collection (CRL-1832; Manassas, VA) and cultured in growth medium (F12, Invitrogen [Carlsbad, CA] #21700-075, supplemented with 0.118% $NaHCO_3$, 4 mM glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.1 mM non-essential amino acids, and 10% fetal calf serum [FCS] or [F12-FCS]), at 37°C in a humid atmosphere of 95% air and 5% CO_2. Cells were seeded at ∼500,000 cells/well in 6-well plates (Laguna Scientific, Laguna Niguel, CA) and cultured for 3 days in F12-FCS. Cells were washed with PBS and added serum-free F12 medium supplemented with 1× ITS (insulin, transferrin, and selenium, BioWhittaker #17-838Z, F12-ITS). After culturing for 1 h, the culture medium was replaced with fresh SF-F12 and the cells were cultured for up to 24 hr. Conditioned media were collected at various time points.

Various cytokines, interleukin 1β (IL1β, 201-LB-005), IL6 (206-1L-010), and tumor necrosis factor α (TNFα, 201-TA-005) were purchased from R&D System. Transforming growth factor β1 (TGFβ1) was from Cell Signaling (#8915), and BMP2 was from Medtronic. The following reagents were from EMD (Calbiochem): Glycosylation inhibitors tunicamycin (TM, #654380), 1-deoxynojirimycin (dNJ, #260684), deoxymannojirimycin (dMJ, #260575), and swainsonine (SW, #574775); Golgi network blocker brefeldin A (BFA, #203729); protease inhibitors 4-dichloroisocoumarin (DCI, #287815), N-[N-(N-acetyl-L-leucyl)-L-leucyl]-L-norleucine (ALLN, #208719), aprotinin (#616370), leupeptin (108976), 2S,3S-trans-(ethoxycarbonyloxirane-2-carbonyl)-L-leucine-(3-methylbutyl) amide (E64d, #324890), and carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132, #474787). All other reagents were from Sigma–Aldrich (St. Louis, MO).

Treatment media were prepared by adding inhibitors or activators to F12-ITS at the following final concentrations: 10 μg/ml TM, 0.2 mg/ml dNJ, 0.1 mg/ml dMJ, 2 μg/ml SW, 10 μg/ml BFA, 0.3 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 50 μM DCI, 2 μM ALLN, 2 μg/ml aprotinin, 100 μM leupeptin, 10 μM E64d, 5 μM MG-132, 1 mM EGTA or EDTA, 0.2 mM iodoacetamide (IAM), 2 mM N-ethylmaleimide (NEM) or dithiothreitol (DTT).

While in treatment media, cells were cultured for 2 h in 5% CO_2 at a reduced temperature of 32°C to allow correct folding of proteins [Lodish and Kong, 1993]. After collecting the culture media, cells were harvested by adding 0.3 ml lysis buffer (10 mM Tris–HCl, pH 7.5, 0.5% Triton X-100, and 0.1 mM AEBSF). The cell suspensions were transferred to Eppendorf tubes, sonicated for 5 s at setting 3 (Sonic Dismembranator 550, Fisher Scientific) and centrifuged for 5 min at 16,000*g*.

SDS–PAGE AND WESTERN BLOTTING

Human serum was purchased from Sigma–Aldrich, and rabbit serum was from a pre-bleed sample (Prosci, Inc., Poway, CA). Commercially purified human A2M was purchased from Athens Research & Technology (#16-16-012013, Athens, GA) and Meridian Life Science (#A500114H, Memphis, TN).

Human or rabbit sera or purified human A2M were diluted 1:6 in Laemmli loading buffer (LB, 67 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, and 0.1% bromophenol blue) or LB containing 1% β-mercaptoethanol (βMSH) and/or boiled for 5 min [Laemmli, 1970]. Cell lysate supernatants (10 μl) or treatment media (20 μl) were mixed with 5 or 10 μl of 3× LB. Unless otherwise stated, all F12-ITS condition media from Hig-82 were assayed under non-reducing conditions in LB or were concentrated ~100 fold by centrifugation in Centricon-10 concentrators (Millipore, Billerica, MA) prior to mixing with reducing LB and assaying under reducing conditions.

All samples were separated by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) as described previously [Zhao et al., 2013b]. All antibodies were purchase from Santa Cruz Biotechnology (Dallas, TX) except anti-A2M which was from Meridian Life Science (Memphis, TN). The antibodies are listed in Table I. They were conjugated to HRP by using EZ-link plus activated peroxidase kit from Pierce (#31489, Rockford, IL) according to the

TABLE I. Anti-Human Antibodies Cross React with Rabbit Proteins from Serum or Hig-82 Medium. All Antibodies Were Raised Against Human Plasma and Matrix Proteins or Their Peptides as Antigens From Respective Species: Sheep[a], Goat[b], Rabbit[c], or Mouse[d]. All Antibodies Were Custom-Conjugated to HRP to Eliminate Non-Specific Bands Caused by the Use of HRP-Conjugated Secondary Antibodies. Catalog Numbers Starting With sc- Were From Santa Cruz Biotechnology and K90039C was From Meridian Life Science, Inc.

		Monomer	GeneBank #		Cross reactivity*		
Proteins (antigens)	Abbr.	M_r (kDa)	(*H. sapiens*)	Catalog #	NR	Red	Red/heat
α_2-macroglobulin	A2M	185[#]	NP_000005.2	K90039C[a]	+++	+	−
Secreted phosphoprotein-24 kDa	Spp24	24	NP_008875	sc-169408[b]	++	+	−
				sc-169409[b]	−	−	−
α_1-Antitrypsin (serpinA1)	A1AT	44	NP_000286.3	sc-80461[d]	+++	+	−
α_1-Antichymotrypsin (serpinA3)	ACT	44	NP_001076.2	sc-69983[d]	+++	+	−
Antithrombin-III (serpinC1)	AT3	58	NP_000479	sc-32453[b]	+	−	−
C1 inhibitor (serpinG1)	C1INH	105	NP_000053.2	sc-67150[c]	+++	+	−
α_2-HS-glycoprotein	AHSG	55	NP_001613.2	sc-9663[b]	+	++	+++
Albumin	ALB	67	NP_000468	sc-271605[d]	+++	+	−
Ceruloplasmin	CP	151	NP_000087	sc-21242[b]	+++	+	−
Transferrin	TF	80	NP_001054	sc-365871[d]	++	+	+
Haptoglobin	Hg	22	NP_005134	sc-374208[d]	++	+	−
Transthyretin	TTR	14	NP_000362	sc-13098[c]	+++	+	−
Serum amyloid A4	SAA4	55	NP_006503.2	sc-20275[b]	+++	+	−
Insulin-like growth factor-1	IGF1	7.6	NP_001104755	sc-9013[c]	++	+	−
Proteoglycan-4 (Lubricin)	PRG4	220	NP_005798.2	sc-50081[b]	+	−	−
Inter-α-trypsin inhibitor H4	ITIH4	120	NP_002209.2	sc-34471[b]	+++	+	−
Matrix metalloproteinase-1	MMP1	54	NP_002412	sc-6837[b]	++	+	−
Matrix metalloproteinase-3	MMP3	54	NP_002413	sc-6839[b]	++	−	−
Metalloproteinase inhibitor -1	TIMP1	23	NP_003245	sc-6832[b]	++	+	−
Transforming growth factor-β1	TGFβ1[†]	10	NP_000651.3	sc-146[c]	+	+	+

*NR, non-reducing; red, reducing; red/heat, reducing and heating. Except for anti-AHSG antibody which preferentially reacts with denatured antigen (AHSG), most antibodies react with native (exposed to 0.1% SDS under non-reducing condition) antigens at a much higher sensitivity than reduced antigens. Thus, the (+) under "Red" indicates a 100-fold concentration of samples prior to their reduction by βMSH. [#]A2M monomer that has been cleaved (activated) at the "bait" region by protease(s) can be resolved into 130 and 50 kDa subunits under reducing SDS–PAGE. [†]Positive reactivity data was obtained from the vendor.

manufacturer's instructions. HRP-conjugated primary antibodies were used at 1:5,000 dilution without the need for a secondary IgG-HRP, which causes non-specific bands in visualizing serum samples.

QUANTITATIVE GENE EXPRESSION ANALYSIS BY qPCR

For qPCR analysis, Hig-82 cells were seeded at a density of 10^5 cells/well in 6-well plates and cultured overnight in F12-FCS medium at 37°C and 5% CO_2. Cytokines were added directly to their respective wells to achieve the final working concentrations indicated: 20 pg/ml IL1β, 2 ng/ml IL6, 100 pg/ml TNFα, 10 ng/ml TGFβ1, or 10 ng/ml BMP2 and cultured for an additional 24 h. RNA isolation, cDNA preparation, and qPCR reaction were carried out according to our established protocols [Zhao et al., 2013a, 2015]. Unique oligonucleotide primers for amplification of rabbit plasma protein genes were designed and chemically synthesized by Invitrogen (Supplementary Table SI). Cycle counts (CT) $\leq$33 were used for statistical analysis to allow potential gene expression changes especially decreases, as CT values >34 are unreliable.

IDENTIFICATION OF HIGH MOLECULAR PROTEIN BAND FROM SERUM-FREE CONDITIONED MEDIUM OF HIG-82 CELLS

The purification and mass spectrometric analysis scheme for identifying the proteins secreted by Hig-82 cell cultured in F12-ITS medium is illustrated in Supplemental Figure S1. Briefly, serum-free conditioned medium (25 ml) from Hig-82 cells cultured in five 10 cm diameter plates at 37°C for 16 h was concentrated to ~100 μl in a Centricon-10. The sample was mixed with 18 μl 6× LB, loaded in four lanes and separated by 4–20% SDS–PAGE (100 V × 1 h). One lane was stained with Coomassie blue, and three lanes corresponding to 360–720 kDa unstained bands were cut out, inserted into a new Centricon-10, and electro-eluted for 500 V × 1 h in transfer buffer in a Hoeffer apparatus. The retained sample was concentrated again to ~50 μl, mixed with 25 μl 3× reducing LB, and boiled for 15 min. The sample was separated again by 4–20% SDS–PAGE (100 V × 1 h) and stained. Gel slices were excised, digested with trypsin, and analyzed for unique peptides by LC-MS/MS as described before [Zhao et al., 2013b].

STATISTICAL ANALYSIS AND DATA REPLICATION

All Western blots and qPCR are representative examples of experiments that were independently repeated multiple times (>3) with similar results. Numerical data are expressed as the mean ± SEM (n = 3). Data were analyzed by Student's *t* test using Microsoft Excel.

RESULTS

ANTIBODIES AGAINST HUMAN PLASMA PROTEINS RECOGNIZE THE CORRESPONDING PROTEINS IN RABBIT SERUM

The ability of antibodies directed against the human serum proteins of interest (e.g., A2M) to cross-react with proteins in rabbit serum was tested under control conditions after adding LB only, after reduction (LB plus β-mercaptoethanol or βMSH) and after reduction and heating (LB plus βMSH, followed by boiling). In order to minimize potential contamination of the control lanes with reducing agent, a "non-loading" or "NL" lane was introduced between the control and βMSH-treated samples. The results are shown in Figure 1 and Table I. Each of the 12 native rabbit proteins, including A2M, Spp24, AHSG, A1AT, α_1-antichymotrypsin (ACT or serpinA3), C1 inhibitor (C1INH or serpinG1), TF, ceruloplasmin (CP), ALB, haptoglobin (Hg), insulin-like growth factor-1(IGF1), and ITIH4 was recognized by the corresponding anti-human protein antibody and the migration position for each resembled that of A2M (360–720 kDa) in spite of their differing molecular weights. Only A2M and AHSG were detected after reduction, and only AHSG was detected after both reduction and heat denaturation. These results suggested that most antibodies react preferentially with the native form of their antigens. The exception was anti-AHSG, which reacts better with the denatured form. All of the proteins tested exist as a high molecular weight form in the native condition.

SYNOVIOCYTES SECRETE PLASMA PROTEINS IN HIGH MOLECULAR WEIGHT FORMS

We then addressed two issues: (1) do these antibodies detect antigens produced by cultured rabbit cells; and (2) is the lack of cross-reactivity between these antibodies and antigens in βMSH-reduced rabbit serum, the result of complete inability of these antibodies to recognize the corresponding proteins. As serum contains a high concentration of plasma proteins (>60 mg/ml) [Putnam, 1975], more than half of which is ALB, simple concentration of serum for increased levels of detection in Western blot is difficult. We used confluent rabbit Hig-82 synoviocytes cultured in F12-ITS medium, to address these two questions. Eight plasma proteins, including A2M, AHSG, Spp24, C1INH, serum amyloid A4 (SAA4), transthyretin (TTR), metalloproteinase 1 (MMP1), and metalloproteinase inhibitor-1 (TIMP1) were detected in Hig-82 conditioned media from cells that had been cultured in F12-ITS for a relatively short period (4–9 h). Media proteins were separated by SDS-PAGE under either unconcentrated/native conditions (Fig. 2, lane 1) or 100-fold concentrated and βMSH-reduced conditions (Fig. 2, lane 3). Both the high molecular weight forms (360–720 kDa) were detectable in the native media, and lower molecular weight forms (45–130 kDa) were detected after concentration and reduction. The lack of detectable signal after reduction by βMSH for most proteins (Fig. 1) may reflect preferential antibody specificity for native proteins over reduced/denatured proteins. In other words, these antibodies react better with native proteins than βMSH-reduced proteins. This is summarized in Table I, where cross-reactivity with the antigens of interest under non-reducing conditions in "neat" (unconcentrated samples) is compared with cross-reactivity under reducing conditions in samples concentrated 100-fold.

In order to demonstrate that plasma proteins detected in the serum-free conditioned media were truly synthesized by Hig-82 cells rather than being carried over from bovine serum-containing medium, we collected F12-ITS media at different time points and analyzed these by Western blot. Without exception, newly synthesized plasma proteins were detected at increasing concentration in a time-dependent fashion from the media. These include A2M, Spp24, AHSG, A1AT, TF, CP, IGF1, and antithrombin III (AT3 or SerpinC1; Fig. 3). Except for abundant proteins such as A2M, TF and AHSG, no other proteins were detectable at 1 h but all were

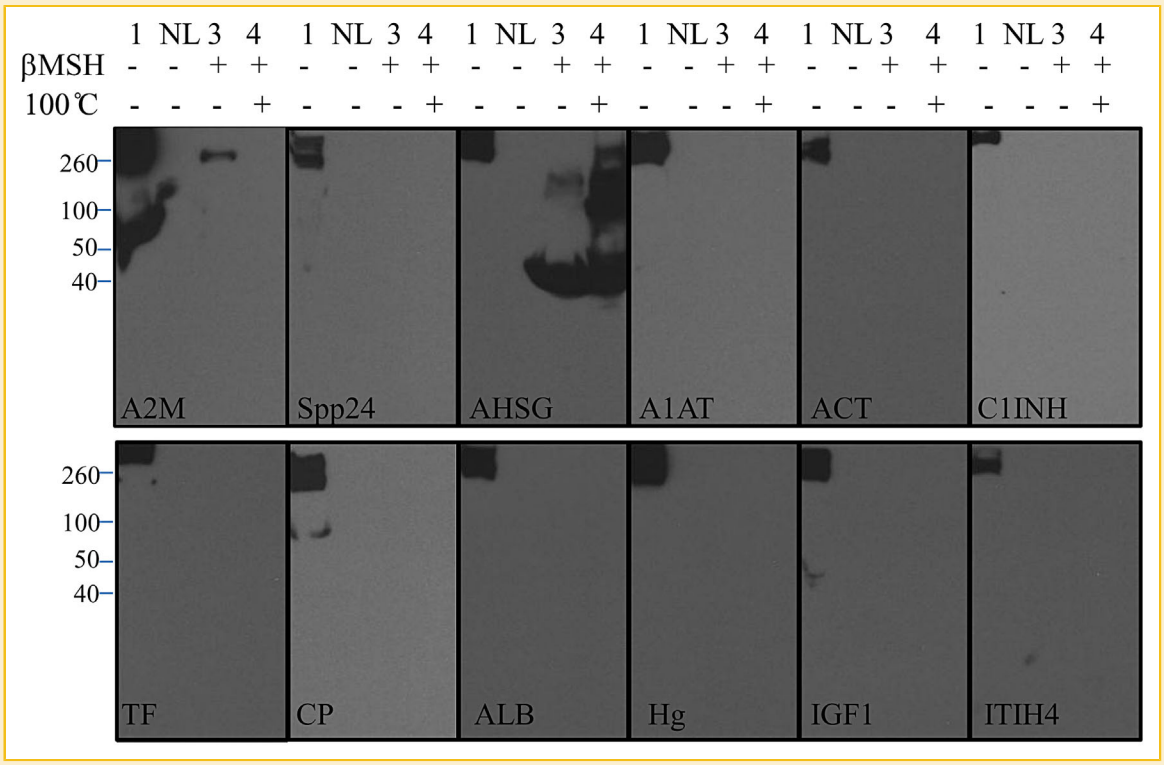

Fig. 1. Cross reactivity of anti-human antibodies with rabbit plasma proteins. Aliquots of rabbit serum (5 μl) were mixed with an equal volume of 2× LB (lane 1), LB-βMSH (lane 3), or LB-βMSH (lane 4) plus boiling for 5 min. All samples were separated by 4–20% SDS–PAGE (a blank well without any sample and labeled as NL was loaded with LB only between neighboring wells to prevent cross-over of βMSH), followed by Western blotting. Exposure times: 10 s for A2M, A1AT, and Spp24; 15 s for AHSG, ACT, TF, ALB, Hg, IGF1, and ITIH4; 30 s for CP; and 5 min for C1INH. A2M, α_2-macroglobulin; Spp24, secreted phosphoprotein 24 kDa; AHSG, α_2-HG-glycoprotein also called fetuin A; A1AT, α_1-antitrypsin; ACT, α_1-antichymotrypsin; C1INH, C1 inhibitor; TF, transferrin; CP, ceruloplasmin; ALB, albumin; IGF1, insulin-like growth factor-1; ITIH4, inter-α-trypsin inhibitor H4.

present within 4 h and increased over time for up to 9 h. This suggested that the detected proteins were not from carry-over serum proteins from prior culture medium, but accumulated after secretion by Hig-82 cells. Furthermore, high molecular weight patterns (360–720 kDa) characteristic of dimeric and tetrameric A2Ms were seen in all of the proteins detected, monomeric and dimeric forms (185–360 kDa) can be seen at several 24 h time point possibly due to turn-over after secretion (Fig. 3).

HIGH MOLECULAR IMMUNOREACTIVITY IS NOT AN ARTIFACT OF NON-SPECIFIC ANTIBODY BINDING TO A2M PROTEIN

In order to exclude the possibility that all of the antibodies tested, except anti-A2M, were non-specifically cross reacting with A2M, the two commercially available A2M proteins from two vendors were separated by SDS–PAGE and probed with antibodies against A2M, C3, Spp24, A1AT, IGF1, and TGFβ1. Both sources of purified A2M (0.1 μg) were detected by anti-A2M antibody, and the A2M from Athens was found to react with anti-C3 antibody when a 50-fold greater quantity of antigen (5 μg) was used, suggesting co-purification of C3 with A2M (Fig. 4). It should be noted that A2M and C3 share similar molecular sizes, structures, and functions. None of the other antibodies show any band when high amounts of A2M antigens were used and longer (overnight) exposure was employed (Fig. 4). These results clearly demonstrated that the high molecular weight bands shown in Figures 1–3 were bona fide plasma proteins recognized by the cross-reactivity with their respective antibodies rather than non-specific cross-reactivity with A2M, the major component of these high molecular weight bands.

PLASMA PROTEIN-SPECIFIC mRNAs ARE DETECTED IN SYNOVIOCYTES AND GENE EXPRESSION IS ACTIVATED BY TGFβ1 AND BMP2

To further examine the source of plasma proteins as being either synthesized in and secreted from synoviocytes or derived from an external source, mRNA corresponding to each of the plasma proteins detected by Western blotting was assayed by RT-qPCR. Furthermore, since certain plasma proteins made by the liver such as A2M, A1AT, and C-reactive protein (CRP) are acute-phase reactants, and are subjected to modulation by inflammatory cytokines, we tested the effects of adding IL1β, IL6, and TNFα in addition to the chondrogenic cytokines TGFβ and BMP2 for comparison. The effects of 24 h of incubation with these cytokines on total RNA levels in Hig-82 synoviocytes are shown in Figure 5 (panel A). Cells treated with TGFβ1 and BMP2 had the lowest levels of total RNA. Several sets of primers were tested for some genes and only data for those that acquire cycle numbers <33 were used for calculation and presentation (supplemental Table SI). Thus, no data are presented for A2M, MMP1, PRG4, SAA4, TF, and TIMP1. The abundance for HAS2 was by far the highest, and HAS1 and C1INH

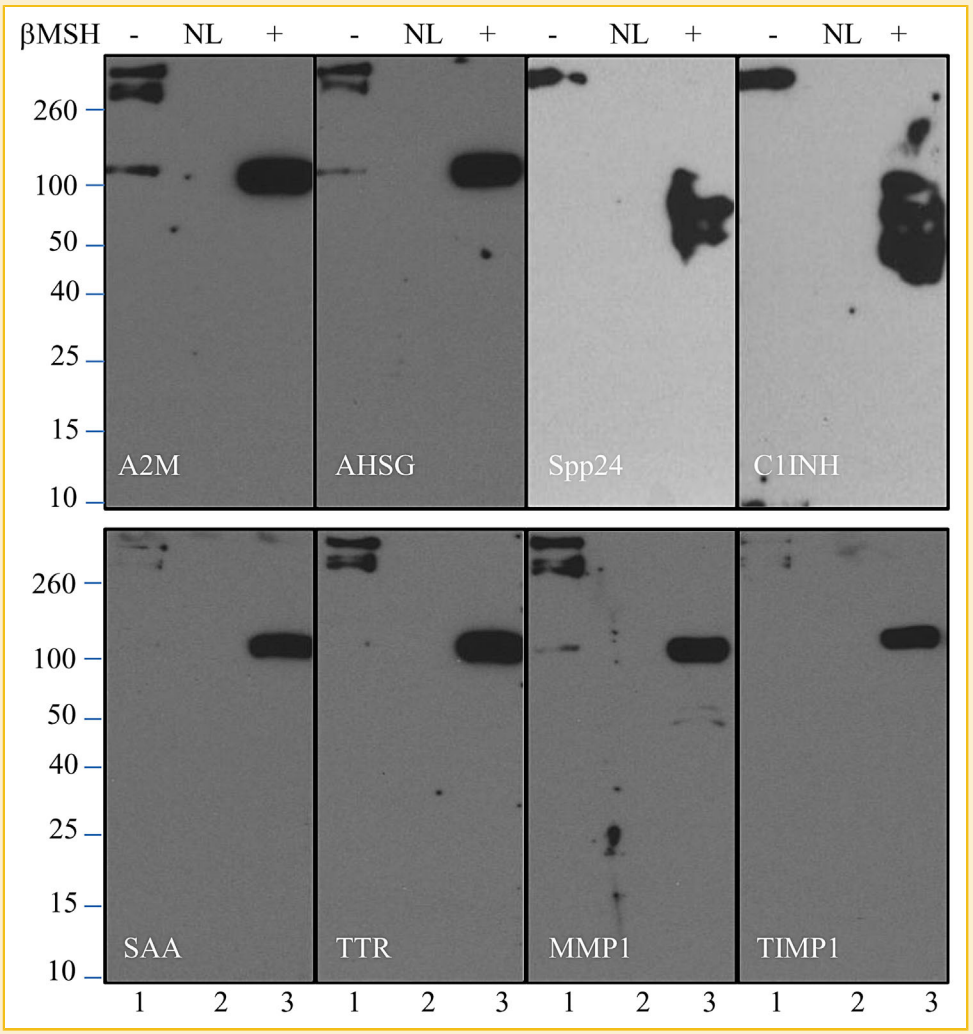

Fig. 2. Antibody reactivity is decreased with βMSH treatment of proteins secreted by Hig-82 synoviocytes. Serum-free conditioned medium was collected after 4–9 h of Hig-82 cell culture. Conditioned medium was used directly and mixed with LB (lane 1) or concentrated 100-fold and mixed with LB/βMSH (lane 3). A lane loaded with LB only was placed between samples to prevent cross-contamination with βMSH (NL, lane 2). All samples were separated on 4–20% SDS-PAGE followed by Western blotting and probed with antibodies conjugated to HRP. A2M, α_2-macroglobulin; AHSG, α_2-HG-glycoprotein also called fetuin A; Spp24, secreted phosphoprotein 24 kDa; C1INH, C1 inhibitor; SAA4, serum amyloid A4; TTR, transthyetin also called prealbumin; MMP1, matrix metalloproteinase-1; TIMP1, matrix metalloproteinase inhibitor-1.

were also clearly present at higher levels than the other genes tested. The effects of treatment of Hig-82 cells with vehicle (control), IL1β, IL6 TNFα, TGFβ1, and BMP2 on the gene expression for Spp24, AHSG, A1AT, IGF1, CRP, HAS1, HAS2, and HAS3 are shown in Figure 5 (panels B–I). TGFβ1 and BMP2 had the greatest effects on seven of the eight proteins but not on HAS1. These results demonstrated that synoviocytes synthesized mRNA for plasma and SF proteins and responded to the trophic cytokines (TGFβ1 and BMP2) to a greater degree than they responded to the inflammatory cytokines (IL1β, IL6, and TNFα).

POSITIVE IDENTIFICATION OF PLASMA PROTEINS IN SERUM-FREE MEDIUM FROM HIG-82 BY LC-MS/MS

To further demonstrate that Hig-82 cells synthesize and secrete plasma proteins, we carried out mass spectrometric analysis of proteins from concentrated serum-free conditioned medium after 16 h of culture. The concentrated proteins were separated by preparative SDS–PAGE, the high molecular weight band corresponding to 360–720 kDa was excised, electroeluted and denatured under reducing condition, and further separated on a reducing SDS–PAGE. Though not all proteins shown in Figures 1–3 were detected due possibly to the masking by abundant proteins, many plasma and matrix proteins, including A2M, TF, LF (lactoferrin), MMP1, MMP2, MMP9, and serpins were found (Table II). In addition to many proteins falling in the size rage of ~50 kDa, smaller sized proteins such as RPF2 (rabbit permeability factor 2-like, 10 kDa), TNFSF10 (tumor necrosis factor ligand superfamily member 10, 19 kDa), IL33 (interleukin-33, 26 kDa), CTRP7 (C1q and tumor necrosis factor related protein 7, 30 kDa), and HTRA1 (high temperature requirement serine protease-1, 51 kDa) appear in both ~200 kDa (R1-R2), and <50 kDa gel slices (R6–R9; Table II). This is in

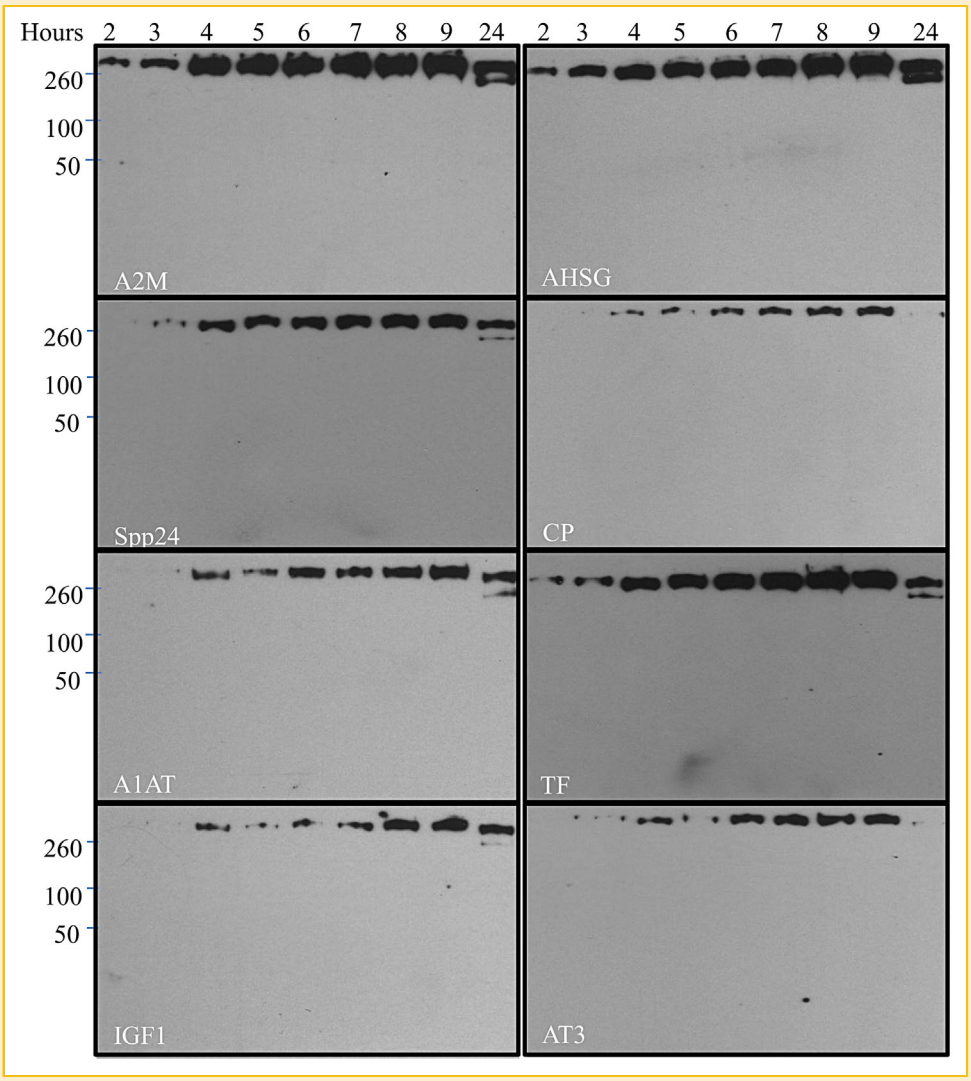

Fig. 3. Time-dependent plasma protein secretions by Hig-82 synoviocytes. Hig-82 rabbit synoviocytes were plated in F12/FCS in 6-well plates and cultured to confluence. The medium was then replaced with F12-ITS for 1 h to purge any exogenous plasma proteins endocytosed by Hig-82 cells. The medium was again exchanged and then collected at the time points indicated above the panels. An aliquot of 20 μl medium was mixed with non-reducing LB and loaded onto 4–20% SDS–PAGE followed by Western blotting. Exposure times: 3 min for A2M, A1AT, and IGF1; 5 m for Spp24, CP, TF, and AT3; and 30 s for AHSG. A2M, α_2-macroglobulin; Spp24, secreted phosphoprotein 24 kDa; A1AT, α_1-antitrypsin; IGF1, insulin-like growth factor-1; AHSG, α_2-HG-glycoprotein also called fetuin A; CP, ceruloplasmin; TF, transferrin; AT3, antithrombin 3.

agreement with Western blotting results under reducing condition as shown in Figure 2.

Spp24~A2M COMPLEX FORMATION OCCURS IN THE ENDOPLASMIC RETICULUM

To examine vesicular trafficking along the secretory pathway, Hig-82 cells were cultured for 2–4 h in the presence of a series of inhibitors of glycosylation and deglycosylation [Elbein, 1987] including TM, dNJ, dMJ, and SW; an inhibitor (BFA) that interferes protein movement from the ER to the CGN or the Golgi apparatus [Lippincott-Schwartz et al., 1989]; as well as three inhibitors of disulfide bond formation including IAM, DTT [Lodish & Kong, 1993], and NEM. The presence of Spp24 and A2M were assayed in cell pellets and conditioned media (Fig. 6).

Spp24 is one of the few non-glycosylated proteins in plasma whereas A2M is known to be a glycoprotein with multiple *N*-linked oligosaccharide side chains. Biosynthesis and secretion of the non-glycosylated Spp24 should be affected by the initial glycosylation step only if A2M, or another glycoprotein, is involved in forming a complex with Spp24 in the ER that is essential for its vesicular transit and secretion. Intracellular Spp24 synthesis and its subsequent secretion were both significantly inhibited by TM (Fig. 6A and B, lanes 2). TM treatment also caused a reduction of cellular dimeric A2M synthesis, but its secretion was actually enhanced (Fig. 6C and D, lanes 2). This indicated that the non-glycosylated Spp24 depends on an *N*-glycosylated partner or chaperone such as A2M for synthesis and secretion, and that such a process takes place in the ER. Post-translational processing of both Spp24 and the partner (A2M)

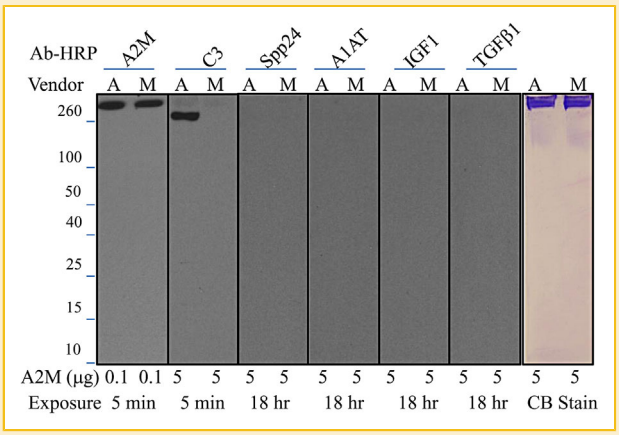

Fig. 4. Reactivity of antibodies with purified human A2M. An aliquot of 0.1 or 5 μg purified human A2M from Athens Research & Technology (A, #16-16-012013) and Meridian Life Science (M, #A500114H) was loaded and separated on 4–20% SDS–PAGE. One set of samples was stained by coomassie blue, and the other sets were transferred to PVDF membrane. After blocking with 5% non-fat milk, the PVDFs were each blotted with anti-human antibodies conjugated to HRP and exposed to X-ray films at various times as indicated. Exposure times on X-ray film are also indicated at the bottom as anestimation of reactivity.

was less affected by inhibitors of trimming glycosidases such as dNJ, dMJ, or SW (Fig. 6A and C, lanes 3–5). However, secretion of Spp24 and A2M was still reduced following treatment with these inhibitors (Fig. 6B and D, lanes 3–5).

In order to clarify when and where such complexes are formed at the subcellular organelle level, BFA was employed. BFA treatment had little or no effect on the cellular synthesis and incorporation of Spp24 into a high molecular complex on Western blot, implying that the complex had been formed prior to entering Golgi apparatus (Fig. 6A, lanes 6). In the medium however, BFA blocked the secretion of Spp24 from the cell (Fig. 6B, lane 6). BFA inhibited the secretion of the Spp24 candidate binding protein, A2M as well (Fig. 6D, lane 6).

A2M commonly forms complexes through a disulfide bonds with its protein ligand(s), which also likely takes place in the ER. Inhibitors of disulfide bond formation that act through derivitization, IAM and NEM, extinguished the persistent presence of Spp24 in the cell pellet (Fig. 6, lanes 7 and 9) whereas the reducing agent, DTT, did not (Fig. 6A, lane 8). All of the treatments prevented the secretion of Spp24 into the media (Fig. 6B). The inhibitors of disulfide bond formation did not prevent the appearance of A2M in either the cell pellet or the media (Fig. 6C and D, lanes 7 and 9) but the reducing agent DTT did dissociate A2M into lower molecular weight components (Fig. 6C and D, lane 8).

Spp24∼A2M COMPLEX FORMATION REQUIRES PROTEASE ACTIVITY

The mechanism of complex formation involving A2M is well established and one key step is the activation of A2M by a protease cleavage of the "bait" region causing a conformational change of the molecule exposing its thioester group [James, 1990; Borth, 1992; Feinman, 1994]. Such activation can also be accomplished by chemical amines such as methylamine in vitro, but is limited to protease(s) possibly residing in the lumen or membrane of the ER in living cells. To examine the role of ER proteases on the formation of the Spp24∼A2M complex, Hig-82 synoviocytes were cultured in the presence of a series of protease inhibitors including AEBSF (serine protease inhibitor), DCI (serine protease inhibitor), ALLN (calpain inhibitor), aprotinin (trypsin inhibitor), leupeptin (cysteine, serine, and threonine protease inhibitor), E64d (calpain inhibitor), MG-132 (proteasome and cysteine inhibitor), and EGTA (metalloprotease inhibitor) and the presence of Spp24 and A2M were assayed in cell pellets and conditioned media (Fig. 7 A and B for Spp24 and C and D for A2M, cell pellets and media, respectively). The amount of the intracellular Spp24∼A2M complex was diminished by treatment with ALLN, leupeptin, E64d, MG-132, and EGTA (Fig. 7A, lanes 4, 6–9) but not by treatment with AEBSF, DCI, and aprotinin (Fig. 7A, lanes 2, 3, and 5). Secretion of the Spp24∼A2M complex was reduced by treatment with AEBSF and MG-132 (Fig. 7B, lanes 2 and 8), while enhanced by treatment with EGTA (Fig. 7B, lane 9). These results suggested that a threonine or cysteine protease is involved in the activation of A2M and that intracellular synthesis is a calcium-dependent process. Persistent intracellular synthesis and secretion of A2M was not affected by any of the inhibitors (Fig. 7C and D), indicating that the bulk of naïve A2M biosynthesis and secretion are not affected, despite decreased complex formation with Spp24 and other ligands.

DISCUSSION

In an animal model of OA, Spp24 was determined to be the protein in SF that was up-regulated the most during the course of OA development [Meng et al., 2005]. In addition, Spp24 is a prominent protein in the SF of human subjects with OA [Balakrishnan et al., 2014b]. In order to establish the role (exacerbating factor, ameliorating response protein, or irrelevant) of Spp24 in the pathological process of OA, we have initiated studies in cultured synoviocytes of the production and secretion of Spp24 in the context of a complex with A2M in a manner similar to its production in the liver, and of the regulation of these processes by cytokines known to be important in bone and cartilage physiology. These studies also provide information pertaining to the origins of synovial fluid proteins in general.

Until very recently, it was assumed that SF was an exudate of plasma. This hypothesis was supported by the anatomy of the joint which showed fenestrated capillary epithelial cells with intracellular openings surrounding the synovium [Chamberlain et al., 1972; Knight and Levick, 1984]. The hypothesis that the proteins of SF were derived from the circulating plasma was also supported to some degree by early physiological studies [Bauer et al., 1933; Rodnan and MacLachlan, 1960], and clinicians tried to identify circulating plasma proteins that are biomarkers for RA disease progressions [Chambers et al., 1983]. It was not until year 2000 that SAA4 was documented to be produced by cultured cells (type B synoviocytes, macrophages, and endothelial cells) from the synovium of RA patients, but not those from healthy controls [O'Hara et al., 2000]. Similarly, a 10 kDa truncated form of thioredoxin (Trx80) was

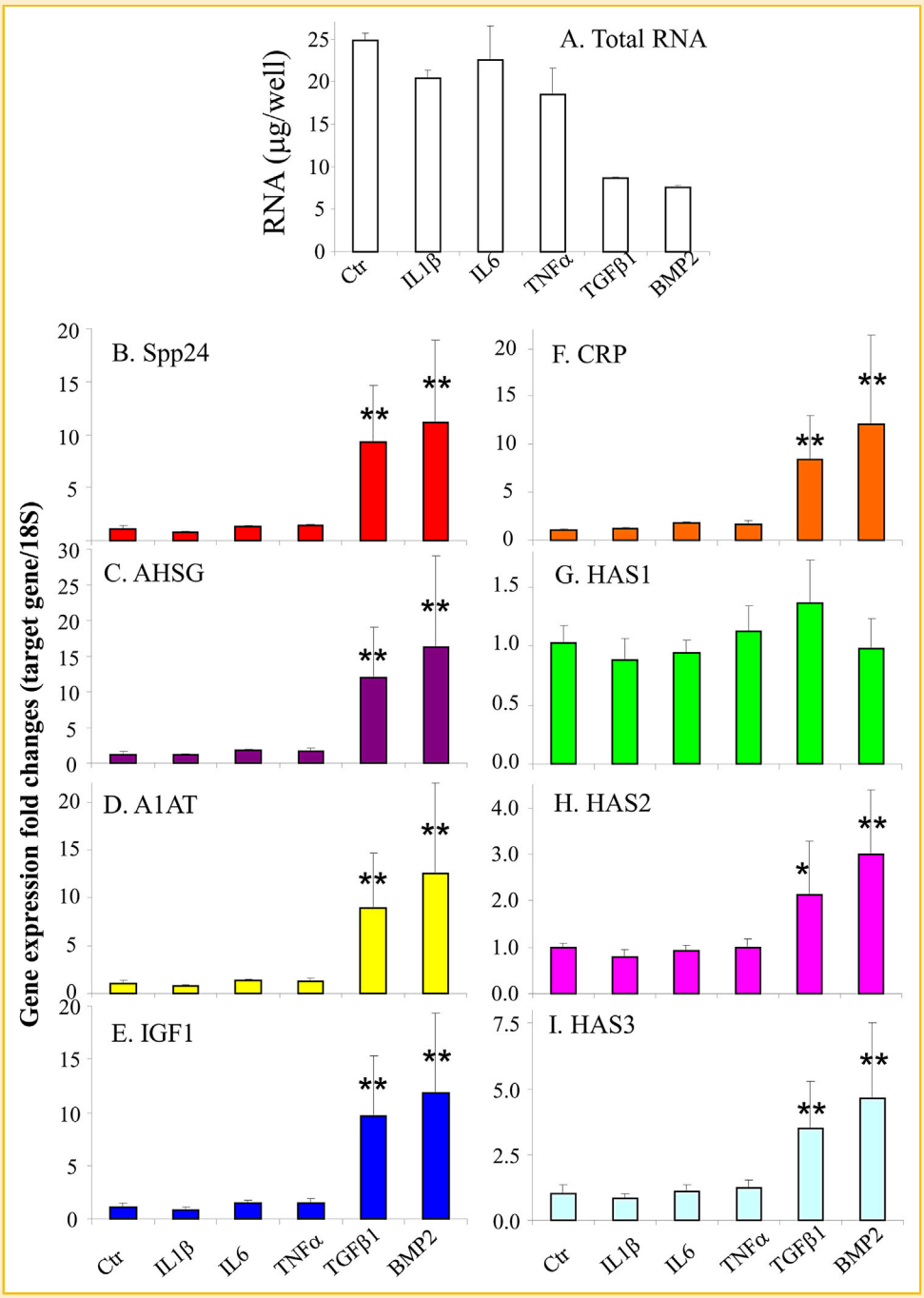

Fig. 5. Changes in levels of expression of selected genes in Hig-82 synoviocytes following treatment with cytokines. Hig-82 synoviocytes were seeded in triplicates at 100,000 cells/well in 6-well plates and cultured for 24 h in F12-FCS. Cytokines were added directly to the media at final concentrations of: 20 pg/ml IL1β, 2 ng/ml IL6, 100 pg/ml TNFα, 10 ng/ml TGFβ1, or 10 ng/ml BMP2. Cells were continued in culture for another 24 h and lysed immediately after removing the conditioned media. RNA extraction, cDNA reverse transcription, and qPCR were performed as detailed in Materials and Methods. Panel A shows total RNA in each treatment group. Panels B–I show gene transcriptsexpressed as cDNA fold changes against their respective ribosomal 18S levels. Mean ± SE, n = 3. In comparison with control, statistically significant differences are indicated by a star ($P < 0.1$) or two stars ($P < 0.05$). Spp24, secreted phosphoprotein 24 kD; AHSG, α_2-HG-glycoprotein also called fetuin A; A1AT, α_1-antitrypsin; IGF1, insulin-like growth factor-1; CRP, C-reactive protein; HAS, hyaluronidase synthase.

detected in cultured synoviocytes from RA patients and levels were slightly increased after treatment with the cytokines IL1β and TNFα [Lemarechal et al., 2007].

Under basal conditions, MH7A synoviocytes from RA patients produce mRNA and enzymatically active MMP1 and MMP13 [Sun and Yokota, 2002]. Cyclic strain reduced the levels of both MMP1 and MMP13 regardless of whether IL1 and TNFα were present or not, but increased the transcription of TIMP1 and TIMP2 [Sun and Yokota, 2002]. In recent years, further evidence of local production of certain SF proteins has emerged. For example, mRNA for HAS2

TABLE II. LC-MS/MS Sequence Analysis of a High Molecular 360–720 kDa Band. Partially Purified 360–720 kDa Bands From Serum-Free Conditioned Medium of Hig-82 Cell Culture Were Analyzed by LC-MS/MS Analysis. Only Secreted and/or Matrix Proteins are Listed Below. Scores are Assigned for Unique Peptides Identified: A = 10–25; B = 6–9; C = 1–5; and "–" = 0. Gel slice R10 was Not Analyzed

		Monomer	Gel slices and molecular weight range								
			R1	R2	R3	R4	R5	R6	R7	R8	R9
Proteins	GenBank #	M_r (kDa)	>200	140–200	100–140	70–100	45–70	38–45	25–38	17–25	12–17
A2M	XP_002712819	185	–	–	C	C	–	C	–	–	–
ACE2	XP_002719891	92	–	–	C	–	–	–	–	–	–
ALB	NP_001075813	67	–	–	C	–	B	C	C	–	C
AFP	XP_002717072	70	–	–	–	–	C	–	–	–	–
Biglycan (PGS1)	NP_001182620	63	A	B	A	A	B	A	A	A	A
BMP1	XP_008248113	111	–	–	–	C	C	–	–	–	C
BRINP3	XP_002717742	88	–	–	C	–	–	–	–	–	–
C1S	XP_002712892	77	–	–	–	–	–	–	–	C	–
C8A	NP_001075724	65	–	–	–	–	–	–	C	–	–
COL1A2	NP_001182597	90	–	–	–	–	–	–	–	C	C
COL3A1	XP_002712379	140	C	–	C	–	–	–	–	–	–
COL6A1	XP_008250068	109	C	C	A	B	C	B	C	C	C
COL6A2	XP_008265474	109	–	–	A	C	–	C	–	–	–
COL6A3	XP_008249075	300	B	–	A	B	C	C	–	C	C
COL7A1	XP_002713393	290	–	–	C	–	–	–	–	–	–
COL11A1	XP_002715509	181	–	–	–	–	–	–	C	C	C
COL12A1	XP_002714579	330	A	A	A	A	A	A	A	A	A
COL14A1	XP_002710799	194	C	–	C	C	C	C	C	–	C
COL15A1	XP_002708200	142	B	C	C	B	C	–	C	C	C
COL21A1	XP_008261289	99	C	–	C	–	C	C	–	–	–
CSF1	XP_008262947	60	–	–	–	–	–	–	–	–	C
CTRP7	XP_002709566	30	C	–	C	C	–	–	–	–	–
DCN	NP_001075799	50	–	C	C	C	–	C	C	–	–
FMOD	XP_002717502	67	–	–	–	C	–	–	–	–	–
HSPG2	XP_002716087	470	A	A	A	A	A	A	A	A	A
HTRA1	XP_008268840	51	–	C	–	–	–	C	C	C	C
IFNG	NP_001075460	60	–	–	–	–	–	–	–	C	–
IGSF8	XP_002715294	70	–	–	–	–	–	–	–	–	C
IIGBP1-like	XP_002715919	65	–	–	–	–	–	–	–	C	C
IL33	XP_008253211	26	C	–	–	–	–	–	–	–	C
LAMC1	XP_002724335	177	–	–	–	C	–	C	–	–	–
LTF	XP_002713370	80	–	–	–	–	–	C	C	–	–
MMP1	NP_001164610	50	–	–	–	–	–	C	–	–	–
MMP2	NP_001075678	72	–	–	–	–	C	–	–	–	–
MMP9	NP_001075672	92	–	–	–	B	B	B	C	C	B
Nephrocan-like	XP_002714821	50	–	–	C	C	C	C	–	–	–
PAMR1	XP_002709265	80	C	–	–	–	–	C	–	–	–
PICA-like	XP_002716595	80	–	–	–	–	C	C	C	–	C
RPF2	XP_002717158	10	–	C	–	–	–	–	–	–	C
SERPINE2	XP_002712550	44	–	–	–	–	–	–	C	C	–
SERPINB7	NP_001164757	50	–	C	–	–	–	C	C	–	–
SERPINA12	XP_002723166	47	–	C	–	–	–	–	–	C	–
SPINK5	XP_008253430	130	–	–	–	–	–	C	–	–	–
SVEP1	XP_002708118	370	B	B	A	A	A	A	A	B	A
TF	NP_001095164	80	–	–	–	–	C	C	C	–	C
TNFSF10	XP_002716472	19	–	C	–	–	–	–	–	–	–

and HAS3, two important enzymes for the production of HA, as well as lubricin was detected in the normal synoviocyte cell line Hig-82 when the cells were subjected to shear stress [Oguchi et al., 2001; Yanagida-Suekawa et al., 2013]. Secretion of lubricin was detected in the medium of Hig-82 cells after culturing 48 h with shear stress [Yanagida-Suekawa et al., 2013]. Most recently, techniques of high throughput proteomics have been applied to the analysis of SF. About 135 proteins were identified in SF from patients with early OA, late OA, or controls and 18 proteins were differentially expressed in OA versus control cells [Gobezie et al., 2007]. Among these, A2M was upregulated in OA. Sohn et al. [2012] identified 108 proteins in the SF of OA patients. Of these, 36% were plasma proteins, and it was concluded that plasma proteins were present in SF but their source could not be conclusively identified [Sohn et al., 2012]. Proteomics techniques have also been applied to the study of cultured synovial fibroblasts from patients with OA, RA, and controls, and 25 proteins have been identified that are differentially expressed [Bo et al., 2009]. However, there are little direct or quantitative data documenting the exact source of SF proteins (systemic vs. local) and the relative contribution of each. Current belief still holds that the higher concentrations of "lower" molecular weight proteins such as ALB and TF and the lower concentrations of "higher" molecular weight proteins such as FN in SF are the result of a size-limiting passive diffusion mechanism of proteins from the circulation [Levick, 1981; Hui et al., 2012]. Our studies have demonstrated that a number of "plasma", SF, and matrix proteins are synthesized at high levels by synoviocytes.

A2M was described as a "protease inhibitor" for decades [Bowman, 1993]. It is present in high molecular weight complexes with other proteins in body fluids such as plasma, seminal fluid, and

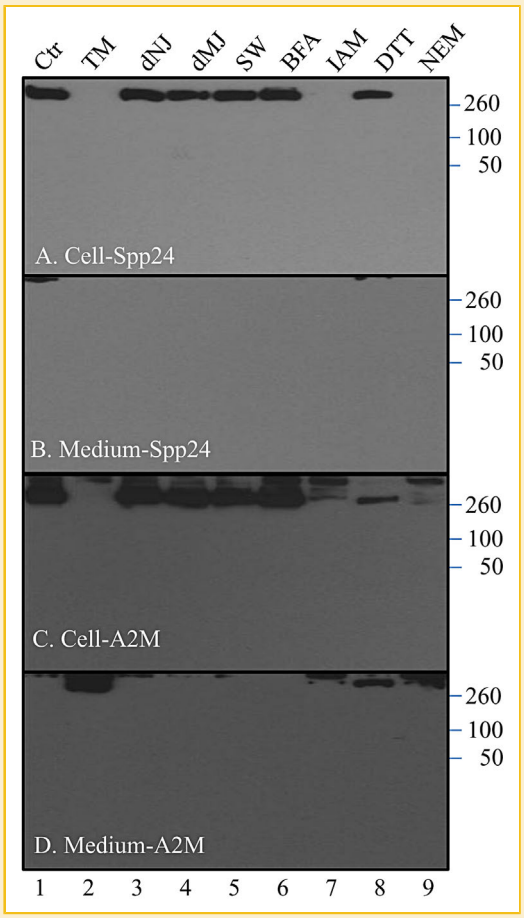

Fig. 6. Inhibition of intracellular and secreted Spp24 and A2M by inhibitors of glycosylation/deglycosylation, Golgi trafficking, and disulfide bond formation. Hig-82 cells were cultured in the absence (lane 1, Ctr) or presence (lanes 2–9) of inhibitors for glycosylation/deglycosylation, Golgi vesicular trafficking, or disulfide formation. Cell pellets (panels A and C) and conditioned media (panels B and D) were collected after 2 h. Proteins were separated in non-reducing 4–20% SDS–PAGE as detailed in the method. HRP-conjugated ntibodies against Spp24 (panels A and B) and A2M (panels C and D) were used as probes. Inhibitor lanes are labeled as tunicamycin (TM, lane 2), deoxynojirimycin (dNJ, lane 3), deoxymannojirimycin (dMJ, lane 4), swaisonine (SW, lane 5), brefeldin A (BFA, lane 6), iodoacetamide (IAM, lane 7), dithiothreitol (DTT, lane 8), and N-ethylmaleimide (NEM, lane 9). Molecular weight markers are indicated as kDa on the right.

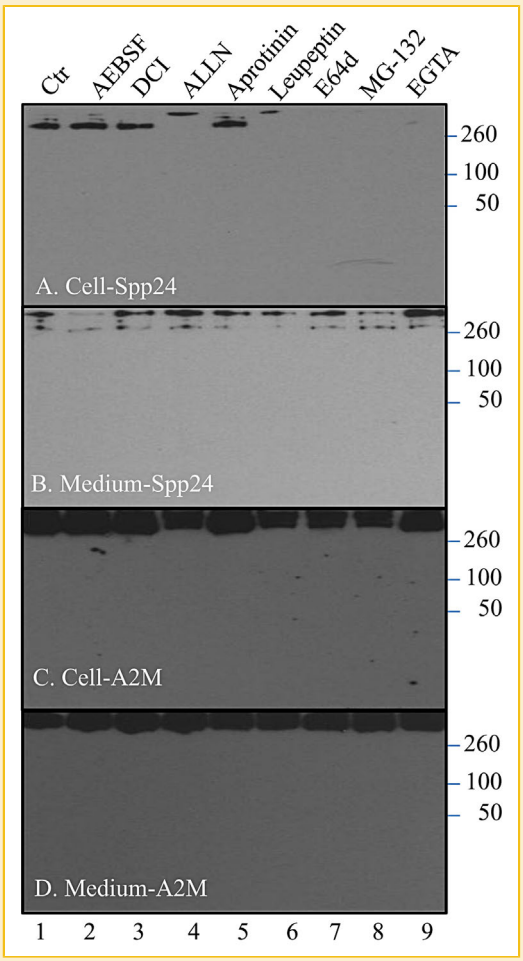

Fig. 7. Inhibition of intracellular and secreted Spp24 and A2M by inhibitors of proteases. Hig-82 cells were cultured in the absence (Ctr, lane 1), or presence (lanes 2–9) of inhibitors for proteases. Cell pellets (panels A and C) and conditioned media (panels B and D) were collected after 2 h. Proteins were separated in non-reducing 4–20% SDS–PAGE as detailed in the method. HRP-conjugated ntibodies against Spp24 (panels A and B) and A2M (panels C and D) were used as probes. Inhibitors used were: AEBSF (lane 2), DCI (lane 3), ALLN (lane 4), aprotinin, (lane 5), leupeptin (lane 6), E64d (lane 7), MG-132 (lane 8), and EGTA (lane 9). Molecular weight markers are indicated as kDa on the right.

SF. More recently, A2M has been identified as an extracellular chaperone for a number of extracellular proteins [Misra et al., 2011; Wyatt et al., 2013]. A2M is activated and undergoes conformational changes after proteolytic cleavage of its "bait" region exposing a reactive cysteinyl and glutamyl residues (thioester) that form covalent bonds with cysteine and lysine residues of protein ligands and/or the attacking protease, respectively [Borth, 1992]. Potential ligands include cytokines (IL1β, IL6, TNFα, TGFβ1, platelet derived growth factor), hormones (growth hormone, insulin), growth factors (IGF1), and enzymes (carboxypeptidase B) [James, 1990; Chu et al., 1991; Valnickova et al., 1996].

Though the process of activation can also be accomplished through treatment with methylamine in vitro [Borth, 1992], activation by a small amine in the ER is very unlikely. We hypothesized that a protease(s) residing in the ER lumen is required for the activation of the small fraction of the A2M population that forms a complex with Spp24, and that activated A2M captures nascent Spp24 via disulfide bond formation. In addition, A2M often captures an attacking protease at its reactive thioester, and an in vitro experiment demonstrated that such a capture may not happen if the attacking protease is immobilized [Björk, 1984]. This observation is consistent with our finding that a cysteine or threonine protease serves as the A2M activator.

By treating Hig-82 cells with various inhibitors of cysteine and serine/threonine proteases, we observed that intracellular formation of Spp24∼A2M dimeric complexes was not affected by serine protease inhibitors such as AEBSF, DCI, and aprotinin, but was significantly

reduced by calpain inhibitors, including ALLN and E64d, the cysteine and serine/threonine peptidase inhibitor leupeptin, the proteasome and calpain inhibitor MG-132, and the Ca^{2+}-dependent metalloprotease inhibitor EGTA. On the other hand, Spp24∼A2M secretion was reduced by AEBSF and MG-132 and enhanced by EGTA treatment. These results suggest that a cysteine or threonine protease(s), which may be part of an ER-associated degradation system [Olzmann et al., 2013] is the candidate enzyme for the catalysis of A2M activation and complex formation. Calcium appears to be essential for the process as well, although it may play a role in ER processing and secretion, rather than acting as an essential cofactor for a protease. Both cellular and secreted levels of total A2M were not substantially affected by any of the protease inhibitors. Taken together, all of these results demonstrate that the secretion of Spp24 by synoviocytes involves formation of a high molecular weight complex with A2M as described in other systems and also strongly suggest that the proteins found in SF are produced primarily by synoviocytes.

We had also evaluated the use of siRNA strategy to suppress A2M translation in synoviocyte culture at the beginning of our project, but did not pursue it mainly for these biological reasons: (1) A2M is one of three proteins (the other two are C3 and PZP) in the family of A2Ms. Suppression of A2M mRNA with siRNA in vitro by 50% may not have any significant effect on its chaperone function, and C3 or PZP may serve as "surrogate" chaperones for Spp24 if A2M production is further reduced. Our decision for not using the siRNA approach was also based on the observation that no clinical manifestation has been documented in A2M-deficient humans [Bergqvist & Nilsson, 1979], implying C3/PZP replacement of A2M functions; (2) The mouse A2M knockout is viable and produced normal sized litters with normal sex ratio, despite an absent of liver mRNA for A2M [Umans et al., 1995]. There was a four fold increase in maternal liver murinoglobulin mRNA in knockout versus control mice at partum, suggesting that A2M can be replaced by other members of its protein family.

Our proposed model for the biosynthesis and secretion of "plasma" proteins by synoviocytes is illustrated in Figure 8. In summary, we have demonstrated that: (1) A number of proteins that are found in the general circulation in a high molecular high complex are also secreted by synoviocytes in a high molecular complex; (2) The formation of the Spp24∼A2M complex most likely occurs in the ER; (3) The formation of the A2M∼Spp24 requires activation of the A2M "bait" region by proteolysis, most likely by a cysteine or threonine protease; (4) TGFβ and BMP2 stimulate synoviocytes to synthesize and secrete a number of proteins including HAS 2, HAS3, and Spp24. It was estimated that >90% A2M forms a "complex" with A1AT in human circulation [Dejgaard et al., 1999]. However, there are still unanswered questions relating to this model. For example, the combined normal concentration of A2Ms in adult plasma (1.5 to 4.2 mg/ml for A2M, 0.9–1.9 mg/ml for C3, and trace amount for PZP which may increase by 500–1,000 times to 0.5–1.0 mg/ml in the third trimester of pregnancy [Putnam, 1975]) may not be enough to capture all of the available ligands, since the A1AT molecule alone (2.0–4.0 mg/ml) may saturate A2Ms considering their sizes and molar ratio (tetramer vs. monomer).

Based on our findings, particularly the sequencing of various plasma and SF proteins in Hig-82 secretion, we hypothesize that

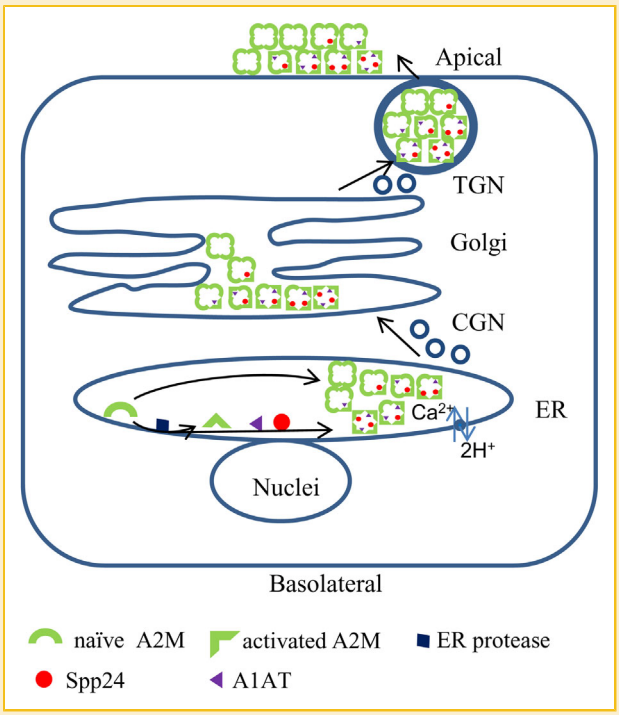

Fig. 8. Proposed model of plasma protein synthesis with A2Ms as chaperones by synoviocytes. Using Spp24 as an example, nascent peptide is synthesized in the lumen of ER, co-synthesis of A2M as an intracellular chaperone. A portion of the nascent A2M polypeptide chain ("bait region") is attacked by an ER resident protease causing a conformational change and exposure of its thioester, which then rapidly reacts with sulfhydryl groups from other newly synthesized plasma proteins such as Spp24 and A1AT. Upto four A2M polypeptide chains in the naïve form or carrying different combinations of other plasma protein ligands can form dimers and/or tetramers. They undergo post-translational processing and modifications through the vesicular pathway in the cis Golgi network (CGN), the Golgi apparatus, and trans Golgi network (TGN). TGN vesicles fuse with the plasma membrane on the apical side of the cell and secrete the A2M complexes into SF. The naïve polypeptide(s) in the complex can continue to serve as extracellular chaperones and react with protein ligands in the SF secreted by cartilage cells and/or macrophages. A2Ms, α_2-macroglobulin; C3, complement, or pregnancy zone protein; Spp24, secreted phosphoprotein 24 kDa; A1AT, α_1-antitrypsin; ER, endoplasmic reticulum; CGN, cis Golgi network; TGN, trans Golgi network.

Spp24 and other plasma proteins are produced locally in normal joints and change as a result of the initiating event in OA and other arthritic diseases. Through its binding properties, Spp24 modulates the relative availability of TGFβ and BMPs, both of which affect osteoblastic and chondrocytic cells. Spp24 may be proteolytically converted to Spp18, or other size forms, and that directly affect a number of cell types through its cytokine agonist properties and/or signal transduction [Zhao et al., 2015]. We cannot ascertain at this time whether increased concentrations of Spp24 in SF are ameliorating or detrimental in terms of disease progression, but other investigators have demonstrated that A2M levels increase in post-traumatic joint fluid and that adding partially purified A2M from normal individuals (which may contain mostly naïve A2M and activated A2M carrying ligands such as Spp24) attenuates the progression of post-traumatic OA [Wang et al., 2014].

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