Published in final edited form as:

Methods Mol Biol. 2020 ; 2043: 195–206. doi:10.1007/978-1-4939-9698-8_16.

Cell-Based Interaction Analysis of ADAMTS Proteases and ADAMTS-Like Proteins with Fibrillin Microfibrils

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Abstract

The extracellular matrix (ECM) is a composite biomaterial that serves as an anchor for cells and provides guidance cues for cell migration, proliferation, and differentiation. However, many details of the hierarchical ECM assembly process and the role of individual protein–protein interactions are not well understood. Here, I describe a cell-culture-based method that allows for determination of the ECM localization of recombinant ADAMTS proteases and ADAMTS-like (L) proteins in relationship to fibrillin microfibrils deposited by human dermal fibroblasts. The method can be readily adapted to study the localization of ECM components other than ADAMTS and ADAMTSL proteins to fibrillin microfibrils and other ECM networks.

Keywords

ADAMTS proteases; ADAMTS-like proteins; Recombinant protein; Coculture assay; Fibrillin microfibrils; Immuno-colocalization; Extracellular matrix

1 Introduction

Fibrillin microfibrils are extracellular supramolecular assemblies that serve as a scaffold for cell attachment, as a reservoir for growth factors such as transforming growth factor (TGF)- β and bone morphogenetic proteins (BMPs), and as a versatile platform for the spatial organization of extracellular matrix (ECM) proteins such as fibulins, ADAMTS proteases, and ADAMTS-like(L) proteins [1]. The specific function of fibrillin microfibrils is determined by each distinct subset of molecules that bind to fibrillin microfibrils at distinct developmental stages or in specific tissues. One group of fibrillin-associated proteins belongs to the ADAMTS/ADAMTS-like (L) family of proteases and nonproteolytic proteins, respectively [2]. The finding in humans, that recessive mutations in genes encoding the proteases *ADAMTS10* or *ADAMTS17* and the nonproteolytic *ADAMTSL2* or *ADAMTSL4* proteins cause similar phenotypes as domain-specific dominant mutations in *FBN1*, the gene encoding fibrillin-1, suggests that these members of the ADAMTS family are functionally linked to fibrillin-1 [3–9]. The genetic consilience and the interplay between ADAMTS and ADAMTSL proteins and fibrillin microfibrils was further supported by biochemical or in vivo experiments, showing direct protein–protein interactions or fibrillin

microfibril dysregulation in tissues from respective knockout mice [4, 10–14]. Conceptually, fibrillin microfibril-associated proteins, such as ADAMTS and ADAMTSL proteins, could define the specific fibrillin microfibril functionality via the following mechanisms: (1) Fibrillin microfibril turnover could be altered by ADAMTS protease activity. ADAMTSL proteins, due to their homology to the ancillary domain of ADAMTS proteases, could modulate ADAMTS protease activity; (2) ADAMTS and ADAMTSL proteins could modulate fibrillin microfibril assembly or influence its tissue-specific iso-type composition (there are three fibrillin isotypes in humans and two in mice, that can form heterotypic fibrillin microfibrils and are differentially regulated on the gene transcription level) [12–18]; (3) the localization of ADAMTS and ADAMTSL proteins in the ECM could require fibrillin microfibrils [10]; or (4) ADAMTS and ADAMTSL proteins could mediate the localization of other ECM proteins to fibrillin microfibrils. The interaction of ADAMTS and ADAMTSL proteins with fibrillin can be studied on multiple levels: Direct protein–protein binding of ADAMTS and ADAMTSL proteins to fibrillin peptides can be studied with biophysical methods [10, 14]. However, to localize ADAMTS and ADAMTSL proteins to fibrillin microfibrils in tissues, specific antibodies are required, which are not always available [12, 13]. Therefore, cell lines that assemble fibrillin microfibrils, can be used as a model system for the analysis of the localization of recombinant, tagged ADAMTS and ADAMTSL proteins to fibrillin microfibrils and can be used to assess their potential roles on the rate of microfibril formation and turnover [10, 12, 13]. Such cell culture systems represent a model for early stages of fibrillin microfibril formation and maturation and are described here in detail. In addition, a purification strategy is described which was used for several ADAMTS and ADAMTSL proteins [10, 14].

2 Materials

2.1 Cell Lines

1. Neonatal human dermal fibroblasts (*see* Note 1).
2. Primary fibroblasts isolated from human foreskin (*see* Note 2).

2.2 Protein Expression and Purification

1. Stable cell lines secreting ADAMTS and ADAMTSL proteins tagged with tandem Myc/His₆ tag to facilitate purification and detection (*see* Note 3).
2. DMEM (high glucose) supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, 0.5 mg/mL G418, with or without 10% fetal bovine serum.
3. Ultrafiltration unit with 10 kDa or 30 kDa molecular weight cutoff membrane.
4. Chromatography buffer A (Buffer A): 20 mM HEPES, 500 mM NaCl, pH 7.2, filtered through a 0.22 µm filter.
5. Chromatography buffer B (Buffer B): 20 mM HEPES, 500 mM NaCl, 500 mM imidazole, pH 7.2, filtered through a 0.22 µm filter.
6. Fast protein liquid chromatography (FPLC) system.
7. Ni-NTA HisTrap-HP columns (1 mL or 5 mL).

8. Gel filtration column (Superose 6 or 12) (optional).
9. Dialysis tube or Slide-A-Lyzer cassette (12,000–14,000 Da molecular weight cutoff).
10. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4.
11. Reagents and equipment for denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

2.3 Fibroblast Cell Culture

1. Humidified cell culture incubator (37 °C, 5% CO₂ atmosphere).
2. DMEM, including 100 units/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum.
3. PBS.
4. 0.25% trypsin–EDTA.
5. Tissue culture flasks.
6. 8-well chamber slides.
7. Cell counter.

2.4 Immunostaining

1. PBS.
2. Acetone–methanol (70%:30%, v/v) (ice-cold) (*see* Note 4).
3. Normal goat serum (10%, reconstituted and diluted in PBS).
4. Anti-fibrillin antibodies: pAB-FBN1-C (1:500) [19], mAB FBN1 11C1.3 (1:200, ThermoFisher Scientific), pAB-FBN2-Gly (1:300) [20].
5. Anti-fibronectin antibodies: pAB 2033 (1:500, EMD Millipore), mAB clone 15 (1:1000, Millipore Sigma).
6. Anti-myc antibodies: mAB 9E10 (1:300, Invitrogen), pAB (1:500, C3956, Sigma).
7. Secondary antibodies: goat anti-mouse Alexa Fluor 488 (1:350) and goat anti-rabbit Alexa Fluor 568 (1:350).
8. ProLong Gold mounting medium with DAPI.
9. Cover slips.
10. Fluorescence microscope.

3 Methods

3.1 Purification of Recombinant ADAMTS and ADAMTSL Proteins

1. Expand HEK293 cells stably expressing the recombinant protein in 5×150 cm dishes and culture in DMEM with FBS in the presence of G418 until confluent.
2. Remove the medium and rinse cell layer carefully with 2×20 mL PBS per dish.
3. Add 25 mL serum-free DMEM without G418 per dish.
4. Collect conditioned medium every 48–72 h and replace with 25 mL fresh serum-free DMEM without G418 per dish. Collect until more than 60–70% of HEK293 cells detached (*see* Note 5).
5. Immediately after each collection, centrifuge conditioned medium at $\sim 4,000 \times g$ for 10 min to remove cell debris (*see* Note 6).
6. Store conditioned medium at -20° and pool collections.
7. Thaw the pooled conditioned medium overnight at RT and filter through a 5 μ m pore filter.
8. Concentrate the medium to <50 mL (loading volume of FPLC super loop) by ultrafiltration with a membrane of 10 kDa or 30 kDa molecular weight cutoff, depending on the size of the desired recombinant protein (*see* Note 7).
9. Transfer concentrated medium in a dialysis tube and dialyze against 1 L Buffer A at 4°C , change buffer once (*see* Note 8).
10. After dialysis, centrifuge concentrated medium at $\sim 20,000 \times g$ for 15 min and inject supernatant in super loop for subsequent loading on the Ni-NTA column.
11. Set the flow rate of the FPLC pump at 1 mL/min for all column preparation and wash-steps. Do not exceed maximum pressure specified for the respective column type.
12. Equilibrate Ni-NTA column with buffer A and load concentrated conditioned medium with a flow rate of 0.2–0.5 mL/min (*see* Note 9).
13. Collect the flow-through as a precaution.
14. Elute bound protein with a 0–500 mM imidazole gradient (0–100% buffer B) over 40 column volumes (*see* Note 10).
15. Collect 1 mL fractions.
16. Evaluate starting material, flow-through and fractions in elution peak(s) with SDS-PAGE followed by Coomassie staining for the presence and purity of the recombinant protein.
17. Pool fractions with purified protein and dialyze against 3×500 mL PBS at 4°C .
18. Determine final protein concentration with a spectrophotometer or colorimetric assay and the purity with SDS-PAGE.

19. Store proteins in aliquots at 80 °C (*see* Note 11).

3.2 Colocalization of Recombinant ADAMTS and ADAMTSL Proteins with Fibrillin Microfibrils in the ECM of Human Dermal Fibroblasts

To determine if recombinant ADAMTS and ADAMTSL proteins localize to fibrillin microfibrils or modulate fibrillin microfibril formation, three methods are described here: (1) Addition of purified recombinant proteins to human dermal fibroblasts (HDFs); (2) Addition of conditioned medium containing ADAMTS and ADAMTSL proteins to HDFs; and (3) coculture of HEK293 cells stably expressing ADAMTS and ADAMTSL proteins with HDFs (*see* Note 12). The methods differ in how the recombinant protein is supplied to the HDFs. However, the immunolocalization protocol is the same for all three methods.

1. Method 1: Seed 50,000 cells per chamber in 8-well chamber slides in a total volume of 0.5 mL DMEM medium with FBS. Mix 10–50 µg/well of purified ADAMTS and ADAMTSL protein with the cells at the time of seeding (Fig. 1). Mix same volume of PBS with cells in a separate well to control for potential dilution effect and to monitor the specificity of the antibodies (*see* Note 13).
2. Method 2: After HDFs attach (~2–4 h after seeding), replace the growth medium with 0.5 mL conditioned medium, containing recombinant ADAMTS and ADAMTSL proteins (*see* Note 14).
3. Method 3: Seed 37,500 HDFs together with 37,500 HEK293 cells stably or transiently expressing recombinant ADAMTS or ADAMTSL proteins in a total volume of 0.5 mL (*see* Note 15).
4. Culture cells for 48–72 h (*see* Note 16).
5. Remove cell culture supernatant and rinse cell layer with 0.5 mL PBS (the volumes indicated below are per well of an 8-well chamber slide) (*see* Note 17).
6. Fix cells with 0.2 mL ice cold acetone–methanol for 5 min at RT. Incubate slide on slow-medium fast orbital shaker.
7. Rinse cells with 0.5 mL PBS.
8. Block with 0.15 mL 10% normal goat serum in PBS for 1 h at RT.
9. Incubate cells with 0.12 mL primary antibody, diluted in blocking buffer, for 2 h at RT.
10. Rinse 3 × 5 min with 0.5 mL PBS.
11. Incubate with 0.12 mL of corresponding secondary antibodies carrying green and red fluorophore for 1.5 h at RT. From here on, cover slide to protect from light (*see* Note 18).
12. Rinse 3 × 5 min with 0.5 mL PBS.
13. Mount stained cells with one drop of ProLong gold including DAPI and cover-slip.
14. Image the slides with a fluorescence microscope (Fig. 1) (*see* Note 19).

4 Notes

1. Neonatal human dermal fibroblasts typically secrete fibrillin-1 and deposit abundant fibrillin-1 microfibrils within 3–5 days in culture. However, fibrillin-2 microfibrils are deposited with greater variability. The quantity of fibrillin-2 microfibrils is typically less when compared to fibrillin-1 microfibrils and fibrillin-2 microfibrils are sometimes not deposited at all. Primary cell batches should be tested individually. FBN1 secretion and deposition tends to be reduced at higher passage numbers (>8). Therefore, we typically use HDFs for no more than 6–8 passages when staining for fibrillin microfibrils. Fibronectin deposition is very strong at early passages and can be already detected after 24 h [21]. At later time points, the fibronectin network appears as a dense woven network and individual fibronectin fibers are difficult to distinguish. Additional cell lines and cell types that produce fibrillin-1 and, to a lesser extent, fibrillin-2 include human nonpigmented ciliary epithelial cells, MG63 osteosarcoma cells, NIH3T3 cells, mouse embryonic fibro-blasts, and fetal bovine nuchal ligament cells [18]. To analyze the localization of ADAMTS and ADAMTSL proteins to a specific fibrillin isoform, fibrillin gene expression can be reduced by siRNA, or *Fbn1*, *Fbn2*, or *Fbn1/Fbn2* double knockout mouse embryonic fibroblasts can be used [21–23].
2. To isolate HDFs from fresh foreskin obtained from circumcisions (institutional IRB regulations have to be followed), collect the specimens in sterile PBS and keep on ice during transport. In a biosafety cabinet, rinse foreskin briefly in 70% ethanol, followed by a rinse and subsequent incubation in sterile PBS until processing. Cut the foreskin in small pieces (1 × 1 mm) in a petri dish using a sterile and sharp scalpel and distribute 20–30 pieces in a 10 cm tissue culture dish. Incubate the foreskin pieces without medium in a humidified cell culture incubator at 37 °C in a 5% CO₂ atmosphere. After 3–5 h carefully add 10 mL DMEM, including 100 units/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum. Observe tissue piece for 1–2 weeks for outgrowth of fibroblasts from the edges and change the medium after 1 week. Once outgrowth is observed and dense cell sheets are formed around the skin pieces, remove the medium and skin pieces with a sterile glass pipette and vacuum suction (cells will adhere and not be removed with the skin pieces). Rinse the cell layer with 10 mL PBS per dish, trypsinize the cells, and reseed on a new 10 cm dish. Once the cell layer is confluent, split in a ratio of 1:3–1:4 (passage 1). Freeze the cells in aliquots (typically 2×1 mL per one 10 cm dish) in 10% DMSO/90% FBS or continue expanding the cells from passage 1 for experimentation.
3. ADAMTS and ADAMTSL proteins can be expressed in HEK293 cells in a pcDNA3.1 MycHis or a pSecTag vector backbone. Stable cell lines should be selected as individual clones or as a batch using 1 mg/mL G418 for several weeks. After verification of recombinant protein secretion by western blotting, cell lines can be maintained in DMEM with FBS in the presence of 0.5 mg/mL

G418. Proteins can also be expressed in any other suitable cell type, such as Chinese hamster ovary (CHO) cells.

4. Store acetone–methanol mixture in explosion-proof -20°C freezer or store at room temperature and cool aliquot on ice before use.
5. HEK293 cells will “contract” into a reticular network upon exposure to serum-free DMEM medium. However, they continue to secrete protein, even if only 40–50% of the dish area is covered. Aliquots of the conditioned medium can be collected for western blot analysis of protein expression and used to determine which collections to include for the purification.
6. At this point protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF) or cOmplete-EDTA free tablets may be added. The stability of any protein should be assessed to decide if protease inhibitors are required. If the ADAMTS protease of interest is degraded by autocatalytic processing, addition of protease inhibitors at that point may not prevent further degradation of the protease [10]. Alternatively, a proteolytically inactive mutant of the ADAMTS protease can be purified to study protein–protein interactions.
7. Ultrafiltration of 1–1.5 L conditioned medium can take 8–24 h or longer. Measure the flow rate and assess if ultrafiltration can proceed overnight. If medium is filtered completely (membrane is dry), add 20 mL of chromatography buffer A and stir for 10–15 min. Protein in buffer A can be centrifuged and loaded directly on the Ni-NTA column. No dialysis is required and yield may not be affected.
8. Dialysis can be performed overnight at 4°C and the buffer changed the next morning. Dialysis does not have to be complete. The dialysis step is intended to reduce the histidine content present in DMEM medium, which may interfere with binding of the His-tag of the recombinant protein to the Ni-NTA resin.
9. The flow rate for the loading step depends on the maximum pressure under which the column can be operated. 0.5 mL/min equals a “contact time” of protein to Ni-NTA resin of 2 min for a 1 mL column. Slower flow rates can be used.
10. ADAMTS and ADAMTSL proteins typically elute with >10% buffer B (>50 mM imidazole). When the recombinant protein elution starts, one may switch to 100% buffer B to concentrate the protein and shorten the chromatography run. Imidazole absorbs at 280 nm and 100% buffer B results in an elevated but stable reading at 280 nm. However, a protein peak can still be distinguished. Depending on the purity after Ni-NTA chromatography, an additional gel filtration step may be required to improve the purity of the recombinant protein and can then be used for buffer exchange instead of dialysis.
11. Storage conditions for the different proteins can vary and need to be determined empirically. Storage at 4°C or at 20°C in the presence of glycerol may be an alternative to freezing to prevent aggregation and precipitation. Before use, protein aggregates should be removed by spinning the sample with a

microcentrifuge at maximum speed for 5 min at RT. Determination of the protein concentration before use is recommended.

12. The different methods to supply the recombinant proteins to cells each have its advantages and disadvantages; for example, addition of purified protein does not recapitulate processes relevant for ECM localization such as cosecretory interactions or cell-surface interactions established during secretion. The transfer of conditioned medium requires control of the expression level of the recombinant protein and must include a vector control conditioned medium. The coculture systems require either the generation of stable cell lines or the prior transient transfection of HKE293 cells with the respective expression plasmids. In addition, protein expression plasmids could be directly introduced in fibrillin microfibril producing cell lines by transfection, transduction, or electroporation. These methods may require special equipment or an institutional protocol for the use of lentiviruses.
13. Recombinant proteins can be added at the time of seeding (dilute cells and recombinant protein in 500 μ L complete DMEM). However, if the specific protein interferes with cell attachment, then HDFs can be allowed to attach for 2–4 h and recombinant protein can be directly added to the culture medium at that time. Sterile-filtration of the recombinant proteins is not required.
14. Expression of recombinant protein in the medium has to be verified by western blotting. Concentration of conditioned medium may be required. To replenish nutrients and growth factors, sterile glucose or fresh FBS may be added to concentrated conditioned medium or the conditioned medium may be diluted with fresh DMEM supplemented with FBS.
15. When observing the cells in the coculture system after cell attachment, HEK293 cells may appear as patches with a network of connected HDFs surrounding them. The two cell types do not mix well so that isolated clusters of each cell type can be observed. If desired, the ratio of HDFs to HEK293 cells can be varied [23]. The ECM networks, including fibrillin microfibrils, do not cross over patches of HEK293 cells but primarily overlap with the fibroblasts (*see* Fig. 1).
16. If longer cell culture periods are desired, the medium including the recombinant protein (for method 1) or conditioned medium (for method 2) should be replaced after 72 h.
17. Medium can be used for western blot analysis, for example to verify the integrity of the recombinant protein after exposure to the HDF cells.
18. Due to a more intense signal, the green fluorescent Alexa Fluor 488 label is recommended for detection of the Myc-tag of the recombinant protein and the Alexa Fluor 564 label for the corresponding ECM protein (fibrillin or fibronectin).
19. The results can be analyzed and interpreted with regards to colocalization of the ADAMTS and ADAMTSL proteins with fibrillin-1, -2, or fibronectin. In

addition, by comparing quantitative measures for the respective ECM network, it can be determined if ADAMTS and ADAMTSL proteins have a role in promoting ECM network formation or modulating the respective fibrillin isoforms. The latter would require simultaneous staining of FBN1 and FBN2 and possibly the recombinant ADAMTS protease or ADAMTSL protein. Gene expression changes for the respective ECM proteins should be monitored using quantitative real-time PCR and/or western blotting.

Acknowledgments

This work was supported by NIH grant AR070748.

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Fig. 1.

Coculture of HDF with HEK293 cells stably expressing proteolytically inactive ADAMTS17^{EA} (a) or HEK293 cells stably expressing empty vector (b). Colocalization of ADAMTS17EA (stained with α -Myc antibody in green) with fibrillin-1 microfibrils (pAB-FBN1 in red) is observed where both cell types are in close proximity (yellow, arrows in a). Patches of HEK293 cells are evident in (b) (star). Note that fibrillin-1 microfibrils formed by HDF cells do rarely cross the HEK293 patches. (c–e) 50 μ g recombinant ADAMTS17-PCD was added to HDF 5 h after seeding [10]. After 48 h, the ECM was stained with α -Myc antibody (green, ADAMTS17-PCD), pAB-FBN1 (fibrillin-1, red) and cells were labeled with DAPI (blue). Note the strong colocalization of ADAMTS17-PCD with fibrillin-1 microfibrils in (e) (yellow, arrows). Scale bars are 25 μ m