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Research article

Isolation and identification of extracellular matrix proteins from oil-based CASPERized mouse brains for matrisomal analysis

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ABSTRACT

The extracellular matrix (ECM) components present within all tissues and organs help to maintain the cytoskeletal architecture and tissue morphology. Although the ECM plays a role in cellular events and signaling pathways, it has not been well studied due its insolubility and complexity. Brain tissue has a higher cell density and weaker mechanical strength than other tissues in the body. When removing cells using a general decellularization method to produce scaffolds and obtain ECM proteins, various problems must be considered because tissues are easily damaged. To retain the brain shape and ECM components, we performed decellularization in combination with polymerization. We immersed mouse brains in oil for polymerization and decellularization via O-CASPER (Oil-based Clinically and Experimentally Applicable Acellular Tissue Scaffold Production for Tissue Engineering and Regenerative Medicine) and then isolated ECM components using sequential matrisome preparation reagents (SMPRs), namely, RIPA, PNGase F, and concanavalin A. Adult mouse brains were preserved with our decellularization method. Western blot and LC-MS/MS analyses revealed that ECM components, including collagen and laminin, were isolated efficiently from decellularized mouse brains using SMPRs. Our method will be useful to obtain matrisomal data and perform functional studies using adult mouse brains and other tissues.

1. Introduction

The extracellular matrix (ECM) is a complex network of cross-linked and glycosylated proteins that provides physical support to cells and tissues and regulates diverse cellular processes including proliferation, differentiation, and migration [1]. A volumetric study using electron microscopy reported that the ECM accounts for 20% of the rat brain [2]. During central nervous system (CNS) development, specific ECM components in the brain are regulated in a temporal and spatial manner [3]. Capillary endothelial

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glycocalyx [4] and pericyte-derived laminin [5] protect the blood–brain barrier (BBB). Collagens participate in axon guidance and synaptogenesis [6]. They are also associated with terminal differentiation of myelinating Schwann cells [7]. Moreover, changes and defects in the structure and functions of the ECM are associated with pathological conditions such as stroke [8], aging [9], autism [10], epilepsy [11], traumatic brain injury [12], Alzheimer's disease [13], and schizophrenia [14]. Reconstitution of ECM with a normal structure and functions is greatly needed for medical, pharmaceutical, and tissue engineering applications [15]. However, this is not easily achieved by conventional chemical and physical methods due to the compositional and structural complexity of the ECM and associated proteins (matrisome).

Liquid chromatography-mass spectrometry (LC/MS) is an analytical chemical technique for proteomics analysis. However, it is challenging to use this technique for proteomics analysis of the ECM because it is applicable for studying relatively abundant proteins, but not lowly abundant insoluble proteins with high complexity, such as those found in the ECM. Recent studies showed that decellularization and polymerization of engineered tissues improve the acquisition of ECM proteins [16–18]. However, this approach is inadequate to obtain ECM proteins because tissues are damaged and not preserved. To develop a suitable decellularization method for brain tissue, several attempts have been made to solve these problems by changing various parameters such as the concentrations of several chemical components, temperature, and treatment duration. CASPER (Clinically and Experimentally Applicable Acellular Tissue Scaffold Production for Tissue Engineering and Regenerative Medicine) has been developed to monitor cell−ECM interactions and to perform surrogate organ transplantation [19], but is insufficient to isolate ECM proteins because it does not preserve the whole brain completely.

In this study, we propose a method to preserve the brain in an intact form by decellularizing fixed brain tissues with 4% hydrogel followed by immersion in oil. Moreover, ECM and matrisomal proteins were successfully isolated from decellularized brains using sequential matrisome preparation reagents (SMPRs), namely, RIPA, PNGase F, and concanavalin A. Therefore, this method can be widely used not only for analyses of the ECM and matrisome and investigation of their functions, but also for various applications, including tissue regeneration, which require tissues with intact shapes.

2. Materials and methods

2.1. Animal maintenance

All animal experiments were performed in accordance with the approved animal protocols and guidelines established by the Korea Brain Research Institute (IACUC-19-00012). C57BL/6J WT mice were purchased from The Jackson Laboratory and used at embryonic day 18.5 (E18.5), postnatal day 1 (P1), postnatal day 5 (P5), and 12 weeks of age. Mice were housed in a specific pathogen-free facility with 12 h of light and 12 h of dark per day at an ambient temperature of 22 °C and a relative humidity of 40 ± 5%. Food and water were provided ad libitum.

2.2. Decellularization and polymerization method

For general decellularization of brain tissue, mice are perfused with phosphate-buffered saline (PBS), brain tissue was collected followed by removing the meninges. The brain tissue was incubated at 1% SDS at 37 °C for 2 weeks with moderate shaking and changed to 1% SDS solutions every day. CASPER was performed as described in a previous study with slight modifications [19]. Briefly, mice were perfused successively in PBS, and a hydrogel monomer solution containing 20% acrylamide, 0.1% bis-acrylamide, 0.25% 2,2′-azobis[2-(2-imidazolin-2-yl) propane] dihydrochloride (VA-044), which is a polymerization initiator, and 0.01% sodium azide prepared in PBS. For CASPERization, hydrogel-infused brains were polymerized for 3 h at 37 °C with or without corn oil (Merck) using the X-Clarity Polymerization system (Logos Biosystems, Korea) followed by shaking at 37 °C in 4% SDS solution or used in electrophoresis at 37 °C for an appropriate duration depending on their size. In case of adult mouse whole brain, it is necessary to be incubated for 2 weeks with shaking or 16 h for electrophoresis. For electrophoresis, the polymerized brains were placed in an electrophoretic tissue decellularization chamber (X-CLARITY™ Tissue Clearing System II, Logos Biosystems) and incubated under a current of 1.5 A at 37 °C for an appropriate time. To remove SDS, all specimens were washed in PBS every hour overnight at room temperature on shaker.

2.3. Isolation and enrichment of ECM proteins

To isolate the ECM, brains decellularized by CASPER or O-CASPER were homogenized with 1% RIPA or 3 M urea buffer containing a protease and phosphatase inhibitor cocktail (Thermo Fisher) and then incubated at 4 °C for 24 h or boiled for 2 h, respectively. After centrifugation at 12,000×*g* for 20 min at 4 °C, supernatants were collected and desalted with spin desalting columns (Pierce). Only desalted samples were used for subsequent experiments. RIPA lysates were supplemented with PNGase F and incubated at 37 °C for 4 h. Lysates obtained using a combination of RIPA and PNGase F were analyzed by LC-MS/MS. To enrich ECM proteins, concanavalin A was pulled down using a Glycoprotein Isolation Kit (Pierce) according to the manufacturer's instructions. Briefly, samples containing 1–1.5 mg of total protein were diluted 4:1 with 5 × Binding/Wash Buffer stock solution and added to a column of a collection tube. After repeated centrifugation and washing, eluted samples were collected and stored for subsequent analyses.

2.4. Coomassie and silver staining

Electrophoresed gels were incubated with gel fixation solution comprising 50% (v/v) ethanol and 10% (v/v) acetic acid at room temperature for 1 h. After washing with HPLC grade water, Coomassie and silver staining were performed with Imperial™ Protein Stain (Pierce) and a Pierce™ Silver Stain Kit (Pierce), respectively, according to the manufacturer's instructions. All stained gels were stored in 5% acetic acid solution at 4 °C until in-gel digestion was performed. Images of gels were acquired using an LAS 4000 imaging system (Fujifilm).

2.5. Immunohistochemical fluorescence staining analysis

CASPERized tissues were embedded in OCT compound, and sectioned (50 μm) using a cryotome Leica CM1860 (Leica Biosystems, Wetzlar, Germany). Naïve brain tissues were processed by general histology protocol and equally sectioned. All sections were stored at −80 °C until use. After cell seeding, recellularized O-CASPER slices are employed for immunohistochemistry fluorescence labeling at 1 and 3 days. The scaffold seeded by cells were fixed with 4% paraformaldehyde for 30min, washed with PBS at room temperature and stored at 4 °C until use. For immunohistochemical fluorescence staining, all samples were washed with PBS 3 times and incubated with the blocking solution consisting of 10% donkey serum, 1% bovine serum albumin (BSA), 0.2% (v/v) Triton X-100 in PBS for 1 h at

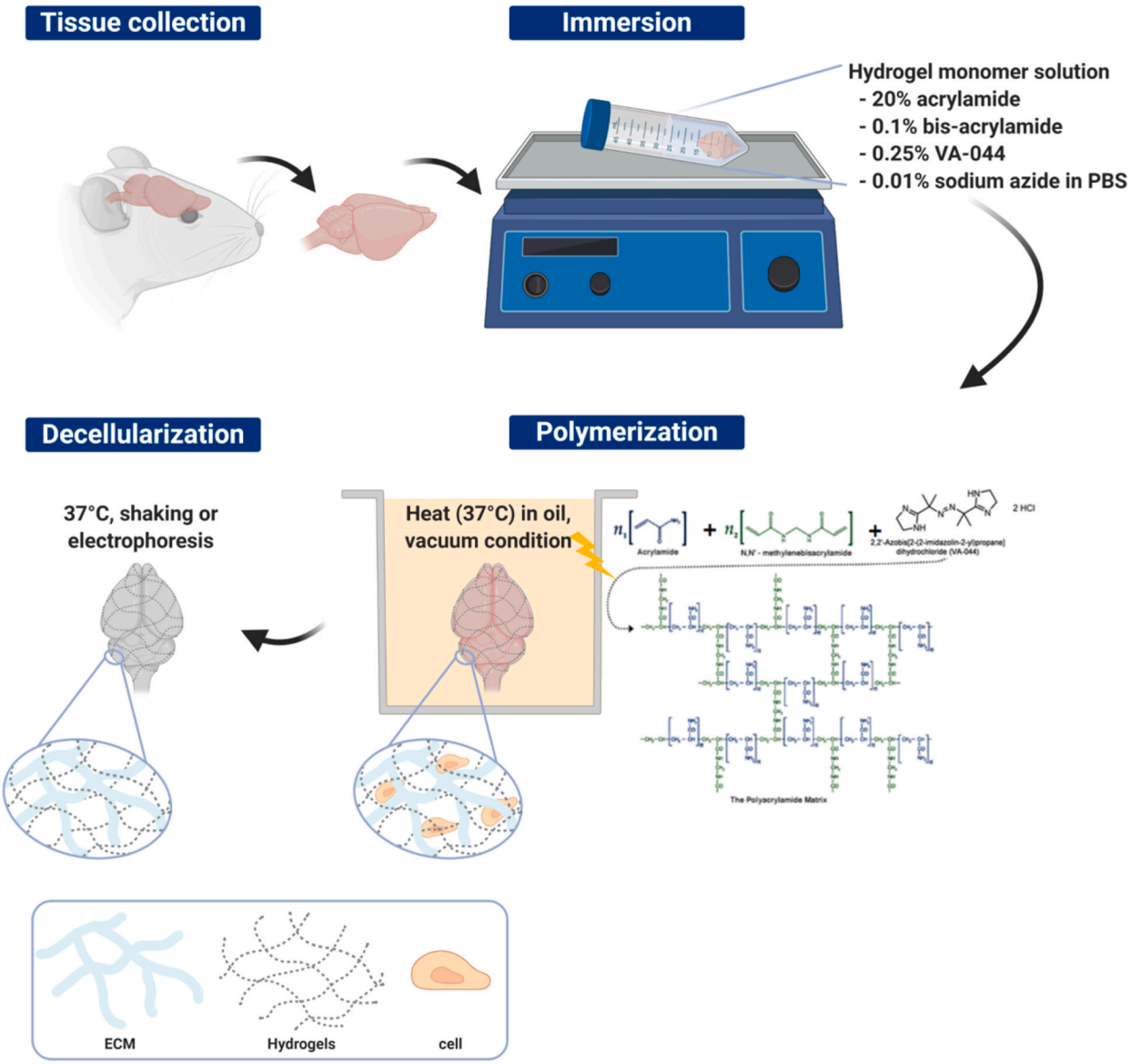


Fig. 1. Workflow for decellularization of brain tissues of 12-week-old mice using O-CASPER.

room temperature. Primary antibodies were incubated overnight at 1:200 dilution, 4 °C. Detailed antibody information as followed; collagen I (ab34710, Abcam, Cambridge, UK), collagen IV (ab6586, Abcam, Cambridge, UK), laminin (L9393, Sigma-Aldrich, MO, U.S.A.), class III beta-tubulin (Tuj1; mms-435p-100, Biolegend, CA, U.S.A). Sections were washed with PBS 3 times, and then incubated 2 h with Alexa Fluor 488 or 594-conjugated anti-IgG (1:500, Invitrogen, MA, U.S.A.) at room temperature. Nuclei counterstaining was performed using 4′,6-diamidino-2-phenylindole (DAPI, 1:1000, Thermo Fisher Scientific, MA, U.S.A.). Sections were washed, and then mounted in Vectashield mounting medium (Vector laboratories, CA, USA) for fluorescence analysis. The images were acquired using a Nikon A1-Rsi Confocal microscope system (Nikon, Tokyo, Japan) equipped with a 10X, 20X lens objective.

2.6. Cell culture and repopulation

Neuro2A murine neuroblastoma cells (CCL-131, ATCC, VA, USA) were grown in DMEM (Gibco, MA, U.S.A.) media with 10% fetal bovine serum (Gibco, MA, U.S.A.) and 1% penicillin and streptomycin (Gibco, MA, U.S.A.). To sterilize, 2 mm coronal sliced O-CASPER were incubated in 70% ethanol overnight. After being rinsed with PBS, the O-CASPERized brain slices were incubated for 1 h in a complete medium. Fifty thousand cells were seeded per O-CASPERized brain slice at 12-well plate. O-CASPERized brain slices were maintained for 1 and 3 days in a humidified 37 °C, 5% CO_2 environment to facilitate cell adhesion following seeding and inclusion of complete media. After 1 or 3days, the scaffold with cells were fixed and processed as described above.

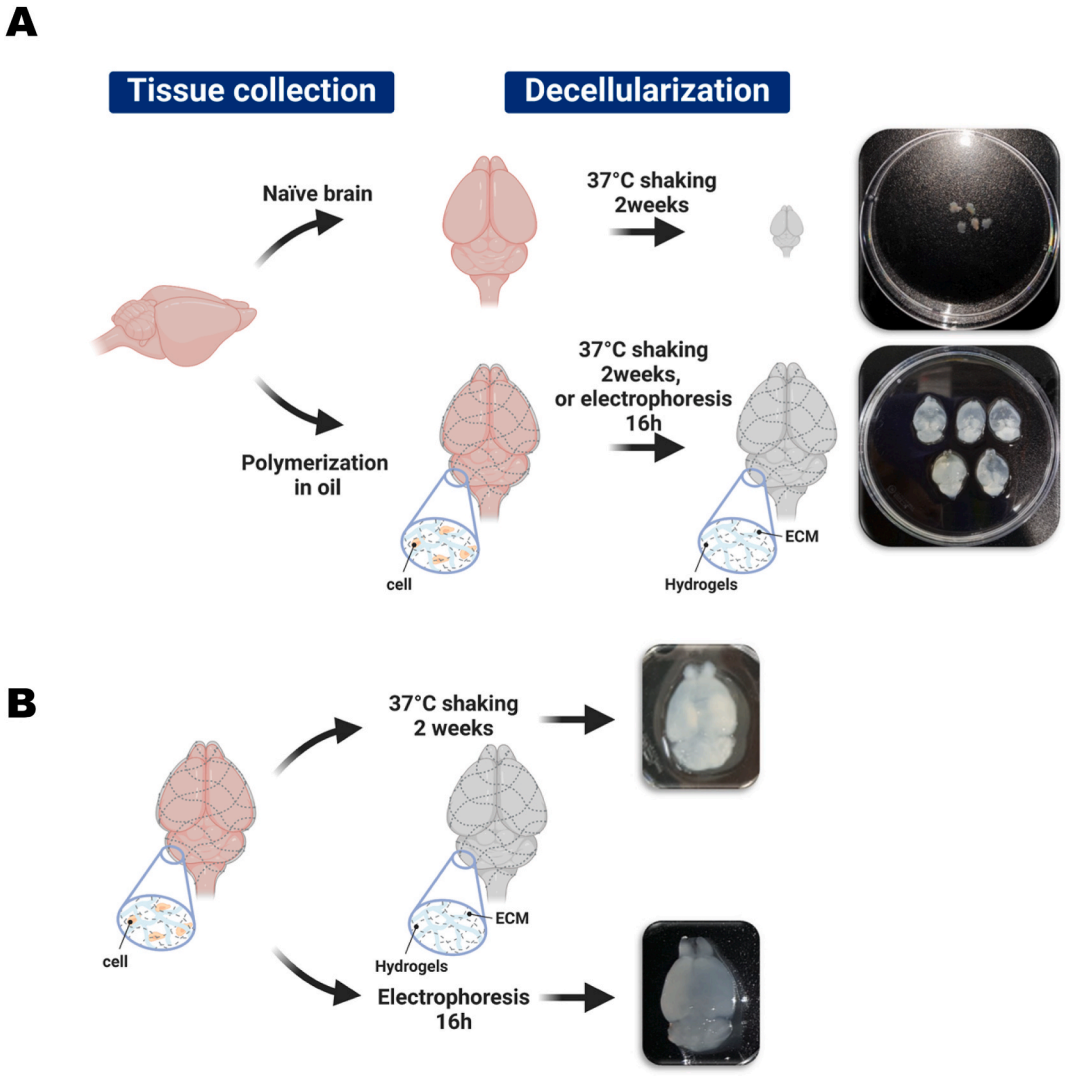


Fig. 2. Preservation of brain morphology after decellularization and polymerization in oil. A. Representative images showing that the sizes and shapes of mouse brains processed by CASPER or O-CASPER. B. Two types of tissue-clearing methods.

2.7. LC-MS/MS analysis

The gel was destained and bands were excised and processed. Briefly, acetylated protein bands were divided into 10 mm sections and subjected to in-gel digestion with trypsin. The tryptic digests were separated by online reversed-phase chromatography using the Thermo Scientific Easy Nano LC 1200 ultra-high performance liquid chromatography system equipped with an autosampler using an Acclaim PepMap™ 100 reversed-phase peptide trap (inner diameter, 75 μm; length, 2 cm) and a PepMap™ RSLC C18 reversed-phase analytical column (inner diameter, 75 μm; length, 15 cm; particle size, 3 μm), both of which were purchased from Thermo Scientific, followed by electrospray ionization at a flow rate of 300 nl/min. The chromatography system was coupled in line with an Orbitrap Fusion Lumos mass spectrometer. Spectra were searched against the UniProt mouse database using Proteome Discoverer Sorcerer 2.2 with the SEQUEST-based search algorithm. Comparative analysis of proteins identified in this study was performed using Scaffold 4 Q + S. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [20] partner repository with the dataset identifier PXD028272 and 10.6019/PXD028272.

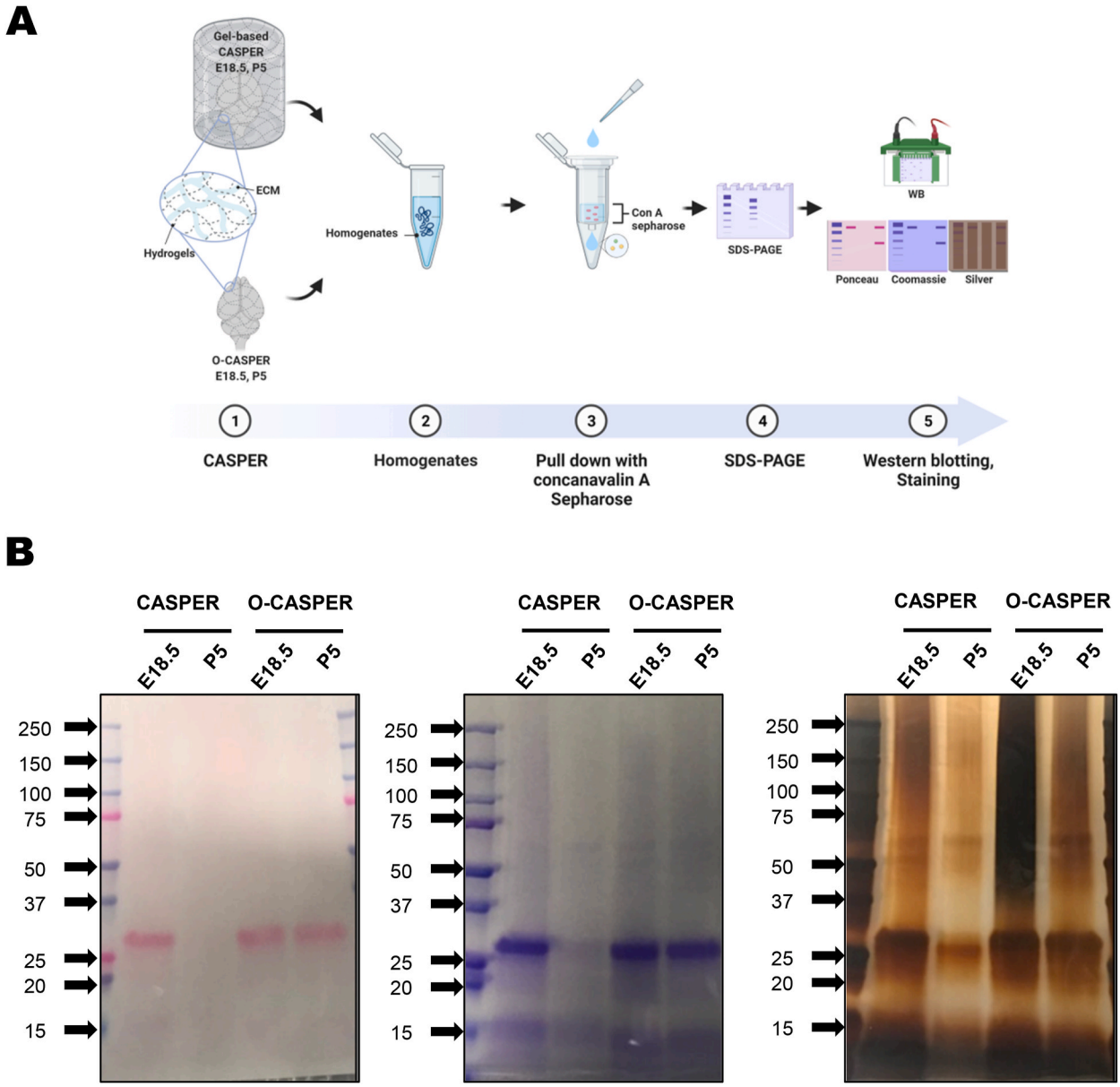
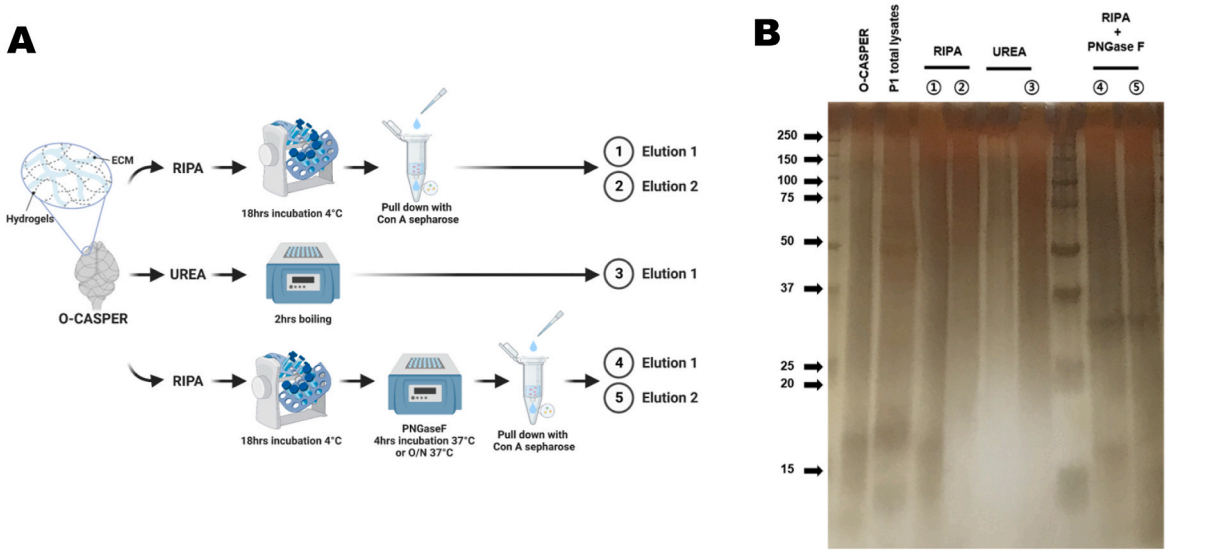
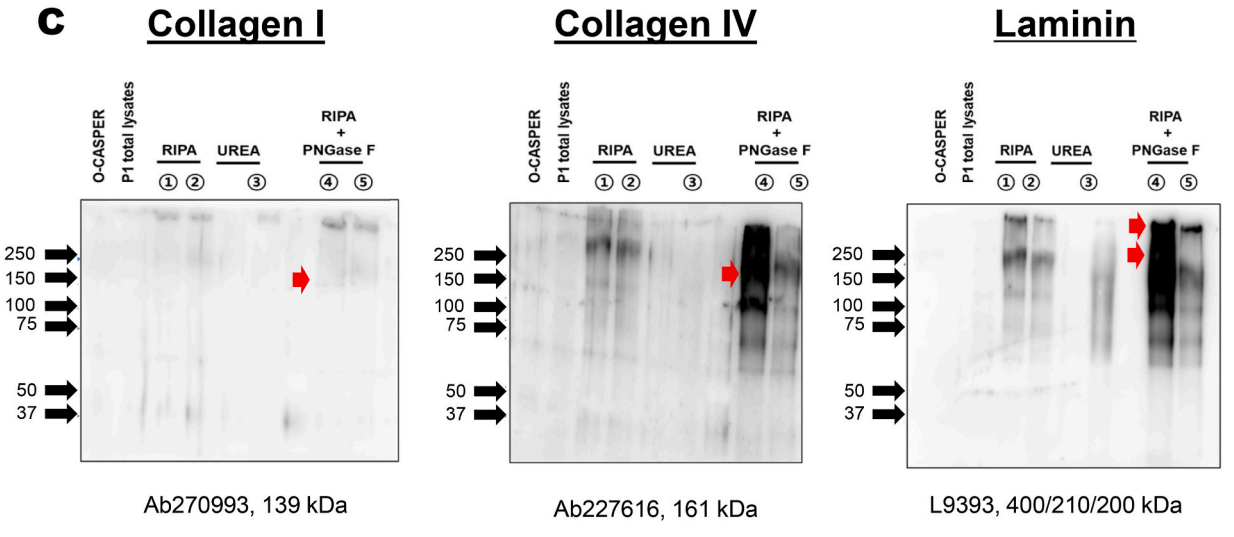


Fig. 3. Acquisition of ECM components from mouse brains processed by CASPER and O-CASPER. A. Experimental scheme showing purification of ECM proteins from brains of E18.5 and P5 mice processed by CASPER and O-CASPER. B. Comparison of N-glycoproteins pulled down using concanavalin A sepharose from E18.5 and P5 mouse brains processed by CASPER and O-CASPER.





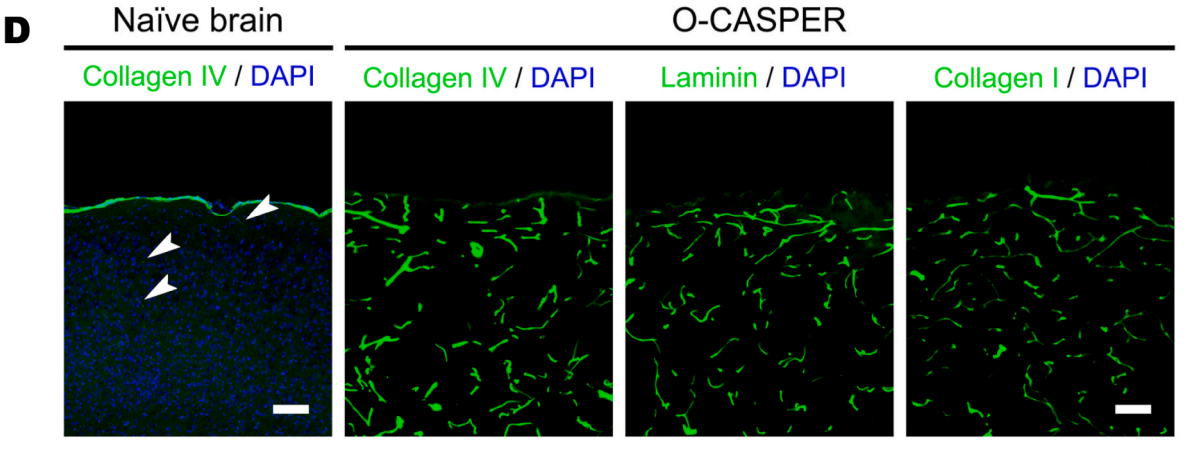


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Fig. 4. Efficient extraction of ECM components from mouse brains using SMPRs A. Schematic illustration of the procedure used to extract ECM proteins from brains of 12-week-old mice. B. Silver-stained gel showing extraction of ECM components using the SMPRs urea, RIPA, and PNGase F. C. Western blot analysis of collagen I, collagen IV, and laminin. Red arrows indicate the original molecular weight of each protein. D. Immunofluorescence staining of collagen I, collagen IV, and laminin from brains of 12-week-old mice processed by O-CASPER. White arrows indicate the collagen I of naïve brains. Scale bar represents 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.8. Western blot analysis

Protein concentrations were determined by the bicinchoninic acid assay (Pierce). Protein samples were loaded onto 8–12% SDS-PAGE gels, electrophoresed, and transferred to polyvinylidene fluoride membranes (Millipore). The membranes were blocked with 5% skim milk for 1 h and then analyzed by western blotting using antibodies against collagen I (ab270993) and IV (ab227616), which were purchased from Abcam, and laminin (L9393), which was purchased from Sigma. Images of western blots were acquired using the LAS 4000 imaging system (Fujifilm). Reducing condition greatly diminished the reactivity and selectivity of the antibodies; therefore, non-reducing electrophoresis was used for western blotting.

3. Results

3.1. Preservation of brain shape by O-CASPER

To obtain an intact brain prior to isolation of ECM components, we established a method for brain decellularization. Fig. 1 shows a schematic of the workflow and depicts the key steps of decellularization. Using the general clearing method (incubation in PBS containing 1% SDS for 5–7 days), we first compared adult brains that were polymerized with oil (O-CASPER) and those that were not (CASPER). Brains not polymerized in oil were easily damaged or decreased in size during decellularization. By contrast, decellularized brains polymerized with oil had an intact shape without any damage (Fig. 2A and Supplementary Figure S1). Moreover, brains were more transparent when subjected to O-CASPER in combination with electrophoresis, which is a crucial step of polymerization for brain clearing (Fig. 2B). These results suggest that O-CASPER, which involves oil-based decellularization and polymerization, completely preserves brain shape.

3.2. Acquisition of ECM components from brains decellularized by O-CASPER and CASPER

The concentrations and structures of ECM components influence tissue transparency [21]. Regardless of which procedure is used to preserve the brain, ECM components must be efficiently extracted from the processed brain. To compare the ECM components in the brains of E18.5 and P5 mice decellularized by O-CASPER or CASPER, we pulled down concanavalin A, which is an α-mannose/α-glucose-binding lectin that recognizes N-glycans, from brain lysates to enrich ECM components (Fig. 3A). Coomassie and silver staining confirmed that ECM components were efficiently acquired from brains processed by O-CASPER compared with those processed by CASPER (Fig. 3B). These results suggest that ECM components are more efficiently isolated from brains using O-CASPER than using CASPER.

3.3. Efficient extraction of ECM components from brains decellularized by O-CASPER using a combination of RIPA and PNGase F

To obtain ECM components, we tested lysis buffers containing 1% RIPA, 3 M urea, or 1% RIPA and PNGase F (Fig. 4A). ECM components in the gel were visualized by silver staining. This revealed that ECM components were not fully extracted from brains decellularized by O-CASPER using RIPA buffer alone or urea alone. Moreover, denser bands and a greater number of bands were obtained with samples of P1 brains using a combination of PNGase F and RIPA buffer than using other conditions (Fig. 4B). The ECM components collagen I, collagen IV, and laminin were analyzed by western blotting. Collagen IV and laminin were detected in samples lysed using a combination of RIPA and PNGase F, and collagen I was weakly detected (Fig. 4C). PNGase F helps to isolate ECM components by cleaving N-linked oligosaccharides from glycoproteins, resulting in a high rate of protein acquisition. These results show that the structure of the ECM and its components are well preserved when brains are decellularized by O-CASPER and that incubation with a combination of RIPA and PNGase F is optimal to isolate ECM and matrisomal components from brains processed by O-CASPER. RIPA, PNGase F, and concanavalin A were used as SMPRs in subsequent analyses. Immunofluorescence staining revealed that brains decellularized by O-CASPER have a well-preserved intact distribution of collagen I, collagen IV, and laminin (Fig. 4D).

3.4. ECM and matrisomal proteins extracted from brains using SMPRs

To evaluate the ability of SMPRs to extract ECM components, we analyzed the brains of E18.5, P1, and 12-week-old mice decellularized using O-CASPER by LC-MS/MS. Proteins identified by a Mascot search and validated by Scaffold 4 Q + S were annotated using a matrisome database (http://matrisomeproject.mit.edu). A total of 576 proteins were identified in the decellularized brains of E18.5, P1, and 12-week-old mice. Proteins from decellularized 12-week-old mice using O-CASPER are the most abundantly detected. However, proteins from E18.5 and P1 are also present in almost similar numbers. Among them, annotated matrisomal proteins account

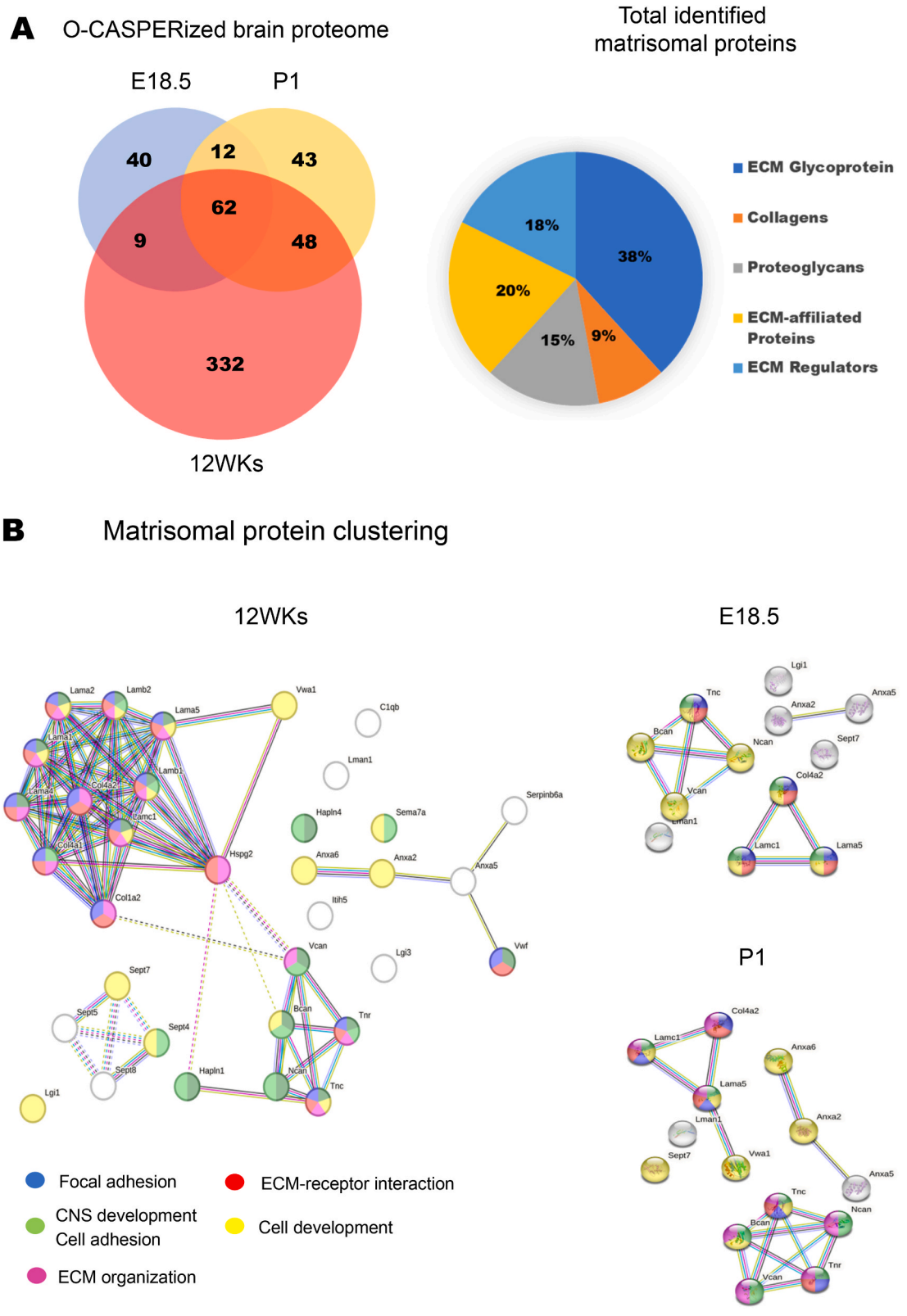

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Fig. 5. Proteomic analysis of O-CASPER mouse brains. A. Venn diagram corresponds to the proteins identified from O-CASPER brains in E18.5 (Embryos, blue), P1 (Neonates, yellow), and 12WKs (Adult, Red) mice in at least 2 biological replicates. The pie chart show identified proteins grouped by matrisome categories. B. Network of known interactions between matrisomal proteins. The indicated colors represent previously established ECM proteins identified by Matrisome DB and projected ECM-interactors. The networks were constructed with high confidence using the STRING database. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

for ECM glycoprotein (38%), collagens (9%), proteoglycans (15%), ECM-affiliated proteins (20%), and ECM regulators (18%) (Fig. 5A). To analyze the functional network of matrisomal proteins, network analysis was performed by STRING online database. The matrisomal proteins network showed that the interactions and numbers between matrisomal proteins involved in focal adhesion (blue), CNS development and cell adhesion (green), ECM organization (purple), ECM-receptor interaction (red), and cell development (yellow) are increased with age (Fig. 5B). Table 1 shows matrisomal proteins commonly identified in brains decellularized by O-CASPER. These were classified as core matrisomal proteins, including ECM glycoproteins, collagens, and proteoglycans, and matrisome-associated proteins, including ECM-affiliated proteins, ECM regulators, and secreted factors [22]. Core matrisomal proteins mainly belonged to the laminin family (Lama1, Lama2, Lama4, Lama5, Lamb1, Lamb2, and Lamc1), collagen family (Col1a2, Col4a1, and Col4a2), and proteoglycan family (Hapln1, Hapln4, Hspg2, Ncan, and Vcan). Matrisome-associated proteins included ECM-affiliated proteins such as semaphorin 7A (Sema7A), ERGIC-53 (Lman1), complement C1q subcomponent subunit B (C1qb), brevican (Bcan), and annexin family proteins (Anxa2, Anxa5, and Anxa6) as well as ECM regulators including a serine (or cysteine) peptidase inhibitor protein (Serpinb6a) belonging to the clade A (Serpin) family, septin family proteins (Sept4, Sept5, Sept7, Sept8), and inter-alpha-trypsin inhibitor heavy chain H5 (Itih5). Moreover, comparative analysis of protein abundance showed that most of the identified matrisomal proteins were found in O-CASPERized brains of adult mice (12 weeks old; C1 and C2) but not in embryonic (E18.5, E) or neonatal (P1) brains (Supplementary Figure S2). These findings demonstrate that isolation of ECM components using

Table 1
List of identified matrisomal proteins in O-CASPERized adult mouse brains.

Gene	Name	Division	Category
Lama1	Laminin, alpha 1	Core matrisome	ECM Glycoproteins
Lama2	Laminin, alpha 2	Core matrisome	ECM Glycoproteins
Lama4	Laminin, alpha 4	Core matrisome	ECM Glycoproteins
Lama5	Laminin, alpha 5	Core matrisome	ECM Glycoproteins
Lamb1	Laminin B1 subunit 1	Core matrisome	ECM Glycoproteins
Lamb2	Laminin, beta 2	Core matrisome	ECM Glycoproteins
Lamc1	Laminin, gamma 1	Core matrisome	ECM Glycoproteins
Lgi1	Leucine-rich repeat LGI family, member 1	Core matrisome	ECM Glycoproteins
Lgi3	Leucine-rich repeat LGI family, member 3	Core matrisome	ECM Glycoproteins
Tnc	Tenascin C	Core matrisome	ECM Glycoproteins
Tnr	Tenascin R	Core matrisome	ECM Glycoproteins
Vwa1	von Willebrand factor A domain containing 1	Core matrisome	ECM Glycoproteins
Vwf	von Willebrand factor homolog	Core matrisome	ECM Glycoproteins
Col1a2	Collagen, type I, alpha 2	Core matrisome	Collagens
Col4a1	Collagen, type IV, alpha 1	Core matrisome	Collagens
Col4a2	Collagen, type IV, alpha 2	Core matrisome	Collagens
Bcan	Brevican	Core matrisome	Proteoglycans
Hapln1	Hyaluronan and proteoglycan link protein 1	Core matrisome	Proteoglycans
Hapln4	Hyaluronan and proteoglycan link protein 4	Core matrisome	Proteoglycans
Hspg2	Perlecan (heparan sulfate proteoglycan 2)	Core matrisome	Proteoglycans
Ncan	Neurocan	Core matrisome	Proteoglycans
Vcan	Versican	Core matrisome	Proteoglycans
Anxa2	Annexin A2	Matrisome-associated	ECM-affiliated Proteins
Anxa5	Annexin A5	Matrisome-associated	ECM-affiliated Proteins
Anxa6	Annexin A6	Matrisome-associated	ECM-affiliated Proteins
C1qb	Complement component 1, q subcomponent, beta polypeptide	Matrisome-associated	ECM-affiliated Proteins
Lman1	Lectin, mannose-binding, 1	Matrisome-associated	ECM-affiliated Proteins
Sema7a	Sema domain, immunoglobulin domain (Ig), and GPI membrane anchor, (semaphorin) 7A	Matrisome-associated	ECM-affiliated Proteins
Itih5	Inter-alpha (globulin) inhibitor H5	Matrisome-associated	ECM Regulators
Serpina5	Serine (or cysteine) peptidase inhibitor, clade A, member 5	Matrisome-associated	ECM Regulators
Serpina7	Serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7	Matrisome-associated	ECM Regulators
Serpinb6a	Serine (or cysteine) peptidase inhibitor, clade B, member 6a	Matrisome-associated	ECM Regulators

SMPRs is suitable for adult mouse brains, but not embryonic or neonatal mouse brains.

3.5. Biocompatibility of O-CASPERized brain in vitro

We tested if O-CASPER brains could be recellularized when cells were added *in vitro* to see if they were biocompatible for a 3D structure that can be used organoid in tissue regeneration or an *in vitro* model for research on how neuronal cells interact with ECM. First, we prepared O-CASPER brain slices of 12-week-old mice (Fig. 6A). After the slices were washed with culture media, neuro2a cells were placed on them, and the whole thing was incubated for 1 and 3 days. During culture, cells are well alive inside O-CASPER brain slices (Fig. 6B). These results show that the O-CASPER brain is a flexible scaffold for many different types of cells.

4. Discussion

Tissue-clearing techniques for the whole brain have remarkably developed over the past decade [23]. Among them, CLARITY, a hydrogel-based method involving acrylamide embedding and electrophoresis for super-resolution imaging, has been widely used [24]. Many studies using modified CLARITY have successfully imaged various organs at high resolution [25–27]. Proteomics analysis of the ECM is very challenging [28,29]. The levels of ECM proteins are often low, depending on the extraction method performed before LC-MS analysis, and many scientists have made great efforts to solve this problem. The brain ECM is a meshwork of highly cross-linked components, many of which are high molecular weight macromolecules that are highly glycosylated [30]. Regular isolation methods used in most proteomics-based studies are more suitable to isolate intracellular proteins than to isolate ECM proteins. In this regard, methods designed to specifically solubilize brain matrix components might help to identify ECM proteins.

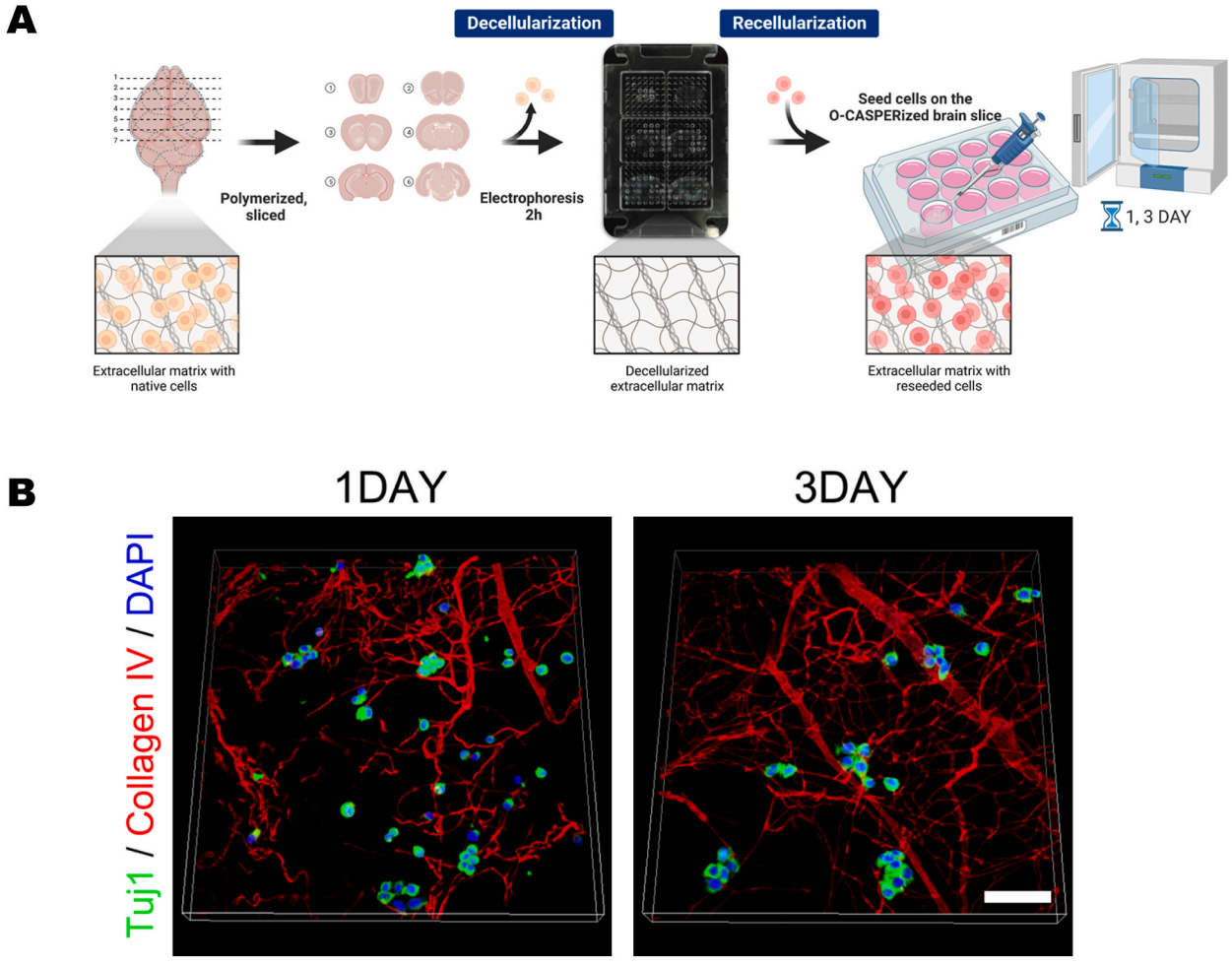

Fig. 6. Recellularization of O-CASPER slice brain *in vitro*. A. Schematic illustration of the procedure used to brain slice culture and recellularization from brains of 12-week-old mice. B. Immunofluorescence staining of collagen IV (Red, an ECM structure) and Tuj-1 (Green, a neuron marker) using Neuro2a cells grown in O-CASPER brain slices for 1 and 3 days. Nucleus is visualized by DAPI (Blue) staining. Scale bar represents 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Although many methods used in recent studies are useful, they are insufficient to obtain ECM components because soft tissues such as the brain are easily damaged, and their contents are lost under harsh decellularization conditions [31]. Decellularization is the removal of all cellular and nuclear components from a tissue or organ while preserving the ultrastructure and composition of the native ECM to delay the onset of an immune response. When developing an efficient decellularization method, it is crucial to consider various factors, including cell density, lipid content, and tissue thickness [32]. Numerous organs, including the brain and embryonic soft tissues, rely on polymerized hydrogels to preserve the intricate architecture and biological functions of ECM components. Given that the recellularization of CASPERized tissues resulted in a tissue-regeneration-like process following implantation without pathogenic inflammation or fibrosis *in vivo*, these tissues may be utilized for monitoring cell-ECM interactions and transplantation of surrogate organs [19]. Using O-CASPER, we developed a decellularization protocol to preserve the ECM components of mouse brains while removing lipids and cellular compartments such as nuclear components. By performing the infusion and hydrogel polymerization processes in oil before the SDS chemical treatment or electrophoresis, we could generate a more stable and faster highly porous O-CASPERised mouse brain matrix while preserving the ECM as well as the shape of brain.

Although it has been suggested that the brain ECM plays a significant role in neurodegenerative diseases [33] and neuronal development [34], this role has not been fully characterized, in part due to the lack of models that incorporate aspects of the structure or composition of the brain's native ECM. Many scientists are studying development and simulating diseases *in vitro* using organoid structures, which are three-dimensional reproductions of organs. The existing techniques for reproducing morphogenetic processes *in vitro* depend on ill-defined and challenging control matrices, ignoring the significance of biochemical and biophysical ECM components in promoting multicellular growth and reorganization. In a 3D microenvironment, complex cell rearrangement and differentiation start to take shape [35]. By dynamically interacting biochemical and biophysical signals, this 3D environment influences cell rearrangement during morphogenesis [36]. Therefore, we propose that the O-CASPERized brain in this study may be employed as a scaffold to imitate most of the 3D structure *in vivo* and to define the role and function of ECM systematically. In addition to being a useful tool for research on the interactions between neuronal cells and ECM, these *in vitro* systems would not be possible without understanding how ECM affects the growth and maturation of neuronal co-cultures. Our method can be generally used to investigate the complex 3D cell-matrix interactions that regulate multicellular growth and differentiation.

The ECM in the tissue microenvironment consists of many heterogeneous proteins that play essential roles in tissue development, homeostasis, and wound healing [37]. ECM components provide structural support as well as biochemical and biophysical signals transferred by cell surface receptors, such as integrins, to induce intracellular signaling events that regulate diverse cellular processes, including proliferation, death, and differentiation [38,39]. Impairment of ECM remodeling, which involves a sequence of quantitative and qualitative alterations such as compositional changes and breakdown of components, is closely associated with several human diseases [40]. For instance, Alzheimer's disease is correlated with abnormal levels of glycans, including heparan sulfate proteoglycans [41]. ECM structural components, including collagens, proteoglycans, and glycosaminoglycan-binding molecules, are upregulated in the brains of Parkinson's disease sufferers [42]. To characterize the ECM composition of normal and disease tissues, recent studies showed how to decellularize, deglycosylate, and digest ECM-enriched protein preparations to generate peptides for subsequent analysis by mass spectrometry [43–45]. However, these studies did not fully consider the optimal conditions according to age or tissue type for analysis of ECM components. Our findings indicate that we successfully enriched and characterized ECM components from mouse brains decellularized by O-CASPER. Many laminins, collagen, annexin a, and septin family proteins were identified by LC-MS/MS. Collagens play structural roles and contribute to the mechanical properties, organization, and shape of tissues. Collagen I is a fibril-forming collagen. Collagen IV is a network-forming collagen [46]. Laminin is widely expressed in the CNS and participates in CNS development, BBB maintenance, neurodegeneration, stroke, and neuroinflammation [47]. Collagen IV and laminin constitute the basement membrane that contributes to the integrity of the BBB [48]. Annexin A belongs to a large family of Ca^{2+}-dependent anionic phospholipid- and membrane-binding proteins. It also regulates tissue integrity through intercellular and cell–ECM interactions [49], and is an essential endogenous regulator of BBB integrity in neurodegenerative diseases [50]. Bcan is a highly abundant proteoglycan in the adult brain. The LC-MS/MS results indicate that our method is more suitable for analyzing adult brains than embryonic and neonatal brains. Although ECM components were well isolated, they were identified more in adult brains than in embryonic and postnatal brains. These findings suggest that it is crucial to preserve brain tissues during decellularization and protein extraction to investigate the matrisome. The composition of the ECM likely changes during brain development. The ECM changes during cellular processes and tissue morphogenesis upon embryonic development [51]. A further investigation is necessary to elucidate how the ECM composition changes during brain development.

A bioinformatics approach revealed that the matrisome is defined as the complex of ECM and ECM-associated proteins [52,53]. The matrisome comprises core matrisomal proteins, including ECM glycoproteins, collagens, and proteoglycans, and matrisome-associated proteins, including ECM regulators, affiliated proteins, and secreted factors [15]. Given that the matrisome contains a very complex mixture of proteins, some studies have further separated these proteins using a combination of decellularization protocols and extraction methods [29,54]. However, the insolubility of matrisomal proteins, which are also often extensively glycosylated, makes them difficult to resolve by SDS-PAGE, leading to suboptimal conditions for subsequent protein digestion, identification, and quantification. In the present study, we report that SMPRs (RIPA, PNGase F, and concanavalin A) improved the isolation of matrisomal proteins from O-CASPERized brains of adult mice, but not of E18.5 embryos or P1 neonates. Although our method helps to preserve brain shape and isolate highly purified ECM components, it is necessary to obtain the optimal condition to isolate ECM components from embryos and neonates in a further study.

In summary, we propose a method to preserve brain shape and improve the isolation efficiency of ECM components that involves polymerization in oil and uses SMPRs (RIPA, PNGase F, and concanavalin A). Our method will be useful to acquire matrisomal data in functional studies using mouse brains and other tissues.

Author contribution statement

Sung-Jin Jeong: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Byung Geun geun Ha; Yu-Jin Jang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

EunSoo Lee; Byung-Gyu Kim; Kyungjae Myung; Woong Sun: Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data associated with this study has been deposited at 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 PXD028272 and 10.6019/PXD028272.

Declaration of interest's statement

We declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e14777.

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