ADAM, ADAMTS AND ASTACIN PROTEASES: CHALLENGES AND BREAKTHROUGHS IN THE -OMICS ERA

EDITED BY: Salvatore Santamaria, Hang Fai Kwok, Kazuhiro Yamamoto, Simone Dario Scilabra and Rens de Groot

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ADAM, ADAMTS AND ASTACIN PROTEASES: CHALLENGES AND BREAKTHROUGHS IN THE -OMICS ERA

Topic Editors:
Salvatore Santamaria, Imperial College London, United Kingdom
Hang Fai Kwok, University of Macau, China
Kazuhiro Yamamoto, University of Liverpool, United Kingdom
Simone Dario Scilabra, Helmholtz Association of German Research Centers (HZ), Germany
Rens de Groot, University College London, United Kingdom

Professor Hang Fai Kwok has two filed patents on ADAM17 antibody applications in cancer and cardiovascular disease. All other Topic Editors declare no competing interests with regards to the Research Topic.

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EDITORIAL
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Editorial: ADAM, ADAMTS and Astacin Proteases: Challenges and Breakthroughs in the -Omics Era

Kazuhiro Yamamoto[1], *Rens de Groot*[2], *Simone Dario Scilabra*[3], *Hang Fai Kwok*[4,5] *and Salvatore Santamaria*[6]*

[1]Institute of Life Course and Medical Sciences, University of Liverpool, Liverpool, United Kingdom, [2]Institute of Cardiovascular Science, University College London, London, United Kingdom, [3]Proteomics Group of Fondazione Ri.MED, Department of Research IRCCS ISMETT, Palermo, Italy, [4]Cancer Centre, Faculty of Health Sciences, University of Macau, Macau, China, [5]MOE Frontiers Science Center for Precision Oncology, University of Macau, Macau, China, [6]Department of Immunology and Inflammation, Imperial College London, London, United Kingdom

Keywords: ADAM, ADAMTS, proteomics, extracellar matrix, astacin

Editorial on the Research Topic

ADAM, ADAMTS and Astacin Proteases: Challenges and Breakthroughs in the -Omics Era

Metzincins are a superfamily of metalloproteinases characterized by the presence of a conserved methionine residue downstream the active site zinc (Cerdà-Costa and Gomis-Rüth, 2014). This Frontiers Research Topic is focused on three metzincin sub-families: ADAMs (A Disintegrin and Metalloproteinases), ADAMTS (A Disintegrin and Metalloproteinase with Thrombospondin motifs) and Astacins. Research into these proteases has seen impressive breakthroughs thanks to the progress of the -Omics era. Genome Wide Association Studies (GWAS) have implicated several ADAM and ADAMTS family members in human disease, for example ADAMTS7 in Coronary Artery Disease (Reilly et al., 2011), and ADAM10 in Alzheimer's disease (Marioni et al., 2018; Schwartzentruber et al., 2021). Although less characterized, the proteolytic activity of Astacins has been linked to digestion, cytokine activation and cancer (Sterchi et al., 2008; Peters and Becker-Pauly, 2019).

ADAMTSs are a family of 19 secreted proteases playing a crucial role in the turnover of the extracellular matrix (ECM). While a fine regulation of their activity ensures tissue homeostasis, several pathological conditions are associated with their aberrant activity. Rose et al. comprehensively review the current knowledge about regulatory mechanisms of ADAMTS activity. Protein levels and activity of ADAMTSs can be modulated at different levels, including transcriptional regulation, alternative splicing, proteolytic activation, endocytosis, and inhibition by tissue inhibitors of metalloproteinases (TIMPs). A deeper understanding of such regulatory mechanisms can lead to new strategies to target the pathological potential of specific ADAMTSs without affecting the physiological activity of other family members. The contribution by Jiang et al. provides updated insights on multiple layers of regulation of one of the best characterized ADAMTS family members, ADAMTS5. ADAMTS5 is an important pharmaceutical target in osteoarthritis (OA) due to its ability to cleave aggrecan, the major proteoglycan in articular cartilage (Santamaria, 2020). Since no disease-modifying OA therapies are currently available, the authors summarize current preclinical approaches to target ADAMTS5. Monoclonal antibodies and small molecule inhibitors against ADAMTS5 were proved to be beneficial pre-clinically. Based on the recent novel RNA therapies that demonstrated potential in OA animal models, the authors emphasize that upstream signaling blockade of ADAMTS5 using mi/siRNAs may represent a feasible therapeutic strategy for OA.

ADAMTS12 is another example of how we are just starting to scratch the surface of ADAMTS complexity. Mohamedi et al. overview this complex and multifunctional metalloproteinase on the

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University of Parma, Italy

***Correspondence:**
Salvatore Santamaria
s.santamaria@imperial.ac.uk

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20th anniversary of its identification. The authors discuss functions of ADAMTS12 in inflammatory processes, arthritis, degenerative intervertebral disc, chondrogenesis, tendon degeneration, cancer, neurological and fertility disorders. Different domains of ADAMTS12 can interact with different ECM components, which in turn modify its proteolytic activity. The authors emphasize that identification of ADAMTS12-binding partners would contribute to a deeper understanding of its biological functions.

A central aspect of this Research Topic is the identification of novel metzincin substrates using state-of-the-art proteomics approach. In the last decade, mass spectrometry-based techniques have dramatically expanded our knowledge of proteases' substrate repertoire, the degradome (López-Otín and Overall, 2002). The most successful techniques have been those targeting the newly formed N-terminus generated following cleavage of the substrate, such as N-Terminal Amine Isotopic Labeling of Substrates (TAILS) (Mintoo et al., 2021). N-TAILS has been instrumental to elucidate the degradome of ADAMTS7 (Colige et al., 2019), 2, 3, and 14 (Bekhouche et al., 2016). While these approaches used fibroblast conditioned media as a source of substrates, Leduc et al. investigated substrates *in vivo* by directly comparing skin from *Adamt2*$^{-/-}$, *Adamts14*$^{-/-}$ and *Adamts2*$^{-/-}$/*Adamts14*$^{-/-}$ knockout mice with those of wild type littermates. Their results confirm the cleavage site preference (P1 and P1′ residues) of these proteases and provide more detail about their role in collagen fibril organization. As often in proteomics, further studies are required to assess the relevance of these intriguing proteolytic events and novel substrates.

The current pandemic has stressed the need for better intervention strategies aiming at the prevention and treatment of infections caused by respiratory viruses. As highlighted by Pedrina and Stambas, the ECM offers novel opportunities for intervention. The proteoglycan versican is a major ECM component and has been shown to be involved in the immune response to viral infection by binding chemokines that guide leukocyte extravasation. Versican also provides a barrier for leukocyte migration, as summarized in the contribution by McMahon et al. ADAMTS4 and ADAMTS5 are the two major versicanases (Santamaria et al., 2019), so it does not come as a surprise that they have been implicated in the pathological ECM remodeling that occurs upon viral infection. However, their role seems to be radically different. ADAMTS5 versicanase activity seems to exert a beneficial role by facilitating leucocyte migration as demonstrated by the delayed virus clearance observed in *Adamts5* knockout mice (McMahon et al., 2016). On the other hand, ADAMTS4 expression positively correlates with severe influenza virus infection in humans (Boyd et al., 2020), suggesting that ADAMTS4 can be a potential target for therapeutic intervention. Several inhibitory antibodies have been described (Santamaria and de Groot, 2019) and these can be deployed to target ADAMTS4 and other metzincins involved in the dysregulated immune response to viral infection. On the other hand, increasing the activity of ADAMTS5 in the context of severe influenza infection may not be trivial. Although increased ADAMTS5 activity has been achieved indirectly by blocking its endocytosis with a monoclonal antibody (Santamaria et al., 2017), a caveat when considering ADAMTSs as therapeutic targets is their complex, multisystemic role, for example their involvement in cardiovascular homeostasis (Santamaria and de Groot, 2020).

Like ADAMs, Astacins can cleave membrane-tethered proteins and therefore act as sheddases (Sterchi et al., 2008; Peters and Becker-Pauly, 2019). Meprin β is perhaps the best characterized Astacin. Meprin β is a type I transmembrane protein whose expression is associated with certain types of cancer, including melanoma. Thus, it is crucial to find the driver mutation(s) of meprin β and its variants to study the impact on the invasiveness of tumor cells. Two genetic variants of meprin β (G45R and G89R) have been found in melanomas. Gellrich et al. characterized these two variants and found that, similar to wild type meprin β, the G45R variant is able to shed a number of specific substrates and promote invasion when expressed in HeLa cancer cells. On the other hand, the G89R variant showed impaired trafficking to the cell surface, reduced shedding of its target substrates and diminished cancer cell invasion. A similar mechanistic approach can be extended to elucidate the activity of other meprin β variants (Peters and Becker-Pauly, 2019).

Overall, the studies included in this Research Topic expand our knowledge of the role and regulation of ADAMs, ADAMTSs and Astacins in a variety of biological processes, something that would not have been possible without the powerful new -Omics tools that have been widely adopted in the last decade.

AUTHOR CONTRIBUTIONS

KY, RdG, SDS, HK and SS planned, wrote, and revised the editorial manuscript. All authors contributed to the article and approved the submitted version.

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In vivo N-Terminomics Highlights Novel Functions of ADAMTS2 and ADAMTS14 in Skin Collagen Matrix Building

Cédric Leduc[1], *Laura Dupont*[1], *Loïc Joannes*[1], *Christine Monseur*[1], *Dominique Baiwir*[2], *Gabriel Mazzucchelli*[2], *Christophe Deroanne*[1], *Alain Colige*[1†] *and Mourad Bekhouche*[1,3,4*†]

[1]*Laboratory of Connective Tissues Biology, GIGA-Cancer, University of Liège, Liège, Belgium,* [2]*GIGA Proteomic Facility, GIGA–Interdisciplinary Cluster for Applied Genoproteomics, University of Liège, Liège, Belgium,* [3]*Tissue Biology and Therapeutic Engineering, Centre National de la Recherche Scientifique/University of Lyon Unité Mixte de Recherche 5305, Lyon, France,* [4]*Faculté d'Odontologie de Lyon, Université de Lyon, Université Lyon 1, Lyon, France*

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Edited by:
Kazuhiro Yamamoto, University of Liverpool, United Kingdom

Reviewed by:
Fransiska Malfait, Ghent University, Belgium
Paripok Phitsuwan, King Mongkut's University of Technology Thonburi, Thailand

***Correspondence:**
Mourad Bekhouche
mourad.bekhouche@univ-lyon1.fr

[†]*These authors share last authorship*

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A disintegrin and metalloproteinase with thrombospondin type I motif (ADAMTS)2 and ADAMTS14 were originally known for their ability to cleave the aminopropeptides of fibrillar collagens. Previous work using N-terminomic approach (N-TAILS) *in vitro* led to the identification of new substrates, including some molecules involved in TGF-β signaling. Here, N-TAILS was used to investigate the substrates of these two enzymes *in vivo*, by comparing the N-terminomes of the skin of wild type mice, mice deficient in ADAMTS2, in ADAMTS14 and in both ADAMTS2 and ADAMTS14. This study identified 68 potential extracellular and cell surface proteins, with the majority of them being cleaved by both enzymes. These analyses comfort their role in collagen matrix organization and suggest their implication in inflammatory processes. Regarding fibrillar collagen, this study demonstrates that both ADAMTS2 and ADAMTS14 are involved in the processing of the aminopropeptide of alpha1 and alpha2 type V collagen. It also revealed the existence of several cleavage sites in the Col1 domain and in the C-propeptide of type I collagens. In addition to collagens and other extracellular proteins, two major components of the cell cytoskeleton, actin and vimentin, were also identified as potential substrates. The latter data were confirmed *in vitro* using purified enzymes and could potentially indicate other functions for ADAMTS2 and 14. This original investigation of mouse skin degradomes by N-terminomic highlights the essential role of ADAMTS2 and ADAMTS14 in collagen matrix synthesis and turnover, and gives clues to better understand their functions in skin pathophysiology. Data are available via ProteomeXchange with identifier PXD022179.

Keywords: ADAMTS, collagen, Ehlers-Danlos Syndrome (EDS), degradomics, TAILS, N-Terminomics

INTRODUCTION

Ehlers-Danlos syndrome (EDS) encompasses a group of inherited diseases caused by mutations affecting genes involved in the homeostasis of connective tissues (Beighton et al., 1998). A significant number of these genes are directly related to the biology of fibrillar collagens. Mutations in type V and type III procollagens are the main causes of, respectively, the "classical" and the "vascular" types of EDS. Missense mutations in type I procollagens can also lead to rare forms of classical and vascular

EDS, while complete or partial skipping of exon six in alpha1 type I (COL1A1) or alpha2 type I (COL1A2) is responsible for arthrochalasia EDS, a condition mainly characterized by congenital bilateral hip dislocation, severe generalized joint hypermobility with multiple dislocations/subluxations and skin hyperextensibility (De Paepe and Malfait, 2012; Malfait and De Paepe, 2014). Electron microscopy of skin specimens shows loosely and randomly organized collagen fibrils with a smaller and variable diameter, and an irregular outline (Colige et al., 2004). The molecular basis behind this clinical picture is the absence of aminopropeptide cleavage of the mutated chain (either alpha1 or alpha2) since exon six encodes the cleavage site cleaved by aminoprocollagen peptidases (ADAMTS2, 3 and 14) (Colige et al., 1999). Null mutations in ADAMTS2, the main aminoprocollagen peptidase, lead also to EDS, but, surprisingly, not to the arthrochalasia EDS as reported for its mutated substrates. Instead, absence of ADAMTS2 activity causes dermatosparaxis EDS presenting with extreme skin fragility, characteristic craniofacial features, redundant skin with excessive skin folds at the wrists and ankles, umbilical hernia and severe bruising with a risk of subcutaneous hematomas and hemorrhages (Colige et al., 1999). In these patients and in animal models of deficiency in Adamts2 ($TS2^{-/-}$) electron microscopy shows collagen fibrils with a highly typical hieroglyphic or ribbon-like pattern. These differences in the morphology of collagen fib rils in arthrochalasia and dermatosparaxis, while persistence of N-propeptides is observed in both diseases, suggested that ADAMTS2 has possibly substrates other than type I procollagens.

Because of their close similarities with ADAMTS2, the consequences of mutations affecting ADAMTS3 and ADAMTS14 have been evaluated in mouse models (Janssen et al., 2016; Dupont et al., 2018). ADAMTS3 has been shown to be required for the formation of lymphatic network by its capacity to cleave pro-VEGF-C into fully active VEGF-C able to bind its receptors (Janssen et al., 2016). Regarding ADAMTS14, KO-mice do not display obvious specific phenotype despite the fact that biallelic null mutations have been predicted to be pathogenic using dedicated browser such as "GnomAD". In the same line, mice deficient for both Adamts2 and Adamts14 ($TS2^{-/-}TS14^{-/-}$) display an aggravated phenotype as compared to $TS2^{-/-}$ mice, again indicating further roles and substrates for ADAMTS14 (Dupont et al., 2018). In order to have first indications about the diversity of the substrates of ADAMTS2, 3 and 14, a proteomic analysis dedicated to the identification of protease substrates (N-TAILS) was performed in cell culture models (Bekhouche et al., 2016). This study showed that the repertoire of substrates of these "so-called" aminoprocollagen peptidases extends beyond fibrillary procollagens. Although most useful because of their relatively low complexity, these *in vitro* models do not recapitulate the *in vivo* situation. As an example, fibrillary procollagens are secreted in the culture medium as uncleaved precursors, whereas in connective tissues *in vivo* they stay concentrated close to the producing cells and are fully matured.

In order to clarify why arthrochalasia and dermatosparaxis EDS display different clinical phenotypes (Van Damme et al., 2016) and to investigate the diverse functions of ADAMTS2 and ADAMTS14 *in vivo* (Dupont et al., 2018), N-Tails analysis has been performed on skin samples from wild-type mice (Wt) and $TS2^{-/-}$, $TS14^{-/-}$ and $TS2^{-/-}TS14^{-/-}$.

MATERIALS AND METHODS

Reagents

The hyperbranched polyglycerol-aldehyde polymer was purchased from Flintbox (University of British Columbia, Vancouver, BC, Canada). Isobaric tag for relative and absolute quantitation (iTRAQ) labels are from AB Sciex (Concord, ON, Canada). Porcine trypsin was purchased from Promega (#V511A; Madison, WI, United States). Ultrafiltration devices were purchased from Merck Millipore. All other reagents were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France).

Cell Lines

Fibroblasts were obtained from the dermis of healthy donors (normal fibroblasts, NF) and of a patient suffering from the dermatosparactic type of the Ehlers-Danlos syndrome (dermatosparactic fibroblasts, DF) (Nusgens et al., 1992). Cells were cultured in DMEM (Lonza) supplemented with 10% fetal bovine serum (FBS, Lonza). For collagen production, cells were cultured in DMEM supplemented with 1% FBS and 50 µg/ml 2-phospho-L-ascorbic acid (Sigma Cat#49752). The conditioned medium was collected after 48h, centrifuged for 15 min at 4,000 rpm at 4°C. The supernatant was conserved at −80°C before collagen purification.

Skin Proteome Preparation

The experiment was conducted in triplicate using twelve 8-week-old adult mice (2 males and one female of each genotype (Wt, $TS2^{-/-}$, $TS14^{-/-}$ and $TS2^{-/-}/TS14^{-/-}$)). The investigation on mice was reviewed and approved by Ethics Committee for Animal Use and Care of the University of Liege (Belgium) (protocol N°1,109). A square (1 cm^2) of shaved skin was collected from the anteroventral part of the mice and incubated 1 h at 4°C under shaking in 3 ml of 50 mM HEPES sodium salt (pH 7.5), 2 mM $CaCl_2$ and 150 mM NaCl containing a cocktail of inhibitors of proteases and phosphatases (MS-SAFE, Sigma). Skin pieces were harvested and crushed with ceramic beads (MagNA Lyser Green Beads, Roche, n°03358,941,001) using the MagNA Lyser Instrument (Roche) in 90% (V/V) tissue protein extraction reagent (Thermo Scientific #78510), 10% (V/V) HEPES sodium salt (pH 7.5), 2 mM $CaCl_2$, 150 mM NaCl containing the MS-SAFE inhibitors cocktail. Crushed samples were centrifuged 5 min at 8,000 rpm at 4°C and the protein concentrations estimated (Bradford assay (Bradford, 1976)) in the collected supernatants and adjusted to 1.0 mg/ml. Samples of 500 µg of proteins for each condition were used for iTRAQ-TAILS labeling (Kleifeld et al., 2011; Bekhouche et al., 2016). Proteomic analyses were performed at the GIGA Proteomic platform on the ESI-Q Exactive (ThermoFisher) coupled with a 2D-RP/RP liquid chromatography (2D-RP/RP NanoAcquity

UPLC, Waters, Milford, United States) for the peptide fractionation in three fractions.

Proteomic Data Analysis

Proteomic data analysis was previously described (Bekhouche et al., 2016). Briefly, in a first step, peptides were identified using Mascot (version 2.2.06; Matrix Science Inc., Boston, MA, United States) and allowing non-tryptic cleavages and two missed cleavages/peptide. Carbamidomethyl cystein was set as a fixed modification, and other modifications were set as variable: N-terminal acetyl, deamidation (NQ), Pyro-glu (N-term E), Pyro-Gln (N-term Q), Oxidation (M), iTRAQ (K), iTRAQ (Y) and iTRAQ (N-term). Peptide tolerance was set at 0.02 Da.

The tandem mass spectrometry (MS/MS) data were analyzed using the TransProteomicPipeline (TPP). The PeptideProphet and ProteinProphet software programs, embedded into TPP, were used to validate protein and peptide assignment. The nontryptic model was omitted in the PeptideProphet parameters. The error rate to validate proteins or peptides was respectively set at 2 and 5%. Then, Clipper software was used to determine the upper and lower cutoffs corresponding to 3-sigma calculated from the normal distribution of the log2(P:C ratio) from natural mature N termini. A Gaussian error function was used to calculate a *p* value that reflects the probability of a peptide to be a false-positive. A peptide with a P:C ratio above or below the 3-sigma cutoff has 99,8% chance to be dependent of the studied protease (auf dem Keller and M Overall, 2012). The cutoffs for each experiment are reported in **Supplementary Table S4**. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD022179 and 10.6019/PXD022179.

Biological Process Analysis

The biological processes have been investigated using the Panther database (PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools (Mi et al., 2019)). Statistical overrepresentation tests were performed using the whole *Mus musculus* genome as reference dataset. The *p*-Values are determined using the Fisher's Exact test corrected by the determination of the false discovery rate (fdr). The fdr was below 0.05 for all the reported biological process.

The Venn diagram were drawn using the BioVenn software (BioVenn—a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams (Hulsen et al., 2008)).

Purification of Recombinant ADAMTS2 and 14

Recombinant ADAMTS2, its inactive mutant, or ADAMTS14 were produced in HEK293 cells, purified and quantified as previously described (Colige et al., 2005). Briefly, recombinant proteases were purified, using Concanavalin A-Sepharose and Heparin-Sepharose columns, from 1 L of serum-free medium conditioned during 48 h. Proteases were recovered in 50 mM Tris, pH 7.5, 1 M NaCl, 2 mM $CaCl_2$.

Analysis of Fibrillar Collagens Degradation

Collagen from dermatosparactic or normal calf skin was extracted and purified according to a reported procedure (Nusgens and Lapiere, 1979; Colige et al., 1995). Collagen from fibroblasts cultures, was concentrated by adding ethanol to the conditioned medium (see above, 2.2) at a final concentration of 33% (V/V). After overnight incubation at 4°C, precipitated collagen was recovered by centrifugation (7,000 rpm, 40 min, 4°C). Pellets were solubilized in 0.1 M acetic acid (pH 2.9) under shaking at 4°C for 18 h. Non-solubilized contaminants were excluded by centrifugation (17,000 rpm, 40 min, 4°C) and the supernatant containing the collagen was neutralized by adding 1 M Tris base solution (at 1/10 of the final volume). Collagen cleavage was analyzed by incubation of 10 µg of type I collagen with recombinant ADAMTS2 or 14 (200 nM for 16 h at 37°C) in 50 mM Tris (pH 7.5), 0.5 M NaCl, 2 mM $CaCl_2$, in the presence or absence of 25 mM EDTA used as inhibitor of ADAMTS metalloproteinase activity. Collagens were used in native form or after thermal denaturation at 95°C for 10 min. Digestion products were separated by SDS-PAGE and stained with Coomassie Blue.

Analysis of Cleavage of Intracellular Substrates

Cell lysates from human normal fibroblasts (NF) were prepared by sonication and incubated with recombinant human ADAMTS2 and/or 14 (200 nM for 16 h at 37°C) in 50 mM Tris (pH 7.5), 0.5 M NaCl, 2 mM $CaCl_2$, in the presence or absence of 25 mM EDTA. Actin and vimentin cleavages were analyzed by Western blot using polyclonal anti-alpha-actin-1 (A2066, Sigma) and polyclonal anti-vimentin (VI008–01, Quartett).

Determination of Cleavage Site Specificity

Amino acid sequence logos, corrected by the natural abundance of amino acids in the human proteome, were generated using the iceLogo software package (Colaert et al., 2009). Analyses were based on the cleavage sites determined by proteomics for all the potential extracellular substrates, without type I collagen cleavage sites or without all fibrillar collagens.

RESULTS

N-Terminomic Analysis of ADAMTS2 and ADAMTS14 Skin Degradome

ADAMTS2 and ADAMTS14 are clearly implicated in several physio-pathological processes (Colige et al., 1999; Kesteloot et al., 2007; Dubail et al., 2010; Dupont et al., 2018; Wang et al., 2020). To better understand their *in vivo* roles in skin physiology and extracellular matrix homeostasis, N-terminomic experiments have been performed on skin of mice, either wild type (Wt), deficient in Adamts2 ($TS2^{-/-}$), deficient in Adamts14 ($TS14^{-/-}$)

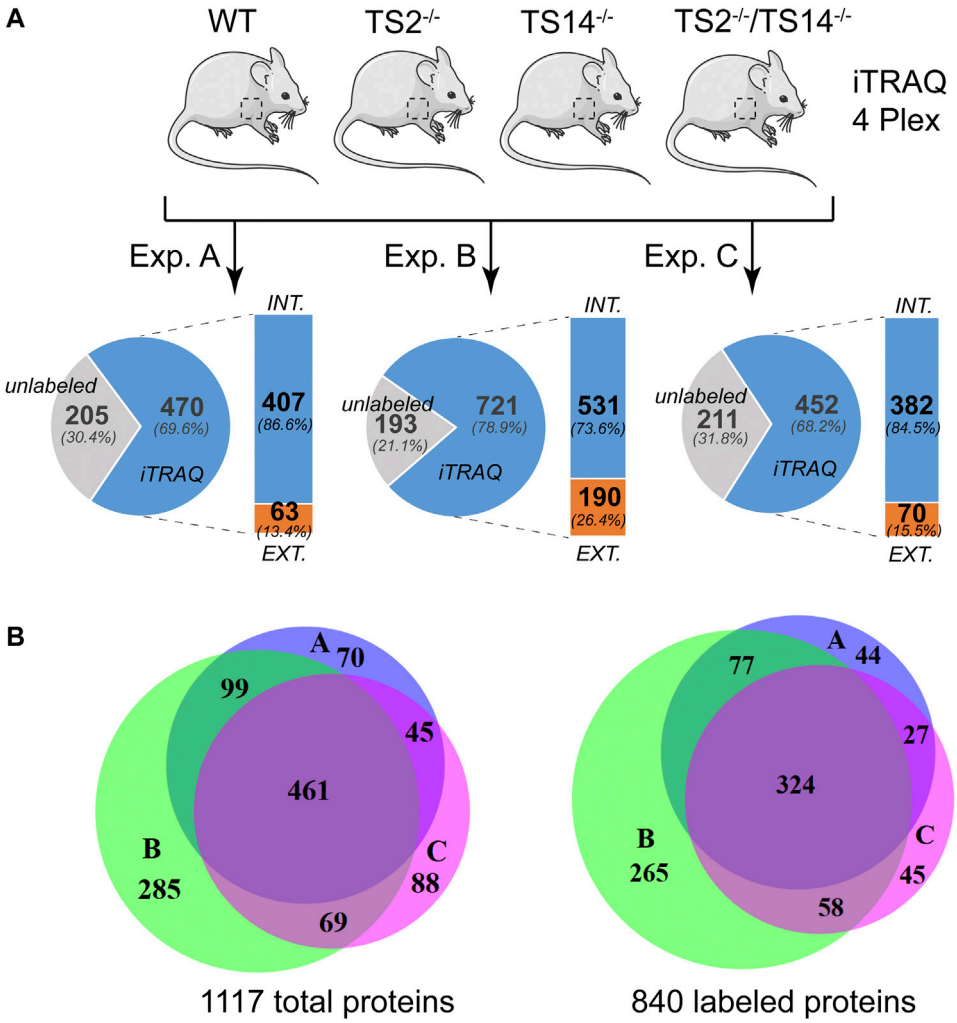


FIGURE 1 | N-terminomic analysis of ADAMTS2 and 14 in mouse skin. **(A)** Schematic view of the experimental method to assess the potential substrates of ADAMTS2 and/or ADAMTS14 in mouse skin and to investigate their biological function *in vivo*. Three independent iTRAQ-TAILS analyses using skin proteome of each genotypes (4-plex) have been performed. As a general procedure, a protease/control (P/C) ratio above or below the three sigma cutoff from the normal distribution of natural N-termini is considered to be related to the studied protease. Here, the ratio Wt/TS2$^{-/-}$ and TS14$^{-/-}$/TS2$^{-/-}$TS14$^{-/-}$ were used to investigate the ADAMTS2 substrates, the ratios Wt/TS14$^{-/-}$ and TS2$^{-/-}$/TS2$^{-/-}$TS14$^{-/-}$ were used to investigate the ADAMTS14 substrates and the ratio Wt/TS2$^{-/-}$TS14$^{-/-}$ was used to investigate the substrates common to ADAMTS2 and ADAMTS14. **(B)** Venn diagram showing the number of specific or common total **(left)** or labeled **(right)** proteins identified in each experiment. Experiment A is in blue, B in green and C in purple. Venn diagrams were generated using the online BioVenn software (Hulsen et al., 2008).

and deficient in both Adamts2 and Adamts14 (TS2$^{-/-}$TS14$^{-/-}$) (**Figure 1A**). For each experiment, proteins extracted from the four genotypes were labeled with specific iTRAQ labels for quantification by mass spectrometry (MS/MS). This 4-plex experiment was performed in triplicate. The proteomic analysis shows that between 68 and 79% of the proteins were labeled and have therefore been used for relative quantification. Among the labeled proteins between 13 and 26% are extracellular. By using Venn diagrams as analytical tool, a total of 1,117 proteins, including 840 with an iTRAQ labeled peptide, were identified in the three experiments, but only 41 and 38% of them were common to the three experiments, for unlabeled and labeled proteins, respectively (**Figure 1B**). As actual substrates might have been missed during the proteomic analyses, especially when their abundance is low, the data of these three experiments can be considered as complementary. In order to take this limitation into account but to generate also high confidence data, a labeled peptide was considered to identify a candidate substrate when it was observed in at least two experiments with a similar Protease/Control (P/C) fold change (either increased or decreased).

The degradomes (proteolytic events related to a proteolytic enzyme) are determined from the Protease/Control (P/C) ratios significantly different from the normal distribution of the natural N-termini. They include the proteins cleaved directly by the studied protease and also indirectly by the activation of other proteases or regulatory pathways. In this study, any N-terminally iTRAQ labeled peptide was considered to be part of the

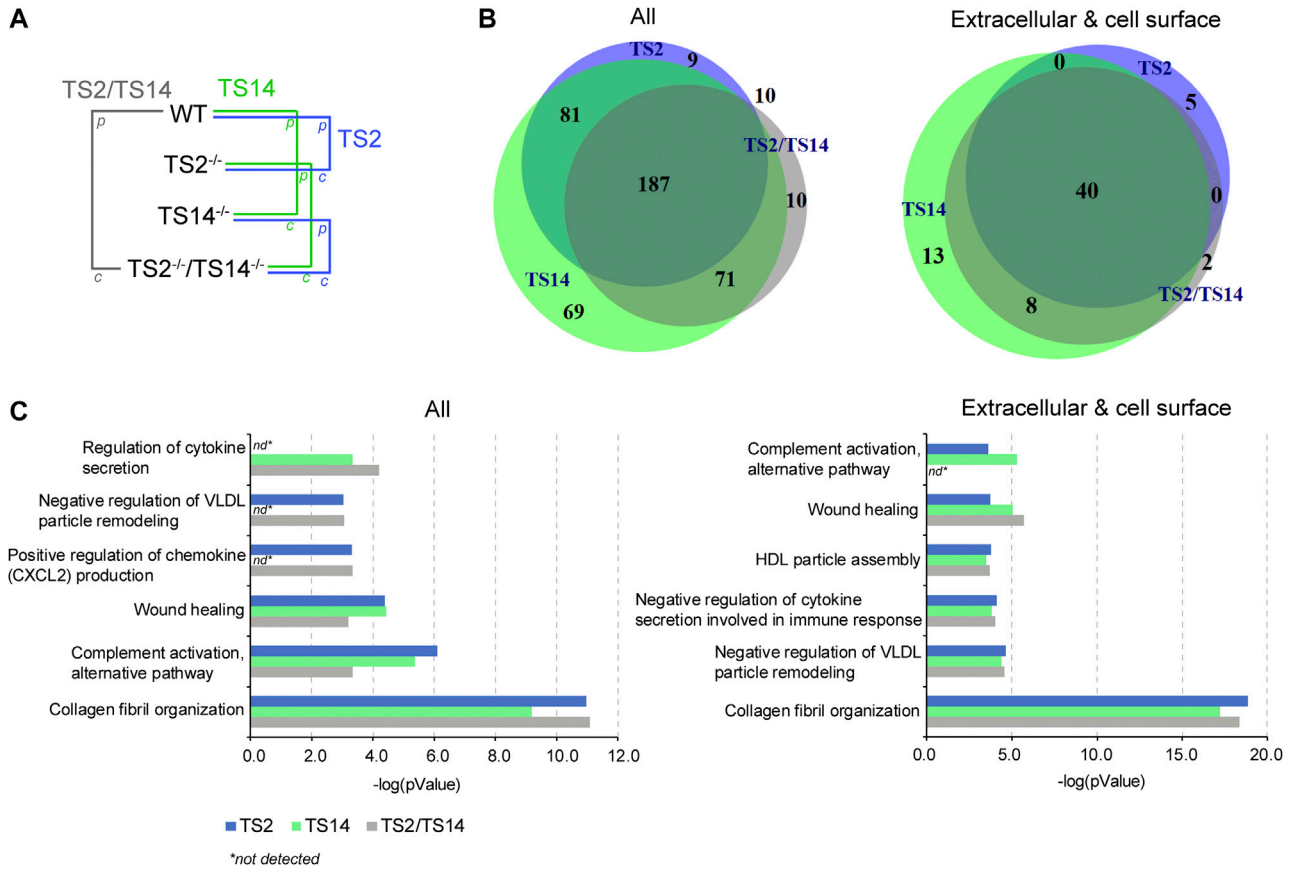


FIGURE 2 | ADAMTS2 and 14 degradomes in mouse skin and their biological processes. **(A)** Schematic view of the multiple comparisons of the P/C ratios analyzed for each iTRAQ-TAILS experiment (n = 3). The function of the ADAMTS2 (in blue) is investigated by the comparison of wild type (Wt) mice with those deficient for ADAMTS2 ($TS2^{-/-}$) and from the comparison of the mice deficient for ADAMTS14 ($TS14^{-/-}$) with those deficient for ADAMTS2 and ADAMTS14 ($TS2^{-/-}TS14^{-/-}$). The potential substrates of ADAMTS14 (in green) were identified from the comparison of Wt mice with the mice deficient for ADAMTS14 ($TS14^{-/-}$) and from the comparison of the mice deficient for ADAMTS2 ($TS2^{-/-}$) with those deficient for ADAMTS2 and ADAMTS14 ($TS2^{-/-}TS14^{-/-}$). The common substrates of ADAMTS2 and 14 (red) were obtained from the comparison between Wt mice and $TS2^{-/-}TS14^{-/-}$ mice, and by combining data obtained specifically for Adamts2 and for Adamts14. **(B)** Venn diagram showing the common and specific N-terminally labeled proteins related to ADAMTS2 (blue), ADAMTS14 (green) or ADAMTS2 and ADAMTS14 (green). All the 437 unique proteins (287 identified by comparing Wt to $TS2^{-/-}$, 408 by comparing Wt to $TS14^{-/-}$ and 278 by comparing Wt to $TS2^{-/-}TS14^{-/-}$) showing a P/C ratio above or below three sigma cutoff from the normal distribution of natural N-termini are reported on the left. The 68 extracellular and cell surface proteins related to ADAMTS2 or ADAMTS14 activities (45 by comparing Wt to $TS2^{-/-}$, 61 by comparing Wt to $TS14^{-/-}$ and 50 by comparing Wt to $TS2^{-/-}TS14^{-/-}$) are reported on the right. **(C)** Biological processes related to all the proteins affected by ADAMTS2 and ADAMTS14 in mouse skin **(left panel)** or related to the extracellular and cell surface substrates **(right panel)**. The biological processes have been identified using the Panther database (Mi et al., 2019). Statistical Overrepresentation test was performed using the whole *Mus musculus* as reference dataset, the pValue is determined using the Fisher's Exact test and corrected by the determination of the false discovery rate (fdr). The fdr is below 0.05 for all the biological processes reported. nd: not detected.

ADAMTS2 and/or ADAMTS14 degradomes when its P/C ratio was above or below a 3-sigma cut-off from the normal distribution of natural N-termini, including those obtained after the removal of the signal peptide. The degradome of ADAMTS2 has been determined from Wt vs $TS2^{-/-}$ and from $TS14^{-/-}$ vs $TS2^{-/-}TS14^{-/-}$ ratios, and that of ADAMTS14 has been determined from Wt vs $TS14^{-/-}$ and from $TS2^{-/-}$ vs $TS2^{-/-}TS14^{-/-}$ ratios, while that of ADAMTS2 and ADAMTS14 together has been determined from Wt vs $TS2^{-/-}TS14^{-/-}$ ratios (**Figure 2A**). In this analysis, the whole degradome of ADAMTS2 and/or ADAMTS14 was found to be composed of 437 proteins, including 68 proteins secreted or anchored at the cell surface. Forty extracellular and cell surface proteins were related to both ADAMTS2 and ADAMTS14 activities, while five are specific of ADAMTS2 and 21 (13 + 8) of ADAMTS14. Of note, two proteins were detected only when comparing Wt and $TS2^{-/-}TS14^{-/-}$, the coagulation factor XIIIa and the mast cell protease 4 (**Figure 2B**). The degradomes of ADAMTS2 and ADAMTS14 are common at 59% (40/68) when considering specifically the extracellular and cell surface proteins, while only a 40% (148/369) overlap is found when considering all the other proteins, showing an enrichment for common extracellular substrates (**Figure 2B**). Investigation of the biological processes (pantherdb.org) related to these degradomes clearly illustrated a fundamental role for these two enzymes in collagen fibril organization, which was

TABLE 1 | Potential extracellular substrates specific or common of ADAMTS2 and/or ADAMTS14.

Name	TS2	TS14	TS2TS14	Name	TS2	TS14	TS2TS14
Inter alpha-trypsin inhibitor	1.2 ± 0.7	1.9 ± 0.7	1.6 ± 0.5	Galectin-1	1.3 ± 0.3	1.9 ± 0.4	2.1 ± 0.4
AE-binding protein 1	0.7 ± 0.2	2.0 ± 0.5	1.2 ± 0.3	Galectin-7	2.0 ± 0.6	2.8 ± 0.8	4.8 ± 1.4
Alpha-2-HS-glycoprotein	0.7 ± 0.3	2.2 ± 1.3	0.9 ± 0.3	Gc-globulin	1.7 ± 0.8	4.1 ± 2.0	6.0 ± 3.7
Alpha-2-macroglobulin	1.7 ± 1.0	2.0 ± 0.6	2.2 ± 0.6	Gelsolin	2.6 ± 0.7	2.4 ± 0.9	5.0 ± 1.7
Annexin-8	6.4 ± 2.2	5.5 ± 1.9	15.0 ± nd[a]	H2-Q9	1.9 ± 1.3	2.7 ± 1.1	2.9 ± 1.1
Apolipoprotein A-I	2.7 ± 1.8	3.3 ± 1.9	4.5 ± 1.8	Hemopexin	1.0 ± 0.4	1.9 ± 0.5	1.5 ± 0.5
Apolipoprotein H	1.6 ± 0.8	2.6 ± 1.2	2.3 ± 0.8	Ig gamma-2B	1.8 ± 0.5	2.3 ± 0.8	3.5 ± 1.2
Beta-globin	2.2 ± 0.6	2.9 ± 1.1	6.7 ± 3.3	Ig gamma-3	1.1 ± 0.4	1.9 ± 0.4	1.8 ± 0.4
Biglycan	2.8 ± 2.4	3.0 ± 1.6	3.6 ± 1.8	Ig kappa chain V-V	1.2 ± 0.8	5.1 ± 2.7	2.9 ± 1.1
Carboxypeptidase A3	1.6 ± 0.4	2.1 ± 0.5	3.2 ± 1.1	Kallikrein j	2.0 ± 0.8	2.4 ± 0.6	5.1 ± 0.6
Cathepsin B heavy chain	1.3 ± 0.6	1.9 ± 0.6	1.6 ± 0.3	Kininogen-1	2.2 ± 0.5	2.9 ± 0.6	5.5 ± 0.2
Cathepsin H	1.2 ± 0.6	2.4 ± 0.8	2.0 ± 0.6	Lumican	1.6 ± 0.7	2.6 ± 1.3	2.3 ± 0.4
Cathepsin S	1.0 ± 0.5	2.2 ± 0.7	1.5 ± 0.4	Mast cell protease 4	1.5 ± 0.2	1.7 ± 0.2	2.4 ± 0.5
Caveolin-1	2.0 ± 0.8	3.1 ± 1.4	3.1 ± 0.0	MIF	1.1 ± 0.6	2.7 ± 1.1	1.8 ± 0.5
Cavin-1	2.1 ± 0.4	3.5 ± 0.9	8.7 ± 0.4	Myelin protein zero	1.6 ± 0.7	2.1 ± 1.1	1.7 ± 0.4
Coagulation factor XIIIa	2.5 ± 1.0	3.8 ± 1.5	7.8 ± nd[a]	Osteoglycin	2.3 ± 0.7	2.3 ± 0.9	4.5 ± 1.6
Collagen alpha-1(I) chain	4.2 ± 0.5	1.7 ± 0.2	6.8 ± 1.0	p24 gamma-1	2.1 ± 1.1	3.3 ± 1.7	1.4 ± 1.0
Collagen alpha-1(III) chain	3.7 ± 2.2	2.8 ± 1.3	5.8 ± 2.7	p35/Annexin A1	2.7 ± 1.2	2.7 ± 0.9	7.4 ± 3.7
Collagen alpha-1(IV) chain	1.6 ± 0.6	1.8 ± 0.6	2.2 ± 0.4	p36/Annexin A2	2.7 ± 1.2	2.7 ± 0.9	7.4 ± 3.7
Collagen alpha-1(V) chain	3.9 ± 1.7	4.1 ± 1.6	9.3 ± 0.8	PCPE-1	2.2 ± 0.4	1.9 ± 0.4	3.6 ± 0.5
Collagen alpha-1(VI) chain	2.2 ± 0.8	1.9 ± 0.6	3.4 ± 0.9	PDI A6	1.8 ± 0.7	2.1 ± 0.7	2.9 ± 0.9
Collagen alpha-1 (XIV) chain	3.6 ± 0.7	2.3 ± 0.5	8.2 ± 2.5	Periostin	1.0 ± 0.5	2.2 ± 0.8	1.3 ± 0.4
Collagen alpha-2(I) chain	3.1 ± 0.7	1.6 ± 0.2	4.6 ± 0.6	Proapolipoprotein A-II	3.5 ± 2.5	4.9 ± 3.2	11.9 ± 7.9
Collagen alpha-2(V) chain	1.8 ± 0.3	1.7 ± 0.3	2.8 ± 0.3	Prolargin	2.9 ± 0.9	2.6 ± 0.8	5.8 ± 1.3
Collagen alpha-2(VI) chain	2.5 ± 0.7	2.2 ± 0.6	4.1 ± 0.0	Protein unc-80 homolog	1.8 ± 0.6	2.0 ± 0.2	3.5 ± 1.4
Collagen alpha-3(VI) chain	4.7 ± 1.3	2.1 ± 0.6	8.0 ± 1.2	Serpin A1c	1.1 ± 0.3	2.4 ± 1.0	2.1 ± 0.5
Complement C3	1.8 ± 1.4	4.3 ± 3.4	2.3 ± 0.8	Serpin A1d	0.8 ± 0.5	2.8 ± 1.3	1.0 ± 0.4
Complement C4-B	0.3 ± 0.1	2.7 ± 1.0	0.6 ± 0.2	Serpin B6	4.7 ± 2.5	3.9 ± 2.4	8.0 ± 1.9
Complement factor B	0.9 ± 0.2	2.0 ± 0.4	1.8 ± 0.5	Protein serpinb6e	1.3 ± 0.3	2.3 ± 0.6	2.5 ± 0.1
Complement factor H	1.0 ± 0.3	1.8 ± 0.5	1.7 ± 0.6	Serum albumin	3.2 ± 1.7	4.2 ± 2.4	12.6 ± 9.7
Corneodesmosin	1.5 ± 0.4	1.7 ± 0.4	2.2 ± 0.4	Siderophilin	2.2 ± 0.7	2.8 ± 1.1	5.2 ± 2.5
Cystatin-3	2.4 ± 0.6	2.7 ± 0.8	7.0 ± 3.7	Stromelysin-1/MMP3	0.4 ± 0.1	1.5 ± 0.3	0.5 ± 0.1
Dermatopontin	2.0 ± 0.4	1.6 ± 0.2	3.1 ± 0.6	Susd 4	2.3 ± 0.7	2.4 ± 1.1	3.9 ± 1.1
Dermokine	1.1 ± 0.3	1.4 ± 0.3	1.4 ± 0.2	Transcobalamin II	0.8 ± 0.5	2.2 ± 1.1	0.8 ± 0.2

[a]*nd: not determined.*

The 68 extracellular and cell surface potential substrates are reported together with the average P/C ratio from at least two experiments for ADAMTS2, ADAMTS14 or ADAMTS2 and ADAMTS14. When several peptides were identified for a protein, the ratios from the peptide giving the highest value are reported for illustration. All the peptides and ratios are reported in supplemental.

expected for ADAMTS2 but was more surprising for ADAMTS14 since collagen fibrils in TS14$^{-/-}$ mice appear to be normal. This analysis also shed light on their implication in lipoprotein regulation and assembly, notably by the cleavage of the proapolipoprotein A-II and apolipoprotein A-I, and in immune response by the regulation of cytokines and chemokines secretion and complement activation, notably through the cleavage of complement proteins (C3C, C4-B) and of the macrophage migration inhibitory factor (**Figure 2C**, **Table 1**, **Supplementary Figure S1**).

Proteomic Analysis of Type I Collagen Processing by ADAMTS2 And/or ADAMTS14 in Mouse Skin

Cleavage of the Aminopropeptide of Fibrillar Collagens

As a positive control assessing the quality and the specificity of our technical approach, we first focused on the cleavages of the aminopropeptides of type I procollagens, the primary substrates of ADAMTS2. For Col1A1 and Col1A2, the highest P/C ratios were detected at sequences corresponding to the published cleavage sites in the NC2 domain: S_{151}-$_{152}$Q for Col1A1 and A_{85}-$_{86}$Q for Col1A2 (**Table 2**). Peptides corresponding to more upstream sequences have P/C ratios largely below 1, evidencing the near absence of intact N-propeptide in Wt skin (**Supplementary Table S2**). These data clearly validate the reliability of our experimental setting.

Surprisingly, several additional potential cleavage sites were also identified in the NC2 domain (**Figure 3A**; **Table 2**), some with a P/C ratio >2. Some were a few amino acids downstream the "canonical" cleavage site and could therefore result from exopeptidase activity, but several others were at longer distance suggesting that ADAMTS2 cleaves within a preferred region and not exclusively at the previously reported single cleavage site.

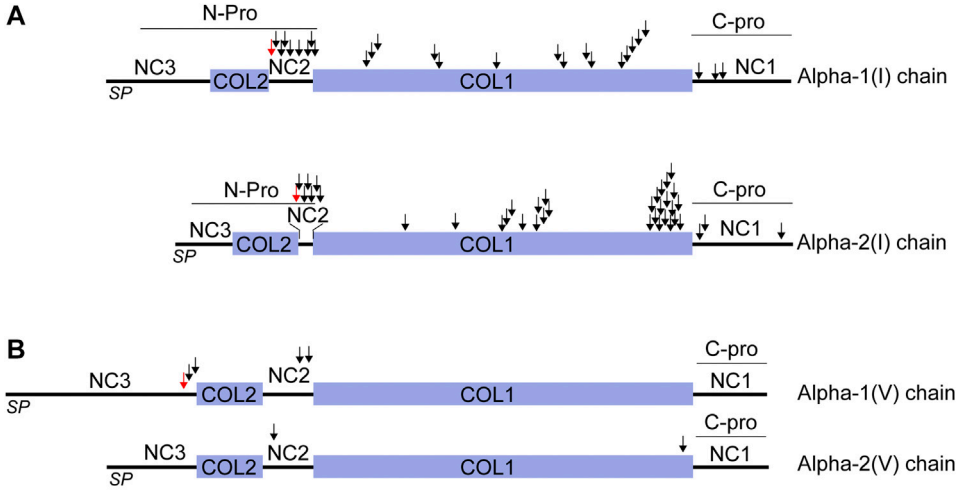
Regarding ADAMTS14, no clear activity could be evidenced at sites cleaved by ADAMTS2, which is in line with previous studies

TABLE 2 | Type I collagen processing in mouse skin.

Collagen α1(I) chain				Collagen α2(I) chain			
Peptide Sequence	**TS2**	**TS14**	**TS2TS14**	**Peptide Sequence**	**TS2**	**TS14**	**TS2TS14**
NC2 (A150-P167)				*NC2 (A75-P96)*			
NFAS(151).(152) QMSYGYDEKSAGVSVPGPMGPSGPR	4.2 ± 0.5	1.7 ± 0.2	6.8 ± 1.0	NFAA(85).(86)QYSDKGVSSGPGPMGLMGPR	3.1 ± 0.7	1.6 ± 0.2	4.6 ± 0.6
FASQ(152).(153) MSYGYDEKSAGVSVPGPMGPSGPR	2.0 ± 0.3	1.5 ± 0.2	2.9 ± 0.4	FAAQ(86).(87)YSDKGVSSGPGPMGLMGPR	2.0 ± 0.3	2.0 ± 0.3	1.5 ± 0.2
QMSY(155).(156) GYDEKSAGVSVPGPMGPSGPR	2.4 ± 1.0	1.6 ± 0.3	3.3 ± 1.1	YSDK(90).(91)GVSSGPGPMGLMGPR	2.9 ± 0.4	1.4 ± 0.2	2.9 ± 0.6
SYGY(157).(158) DEKSAGVSVPGPMGPSGPR	3.0 ± 1.0	1.8 ± 0.3	5.2 ± 2.0	SDKG(91).(92)VSSGPGPMGLMGPR	1.9 ± 0.3	1.4 ± 0.2	2.6 ± 0.3
DEKS(161).(162)AGVSVPGPMGPSGPR	2.0 ± 0.3	1.5 ± 0.2	2.9 ± 0.5	DKGV(92).(93)SSGPGPMGLMGPR	1.9 ± 0.2	1.5 ± 0.1	2.8 ± 0.1
EKSA (162).(163)GVSVPGPMGPSGPR	1.9 ± 0.3	1.4 ± 0.1	2.6 ± 0.4	*Triple helix (G97-G1113)*			
KSAG(163).(164)VSVPGPMGPSGPR	3.7 ± 0.3	1.4 ± 0.1	5.1 ± 0.2	SSGP(96).(97)GPMGLMGPR	1.9 ± 0.3	1.5 ± 0.2	2.7 ± 0.5
Triple helix (<I>G168-P1181</I>)				GPGP(98).(99)MGLMGPR	2.5 ± 0.8	1.8 ± 0.5	3.9 ± 1.6
VSVP(167).(168)GPMGPSGPR	1.8 ± 0.5	1.6 ± 0.4	2.5 ± 0.6	RGIP(336).(337)GPAGAAGATGAR	2.5 ± 0.7	1.7 ± 0.4	3.6 ± 1.0
VPGP(169).(170)MGPSGPR	1.8 ± 0.5	1.5 ± 0.4	2.6 ± 1.0	RPGP(485).(486)IGPAGPR	2.4 ± 0.7	1.6 ± 0.4	3.5 ± 1.3
TGPP(332).(333) GFPGAVGAKGEAGPQGAR	2.1 ± 0.6	1.7 ± 0.3	3.5 ± 1.1	RGTP(600).(601)GESGAAGPSGPIGSR	2.1 ± 0.6	1.6 ± 0.3	3.2 ± 1.1
PPGF(334).(335)PGAVGAKGEAGPQGAR	2.3 ± 0.8	1.7 ± 0.5	3.5 ± 1.7	GESG(604).(605)AAGPSGPIGSR	2.0 ± 0.2	1.3 ± 0.1	2.5 ± 0.4
PGFP(335).(336)GAVGAKGEAGPQGAR	2.4 ± 0.8	1.9 ± 0.5	4.0 ± 1.3	ESGA(605).(606)AGPSGPIGSR	2.0 ± 0.4	1.4 ± 0.2	2.7 ± 0.5
RGFP(485).(486)GADGVAGPKGPSGER	2.6 ± 1.0	1.7 ± 0.4	3.8 ± 1.5	VGAP (636).(637)GSAGASGPGGLPGER	2.3 ± 0.8	1.7 ± 0.4	3.5 ± 1.2
GFPG(486).(487)ADGVAGPKGPSGER	1.8 ± 0.3	1.4 ± 0.2	2.3 ± 0.2	SGDR(699).(700)GEAGAAGPSGPAGPR	1.9 ± 0.4	1.6 ± 0.3	3.0 ± 0.7
AGAQ(608).(609)GAPGPAGPAGER	2.2 ± 0.4	1.6 ± 0.3	3.1 ± 0.3	GDRG(700).(701)EAGAAGPSGPAGPR	1.9 ± 0.3	1.5 ± 0.2	2.8 ± 0.3
PGPI(842).(843)GNVGAPGPKGPR	2.4 ± 0.3	1.9 ± 0.3	4.3 ± 0.2	DRGE(701).(702)AGAAGPSGPAGPR	2.4 ± 0.6	1.7 ± 0.4	3.8 ± 1.1
PPGP(889).(890)VGKEGGKGPR	2.4 ± 0.6	1.4 ± 0.3	3.1 ± 0.8	GEAG(703).(704)AAGPSGPAGPR	2.0 ± 0.2	1.3 ± 0.1	2.5 ± 0.4
AGSP(935).(936)GTPGPQGIAGQR	2.2 ± 0.6	1.6 ± 0.3	3.1 ± 1.0	EAGA(704).(705)AGPSGPAGPR	2.1 ± 0.4	1.5 ± 0.2	3.0 ± 0.7
PGTP(938).(939)GPQGIAGQR	2.6 ± 0.8	1.6 ± 0.4	3.8 ± 1.3	AGAP(969).(970)GPHGSVGPAGKHGNR	2.2 ± 0.5	1.6 ± 0.3	3.4 ± 0.7
KNGD(1,054).(1,055) RGETGPAGPAGPIGPAGAR	2.1 ± 0.5	2.0 ± 0.4	3.6 ± 0.1	RGEP(987).(988)GPAGSVGPVGAVGPR	2.5 ± 0.8	1.6 ± 0.4	3.7 ± 1.4
NGDR(1,055).(1,056) GETGPAGPAGPIGPAGAR	2.0 ± 0.4	1.4 ± 0.2	2.6 ± 0.5	EPGP(989).(990)AGSVGPVGAVGPR	2.2 ± 0.6	1.5 ± 0.3	3.2 ± 1.1
RGET (1,058).(1,059) GPAGPAGPIGPAGAR	1.7 ± 0.2	1.2 ± 0.1	2.1 ± 0.1	PGPA(990).(991)GSVGPVGAVGPR	1.9 ± 0.2	1.3 ± 0.1	2.4 ± 0.4
ETGP(1,060).(1,061)AGPAGPIGPAGAR	2.4 ± 0.7	1.6 ± 0.4	3.5 ± 1.1	AGSV(993).(994)GPVGAVGPR	2.3 ± 0.5	1.5 ± 0.2	3.3 ± 0.9
PAGP(1,063).(1,064)AGPIGPAGAR	2.6 ± 0.8	1.5 ± 0.4	3.7 ± 1.5	SVGP(995).(996)VGAVGPR	1.7 ± 0.2	1.6 ± 0.2	2.6 ± 0.3
NC1 (S1182-V1453)				LKGY(1,031).(1,032) SGLQGLPGLAGLHGDQGAPGPVGPAGPR	2.2 ± 0.6	1.9 ± 0.5	3.6 ± 1.1
GYDF(1,187).(1,188) SFLPQPPQEKSQDGGR	4.4 ± 0.4	9.5 ± 3.6	24.3 ± 7.6	YSGL(1,034).(1,035) QGLPGLAGLHGDQGAPGPVGPAGPR	2.6 ± 1.2	1.7 ± 0.5	3.1 ± 1.0
DTTL(1,224).(1,225)KSLSQQIENIR	2.5 ± 0.9	2.0 ± 0.9	3.4 ± 0.8	GLPG (1,039).(1,040) LAGLHGDQGAPGPVGPAGPR	2.9 ± 0.6	1.9 ± 0.5	5.6 ± 2.4
LKSL(1,227).(1,228)SQQIENIR	2.3 ± 0.6	1.7 ± 0.5	3.2 ± 0.8	LPGL(1,040).(1,041) AGLHGDQGAPGPVGPAGPR	2.3 ± 0.4	1.3 ± 0.2	2.8 ± 0.3
				PGLA(1,041).(1,042) GLHGDQGAPGPVGPAGPR	2.8 ± 1.2	2.5 ± 1.2	6.1 ± 2.9
				LAGL(1,043).(1,044)HGDQGAPGPVGPAGPR	2.0 ± 0.3	1.6 ± 0.2	2.9 ± 0.5
				GLHG(1,045).(1,046)DQGAPGPVGPAGPR	1.5 ± 0.4	1.8 ± 0.2	2.7 ± 0.9
				LHGD(1,046).(1,047)QGAPGPVGPAGPR	2.2 ± 0.3	1.5 ± 0.2	3.2 ± 0.1
				HGDQ(1,047).(1,048)GAPGPVGPAGPR	1.8 ± 0.2	1.3 ± 0.1	2.4 ± 0.4
				GDQG(1,048).(1,049)APGPVGPAGPR	2.0 ± 0.2	1.4 ± 0.2	2.7 ± 0.5
				RSGQ(1,076).(1,077)PGPVGPAGVR	2.2 ± 0.3	1.5 ± 0.2	3.2 ± 0.1
				NC1 (Y1114-K1372)			
				DATL(1,145).(1,146)KSLNNQIETLLTPEGSR	3.9 ± 3.4	6.0 ± 5.6	3.2 ± 1.0
				LKSL(1,148).(1,149)NNQIETLLTPEGSR	1.6 ± 0.4	2.0 ± 0.6	2.7 ± 0.8
				RLPF(1,347).(1,348)LDIAPLDIGGADQEFR	3.0 ± 0.7	2.0 ± 0.6	5.3 ± 1.2

Cleavage sites observed by proteomics of ADAMTS2 (TS2) and ADAMTS14 (TS14) within α1 and α2 chains of type I collagen. Each cleavage site has been observed at least in two experiments. The sequences of the cleavage sites are reported in bracket according to the UniprotKB numbering. The classical N-propeptide cleavage sites are highlighted in grey.

(Dupont et al., 2018) showing that type I collagen is fully processed in the $TS14^{-/-}$ mice, and further confirming the specificity of our analyses. Most interestingly, the P/C ratios were, however, higher in $TS2^{-/-}TS14^{-/-}$ than in $TS2^{-/-}$ mice suggesting that, in the absence of ADAMTS2, ADAMTS14 can display some aminoprocollagen peptidase activity *in vivo* (**Table 2**).



C

Cleavage sites	TS2	TS14	TS2TS14
Alpha-1(V) chain			
PGMP(435).(436)ANQD	3.9 ± 1.7	4.1 ± 1.6	9.3 ± 0.8
MPAN(437).(438)QDTI	2.5 ± 0.6	1.9 ± 0.4	3.9 ± 0.3
QDTI(441).(442)FEGI	2.2 ± 0.6	1.8 ± 0.5	3.4 ± 0.7
AGSK(535).(536)GPMV	1.5 ± 0.4	2.1 ± 0.5	3.0 ± 0.8
MVSA(541).(542)QESQ	1.6 ± 0.3	1.8 ± 0.3	2.7 ± 0.7
Alpha-2(V) chain			
SRPF(189).(190)SAQM	1.8 ± 0.3	1.7 ± 0.3	2.8 ± 0.3
QGPP(903).(904)GATG	2.6 ± 0.9	1.8 ± 0.4	4.3 ± 1.6

FIGURE 3 | ADAMTS2 and/or ADAMTS14 cleavages of type I and V collagens. Schematic view of the pro alpha-1(I) and alpha-2(I) chains showing the signal peptides (PS), the main triple helix collagenous domain (COL1) and the short triple helix collagenous domain (COL2) and the N- and C-propeptides (N- and C-Pro) **(A)**. Schematic view of the pro alpha-1(V) and alpha-2(V) chains showing the collagenous (COL) and non collagenous (NC) domains **(B)**. The ADAMTS2 and ADAMTS14 cleavage sites are indicated by arrows. The red arrows correspond to the already reported ADAMTS2 cleavage sites (Colige et al., 2005; Bekhouche and Colige, 2015). Table showing the P/C ratio for type V collagens, according to the cleavage sites observed by proteomic. Ratios above the average 3σ cut-offs are highlighted in green. The previously described ADAMTS2 cleavage site within alpha-1(V) chain is in bold **(C)**.

The processing of the aminopropeptide of type V procollagens is still a matter of controversy, especially *in vivo*. Processing of Col5A1 has been reported to be performed by BMP1 (within the NC3 domain) and by ADAMTS2 (within the NC3 domain upstream of the Col2 domain) (**Figure 3B**). However, no cleavage has been reported in the NC2 domains of Col5A1 or Col5A2, while their sequence and localization between two collagen domains are similar to those of type I, II and III fibrillar procollagens which are all processed in this region. Several peptides N-terminally iTRAQ labeled were identified in the large N-terminal domain of Col5A1 encompassing the Col2, NC2 and NC3 domains (**Figure 3B**). As opposed to what was observed for type I procollagens, the P/C ratios were usually similar for ADAMTS2 and ADAMTS14, suggesting that both enzymes can process Col5A1 with the same efficacy (**Figure 3C**). Accordingly, the P/C ratios were much higher when comparing Wt and $TS2^{-/-}TS14^{-/-}$ samples. The main processing site ($P_{435\cdot436}A$, upstream of the Col2 domain) was identical to that described previously using recombinant proteins (Colige et al., 2005). As for Col1A1 and Col1A2, other cleavage sites were also identified in this region of Col5A1, two with P/C > 2 ($N_{437\cdot438}Q$ and $I_{441\cdot442}F$) and one with a P/C of 0.4 ($Q_{438\cdot439}D$) reflecting a preferential cleavage at $N_{437\cdot438}Q$. The P/C of the other labeled peptides was not affected by the presence or absence of ADAMTS2 and ADAMTS14, which confirms the specificity of the cleavages with a P/C ratio >2.

Most interestingly, two cleavage sites were also identified in the NC2 domain of Col5A1, including one at an A. Q site as in Col1A2 (**Figure 3B**). Altogether these data indicate that ADAMTS2 and ADAMTS14 can process the N-terminal portion of Col5A1 at three different sites, meaning that Col5A1 is present in the skin under forms (including that generated by BMP1 cleavage) having N-terminal non-collagenous extremity of different size and bulkiness.

N-terminally iTRAQ-labeled peptides corresponding to the NC2 domain of Col5A2 were also detected. Although the P/C

ratios were lower than for Col1A1, Col1A2 and Col1A5, it clearly indicates that the N-propeptide of Col5A2 can be processed by ADAMTS2 and ADAMTS14, but probably at a reduced rate.

Similar to our previous study *in vitro*, the cleavage of the N-propeptide of Col3A1 was not seen in this experiment, probably because the corresponding peptide was not isolated and therefore not analyzed during the MS/MS step.

Cleavages Outside of the Aminopropeptide

Having confirmed and further documented the processing of the aminopropeptides of fibrillar collagens, we were also intrigued by the presence of potential cleavage sites elsewhere in type I collagen chains. In a previous work performed *in vitro* using human cells (Bekhouche et al., 2016), we identified several peptides corresponding to the Col1 domain and the C-propeptide of Col1A1 and Col1A2 that were possibly generated by ADAMTS2 or ADAMTS14 (**Supplementary Table S3**). However, we hypothesized that this could be an artifact linked to the *in vitro* conditions, such as incomplete folding of the triple helical domains leading to an increased sensitivity to proteases. Here, dozens of peptides corresponding to the Col1 domain of Col1A1 and Col1A2 were found to be N-terminally iTRAQ labeled. For both Col1A1 and Col1A2, the P/C ratios were, on average, between 1.5 and two when considering ADAMTS2, and between 1.0 and 1.5 for ADAMTS14 (**Supplementary Tables S2, S3**). However, similarly to what was observed for the processing of the aminopropeptides, these ratios were significantly higher when comparing Wt and $TS2^{-/-}TS14^{-/-}$ skin samples, suggesting that ADAMTS2 and ADAMTS14 can cleave at identical sites. Some "hot spot" regions characterized by several contiguous cleavage sites were found, as, for example, at positions 333 to 337 and 827 to 838 of Col1A1 as well as at positions 988 to 996 of Col1A2 (**Table 2**). They can be generated by direct cleavages operated by ADAMTS2 and 14 in more sensitive domains or, alternatively, by a single cleavage followed by progressive degradation by exopeptidases. In both cases however, it means that the observed individual P/C ratios give an underestimation of the actual cleavage activity in the concerned region.

Finally, peptides corresponding to cleavages in the C-propeptides were also found. As opposed to our observations for Col1 domain, the P/C ratios were similar for both ADAMTS2 and ADAMTS14, again illustrating the specificity in the identification of potential cleavage sites. For Col1A1, one site ($F_{1187\text{-}1188}S$) is located upstream of the C-propeptide cleavage site by BMP1 ($A_{1207\text{-}1208}D$) while the two others are located about 20 amino acids downstream. For Col1A2, three sites were identified, all after the cleavage site for BMP1 (**Table 2**).

Confirmation of the Cleavage of Type I Collagen Within the Col1 Domain and the C-Propeptide

To confirm our iTRAQ data showing multiple cleavages in the Col1 triple helical domain and in the C-propeptide of type I collagens, we performed *in vitro* assays using recombinant enzymes and collagen purified from the skin of dermatosparactic calf which is characterized by the persistence of the aminopropeptides in about 80% of type I collagen molecules (**Figure 4A**, lane 1). This particular substrate was chosen because it provides the opportunity to have an internal control consisting in the processing of the aminopropeptide of Col1A1 and Col1A2. This cleavage (conversion of pNa1 and pNa2 into a1 and a2 chains) was almost complete in the presence of recombinant ADAMTS2, but much reduced in the presence of ADAMTS14 in accordance with its lower aminoprocollagen peptidase activity (**Figure 4A**, lanes 2 and 4, respectively). When looking at lower molecular weight products (**Figures 4C,E**), the released pNa1 propeptide was observed with an apparent 30 kDa MW and was mainly found in the presence of recombinant active ADAMTS2 (lanes 2 and 6). The presence of other bands and of "trails", covering the entire migration lanes and corresponding to cleavages of collagen in multiple sites, were also identified (lane 2), including in the presence of recombinant ADAMTS14 (lane 4). Since ADAMTS14 has only a reduced aminoprocollagen peptidase activity, it shows that the two types of activity are not related.

The same type I collagen preparation was also used as substrate after heat denaturation to verify whether disruption of the triple helical folding impacts the sensitivity to cleavage. Processing of the aminopropeptides by ADAMTS2 or ADAMTS14 was still observed, but marked differences were also visible as compared to gels obtained with native collagen (compare panels a, c, e to panels b, d, f of **Figure 4**), such as the presence of products at 97 and 110 kDa observed in the presence of ADAMTS2 and 14 (lanes 2, 4 and 6 on each panel), and at 105 kDa specifically in the presence of ADAMTS14 (lanes four on each panel). Additional discrete degradation products of lower MW were also identified as well as more pronounced degradation trails along the entire migration lanes (**Figure 4**, lanes 2, 4, 6; panels d and f).

The presence of cleavage sites within the C-propeptide of Col1A1 and Col1A2 was also evidenced by our iTRAQ analyses on *in vitro* assays. Since collagen purified from dermatosparactic skin lacks the C-propeptide, we used collagen produced by human dermatosparactic fibroblasts in culture which is mainly secreted as complete procollagen still retaining its two propeptides (pro-alpha one and pro-alpha 2). After incubation with active ADAMTS2 (**Figure 5**, lane 2), proa1 and proa2 are converted into pCa1 and pCa2, respectively, as a result of the aminoprocollagen peptidase activity of ADAMTS2. Fully processed alpha1 was also observed while similar amount of pNa1 was absent in the control (lane 1) suggesting that it is produced by cleavage of the N- and C-propeptides of the proa1 chain. In line with this hypothesis, the amount of alpha2 chain recovered after incubation with ADAMTS2 exceeded the amount of pNa2 in the control. Moreover, the relative intensity of pCa2 was lower after incubation with ADAMTS2 (lane 2) than expected from the intensity of proa2 in the control. These two observations clearly suggest some cleavage of the C-propeptide of proa2.

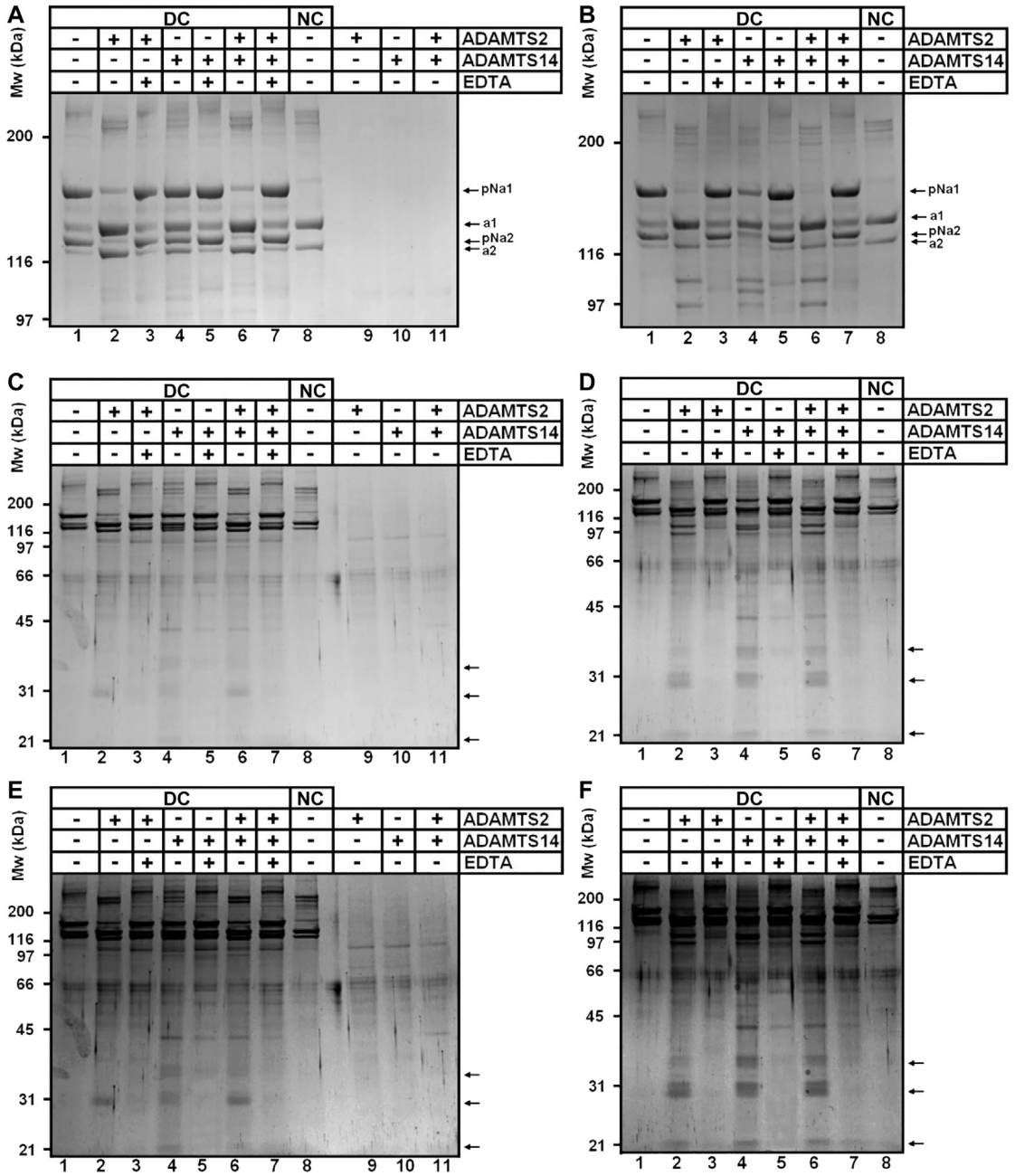


FIGURE 4 | Collagen digestion from calf skin by recombinant human ADAMTS2 and/or ADAMTS14 **(A–F)** Collagen from dermatospatactic calf (DC) skin has been heat denatured (10 min at 95°C) **(B, D, F)** or not **(A, C, E)** before addition of recombinant human TS2 and/or TS14 overnight at 37°C. Digestion products have been analyzed by 6.25% **(A, B)** or 10% **(C–F)** SDS-PAGE stained by Coomassie blue. Pictures at **(E)** and **(F)** correspond respectively to **(C)** and **(D)** with a higher contrast to emphasize the degradation trail. Collagen from normal calf skin (NC) have been used as control for the identification of the fully processed α1 and α2 chains. Products at 31 kDa **(C, E)** correspond to the N-terminal propeptide released mainly by ADAMTS2 when type I collagen is in its native form. Additional low MW products can be observed after incubation of denatured collagen with ADAMTS14 and ADAMTS2.

Regarding ADAMTS14, only a low aminoprocollagen peptidase activity was observed, as expected, as illustrated by the presence of low amounts of pCa2 generated from proa2 (lane 3). However, accumulation of pNa2 was clearly observed indicating that ADAMTS14 can cleave the C-propeptide of Col1A2 more efficiently than its N-propeptide.

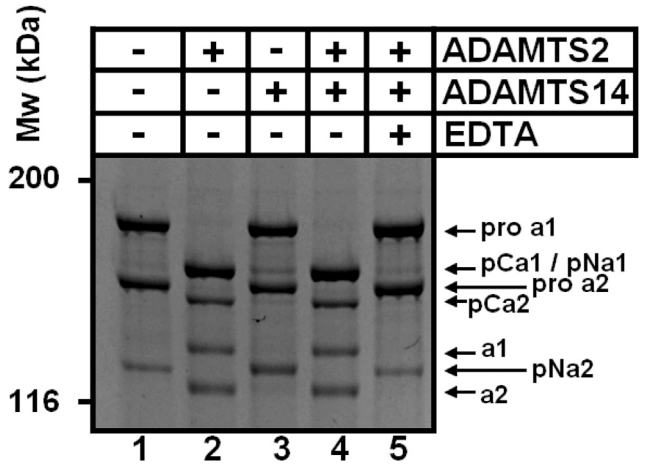


FIGURE 5 | Cleavage of type I procollagen. The collagen substrate, mainly in the form of pro-α1I and pro-α2I, was recovered from culture medium conditioned by dermatosparactic fibroblasts, and then incubated with recombinant human ADAMTS2 and/or ADAMTS14 during 18 h at 37°C. Digestion products were analyzed by 6.25% SDS-PAGE stained by Coomassie blue. The cleavage of pro-α2 into pCα2 and mature α2 demonstrates that ADAMTS2 displays a procollagen C-proteinase activity in addition to its aminoprocollagen peptidase property.

Other Potential Substrates Involved in Collagen Fibril Formation

Several proteoglycans, non-fibrillar collagens and matricellular proteins are known to be involved in the regulation of collagen fibril formation and functions. Some of them were found to be potential substrates of ADAMTS2 and/or ADAMTS14 based on P/C ratios significantly >2 (**Table 1**). They are therefore mentioned, although cleavages were not confirmed by other methods, since their processing could be involved in the clinical features found in dermatosparactic EDS and other connective tissue disorders.

Two sites of cleavage in type XIV collagen were found for both ADAMTS2 and ADAMTS14: at positions 630 (AQQY.LEID) within the fifth fibronectin type-III domain and 1,636 (MARY.TAIL) within the third collagen like domain. Identical sites for both enzymes strongly suggest that the cleavages are real and that type XIV collagen is a true substrate. Potential cleavages were also found in COL4A1 ($G_{1438\cdot1439}T$), at a position corresponding to the release of the arresten cryptic bioactive fragment and in type VI collagens: (1) in the first VWFA domain of COL6A1 ($F_{186\cdot187}S$); (2) in the VWFA1 and VWFA2 domains of COL6A2 ($F_{116\cdot117}S$ and $F_{141\cdot142}A$) and (3) in the VWFA6 domain of COL6A3 ($F_{1051\cdot1052}A$). Besides collagens, potential proteolytic cleavages were also found in PCPE-1 (a co-factor stimulating the processing of the C-propeptide of fibrillar collagens by BMP-1) and in three proteoglycans regulating matrix assembly: biglycan, lumican and osteoglycin. Finally, the degradomes of ADAMTS2 and ADAMTS14 in mouse skin point out several proteins involved in the immune system such as immunoglobulins, complement proteins (C3, C4-B, factor B, factor H), the macrophage inhibitory factor or annexins A8, A1 and A2 known to be involved in leukocyte recruitment and activation (Swisher et al., 2007; Poeter et al., 2014; Sugimoto et al., 2016).

Potential Intracellular Cleavages

This work was primarily focused on extracellular and cell surface degradome since ADAMTS2 and ADAMTS14 are secreted proteases. Unexpectedly however, numerous cleavage sites related to the presence/absence of ADAMTS2 and/or ADAMTS14 were found in intracellular proteins (**Supplementary Table S1**), including actins and vimentin (**Figure 6A**). On average, P/C ratios for actin were higher for ADAMTS14 than for ADAMTS2, as opposed to what was seen for the N-propeptides cleavages, and even higher when comparing Wt skin and $TS2^{-/-}TS14^{-/-}$ skins. Of note also, cleavage sites in actin-2, also known as gamma-actin, are identical to the corresponding cleavage sites in actin-1 which suggests their specificity. In order to confirm these surprising observations, fibroblasts extracts were incubated in the presence of purified ADAMTS2 and/or ADAMTS14 with or without a saturating concentration of EDTA used as inhibitor (**Figure 6B**). In the absence of ADAMTS and EDTA, actin was detected as a single band of 42 kDa with an antibody raised against the C-terminal decapeptide of alpha-actin-1, demonstrating absence of cleavage by intracellular endogenous proteases, while a second product of about 40 kDa was observed after incubation with ADAMTS2, which would correspond to cleavages at positions $V_{19\cdot20}K$ and/or $A_{21\cdot22}G$ in alpha-actin-1 (**Figure 6A**). This product was also seen with ADAMTS14, in addition to a second one at about 34–35 kDa which was not observed with ADAMTS2. Remarkably, only discrete bands were seen, with no trace of smear which would indicate multiple cleavages at specific positions.

Regarding vimentin, several peptides with N-terminal iTRAQ labeling were detected, some with P/C ratio slightly but significantly higher in the presence of ADAMTS2 or ADAMTS14 (**Figure 6A**). For confirmatory purposes Western blot analysis was performed on fibroblast extracts incubated or not with purified ADAMTS2 or ADAMTS14. In the control samples, vimentin appeared as two major bands at 48 kDa and at 58 kDa the latter likely corresponding to the full-length protein. Additional minor products at 53, 50, 46 and 39 kDa were also visible (Romano et al., 2020) (**Figure 6C**). Upon incubation with ADAMTS2 or 14, the intensity of bands at 58 and 53 kDa were markedly reduced, accompanied by an increased intensity of the products at 48 and 39 kDa and the presence of additional bands at 37 and 32 kDa. These data clearly suggest the existence of several cleavage sites by ADAMTS2 and ADAMTS14 but were not investigated further.

Cleavage Site of ADAMTS2 and ADAMTS14 in Mouse Skin

The cleavage sites observed by N-terminomics were used to evaluate cleavage sites enrichment linked to the presence of ADAMTS2 and ADAMTS14. All the potential cleavage sites were used, in a first step, to establish the privileged consensus

A

Cleavage sites	TS2	TS14	TS2TS14
Alpha-ACTIN-1			
VCDN(14).(15)GSGL	1.4 ± 0.3	2.1 ± 0.6	2.5 ± 0.5
SGLV(19).(20)KAGF	2.1 ± 0.4	2.2 ± 0.4	4.5 ± 1.1
LVKA(21).(22)GFAG	2.1 ± 0.7	2.2 ± 0.8	3.6 ± 0.8
PTLL(107).(108)TEAP	2.0 ± 1.0	3.9 ± 2.4	3.2 ± 3.1
KDLY(296).(297)ANNV	1.8 ± 0.6	2.4 ± 0.9	3.5 ± 1.1
KQEY(364).(365)DEAG	2.4 ± 0.7	2.6 ± 0.6	5.8 ± 1.7
Gamma-ACTIN			
KDLY(294).(295)ANTV	1.6 ± 1.0	3.5 ± 1.6	2.7 ± 2.0
QQMW(356).(357)ISKQ	1.8 ± 0.6	1.9 ± 0.5	2.6 ± 0.4
KQEY(362).(363)DESG	1.8 ± 0.3	1.9 ± 0.3	3.4 ± 0.2
VIMENTIN			
SNRS(29).(30)YVTT	1.7 ± 0.5	1.7 ± 0.5	3.0 ± 1.5
RSLY(53).(54)SSSP	1.9 ± 0.5	1.9 ± 0.5	3.8 ± 0.6
SVDF(86).(87)SLAD	1.1 ± 0.3	1.9 ± 0.5	1.8 ± 0.6
DFSL(88).(89)ADAI	1.2 ± 0.4	1.7 ± 0.6	1.3 ± 0.1
SLAD(90).(91)AINT	1.4 ± 0.5	1.5 ± 0.5	1.6 ± 0.4
NKIL(131).(132)LAEL	1.7 ± 0.6	1.9 ± 0.6	2.4 ± 0.8
KILL(132).(133)AELE	1.8 ± 0.6	2.3 ± 0.5	3.4 ± 0.5
LQSF(206).(207)RQDV	2.1 ± 0.5	2.0 ± 0.5	3.5 ± 0.2
KSKF(295).(296)ADLS	1.6 ± 0.7	2.0 ± 0.6	2.2 ± 0.7
SLTC(328).(329)EVDA	2.8 ± 1.0	2.2 ± 0.7	4.7 ± 0.5

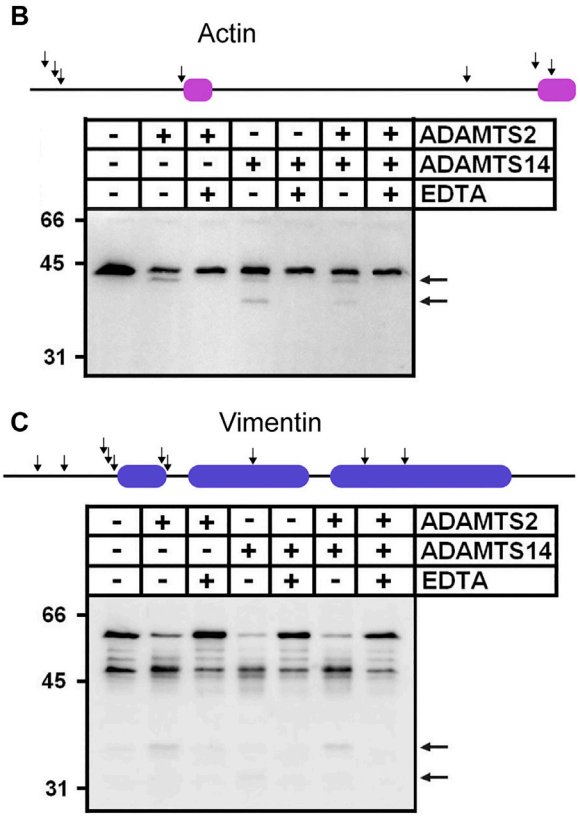


FIGURE 6 | *In vitro* cleavage of actin and vimentin by ADAMTS2 and ADAMTS14. Table showing the P/C ratios according to the cleavage sites observed by proteomic. Ratios above the average 3σ cut-offs are highlighted in green **(A)**. Western blot analysis of *in vitro* cleavage of actin **(B)** and vimentin **(C)** by ADAMTS2 and/or ADAMTS14. The schematic representation of actin and vimentin are reported on the top of the western blots with their respective actinin binding (magenta) and coiled coil (blue) domains. Human fibroblasts lysates were incubated in the presence of recombinant ADAMTS2 and/or ADAMTS14 (200 nM, 16 h at 37°C). EDTA was used as an inhibitor of metalloproteinases. Degradation products are indicated by black arrows.

cleavage sites (**Figure 7**, upper panel). This revealed an overrepresentation of G and P that could be linked, at least in part, to the abundance of these amino acids in collagens. In addition, Y, F and A were often found at P1 and A and S at P1', which is similar to previously described cleavage sites for aminoprocollagen peptidases (Bekhouche et al., 2016; Janssen et al., 2016) (**Figure 7**, upper panel). Considering all the potential extracellular substrates, excluding type I collagen (middle panel) or all fibrillar collagens (lower panel), it was shown that ADAMTS2 and ADAMTS14 display common preferential cleavage sites, enriched in small nonpolar, amphipatic or slightly hydrophobic amino acids (G, P, F, Y, A, L and M). Of note also was the presence of acidic amino acids at P2', P3' and P4'.

DISCUSSION

The procollagen N-proteinases were originally described only for their ability to specifically excise the N-terminal propeptide of fibrillar collagens (Bekhouche and Colige, 2015). However, more recently, we and others have demonstrated their roles in TGFβ signaling (Bekhouche et al., 2016) and in lymphangiogenesis through the proteolytic activation of pro-VEGF-C into VEGF-C able to interact with its receptors (Janssen et al., 2016; Brouillard et al., 2017; Dupont et al., 2018; Wang et al., 2020). The existence of marked differences between the phenotype of patients with arthrochalasia EDS (caused by the absence of the ADAMTS2 cleavage sites in type I collagen) and the phenotype of patients with dermatosparactic EDS (null mutations in ADAMTS2) is another observation arguing for additional functions of ADAMTS2. Finally, the peculiar atopic dermatitis-like skin phenotype of mice deficient in both ADAMTS2 and ADAMTS14 (Dupont et al., 2018) also points to yet to be discovered new substrates of these two related enzymes. In order to search for such potential substrates using a large-scale unbiased approach, we have compared N-terminomes of the skin of Wild type, $TS2^{-/-}$, $TS14^{-/-}$ and $TS2^{-/-}TS14^{-/-}$ mice, an experimental approach never tried before because its inherent difficulties, such as the dynamic nature of the cleavage events that could be hidden by cellular uptake, rapid degradation of the generated peptides, and potential variations in protein composition or cell abundance. Moreover, the deficiency of a protease *in vivo* can be compensated by another protease cleaving near or at the same cleavage site (Fortelny et al., 2014). As an additional challenge, skin is formed by several

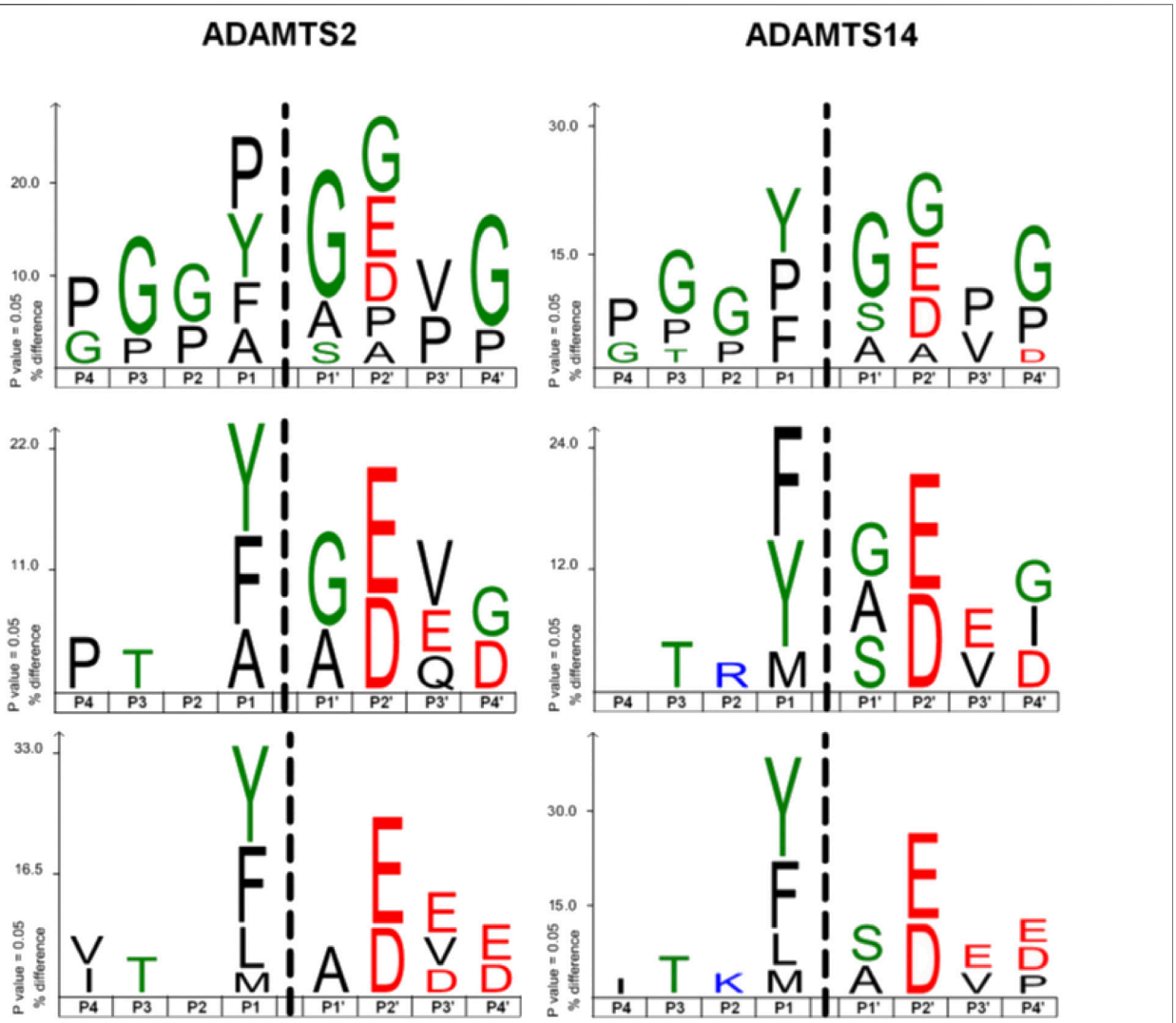


FIGURE 7 | Cleavage site specificity of ADAMTS2 and ADAMTS14 in mouse skin. The cleavage site specificity of ADAMTS2 **(left panels)** or ADAMTS14 **(right panels)** was determined using all the extracellular or extracellular transmembrane cleavage sites **(top)**, without type I collagen **(middle panels)** or without all the fibrillar collagens **(down panels)**. The amino acid sequence logos, corrected by the natural abundance of amino acids in the human proteome, were generated using the iceLogo software package (Colaert et al., 2009). The height of the amino acids represents their frequency regarding their natural abundance; the color reflects its physicochemical properties.

compartments such as *epidermis*, dermis, and muscular, nerve and blood vessels elements which may vary in abundance, a situation susceptible to induce modifications in the relative abundance of a specific protein. The use of a high throughput method and the complexity of the skin samples led to variations between the three individual experiments which prevented the identification of potential gender differences. In this study, three individual experiments have therefore been analyzed independently, in order to increase the likelihood of identifying new substrates, and have been compared between each other in a second step for validations purposes. Potential substrates were identified by taking into account only peptides harboring a P/C ratio above or below the 3-sigma cutoff from the normal distribution of natural N-termini, which constitutes a very stringent threshold. Sixty-eight proteins located at the cell surface or being part of the extracellular matrix were identified as potential substrates of ADAMTS2 and/or ADAMTS14, from which fibrillary collagens were the most represented in terms of identified peptides.

The previously described cleavage sites by ADAMTS2 of the N-propeptide of COL1A1 and COL1A2 were clearly identified, confirming the relevance of our experimental model. Of notes, however, these cleavages seem to occur within a preferential sequence rather than at a specific cleavage site as always reported so far, which illustrates that ADAMTS2 is capable to cleave multiple peptide bonds. As an additional evidence of the specificity of the N-TAILS data, cleavages of the N-propeptide of COL1A1 and COL1A2 by ADAMTS14 were only marginal, which was expected from previous *in vitro* and *in vivo* data showing that ADAMTS14 displays only a very low aminoprocollagen peptidase activity (Colige et al., 2002; Dupont et al., 2018). Having clearly demonstrated the reliability of our experimental design, we then

investigated the cleavages of the N-propeptides of type V procollagens which are still incompletely defined. For COL5A1, a cleavage by BMP1 was previously identified within the NC3 domain (Imamura et al., 1998; Bonod-Bidaud et al., 2007) but could not be identified here as expected when using models comparing Wt to Adamts2 and/or Adamts14 deficient skins. The $P_{435\text{-}436}$A site previously found for ADAMTS2 at the end of the variable domain (Colige et al., 2005) was identified here for Adamts2 but also for Adamts14. Accordingly, the P/C ratio was much higher by comparing Wt skin to $TS2^{-/-}TS14^{-/-}$ skin, indicating that both enzymes display this activity *in vivo* and, therefore, identifying a new function for ADAMTS14. Some cleavages by both enzymes were also identified in the NC2 domain of COL5A1 and COL5A2 separating the small and the large central triple helical domains, at a location similar to the ones reported for type I, type II and type III procollagens, which was never described before. The existence of several cleavage sites generating type V collagens with N-propeptides of different size and bulkiness is a new observation and is probably part of the regulation operated by type V collagen on collagen fibril formation (Linsenmayer et al., 1993; Marchant et al., 1996; Wenstrup et al., 2004) as observed *in vitro* and *in vivo* in EDS. It would also explain why arthrochalasic and dermatosparactic EDS have different clinical manifestations since both type I and type V collagen processing are altered in dermatosparaxis while only type I collagen is affected in arthrochalasia.

Besides cleavages of the N-propeptides, N-TAILS identified also several cleavages in the central COL1 domain of both alpha one and alpha 2 type I collagens. While some sites were not, or only barely, affected by the presence of ADAMTS2 or 14 (P/C < 1.5), some others were characterized by higher ratios, especially in some clusters, and when comparing Wt to $TS2^{-/-}TS14^{-/-}$ skin, which suggested specificity in these cleavages. This hypothesis was evaluated *in vitro* using purified enzymes and purified collagen, either native or heat denatured. It showed that ADAMTS2 and ADAMTS14 can cleave within the COL1 triple helical domain, but mainly (or only) when collagen is unfolded. This "collagenase" activity was similar for ADAMTS2 and ADAMTS14, while ADAMTS14 displays only a limited capacity to cleave the aminopropeptide of non-denatured procollagen which demonstrates that the two activities are independent. The existence of such function was never described before and its biological relevance will have to be further investigated. An attractive hypothesis would be that, when ADAMTS2 (or ADAMTS14) meets and interacts with its type I procollagen substrate, it cleaves the N-propeptide but it also degrades collagen trimers that are not correctly folded, therefore preventing their integration in fibrils. This quality control function of aminoprocollagen peptidases, if confirmed, would also explain why collagen fibrils have a so irregular and abnormal shape in dermatosparaxis. Indeed, in the absence of ADAMTS2, aminoprocollagen but also collagen with defects in the triple helix domains can both accumulate in fibrils and hamper the highly organized polymerization process of collagen fibrils.

Excision of the C-propeptide of type I procollagen was originally attributed to BMP1, and later extended to meprins α and β (Kessler et al., 1996; Jefferson et al., 2013). Here, our degradomic analysis has revealed major cleavage sites for ADAMTS2 and ADAMTS14 in the C-propeptide of both COL1A1 and COL1A2. This was also confirmed *in vitro* using purified enzymes and type I procollagen, and is reminiscent to what was already demonstrated for type III procollagen (Bekhouche et al., 2016). However, the functional relevance of these observations *in vivo*, when BMP1 is present and active, is not clear yet since, except for one site in COL1A1, they are located downstream of the BMP1 site. In these conditions, these ADAMTS-dependent cleavages should not affect the maturation of type I collagen and its polymerization. Since the C-propeptide of type I collagen can inhibit collagen synthesis when released in the extracellular space (Mizuno et al., 2000), an intriguing hypothesis would be that its cleavage by ADAMTS2 or 14 would affect its regulatory function.

The main purpose of this study was to get a better insight into the overall implications of ADAMTS2 and ADAMTS14 in collagen fibril homeostasis. In addition of cleavages at multiple sites in fibrillary collagens, other proteins regulating collagen fibrillogenesis were also found to be potential substrates, similarly to what was found previously regarding the cleavage of Lox (Rosell-García et al., 2019), a crucial enzyme for the formation of crosslinks stabilizing collagen fibrils. Two cleavages were found in COL14A1, a FACIT (Fibril Associated Collagen with Interrupted Triple helix) known for its capacity to interact with collagen fibrils and regulate their formation (Young et al., 2002; Agarwal et al., 2012). Similarly, type VI collagen was also identified as a likely substrate regulating fibril formation (Lamandé and Bateman, 2018; Wu and Ge, 2019). However, the functional relevance of these cleavages was not investigated further but would deserve additional characterizations.

Lumican, biglycan and osteoglycin are members of the Small Leucin-rich Proteoglycan (SLRP) family (Frikeche et al., 2016). They are considered as collagen fibril-regulating proteins, and are also emerging as factors controlling immune response. It is interesting to note that these three proteoglycans have been identified here as potential substrates of ADAMTS2 which is also implicated in immune response as previously described (Hofer et al., 2008) and further illustrated here by the specific enrichment in biological processes linked to complement activation and regulation of cytokines involved in immune response. Although still to be considered as a working hypothesis and having to be confirmed by complementary approaches, these observations could pave the way to better understand the multiple roles of ADAMTS2 and 14 in extracellular matrix formation and functions.

Analysis of the skin degradome also identified cytoplasmic proteins, such as actin and vimentin, as potential substrates of ADAMTS2 and 14. These surprising data were confirmed *in vitro* using skin fibroblasts extracts incubated with recombinant purified enzymes, but not by comparing the electrophoretic pattern of actin and vimentin in Wt and ADAMTS2-deficient fibroblasts. This could suggest that these cleavages occur only after secretion or release in the extracellular space, as observed after cell death for example. We cannot however completely rule out the possibility that ADAMTS2 and 14 could display intracellular activities, either during the secretion process, as described for procollagen processing, or after their internalization when bound to cell membrane (Dubail et al.,

2010). The proteomic identification of intracellular substrates has already been reported for MMP2(auf dem Keller et al., 2013) and by studies reporting the intracellular functions of MMPs(Jobin et al., 2017), such as α-actinin cleavage, or the transcriptional regulation of NF-kappa-B inhibitor alpha by MMP12(Marchant et al., 2014). Interestingly, these intracellular activities are related to the innate immunity system (Marchant et al., 2014) with a propensity for MMPs to regulate negatively the pro-inflammatory response (Dufour and Overall, 2013; Khokha et al., 2013). Further studies are needed to precisely assess the role the cleavage of proteins by ADAMTS2 and ADAMTS14 either in the extracellular and/or in the intracellular space, with a potential implication in cytoskeleton dynamic, gene expression and inflammatory response.

CONCLUSION

Our N-TAILS analysis of mouse skin degradomes has extended our knowledge regarding the roles of ADAMTS2 and 14 well beyond the previously known cleavage of the N-propeptide of type I, type II and type III procollagens. The identification *in vivo* of several cleavage sites in the N-terminal region of type V collagen, generating N-terminal propeptides of different size and bulkiness, is a new finding possibly explaining how type V collagen can finely tune collagen fibril formation and structure. In the same context, other molecules involved in fibrillogenesis, such as SLRPs and non-fibrillar collagens, have also been identified as likely substrates of ADAMTS2 and 14.

Another intriguing observation is the potential quality control activity leading to the degradation of incorrectly folded collagen trimers before their assembly in collagen fibrils. If this hypothesis is confirmed, this would represent a key activity of ADAMTS2 and 14 for maintaining the structural integrity of collagen fibers and connective tissues.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee for Animal Use and Care, University of Liège, Belgium.

AUTHOR CONTRIBUTIONS

Conceptualization, AC and MB; methodology, MB; formal analysis, AC DB and MB; investigation, CL CM LD CD and LJ; data curation, DB GM and MB; writing original draft preparation, MB and CL; writing review and editing, AC and MB; visualization, MB and AC; supervision, MB and AC; project administration, AC and MB; funding acquisition, AC and MB. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

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frontiers in Molecular Biosciences

REVIEW
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ADAMTS-12: Functions and Challenges for a Complex Metalloprotease

Yamina Mohamedi[1], *Tania Fontanil*[1,2], *Santiago Cal*[1,3], *Teresa Cobo*[4,5]*† and *Álvaro J. Obaya*[3,6]*†

[1]Departamento de Bioquímica y Biología Molecular, Universidad de Oviedo, Oviedo, Spain, [2]Departamento de Investigación, Instituto Ordóñez, Oviedo, Spain, [3]Instituto Universitario de Oncología, IUOPA, Universidad de Oviedo, Oviedo, Spain, [4]Departamento de Cirugía y Especialidades Médico-Quirúrgicas, Universidad de Oviedo, Oviedo, Spain, [5]Instituto Asturiano de Odontología, Oviedo, Spain, [6]Departamento de Biología Funcional, Área de Fisiología, Universidad de Oviedo, Oviedo, Spain

Nineteen members of the ADAMTS family of secreted zinc metalloproteinases are present in the human degradome. A wide range of different functions are being attributed to these enzymes and the number of their known substrates is considerably increasing in recent years. ADAMTSs can participate in processes such as fertility, inflammation, arthritis, neuronal and behavioral disorders, as well as cancer. Since its first annotation in 2001, ADAMTS-12 has been described to participate in different processes displayed by members of this family of proteinases. In this sense, ADAMTS-12 performs essential roles in modulation and recovery from inflammatory processes such as colitis, endotoxic sepsis and pancreatitis. ADAMTS-12 has also been involved in cancer development acting either as a tumor suppressor or as a pro-tumoral agent. Furthermore, participation of ADAMTS-12 in arthritis or in neuronal disorders has also been suggested through degradation of components of the extracellular matrix. In addition, ADAMTS-12 proteinase activity can also be modified by interaction with other proteins and thus, can be an alternative way of modulating ADAMTS-12 functions. In this review we revised the most relevant findings about ADAMTS-12 function on the 20th anniversary of its identification.

Keywords: ADAMTS-12, matrix metalloprotease, inflammation, arthritis, schizophrenia, cancer

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***Correspondence:**
Teresa Cobo
teresa@iaodontologia.es
Álvaro J. Obaya
ajobaya@uniovi.es

†These authors have contributed equally to this work and share last authorship

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INTRODUCTION

Since its first annotation in the human genome 20 years ago (NCBI Gene ID: 81792), ADAMTS-12 has been associated to different biological processes in both physiological and pathological conditions, including development, cancer, osteoarthritis, neurological disorders and inflammation. Following ADAMTS-12 initial identification and characterization, eight more members of the ADAMTS (*A Disintegrin And Metalloprotease with ThromboSpondin domains*) family of proteinases have been identified, making a total of nineteen the number of members of this family of metalloproteases in mammals (Kelwick et al., 2015). In general, all the members share a complex multidomain architecture. This organization includes a signal peptide, a prodomain, a catalytic domain (proteinase domain characterized by an aspartic residue at the end of the consensus Zn^{2+}-chelating motif HExxHxxGxxHD), a disintegrin-like domain, a central thrombospondin like type-1 (TSP-1) motif, a cysteine-rich domain, a spacer region and a variable number of TSP-1 repeats. Most of the members differ in the number of TSP-1 repeats at the carboxy-end region and

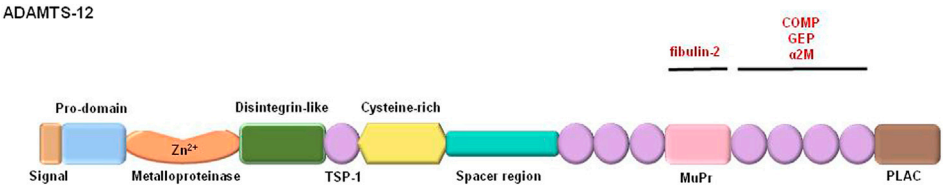

FIGURE 1 | Schematic representation of human ADAMTS-12. In black are shown the main domains of the protein; TSP-1: thrombospondin-like type-1 domain; MuPr: spacer two region. In red are shown proteins known to interact with ADAMTS-12; COMP: cartilage oligomeric matrix protein; GEP: granulin epithelial precursor; α2-macroglobulin.

also by the presence of additional motifs like PLAC, MuPr, *cub* or GON-1 domains (Porter et al., 2005; Kelwick et al., 2015).

Human ADAMTS12 gene maps at position 5p13 and spans approximately 368.45 Kb of genomic DNA containing a total of 24 exons (HGNC: 14605). Conceptual translation of human ADAMTS12 cDNA indicates that it encodes for a 1594 amino acid extracellular protein with an estimated molecular weight of 178 kDa (UniProtKB–P58397). ADAMTS12 expression was detected in a variety of tissues like cartilage, lung, kidney, liver, synovium, tendon, skeletal muscle, adipose (Liu, 2009; El Hour et al., 2010; Moncada-Pazos et al., 2012). It is also widely expressed in tumors from different origins and mainly associated with the gastrointestinal tract, suggesting that this enzyme could also participate in the development or progression of this type of tumors (Liu et al., 2006b; Moncada-Pazos et al., 2009; Wang et al., 2011; Fontanil et al., 2014; Liang et al., 2020; Rabadan et al., 2020).

Several studies performed to characterize ADAMTS-12 function suggest its role as a host-protective enzyme with antitumor properties (Llamazares et al., 2007; Moncada-Pazos et al., 2009; El Hour et al., 2010; Wang et al., 2011; Rabadan et al., 2020). ADAMTS-12 also participates in other pathological situations in which a common trail of an inflammatory outburst is present such as the allergen-induced hyperresponsiveness detected in asthma or the extracellular matrix degradation observed in osteoarthritic processes (Liu, 2009; Moncada-Pazos et al., 2012; Nah et al., 2012; Paulissen et al., 2012). Furthermore, in support of previous observations, by different genomic approximations the ADAMTS-12 locus has been associated with several human pathologies like asthma, schizophrenia or predisposition to pediatric stroke (Kurz et al., 2006; Arning et al., 2012; Bespalova et al., 2012). In recent years, genome-wide and bioinformatics analysis techniques have been used with results supporting ADAMTS12 participation in those pathological processes and also in new situations with the benefit of the possibility of being used as a prognosis biomarker (Hu et al., 2020; Liang et al., 2020; Zhang et al., 2020). However, besides its participation in pathological situations much more research is needed to identify ADAMTS-12 interacting partners and its substrates in normal or pathological processes. This fact relies in the complex structure of ADAMTS-12 in which multiple domains can be identified and thus, multiple interacting regions are possible for other components of the extracellular matrix (ECM) (**Figure 1**).

The main processes in which ADAMTS-12 has been proposed to participate will be discussed in the following sections, focusing in the importance of those biological processes in which the associations with ADAMTS-12 have been described.

ADAMTS-12 in Inflammation

Initial studies aimed to decipher ADAMTS-12 biological function indicate its role as a critical mediator in inflammation processes. Thus, in the human genome screening program ADAMTS12 was identified as a putative asthma associated gene (Kurz et al., 2006). Screening was made in two different populations to identify a chromosome 5-linked asthma or bronchial hyperresponsiveness locus by genotyping 89 single nucleotide polymorphisms (SNPs) in 22 genes and in two different populations (Kurz et al., 2006). Further, *in vivo* studies were made taking advantage of the availability of the *Adamts12* deficient mice and the use of different functional approaches (El Hour et al., 2010; Moncada-Pazos et al., 2012; Paulissen et al., 2012). Two different models of allergic airways disease were used to challenge the *Adamts12* deficient mice, ovalbumin (OVA) and house dust mite asthma-induced models. In absence of Adamts-12, an elevated eosinophilic inflammation, together with increased levels of mast cells and IL-33, were observed in the alveolar environment suggesting a protective role of this proteinase against this inflammatory process (Paulissen et al., 2012).

Other models of inflammation were performed in the same genetic background and compared to normal mice such as colitis, pancreatitis and endotoxic sepsis. Overall, absence of *Adamts12* in mice resulted in a more severe inflammatory phenotype. As mentioned before, a protective role of ADAMTS-12 in inflammatory processes is suggested for these three models which, at the same time, showed some common cellular and molecular patterns. Mice lacking Adamts-12 exhibited an increased tissue damage subsequent to inflammation, accompanied by high levels of pro-inflammatory cytokines like IL-6, IL-11, GCSF, S100A8, S100A9 and also hemopexin, a free heme scavenger protein known for delaying apoptosis of neutrophils (Arruda et al., 2004). Furthermore, neutrophil accumulation has been observed in all the models and in the insulted organs of *Adamts12* deficient mice which suggested a required key role of Adamts-12 in neutrophil clearance and subsequent resolution of the inflammatory process (Moncada-Pazos et al., 2012).

All together these data suggest that ADAMTS-12 has a protective role or acts as an intermediate factor in the resolution in these inflammatory processes, In addition,

absence of this proteinase results in a sustain inflammatory phenotype.

ADAMTS-12 in Orteoarthritis

Osteoarthritis (OA) and rheumatoid arthritis (RA) are believed to be the most prevalent rheumatic diseases, affecting approximately 11% (10% OA and 1% RA) of the world population (Frangos and Maret, 2020). Both, RA and OA are debilitating degenerative diseases affecting articular joints which are characterized by a deregulation in joint or bone homeostasis. Physiological balance is altered and results in the degradation of articular cartilage, alterations in the subchondral bone mass, and also it is also characterized by the presence of localized inflammatory events (Dancevic and McCulloch, 2014; Perez-Garcia et al., 2019a). In addition, this local inflammation is associated with an increase in the production of zinc-dependent matrix metalloproteases which are responsible for the degradation of main components of the extracellular matrix of the cartilaginous tissue (Kevorkian et al., 2004). MMPs and ADAMTSs are known proteases that participate in this process by cleaving some of these components; such is the case of COMP (Cartilage Oligomeric Matrix Protein) and the hyalectan aggrecan (Dickinson et al., 2003; Sandy, 2006). Among them, the association of ADAMTS-12 with arthritis and osteoarthritis has been described in several studies along with other members of the ADAMTS family such as ADAMTS-7, and the aggrecanases ADAMTS-4 and ADAMTS-5 (Lin and Liu, 2009; Lin and Liu, 2010).

ADAMTS-12 and ADAMTS-7 share a very similar structure, showing unique domains and thus forming their own subgroup within the ADAMTS family (Kelwick et al., 2015). It is suggested that both enzymes may play key roles in the onset of arthritic events through the ability of cleaving COMP, a pentameric glycoprotein that is part of the extracellular matrix and is used as an indicator of accelerated joint erosion (Liu et al., 2006a; Liu et al., 2006b; Kullich et al., 2007; Liu, 2009). The cleavage of COMP requires an interaction of the EGF-like domain of COMP with the four carboxy-end type 1-thrombospondin repeats found in both ADAMTS-7 and ADAMTS-12 (Guo et al., 2010; Lin and Liu, 2010). The recognition of these binding sites was initially observed by a yeast-two-hybrid genomic screening and subsequently demonstrated by co-immunoprecipitation techniques and *in vivo* detection (Liu et al., 2006a; Liu et al., 2006b). In fact, ADAMTS-12 participation in arthritic processes has been mainly related to its ability to degrade COMP because fragments generated following its cleavage were found in diseased cartilage, synovial fluid and in serum of patients with knee injuries, osteoarthritis and rheumatoid arthritis (Neidhart et al., 1997; Dickinson et al., 2003; Lin and Liu, 2009). Furthermore, COMP fragments observed in cartilage explants of osteoarthritis patients showed similar size to those identified from *in vitro* studies (Luan et al., 2008). Elevated levels of ADAMTS-12 in OA and RA, and ADAMTS-7 in RA were detected in the same tissues in which products generated by the cleavage of COMP were identified suggesting that COMP and these enzymes may co-localize (Liu et al., 2006a; Liu et al., 2006b; Luan et al., 2008; Lin and Liu, 2009). In 2008 Luan *et al.* demonstrated that the COMP fragments found in the cartilage of six patients with OA showed the same size (110 kDa) than those obtained by *in vitro* degradation of COMP by both proteases. In addition, in cartilage explants the expression of both enzymes increased with the expression of TNF-α and IL-1β, therefore favoring COMP cleavage. Moreover, inhibition with antibodies that block either ADAMTS-7 or ADAMTS-12 as well as the use of specific siRNAs kept COMP unprocessed in both, the explants and human chondrocytes (Luan et al., 2008). Additional finding linking ADAMTS-12 with osteoarthritis pathologies came from the identification of ADAMTS12 polymorphisms associated with RA. More specifically three SNPs: rs1364044, intron C/T; rs10461703, intron C/T; rs25754, and a missense Thr1495Ile) were identified in this pathology (Nah et al., 2012). All of it together with the fact that *ADAMTS12* is one of the genes up-regulated in OA with high levels in cartilage, synovial fluid and serum of arthritic patients supports the idea of *ADAMTS12* to be considered as a *bona fide* gene for the development of anti-RA therapies (Kevorkian et al., 2004; Liu et al., 2006b; Nah et al., 2012).

Further confirmation of ADAMTS-12 participation in osteoarthritis processes was recently demonstrated through the employment of an *in vivo* collagen-induced arthritis (CIA) model by using *Adamts12* deficient mice (Wei et al., 2018). Mice lacking Adamts-12 developed accelerated inflammatory arthritis when compared to their control littermates. Bone destruction, increase in synovitis, higher cartilage loss, and increased osteoclastogenesis were the main alterations observed in *Adamts12* deficient mice after CIA. This work also pointed out that ADAMTS-12 participation in the arthritic phenotype may be in part caused through proteolytic processing of Connective Tissue Growth factor (CTFG) by ADAMTS-12. The observed data also suggested that CTFG may be a downstream mediator of ADAMTS-12 participation in the inflammatory process (Wei et al., 2018). In this context, ADAMTS-12 is suggested to act as a factor that prevents inflammatory arthritis. In fact, absence of CTGF degradation in *Adamts12* deficient mice after IL-1β stimulation led to an increase of pro-inflammatory markers like NOS-2, IL-6 and COX-2 and a reduction of IL-10, a known anti-inflammatory molecule (Wei et al., 2018). Therefore, this data suggests again a protective role for ADAMTS-12 in arthritic events through modulation of the inflammatory landscape.

Interactions between ADAMTSs and different ECM components are of important relevance regarding the participation of these metalloproteases in the regulation of extracellular matrix homeostasis. In fact, degradation of COMP by ADAMTS-7 and ADAMTS-12 is naturally inhibited by the presence of alpha-2-macroglobulin (α2M), which is also an endogenous substrate of both proteases (Luan et al., 2008; Wan et al., 2012). ADAMTS-7 and ADAMTS-12 have also been described to interact and co-localize with Granulin-Epithelin Precursor (GEP), an autocrine growth factor expressed in different pathologies, including RA and OA (Justen et al., 2000). In turn, GEP is also able to block COMP degradation by these two enzymes (Bai et al., 2009a; Guo et al., 2010). GEP binding to either ADAMTS-7 or ADAMTS-12 matches the same domains to which COMP interaction occurs and thus can be

considered as a competitive inhibitor. GEP and COMP bind to the four C-terminal TSP-I like motifs of both ADAMTSs and it does so in a dose dependent manner. GEP interaction resulted in a GEP proteolytic cleavage that releases several fragments of ~6 kDa known as granulins that can attach to the carboxy-end of both enzymes (Davidson et al., 2004). GEP not only competes directly with COMP for ADAMTS binding but also reduces the levels of both, ADAMTS-7 and ADAMTS-12 through downregulation of TNF-α (Luan et al., 2008).

Cartilage remodeling by uncontrolled activity of chondrocytes is not the unique hallmark in the development of OA since other processes, such as synovial inflammation can also occur (Di Nicola, 2020; Rocha et al., 2021). In this sense, synovial fibroblasts (SF) can also be included as important players in joint destruction in both RA and OA with an invasive phenotype. SF, in an inflammation environment, are known to produce and secrete various members of the ADAMTS family which are able to degrade cartilage components such as aggrecan and COMP (Lefevre et al., 2015; van Nie et al., 2020). Moreover, inflammatory cytokines like IL-1β as well as fibronectin fragments can induce an increase of ADAMTS-12 expression, both at the mRNA and protein levels, in SF from OA patients when compared with healthy synovial fibroblasts and therefore increase the degradation of COMP. Something similar occurs with ADAMTS-7 with the difference that in this case it occurred in both types of fibroblasts (Perez-Garcia et al., 2016).

Mechanism by which IL-1β mediates ADAMTS-7 and ADAMTS-12 expression in SF was recently described and involves two different pathways, Runx2 and Wnt/β-catenin. Presence of specific inhibitors for both pathways in SF co-cultured with cartilage explants from patients with OA were used to demonstrate that ADAMTS-7 expression depends on the Wnt/β-catenin pathway, whereas ADAMTS-12 depends on the ERK pathway and ultimately in the activation of Runx2. It was also shown that IL-1 β can induce expression of ADAMTS-7 and ADAMTS-12 which led to COMP degradation. However, while IL-1β can induce both ADAMTSs, the presence of fibronectin fragments was only able to induce ADAMTS-12 expression and the consequent COMP degradation (Perez-Garcia et al., 2019b).

In summary, involvement of ADAMTS-12 in arthritic processes has been demonstrated by its ability of degrading essential components such as COMP. Production of inflammatory mediators (for example IL-1β) induces ADAMTS-12 expression which through COMP cleavage releases other component of the extracellular matrix with proinflammatory effects. As a result, all these molecular interactions generate a positive feedback loop that increases joint damage which is a characteristic observed in osteoarthritic processes (Liu et al., 2006b; Perez-Garcia et al., 2016; Perez-Garcia et al., 2019b). In addition, ADAMTS-12 seems to be also responsible for limiting inflammatory arthritis by modulating pro- and anti-inflammatory molecules. Degradation of other ECM molecules like CTFG can be considered part of an important mechanism for the resolution of the arthritic inflammatory phenotype.

ADAMTS-12 in Chondrogenesis

ADAMTS-12 plays an important role in the regulation of chondrogenesis and cartilage development. Levels of ADAMTS-12 have been detected strongly upregulated during chondrogenesis and also in proliferating and hypertrophic chondrocytes (Jin et al., 2007; Bai et al., 2009a; Bai et al., 2009b; Jahangir et al., 2020). ADAMTS-12 role in chondrogenesis is related to an important chondrogenic regulator, Parathyroid Hormone-Related Peptide (PTHrP) (Rajagopal et al., 2020). A feedback loop beween PTHrP and ADAMTS-12 has been observed in terms that ADAMTS-12 induces PTHrP and ADAMTS-12 is barely detectable in PTHrP -/- growth plate chondrocytes since PTHrP, at the same time induces ADAMTS-12 expression. The effects caused by these two molecules result in the inhibition of chondrocyte differentiation through the inhibition of genes such as type II collagen (Col II) or Sox9, markers of chondrocyte differentiation (Bai et al., 2009b).

On the other hand, genetic studies in a family of patients with autosomal-dominant Brachydactyly Type E disorder had led to the identification of a (8; 12) (q13; p11.2) translocation that affected the PTHLH gene which encodes for PTHrP (Maass et al., 2010). Since this translocation affects PTHrP locus, it also has consequences on its downstream targets, ADAMTS-7 and ADAMTS-12, therefore affecting normal chondrogenic differentiation.

Another molecule that regulates the activity of ADAMTS-12 in chondrogenesis is the transcription factor c-Maf, a member of the Maf family of basic ZIP transcription factors (bZIP). Among other functions, c-Maf is known for its involvement in chondrogenesis (Hong et al., 2011; Hong et al., 2013). Thus, expression of c-Maf is maximal in hypertrophic chondrocytes during embryonic development and postnatal growth. On the other hand, ADAMTS-12 expression increases during *in vitro* chondrogenesis of human mesenchymal stem cells as well as during mouse embryonic limb development (Bai et al., 2009b; Hong et al., 2011), A "Maf recognition element" (MARE) has been identified in the ADAMTS12 proximal promoter where, through the use of luciferase reporter assays, c-Maf binding has been demonstrated to induce ADAMTS-12 expression. Therefore, c-Maf effects on differentiation and hypertrophy of chondrocytes might be in part through up regulation of ADAMTS-12 (Hong et al., 2013).

ADAMTS-12 in Degenerative Intervertebral Disc

Between each vertebral body of the spine are pads of fibrocartilage-based structures that provide support, flexibility, and a better load distribution known as intervertebral discs. Each of them also contains a soft tissue within the interior which is known as nucleus pulposus (Donnally et al., 2021). As happens in arthritic processes and other inflammatory disorders, the intervertebral discs can be subjected to degradation by molecules capable of processing extracellular matrix components as is the case of MMPs and ADAMTSs (Le Maitre et al., 2007). In this regard, high levels of ADAMTS-7

and ADAMTS-12 have been observed in nuclei pulposus extracted from rats in which disc degeneration was generated through the use of an external compression device (Yu and Zhu, 2012). These results were accompanied by an increase in COMP fragments (Liu et al., 2006b; Yu and Zhu, 2012). In addition, levels of both proteases are increased in endplate cells isolated from patients with IVD suggesting their participation in this pathological process through degradation of the ECM components (Zhang et al., 2012).

All of these data would indicate that ADAMTS-7 and ADAMTS-12 contribute to intervertebral disc degeneration through a molecular mechanism similar to that described for the osteoarthritic disorders (Yu and Zhu, 2012; Zhang et al., 2012).

ADAMTS-12 in Ossification and Tendon Degeneration

Presence of higher levels of ADAMTS-7 and ADAMTS-12 at mRNA and protein levels in degenerative IVD when compared with normal IVD seems to be associated with low levels of Col II, Sox9 and Col X, known chondrogenesis marker genes (Zhang et al., 2012). ADAMTS-7 and ADAMTS-12 are associated to cartilage destruction and endochondral ossification (Lin and Liu, 2009). *Adamts7* and *Adamts12* deficient mice are viable with no apparent phenotype. *Adamts7* knockout was initially used to validate the association of Adamts-7 with atherogenesis while *Adamts12* knockout served as a model to describe Adamts-12 antitumoral function (El Hour et al., 2010; Bauer et al., 2015). Both proteases share an important structural homology and have shown mutual compensation in hindlimb tendons, with upregulation of one of them when the other one is absent (Mead et al., 2018). Double knockout of both ADAMTSs has been generated to prove their participation in skeletal development and more specifically, in heterotopic ossification (HO) of tendons and ligaments (Mead et al., 2018). Furthermore, these mice developed normally, with no skeletal anomalies till the age of 4 months when HO was identified within the quadriceps tendon, Achilles tendon and menisci (Mead et al., 2018). Extension of the study to human pathologies showed that reduction of ADAMTS-7 and ADAMTS-12 immunostaining was evident in human degenerative biceps tendons although only ADAMTS7 mRNA was reduced significantly (Mead et al., 2018).

Participation of ADAMTS-12 and ADAMTS-7 in ECM remodeling in musculoskeletal tissues is important for homeostasis maintenance. The above studies indicate that both proteases are basally expressed in bone, cartilage, synovium, ligament and tendons and thus being implicated in normal turnover of these tissues. Alterations on their expression could lead to the development of different pathologies like arthritis, IVD degeneration and ossification. Furthermore, the structural similarity of ADAMTS-7 and ADAMTS-12 can generate compensatory mechanisms between these proteases in those body compartments in which both are normally expressed.

ADAMTS-12 in Neurological Disorders

In 2012, Bespalova et al. (Bespalova et al., 2012) identified a schizophrenia susceptibility locus on chromosome 5p13. This region contains the ADAMTS12 gene and to study its potential involvement in the disease, a mutation analysis was performed in Puerto Rican patients of Spanish descent. That analysis revealed that one intronic variant and two SNP haplotypes were closely linked to the susceptibility of developing schizophrenia. Previously, sequence variants in the ADAMTS12 gene had also been associated to bipolar disorder and narcolepsy using genome-wide association studies (GWAS) in Japanese population (Hattori et al., 2009; Koike et al., 2009). GWAS technique has also allowed to include ADAMTS12 gene as functionally linked to Alzheimer´s disease (Wang et al., 2015). Taking together, those studies showed clear evidence that genetic variations of the ADAMTS12 gene could underlie mental health disorders.

Also in 2012, a study was published highlighting the association between the ADAMTS12 gene and pediatric stroke using GWAS (Arning et al., 2012). Further evidence that ADAMTS12 could display a role in pediatric stroke was obtained through the sequencing and fine mapping of ADAMTS12 gene variants (Witten et al., 2020). Similar type of analysis was performed to determine that an ADAMTS-12 variant could offer a protective effect against cerebral aneurysm (Arning et al., 2016). These results suggest that ADAMTS12 could be considered a very promising factor to analyse in relation to its capacity to associate to other ECM components of the CNS. Moreover, ADAMTS-12 might also potentially induce modifications in ECM components through its catalytic activity. In consequence, an altered ADAMTS-12 metalloprotease could lead to a disrupted ECM. These studies also open the possibility of ADAMTS-12 could be a potential diagnostic and prognostic biomarker in cerebrovascular diseases. However, it would be necessary to explore the functional mechanisms associated to ADAMTS-12 activities in neurological system.

In order to shed light about the functional role of ADAMTS12 in the central nervous system, we recently studied its expression in different areas of mouse brain at different developmental stages (Fontanil et al., 2019). Expression was detected at different levels in embryonic stages, but was very low or undetected in the postnatal or adult stages analyzed. As a potential substrate of ADAMTS-12 in brain, neurocan expression was also evaluated with the finding that this hyalectan displayed a similar expression pattern as that shown by the metalloprotease. Gene expression analysis indicates that both, neurocan and ADAMTS12, can be detected in the olfactory bulb in the brain of adult mice. *Adamts12* knockout mice develop normally with no apparent behavior deficiencies or CNS alterations. However, the absence of Adamts-12 results in neurocan accumulation in specific areas of the CNS such as olfactory bulb, hypothalamus and spinal ganglia of new born mouse (Fontanil et al., 2019). This accumulation potentially excludes neurocan processing by other proteases. Neurocan cleavage not only by ADAMTS-12 but also by other ADAMTS family members could be crucial for normal maintenance of brain function (Tauchi et al., 2012; Gottschall and Howell, 2015; Fontanil et al., 2021). In this regard, neurocan

has been related with important functions in brain development. For instance, it has been recently reported that neurocan contributes to the perineuronal nets formation during postnatal development in mouse (Schmidt et al., 2020). Moreover, neurocan levels increase during traumatic brain injury (Asher et al., 2000), which could contributed to generate a three-dimensional net together other ECM components for cells in order to repair the damage (Fontanil et al., 2019). It is also noteworthy that gene encoding neurocan, NCAN, has been associated with mental health diseases (Avram et al., 2014) including schizophrenia and bipolar disorder (Muhleisen et al., 2012; Oruc et al., 2012; Wang et al., 2016) or related with the etiology of mania (Miro et al., 2012). Different studies have connected the rs1064395 SNP within NCAN gene with those disorders (Raum et al., 2015; Wang et al., 2016). It has been recently shown that the NCAN rs1064395 A allele is linked to lower hippocampus-dependent memory function (Assmann et al., 2020). Furthermore, that SNP influences the expression of the neighboring HAPLN4 gene (Assmann et al., 2020). This gene codes for the ECM link protein Hapln4/Bral2, which show a strong expression in human cortex (Bekku et al., 2003; Spicer et al., 2003). These studies point to the strong influence of this genetic variant of the NCAN gene on the ECM composition, highlighting the influence of neurocan in both normal and pathological conditions of the CNS. In addition, a growing body of evidence suggests that ADAMTS-12 could influence brain function through different mechanisms, including its functional relationship with neurocan.

ADAMTS-12 in Cancer

ADAMTS-12, like many other members of the ADAMTS family, may have a pro- or anti-tumor role in various types of cancers. Initially, ADAMTS-12 was described as a tumor suppressor protein, as the exogenous expression of ADAMTS-12 in Madin-Darby Canine Kidney (MDCK) cells prevents the phenotypic changes associated with renal carcinogenesis caused by the growth factor of hepatocytes (HGF). This effect is due to the thrombospondin motifs that this enzyme exhibits at the C-terminal end, and which block the Ras-MAPK signalling pathway (Llamazares et al., 2007). MDCK cells expressing a truncated form of ADAMTS-12 lacking the TSP-1 repeats also showed tumorigenic effects compatible with those previously described. ADAMTS-12 overexpression in A549 lung adenocarcinoma cells inhibits subcutaneous tumor formation in immunodeficient SCID mice in comparison with animals injected with A549 parental cells. In addition, ADAMTS12 gene is epigenetically silenced by hypermethylation of its promoter in tumors of different sources, which reinforced the hypothesis of ADAMTS12 as a tumor suppressor (Moncada-Pazos et al., 2009). Furthermore, in colon tumors there is a dual regulation of ADAMTS-12 expression: first, an epigenetic inactivation in colon cancer cells due to high levels of methylation of ADAMTS12 promoter. And second, an overexpression of ADAMTS-12 by the fibroblasts surrounding neoplastic cells was detected, which suggest a protective stromal response to reduce tumor progression. Other studies support the protective role of ADAMTS-12 in colorectal cancer (Wang et al., 2011; Zheng et al., 2019). However, it has also been described that ADAMTS-12 can promote the migration and proliferative properties of HCT116 colorectal carcinoma cells by activating the Wnt/β-catenin signaling pathway (Li et al., 2020a). On the other hand, the phenotypic analysis of the *Adamts12* deficient mouse indicated that this protein plays a protective role against angiogenesis and tumor growth. In fact, mice lacking an active Adamts-12 showed high levels of vascularization and tumor invasion after malignant keratinocyte transplantation (El Hour et al., 2010). These mice also showed a greater number of lung tumors when compared with their normal littermates after administration of the carcinogen urethane. In addition, *in vitro* studies using M-38 lung carcinoma cells revealed an increase in their proliferative and invasive potential after being depleted of ADAMTS-12 using RNA interference techniques. Protective role of ADAMTS-12 in lung cancer was also supported through the employment of bioinformatic approaches. This analysis would indicate that patients with lung adenocarcinoma in which truncating mutations in the ADAMTS12 gene were detected, would have a worse prognosis than those with the unaltered gene (Rabadan et al., 2020).

A protective role of ADAMTS-12 has also been found in breast cancer through its association with fibulin-2 (Fontanil et al., 2014). In this case, fibulin-2/ADAMTS-12 interaction was demonstrated after yeast-two-hybrid screening and inmunoprecipitation studies. ADAMTS-12 exogenously expressed in breast cancer cells increases the formation of subcutaneous tumors in immunodeficient SCID mice, and the capacity of migration, invasion, and mammosphere formation of these cancer cells, which can be associated to a pro-tumoral effect. However, presence of fibulin-2 and ADAMTS-12 is able to block those properties suggesting a protective role induced by that interaction (Fontanil et al., 2014). In fact, fibulin-2 can be degraded by ADAMTS-4 and ADAMTS-5 under the same experimental conditions, which results in an increase of the tumoral properties of breast cancer cells (Fontanil et al., 2017). The interaction of ADAMTS-12 with fibulin-2 results in a blockade of the proteolytic degradation of fibulin-2 by ADAMTS4 and ADAMTS-5 and therefore, inhibition of the tumoral properties of breast cancer cells (Fontanil et al., 2017).

In a stark contrast, ADAMTS-12 has also been associated to protumoral effects. Thus, it has been shown that the overexpression of ADAMTS-12 in human trophoblastic cells enhances their invasive phenotype through regulating the expression and function of the integrin $\alpha_v\beta_3$ (Beristain et al., 2011). In addition, ADAMTS-12 plays a pro-tumoral role in breast cancer, by increasing the formation of subcutaneous tumors in immunodeficient SCID mice, and the capacity for migration, invasion, and mammosphere formation in breast cancer tumor cells. However, when ADAMTS-12 interacts with fibulin-2, these effects are reversed, promoting an antitumor role (Fontanil et al., 2014). Other studies seem to suggest the tumorigenic function of this protein since the gene encoding for ADAMTS12 has been identified as one of the genes overexpressed in ovarian cancer patients with intestinal metastases (Mariani et al., 2019), in patients with metastatic

renal carcinoma (Ho et al., 2017), gastric carcinoma with poor prognosis (Liang et al., 2020) and esophageal squamous cell carcinoma (Li et al., 2020b).

With the previous data on mind, analysis and correlation studies are of key importance to decipher the precise role of ADAMTS-12 in cancer development. In fact, ADAMTS-12 seems to be preferentially associated with cancer associated fibroblasts (CAFs) and immune cells (macrophages) in colorectal cancer (Moncada-Pazos et al., 2009; Wang et al., 2011). ADAMTS-12 has been specifically detected in activated fibroblasts in the proximity of colon cancer cells. Furthermore, co-cultured fibroblasts are able to induce colon cancer cells death by apoptotic mechanisms (Moncada-Pazos et al., 2009). In the urethane-induced lung cancer model, ADAMTS-12 is present in cells surrounding the highly proliferative cells within the tumor (Rabadan et al., 2020). In breast cancer, ADAMTS-12 expression is localized preferentially surrounding the tumoral tissue and high expression is correlated with good prognosis of these patients (Fontanil et al., 2014). On the other hand, expression analysis in gastric cancer has revealed high levels of ADAMTS-12 in tumoral samples when compared to adjacent tissue and a correlation with poor prognosis (Liang et al., 2020). In this sense, the availability of gene expression databanks can be a great tool to analyze gene participation in cancer. However, care must be taken in order to define gene groups when analyzing complex samples from tumors in which types of cells from different origin can be found (fibroblasts, cancer cells, endothelial, muscle cells and immune cells among others). In addition, ADAMTS-12 participation in the resolution of inflammatory processes should also be taking into account in local cancer development. Inflammatory molecules and the immune response have been described to be drivers and to potentiate cancer development (Suarez-Carmona et al., 2017; Gupta et al., 2018). Pro-inflammatory cytokines are also potent chemoattractants for immune cells that, consequently, increase the local inflammatory environment. ADAMTS-12 participation in modulation and resolution of inflammatory processes might as well be part of its antitumoral effect. In fact, ADAMTS-12 can reduce levels of inflammatory molecules by degradation of CTFG and, at the same time it is also able to induce neutrophil clearance by inducing cell apoptosis (Moncada-Pazos et al., 2012; Wei et al., 2018).

In conclusion, ADAMTS-12 can be related to act as a tumoral suppressor and also to act as a protumoral agent. In this sense, ADAMTS-12 function may depend on several factors, type of cells in which it is expressed (cancer cells, inmune or stromal cells), the appearance of ADAMTS-12 mutants (point mutations, truncated forms, protein processing) as well as the interactions with other components of the ECM.

ADAMTS-12 in Fertility Disorders

The initial phenotypic analysis of *Adamts12* deficient mice showed no alterations in reproduction, with normal gestation periods and fertility. However, the expression of *Adamts12* in wild type mice was detected in tissues such as the mammary glands, uterus and ovary, but not in testes (El Hour et al., 2010). In addition, ADAMTS-12 plays a role in angiogenesis and inflammation, which may imply that this protein can be involved in processes such as the formation of the placenta and problems associated with pregnancy as is the case of pre-eclampsia. Moreover, ADAMTS-12 affects the invasion of trophoblastic cells and their adhesion to components of the extracellular matrix by regulating the expression of the integrin $\alpha_v\beta_3$ (Beristain et al., 2011). In fact, high levels of ADAMTS-12 are detected in placenta during the first three months of pregnancy, which highlights the important role that this protein plays in the implantation of this organ. Regarding the role of ADAMTS-12 in pre-eclampsia, data are controversial, as a decrease in ADAMTS-12 levels have been detected in women with pre-eclampsia (Eda Gokdemir et al., 2016). However, other studies found an increase in ADAMTS-12 levels in placenta (but not in maternal blood or umbilical cord) in patients with pre-eclampsia (Namli Kalem et al., 2018). Finally, other studies did not detect differences in these groups (Daglar et al., 2016; Kirbas et al., 2016; Oztas et al., 2016) but a decrease in levels of ADAMTS-12 in placenta during pregnancy-associated cholestasis (Oztas et al., 2016). Recently, it has also been reported that women with spontaneous preterm birth present hypomethylation of ADAMTS12 promotor in the placenta tissue (Mani et al., 2019).

Perspectives in ADAMTS-12 Functional Biology

During the past two decades several studies have associated ADAMTS-12 with different pathologies as well as being part of physiological processes (**Figure 2**). ADAMTS-12 has been initially identified as a member of the wide family of Zn^{2+}-metalloproteases. However, ADAMTS-12 needs to be considered much more than a simple proteinase due to its complex structure that contains multiple different domains able to interact with other components of the ECM. These interactions not only could be important for the proteolytic activity of the enzyme but can also sequester different factors, bring to proximity components of the extracellular matrix or trigger cellular responses. For instance, it is known that ADAMTS-12 interacts with other ECM components like fibulin-2, CTFG, COMP, GEP, α2M and can also interact with growth factors such as VEGF (Liu et al., 2006b; Llamazares et al., 2007; Luan et al., 2008; Wan et al., 2012; Fontanil et al., 2014; Wei et al., 2018). In addition, ADAMTS-12 is known to be processed which generates two fragments, one with the proteolytic domain (amino-terminal end) and the other one containing different anchoring domains (mainly TSP-1 repeats) (carboxy-terminal end) which, in turn, can generate the appearance of different activities (Llamazares et al., 2007; Beristain et al., 2011; Wei et al., 2018). All these facts could be relevant to ADAMTS-12 biological function since it is generally accepted that C-terminal domains of metalloproteinases are often important for determining substrate specificity (Martel-Pelletier et al., 2001).

ADAMTS-12 participates in different processes related to an inflammatory response such as asthma, colitis, sepsis, pancreatitis and osteoarthritis. In this sense, mechanisms about ADAMTS-12 participation and regulation of these inflammatory events are

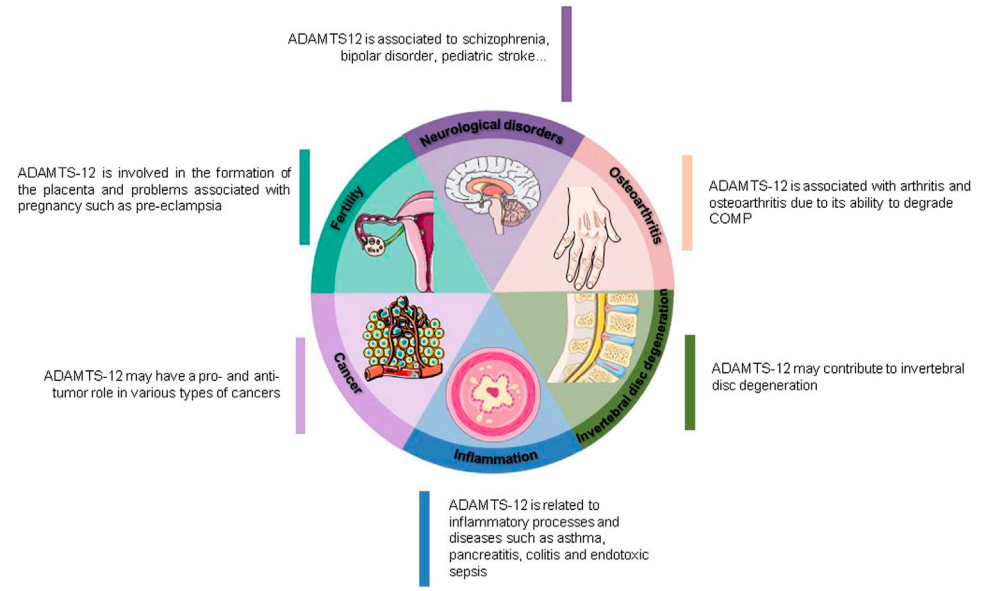

FIGURE 2 | ADAMTS-12 biological implications.

known to be through interaction and degradation of different components of the ECM. It is interesting to note that this enzyme is able to cleave key proteins of cartilage like COMP and, at the same time, degrade other components like CTGF which seems to be required for reducing the inflammatory response (Liu et al., 2006b; Wei et al., 2018). In addition, ADAMTS-12 may have other anti-inflammatory actions by blocking the appearance of elevated levels of pro-inflammatory molecules as well as a role in neutrophil clearance (Moncada-Pazos et al., 2012). Therefore, ADAMTS-12 can be considered an important player in mediating and resolving the inflammatory response and, its absence may result in pathologies known to be due in part to a not well resolved inflammatory process like can be the case of schizophrenia (Asher et al., 2000; Fontanil et al., 2019).

In summary, ADAMTS-12 can be a key player in the control and regulation of different biological processes, both normal and pathological. It is clearly associated with inflammatory processes but also seems to have an important role in cancer, neurological disorders as well as being related with fertility disorders. Further studies on patients with infrequent anomalies as well as those that have been treated with radioisotopes have found that ADAMTS-12 can also be part of the biological mechanism of Freiberg-s infraction, brachydactyly A1B as well as salivary gland damage (Koca et al., 2013; Mysliwiec et al., 2015; Sadic et al., 2016).

As mention in this review, ADAMTS-12 has a complex architecture which can support different activities and hence, be associated to different physiological a pathological processes. The availability of mice deficient in this protease is a useful tool in order to demonstrate its participation in those processes through the use of different experimental models. Furthermore, bioinformatics applications are also being widely used to make associations of ADAMTS-12 with several human diseases. However, more bench work is needed to solve ADAMTS-12 biological function, its interactions with other components of the ECM, the existence of specific substrates and the implications of the different parts of the molecule in regulating those functions. It would be also crucial to identify new interacting partners of this complex metalloprotease within the ECM. This would open the possibility to clarify controversial aspects about the functional relevance of ADAMTS-12.

AUTHOR CONTRIBUTIONS

Conceptualization, TC and ÁO; data collection, YM, TF, and SC; manuscript editing, SC, TC and ÁO; figure design, YM, TF. All authors have read and agreed to the published version of the manuscript.

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REVIEW
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Regulation of ADAMTS Proteases

*Keron W. J. Rose, Nandaraj Taye, Stylianos Z. Karoulias and Dirk Hubmacher**

Orthopaedic Research Laboratories, Leni and Peter W. May Department of Orthopaedics, Icahn School of Medicine at Mount Sinai, New York, NY, United States

A disintegrin and metalloprotease with thrombospondin type I motifs (ADAMTS) proteases are secreted metalloproteinases that play key roles in the formation, homeostasis and remodeling of the extracellular matrix (ECM). The substrate spectrum of ADAMTS proteases can range from individual ECM proteins to entire families of ECM proteins, such as the hyalectans. ADAMTS-mediated substrate cleavage is required for the formation, remodeling and physiological adaptation of the ECM to the needs of individual tissues and organ systems. However, ADAMTS proteases can also be involved in the destruction of tissues, resulting in pathologies such as arthritis. Specifically, ADAMTS4 and ADAMTS5 contribute to irreparable cartilage erosion by degrading aggrecan, which is a major constituent of cartilage. Arthritic joint damage is a major contributor to musculoskeletal morbidity and the most frequent clinical indication for total joint arthroplasty. Due to the high sequence homology of ADAMTS proteases in their catalytically active site, it remains a formidable challenge to design ADAMTS isotype-specific inhibitors that selectively inhibit ADAMTS proteases responsible for tissue destruction without affecting the beneficial functions of other ADAMTS proteases. In vivo, proteolytic activity of ADAMTS proteases is regulated on the transcriptional and posttranslational level. Here, we review the current knowledge of mechanisms that regulate ADAMTS protease activity in tissues including factors that induce ADAMTS gene expression, consequences of posttranslational modifications such as furin processing, the role of endogenous inhibitors and pharmacological approaches to limit ADAMTS protease activity in tissues, which almost exclusively focus on inhibiting the aggrecanase activity of ADAMTS4 and ADAMTS5.

Keywords: extracellular matrix, arthritis, small molecule inhibitor, posttranslational modifications, alternative splicing, cartilage, aggrecan

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Dylan Edwards, University of East Anglia, United Kingdom

****Correspondence:***
Dirk Hubmacher
dirk.hubmacher@mssm.edu

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INTRODUCTION

The a disintegrin and metalloprotease with thrombospondin type I motifs (ADAMTS) protease family comprises 19 secreted metalloproteases with a broad substrate and functional spectrum that is ever expanding due to recent advances in mass spectrometry-based substrate identification and due to the characterization of knock-out mouse models for most of the ADAMTS proteases (Puente et al., 2003; Kleifeld et al., 2011; Dubail and Apte, 2015; Savickas and Auf dem Keller, 2017; Apte, 2020; Satz-Jacobowitz and Hubmacher, 2021). Commensurate with the broad substrate and functional spectrum, ADAMTS proteases play major roles in organ development and tissue homeostasis by regulating extracellular matrix (ECM) formation, remodeling and homeostatic adaptation. Well characterized examples include the promotion of collagen fibrillogenesis by ADAMTS2, which cleaves the N-terminal propeptide of procollagen, or the remodeling of proteoglycan-rich ECMs by

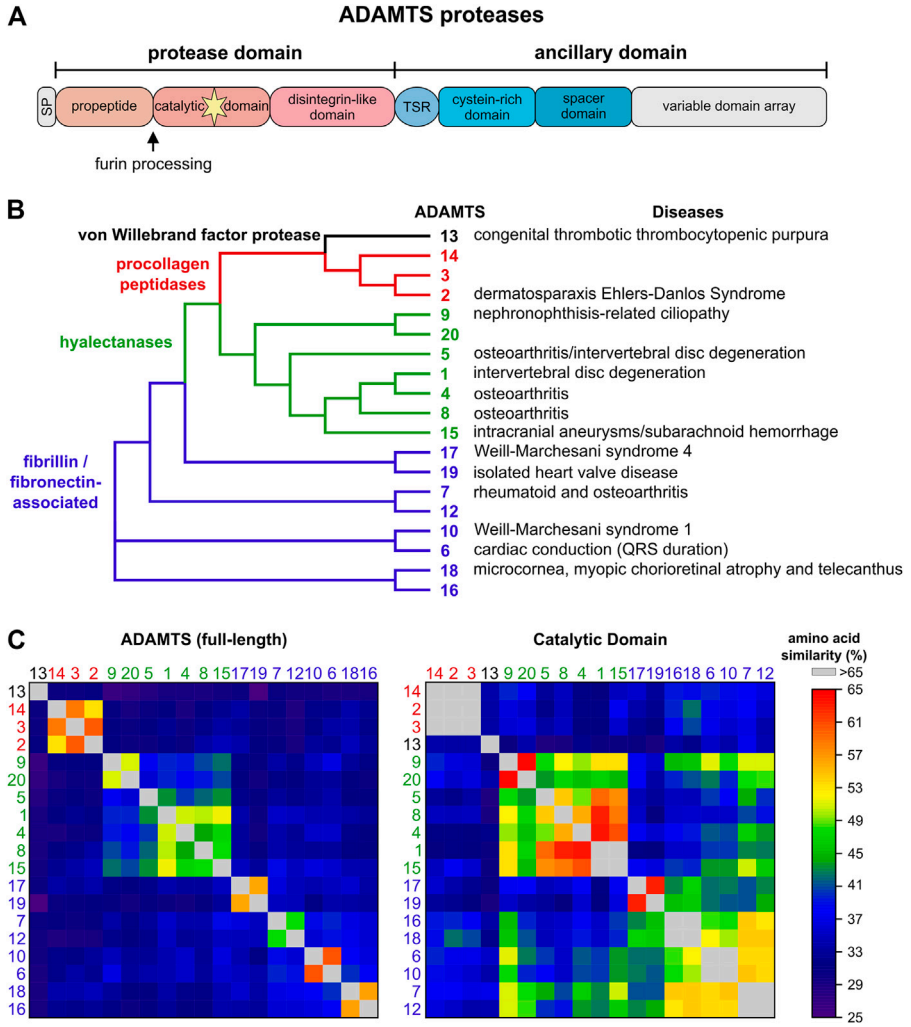

FIGURE 1 | The human ADAMTS protease family. **(A)** Domain organization of ADAMTS proteases. ADAMTS protease show identical domain organization of the protease domain and parts of the ancillary domain. The C-terminal variable domain arrays include between 0 (ADAMTS4) - 14 (ADAMTS9, ADAMTS20) thrombospondin type I motif (TSR) domains interspersed with additional domains unique to ADAMTS protease pairs, such as GON1, PLAC or CUB domains. **(B)** Phylogenetic tree of the human ADAMTS proteases generated with Clustal Omega using the full-length protein sequences of the ADAMTS protease (Madeira et al., 2019). Four ADAMTS subfamilies are evident: ADAMTS13 (black), the procollagen peptidases (red), the hyalectanases (green), and ADAMTS proteases associated with cleavage and/or binding to fibrillin and/or fibronectin (blue). The latter subfamily consists of four distinct pairs of ADAMTS proteases. Disorders associated with individual ADAMTS proteases are indicated on the right. **(C)** Heat map showing the amino acid similarities of full-length ADAMTS proteases (left) and the respective catalytic domains (right). The full-length ADAMTS proteases cluster in the same groups as indicated in A with little similarities to proteases outside of these groups. These clusters are mainly defined by the ancillary domain and the propeptide domain. However, a similar analysis of the catalytic domain shows that amino acid similarity still separates ADAMTS13 and the procollagen peptidases but that the boundaries that separated the hyalectanases and the fibrillin/fibronectin associated ADAMTS protease are now less well defined. This underscores the challenge of generating specific inhibitors for individual ADAMTS proteases by targeting the catalytic domain.

ADAMTS5, 9 and 20 during interdigital web regression and palate closure (Colige et al., 1999; Le Goff et al., 2006; McCulloch et al., 2009; Enomoto et al., 2010; Dubail et al., 2014). On the other hand, ADAMTS proteases are also involved in the pathogenesis of acquired and congenital connective tissue disorders, most prominently in arthritis, where ADAMTS4 and ADAMTS5 degrade aggrecan and contribute to the erosion of articular cartilage and joint degeneration (Glasson et al., 2005; Ilic et al., 2007; Song et al., 2007; Verma and Dalal, 2011; Santamaria, 2020). Examples of inherited connective tissue disorders that are caused by mutations in ADAMTS proteases, which likely reduce protease activity in the ECM, include Weill-Marchesani syndrome (*ADAMTS10*, *ADAMTS17*), dermatosparaxis Ehlers Danlos syndrome (*ADAMTS2*), isolated heart valve disease (*ADAMTS19*), or congenital thrombotic thrombocytopenic purpura (*ADAMTS13*) (Colige et al., 1999; Dagoneau et al., 2004; Sadler, 2008; Morales et al., 2009; Evans et al., 2020; Karoulias et al., 2020; Wunnemann et al., 2020).

ADAMTS proteases can be divided into four groups based on their substrate spectrum: ADAMTS13, procollagen peptidases, hyalectanases and ADAMTS proteases associated with fibrillin

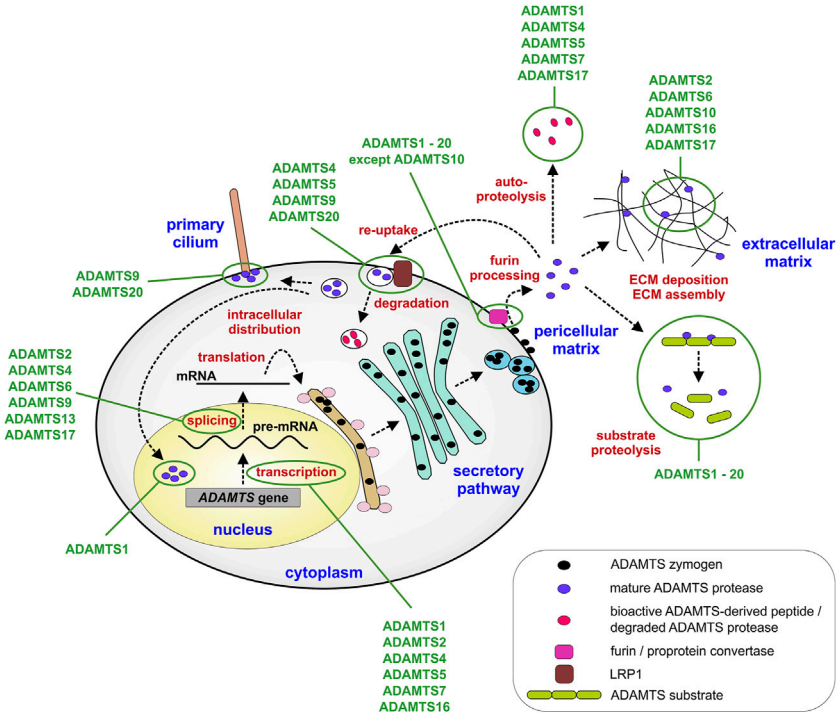

FIGURE 2 | Steps that regulate ADAMTS protease expression and activity. ADAMTS proteases can be regulated transcriptionally, during mRNA splicing or translation into protein. Major posttranslational regulatory steps include furin-mediated activation of ADAMTS proteases and the localization of ADAMTS protease activity in the pericellular or extracellular matrix. In addition, re-uptake of active ADAMTS proteases mediated by LRP1 results in the localization of ADAMTS protease activity to intracellular compartments, such as the primary cilium or the nucleus or in the clearance of protease activity from the extracellular matrix. Cellular and extracellular compartments are labeled in blue and points of regulation in red. Some ADAMTS proteases discussed in the review are depicted. Exceptions, such as activation of ADAMTS proteases in the secretory pathway or the ECM or absence of furin processing are described in the text only.

and fibronectin (**Figures 1A,B**). This is reflected in the phylogenetic tree based on the amino acid similarity of the 19 human ADAMTS proteases (**Figure 1B**). The grouping of ADAMTS proteases is mainly driven by their ancillary domains, since a similar analysis with the catalytic domain shows much higher amino acid conservation and the identity of the hyalectanases and the fibrillin/fibronectin-associated ADAMTS proteases is almost lost (**Figure 1C**). The individual ADAMTS subgroups were generated likely by gene duplication events during vertebrate evolution and originate from six *ADAMTS* genes that were identified in the basic chordate *Ciona intestinalis* (Huxley-Jones et al., 2005). The fibrillin/fibronectin-associated ADAMTS proteases are subdivided into four pairs and *ADAMTS*13, 17, and 19 do not have orthologues in *C. intestinalis*, which suggests that these *ADAMTS* proteases evolved in vertebrates (Huxley-Jones et al., 2005). The phylogenetic tree also indicates that individual ADAMTS proteases have a "sister" protease, with the exception of *ADAMTS5*, 8, 13, and 14. Functional redundancy or genetic interactions were demonstrated for several of these ADAMTS pairs (McCulloch et al., 2009; Mead et al., 2018; Mead et al., 2021). The *ADAMTS9*/*ADAMTS20* pair is conserved down to the worm *Caenorhabditis elegans*, where *Gon-1* is the only ADAMTS orthologue (Blelloch et al., 1999; Blelloch and Kimble, 1999). Overall, the evolutionary expansion of the ADAMTS protease family likely reflects the evolution of the ECM, which gained tremendous structural and functional complexity when transitioning from invertebrates to vertebrates (Nicholson et al., 2005; Hynes, 2012; Brunet et al., 2015).

While it is certainly true that most of the biology of ADAMTS proteases, or any protease for that matter, is ultimately defined by the consequences of substrate cleavage *in vivo*, it is equally important to elucidate the mechanisms by which ADAMTS protease activity is regulated during tissue development and maturation to fully understand how ADAMTS proteases work (Jones and Riley, 2005; Apte, 2020; Satz-Jacobowitz and Hubmacher, 2021). By regulating ADAMTS protease activity itself, the fate of entire groups of ECM substrates can be simultaneously altered and therefore, these proteases could be seen akin to nodes in a regulatory network that could be targeted in disorders such as arthritis, where ADAMTS protease activity is pathologically upregulated. The regulation of ADAMTS protease activity can be achieved on multiple levels, such as transcriptional regulation, posttranscriptional and posttranslational modifications or on the level of ADAMTS activation by furin processing (**Figure 2**). Further, regulating the access and binding of ADAMTS proteases to their substrates and ECM scaffolds can add additional layers of complexity to spatially control ADAMTS protease activity in the ECM. Finally, endogenous and exogenous inhibitors can be deployed to balance ADAMTS protease activity

in tissues with the translational goal to prevent connective tissue destruction and ultimately tissue failure.

Several aspects of ADAMTS proteases have been reviewed recently, including structural considerations, evolutionary aspects, the implication of ADAMTS proteases in inherited connective tissue disorders and the importance of substrate identification in understanding ADAMTS protease biology (Nicholson et al., 2005; Brunet et al., 2015; Takeda, 2016; Mead and Apte, 2018; Apte, 2020; Satz-Jacobowitz and Hubmacher, 2021). Here, we summarize recent insights into the mechanisms that regulate ADAMTS protease activity that could potentially be harnessed to modulate ADAMTS-mediated substrate cleavage, specifically in the context of degenerative connective tissue disorders. We will also provide an overview of past and current efforts to develop ADAMTS isotype-specific inhibitors that mainly target the aggrecanases ADAMTS4 and ADAMTS5 and are currently being pursued as potential disease-modifying arthritis drugs.

TRANSCRIPTIONAL AND POSTTRANSCRIPTIONAL REGULATION OF ADAMTS PROTEASES

ADAMTS mRNA abundance can be regulated on the transcriptional level and through microRNAs, which may also interfere with *ADAMTS* mRNA translation. In addition, several posttranscriptional and posttranslational mechanisms including alternative splicing and furin-mediated ADAMTS protease activation can determine tissue-specific ADAMTS isoform composition with possibly distinct proteolytic activities and/or substrates.

Transcriptional Regulation of ADAMTS Proteases

Despite the importance of ADAMTS proteases in developmental and homeostatic processes, little is known about their transcriptional activation or repression. Due to their prominence in arthritis, the regulation of *ADAMTS4* and *ADAMTS5* gene expression by pro-inflammatory cytokines has been studied to some extent (Malfait et al., 2002; Glasson et al., 2005; Bondeson et al., 2008; Kapoor et al., 2011). For example, when human chondrocytes were exposed to oncostatin or interleukin (IL)-1β, *ADAMTS4* and matrix metalloproteinase (MMP) 13 gene expression and protease activity was upregulated (El Mabrouk et al., 2007). The IL-1β signal was transduced through a combination of ERK1/2, JAK3-STAT1/3, PI3 kinase and Akt signaling suggesting a complex, possibly chondrocyte-specific signal transduction network that resulted in the induction of ADAMTS4. Tumor necrosis factor (TNF) α and IL-6 were also able to induce *ADAMTS4* and *ADAMTS5* mRNA expression in synovial cells consistent with a regulation of these two ADAMTS proteases by pro-inflammatory cytokines (Mimata et al., 2012; Uchida et al., 2017). Signal transduction of TNFα in synovial cells involved TAK1, a MAP kinase (MAPK) that can transduce signals originating from several cytokines (Xu and Lei, 2020). IL-6 signaling was mediated by MAPK, ERK1/2 and MEK. Regulation of *ADAMTS4* and *ADAMTS5* gene expression by pro-inflammatory cytokines is not restricted to arthritis and was also described in the heart and the intervertebral disc. Increased *ADAMTS4* and *ADAMTS8* mRNA expression was observed in neonatal cardiomyocytes and cardiac fibroblasts when stimulated with TNFα and IL-1β (Vistnes et al., 2014). *ADAMTS4* and *ADAMTS5* were induced in degenerating intervertebral discs due to increased levels of TNFα and IL-1β (Zhao et al., 2011; Tian et al., 2013). Stimulation of nucleus pulposus cells, which represent the inner aggrecan-producing cells of the intervertebral disc, with TNFα and IL-1β increased mRNA levels of both aggrecanases, *ADAMTS4* and *ADAMTS5*.

Outside of inflammatory regulators there is evidence that *ADAMTS1* is regulated by progesterone and luteinizing hormone during ovulation, where it may play a role in versican remodeling in the ECM surrounding the cumulus-oocyte complex (Doyle et al., 2004). Consistent with this finding, *Adamts1* knock-out mice showed abnormal ovaries and reduced fertility (Shindo et al., 2000). *ADAMTS16* regulation was also described in the genitourinary system. Follicle-stimulating hormone and forskolin, an adenylyl cyclase activator, induced *ADAMTS16* expression in fully differentiated granulosa cells, which are part of the cumulus complex that surrounds oocytes likely through stimulation of the cAMP pathway (Gao et al., 2007). In the kidneys, *ADAMTS16* was directly regulated by the zinc-finger transcription factor WT1 (Jacobi et al., 2013). ADAMTS16 plays a role in branching morphogenesis of kidneys, possibly through proteolysis of fibronectin (Schnellmann et al., 2018). In addition, *ADAMTS16* is positively regulated by the transcription factors EGR1 and SP1 and transforming growth factor (TGF) β in chondrocyte cell lines (Surridge et al., 2009). *ADAMTS2* was upregulated in osteoblastic cell lines, such as MG63 or Saos-2 when treated with IL-6. The upregulation of *ADAMTS2* mRNA by IL-6 was mediated by the JNK pathway as treatment of Saos-2 cells with a JNK specific inhibitor suppressed IL-6 induced *ADAMTS2* mRNA expression (Alper and Kockar, 2014). *ADAMTS2* expression was also induced by glucocorticoids in macrophages (Hofer et al., 2008). Since the primary function of ADAMTS2 is the processing of the N-terminal procollagen propeptide, the functional significance for *ADAMTS2* induction in macrophages during wound repair remains to be established. Recently, additional substrates for ADAMTS2 were identified in the skin, including fibronectin, which is an important part of the provisional ECM formed after injury, and several proteins linked to inflammation (Bekhouche et al., 2016; Leduc et al., 2021). Therefore, regulation of ADAMTS2 in non-collagen producing cell types could hint to a broader substrate spectrum as previously anticipated *in vivo*. Alternatively, macrophage-derived ADAMTS2 could enhance collagen fibrillogenesis during wound healing in-trans and thus augment the capacity of collagen-producing cell types to deposit collagen fibrils, if ADAMTS2-mediated removal of the collagen propeptide is a rate-limiting step. *ADAMTS7*, a potent chondrocyte differentiation inhibitor, was directly regulated by PTHrP, where it mediated the inhibition of chondrocyte hypertrophy (Bai et al., 2009).

In several cancer cell lines, expression of ADAMTS proteases was silenced through epigenetic mechanisms (Wagstaff et al., 2011; Redondo-Garcia et al., 2021). In most instances, including for *ADAMTS1*, 5 and 8, epigenetic regulation was achieved through increased methylation of the respective promotor regions (Choi et al., 2008; Kim et al., 2011; Choi et al., 2014). *ADAMTS12* is an interesting example, since it was silenced through promoter hypermethylation in colon cancer cells but it was transcriptionally activated in the surrounding stromal cells (Moncada-Pazos et al., 2009). The authors speculated that the upregulation of *ADAMTS12* in the stroma may be a response to restrain the growing *ADAMTS12*-negative tumor. In a more recent study it was indeed shown that *ADAMTS12* depletion in a lung cancer cell line resulted in increased proliferation and invasion and that *Adamts12*-deficient mice had a 5-fold increase in lung tumor burden after urethane exposure (Rabadan et al., 2020). In contrast to malignant cell types, much less is known about the regulation of ADAMTS gene expression through chromatin alterations and epigenetic mechanisms in other cell types or tissues. In cardiac development, *ADAMTS1* is repressed by BRG1-mediated chromatin remodeling until trabecular growth is completed (Stankunas et al., 2008). Upon de-repression, i.e. induction of *ADAMTS1* and its versicanase activity, the cardiac jelly, which is permissive for trabeculation, is remodeled and degraded and trabeculation ceases. Epigenetic dysregulation of several members of the ADAMTS family was identified in placentas from preterm births compared to term birth placentas (Mani et al., 2019). Specifically, *ADAMTS12* and *ADAMTS16* displayed multiple differential methylation sites where *ADAMTS12* and *ADAMTS16* were hypo- and hypermethylated in the preterm cohort, respectively. Based on gene expression and functional data the authors concluded that both proteases may be important for trophoblast invasion and the proper anchoring of the placenta. With the current pace in the development of high throughput methodology to identify epigenetic modifications and determine tissue and cell level gene expression changes, it will be exciting to define the epigenetic regulatory networks that determine ADAMTS protease activity and to explore if these mechanisms can be harnessed to either activate desired ADAMTS proteases or to silence deleterious ADAMTS protease activity *in vivo* (Li, 2021).

Collectively, regulation of *ADAMTS* gene expression through transcriptional and epigenetic mechanisms is complicated and likely tissue and cell-type specific. Despite the complexity, it appears that a subgroup of *ADAMTS* proteases respond to pro-inflammatory cytokines while other ADAMTS proteases are regulated by sex-hormones during ovulation. It will be interesting to discover the gene-regulatory networks in which *ADAMTS* proteases participate and to elucidate how these networks are spatiotemporally regulated during normal development and homeostasis or possibly dysregulated in acquired diseases. It is worth mentioning that individual ADAMTS proteases can compensate for each other and in some instances this compensation is attributed to the induction of gene expression of the respective sister protease (McCulloch et al., 2009; Mead et al., 2018; Nandadasa et al., 2019). However, nothing is known about the signals that regulate these compensatory gene expression changes, which may involve signaling through the respective ADAMTS substrates themselves.

Alternative Splicing and Posttranslational Modifications of ADAMTS Proteases

Alternative splicing events have the potential to augment the proteome, i.e., to increase the number of individual protein species compared to the number of protein-coding genes with predictions of up to 200,000 potentially protein-coding transcripts (Hu et al., 2015). However, for a large majority of protein isoforms, the functional role for the alternatively spliced gene products is unclear. Alternative splicing and the existence of individual *ADAMTS* isoforms was reported for *ADAMTS2*, 4, 6, 7, 9, 13, and 17, but functional differences between individual ADAMTS isoforms were only described in a few reports (Colige et al., 1999; Bevitt et al., 2003; Bevitt et al., 2005; Balic et al., 2021). A splice variant of *ADAMTS4*, which resulted in the removal of the spacer domain and the inclusion of a unique C-terminus, was detected in the synovium of arthritis patients (**Figure 3A**) (Wainwright et al., 2006). As a consequence, aggrecanase activity of ADAMTS4 was enhanced and it was suggested that the alternatively spliced isoform of ADAMTS4 could contribute to cartilage erosion in the arthritic joint (Hashimoto et al., 2004; Kashiwagi et al., 2004). However, targeted deletion of the spacer domain in ADAMTS4 and ADAMTS5 reduced or abolished aggrecanase and versicanase activity *in vitro* suggesting that the spacer domain is required for cleavage of aggrecan and versican (Fushimi et al., 2008; Santamaria et al., 2019). One factor which could explain these discrepancies may be the degree of contamination with heparin during ADAMTS4 preparations. Heparin specifically inhibited the aggrecanase activity of full-length ADAMTS4 and may have resulted in an underestimation of its aggrecanase activity, compared to the activity of alternatively spliced ADAMTS4 (Fushimi et al., 2008). Alternatively, the ADAMTS4 isoform without the spacer domain may have lost its capability to be sequestered to the cell-surface and as a consequence was released into the ECM/synovium, where the spacer-less ADAMTS4 isoform now could reach its substrate aggrecan (Gao et al., 2004). The alternative splicing events reported for *ADAMTS17* also occurred in the spacer domain and resulted not in the removal of the entire domain but in the deletion and insertion of shorter peptides that altered the structure of the domain itself (Balic et al., 2021). Functionally, alternative splicing of *ADAMTS17* affected secretion of the shorter isoform and altered the autocatalytic properties of ADAMTS17 and potentially the recognition of ADAMTS17 substrates. However, the *in vivo* consequences of alternative *ADAMTS17* splicing will need to be determined in future experiments. Besides the questions surrounding altered functions of the ADAMTS protease isoforms, how ADAMTS isoform generation through alternative splicing itself is regulated could warrant further investigation (Goncalves et al., 2017). This could be of special interest since alternative splicing events can be targeted with antisense oligonucleotides. For example exon-skipping therapy could potentially prevent the generation of detrimental

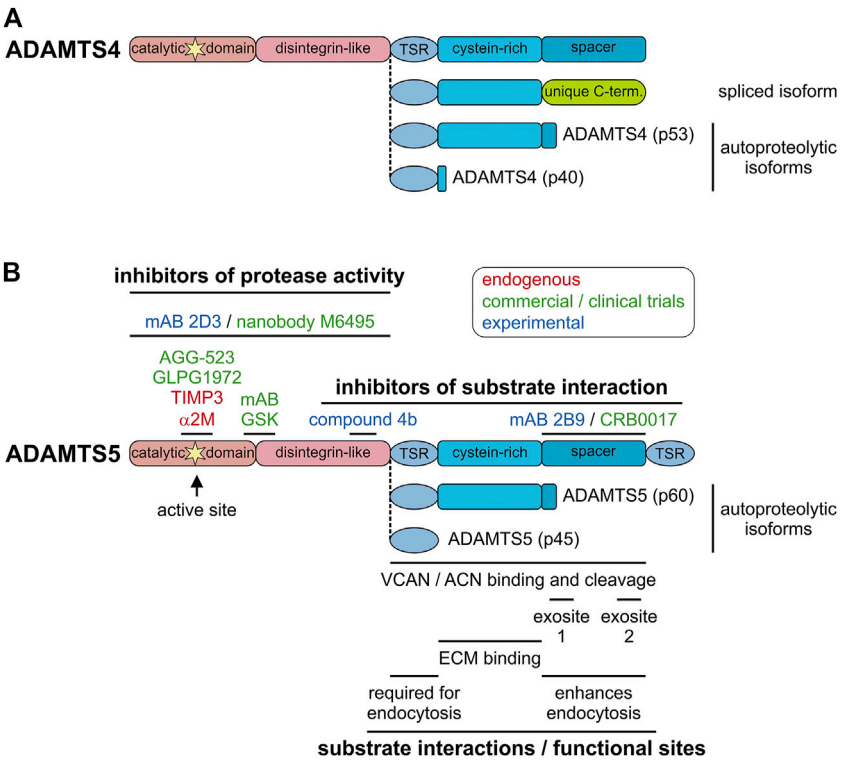

FIGURE 3 | Endogenous and pharmacological inhibitors of ADAMTS proteases. **(A)** Domain organization of furin-processed mature ADAMTS4 and the organization of the ancillary domain of spliced and autoproteolytic isoforms showing the similarity to ADAMTS5 and its isoforms. **(B)** Domain organization of furin-processed mature ADAMTS5 depicting the location of endogenous and pharmacological inhibitor epitopes **(top)** and relevant functional and substrate interaction sites of ADAMTS5 **(bottom)**. The ancillary domains of the autoproteolytic isoforms p60 and p45 are depicted. Endogenous inhibitors are indicated in red, inhibitors that went through clinical trials or are in commercial product pipelines are indicated in green and experimental inhibitors reported in the literature are indicated in blue.

ADAMTS4 isoforms without affecting other functions of ADAMTS4 (Dzierlega and Yokota, 2020). Such an approach however, requires the exact knowledge about which ADAMTS domains are involved in the recognition of and binding to individual substrates.

Proteolytic Processing of ADAMTS Proteases

ADAMTS proteases are initially synthesized as inactive zymogens and generally require a furin/proprotein convertase-mediated cleavage event that results in the removal of the propeptide, which then activates the protease. Most ADAMTS proteases contain one or more furin-consensus sites in their propeptide, with the one closest to the catalytic domain being cleaved during the activation step. If furin-processing at the other sites is required for zymogen maturation of ADAMTS proteases, as it was suggested for members of the related ADAM protease family, is unclear (Wong et al., 2015). For ADAMTS1 and ADAMTS5 it was shown that only one of the furin consensus sites was cleaved, while there is *in vitro* evidence for processing at the additional furin sites of ADAMTS9 and ADAMTS17 (Longpre and Leduc, 2004; Koo et al., 2007; Longpre et al., 2009; Hubmacher et al., 2017). The subcellular localization for furin-mediated activation differs for individual ADAMTS proteases. ADAMTS1 and ADAMTS4 are activated in the Golgi apparatus during secretion and the active enzyme was released into the cell culture medium (Longpre and Leduc, 2004; Wang et al., 2004). In contrast, ADAMTS5 was activated after secretion since mature ADAMTS5 was only detected in conditioned medium but not in cell lysate (Longpre et al., 2009). A third possible location for furin processing was described for ADAMTS9, where the zymogen was detected at the cell surface and mature ADAMTS9 was released into the medium after furin processing (Koo and Apte, 2010). ADAMTS7 was shown to be processed inside the cell, likely in the secretory pathway, and at the cell surface (Somerville et al., 2004b). The biological relevance of these different subcellular locations of zymogen processing remains unclear, but it could suggest that some ADAMTS proteases may cleave respective substrates already in the secretory pathway (ADAMTS1, ADAMTS4) while the activity of other ADAMTS proteases may be detrimental in this location and activation occurs outside of the cell restricting proteolytic activity to the cell surface or the ECM (ADAMTS5, ADAMTS9).

Exceptions to the generalized observation that propeptide removal is required to activate ADAMTS proteases are ADAMTS9, 10, 13, and 17. For ADAMTS9 it was shown that

the pro-form, obtained by mutating all three furin sites or using a proprotein convertase inhibitor, was able to cleave its substrate versican even more efficiently then mature furin-processed ADAMTS9 and it was proposed that furin-processing of ADAMTS9 reduced its catalytic activity towards versican (Koo et al., 2007). Since the ADAMTS9 propeptide remained attached to the mature enzyme and a construct lacking the propeptide was not secreted at all, it was suggested that the ADAMTS9 propeptide was acting as a potential chaperone. In a very similar manner, ADAMTS17 retained its autocatalytic properties when the key furin processing site was mutated and ADAMTS17 secretion was abolished when the propeptide sequence was removed (Hubmacher et al., 2017). The sequence of ADAMTS13 includes only a very short propeptide, which does not seem to play a role as a chaperone during secretion or in modulating the catalytic activity of ADAMTS13 (Majerus et al., 2003). ADAMTS10 undergoes very inefficient furin processing due to the presence of a degenerated furin consensus sequence at the critical junction of the propeptide domain and the catalytic domain (Somerville et al., 2004a). Poorly furin-processed ADAMTS10 cleaved fibrillin-1 inefficiently *in vitro* (Kutz et al., 2011). However, upon restoration of the furin-consensus sequence by mutagenesis ADAMTS10 was activated, fibrillin-1 cleavage was enhanced and fibrillin-2 was now also cleaved (Wang et al., 2019a). Together with evidence that ADAMTS10 was size-shifted by α2-macroglobulin, which requires protease activity, ADAMTS10 could potentially work as a true protease if activated, potentially by non-furin proteases (Somerville et al., 2004a). However, if ADAMTS10 indeed functions as a true protease *in vivo* or through protease-independent mechanisms, such as modulation of fibrillin-1 assembly remains to be established (Kutz et al., 2011; Mularczyk et al., 2018; Wang et al., 2019a). With the advent of CRISPR/Cas9 gene editing, the introduction of point mutations in mice is greatly facilitated and it will be interesting to test the role of furin processing *in vivo* by introducing point mutations in furin processing sites or reestablishing a furin consensus site in *Adamts10*.

Autoproteolysis, the process of "self-cleavage" of ADAMTS proteases, adds an additional layer of complexity in the regulation of ADAMTS protease activity. Autocatalysis was described for several ADAMTS proteases including ADAMTS1, 4, 5, 7, and 17 with different consequences. Autocatalysis of ADAMTS1 results in two distinct ~21 kDa peptides and can be prevented by the addition of heparin/heparan sulfates (Liu et al., 2006). ADAMTS4 can cleave itself in the Cys-rich and spacer domain through an intramolecular mechanism, resulting in distinct truncated ADAMTS4 proteases (**Figure 3A**) (Flannery et al., 2002). While the ability of truncated forms of ADAMTS4 to cleave bovine aggrecan were preserved, the affinities for sulfated glycosaminoglycans was reduced. This suggested the possibility that ECM-bound or cell surface-bound ADAMTS4 may be mobilized through autocatalysis, which now allows ADAMTS4 to reach its substrate aggrecan and contribute to its degradation in cartilage resulting in arthritis. Interestingly, similar proteolytic peptides were found for ADAMTS5 *in vitro* as well as in human cartilage and synovial tissue biopsies (**Figure 3B**) (Vankemmelbeke et al., 2001; Malfait et al., 2002; Zeng et al., 2006). It is currently unclear if these shortened ADAMTS5 isoforms were generated by autocatalytic processing or by ambient synovial proteases. Autoproteolysis of ADAMTS7 resulted in cleavage in the spacer domain, which may be involved in ADAMTS7 substrate recognition. It will be interesting to investigate, if the autocatalytic cleavage product of ADAMTS7 can still recognize its substrates (Colige et al., 2019). ADAMTS17 was autocatalytically cleaved in multiple locations, including in the catalytic domain itself and full-length mature ADAMTS17 was almost undetectable in conditioned medium (Hubmacher et al., 2017). In this case, autoproteolysis may be a mechanism to restrict ADAMTS17 protease activity to the cell surface or to generate bioactive peptides that are released from the cell surface. In addition to autocatalysis, ADAMTS proteases can be cleaved and/or activated by other proteases in the ECM. For example, ADAMTS4 was activated by MMP9, MMP13 and trypsin *in vitro* and ADAMTS1 was cleaved by MMP2, MMP8 and MMP15 (Rodriguez-Manzaneque et al., 2000; Gao et al., 2002; Tortorella et al., 2005). The relevance of these protease cascades *in vivo* remains to be established.

Differential Cellular Localization of ADAMTS Proteases

ADAMTS-mediated substrate cleavage can only occur when ADAMTS proteases and their respective substrates are in close vicinity. Therefore, restricting ADAMTS protease activity to distinct cellular or subcellular compartments or signaling-induced relocation of ADAMTS proteases could shift their accessibility to different sets of substrates. As previously described, alternative splicing and autoproteolysis of ADAMTS4 could result in its release from the ECM (Gao et al., 2004). Additionally, alternative splicing and extensive autoproteolysis can spatially restrict ADAMTS17 protease activity to the cell surface, the adjacent pericellular matrix or an intracellular compartment that has yet to be identified (Hubmacher et al., 2017; Balic et al., 2021). The activation of ADAMTS5 outside of the cell may restrict its activity to the ECM and prevent substrate cleavage at the cell surface (Longpre et al., 2009). However, additional versican proteolysis by ADAMTS5 in the pericellular matrix was also reported (Hattori et al., 2011; Stupka et al., 2013).

ADAMTS protease activity can also be targeted to the ECM by binding of ADAMTS proteases to individual ECM proteins. This was demonstrated for example for ADAMTS1, 9, 10, and 17, which localized to fibronectin and fibrillin microfibrils in the ECM of cultured cells (Kuno and Matsushima, 1998; Kutz et al., 2011; Hubmacher et al., 2017; Wang et al., 2019b; Balic et al., 2021). In addition, ECM localization by binding to heparan-sulfate proteoglycans was demonstrated for ADAMTS1, 2, 4, and 5 (Kuno and Matsushima, 1998; Colige et al., 2005; Gendron et al., 2007; Fushimi et al., 2008). In these cases, addition of heparin to cells expressing the respective recombinant ADAMTS proteases resulted in their release into the medium. For ADAMTS2 it was shown that heparin increased the affinity of

ADAMTS2 for TIMP3 (Wang et al., 2006). In the case of ADAMTS4 and ADAMTS5 addition of heparin inhibited the aggrecanases activity without affecting ADAMTS4 activity against other substrates (Vankemmelbeke et al., 2001; Fushimi et al., 2008). It will be interesting to see if binding of ADAMTS4 and ADAMTS5 to heparan-sulfate or other types of proteoglycans indeed inhibits aggrecanase activity *in vivo* and if this is a general property of the proteoglycanase subgroup to modulate substrate-specificity.

In an interesting experimental model ADAMTS9 was unintentionally trapped at the cell surface (Nandadasa et al., 2015). As a result, ADAMTS9 protease activity was restricted to the pericellular matrix while absent in the ECM. Several phenotypes were lacking when these mice were compared to the full ADAMTS9 knockout, suggesting that ADAMTS9 substrates in the pericellular matrix and the ECM are distinct and that ADAMTS9 is required in both compartments for normal development and tissue homeostasis. In addition, ADAMTS9 was identified inside the cell at the base of the primary cilium (Nandadasa et al., 2019). However, ADAMTS9 was not diverted from the secretory pathway, but was first secreted and then re-internalized. Since a short cilium phenotype could be restored by transfection with ADAMTS9, but not catalytically inactive ADAMTS9, a proteolytic function for ADAMTS9 in ciliogenesis was suggested.

The cell-surface receptor low-density lipoprotein receptor-related protein 1 (LRP1) was invoked as a mechanism for the re-uptake of secreted ADAMTS9 (Nandadasa et al., 2019). This is consistent with the LRP1-mediated uptake of ADAMTS4, ADAMTS5 and TIMP3, which has been described previously (Scilabra et al., 2013; Yamamoto et al., 2013; Yamamoto et al., 2014). In contrast to ADAMTS9, where LRP1-mediated uptake results in the shuttling of active ADAMTS9 to a new intracellular destination, it is thought that LRP1-mediated uptake of ADAMTS4 and ADAMTS5 is important to clear ADAMTS4 and ADAMTS5 from the cellular microenvironment and thus reduce, for example aggrecanase activity in cartilage. Interestingly, the domain requirements for binding to LRP1 differ between ADAMTS4 (cysteine-rich and spacer domain) and ADAMTS5 (spacer and TSR domain) and ADAMTS5 can compete with ADAMTS4 for binding to LRP1 (Yamamoto et al., 2014). In addition, it was demonstrated that LRP1 can be shed by ADAM17 and MMP14 in cartilage and the resulting soluble form of LRP1 prevented the uptake of ADAMTS5 without interfering with its catalytic activity. Since LRP1 shedding was increased in osteoarthritis, soluble LRP1 further augmented the aggrecanase activity in cartilage by preventing its cellular uptake (Yamamoto et al., 2017). As such, LRP1 and potentially other cell-surface receptors, are important regulators of ADAMTS recycling and the modulation of extracellular ADAMTS protease activity.

An unusual intracellular localization was described for ADAMTS1, which was found in the nucleus of a normal mammary cell line and two breast cancer cell lines (Silva et al., 2016). Since it was found together with aggrecan, the authors suggested that ADAMTS1 may play a proteolytic role in the nucleus. However, no nuclear substrates have been reported. A possible function for secreted ADAMTS proteases in intracellular compartments, such as the nucleus, raises many interesting questions, which have been partially answered for several MMPs that were identified in different intracellular compartments (Jobin et al., 2017).

Together, restricting ADAMTS protease activity to distinct subcellular and extracellular compartments represents an interesting way to regulate protease activity and substrate cleavage depending on the specific location. Conceptually, it is also possible that binding of ADAMTS proteases to distinct ECM proteins regulates the activity of these proteases by modulating binding affinities to individual substrates or by blocking access to substrate binding sites, adding yet an additional layer of complexity in spatially regulating ADAMTS protease activity.

INHIBITORS OF ADAMTS PROTEASES

There has been a great interest in developing inhibitors to explore the feasibility of targeting ADAMTS protease isotypes to alter disease progression where aberrant protease activity results in tissue destruction (Yang et al., 2017; Santamaria, 2020). Limiting ADAMTS protease activity can in principle be achieved by decreasing ADAMTS protease expression, preventing ADAMTS protease activation, blocking their proteolytic activity or preventing the cleavage of specific ADAMTS substrates. Transcriptional targeting through modulation of signaling pathways or targeting of the proprotein convertase-mediated activation step are likely non-selective and will reduce the protease activity of several, if not all ADAMTS proteases with possible undesired side effects. Therefore, selectively targeting individual ADAMTS proteases with pharmacological or biological inhibitors, or preventing the cleavage of individual ADAMTS protease substrates will probably represent the most promising approaches to reduce ADAMTS protease activity in pathological settings. The most fruitful ventures in this field have been through the discovery and study of both endogenous and pharmacological inhibitors of specific ADAMTS proteases. Therefore, we will first describe the regulation of ADAMTS protease activity by endogenous inhibitors, focusing on tissue inhibitor of metalloproteinases (TIMP) 3 and α2-macroglobulin (α2M) and then summarize current strategies aimed at developing pharmacological inhibitors for specific ADAMTS proteases and substrate cleavage events. We will conclude with a summary of the clinical trials conducted for some of these inhibitors.

Endogenous ADAMTS Protease Inhibitors

Homeostatic net protease activity in tissues can be controlled by carefully balancing the amount of proteases with the amount of endogenous protease inhibitors. An excess amount of proteases will lead to increased anabolic or catabolic ECM proteolysis while increasing the amount of protease inhibitors, such as TIMPs, will decrease or stop ECM proteolysis and protect tissues from excessive ECM turnover. There are four TIMP isotypes, which can inhibit virtually all metalloproteinases including MMPs, the pericellular ADAM proteases and ADAMTS proteases, albeit with different specificities and efficiencies for the individual

protease families (Brew and Nagase, 2010; Arpino et al., 2015; Fan and Kassiri, 2020). For ADAMTS proteases, TIMP3 appears to be the most potent TIMP *in vivo*. In seminal biochemical studies it was shown that TIMP3 can inhibit ADAMTS4 and ADAMTS5 (Kashiwagi et al., 2001). A protective role for TIMP3 in articular cartilage homeostasis was identified in TIMP3 knockout mice and supports a role for TIMP3 as an important endogenous inhibitor for ADAMTS4 and ADAMTS5 (Sahebjam et al., 2007). More recently, transgenic overexpression of TIMP3 showed a protective effect toward articular cartilage degradation in a surgical osteoarthritis model (Nakamura et al., 2020). Interestingly, transgenic overexpression of a mutant TIMP3, which included an extra alanine-residue in its N-terminus that shifted the inhibitory profile for TIMP3 from "MMP plus ADAMTS" to a more selective "ADAMTS only" inhibition was even more efficient in preventing articular cartilage degradation (Lim et al., 2010; Nakamura et al., 2020). It will be fascinating to see how far the specificity of TIMP3 for ADAMTS4 and ADAMTS5 can be increased through systematic mutagenesis screens without losing the overall potency of TIMP3 as an ADAMTS protease inhibitor. More recently, TIMP4 was shown to inhibit ADAMTS7 even more efficiently then TIMP2 or TIMP3 (Colige et al., 2019). This raises the possibility that TIMP2 and TIMP4 are inhibitors for specific ADAMTS protease and the TIMP inhibition profile of ADAMTS proteases may need to be investigated on a protease-by-protease basis.

α2M is a large glycoprotein that is present in serum and tissues and that can inhibit almost all types of proteases in a "bait-and-trap" mechanism (Cuellar et al., 2016). The fact that α2M remains associated with proteases after cleavage, including the ADAMTS proteases, can be used to determine protease activity by gel-shift assays (Somerville et al., 2004a; Somerville et al., 2004b; Tortorella et al., 2004). ADAMTS1 was the first ADAMTS protease shown to bind, cleave and be inhibited by α2M (Kuno et al., 1999). Among ADAMTS proteases involved in arthritis, α2M was shown to inhibit both ADAMTS4 and ADAMTS5 in a dose-dependent manner (Tortorella et al., 2004). In addition, the cartilage oligomeric matrix protein (COMP)-processing activities of ADAMTS7 and ADAMTS12 were inhibited by α2M, suggesting that dysregulation of the ADAMTS7 and ADAMTS12 protease activity through α2M could be involved in arthritis (Luan et al., 2008). In the context of arthritis, α2M was found in similar concentrations as TIMP3 in the synovial fluid of joints and it rapidly bound to collagenase (Cawston et al., 1987). α2M was then identified as a key regulator of several cartilage degenerating factors and intra-articular injections of α2M could ameliorate osteoarthritis progression (Wang et al., 2014). In a similar approach as mentioned for TIMP3, the sequence of α2M was altered to increase its protective efficacy against cartilage degradation (Zhang et al., 2017). However, since α2M has many functions it is unclear to what extent the arthritis-protective effects can be related to the direct inhibition of ADAMTS proteases or the interaction of α2M with inflammatory cytokines, such as IL-1β or tumor necrosis factor TNFα (Wollenberg et al., 1991; Legres et al., 1994; Rehman et al., 2013).

In summary, it can be inferred that the presence of both TIMP3 and α2M are vital endogenous regulatory factors limiting ADAMTS protease activity within the joint and various other tissues. Any homeostatic imbalance between these two endogenous inhibitors and ADAMTS proteases can possibly lead to the onset or acceleration of degenerative diseases resulting in tissue destruction. Conversely, TIMP3 and α2M could potentially be used as a template to develop ADAMTS isotype specific peptide inhibitors.

Pharmacological Inhibitors

The development of inhibitors for ADAMTS proteases is primarily focused on inhibiting ADAMTS4 and ADAMTS5 due to their prominent role in cartilage destruction in arthritis where both proteases degrade aggrecan, a major structural proteoglycan in the articular cartilage (Verma and Dalal, 2011). Conceptually, to inhibit ADAMTS4 and ADAMTS5, one could block their active site, prevent their binding to aggrecan or promote removal from the target tissue. All three approaches have been or are actively pursued in the quest to develop a specific inhibitor of aggrecanase activity to halt joint erosion in arthritis. However, a major complication in directly targeting ADAMTS4 and ADAMTS5 protease activity with small molecules is the high degree of sequence and structural conservation of the active site, not only within members of the ADAMTS protease family, but also between the ADAMTS, MMP, and ADAM protease families (**Figure 1C**) (Yiotakis and Dive, 2008; Kelwick et al., 2015). Therefore, unintended cross-inactivation of several metalloproteinases with possible short- and long-term side effects are likely when inhibitors are administered systemically. This may have been one of the reasons why early MMP inhibitors have failed as cancer therapeutics in clinical trials (Coussens et al., 2002; Winer et al., 2018). However, an ADAMTS5-targeting monoclonal antibody (mAb) was developed that bound at the interface of the catalytic and the disintegrin-like domain and appeared to reduce the structural flexibility of the active site, thus reducing ADAMTS5 activity (**Figure 3B**) (Larkin et al., 2015). The binding site of this ADAMTS5 mAb does not coincide with the aggrecan/versican substrate recognition site, which is located in the cysteine-rich and spacer domains (Santamaria et al., 2019). Very recently, a Zn-coordinating small molecule active site inhibitor was described for ADAMTS5 (GLPG 1972) that displayed strong selectivity against ADAMTS1 and other MMPs with the exception of ADAMTS4, where the selectivity was only ~8 fold (Brebion et al., 2021). This selectivity is much lower compared to mAB-based exosite inhibitors against ADAMTS5, which did not bind to ADAMTS4 nor did they inhibit the protease or aggrecanase activity of ADAMTS4 (Santamaria et al., 2015). However, simultaneous inhibition of ADAMTS4 and ADAMTS5 may be desired in modifying arthritis progression. The ADAMTS5-inhibiting activity of GLPG1972 in articular cartilage has been studied extensively in both mouse and human cartilage tissue explants. In these studies, GLPG1972 inhibition of ADAMTS5 substantially reduced proteoglycan

loss from femorotibial cartilage. Based on its performance in prior studies, GLPG1972 displays potent and selective inhibition of ADAMTS5, which may protect cartilage. However, a recently completed phase two clinical trial with GLPG1972 yielded disappointing results (see below).

As an alternative to targeting the active site the binding of ADAMTS proteases to its substrate could be blocked. The feasibility of such a strategy is further encouraged by the finding that epitopes in the ADAMTS ancillary domain, which is catalytically inactive and diverse in domain composition, contribute to substrate recognition through so-called "exosites" (Chiusaroli et al., 2013; Larkin et al., 2015; Santamaria et al., 2015). For example, it was shown that ADAMTS5 lacking the C-terminal ancillary domain was a poor aggrecanase when compared to full-length ADAMTS5 (Fushimi et al., 2008). In an extreme case, substrate preference for ADAMTS5 was switched from aggrecan to von Willebrand factor when its ancillary domain was swapped with the ancillary domain of ADAMTS13, the native ADAMTS protease that cleaves von Willebrand factor (Gao et al., 2012). However, the aggrecanase activity of ADAMTS5 could not be transferred to ADAMTS13 by fusing the ADAMTS5 ancillary domain with the ADAMTS13 catalytic domain. This may be explained by an unusual latent structure of the ADAMTS13 protease domain that requires allosteric activation by binding to von Willebrand factor, which differs from the ADAMTS5 protease domain (Petri et al., 2019). This suggests that not only the ancillary domain of ADAMTS5 but also the catalytic domain contributes specificity to substrate recognition and cleavage. Based on this rationale, a phage display screen identified mAbs against ADAMTS4 and ADAMTS5, which reacted with the catalytic/disintegrin domain outside of the active site, the spacer domain or the interface between the catalytic/disintegrin domain and the first TSR domain (**Figure 3B**) (Larkin et al., 2015; Santamaria et al., 2015). These mAbs reduced ADAMTS5 protease activity measured through aggrecan degradation. Additionally, when testing possible synergistic effects of combining both ADAMTS4- and ADAMTS5-inhibiting mAbs, ADAMTS5 appeared to be the dominant ADAMTS protease mediating cartilage degeneration in human cartilage explants or in primary human chondrocytes. As an alternative to mAbs, an ADAMTS5 exosite inhibitor based on a glycoconjugated arylsulfonamide was developed recently, which showed selectivity over ADAMTS4 and inhibited both, versican and aggrecan cleavage (Santamaria et al., 2021). It will be interesting to see how these inhibitors will perform *in vivo* since both, versicanase and aggrecanase activity, are blocked, or if exosite inhibitors can be developed that can discriminate between versican and aggrecan recognition.

A third strategy targeted the endogenous clearance pathway of ADAMTS4 and ADAMTS5, which involves LRP1. Both ADAMTS proteases are rapidly cleared in cartilage explants and articular chondrocytes by LRP1-mediated endocytosis (Yamamoto et al., 2013; Yamamoto et al., 2014). The interaction of ADAMTS4 and ADAMTS5 with LRP1 is mediated by the TSR1 domain and the spacer domain. Blockage of ADAMTS4 and ADAMTS5 clearance with an inhibitor resulted in increased aggrecan degradation. Interestingly, shedding of LRP1 was increased in osteoarthritic cartilage tissue, resulting in enhanced aggrecanase activity and inhibition of LRP1 shedding could potentially reduce degradation of cartilage ECM (Yamamoto et al., 2017). More recently, a role for TIMP3 in promoting LRP1-based protease clearance raises the possibility of identifying peptides that promote both, the binding and clearance of destructive ADAMTS proteases in cartilage to modify disease progression in arthritis (Carreca et al., 2020).

Pharmaceutical Aggrecanase Inhibitor Projects and Clinical Trials

Several clinical trials have been conducted to test the safety and efficacy of arthritis-modifying candidate drugs that reduced aggrecanase activity, i.e., inhibited ADAMTS4 and ADAMTS5 in preclinical studies. However, none of these candidate drugs have advanced beyond phase II trials. The first aggrecanase inhibitor (AGG-523, Wyeth/Pfizer) was evaluated in phase I clinical trials (NCT00454298, NCT00454298) but results were not reported and further development of AGG-523 was discontinued, probably due to poor pharmacokinetics (Chockalingam et al., 2011; Malfait and Tortorella, 2019). Another small molecule inhibitor for ADAMTS5 (GLPG 1972, Galapagos) was tested in phase I/II trials (e.g. NCT03595618, NCT04136327) (Brebion et al., 2021). However, the phase II ROCCELLA trial (NCT03595618) with cartilage thickness as the primary endpoint did not improve outcome in patients with osteoarthritis (ThePharmaLetter, 2021). Several antibodies that are in commercial drug development pipelines were tested in phase I clinical studies. Safety and dose escalation studies were recently completed for an ADAMTS5 nanobody (M6495, Merck KGaA) (NCT03583346, NCT03224702) demonstrating safety, tolerability and a reduction in aggrecan proteolysis (Siebuhr et al., 2020). Further phase II clinical trials will be performed by Novartis (Merck, 2021). A humanized mAb (GSK2394002, GlaxoSmithKline), which was successful in modifying arthritis in preclinical studies, did not reach the clinical trial stage primarily due to unexpected and sustained cardiovascular side effects after a single dose injection of GSK2394002 in cynomolgus monkeys (Larkin et al., 2014; Larkin et al., 2015). An ADAMTS5 antibody against an epitope in the ancillary domain (CRB0017, Rottapharm) also ameliorated arthritis disease progression in knee joints of mice (Chiusaroli et al., 2013). However, the antibody is currently not listed as part of the Rottapharm drug pipeline and its clinical fate is unclear (RottapharmBiotech, 2021).

Novel Technologies to Regulate ADAMTS Protease Activity

RBM-010 is an ADAMTS5-inhibiting aptamer developed by Ribomic, which awaits preclinical and clinical testing (Ribomic, 2021). Aptamers are single stranded RNA-based molecules that are selected from a randomized pool by their

high binding affinity to a target protein and that have several advantages over other biomolecules as therapeutics (Zhang et al., 2019). An alternative method to reduce ADAMTS5 protease activity is the use of inhibitory RNA technologies to selectively degrade *ADAMTS5* mRNA. This could be achieved by delivering stabilized *ADAMTS5* antisense oligonucleotides directly into the knee joint. As a proof of principle, stabilized siRNA targeting *ADAMTS5* mRNA was injected into the nucleus pulposus after intervertebral disc puncture injury and subsequently delayed progression of intervertebral disc degeneration (Seki et al., 2009). More recently, a fibrin-hyaluronic acid hydrogel-based delivery system for *ADAMTS5* antisense oligonucleotides was described with the goal to develop a cartilage repair tissue that would inhibit ADAMTS5 activity within the co-delivered chondrocytes and the surrounding tissue (Garcia et al., 2019). It remains to be established if inhibitory RNA-based ADAMTS5 inactivation can modulate arthritis progression *in vivo*. CRISPR/Cas9-mediated gene deletion is an attractive approach to specifically delete target genes in select tissues. In combination with adeno-associated virus (AAV)-mediated gene delivery, one could envision that ADAMTS5 activity could be significantly reduced in knee joints affected by arthritis and halt disease progression (Evans et al., 2018; Fitzgerald, 2020). In a recent study, disease modification was described in a mouse model for osteoarthritis where CRISPR/Cas9-mediated simultaneous deletion of *NGF*, *IL-1β* and *MMP13* was achieved (Zhao et al., 2019). However, *ADAMTS5* was not targeted directly, but its gene expression was reduced.

CONCLUSION AND OUTLOOK

In this review, we showcased the complexity of ADAMTS protease regulation, which ranges from transcriptional regulation to protease-mediated protease activation to balancing proteases and their endogenous inhibitors. However, several remaining questions need to be addressed in the future to fully understand the regulatory networks involving ADAMTS proteases and to fully harness the potential of inhibiting individual ADAMTS proteases to modify outcomes in degenerative diseases such as arthritis. For example, there is an ongoing quest to identify and validate the entire *in vivo* substrate spectrum for each individual ADAMTS protease and to determine the consequences of altered substrate cleavage (Schilling and Overall, 2007; Savickas and Auf dem Keller, 2017; Apte, 2020; Satz-Jacobowitz and Hubmacher, 2021). This is relevant to fully understand the biological function of individual proteases and to predict potential undesired side effects when designing isotype-specific inhibitors. An example is ADAMTS5, which can cleave aggrecan and versican but also the small leucine-rich proteoglycan decorin, biglycan and fibromodulin and possibly other proteins (Stanton et al., 2005; Gendron et al., 2007; Longpre et al., 2009). Some of these cleavage events may be beneficial and may need to be preserved in the presence of an ADAMTS5 inhibitor. In addition, inhibition of aggrecan cleavage to prevent tissue degradation may be desired in one tissue, such as the articular cartilage but simultaneously may have adverse effects in other tissues, such as tendon, where aggrecan accumulation resulted in decreased mechanical properties, or the aorta, where aggrecan and versican accumulated in thoracic aortic aneurysms potentially promoting aortic dissection and rupture (Velasco et al., 2011; Wang et al., 2012; Cikach et al., 2018). Therefore, an ideal small molecule or biologic inhibitor for ADAMTS5 as a disease modifying arthritis drug would be, one, highly specific for ADAMTS5; two, spare substrates other than aggrecan; and, three, deliverable to or become selectively activated in articular cartilage or the synovium. Since mAB 2B9 inhibited versicanase and aggrecanase activity of ADAMST5, the same exosites seem to be required for versican and aggrecan recognition and the design of such an "ideal" inhibitor may not be feasible.

Another question revolves around the regulatory networks in which ADAMTS proteases participate. These involve in one aspect transcriptional programs that induce distinct sets of genes, including ADAMTS proteases and that are regulated by specific signaling inputs, such as pro-inflammatory cytokines (Bondeson et al., 2008). By regulating critical nodes in the inflammatory cascade, ADAMTS protease could potentially be co-regulated together with other disease modifying genes. Additionally, it will be important to determine, if other ADAMTS sister proteases could compensate for an inhibited or downregulated ADAMTS protease as it was shown for ADAMTS9/ADAMTS20, ADAMTS7/ADAMTS12 or ADAMTS6/ADAMTS10 (McCulloch et al., 2009; Dubail et al., 2014; Mead et al., 2018; Mead et al., 2021).

Lastly, it will be interesting to explore novel technologies to regulate ADAMTS protease activity *in vivo*, for example by using antisense oligonucleotides, CRISPR/Cas-9 gene editing or by tissue-specific inhibitors. Such approaches may contribute to achieve the Holy Grail of ADAMTS isotype-specific and substrate-specific inhibitors that can then be deployed to modulate disease progression in degenerative conditions, such as arthritis.

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Targeting the Host Response: Can We Manipulate Extracellular Matrix Metalloproteinase Activity to Improve Influenza Virus Infection Outcomes?

*Jess Pedrina and John Stambas**

Faculty of Health, School of Medicine, Deakin University, Geelong, VIC, Australia

Each year, hundreds of thousands of individuals succumb to influenza virus infection and its associated complications. Several preventative and therapeutic options may be applied in order to preserve life. These traditional approaches include administration of seasonal influenza vaccines, pharmacological interventions in the form of antiviral drug therapy and supportive clinical approaches including mechanical ventilation and extracorporeal membrane oxygenation. While these measures have shown varying degrees of success, antiviral therapies and vaccination are constrained due to ongoing antigenic drift. Moreover, clinical approaches can also be associated with complications and drawbacks. These factors have led to the exploration and development of more sophisticated and nuanced therapeutic approaches involving host proteins. Advances in immunotherapy in the cancer field or administration of steroids following virus infection have highlighted the therapeutic potential of targeting host immune responses. We have now reached a point where we can consider the contribution of other "non-traditional" host components such as the extracellular matrix in immunity. Herein, we will review current, established therapeutic interventions and consider novel therapeutic approaches involving the extracellular matrix.

Keywords: immunity, extracellular matrix, influenza, therapeutics, metalloproteinases

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****Correspondence:***
John Stambas
john.stambas@deakin.edu.au

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INTRODUCTION

Influenza virus infections, known colloquially as "the flu" are acute infections of the respiratory tract caused by members of the influenza virus genera in the *Orthomyxoviridae* family. Though influenza A, B, and C viruses are capable of infecting humans, seasonal epidemics are typically caused by influenza A and B viruses (Kawaoka and Neumann, 2012). At the moment, only influenza A viruses are considered to have pandemic potential. While the majority of these infections are self-limiting in healthy individuals, when susceptible members of the population become infected, the prognosis may be far less favourable. Those particularly at risk of influenza virus complications are predominantly the very young, the elderly and the immunocompromized. When these individuals become infected, there is a significantly greater likelihood of complications including viral pneumonia, secondary bacterial pneumonia, acute respiratory distress syndrome (ARDS), sepsis, encephalitis, pericarditis, and myocarditis, just to name a few (reviewed by (Kalil and Thomas, 2019)). It is in these instances that medical intervention is required. In addition to the risk posed by seasonal influenza virus epidemics to vulnerable populations, it is also vital that we have effective

TABLE 1 | Evaluation of current and emerging therapeutics for influenza virus infection.

Strategy	Current/emerging	Prophylactic/ therapeutic	Strengths	Limitations
Seasonal vaccination	Current	Prophylactic	Offers a range of protection against seasonal strains of influenza virus (Hoft et al. (2011), Estrada and Schultz-Cherry (2019)	Negatively impacted by antigenic drift and shift Demicheli et al. (2018), Sautto et al. (2018), Estrada and Schultz-Cherry (2019)
Universal vaccination	Emerging	Prophylactic	Antigenic drift has less impact and can be applied to pandemic strains Ellebedy and Webby (2009), Ellebedy et al. (2014), Jang and Seong (2019)	Still under development and uncertainty remains regarding effectiveness Estrada and Schultz-Cherry (2019)
Public health measures	Current	Prophylactic	Reduces the transmission of influenza virus Yeoh et al. (2020)	Not socially or economically sustainable Dubey et al. (2020)
Antiviral drug therapy	Current	Both	May be used in a prophylactic or therapeutic context Amarelle et al. (2017), Higashiguchi et al. 2018, Gaitonde et al. (2019)	Emergence of antiviral drug resistance that limits efficacy Lackenby et al. (2011), Hussain et al. (2017), Goldhill et al. (2018)
Corticosteroid therapy	Current	Therapeutic	May reduce lung pathology Li et al. (2017)	May contribute to increased mortality Viasus et al. (2011), Ni et al. (2019), Ye et al. (2020), Zhou et al. (2020)
Clinical interventions	Current	Therapeutic	Potentially lifesaving Davies et al. (2009), Popat and Jones (2012)	Invasive, potential for increased viral transmission Davies et al. (2009), Arulkumaran et al. (2020), Ng et al. (2020), Al Lawati et al. (2021)
ECM manipulation	Emerging	Unknown	Newly identified roles for zinc proteases in virus infection. Wu et al. (2007), McMahon, et al. (2016), Rojas-Quintero et al. (2018), Boyd et al. (2020)	New field of research – many unknowns
Small molecule inhibitors	Emerging and current (in other disorders)	Unknown	Evidence of successful MMP, ADAM and ADAMTS protein inhibition Malemud (2019), Santamaria et al. (2021)	Pre-clinical stages
Antibody-based inhibition	Emerging and current (in other disorders)	Unknown	Evidence of *in vitro, in vivo* and human *ex vivo* inhibition Larkin et al. (2015), Santamaria et al. (2015)	May lack binding specificity Santamaria et al. (2021)

intervention strategies available in the event of an influenza virus pandemic. As demonstrated by the 1918 "Spanish Flu," the impact on the the aforementioned susceptible populations was less Hoffman (2011), Short et al. (2018), Auladell (2019) than that seen on younger, healthy members of the population. Furthermore, the ongoing COVID-19 pandemic has highlighted global vulnerabilities when dealing with large-scale respiratory virus outbreaks. It is therefore necessary to re-evaluate current strategies, whilst also developing alternative approaches that can be applied to a variety of infectious agents. This will be discussed throughout this review. A summary of current and emerging strategies can be found in **Figure 1** and **Table 1**.

PREVENTATIVE STRATEGIES

As with any infectious agent, the best course of prevention is to ensure the virus doesn't cause disease in the first place. This may be achieved through vaccination, whereby you prevent the virus gaining access and entry to target cells or through behavioral modifications that reduce the likelihood of an individual being exposed to the virus.

Seasonal Vaccination

The most common method of preventing infection with a viral pathogen is vaccination, and for influenza viruses this is required on a seasonal basis. At present, there are two approved platforms for influenza vaccines–1) live, attenuated influenza vaccines (LAIV), and 2) inactivated influenza vaccines (Fiore et al., 2010). While both vaccines illicit a robust hemagglutinin-directed antibody response, LAIV also has the capacity to activate T cell immunity (Hoft et al., 2011; Estrada and Schultz-Cherry, 2019). Despite broad use, vaccine efficacy is variable and susceptible to changes is circulating strains. As the vaccines predominately target the hypervariable viral hemagglutinin head, they are vulnerable to mutations (genetic drift) and as such need to be reformulated on an annual basis (Demicheli et al., 2018; Sautto et al., 2018; Estrada and Schultz-Cherry, 2019). Furthermore, the time required to manufacture vaccines may impact responses during an evolving influenza virus pandemic, highlighting the need for development of a more broadly protective universal influenza vaccine (Ellebedy and Webby, 2009; Krammer and Palese, 2013; Sparrow et al., 2021).

Universal Influenza Virus Vaccines

In an effort to circumvent issues associated with current seasonal influenza vaccines, "universal vaccine" platforms have been under development for many years. Current vaccines for influenza viruses target the highly variable head of the viral hemagglutinin (HA) protein (Ellebedy and Webby, 2009; Xu et al., 2010; Sautto, Kirchenbaum et al., 2018; Estrada and Schultz-Cherry, 2019). By targeting highly conserved regions, we may reduce the need for seasonal vaccines (Ellebedy et al., 2014; Jang and Seong, 2019). Universal vaccines that have focused on the conserved HA stalk region have been developed in recent years for influenza A viruses (Krammer and Palese, 2013). As such,

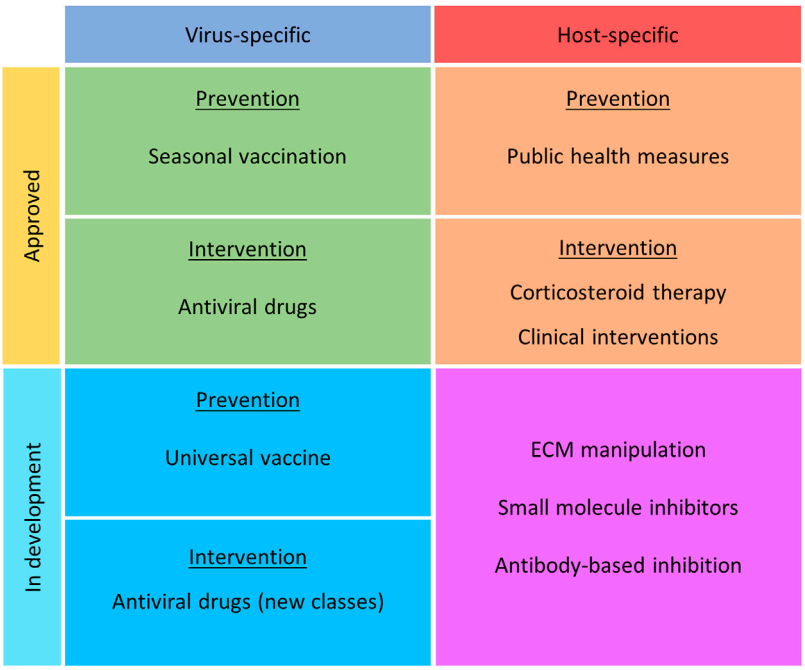

FIGURE 1 | Current and emerging intervention strategies for the prevention and treatment of influenza virus infection. Strategies have been noted as virus or host specific, approved or in development, and nature of treatment (prevention or intervention).

stalk-based targets appear to be a promising candidate in the hunt for a universal vaccine (Mallajosyula et al., 2015). Another highly conserved potential target is the ectodomain matrix protein 2 (M2e) found on influenza A viruses. Unlike other targets, M2e does not generally stimulate the expression of neutralizing antibodies, but instead induces non-neutralizing antibodies that trigger antibody-dependent cell-mediated cytotoxicity (Simhadri et al., 2015). There has been significant activity in the universal vaccine space and candidates are under development or in clinical trial, with only limited knowledge as to the degree and longevity of protection they offer (Corder et al., 2020).

Public Health Measures

During the current coronavirus disease (COVID-19) pandemic, governments around the world have introduced necessary public health measures in an effort to reduce the rate of transmission of SARS-CoV-2 virus. While the specific nature of these measures varied, curfews, lockdowns, social distancing and the implementation of mask-wearing have all been introduced to mitigate spread of disease. As a consequence of these measures, there has been a significant decline in the number of laboratory-confirmed influenza virus infections observed (Yeoh et al., 2020). The most likely explanation for this marked reduction is not fewer diagnostic tests, but rather the similarities in transmission route for both respiratory viruses (Wu et al., 2020; Yeoh et al., 2020). Therefore, by implementing public health measures to stem the rate of coronavirus infection, a positive byproduct has been a reduction in transmission of influenza viruses. Despite the success of these measures, it should be noted that curfews and lockdowns are simply not sustainable beyond a pandemic setting, due to the significant economic and psychosocial burden they create (Dubey et al., 2020).

PHARMACOLOGICAL INTERVENTION

Though the best course of action is to prevent influenza virus infection, as with many infectious agents, this is not always feasible. In these cases, it is necessary to switch from a preventative model to one of harm minimization. There are a range of pharmacological interventions that may be implemented as supportive therapies in order to reduce the rate of viral replication, as well as to ameliorate the damaging effects of immunopathology on tissue during the process of viral clearance. The most common of these treatments are discussed further throughout this section.

Antiviral Drug Therapies

Following infection with influenza viruses there are several antiviral drugs that may be administered as supportive therapies. Of these drugs, there are currently three major classes approved for use in a clinical setting–M2 ion channel inhibitors, neuraminidase inhibitors, and transcriptional inhibitors (Amarelle et al., 2017; Hayden et al., 2018). It should also be noted that there are a number of drugs targeting various components of influenza viruses or the human immune system, that are currently under development and are not discussed as they are beyond the scope of this review.

M2 Ion Channel Inhibitors

The oldest group of antiviral drugs approved for the treatment of influenza A virus infections are the M2 ion channel inhibitors. The drugs amantadine and rimantadine inhibit the function of the M2 ion channel of influenza A viruses, preventing viral uncoating and the transfer of viral genomic material into the host cell (Hay et al., 1985; Lampejo, 2020). Due to their mechanism of action, these drugs are only effective against influenza A viruses, as all other influenza viruses lack M2 ion channels. The M2 ion channel inhibitors are no longer recommended for administartion due to the large percentage of circulating influenza A strains where resistance has been identified (Hussain et al., 2017).

Neuraminidase Inhibitors

Neuraminidase inhibitors are among the most effective antiviral drugs used in the clinic for the treatment of influenza virus infection. Mechanistically, these antiviral therapeutics block the enzymatic activity of the viral neuraminidase, preventing cleavage of sialic acid residues and release of new virions from the host cell (Sidwell et al., 1998). Within this class, of drugs there a currently two approved antivirals administered for the prevention and treatment of influenza A and B infection; oseltamivir (oral), and zanamivir (inhaled), and two approved for treatment only; peramivir (intravenous), and laninamivir (inhaled) (Amarelle et al., 2017; Higashiguchi et al., 2018; Gaitonde et al., 2019). Despite their success in the past, emergence of viral resistance to these drugs is becoming an increasing problem (though not to the same extent as the M2 ion channel inhibitors), and these drugs will need to be used responsibly to ensure their effectiveness is maintained (Lackenby et al., 2011; Li et al., 2015).

Transcriptional Inhibitors

Transcriptional inhibitors are a broad category of antiviral drugs that inhibit viral replication pathways, by blocking viral transcription. This category includes the viral RNA-dependent RNA polymerase inhibitor, favipiravir, as well as the more recent selective cap-dependent endonuclease inhibitor baloxavir marboxil (Furuta et al., 2013; Takashita et al., 2018). While both these drugs are approved for the treatment of influenza A and B infections (pandemic use only in the case of favipiravir), there is little evidence in the literature that supports their potential as prophylactics (Lampejo, 2020). Furthermore, as with other antivirals on the market, there is strong evidence to suggest influenza viruses have the potential to develop resistance to these treatments, rendering them ineffective (Goldhill et al., 2018; Takashita et al., 2019).

Corticosteroid Treatment

Of the pharmacological interventions currently available for treatment of acute respiratory infections such as influenza viruses and SARS-CoV-2, corticosteroid therapy is possibly one of the most controversial. During infection, the immune system may respond excessively in an attempt to clear the infectious agents. In doing so, tissue may become damaged as a consequence of excessive production of proinflammatory cytokines Li et al. (2017), leading to further complications. The principle aim of this type of therapy is to counteract excessive inflammation and reduce immunopathology (La Gruta et al., 2007). Although corticosteroids have been used in the treatment of severe viral respiratory infections and associated complications for some time, evidence is beginning to emerge that administration of corticosteroids such as dexamethasone during influenza virus infection does not reduce the risk of further complications, and may in fact be contributing to increased mortality (Viasus et al., 2011; Ni et al., 2019; Ye et al., 2020; Zhou et al., 2020).

CLINICAL INTERVENTIONS

Despite the availability of pharmacological therapeutics for the treatment of influenza virus infection, in severe cases or in individuals with secondary complications, more invasive clinical interventions may be necessary to maintain respiratory function. The most common of these supportive measures are noninvasive mechanical ventilation, invasive mechanical ventilation, and extracorporeal membrane oxygenation (ECMO). Noninvasive mechanical ventilation refers to the provision of respiratory support without direct tracheal involvement, in the form of oxygen delivery *via* face mask, helmets, mouth pieces etc. (Popat and Jones, 2012). Conversely, invasive mechanical ventilation involves direct access to the trachea, typically via intubation (Popat and Jones, 2012). Although both measures may be necessary to preserve life, they are associated with increased risk of transmission of respiratory viruses to healthcare workers, due to the production of aerosols (Esquinas et al., 2014; Arulkumaran et al., 2020; Ng et al., 2020; Al Lawatis et al., 2021). If hypoxia continues in the presence of invasive mechanical ventilation, extracorporeal membrane oxygenation (ECMO) may be implemented as a last resort. ECMO acts as an artificial heart and lung, externally oxygenating the blood, then returning it to the circulatory system. Given the extremely invasive nature of the procedure and the many associated risks, ECMO is only ever used in life-threatening infections when all other measures have failed (Davies et al., 2009; Kowalewski et al., 2020).

THERAPEUTIC POTENTIAL OF THE EXTRACELLULAR MATRIX

As highlighted throughout this review, current preventative strategies and therapeutic treatments are all either not sustainable, highly invasive, or are susceptible to being rendered ineffective through constant mutations in the genome of these viruses. These factors clearly necessitate the development of novel therapeutics that counteract the vulnerabilities described throughout this review. Although the host immune response has been studied in depth as a potential source of therapeutic targets, "non-traditional" elements of the response are often overlooked.

Extracellular Matrix

One such element often overlooked is the extracellular matrix, or ECM. The ECM is a highly dynamic, non-cellular structure that plays a key role in cellular signaling, growth, repair, migration, and general homeostasis (Hynes, 2009; Bonnans et al., 2014; Theocharis et al., 2019). In mammalian systems, the ECM is comprised of hundreds of different proteins including glycoproteins, collagens, and proteoglycans, just to name a few (Hynes and Naba, 2012). While historically viewed as a rigid network with a purely structural role, the ECM is now understood to be a highly complex and vital component of tissues. More recently, the ECM has been found to have both positive and negative associations with different disease states, depending on the degree of remodeling by metalloproteinases (Bradley et al., 2012; Klose et al., 2013; Cal and Lopez-Otin 2015; Binder et al., 2017; Herrera et al., 2018). It is therefore a logical progression to investigate the ECM and the enzymes that remodel it as a potential therapeutic target to enhance the immune response against infectious disease, including influenza virus infections.

A Disintegrin-like and Metalloproteinase with Thrombospondin Motifs

Of these ECM remodeling enzymes, the a disintegrin-like and metalloproteinase with thrombospondin motifs (ADAMTS) family is one of the more recently discovered, with its first member ADAMTS1, identified in 1997 (Kuno et al., 1997; Porter et al., 2005). Since that time, an additional 18 mammalian members have been reported (Apte, 2009; Dubail and Apte, 2015). The ADAMTS family are responsible for cleaving a variety of substrates from proteoglycans (proteoglycanase clade), to clotting enzymes (ADAMTS13), to yet unidentified substrates (orphan clade) (Kelwick et al., 2015). As our knowledge of these enzymes has become more sophisticated, their importance in disease states has become more apparent. This has been eloquently presented in reviews by (Mead and Apte, 2018; Santamaria, 2020). While the role of these enzymes in cancer biology has been known for some time Kumar et al. (2012a), Kumar et al. (2012b), Cal and Lopez-Otin (2015), Chen et al. (2018), very little was known about their function in the immune response to viral infection. In 2016, a study by McMahon et al. (2016) found that expression of ADAMTS5 was necessary for optimal $CD8^+$ T cell immunity at effector sites in the periphery, as mice lacking this enzyme displayed increased disease severity and poor viral clearance associated with an accumulation of the ADAMTS 5 substrate versican in draining lymph nodes following influenza virus infection. In contrast, a recent study by Boyd et al. (2020) demonstrated that expression of ADAMTS4 in lung fibroblasts was associated with immunopathology and poor disease outcomes in mice and humans (Boyd et al., 2020). Furthermore, systems biology approaches have indicated that overexpression of versican, an ADAMTS enzyme substrate, may be associated with hyperinflammatory responses following severe COVID-19 infection (Jung et al., 2021). These findings suggest that ADAMTS enzymes and related pathways may be attractive therapeutic targets and further investigation is warranted. In support of these findings a number of antibodies and small molecule inhibitors (for example GSK2394000, GSK2394002, 237-53, sugar-based arylsulfonamide 4b, and Agg-523) are currently available and in clinical and preclinical trials that can be used to target ADAMTS enzymes (Dancevic and McCulloch, 2014; Larkin et al., 2015; Santamaria et al., 2015; Shiraishi et al., 2016; Balchen et al., 2018; van der Aar et al., 2018; Malemud, 2019; Santamaria and de Groot, 2019; Santamaria, 2020; Santamaria et al., 2021). It should be noted however that over-expression of ADAMTS enzymes may also improve disease outcomes (McMahon et al., 2016).

Matrix Metalloproteinases

In addition to the ADAMTS family, another key remodeling enzyme family in the ECM are the matrix metalloproteinases (MMPs). Unlike the ADAMTS family, the MMPs have been studied for quite some time with proteolytic activity first being described in 1962 (Gross and Lapiere, 1962). Of the 28 reported MMPs, 23 are expressed in humans Cui et al. (2017), Raeeszadeh-Sarmazdeh et al. (2020), and are grouped into broad clades based on structure, function and substrate preference (Raeeszadeh-Sarmazdeh et al., 2020). Unlike ADAMTS enzymes, the role of MMPs is well-documented in immune responses, and has been specifically characterized following influenza virus infection. While some family members have been associated with protection, other family members have been associated with poor outcomes following influenza virus infection. A recent study by Rojas-Quintero et al (2018) found that an absence of MMP9 in mice resulted in increased viral clearance from the lung, likely due to the induction of more effective adaptive immune responses and reduced lung damage associated with reduced E-cadherin shedding (Rojas-Quintero et al., 2018). Conversely, by inhibiting the wnt pathway and reducing the expression of MMP2 and MMP9, the extravasation of T cells is greatly impaired (Wu et al., 2007). While this may be beneficial in certain disease states where an anti-inflammatory environment is desired, in the context of influenza virus infection, reduced T cell migration can lead to delayed viral clearance and poor disease outcomes (McMahon et al., 2016). MMP inhibitors have also been tested in phase I clinical trials and have the potential to be utlized to counteract poor virus infection outcomes (Brown 1999; Fingleton 2008; Li, Saji et al., 2013).

CONCLUSION

As demonstrated throughout this review, there are significant gaps and controversies surrounding current influenza virus prophylactics and therapeutics. A common theme highlighted herein is the vulnerability of our current strategies due to our overreliance of targeting influenza virus structures and replication. If we are to overcome these vulnerabilities, the development of novel therapeutics that target the host and work in consert or complement existing strategies is critical in order to prepare us for future epidemics or pandemics. The untapped potential of the ECM and its remodeling enzymes has been demonstrated herein, and further investigation of possible applications to viral immunity is warranted.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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ADAMTS5 in Osteoarthritis: Biological Functions, Regulatory Network, and Potential Targeting Therapies

Lejian Jiang[1,2†], Jiachen Lin[3,4,5†], Sen Zhao[3,4,5], Jiaqian Wu[1], Yongming Jin[1], Li Yu[6], Nan Wu[3,4,5], Zhihong Wu[4,5,7], Yue Wang[1,2] and Mao Lin[1,2,4,7]**

[1]Department of Orthopedic Surgery, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China, [2]Spine Lab, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China, [3]State Key Laboratory of Complex Severe and Rare Diseases, Department of Orthopedic Surgery, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China, [4]Beijing Key Laboratory for Genetic Research of Skeletal Deformity, Beijing, China, [5]Medical Research Center, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China, [6]Department of Operating Room, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China, [7]Key Laboratory of Big Data for Spinal Deformities, Chinese Academy of Medical Sciences, Beijing, China

ADAMTS5 is involved in the pathogenesis of OA. As the major aggrecanase-degrading articular cartilage matrix, ADAMTS5, has been regarded as a potential target for OA treatment. We here provide an updated insight on the regulation of ADAMTS5 and newly discovered therapeutic strategies for OA. Pathophysiological and molecular mechanisms underlying articular inflammation and mechanotransduction, as well as chondrocyte hypertrophy were discussed, and the role of ADAMTS5 in each biological process was reviewed, respectively. Senescence, inheritance, inflammation, and mechanical stress are involved in the overactivation of ADAMTS5, contributing to the pathogenesis of OA. Multiple molecular signaling pathways were observed to modulate ADAMTS5 expression, namely, Runx2, Fgf2, Notch, Wnt, NF-κB, YAP/TAZ, and the other inflammatory signaling pathways. Based on the fundamental understanding of ADAMTS5 in OA pathogenesis, monoclonal antibodies and small molecule inhibitors against ADAMTS5 were developed and proved to be beneficial pre-clinically both *in vitro* and *in vivo*. Recent novel RNA therapies demonstrated potentials in OA animal models. To sum up, ADAMTS5 inhibition and its signaling pathway–based modulations showed great potential in future therapeutic strategies for OA.

Keywords: ADAMTS5, osteoarthritis, signaling pathways, monoclonal antibody, small molecule inhibitors, RNA therapies

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***Correspondence:**
Mao Lin
mao.lin@zju.edu.cn
Yue Wang
wangyuespine@zju.edu.cn

†*These authors have contributed equally to this work*

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INTRODUCTION

Osteoarthritis (OA) is one of the most common chronic joint lesions, mainly affecting people aged 50–75 years, with an approximate prevalence of 4–5% in the hand, 6% in the hip, and 16–17% in the knee in the general population (Hunter and Bierma-Zeinstra, 2019). OA is characterized by articular cartilage loss, subchondral bone sclerosis, and osteophyte formation (Loeser et al., 2012). Etiologically, primary OA is driven by a combination of inheritance, aging, obesity, inflammation, and biomechanical risk factors. Dysregulation of signaling pathways, especially the activation of proinflammatory pathways, promotes the overactivation of matrix-degrading

enzymes and exacerbates the degradation of cartilage extracellular matrix (ECM) (Glyn-Jones et al., 2015). Collagens and aggrecan are both pivotal structural components of cartilage ECM, and their degradation is a significant event at the early stage of OA (Maldonado and Nam, 2013). It has been documented that matrix metalloproteinases (MMPs, especially MMP-13) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs, especially ADAMTS4 and ADAMTS5) facilitate type II collagen and aggrecan degradation, respectively (Verma and Dalal, 2011).

ADAMTSs are a family of zinc metalloendopeptidases that participate in diverse biological processes, such as procollagen processing, ECM remodeling, inflammation, cell migration, and vascular biological processes (Kelwick et al., 2015). In particular, ADAMTS5 (aggrecanase-2) overexpression is a key risk factor in degenerative joint diseases and intervertebral disc degeneration (Wu et al., 2014; Santamaria, 2020).

ADAMTS4 and ADAMTS5 are thought to analogously mediate aggrecan cleavage. However, the protective effects of *Adamts5* gene knockout and ADAMTS5-specific antibodies in surgically induced OA mouse models emphasize that ADAMTS5 is the major aggrecan-degrading enzyme in OA (Glasson et al., 2004; Glasson et al., 2005; Apte, 2016). Therefore, ADAMTS5 has long been regarded as a potential target for OA treatment. However, as the balance between matrix synthesis and degradation is critical for ECM structure and tissue homeostasis, direct inhibition of ADAMTS5 has aroused great concern. For instance, ADAMTS5 knockout can lead to deleterious accumulation of proteoglycan in the adult cardiovascular system and disrupt aortic wall mechanics in mice (Dupuis et al., 2011; Dupuis et al., 2013; Fava et al., 2018; Dupuis et al., 2019). Recently, updated knowledge regarding ADAMTS5 regulatory factors and the preclinical discovery of potential disease-modifying drugs have provided more options for OA treatment. Thus, comprehensive insight into the biological functions and molecular regulation of ADAMTS5, supplemented by the current developmental stages of diverse classes of drugs, may be necessary to better understand the involvement of ADAMTS5 in OA and identify future therapeutic strategies.

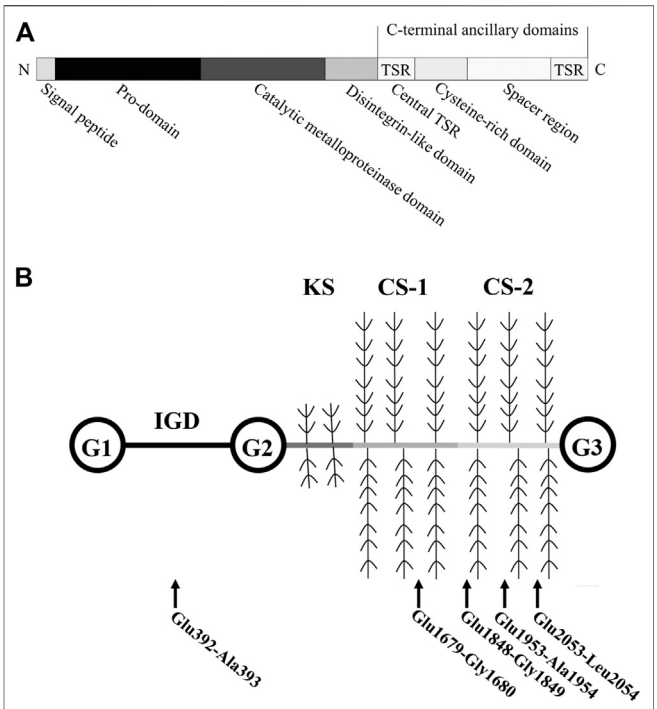

FIGURE 1 | Structure of human ADAMTS5 and Aggrecan. **(A)** Human ADAMTS5 domain structure. Thrombospondin type 1 sequence repeat, central TSR. **(B)** Structure of Aggrecan. ADAMTS5-mediated cleavage within the aggrecan occurs at (glutamate) Glu-Xaa (where Xaa = alanine, glycine, and leucine) recognition motifs. Abbreviations: globular domains, G1-3; interglobular domain, IGD; keratan sulfate attachment domain, KS; chondroitin sulfate attachment domains, CS-1 and -2.

Functions and Regulation of ADAMTS5 in Normal Cartilage Extracellular Matrix

Aggrecan is a major component of cartilage and protects collagens against degradation (Pratta et al., 2003). Aggrecan glycosaminoglycan chains provide a gel-like structure and mechanical resistance in joints (Kiani et al., 2002). Increased levels of aggrecan fragments are a typical pathological change in cartilage and may serve as a severity indicator for OA (Roughley and Mort, 2014).

Aggrecan can be cleaved by ADAMTS family members, including ADAMTS1, ADAMTS4, ADAMTS5, ADAMTS8, and ADAMTS15 (Santamaria, 2020). Among these ADAMTSs with aggrecan-degrading activity, ADAMTS4 and ADAMTS5 tend to be most efficient (Tortorella and Malfait, 2008). These two aggrecanases are regarded as critical factors in metabolism, homeostasis, and pathological changes of joint ECM. Structurally, from the N- to C-terminus, ADAMTS5 is composed of a signal peptide, a pro-domain, a catalytic metalloproteinase domain, a disintegrin-like domain, and other C-terminal ancillary domains (a central thrombospondin type 1 sequence repeat (central TSR) motif, a cysteine-rich domain, a spacer region, and an additional TSR motif) (Kelwick et al., 2015) (**Figure 1A**). Extracellular excision of the pro-domain by proprotein convertases, specifically furin and furin-like enzymes, is essential for ADAMTS5 activation (Longpré et al., 2009). The catalytic metalloproteinase domain alone has little proteolytic activity, and the combination of its C-terminal ancillary domains increased its proteolytic activity (Gendron et al., 2007). The cysteine-rich domain is critical for the localization of ADAMTS5 and its binding to the cell surface and ECM (Gendron et al., 2007). Similar to ADAMTS4, the cysteine-rich domain of ADAMTS5 is also critical for its interaction with the glycosaminoglycan chains of aggrecan, and the central TSR motif is necessary for aggrecan recognition and cleavage (Tortorella et al., 2000; Flannery et al., 2002; Fushimi et al., 2008). Exosites in the spacer region are responsible for substrate recognition and proteolysis (Santamaria et al., 2019).

ADAMTS5 is expressed at low levels in various tissues, including the placenta, heart, lung, skeletal muscle, tendon, cartilage, and synovium (Fagerberg et al., 2014). Breakdown

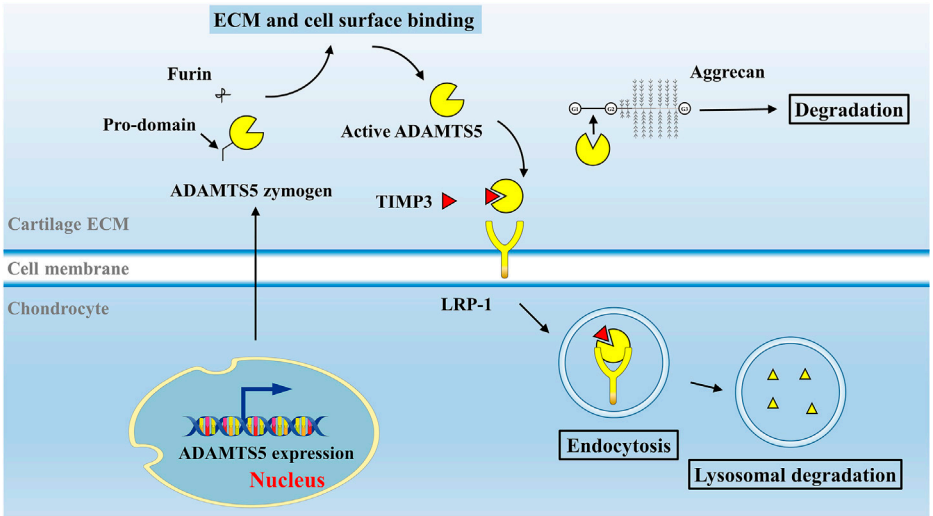

FIGURE 2 | Activation and degradation mechanism of ADAMTS5 *in vivo*. In normal cartilage, ADAMTS5 is activated under the stimulation of inflammatory factors or breakdown products in cartilage ECM. After the removal of its pro-domain by furins, activated ADAMTS5 cleaves the aggrecan core protein at its specific Glu-Xaa recognition motifs. ADAMTS5 can be inhibited by its endogenous inhibitor, TIMP3. ADAMTS5, together with TIMP3 are subsequently endocytosed by chondrocyte via LRP-1 receptor and degraded. Abbreviations: tissue inhibitor of metalloproteinase 3, TIMP3; lipoprotein receptor–related protein 1, LRP-1.

products of cartilage ECM can enhance MMP-13 and ADAMTS5 expression and activation (Jung et al., 2019). ADAMTS5 zymogen in the ECM is inactive and can be activated extracellularly by removal of its pro-domain (Longpré et al., 2009). Activated ADAMTS5 cleaves the aggrecan core protein at its specific recognition motifs, for example, the glutamate (Glu) 373-alanine (Ala) 374 bond (Glu392-Ala393 bond in the modern nomenclature, UniPort ID P16112) in its interglobular domain, as well as other specific sites, leading to loss of integrity of aggrecan molecules (Kiani et al., 2002; Little et al., 2007) (**Figure 1B**). Under physiological conditions, the aggrecanase activity of ADAMTS5 in cartilage can be inhibited by its endogenous inhibitor, tissue inhibitor of metalloproteinase 3 (TIMP3) (**Figure 2**). TIMPs are expressed in connective tissues and play an important role in the inhibition of MMPs (Brew et al., 2000). TIMP3, with its distinct N-terminal inhibitory domain, has a strong inhibitory effect on ADAMTS4 and ADAMTS5 (Kashiwagi et al., 2001). After forming a complex with TIMP3, ADAMTS5 can therefore be cleared by chondrocytes through lipoprotein receptor-related protein 1 (LRP-1)–mediated endocytosis in cartilage tissue (Yamamoto et al., 2013) (**Figure 2**). In cartilage, the cysteine-rich domain and the spacer region of ADAMTS5 are involved in effective binding to the sulfated proteoglycans at the cell surface or the ECM (Gendron et al., 2007). The central TSR motif and the spacer region can also be identified by LRP-1, leading to ADAMTS5 clearance. Thus, ADAMTS5 and TIMP3 can be endocytosed independently or as a complex. LRP-1 is an important regulator of normal cartilage homeostasis, and the location and activity of ADAMTS5 are determined by competition between the ECM and LRP-1.

ADAMTS5 in the pathogenesis of OA *In Vitro* and *In Vivo*.

Cytological studies and animal models recapitulating OA enhance the understanding of disease progress and the evaluation of therapeutic modalities. Desirable biomarkers of OA can effectively assist indications for OA stages and monitor treatment responses (Gu et al., 2019). Since aggrecan destruction in synovial fluid is a hallmark at the early stage of OA, the major aggrecanase, ADAMTS5, is identified as a potential biomarker for the prediction of OA progression (Saberi Hosnijeh et al., 2019).

Genetic polymorphisms in *ADAMTS5* in different populations were also identified to be associated with susceptibility to OA. Bioinformatic analysis on 2,715 patients with OA and 1,185 controls in a European Caucasian population identified two single-nucleotide polymorphisms at *ADAMTS5* gene loci (Rodriguez-Lopez et al., 2008). These two nonsynonymous variants appeared clustered in patients with severe OA and resulted in an aberrant amino acid sequence of encoded ADAMTS5. Furthermore, another genetic variant in *ADAMTS5*, rs2830585, was identified in a Chinese population with 300 pairs of OA patients and control subjects (Zhou et al., 2019).

ADAMTS5, as one of the key downstream responders, was upregulated in OA models *in vitro* and *in vivo* (Song et al., 2007; Johnson et al., 2016). Moreover, cartilage destruction was rescued in *Adamts5* knockout mice with posttraumatic OA, while mice with deletion of *Adamts4* developed OA (Glasson et al., 2004; Glasson et al., 2005). In the joints and serum of rats with surgery-induced OA, the expression of ADAMTS5 was markedly increased along with OA progression (Elsadek et al., 2019).

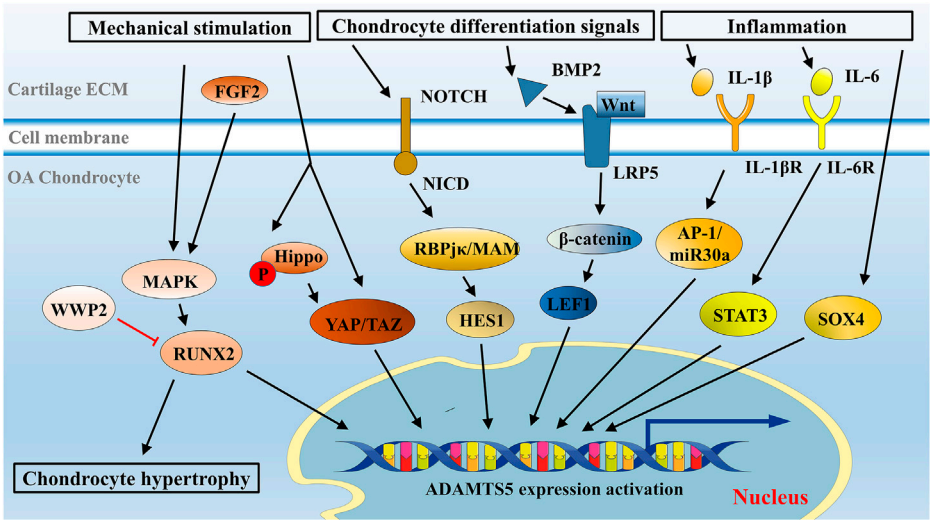

FIGURE 3 | Overview of signaling network in ADAMTS5 regulation in chondrocytes. Mechanical stimulation, cell differentiation signals, and inflammatory environment are primary initiators to ADAMTS5 overexpression in OA. Signaling pathways were illustrated with recent insights, such as Runx2 signaling, Notch signaling, Wnt/β-catenin signaling, and cytokine-mediated signaling pathways, and some newly discussed signaling pathways, such as YAP/TAZ signaling and Sox4 signaling are presented in this schematic presentation.

These studies suggested a critical role of ADAMTS5 in OA development and implied that ADAMTS5 can serve as not only a predictive biomarker of OA staging and prognosis but also a potential target for OA therapy. Thus, a comprehensive understanding of ADAMTS5 regulatory pathways is required.

Signaling Pathways Regulating ADAMTS5 Expression in the OA Pathological Process

Several signaling pathways are involved in ADAMTS5 modulation in the pathophysiological process of OA, such as Runx2 signaling, Fgf2 signaling, Notch signaling, Wnt signaling, YAP/TAZ signaling, and inflammatory signaling pathways (Chia et al., 2009; Hosaka et al., 2013; Ji et al., 2016a; Deng et al., 2018; Catheline et al., 2019; Wang et al., 2019). The regulation of ADAMTS5 and the crosstalk of each signaling pathway are discussed below.

Runx2 Signaling and Fgf2 Signaling

Runt-related transcription factor 2 (RUNX2) is a key transcription factor in osteoblast proliferation and differentiation (San Martin et al., 2009). RUNX2 is strictly expressed in the nucleus of osteoblasts and regulates the cell cycle via its oscillating level of expression (San Martin et al., 2009). Moreover, RUNX2 can respond to mechanical signals and affect bone homeostasis (Kanno et al., 2007). In human OA cartilage, high expression of RUNX2 was detected (Zhong et al., 2016; Chen et al., 2020). RUNX2 is also responsible for hypertrophic differentiation of chondrocytes, which is a characteristic change in the development of OA (Dreier, 2010; Catheline et al., 2019).

Analysis of the *ADAMTS5* promoter sequence identified four binding sites for the RUNX family, among which RUNX2 exhibited strong affinity (Thirunavukkarasu et al., 2007). In mechanical stretch-exposed OA chondrocytes, the expression of ADAMTS5 was overactivated by RUNX2 (Tetsunaga et al., 2011). In this literature, RUNX2 might have a role as a key downstream mediator of MAPK and p38 to regulate mechanical stress–induced ADAMTS5 expression (**Figure 3**). This trend was also confirmed in surgically induced OA mice: the progression of OA was significantly decelerated in *Runx2* knockout mice compared with control mice (Liao et al., 2017). A decrease in the expression of ADAMTS5 was also confirmed by immunohistochemical analysis in this study (Liao et al., 2017). Recently, WW domain-containing protein 2 (WWP2), a kind of E3 ubiquitin ligase in osteoblasts, was shown to inhibit the expression of *ADAMTS5* through ubiquitination and degradation of RUNX2 (Mokuda et al., 2019).

Fibroblast growth factor 2 (FGF2), a growth factor involved in many biological processes, is implicated in chondrocyte differentiation and maintaining cartilage homeostasis and is highly associated with the severity of OA (Ellman et al., 2013; Yan et al., 2012). RUNX2 can be activated by FGF2 (Qi et al., 2020; Ji et al., 2016b). FGF2 molecules can elicit RUNX2 activation through the MAPK/ERK pathway and eventually modulate ADAMTS5 (Ji et al., 2016b; Xiao et al., 2002) (**Figure 3**). Notably, in human OA chondrocytes treated with FGF2 for a short time (mostly less than 1 h), FGF2 was shown to inhibit *ADAMTS5* expression and thus retard cartilage destruction (Sawaji et al., 2008), while after long-term treatment (more than 2 h), FGF2 was likely to activate RUNX2-mediated ADAMTS5 upregulation (Ji et al., 2016b). This effect may be responsible for the temporal expression pattern of ADAMTS5 in OA, although the detailed mechanism remains unclear.

Yes-Associated Protein/TAZ Signaling

Yes-associated protein (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ) signaling are important not only for mediating tissue growth, cell fate, and tissue morphogenesis but also in the development of cartilage (Vanyai et al., 2020). YAP signaling is regulated by upstream Hippo-dependent and independent signaling, such as mechanical cues, metabolic signals, and other signaling pathways (Dupont, 2016). YAP can inhibit chondrocyte maturation by suppression of Collagen type X alpha 1 chain (*COL10A1*) expression through interaction with RUNX2 (Deng et al., 2016).

Overexpression of YAP was observed in cultured chondrocytes and surgery-induced animal OA models (Gong et al., 2019). Furthermore, inhibition of YAP can reduce interleukin-1β (IL-1β)–induced expression of MMP13 and ADAMTS5 and retard cartilage degradation in OA mice (Gong et al., 2019). While inhibition of YAP expression can ameliorate osteoarthritic cartilage degradation, other studies have revealed that YAP plays a protective role as an inflammatory inhibitor in the progression of OA (Deng et al., 2018). In addition, both *Yap* knockout and overexpression of YAP promote cartilage disruption, indicating that YAP regulates cartilage homeostasis in a biphasic manner (Deng et al., 2018; Zhang et al., 2019; Vanyai et al., 2020).

Hippo signaling is triggered by mechanical stimulation and phosphorylates its downstream effectors YAP/TAZ. Mechanical stimulation can also mediate YAP activity in the Hippo-independent signaling pathway, which requires Rho GTPase activity and tension of the actomyosin cytoskeleton (Dupont et al., 2011). The opposite effects triggered by mechanical inputs converge on the regulation of YAP/TAZ. Unphosphorylated YAP/TAZ is transported into the nucleus to promote downstream ADAMTS5 transcription, while phosphorylated YAP/TAZ is degraded in cytoplasm (Zhao et al., 2011) (**Figure 3**).

Notch Signaling

As a juxtacrine cellular signaling pathway, Notch signaling modulates cell differentiation and adult tissue homeostasis, including cartilage formation and pathology (Bray, 2016; Dowthwaite et al., 2004). In mice with surgically induced OA, Notch signaling is overactivated and participates in OA development (Saito and Tanaka, 2017). Generally, Notch signaling is initiated when the NOTCH receptor is cleaved by related proteinases after receiving signals from NOTCH ligands on adjacent cells (Kopan and Ilagan, 2009). The NOTCH receptor is a single-pass transmembrane receptor on the cell surface that is composed of an extracellular fragment, a membrane-tethered fragment and the NOTCH intracellular domain (NICD) (Chillakuri et al., 2012). Two main proteinases take part in the cleavage of the NOTCH receptor, a disintegrin and metalloproteinase 10 (ADAM10) and γ-secretase, which release NICD from NOTCH receptors. Gene transcription in the nucleus is subsequently regulated by the interaction of NICD with *trans*-acting elements, such as recombination signal binding protein for Ig kappa J (RBPjκ) and the coactivator Mastermind (MAM) (Nam et al., 2006; Wilson and Kovall, 2006) (**Figure 3**). Specifically, a downstream transcriptional repressor of Notch signaling, HES1, is upregulated (Kageyama et al., 2007). Once upregulated, HES1 is switched to an activator by its cofactor and directly upregulates the transcription of *Adamts5* and *Mmp13* in OA (Sugita et al., 2015) (**Figure 3**). In *Hes1* knockout OA mice, the expression level of ADAMTS5 and MMP13 was downregulated, and no significant histomorphometric difference was observed between OA mice and controls (Sugita et al., 2015). In addition, the joint cartilage of *Rbpjκ* knockout mice also presented OA-like histological changes, indicating a requisite role of Notch signaling in articular cartilage and joint maintenance (Hosaka et al., 2013; Mirando et al., 2013).

Wnt Signaling

The Wnt/β-catenin signaling pathway is involved in physiological and pathological changes in articular cartilage and is also regarded as a potential therapeutic target of OA (Wang et al., 2019). Wnt comprises a diverse family of extracellularly secreted glycoproteins with various receptors. Canonical Wnt/β-catenin and noncanonical signaling pathways participate in numerous biological processes, such as cell proliferation, differentiation, cell fate determination, and tissue homeostasis (Steinhart and Angers, 2018). Accumulating evidence implies an important role for Wnt signaling in OA pathogenesis. In transgenic surgery-induced OA mice with constitutive activation of β-catenin, sustained expression of ADAMTS5 was observed (Rockel et al., 2016).

Reportedly, activation of Wnt/β-catenin signaling by bone morphogenetic protein 2 (BMP2) contributed to upregulation of ADAMTS5 and severe conditions of OA (Papathanasiou et al., 2012). The results of this study suggested that BMP2 was able to activate Wnt signaling via low-density lipoprotein receptor–related protein 5 (LRP-5), a key component involved in the canonical Wnt pathway. This Wnt pathway signaling promotes the binding of its downstream factor, lymphoid enhancer factor-1 (LEF1), to the *ADAMTS5* promoter and initiation of *ADAMTS5* transcription (**Figure 3**).

Inflammatory Signaling Pathways

Cultured human chondrocytes and cartilage explants could be induced as *in vitro* OA models by inflammatory factors, such as IL-1β, tumor necrosis factor-α (TNF-α), and nuclear factor-κB (NF-κB). In those human OA models, the expression of ADAMTS5 was not significantly changed (Tortorella et al., 2001; Bau et al., 2002), suggesting constitutive expression of ADAMTS5 (Verma and Dalal, 2011; Bondeson et al., 2006; Bondeson et al., 2008). However, some studies in murine chondrocytes revealed that ADAMTS5 expression could be promoted by IL-1 (Ji et al., 2016a; Stanton et al., 2005). In human and mouse chondrocytes, ADAMTS5 might be differentially regulated. A recent study elucidated that IL-1β induced the overexpression of ADAMTS5 via the AP-1/microRNA-30a (miR-30a) axis (Ji et al., 2016a) (**Figure 3**). Notably, miR-30a belongs to a family of small endogenous noncoding RNAs, which play a role in posttranscriptional repression of gene expression (Miyaki and Asahara, 2012). Activator protein 1 (AP-1) elicited by IL-1β molecules can bind to the promoter of miR-30a and initiate its expression (Ji

et al., 2016a). In the chondrogenic ATDC5 cell line, enhanced ADAMTS5 expression was also elicited under IL-1β treatment (Kobayashi et al., 2013).

Interleukin 6 (IL-6) is a cytokine with pleiotropic functions and is an essential initiator of inflammation and immunity (Tanaka et al., 2012). The continual overexpression of IL-6 is responsible for chronic inflammation and autoimmunity (Tanaka et al., 2014). Signal transducer and activator of transcription (STAT), mainly STAT3, is the main downstream effector element triggered by IL-6 molecules (Mihara et al., 2012). The expression level of IL-6 in serum and synovial fluid is associated with OA, and treatment of chondrocytes with IL-6 is a common method in OA model establishment (Tsuchida et al., 2012). A significant increase in ADAMTS5 expression was observed in IL-6-stimulated chondrocyte culture, as well as in mice with intra-articular injection of IL-6 (Legendre et al., 2005; Ryu et al., 2011). Recently, Latourte et al. (2017) found that IL-6 upregulated the expression of ADAMTS5 via the activation of downstream STAT3 *in vitro* (Latourte et al., 2017) (**Figure 3**). In addition, decreased expression levels of ADAMTS5 and decreased severity of OA were observed in both systemic inhibition of IL-6 and STAT3 blockade in a surgically induced OA mouse model (Latourte et al., 2017). This study provided strong evidence that ADAMTS5 can be upregulated via the IL-6/STAT3 pathway under the inflammatory conditions in OA.

NF-κB is a transcription factor stimulated by cytokines and ECM fragments in OA. NF-κB has long been recognized as a potential therapeutic target in OA (Rigoglou and Papavassiliou, 2013). There are three NF-κB binding motifs in the promoter of *ADAMTS5*, −1,196/−1,187 bp region, −896/−887-bp region, and −424/−415-bp region (Kobayashi et al., 2013). p65, also known as RelA, is one of the five components that form the NF-κB transcription factor family (Chen and Greene, 2004). Specific binding between p65 and NF-κB binding motifs in the *ADAMTS5* promoter suggested a transcriptionally induction of *ADAMTS5* expression during osteoarthritis development (Kobayashi et al., 2013). While NF-κB signaling is known to take part in inflammation in OA, it also responds to excessive mechanical loading and accelerates OA progression. Gremlin-1 is an inhibitor of BMPs and can be induced by mechanical stretch. Gremlin-1 activated by excessive mechanical loading can activate NF-κB signaling, resulting in the induction of ADAMTS5 (Chang et al., 2019).

Other Involved Pathways

Sex-determining region Y-box 4 (SOX4) belongs to the SOXC subgroup of the SOX family and is a transcription factor involved in embryonic development and cell fate determination (Moreno, 2020). It has been reported that SOXC family members play a role in skeletal development (Lefebvre and Bhattaram, 2016). Overexpression of ADAMTS4 and ADAMTS5 can be induced by SOX4 in an inflammatory environment and mechanical stress in chondrogenic cell lines (Takahata et al., 2019). Chromatin immunoprecipitation assays showed that SOX4 molecules directly bound to the promoter sequences of *ADAMTS4* and *ADAMTS5* and modulated their transcription (Takahata et al., 2019). In skeletogenesis, SOX4 is involved in the promotion of canonical and noncanonical Wnt signaling, which is vital in OA (Bhattaram et al., 2014; Kato et al., 2015). Retinoic acid, with the ability to potentiate inflammatory cytokines, is commonly used to mimic OA in chondrocyte cell lines (Davies et al., 2009). In superficial zone cells of articular cartilage treated with retinoic acid, SOX4 expression was markedly induced (Takahata et al., 2019). However, *trans*-acting elements of *SOX4* in OA have not been identified. Further studies are required to explore the mechanisms controlling SOX4 in OA.

These signaling pathways are not mutually independent but form a complex network in ADAMTS5 regulation through their interactions. For example, inhibition of YAP significantly enhances the expression of RUNX2 in chondrocyte differentiation, while YAP overexpression significantly downregulates the expression of RUNX2 (Zhang et al., 2019). TAZ also participates in FGF2 signaling and activates RUNX2-mediated transcription of targeted genes (Byun et al., 2014). The Wnt/β-catenin pathway and Hippo/YAP signaling pathway can both be activated by Piezo1/2-mediated mechanical signals in joints (Zhou et al., 2020).

In this section, we assume that the activation of ADAMTS5 is a converged output of a complex molecular network including mechanical loading responses, chondrocyte differentiation, and inflammatory responses (**Table 1**).

Potential Therapies in OA Targeting ADAMTS5

In healthy articular cartilage, the balance between matrix synthesis and degradation is dynamically maintained. Overactivation of matrix remodeling and the inflammatory response are major events in synovial joints in the context of senescence, mechanical stress, and proinflammatory cytokines. In addition, analgesics and nonsteroidal anti-inflammatory drugs are still clinical choices to relieve symptoms of OA (Alcaraz et al., 2019). No disease-modifying OA drugs have ever been applied in clinical treatment. Notably, in recent years, therapeutic options designed to modulate the expression and activity of ADAMTS5, for instance, monoclonal antibodies, small synthetic molecule inhibitors, small interfering RNAs (siRNAs), miRNAs, and injectable agents for ADAMTS5 blockade, have arisen as potential alternatives for OA treatment (**Table 2**).

Monoclonal Antibodies and Small Molecule Inhibitors

Selective and high-affinity antibodies have been evaluated as direct attempts to block ADAMTS5 catalytic activity and reduce cartilage damage (Santamaria and de Groot, 2019). Antibody-based inhibitors, such as CRB0017, GSK2394002, and M6495, were selected and exhibited the efficacy of ADAMTS5 inhibition *in vivo* (Chiusaroli et al., 2013; Larkin et al., 2015; Santamaria et al., 2015; Siebuhr et al., 2020). M6495, which has completed phase 1 clinical trials, is an antibody that selectively binds to the catalytic metalloproteinase domain and inhibits ADAMTS5 *in vitro*, reducing aggrecan cleavage in OA joints (Siebuhr et al., 2020). However, due to potential side effects,

TABLE 1 | Signaling pathways that involve in ADAMTS5 regulation in the pathophysiological process of OA.

Signaling	Biological processes	Mechanism	Reference
Runx2 signaling	Mechanical stimulationHypertrophic differentiation	RUNX2 is a downstream target of p38 and MAPK, and can bind to the promoter sites of *ADAMTS5* and regulate its expression	Kanno et al. (2007)
		WWP2 can repress the expression of ADAMTS5 through ubiquitination and degradation of RUNX2 in osteoblasts	Mokuda et al. (2019)
FGF2 signaling	Chondrocyte differentiation	FGF2 can elicit RUNX2 activation through MAPK/ERK pathway and modulate ADAMTS5 expression	Ji et al. (2016a)
YAP/TAZ signaling	Mechanical stimulation	Unphosphorylated YAP/TAZ, mediated by both hippo-dependent and independent signaling pathways, is transported into the nucleus to promote downstream *ADAMTS5* transcription	Zhao et al. (2011)
	Chondrocyte differentiation		Gong et al. (2019)
Notch signaling	Chondrocyte differentiation	RBPjκ and MAM, which are activated by NICD from NOTCH receptors, can upregulate the expression of *HES1* and following *ADAMTS5*	Sugita et al. (2015) Saito and Tanaka, (2017)
Wnt signaling	Chondrocyte differentiation	BMP2-induced Wnt/β-catenin signaling promotes its downstream factor, LEF1, to bind to *ADAMTS5* promoter and to initiate its transcription	Papathanasiou et al. (2012)
IL-1 signaling	Inflammatory response	IL-1β can induce the overexpression of ADAMTS5 via AP-1/microRNA-30a (miR-30a) axis	Ji et al. (2016b)
IL-6 signaling	Inflammatory response	IL-6 can upregulate the expression of ADAMTS5 via the activation of downstream STAT3	Latourte et al. (2017)
NF-κB signaling	Inflammatory response	NF-κB, especially p65, stimulated by cytokines and ECM fragments can bind to the promoter of *ADMATS5* and upregulate its expression	Kobayashi et al. (2013)
	Mechanical stimulation	Gremlin-1 activated by excessive mechanical loading can activate NF-κB signaling, resulting in induction of ADAMTS5	Chang et al. (2019)
SOX4 signaling	Mechanical stimulation Inflammatory response	SOX4 molecules induced by retinoic acid can directly bind to the promoter sequences of *ADAMTS5* and modulate its transcription	Takahata et al. (2019)

TABLE 2 | Potential drugs targeting ADAMTS5 in OA therapy.

Drug type	Drug name	Mechanism	Status	Reference
Monoclonal antibodies	CRB0017	CRB0017 binds to the spacer domain of ADAMTS5 and reduce its proteolytic activity	Preclinical	Chiusaroli et al. (2013)
	GSK2394002	GSK2394002 binds to catalytic/disintegrin-like domains	Preclinical	Larkin et al. (2015)
	2D3, 2D11, 2D5, and 2B9	2D3 and 2D11 react with epitopes in the catalytic/disintegrin-like domains of ADAMTS5 2D5 binds to thrombospondin type 1 motif and 2B9 binds to the spacer domain	Discovery	Santamaria et al. (2015)
	M6495	M6495 binds to the catalytic and/or disintegrin-like domain	Clinical (phase 1)	AS Siebuhr et al. (2020)
	Sheddase antibodies	Monoclonal antibodies selectively inhibit the LRP-1 sheddases to promote the endocytosis of ADAMTS5	Preclinical	Yamamoto et al. (2017)
	Syndecan 4 specific antibody	Injection of syndecan 4 specific antibody blockes ADAMTS5 protein maturation	Preclinical	Echtermeyer et al. (2009)
Small molecule inhibitors	AGG-523	A reversible, non-hydroxamate, zinc-binding selective inhibitor to both ADAMTS5 and ADAMTS4 developed by Wyeth/Pfizer	Discontinued (phase 1)	Chockalingam et al. (2011)
	Compound[a]	A series of compounds with carboxylate zinc-binding group	Discovery	Shiozaki et al. (2011)
	Compound 15f, 13g, 13e[b]	A series of nonclassical zinc-binding group compounds selected via encoded library technology	Discovery	Deng et al. (2012)
	Compound 7	A compound with zinc-binding group moieties in hydantoin series	Preclinical	Durham et al. (2017)
	GLPG1972	A compound with zinc-binding group moieties in hydantoin series	Clinical (phase 2)	Brebion et al. (2021)
	Glycoconjugated arylsulfonamide	A compound with positively charged residue-binding ability to the disintegrin-like domain of ADAMTS5	Discovery	Santamaria et al. (2021)
RNAs	ADAMTS5 siRNA	ADAMTS5 siRNA silences *ADAMTS5* gene by interfering with its mRNA translation	Preclinical	Chu et al. (2013) Hoshi et al. (2017)
	miRNA-140	miRNA-140 is located in one intron of *WWP2* gene and is a regulator of cartilage homeostasis	Preclinical	Si et al. (2017)
	WWP2 mRNA	WWP2 mRNA suppresses *ADAMTS5* upstream Runx2 signaling	Preclinical	Mokuda et al. (2019)
	ROR2 siRNA	ROR2 siRNA suppresses *ADAMTS5* upstream YAP/TAZ signaling	Preclinical	Thorup et al. (2020)
	Antisense oligonucleotides	Antisense oligonucleotides silences *ADAMTS5* gene by interfering with its mRNA translation	Preclinical	Garcia et al. (2019)

[a]*(1S,2R, 3R)-2,3-Dimethyl-2-phenyl-1-sulfamidocyclopropanecarboxylates.*

[b]*The core structure of these compounds is triazine pyrrolidine (4-n-propanephenyl)sulfonamide.*

most antibodies fail to progress beyond preclinical expectations, and only a few are undergoing or have progressed further than phase 1 clinical trials (Santamaria, 2020) (**Table 2**). For example, the risk of cardiovascular side effects was increased upon systemic administration of GSK2394002 in mice (Larkin et al., 2014). Since ADAMTS5 also exerts a role in cardiovascular and limb development (McCulloch et al., 2009; Dupuis et al., 2011), the long-term impacts of these antibodies need to be investigated before clinical trials.

Apart from ADAMTS5-specific antibodies, other antibodies that block ADAMTS5 maturation and function also presented protective outcomes preclinically. Monoclonal antibodies that selectively inhibit LRP-1 (ADAMTS5 endocytic receptor) sheddases reversed OA cartilage degradation (Yamamoto et al., 2017). Intra-articular injection of inhibitors that block posttranslational modifications of the ADAMTS5 proprotein was also used to treat OA mice (Echtermeyer et al., 2009).

Compared with monoclonal antibodies, most small molecule inhibitors of ADAMTS5 are orally bioavailable (Shiozaki et al., 2011), while the specificity of small molecule inhibitors is not that exquisite. Small molecule inhibitors were selected based on the structure of the ADAMTS5 protein, and the majority of inhibitors were developed based on the catalytic metalloproteinase domain (Shiozaki et al., 2011; Chockalingam et al., 2011; Deng et al., 2012; Durham et al., 2017; Brebion et al., 2021; Nuti et al., 2013). The zinc-binding group in the catalytic metalloproteinase domain is the distinguishing structure of these inhibitors, such as hydroxamate and carboxylate (Shiozaki et al., 2011; Deng et al., 2012). However, zinc-binding domains widely exist in many metalloproteinases, which may lead to cross-inhibition of these drugs (Bakali et al., 2014). Many small molecule inhibitors were only tested at the discovery/preclinical stage or discontinued in phase 1 clinical trials (**Table 2**). GLPG1972 is a compound with zinc-binding group moieties and belongs to the hydantoin series (Brebion et al., 2021). After screening, *structure–activity relationship optimization* was used to improve its potency and eventually led to its discovery. GLPG1972 displayed high potency against ADAMTS5 in cultured cartilage explants and is now under phase 2 clinical trials with a high degree of selectivity (Brebion et al., 2021).

Since it is challenging to select suitable drugs among classical compounds with zinc-binding groups, new strategies are proposed to circumvent these drawbacks. Specific amino acid residues in the ancillary domains were targeted for drug development. For example, glycoconjugated arylsulfonamide was identified to target the disintegrin-like domain of ADAMTS5 with its positively charged residue-binding ability (Santamaria et al., 2021). This exosite inhibitor presented amenable selective inhibition of ADAMTS5 activity and indicated the prospects of a novel class of OA drugs.

Posttranscriptional Suppression and Upstream Signaling Blockade of ADAMTS5 Using miRNAs and siRNA in OA Therapies.

Many antibodies and small molecule inhibitors failed to exhibit the expected results after preclinical testing. Thus, drugs with less cross-inhibition and off-target damage are required. miRNAs are a class of small, noncoding RNAs that specifically bind to messenger RNAs (mRNAs) and posttranscriptionally regulate protein expression level. miR-140 is an endogenous RNA abundantly expressed in chondrocytes and is located in one intron of the *WWP2* gene (Nakamura et al., 2008). Similar to the WWP2 protein, miRNA-140 helps maintain the homeostasis of cartilage, and miRNA-140 knockout mice showed OA-related changes (Miyaki et al., 2009; Miyaki et al., 2010). Multiple downstream factors, including ADAMTS5 and MMP13, were shown to be downregulated by miRNA-140 in OA model (Liang et al., 2016). Direct intra-articular injection of miRNA-140 has shown significant improvement of histological score of articular cartilage and significantly decreased expression levels of ADAMTS5 and MMP13 (Si et al., 2017). However, miRNA-140 can be degraded by nucleases under inflammatory conditions in OA. Chemical modifications, exosomes, viruses, and liposomes have been designed for the transport of miRNAs with the benefits of accurate delivery to targeted cells and slow release for cellular uptake (Duan et al., 2020). Tentatively, chitosan-mediated miRNA-140 and insulin-like growth factor 1 overexpression *in vivo* can significantly reduce ADAMTS5 and improve the repair of articular cartilage in OA (Zhao et al., 2019).

Similar to miRNAs, siRNAs are a class of double-stranded noncoding RNAs that bind to complementary mRNAs and promote their degradation. Lentivirus-mediated siRNA is used to knock down target genes (Tiscornia et al., 2003). The expression of ADAMTS5 was significantly decreased after injection of lentivirus-mediated ADAMTS5 siRNA *in vivo* and *in vitro* in a surgically induced OA mouse model (Chu et al., 2013). In addition, injection of ADAMTS5 siRNA without viral vectors also attenuated articular cartilage degeneration in an OA mouse model (Hoshi et al., 2017). Double-stranded siRNA needs to be unwound into a single-stranded component before binding to the target mRNA sequence (Chery, 2016). Antisense oligonucleotides, a class of single-stranded nucleic acids, are also introduced for posttranscriptional modification due to their higher affinity and selectivity, and lower toxicity after chemical modifications (Kole et al., 2012). Sustained local release of antisense oligonucleotides from a fibrin-hyaluronic acid hydrogel also resulted in long-term silencing of *ADAMTS5* in OA chondrocytes (Garcia et al., 2019).

In addition to directly knockdown ADAMTS5 translation, siRNAs that suppress ADAMTS5 upstream signaling were also used in OA treatment. Receptor tyrosine kinase–like orphan receptor 2 (ROR2) belongs to the tyrosine kinase receptor family and is involved in skeletal development (DeChiara et al., 2000). Thorup et al. (2020) demonstrated that blocking the activity of ROR2 can retard cartilage degradation in an OA mouse model by inhibiting YAP signaling (Thorup et al., 2020). ROR2 blockade also suppressed the expression of ADAMTS5 and protected mice from loss of cartilage integrity. In chondrocytes, ROR2 can facilitate YAP nuclear translocation and elevate BMP2 expression (Blaney Davidson et al., 2015). The results showed that decreased expression of ROR2 by intra-articular injection of ROR2 siRNA decreased downstream YAP signaling and ADAMTS5 expression. In addition to articular

cartilage–protecting effect, ROR2 blockade achieved OA-induced pain relief and absence of side effects, at least until the mice were euthanized 22 weeks after birth (Thorup et al., 2020). Furthermore, WWP2 mRNA-treated chondrocytes also presented protective results via Runx2 signaling inhibition (Mokuda et al., 2019).

Compared with antibodies and small molecule inhibitors, miRNA- and siRNA-mediated ADAMTS5 inhibitory effects are specific due to complementary pairing. Considering that ADAMTS5 is involved in multiple regulatory mechanisms, nonspecific blockade of ADAMTS5 is not worth considering for OA therapy. Intra-articular injection of miRNA and siRNA can precisely knock down ADAMTS5 expression in imbalanced joints but also confines the drug to a limited space. Moreover, investigation on the specificity and bioavailability of RNA-based therapeutics in OA treatment are still challenging (Winkle et al., 2021). The off-target effects on tissues, cells, and genes may lead to severe toxicity or autoimmune responses (Hong et al., 2020). Besides, the instability and inefficient delivery of unmodified RNAs *in vivo* limit the improvement of therapeutic effect. To be noted, no clinical trials on OA have been registered so far (https://clinicaltrials.gov). However, recent years have seen a growing number of approvals for commercial use RNA therapies in treating liver, muscle, or the central nervous system diseases, shedding lights on further investigation of OA treatment (Crooke et al., 2019). The robustness of subcutaneous, intravitreal, and intrathecal delivery in hereditary transthyretin amyloidosis, cytomegalovirus retinitis, and spinal muscular atrophy treatment has provided perfect examples for a safe and efficient delivery of the therapeutic construct (Group, 2002; Aartsma-Rus, 2017; Wood, 2018; Gillmore et al., 2021). Progress in exploits of the molecular mechanisms of OA may facilitate the development and deployment of novel RNA therapeutics in future clinical trials.

SUMMARY AND OUTLOOK

In this review, we comprehensively discussed the roles of ADAMTS5 in OA development. ADAMTS5 is the main aggrecanase in the pathogenesis of OA and is the chief cause of articular cartilage breakdown and matrix loss. Under stimulation by inflammatory factors and mechanical stress overload, upstream signaling pathways function improperly, leading to dysregulation of ADAMTS5. A complex molecular signaling regulatory network modulates ADAMTS5-related OA pathogenesis. Since analgesics and nonsteroidal anti-inflammatory drugs are still first-line options in OA therapy, disease-modifying OA drugs that inhibit ADAMTS5 expression and activity are required for OA therapies.

AUTHOR CONTRIBUTIONS

Conceptualization: LJ, JL, and ML; data collection: SZ, JW, YJ, and LY; manuscript editing: LJ, JL, and ML; figure design, NW, ZW, and YW. All authors have critically read and agreed to the published version of the manuscript.

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Extracellular Matrix Enzymes and Immune Cell Biology

*Meagan McMahon, Siying Ye, Jess Pedrina, Daniel Dlugolenski and John Stambas**

Faculty of Health, School of Medicine, Deakin University, Geelong, VIC, Australia

Remodelling of the extracellular matrix (ECM) by ECM metalloproteinases is increasingly being associated with regulation of immune cell function. ECM metalloproteinases, including Matrix Metalloproteinases (MMPs), A Disintegrin and Metalloproteinases (ADAMs) and ADAMs with Thombospondin-1 motifs (ADAMTS) play a vital role in pathogen defence and have been shown to influence migration of immune cells. This review provides a current summary of the role of ECM enzymes in immune cell migration and function and discusses opportunities and limitations for development of diagnostic and therapeutic strategies targeting metalloproteinase expression and activity in the context of infectious disease.

Keywords: immunity, extracellular matrix, metalloproteinases, a disintegrin and metalloproteinases with thombospondin-1 motifs, a disintegrin and metalloproteinases

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****Correspondence:***
John Stambas
john.stambas@deakin.edu.au

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INTRODUCTION

The extracellular matrix (ECM) forms the structural architecture surrounding cells and plays a key role in supporting tissue integrity and cellular functions. Remodelling of the ECM by metalloproteinase enzymes has being increasingly linked to immunity, especially immune cell migration (Bradley et al., 2012; McMahon et al., 2016; Chen et al., 2018; Boyd et al., 2020). Immune cell migration is critical for resolution of infectious disease. Large populations of mature immune cells do not normally reside at the site of disease and are instead located in the circulation or secondary lymphoid organs where they can be called upon when required. For example, following influenza virus infection, T cells become activated, proliferate in the mediastinal lymph node and then migrate to the lung to assist with clearance of virus. Using a murine model of influenza virus infection, one study has proposed that A Disintegrin-like and metalloproteinase with Thrombospondin-1 Motifs -5 (ADAMTS-5), an ECM metalloproteinase, facilitates migration of influenza virus-specific $CD8^+$ T cells from the mediastinal lymph node (MLN) to the lung following infection (McMahon, Ye et al., 2016). In the absence of ADAMTS-5, viral clearance was disrupted. Conversely, a study using an alternative murine influenza virus infection model demonstrated that a closely related ECM enzyme, ADAMTS-4, when expressed in fibroblasts, contributed to increased lung damage following infection (Boyd, Allen et al., 2020). These studies emphasise the importance of the ECM and ECM remodelling in disease outcomes following virus infection and highlight the potential for development of ECM-based therapeutics. Recent advances in the fields of the ECM biology, metalloproteinase biology and immune cell migration, and the potential application of this new knowledge to the treatment of infectious disease will be discussed in detail throughout this review.

THE EXTRACELLULAR MATRIX COMPONENTS

There are four major ECM components that contribute to structural integrity. These include proteoglycans, non-proteoglycan polysaccharides, fibres and multi-adhesive proteins. Each of these components has been shown to affect immune cell migration and will be discussed below.

Proteoglycans

Proteoglycans are generated by most eukaryotic cells and consist of repeating units of covalently bound glycosaminoglycans (GAGs). GAGs provide adhesion points for a range of ECM molecules, including chemokines (Rot 1992; Wight 2002; Rot 2010). Chemokines have chemoattractant properties that drive leukocyte extravasation and migration through the ECM. The interaction between chemokines and GAGs is critical for immune cell recruitment and migration (Spillmann et al., 1998; Kuschert et al., 1999; Hirose, Kawashima et al., 2001; Li et al., 2002; Proudfoot et al., 2003). Versican, an ECM proteoglycan, binds C-C chemokine ligand (CCL)−2, −5, −8 and −21 (Hirose, Kawashima et al., 2001), all of which act as chemoattractants for immune cell migration (Murooka et al., 2008; Murphy 2010; Fang et al., 2012). GAGs also provide adhesion points for immune cells along migratory pathways (Gotte, 2003; Parish, 2006). Lumican, an ECM proteoglycan encoding a GAG domain, binds neutrophils helping them traverse the endothelial cell layer. It also promotes neutrophil migration via its interactions with β2-integrins (Lee et al., 2009).

ECM proteoglycans can also inhibit migration of immune cells. Versican has been shown to bind hyaluronan, a non-sulphated GAG widely distributed in connective tissue that acts to maintain ECM integrity (Suwan et al., 2009). Activation of T cells with poly I:C increased the viscosity of the ECM by facilitating versican-hyaluronan interactions, leading to the inhibition of $CD4^+$ T cell migration (Evanko et al., 2012). In support of this proposition, analysis of versican expression in human cervical cancer samples has suggested that increased versican expression in stromal cells inhibited $CD8^+$ T cell invasion, preventing $CD8^+$ T cell clearance of tumorigenic cells (Gorter et al., 2010). Furthermore, a build-up of versican in the mediastinal lymph nodes of *Adamts5*$^{-/-}$ mice was associated with accumulation and poor migration of virus-specific $CD8^+$ T cells to the periphery following influenza-virus infection (McMahon et al., 2016). In contrast, versican expression in a poly (I:C)-induced acute lung injury mouse model has been shown to encourage leucocyte infiltration and accumulation into lungs (Chang et al., 2014; Kang et al., 2016). These studies highlight the need for additional research to understand the contribution of ECM proteoglycans to immune cell migration.

Non-Proteoglycan Polysaccharides

Hyaluronan is an ECM polysaccharide that consists of repeating units of glucuronic acid and N-acetyl glucosamine that can adhere to cell surface molecules such as CD44 (Lesley et al., 1994; Karvinen et al., 2003; Ruffell and Johnson, 2008; Suwan et al., 2009, Stephen P.; Evanko et al., 2012). The interaction between CD44 and hyaluronan facilitates mononuclear leukocyte adhesion to mucosal smooth muscle colon cells *in vitro* following inflammatory (poly I:C) stimulus (de la Motte et al., 2003). Hyaluronan also interacts with CD44 on the surface of T cells and is important for migration and activation of these cells (Lesley et al., 1994). However, the link between hyaluronan, cell surface molecules and immune cell migration needs to be further explored.

Fibres

Collagen and elastin are the main structural fibres of the ECM, contributing to ECM stiffness and supporting tissue structure. Expression of collagen has been shown to alter migratory patterns of macrophages and T cells (Applegate et al., 1990; Li et al., 2003; Klose et al., 2013; Murray et al., 2013). Elastin can be degraded by metalloproteinase expressing macrophages and is critical for migration (Varga et al., 1997; Brassart et al., 1998; Hance et al., 2002; Antonicelli et al., 2007). Moreover, elastin degradation peptides encourage recruitment of mononuclear phagocytes (Varga et al., 1997; Brassart et al., 1998; Hance et al., 2002; Antonicelli et al., 2007). These studies highlight a key role for ECM fibres in immune cell migration. However, targeting ECM fibres for therapeutic use may prove difficult given the importance of these fibres in tissue structure.

Multi-Adhesive Proteins

The ECM also contains ligands, such as fibronectin and laminin, that provide structural attachment sites for migrating immune cells. Laminin is a fibrous protein present within the basal lamina of epithelial tissue. It forms an intricate protein network for cellular contact to enhance structural integrity of tissues. Laminin α4 (a sub-type of laminin) knockout mice (*Lamα4*$^{-/-}$) have been successfully used to determine the contribution of this ECM component to immune cell migration. Croton-oil administration to the skin of *Lamα4*$^{-/-}$ mice showed reduced neutrophil and monocyte infiltration towards the inflammatory stimulus (Wondimu et al., 2004; Kenne et al., 2010). Moreover, in the absence of Laminin α4, reduced T cell infiltration into the brain was observed in an experimental autoimmune encephalomyelitis mouse model (Wu et al., 2009).

FUNCTION, STRUCTURE AND REGULATION OF ZINC-DEPENDENT METALLOPROTEINASES

Function

In mammals, the zinc-dependent metalloproteinase (metzincin) superfamily includes 24 Matrix Metalloproteinases (MMPs) (Klein and Bischoff 2011), 40 A Disintegrin and Metalloproteinases (ADAMs) and 19 ADAMTS metalloproteinases (Brocker et al., 2009; Duffy et al., 2009; Mead and Apte, 2018). Of these, only 23 MMPs and 21 ADAMs have been identified in humans, while all 19 ADAMTS family members are found in humans (Raeeszadeh-Sarmazdeh et al., 2020). Metalloproteinases (MMPs, ADAMs and ADAMTSs) collectively cleave a large array of ECM substrates including proteoglycans, collagens and membrane-associated protein substrates such as cytokines (Black et al., 1997; Khatwa et al., 2010). Functionally, MMPs are responsible for regulating and degrading a variety of ECM components including collagen, elastin, and gelatin and contribute to regulation of cytokine expression (Ra and Parks, 2007; Siddhartha and Garg, 2021). Similarly, ADAMs cleave and release soluble factors like chemokines (Reiss and Saftig, 2009), mediate shedding of membrane-associated proteins into their active

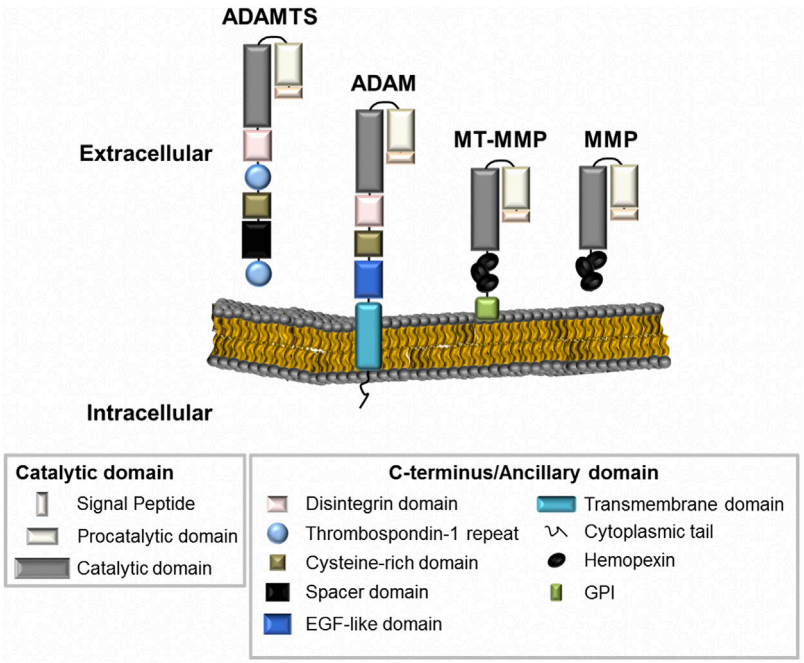

FIGURE 1 | The metalloproteinase superfamily. The basic structural organisation of ADAMTS, ADAM and MMPs family members (including MMPs with a transmembrane domain, MT-MMP). Metalloproteinases generally contain similar proteinase domains. Differences in structure can be seen at the C-terminus or at the ancillary domain where ADAMTS enzymes contain Thrombospondin repeats; ADAMs are membrane-anchored through a transmembrane domain; and MMPs contain haemopexin-like regions.

forms (i.e. TNF-α) (Black et al., 1997) or regulate gene expression through the generation of molecules that potentially act as transcription factors following intramembrane proteolysis and translocation to the nucleus (Reiss and Saftig, 2009). ADAMTSs are categorized based on cleavage substrates within the ECM–substrates include proteoglycans, pro-collagen N-pro-peptides, cartilage oligomeric matrix protein (COMP), and unknown or "orphan substrates" (Kelwick et al., 2015). A group of ADAMTS enzymes (ADAMTS−1, −4, −5, −8, −9, −15 and −20) can cleave aggrecan, versican, brevican, and neurocan and are termed "hyalectinases" (Abbaszade, Liu et al., 1999; Boerboom et al., 2011; Dancevic et al., 2013; Dancevic and McCulloch 2014). Cleavage of these ECM substrates allow metalloproteinases to play a key functional role in migration, proliferation and differentiation of cells. The role of metalloproteinases in the migration of immune cells will be discussed in detail below.

Structure

All metzincins are synthesized as zymogens that contain a pro-domain and a catalytic domain for enzymatic activity, along with a distinctive C-terminus or ancillary domain (Massova et al., 1998; Nagase et al., 2006) (**Figure 1**). MMPs can be distinguished by the presence of a haemopexin-like domain that facilitates adhesion to their various substrates. MMPs are divided into three main categories–collagenases, gelatinases, and stromelysins based on their substrate specificity. They can also be categorized by the presence or absence of a transmembrane domain, allowing them to exist either as membrane-anchored or secreted metalloproteinases (**Figure 1**) (Klein and Bischoff, 2011). ADAMs and ADAMTS are distinguished from MMPs by the presence of a disintegrin or disintegrin-like domain, respectively. Metalloproteinase structure has been reviewed extensively and further information for these enzyme families can be found in the following review articles (Birkedal-Hansen, 1988; Birkedal-Hansen et al., 1993; Massova et al., 1998; Tang, 2001; Apte, 2009).

Activation and Regulation

Metalloproteinases contain a conserved methionine residue at the active site and use a zinc ion for catalysis in enzymatic reactions (Bode et al., 1993). Activation of metalloproteinases varies depending on which type of zinc protease they represent; MMP, ADAM or ADAMTS. MMPs are synthesized and secreted as inactive enzymes. They remain in a latent state until they undergo catalytic activation by pro-protein convertases, such as furin (Ra and Parks, 2007). Catalytic activation of ADAMs occurs mostly intracellularly prior to secretion where they are found in their active form (Lum et al., 1998; Roghani et al., 1999; Kang et al., 2002). ADAMTSs share similarities with both ADAMs and MMPs and can be secreted in their inactive "pro" form or can be activated intracellularly and then secreted (Longpre et al., 2009; Kelwick et al., 2015). Metalloproteinase-mediated catalytic activity can be up-regulated or inhibited. Regulation and inhibition by host factors including reversion-inducing cysteine-rich protein with Kazal motifs, α-macroglobulin and

the tissue inhibitors of metalloproteinases (TIMPs) has been characterized (Brew and Nagase, 2010). All four of the TIMP (TIMP-1–4) family members broadly inhibit MMPs (Greene et al., 1996; Ikonomidis et al., 2005; Jacobsen et al., 2008; Brew and Nagase, 2010; Kveiborg et al., 2010), with TIMP-3 inhibiting all MMPs, ADAM-10, −12, −17, −28 and −33, as well as ADAMTS−1, −2, −4 and −5 (Amour et al., 2000; Kashiwagi et al., 2001; Wang et al., 2006). These regulators of metalloproteinase activity can therefore be used to study disease processes.

METALLOPROTEINASE REGULATION OF IMMUNE CELL MIGRATION

As discussed above, ECM molecules are capable of inhibiting and supporting migration of immune cells. Immune cells (or relevant surrounding cells) express distinct metalloproteinases that interact with components of the ECM such as collagen and proteoglycans, to inhibit or promote immune cell migration.

Neutrophils

Neutrophils secrete a range of pro-inflammatory molecules and immune mediators (reactive oxygen species, defensins and TNF-α), which have potent antiviral and antibacterial activity against infected cells. However, excessive neutrophil infiltration following inflammatory stimulus can lead to tissue damage and exacerbation of disease. Neutrophil infiltration into the lungs of mice infected with a laboratory adapted influenza virus (A/Puerto Rico/8/1934 (H1N1)) correlates with increased expression of MMP-2 and MMP-9, leading to pathology associated with enhanced cellular infiltrates and destruction of lung architecture (Bradley et al., 2012). In support of this, influenza virus infection results in MMP-9 secretion by neutrophils to facilitate infiltration into alveoli of the lung, which can be associated with poor disease outcomes in these mice (Wang et al., 2010; Narasaraju et al., 2011; Bradley et al., 2012). However, Bradley et al (2012), also demonstrate that MMP-9 expression is necessary for normal, protective neutrophil infiltration associated with viral clearance (Bradley et al., 2012).

Macrophages

To enter tissue, macrophages must first traverse the basement membrane (Kelley et al., 2014; Tsuji et al., 2018). Macrophage-induced MMP-2, MMP-9, and MMP-14 (MT1-MMP) enzymatic activity facilitates infiltration and degradation of collagen in the basement membrane for a number of diseases, including fibrosis, vasculitis, and dermatitis. (Ray et al., 2004; Nishida et al., 2007; Gong et al., 2008; Klose et al., 2013; Watanabe et al., 2018). Indeed, depletion of plasminogen, which normally activates MMP-9 into its catalytically active form, results in reduced macrophage infiltration and a decreased likelihood of aortic aneurysm in a murine model of abdominal aortic aneurysm (Gong et al., 2008). In addition, reduced renal fibrosis was observed in *Mmp-2*$^{-/-}$ mice following unilateral ureteral obstruction (Du et al., 2012). MMP-14 has also been shown to be involved in macrophage infiltration in a murine model of contact dermatitis, where in *Mmp-14*$^{-/-}$ mice showed reduced macrophage infiltration at the site of dermatitis (Klose et al., 2013). These studies highlight the importance for MMPs in macrophage migration. In addition, related enzymes including ADAMs (ADAMs−8, −9, −15 and −19) and ADAMTS (ADAMTS−1, −4, −5 and −8) are found to be highly expressed in macrophage-rich areas in atherosclerosis. It is currently unclear if expression of these enzymes enhances macrophage infiltrating potential (Wågsäter et al., 2008; Salter et al., 2011).

Dendritic Cells

Efficient dendritic cell (DC) migration is critical for initiating adaptive immune cell responses. In the absence of DC signaling, adaptive immune cell activation is severely impaired. The role of metalloproteinases in DC migration is currently under-studied. *In vitro* migration assays indicate that DCs isolated from *Mmp9*$^{-/-}$ mice show reduced migration when compared to their WT counterparts (Yen et al., 2008). To further expand on the role of *MMP-9* in DC migration, DC trafficking during allergen-induced airway inflammation in *Mmp9*$^{-/-}$ mice was assessed (Vermaelen et al., 2003). In the absence of MMP-9, inflammatory migration of DCs into the airway lumen was restricted, preventing the development of allergic airway inflammation. These studies highlight the importance of further defining the role of metalloproteinase mediated migration of DCs in acute and chronic disease.

T Cells

Effector T cells do not normally reside at sites of disease. They are activated by DCs in lymph nodes and migrate to sites where they are required to perform their function. Indeed, migrating T cells in the high endothelial venules of lymph nodes require MMP-2 and -9 for normal migration (Faveeuw et al., 2001). Peripheral blood mononuclear cells derived from multiple sclerosis patients have been used to determine differences in migration of CD4$^+$ T cell subpopulations *in vitro* using a transwell migration assay. T helper 1 (Th1) CD4$^+$ T cells isolated from the aforementioned multiple sclerosis patients secreted higher amounts of MMP−2 and −9 when compared to Th2 CD4$^+$ T cells, which was reflected through increased mobility in the transwell system (Abraham et al., 2005). Furthermore, inhibition of the Wnt pathway (a regulator of MMP−2 and −9 expression) results in reduced MMP−2 and −9 expression, leading to collagen accumulation and inhibition of T cell extravasation (Wu et al., 2007). The absence of MMP−2 and −9 in mice disrupts cleavage of collagen type IV and T cell movement through the ECM (Wu et al., 2007). Other ECM enzymes have also been shown to affect T cell migration. In human myeloma biopsy samples, one study has shown that samples containing high numbers of CD8$^+$ T cells also demostrated elevated versican proteolysis via ADAMTS enzymes, suggesting these enzymes are important for T cell clearance of tumours (Hope et al., 2016). Mice lacking ADAMTS-5 (*Adamts5*$^{-/-}$ mice) show reduced movement of virus-specific CD8$^+$ T cells following influenza virus infection (McMahon et al., 2016). Mechanistic analyses suggested that versican

accumulation in the draining mediastinal lymph node interrupted egress of $CD8^+$ T cells from the mediastinal lymph node to the periphery (McMahon et al., 2016). This result was further supported *in vitro,* where Jurkat $CD4^+$ T human cells treated with an anti-ADAMTS5 antibody also showed impaired migration through versican in a transwell migration assay (McMahon et al., 2016). While the absence of ADAMTS-5 resulted in poor virus clearance and increased disease severity that was attributed to T cell migration (McMahon et al., 2016), a recent study by (Boyd et al., 2020) has demonstrated that the absence of a closely related family member, ADAMTS-4 in fibroblasts, leads to reduced lung immunopathology and improved lung function following lethal influenza virus infection (Boyd et al., 2020). This highlights the potential of these enzymes to influence outcomes of infection and emphasizes the need for further studies.

Metalloproteinase-Mediated Cleavage of Cytokines to Promote Immune Cell Migration

Chemokines are a group of signaling molecules that are secreted by cells to promote migration of immune cells to the site of inflammation or disease. Neutrophils are typically the first cell type to respond to infection and infiltration may be supported by metalloproteinase activity. Nasal biopsies taken from allergic rhinitis patients, show that upregulated ADAM-12 in airway epithelial cells results in cleavage and release of the neutrophil chemoattractants, CXCL-1 and -8, from the ECM, which assist in the recruitment of neutrophils into the nasal cavity (Estrella et al., 2009). The use of *Mmp* knockout mouse models has also identified roles for MMP−7 and −8 in creating neutrophil chemotactic gradients. In $Mmp7^{-/-}$ mice, lung injury induced by bleomycin treatment resulted in a reduced transepithelial gradient of the chemokine KC (CXCL1), leading to reduced neutrophil influx (Li et al., 2002). Additionally, using a mouse model of acute colon injury Swee et al showed that the reduced neutrophil influx observed in $Mmp7^{-/-}$ mice due to changes in chemotactic gradients protected them from succumbing to colon injury, but that repair of colon tissue was delayed in these mice (Swee et al., 2008). These studies highlight the importance of metalloproteinase-mediated cleavage of cytokines in disease and exemplify an important role for this cleavage in promoting and controlling immune cell migration.

Emerging and Future Areas of Interest

Metalloproteinases are attractive therapeutic targets where modulation of immune responses is required. They are essential for immune cell infiltration, cytokine regulation and tissue repair (Davey et al., 2011) and have the potential to be used as targets through the use of currently approved therapeutics or can be targeted by compounds under development; both of which have been eloquently reviewed by Raeeszadeh-Sarmazdeh et al., 2020 and Santamaria and de Groot, 2019 (Santamaria and de Groot, 2019; Raeeszadeh-Sarmazdeh et al., 2020). Of these potential therapeutics, the most widely studied include, small molecule inhibitors, antibody-based inhibitors, and tissue inhibitors of metalloproteinases (TIMPs), which are the natural regulators of metalloproteinase activity.

Small molecule inhibitors can elicit their inhibitory effects by targeting specific sites of the enzyme itself (catalytic or otherwise) and have been shown to target MMPs, ADAMs and ADAMTSs (Dufour et al., 2011; Remacle et al., 2012; Raeeszadeh-Sarmazdeh et al., 2020; Santamaria, 2020; Santamaria et al., 2021). While many of these compounds have shown potential in preclinical and clinical settings, off-target effects, lack of selectivity and specificity, and toxicity are all issues that need to be addressed (Cathcart et al., 2015; Mushtaq et al., 2018). In comparison to small molecule inhibitors, antibody-based inhibition is typically associated with less toxicity and immunogenicity (Fischer and Riedl 2019; Raeeszadeh-Sarmazdeh et al., 2020). As with small molecule inhibitors, antibodies have demonstrated varying degrees of success in targeting metalloproteinases in preclinical and clinical trials. Studies currently underway are assessing their use in the context of human health. (Dancevic and McCulloch, 2014; Santamaria et al., 2015; Shiraishi et al., 2016; Balchen et al., 2018; Santamaria and de Groot 2019; Raeeszadeh-Sarmazdeh et al., 2020). Manipulation of TIMPs may offer an alternative therapeutic approach. Given these proteins are natural regulators of MMPs, ADAMs and ADAMTSs, manipulation of TIMPs is a logical extension of inquiry. However, previous studies have highlighted both positive and negative outcomes to disease progression as reviewed by Raeeszadeh-Sarmazdeh et al., 2020 (Raeeszadeh-Sarmazdeh et al., 2020). This highlights the need for detailed understanding of TIMP regulation of metalloproteinase activity to inform development of future therapeutic options.

Conversely, as described throughout this review, enhanced expression of metalloproteinases may prove beneficial in certain disease states when increased immune cell migration is desired. While there are no therapeutics currently available that facilitate overexpression of these enzymes in clinical trial, to the best of our knowledge, the vast array of emerging inhibitors suggests that the same effect may be achieved via inhibition of the negative regulators themselves ie TIMPs. It is important to note however, that over-active metalloproteinase activity in other disease contexts e.g arthritis, has been shown to cause inflammation, leading to tissue destruction and poor outcomes in the host as observed in both human and animal studies (Davey et al., 2011; Syed et al., 2021).

Finally, although manipulating individual metalloproteinases seems reasonable, we are yet to fully elucidate how metalloproteinases act synergistically, and need to consider compensatory metalloproteinase activity in such instances. Moreover, many studies examining the function of multiple metalloproteinases in immunity have only been employed *in vitro* models, which do not take into account multi-faceted defence mechanisms elicited by a whole organism towards disease. Similarly most, *in vivo* metalloproteinase knock-out animal models have only dissected the role of individual metalloproteinases in disease processes although use of $Adamts7^{-/-}xAdamts12^{-/-}$ mice and $Adamts4^{-/-}xAdamts5^{-/-}$ mice have been reported (Majumdar et al., 2007; Mead et al., 2018). Further *in vivo* studies are required to understand how metalloproteinases act individually and synergistically before

therapeutic intervention becomes a realistic treatment in the clinic.

CONCLUSION

Metalloproteinases play an important role in regulating remodeling of the ECM to facilitate immune cell activity. Understanding how metalloproteinase activity is regulated and how family members act synergistically to influence immunity is critically important in order to develop novel therapeutic strategies.

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Characterization of the Cancer-Associated Meprin Beta Variants G45R and G89R

*Antonin Gellrich[1†], Franka Scharfenberg[1†], Florian Peters[2], Martin Sammel[1], Ole Helm[3], Fred Armbrust[1], Frederike Schmidt[1], Juliane Lokau[4], Christoph Garbers[4], Susanne Sebens[3], Philipp Arnold[5] and Christoph Becker-Pauly[1]**

[1]Biochemical Institute, Kiel University, Kiel, Germany, [2]Department of Ophthalmology, Laboratory for Retinal Cell Biology, University Hospital Zurich, Zurich, Switzerland, [3]Institute for Experimental Cancer Research, Kiel University, Kiel, Germany, [4]Institute of Pathology, Otto-von-Guericke University Magdeburg, Magdeburg, Germany, [5]Institute of Functional and Clinical Anatomy, FAU Erlangen, Erlangen, Germany

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Helmholtz Association of German
Research Centers (HZ), Germany

***Correspondence:**
Christoph Becker-Pauly
cbeckerpauly@biochem.uni-kiel.de

†These authors have contributed
equally to this work

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Meprin β is a metalloprotease associated with neurodegeneration, inflammation, extracellular matrix homeostasis, transendothelial cell migration, and cancer. In this study, we investigated two melanoma-associated variants of meprin β, both exhibiting a single amino acid exchange, namely, meprin β G45R and G89R. Based on the structural data of meprin β and with regard to the position of the amino acid exchanges, we hypothesized an increase in proteolytic activity in the case of the G45R variant due to the induction of a potential new activation site and a decrease in proteolytic activity from the G89R variant due to structural instability. Indeed, the G89R variant showed, overall, a reduced expression level compared to wild-type meprin β, accompanied by decreased activity and lower cell surface expression but strong accumulation in the endoplasmic reticulum. This was further supported by the analysis of the shedding of the interleukin-6 receptor (IL-6R) by meprin β and its variants. In transfected HEK cells, the G89R variant was found to generate less soluble IL-6R, whereas the expression of meprin β G45R resulted in increased shedding of the IL-6R compared to wild-type meprin β and the G89R variant. A similar tendency of the induced shedding capacity of G45R was seen for the well-described meprin β substrate CD99. Furthermore, employing an assay for cell migration in a collagen IV matrix, we observed that the transfection of wild-type meprin β and the G45R variant resulted in increased migration of HeLa cells, while the G89R variant led to diminished mobility.

Keywords: metalloprotease, meprin, sheddase, IL-6, CD99, APP, ADAM

INTRODUCTION

Meprin β is a metalloprotease of the astacin family of zinc endopeptidases. As a multidomain homodimer and a type I transmembrane protein, it is tethered to the cell surface or it can be shed from the plasma membrane by other proteases such as ADAMs (a disintegrin and metalloproteases) (Broder and Becker-Pauly 2013; Wichert et al., 2017; Scharfenberg et al., 2019). Expressed as a zymogen, meprin β must be activated by proteases with tryptic specificity due to an arginine residue at the P1 position of the activation site. At the cell surface, the activation of meprin β can be achieved by membrane-bound matriptase 2 (Jackle et al., 2015) or as a shed protein by soluble serine proteases,

such as kallikrein 4/5 or pancreatic trypsin (Ohler et al., 2010). Of note, once activated at the plasma membrane, meprin β cannot be shed from the cell surface anymore (Wichert et al., 2017). Hence, meprin β occurs as a membrane-bound sheddase or soluble protease with access to different protein substrates.

Proteolysis of extracellular matrix and adhesion molecules is a very important factor in the context of cancer progression and metastasis. Meprin β is capable of cleaving off the prodomains of fibrillar collagens I and III, thereby inducing a collagen fibril assembly and deposition (Kronenberg et al., 2010; Broder et al., 2013). In this regard, meprin β has been associated with fibrotic conditions of the skin and in the lungs (Becker-Pauly et al., 2007; Biasin et al., 2014). On the other hand, meprin β is able to degrade collagen IV, an important component of the basal membrane, and thus may contribute to cancer cell metastasis (Kruse et al., 2004). Furthermore, meprin β has been shown to cleave different cell adhesion molecules. The expression of E-cadherin, a major adhesion molecule of the epithelium, was shown to be decreased compared to carcinoma and adenoma (Perl et al., 1998). Interestingly, E-cadherin is a substrate of meprin β (Huguenin et al., 2008), which could lead, in the case of dysregulation in the expression and functionality due to mutations, to tumor progression from adenoma to carcinoma. The adhesion molecule CD99 is overexpressed in many types of cancer, particularly in Ewing sarcoma and specific subtypes of leukemia (Manara et al., 2018; Pasello et al., 2018). CD99 is crucial for the transendothelial migration (TEM) of leukocytes promoting the final step of cell extravasation. The type I transmembrane protein is expressed on hematopoietic and endothelial cells (Jefferson et al., 2013; Bedau et al., 2017a; Bedau et al., 2017b). Being a substrate of meprin β, the cleavage of CD99 could influence tumor metastasis.

Another substrate of meprin β is the interleukin-6 receptor (IL-6R), which in its shed soluble form can induce a strong proinflammatory stimulus *via* the so-called IL-6 trans-signaling (Rose-John, 2012). Inflammatory processes and cancer progression are highly connected, mediated by cytokines like the IL-6R and other immunomodulatory molecules in the tumor microenvironment (Balkwill and Mantovani 2001; Fisher et al., 2014). It was observed that a selective blockage of IL-6 trans-signaling with the sgp130Fc protein (Olamkicept) had a suppressive effect on the tumorigenesis and metastasis of colorectal cancer (Schmidt et al., 2018; Schumacher and Rose-John 2019).

Meprin β has been associated with certain types of cancer, and its expression was observed in pancreatic and neuroendocrine tumors (Carr et al., 2013). Searching the BioMuta database (Dingerdissen et al., 2018), several single nucleotide variants (SNVs) of the Mep1b gene can be found in different cancer entities identified by multiple genomic studies, with the largest number identified in melanoma, uterine cancer, and lung cancer (Peters and Becker-Pauly, 2019). In this study, we characterized the melanoma-associated meprin β variants G45R and G89R with regard to cell surface expression, shedding activity, cell proliferation, and tumor cell invasion.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade and obtained from Carl Roth GmbH + Co. KG, Merck KGaA, and Sigma-Aldrich Inc., and Thermo Fisher Scientific Inc., if not stated otherwise.

Cells, Transfection, Plasmids, and Antibodies

ADAM10$^{-/-}$; 17$^{-/-}$ HEK293T cells (Riethmueller et al., 2016), deficient for the metalloproteases ADAM10 and ADAM17, were kindly provided by Björn Rabe, University of Kiel. HeLa and COS-7 cells were obtained from DSMZ GmbH (Braunschweig, Germany), and Ba/F3-gp130 cells (Gearing et al., 1994) were obtained from Immunex (Seattle, WA, United States). All cells were grown in DMEM (Dulbecco's modified Eagle's medium) and high glucose culture medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS), L-glutamine, and 1% penicillin and streptomycin (Thermo Fisher Scientific) and cultured at 37°C in 5% CO_2 atmosphere and at 95% relative humidity. Ba/F3-gp130 cells were cultured using 10 ng/ml recombinant hyper-IL-6, which was expressed and purified as described previously (Fischer et al., 1997). Transient transfection was performed with polyethylenimine according to the manufacturer's instructions. The following plasmids were used: human meprin β in the pcDNA4/TO-3x-Flag vector, human meprin β G45R in the pcDNA4/TO-3x-Flag vector, human meprin β G89R in the pcDNA4/TO-3x-Flag vector, human IL-6R, human CD99-Myc in pCMV6, APP695 in pCI-neo, and pcDNA3.1 as the empty vector control. The following antibodies were used: polyclonal anti–meprin β (generated against the ectodomain peptide CGMIQSSGDSADWQRVSQ, Pineda Antibody-Service, Berlin, Germany), monoclonal anti-IL-6R (4–11, generated against the D1 domain), monoclonal anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (2,118, 14C10; Cell Signaling Technology, Danvers, MA, United States), monoclonal anti-Flag (M2, F1804; MilliporeSigma), polyclonal anti-transferrin receptor (ab84036; Abcam, Cambridge, United Kingdom), monoclonal anti-Myc (9B11, 2,276; Cell Signaling Technology), phosphorylated signal transducer and activator of transcription 3 (pSTAT3) (9,131, Y705, Cell Signaling Technology, Danvers, MA, United States), STAT3 (9,139, 124H6, Cell Signaling Technology, Danvers, MA, United States), monoclonal anti–N-terminal APP (22C11; MilliporeSigma), polyclonal C-terminal anti-APP (CT15) and monoclonal anti–soluble APPα (6E10; Covance, Princeton, NJ, United States), and anti-PDI6 (ab11432; Abcam, Cambridge, United Kingdom).

Generating Meprin β Variants G45R and G89R

Using the meprin β pcDNA4/TO construct with a C-terminal FLAG-tag as the template, the two variants G45R and G89R were generated. For exchanging single nucleotides, we applied

appropriate primers (meprin β G45R: 5′-CAATGAAGGTTT GAGACTGGATCTTTTTGAGGG-3′ and 5′-CCCT CAAAAA GATCCAGTCTCAAACCTTCATTG-3′ and meprin β G89R: 5′-GGAAATG AATGCTAAG CGAGTTATCCTCAATGC-3′ and 5′-GCATTGAGGATAACTCGCTTAGCATTCATTTCC-3′) and the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, United States) following the manufacturer's instructions. To validate the correct single nucleotide exchanges of variants G45R and G89R, the cDNA of the Mep1b gene was sequenced by GATC Biotech, Konstanz, Germany.

Cell Lysis, SDS-PAGE, and Western Blot Analysis

Transfected cells were harvested 48 h after transfection. For C-terminal fragment analysis, cells were treated with 1 μM γ-secretase inhibitor DAPT overnight prior to cell lysis. PBS-washed cells were lysed in 1% Triton X-100 and the protease inhibitor tablet with EDTA (Roche) in PBS. The protein concentration was determined using the BCA protein assay kit (Thermo Fisher Scientific) following the manufacturer's instructions. Samples were heated in sample buffer including DTT for 10 min at 95°C. The total protein lysate (30 μg) was separated by SDS-PAGE and transferred onto PVDF or nitrocellulose membranes (GE Healthcare, Waukesha, WI, United States). Membranes were blocked with 5% dry milk or 3% BSA diluted in TBS for 1 h at room temperature, incubated overnight with the primary antibody in milk at 4°C, washed three times with TBS, and incubated with horseradish peroxidase–conjugated secondary antibody in TBS at room temperature. After further washes, membranes were developed with SuperSignal West Femto (Thermo Fisher Scientific) in a chemiluminescence detection system (LAS-3000; Fujifilm, Tokyo, Japan).

Protein Deglycosylation

The total protein lysate (60 μg) of transfected ADAM10$^{-/-}$; 17$^{-/-}$ HEK293T cells was deglycosylated using PNGase F according to the manufacturer's instructions (P0704; New England Biolabs, Ipswich, MA, United States).

Cell Surface Biotinylation

ADAM10$^{-/-}$; 17$^{-/-}$ HEK293T cells were transiently transfected with meprin β variants or empty vectors and incubated for 24 h. Cells were washed twice with ice-cold PBS–$CaCl_2$ and $MgCl_2$ (CM) (CM: 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ in PBS) and treated with 1 mg/ml biotin solution (Sulfo-NHS-SS-Biotin; Thermo Fisher Scientific) in PBS-CM for 30 min at 4°C. The biotin solution was removed, and cells were incubated with quenching buffer (50 mM Tris-HCl in PBS-CM, pH 8) for 10 min at 4°C, washed three times with PBS-CM, and harvested. Subsequently, cells were lysed and the protein concentration was determined using the BCA assay kit following the instruction manual. For the purification of biotin-labeled proteins, streptavidin-coated magnetic beads (88,816, Thermo Fisher Scientific) were used according to the manufacturer's instructions.

Immunofluorescence Microscopy

The immunofluorescence staining of Cos-7 cells, which were seeded on coverslips, was performed 24 h after transfection as described previously (Peters et al., 2019). In brief, cells were washed three times with PBS and fixed with 4% (w/v) paraformaldehyde in PBS for 10 min. After cell permeabilization with 0.2% (w/v) saponin, cells were incubated with the primary antibody (anti-Flag 1:2000 and anti-PDI6 1: 1000 in 1xPBS with 10% FCS) for 1 h, washed, and then incubated with the respective secondary antibody (Alexa Fluor 488 donkey anti-rabbit and Alexa Fluor 594 donkey anti-mouse, 1:300; Thermo Fisher Scientific). The excessive antibody was removed by five washes in 0.2% (w/v) saponin and PBS and two washes in double-distilled H_2O. The coverslips were mounted onto slides with a mixture of 17% (w/v) Mowiol, 33% (v/v) glycerol, and 50 mg/ml 1,4-diazabicyclo[2.2.2]octane (MilliporeSigma) supplemented with 1 μg/ml DAPI for nuclear staining. Images were acquired on an Olympus FV 1000 confocal laser scanning microscope (Olympus, Hamburg, Germany).

Quenched Fluorogenic Peptide Cleavage Assay

To quantify meprin β activity in cell lysates and at the cell surface, a highly specific fluorogenic peptide substrate for meprin β [7-methyloxycoumarin-4-yl (mca)–EDEDED–K-ε-2,4-dinitrophenyl (dnp), Genosphere Biotechnologies, Paris, France] was used in a final concentration of 50 μm (Jackle et al., 2015). For meprin β cell surface activity, transfected ADAM10$^{-/-}$; 17$^{-/-}$ HEK293T cells were washed three times in PBS and activity measurements were performed with 0.5×10^6 cells in 48-well plates in a total volume of 300 μl. For cell lysate activity, cells were lysed using EDTA free lysis buffer, and a total amount of 50 μg protein was used for the activity assays. For trypsin activation, cells and lysates were incubated with 10 μg/ml of recombinant trypsin (Sigma-Aldrich) for 30 min at 37°C prior to measurement. Remaining cell suspensions were used for Western blot analyses. All activity assays were carried out in duplicates at 37°C. Proteolytic activity was measured as relative fluorescence units (RFUs) every 30 s for 120 min at an excitation of 405 nm and emission of 320 nm using the Tecan Infinite® F200 PRO plate reader (Tecan Trading AG). The activity was determined from the slope of the linear range of the curve normalized to the initial point of the measurement and presented as a bar graph.

Ba/F3-gp130 STAT3 Phosphorylation Assay

The biological activity of the sIL-6R was analyzed as described previously (Sammel et al., 2019). In brief, after washing with PBS, Ba/F3-gp130 cells were incubated in serum-free medium for 2 h. Cells were incubated with an ultracentrifuged (186,000 x g for 2 h at 4°C), conditioned cell culture supernatant of ADAM10$^{-/-}$; 17$^{-/-}$ HEK293T cells co-transfected with meprin β variants and the IL-6R; 150 ng recombinant IL-6 was added. 150 ng hyper-IL-6 (Fischer et al., 1997; Schroers et al., 2005) served as positive control. Cells were incubated at 37°C and were shook for 15 min at 500 rpm followed by centrifugation at 1000 x g for 5 min at

room temperature. After discarding the supernatant, cells were lysed in sample-buffer for 10 min at 95°C. Phosphorylated signal transducer and activator of transcription 3 (p-STAT3) levels were detected by Western blot analysis. STAT3 served as the control.

Real Time Cell Analysis Invasion Assay

A real-time cell analysis (RTCA) system was used for testing the invasiveness of cells overexpressing meprin β and its variants G45R and G89R. RTCA (ACEA Bio, San Diego, CA, United States) is a method measuring the density-dependent variation of cell population growth using a sensitive micro-sensor system. The CIM-plate 16 used in this experiment is a two-chamber-well within a micro-sensor plate beneath a membrane with 8 µm pores. The top of the membrane was coated with a layer of collagen IV (Sigma-Aldrich C5533, 12 µg per well). The medium was measured to detect the background impedance. HeLa cells were transiently transfected with the meprin β variants, and 40.000 cells in a volume of 100 µl medium with 1% FCS were transferred into the upper chamber onto the layer of collagen IV. To prevent the cells from evaporating, the plate around the wells was filled with sterile PBS and covered with a lid. Pre-incubation of 1 h enabled all cells to adhere onto the layer of collagen IV before starting the RTCA experiment. RTCA was run for 24 h, measuring the cell index every 30 min at 37°C and 5% CO_2. The cells passing through the layer of collagen IV touching the microelectrode increased the impedance. This was calculated automatically in the dimensionless cell index. The equation for the cell index is as follows: $CI = (Z_i–Z_0)/15\ \Omega$. Z_i is the individual impedance of each well to a certain time point and Z_o is the impedance of the time point at 15 min at the beginning. To be able to ignore a possible variability of cell numbers between the wells, a delta cell index was calculated and added. The formula for the delta cell index is as follows: $DCI_{ti} = CI_{ti} + (DCI_{reference}–CI_{Delta\_time})$. Therefore, the delta cell index is a constant number for each well representing the difference between reference DCI and the cell index. For statistical analysis, the area under the curve (AUC) or the end-point number was used.

Homology Modeling

The model of the membrane-bound meprin β dimer was built based on the crystal structure of human pro-meprin β (PDB ID: 4GWM) and a molecular model of the membrane-bound form, containing the EGF-like domains, the transmembrane helix, and the C-terminal tail (Arolas et al., 2012), using the SWISS-MODEL workspace (Waterhouse et al., 2018). Structure visualization was carried out using PyMOL (Schrödinger, New York, NY, United States).

Statistical Analysis

All statistical analyses were performed using Prism 8 software (GraphPad Software, La Jolla, CA, United States) for a one-way ANOVA followed by Tukey's post-test. Values are expressed as means ± sd. The null hypothesis was rejected at a value of $p < 0.05$.

RESULTS

Localization and Cell Surface Expression of Meprin β Variants G45R and G89R

Based on the crystal structure of meprin β, we hypothesized a decrease in proteolytic activity for the G89R variant, having an additional arginine at the back of the catalytic domain at the interface to the TRAF domain, which may disturb proper folding (**Figure 1A**). The second meprin β variant G45R could insert a potential cleavage site into the propeptide, which likely promotes faster activation of the protease (**Figure 1A**). Since meprin β can be activated at the cell surface or in its soluble shed form, it was important to first investigate whether the cancer-associated variants G45R and G89R are correctly transported to the cell surface. Therefore, we performed cell surface biotinylation experiments of transfected HEK293T cells, deficient for ADAM10 and ADAM17, to prevent physiological ADAM-mediated shedding of meprin β. The biotin pulldown showed comparable cell surface levels for wild-type meprin β and the G45R variant, whereas clearly less levels of the meprin β variant G89R were detected at the cell surface (**Figure 1B**). However, it has to be considered that, on the one hand, the overall expression level of the meprin β variant G89R was clearly reduced in comparison to wild-type meprin β and G45R. On the other hand, the relative amount of the less glycosylated ER-form of meprin β detectable at about 100 kDa (Peters et al., 2019) was increased compared to the fully glycosylated form at about 130 kDa in the cell lysates of meprin β G89R (**Supplementary Figure S1**).

Within the biotinylated fractions of the meprin β variants, the majority of the detected meprin β corresponds to the higher molecular mass in the case of wild-type meprin β and its G45R variant, whereas only a similar trend was observed for meprin β G89R (**Figure 1B**). This suggests that the G89R mutation has a fold-impairing effect for meprin β that also affects its efficient N-glycosylation and transport to the cell surface.

The results were confirmed employing immunofluorescence microscopy of Cos-7 cells overexpressing the different meprin β variants (**Figure 1C**). Cos-7 cells were used instead of HEK293T cells due to their large size and better visibility of cellular compartments. While the G45R variant showed cell surface expression comparable to wild-type meprin β, the majority of meprin β G89R strongly co-localized with the endoplasmic reticulum marker protein disulfide isomerase (PDI). Overall, these results indicate that the G89R variant is, indeed, stuck on the secretory pathway probably due to impaired folding, causing a less efficient glycosylation.

Activation and Proteolytic Activity of Meprin β Variants G45R and G89R Using a Specific Fluorogenic Peptide Substrate

In order to investigate the functional consequences on the activation and proteolytic activity of the meprin β variants G45R and G89R, we

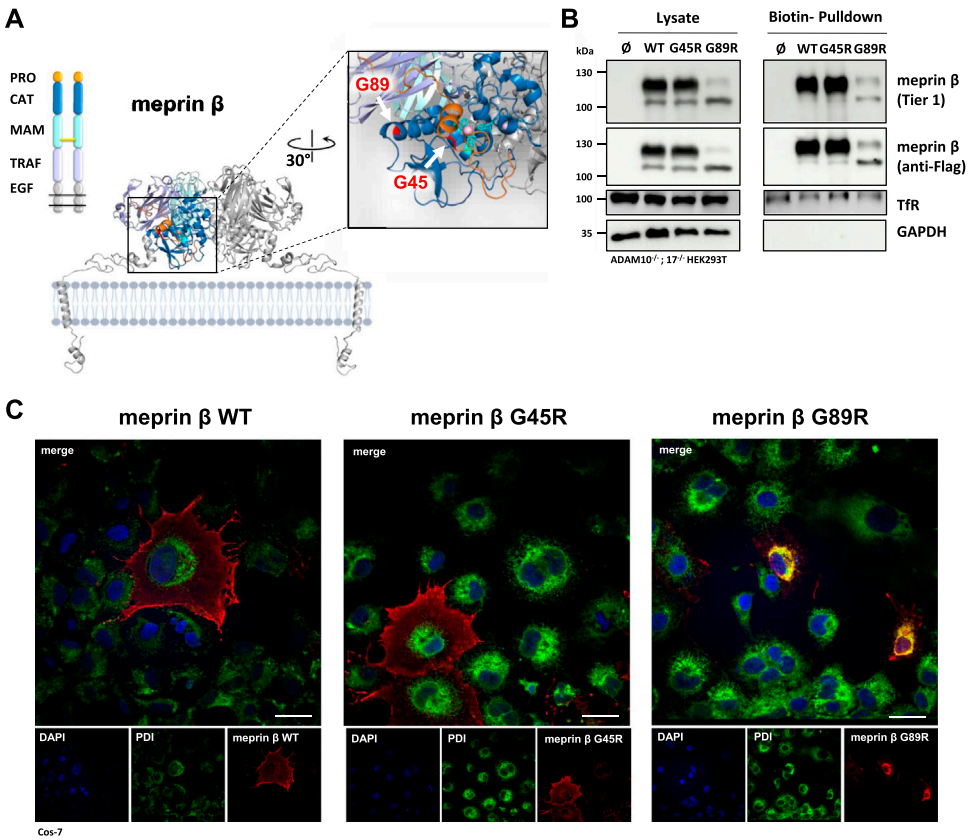

FIGURE 1 | Expression and localization of G45R, G89R, and wild-type meprin β. **(A)** Domain composition (left) and the dimeric structure of the metalloprotease meprin β (right). The cartoon representation of membrane-bound meprin β based on the crystal structure of the ectodomain of human pro-meprin β (PDB: 4GWM), with one monomer colored according to the domain composition and the other one in light gray. The close-up view on the catalytic domain (blue) in the right panel shows the pro-peptide (orange) and the three active site histidine residues (cyan) that coordinate the zinc ion (pink). The positions of the two amino acid exchange variants G45R and G89R are highlighted in red. Pro-peptide, CAT: catalytic domain, MAM: meprin A5 protein tyrosine phosphatase μ domain, TRAF: tumor-necrosis-factor-receptor-associated factor domain, EGF: epidermal growth factor–like domain. The disulfide bridge between the MAM domains responsible for dimerization is indicated as a yellow bar. **(B)** Transfection of ADAM10$^{-/-}$; 17$^{-/-}$ HEK293T cells with the indicated meprin β variants. Cell surface proteins were labeled by primary amine biotinylation, pulled down with streptavidin sepharose beads, and analyzed *via* immunoblotting. GAPDH and transferrin receptor (TfR) served as the loading controls. **(C)** Immunofluorescence images of Cos-7 cells expressing the indicated meprin β variants (red), with the endoplasmic reticulum marker protein disulfide isomerase (PDI, green) and DAPI-stained nuclei (blue). Scale bars, 20 µm.

initially performed activity assays in cell lysates and at the cell surface of transiently transfected ADAM10$^{-/-}$; 17$^{-/-}$ HEK293T cells (**Figure 2A**) using a meprin β–specific fluorogenic peptide (**Figure 2B**). Indeed, the meprin β G45R variant with a potential additional activation site did show significantly increased basal activity in cell lysates, while the cell surface activity was comparable to wild-type meprin β (**Figure 2C**). However, treating cell lysates and cells with the described pro-meprin β activator trypsin (Ohler et al., 2010; Jackle et al., 2015) did not result in increased proteolytic activity of meprin β G45R (**Figure 2C**). In the case of the G89R variant, the basal proteolytic activity in cell lysates and at the cell surface appeared to be very low (**Figure 2C**), which is likely also a result of the reduced expression rate. Nevertheless, the addition of trypsin showed that this meprin β variant can be activated (**Figure 2C**), indicating that the amino acid exchange of G89R does not lead to a complete loss of function mutation.

The Proteolytic Activity of Meprin β G45R and G89R Toward IL-6R, CD99, and APP

In addition to the fluorogenic peptide-based activity assays, we, in a next step, analyzed the proteolytic activity of the meprin β variants G45R and G89R toward three known membrane-bound meprin β protein substrates, namely, IL-6R, CD99, and APP.

IL-6R Shedding by Meprin β Variants

The soluble IL-6R plays an important role in cell proliferation and inflammation through trans-signaling (Rose-John, 2012). Not only ADAM10 and ADAM17 are capable of shedding the IL-6R (Mullberg et al., 1992) but also several other proteases, including meprin β (Arnold et al., 2017; Sammel et al., 2019). Similar to the single transfection of the three meprin β variants, the overall expression of meprin β G89R was also markedly reduced upon co-transfection with the IL-6R compared with the

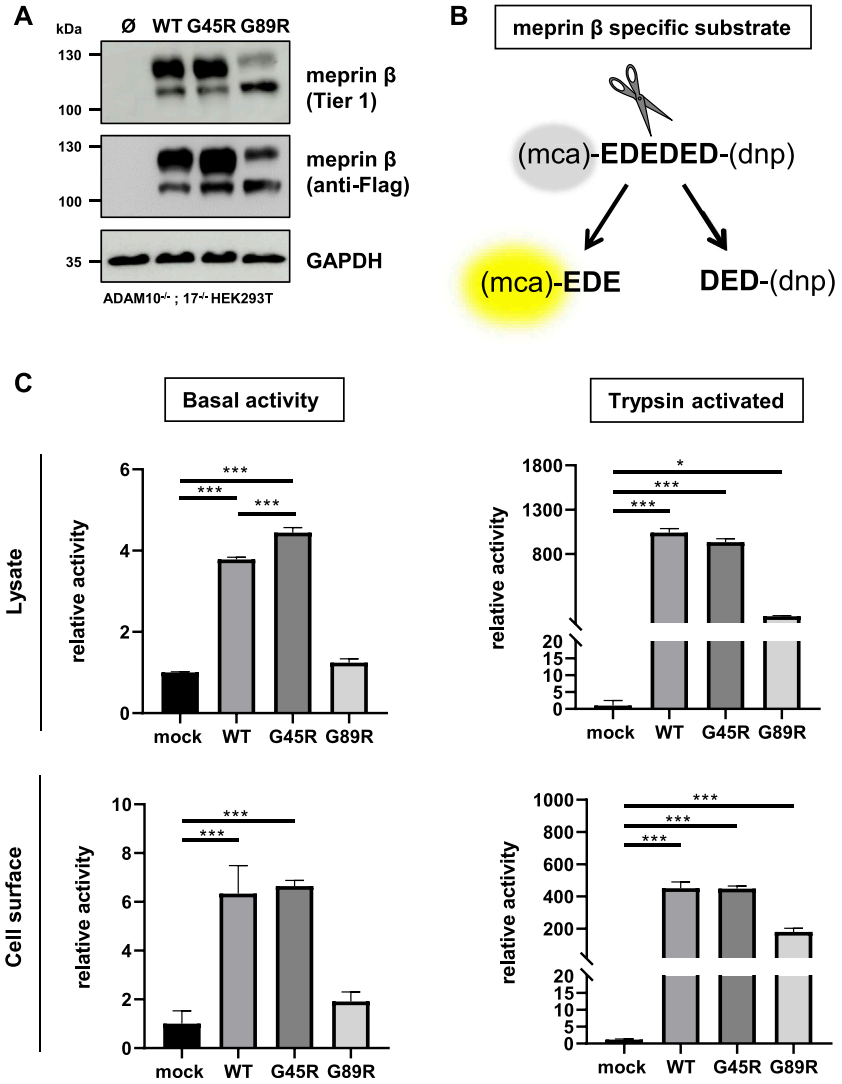

FIGURE 2 | Proteolytic activity of G45R, G89R, and wild-type meprin β toward a fluorogenic peptide. ADAM10$^{-/-}$; 17$^{-/-}$ HEK293T cells were transiently transfected with a control plasmid (Ø) or one of the indicated meprin β variants. **(A)** Representative Western blot analysis of cell lysates. **(B)** Representation of the meprin β-specific quenched fluorogenic peptide used for activity assays. **(C)** Cell lysate and cell surface meprin β activity in the absence and presence of trypsin. Data are presented as means ± sd, and statistical analysis was assessed by the one-way ANOVA followed by Tukey's post-test from three biological replicates. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

other two meprin β variants (**Figure 3A**, **Supplementary Figures S2A,B**). In line with the observed increased activity measured in the cell lysates (**Figure 2C**), co-transfection of meprin β G45R and the IL-6R in ADAM10$^{-/-}$; 17$^{-/-}$ HEK293T cells resulted in an increased release of the soluble IL-6R (sIL-6R) compared to wild-type meprin β (**Figure 3A**, **Supplementary Figures S2A,B**). Interestingly, meprin β G89R was also able to shed the IL-6R without further activation, albeit markedly less efficient than wild-type meprin β (**Figures 2C**, **3A**; **Supplementary Figures S2A,B**). Normalizing the amount of generated sIL-6R to the IL-6R/meprin β levels of the cell lysate even suggests a similar proteolytic activity of meprin β G89R as wild-type meprin β (**Supplementary Figure S2**). However, this analysis might be rather misleading due to the obvious strongly reduced overall expression level of meprin β G89R. Nonetheless, the results suggest that the substrate binding to this meprin β variant has a folding stabilizing effect that renders the protease as proteolytically active.

In order to analyze the biological activity of the generated sIL-6R by the different meprin β variants, Ba/F3-gp130 cells were used. This murine pro-B-cell line (Ba/F3) is stably transfected with the signal transducing receptor gp130 and does not express the IL-6R endogenously (Taga and Kishimoto 1997). Therefore, these cells require either the sIL-6R and IL-6 or hyper-IL-6 (a chimeric fusion protein of the sIL-6R and IL-6) to induce trans-signaling by the JAK/STAT-pathway *via* phosphorylation of

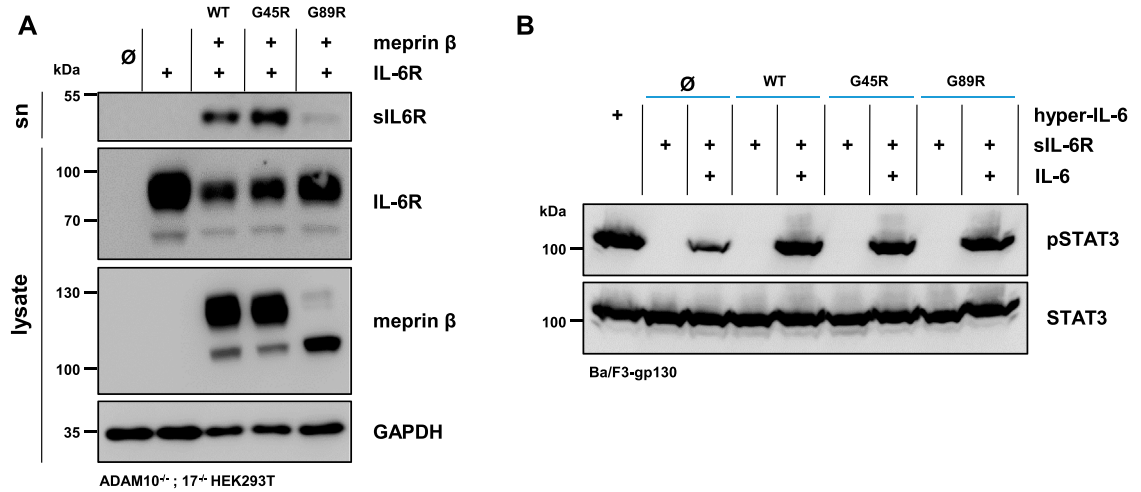

FIGURE 3 | Meprin β-dependent shedding of the IL-6R and induced trans-signaling. **(A)** Immunoblots of ADAM10$^{-/-}$; 17$^{-/-}$ HEK293T cells transiently transfected with a control plasmid (Ø), IL-6R alone, and together with one of the indicated meprin β variants. Supernatants (sn) were ultracentrifuged and analyzed for sIL-6R. IL-6R and sIL-6R were detected with an antibody directed against the D1-domain of IL-6R and meprin β with an anti-Flag antibody. GAPDH served as the loading control. Of note, the amount of sIL-6R in the supernatant was increased upon the co-expression of G45R compared with meprin β WT. **(B)** Phosphorylation of STAT3 in Ba/F3-gp130 cells stably transfected with gp130. Cells were treated with ultracentrifuged supernatants from the experiments in **(A)** and the phosphorylation of STAT3 was analyzed in the presence and absence of 150 ng recombinant IL-6. The same amount of the fusion protein consisting of soluble IL-6R and IL-6 (hyper-IL-6) served as the positive control. Phosphorylation was detected with an antibody raised against phosphorylated STAT3 (pSTAT3). Total STAT3 protein served as the loading control. Western blot quantification of generated sIL-6R and pSTAT3 from three biologic replicates is presented in **Supplementary Figure S2**.

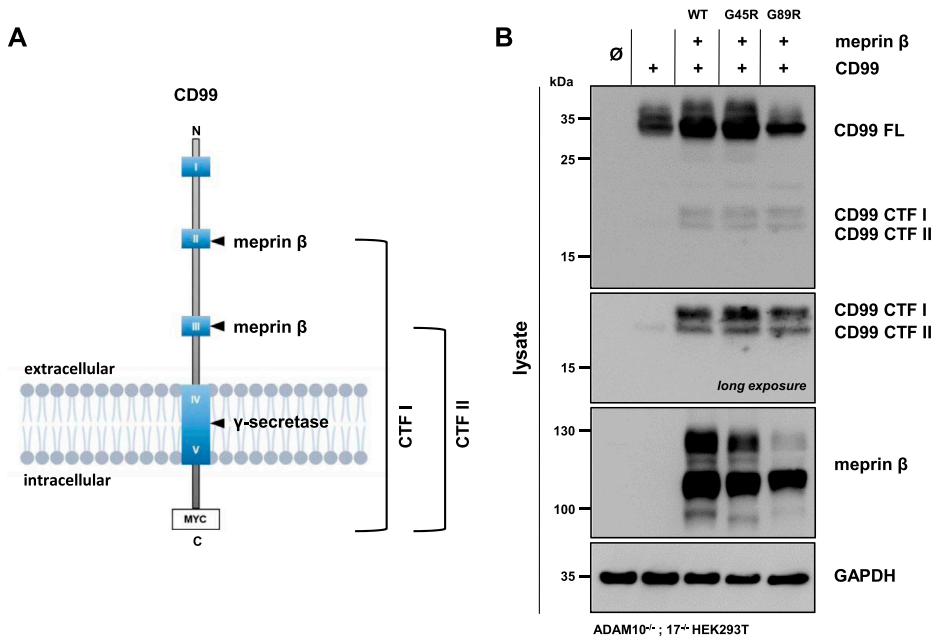

FIGURE 4 | CD99 shedding by the meprin β variants G45R and G89R. **(A)** CD99 construct used in this experiment, highlighting the highly conserved acidic regions (HCRs) as blue boxes with roman numerals, the two known cleavage sites of meprin β and the C-terminal Myc-tag. **(B)** ADAM10$^{-/-}$; 17$^{-/-}$ HEK293T cells were transiently transfected with a control plasmid (Ø), CD99 alone, and together with one of the indicated meprin β variants. Cell lysates were analyzed by Western blotting using an anti-Flag antibody for meprin β and an anti-Myc antibody for CD99 detection (CD99 FL: CD99 full length; CD99 CTF I and CTF II: CD99 C-terminal fragments I and II). GAPDH served as the loading control. To determine the accumulation of γ-secretase–dependent cleavage products, the specific inhibitor DAPT was applied. Western blot quantification of generated CD99 CTFs from three biologic replicates is presented in **Supplementary Figure S3**.

STAT3 (signal transducer and activator of transcription 3). In accordance with the immunoblot analyses, Ba/F3-gp130 cells were incubated with conditioned media from ADAM10$^{-/-}$; 17$^{-/-}$ HEK293T cells co-transfected with the IL-6R, and each of the three meprin β variants significantly induced STAT3 phosphorylation in dependence of the IL-6 in comparison to the mock control (**Figure 3B**, **Supplementary Figure S2C**).

CD99 Shedding by Meprin β Variants

Meprin β was shown to shed the adhesion molecule CD99, thereby promoting the transendothelial cell migration (TEM) of Lewis lung carcinoma (LLC) cells (Bedau et al., 2017a). The cleavage sites were identified in highly conserved acidic regions of CD99 (Bedau et al., 2017a). Comparable to JAM-A, ICAM-1, and L-selectin, CD99 controls the transendothelial migration of cells from the vessel lumen toward the interstitium for neutrophils, monocytes, and lymphocytes (Schenkel et al., 2002; Lou et al., 2007). Here, we investigated whether the meprin β variants G45R and G89R show altered cleavage efficiency of CD99. Therefore, ADAM10$^{-/-}$; 17$^{-/-}$ HEK293T cells were transfected with a C-terminally tagged CD99 alone and together with the meprin β variants. CD99 shedding was analyzed by the accumulation of C-terminal fragments CTF I and CTF II in cell lysates *via* immunoblotting, as described previously (Bedau et al., 2017a) (**Figure 4**, **Supplementary Figure S3**). To block further processing of the CTF II by the γ-secretase, the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L- alanyl]-S-phenylglycine t-butyl ester (DAPT) was added to the transfected cells. As for the IL-6R, we observed that all variants of meprin β are capable of shedding CD99 and generating the fragments CTF I and CTF II (**Figure 4B**, **Supplementary Figure S3**). Similarly, the G45R variant showed a tendency of more efficient CD99 cleavage than wild-type meprin β, visualized by slightly increased levels of CTF I (**Figure 4B**, **Supplementary Figure S3**). In contrast, the G89R variant showed similar CD99 shedding capacity compared with wild-type meprin β, even without considering the normalization of the reduced expression of G89R relative to CD99 (**Figure 4B**, **Supplementary Figure S3**).

APP Shedding by Meprin β Variants

The altered glycosylation pattern of the meprin β variant G89R in cell lysates and biotinylated cell surface fractions together with its significantly reduced cell surface localization as judged by immunofluorescence microscopy has been similarly described for the meprin β variant D204A (Arnold et al., 2015). This mutation likewise results in the expression of a less glycosylated meprin β ER-form and shows only little cell surface activity compared to wild-type meprin β. However, it has a very high β-secretase activity toward the well-described meprin β substrate, amyloid precursor protein (APP), demonstrated by increased Aβ peptide generation (Arnold et al., 2015). Of note, APP processing by meprin β, either at the β-secretase site or at the N-terminus releasing N-APP fragments, has been observed to take place at the cell surface and even on the secretory pathway (**Figure 5A**) (Schonherr et al., 2016; Scharfenberg et al., 2019). Additionally, at least meprin β activity in transiently transfected HEK293 cells correlates negatively with sAPPα levels (Armbrust et al., 2021). The observed proteolytic activity of the meprin β G89R variant towards CD99 and the IL-6R suggests substrate binding and processing already on the secretory pathway. Therefore, APP

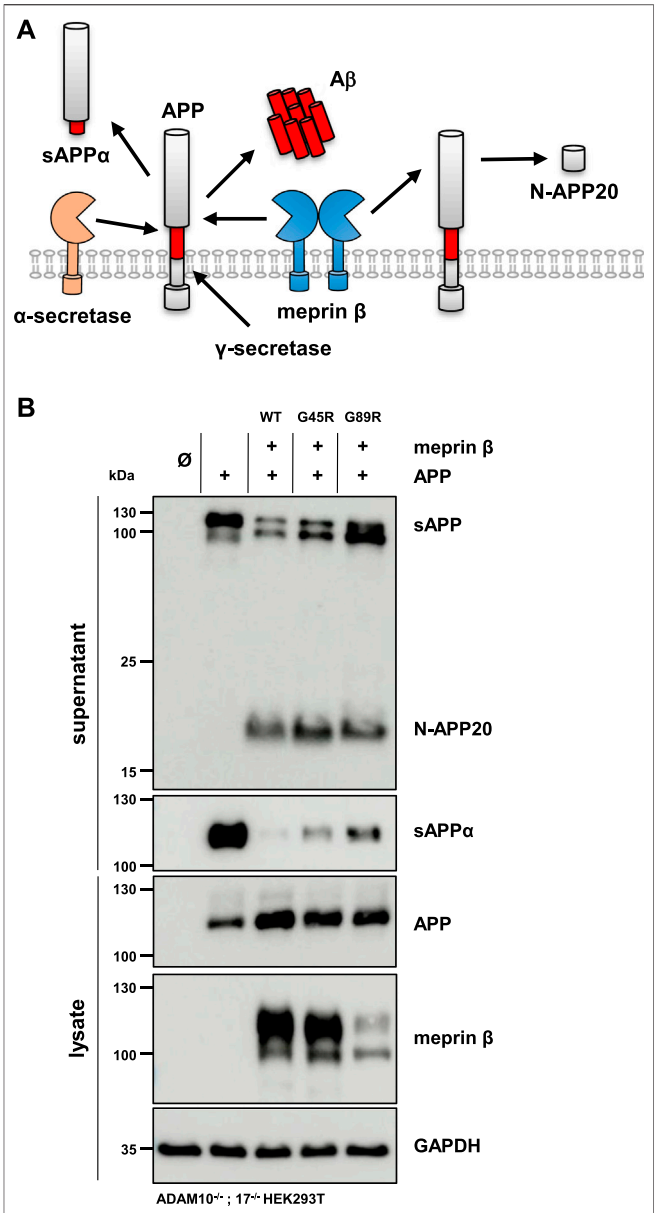

FIGURE 5 | APP processing shedding by the meprin β variants G45R and G89R. **(A)** APP is cleaved by meprin β in two distinct ways. On the one hand, non-amyloidogenic N-APP fragments are produced, and on the other hand, meprin β acts as a β-secretase, competing with α-secretase cleavage at the cell surface. **(B)** ADAM10$^{-/-}$; 17$^{-/-}$ HEK293T cells were transiently transfected with a control plasmid (Ø), APP alone, and together with one of the indicated meprin β variants. Cell lysates and supernatants were analyzed by immunoblotting using an anti-Flag antibody for meprin β and specific APP antibodies (N-APP: 22C11, sAPPα: 6E10, and total APP in lysates: CT15). GAPDH served as the loading control. Western blot quantification of generated N-APP and sAPPα from three biologic replicates is presented in **Supplementary Figure S4**.

represented an ideal substrate to verify this hypothesis. In order to do so, ADAM10$^{-/-}$; 17$^{-/-}$ HEK293T cells were transfected with APP alone or together with the meprin β variants. Even though these cells are deficient for the two α-secretases ADAM10 and ADAM17, APP processing at the α-secretase cleavage site is observed, most likely by a

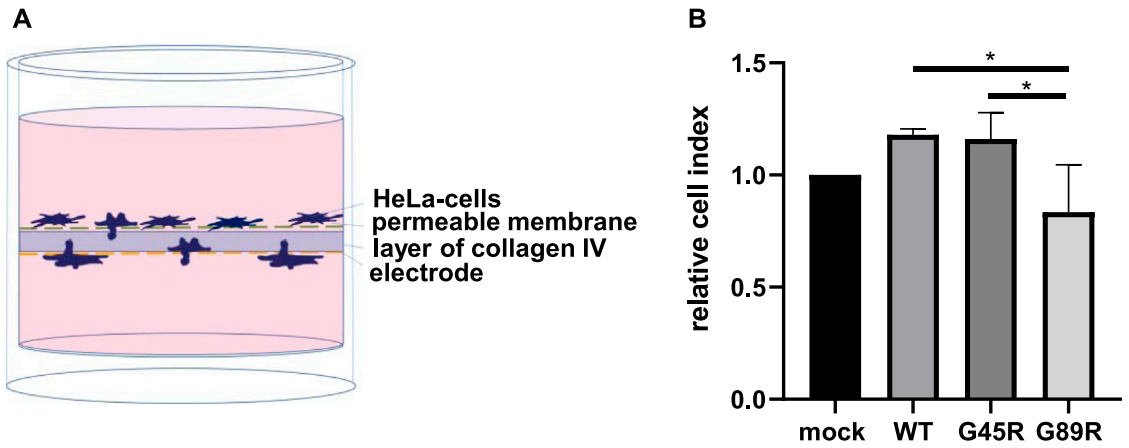

FIGURE 6 | Cancer cell invasion assay through collagen IV matrix. **(A)** HeLa cells were transfected and seeded into the upper chamber of the well, and the impedance of the electrode located at the bottom of the well was measured for 24 h. An increase in impedance was equal to cell invasion through the layer of collagen IV. **(B)** Quantification of the results demonstrates that the expression of G89R reduced the invasiveness significantly. Values of the different meprin β variants were normalized to values of mock-transfected cells, which were set as 1. Data are presented as means ± sd, and statistical analysis was assessed by the one-way ANOVA followed by Tukey's post-test from three biologic replicates. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

compensatory endogenous alternative α-secretase like, e.g., ADAM9 (Asai et al., 2003). However, co-expression of wild-type meprin β and its variants resulted in the expected meprin β-dependent APP cleavage pattern (**Figure 5B**), with a comparable increase of N-APP20 fragments in the supernatant and decreased sAPPα levels (**Figure 5B**). Similarly, as observed for meprin β D204A, APP processing by meprin β G89R resulted in N-APP20 cleavage. However, in the case of meprin β G89R, sAPPα cleavage was higher compared to meprin β wild-type and G45R (**Figure 5B**). Similarly, as observed for CD99 shedding, the normalization of meprin β G45R proteolysis to overall expression levels would suggest that meprin β G45R is even more active than wild-type meprin β (**Supplementary Figure S4B**). Taken together, these findings suggest that meprin β G89R is, indeed, mainly proteolytically active on the secretory pathway.

Meprin β G89R Diminishes the Invasion of Cancer Cells Through Collagen IV

Since invasiveness is an important characteristic of cancer cells, we investigated whether meprin β variants identified in certain tumors have an influence on this process. Collagen IV is a major component of the basal lamina and thus part of an important barrier separating the epithelia from underlying tissues. It has been observed that meprin β is capable of cleaving collagen IV, which may contribute to cancer cell invasiveness (Kruse et al., 2004). Therefore, we analyzed the invasive mobility of cells passing through a semipermeable membrane coated with collagen IV (**Figure 6**). HeLa cells transfected with meprin β WT, G45R, and G89R or mock as negative control were transferred into wells with two chambers, divided by a layer of collagen IV (**Figure 6A**). Cells passing through that layer changed the impedance of the electrode placed on the bottom side. Therefore, the change in impedance was equal to the number of cells, which had passed through the layer of collagen IV. In contrast to the cell surface–expressed meprin β WT and G45R, the expression of the meprin β G89R variant significantly diminished the invasiveness of HeLa cells (**Figure 6B**).

DISCUSSION

The dysregulation of proteolytic enzymes is often associated with tumor progression and metastasis, for instance, by matrix metalloproteases (MMP) degrading extracellular matrix (Winer et al., 2018). This is often caused by an increased expression of proteases by certain cancers, which may induce proliferation or mobility of these cells. Latest genomic approaches revealed that malignant hyperplasia exhibits a number of different driver or bystander mutations and several of these were identified in protease genes. Here, we investigated two cancer-associated variants of the metalloprotease meprin β, namely, G45R and G89R, both leading to an exchange of the amino acid glycine to arginine. We functionally characterized these variants in biochemical and cell biological approaches to determine possible consequences for cancer cell invasiveness.

Based on the crystal structure of meprin β (Arolas et al., 2012), we decided to investigate the biochemical properties and enzymatic functions of the meprin β variants G45R and G89R. The G45R mutation is located within the propeptide of meprin β. Knowing that the cleavage of the propeptide occurs C-terminal of the arginine at position 61 (Jackle et al., 2015), the insertion of another arginine in close proximity could lead to an enhanced activation of meprin β. For the G89R variant, we presumed that the insertion of arginine with a big positively charged sidechain into the very tight area between the catalytic domain and the TRAF domain would have a negative effect on the proteolytic activity due to impaired folding of the protein.

Indeed, we observed decreased levels of fully glycosylated G89R in cell lysates and at the plasma membrane compared to wild-type meprin β. Along the same lines, we detected higher amounts of the less glycosylated ER-form of meprin β in the case of the G89R variant. This indicates that the G89R variant was partially stuck on the

secretory pathway, which could be confirmed by the immunofluorescence analysis where an ER-marker (PDI) showed strong co-localization with G89R. This observation was further supported by a markedly reduced proteolytic activity of this meprin β variant on the plasma membrane of living cells. Nonetheless, considering the protein substrate analyses including APP processing, it turned out that substrate binding on the secretory pathway seems to have a folding stabilizing effect, which enables G89R activation and, therefore, proteolytic activity toward substrates before reaching the cell surface.

Analyzing the meprin β G45R variant, we expected the protease to be faster activated due to the inserted arginine in position 45, representing a potential additional activation site, besides the arginine in position 61 that is cleaved by pancreatic trypsin and matriptase 2 (Ohler et al., 2010; Jackle et al., 2015). This was, indeed, true for the basal activity; however, we did not observe increased proteolytic activity on cells expressing the G45R variant when incubated with trypsin. In studies investigating a meprin β G32R variant, the additional arginine, indeed, resulted in increased cell surface activity of meprin β (Schäffler et al., 2019). Analyzing the proteolytic processing of protein substrates, G45R showed a tendency of increased activity compared to wild-type meprin β. One explanation why G45R is less prone to an increased activation than G32R could be the position of the mutation within the propeptide. In pro-meprin β, the propeptide interacts *via* two salt bridges on the prime site (D30-R146 and D34-R146) and two on the non-prime site (R54-E137 and D56-R131) with the catalytic domain (Arolas et al., 2012). Therefore, it is possible that G32R has a more destabilizing impact by disrupting the prime site interaction than G45R, which is located in the middle of the two important interaction sites. Consequently, G32R leads to more pronounced meprin β activity at the cell surface, while the effect of G45R is rather moderate.

IL-6 classic- and trans-signaling are important mechanisms in immunomodulation, promoting immune cell differentiation and proliferation (Rose-John, 2012). For trans-signaling cleavage of the IL-6 receptor by ectodomain sheddases, such as ADAM17 or meprin β, it is mandatory to act on cells that do not express the receptor endogenously (Arnold et al., 2017; Sammel et al., 2019). The pro-B-cell line Ba/F3-gp130 is dependent on IL-6 trans-signaling as an inducer of proliferation (Sammel et al., 2019). In this study, we could show that the IL-6R cleavage was increased by the meprin β variant G45R in comparison to the wild-type enzyme, which could influence cancer and immune cell proliferation.

The meprin β substrate CD99 was observed to promote cancer cell extravasation (Bedau et al., 2017a; Bedau et al., 2017b). However, whether the persistent cleavage by the G45R and G89R variants as shown in this study has influence on cell migration in melanoma has to be further demonstrated (Wilkerson et al., 2006).

Natural tissue barriers such as the basal membrane are important factors for tumor progression and tumor metastasis (Tanjore and Kalluri 2006). Meprin β has been shown to hydrolyze collagen IV (Kruse et al., 2004), a major component of the basement membrane. Therefore, we investigated whether one of the protease variants would have an impact on the invasiveness of tumor cells *in vitro*. Testing the invasiveness of Hela cells expressing the different variants of meprin β revealed that meprin β in its active form on the cell surface (variant G45R und WT) is more capable of passing through a layer of collagen IV. Although the difference was not huge, an alternation of the proteolytic activity of meprin β likely affects the invasiveness of tumor cells. This result was in line with the results of the G32R variant (Schäffler et al., 2019), thus indicating a possible pro-metastatic function of meprin β.

Taken together, here we characterized biochemically and in cell-based assays the two annotated cancer-associated meprin β amino exchange variants G45R and G89R, indicating controversial functions on cancer progression. On the one hand, we identified potentially cancer-promoting functions of G45R, such as increased proteolytic activity at the cell surface. On the other hand, G89R showed overall reduced expression levels and was mainly active on the secretory pathway and only partially reached the plasma membrane. This rather indicates antitumorogenic properties particularly for meprin β substrates, which can be processed solely at the plasma membrane or substrates of the extracellular matrix, as shown by the cell invasion assay. Hence, future studies will show if the characterized meprin β variants are bystanders or driver mutations in melanoma.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AG, FS, MS, FreS, PA, and FP designed and performed experiments. AG, FS, and CB-P analyzed the data and wrote the manuscript. OH, SS, FA, JL, and CG provided material and technical support.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2021.702341/full#supplementary-material

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