Matrix Biology Plus 23 (2024) 100158

Contents lists available at ScienceDirect

Matrix Biology Plus

journal homepage: www.sciencedirect.com/journal/matrix-biology-plus

Proteomic profiling of the extracellular matrix in the human adrenal cortex

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ARTICLE INFO

Keywords:
Extracellular matrix
Human adrenal gland
Cortex
Matrisome

ABSTRACT

The extracellular matrix (ECM) comprises macromolecules that shape a complex three-dimensional network. Filling the intercellular space and playing a crucial role in the structure and function of tissues, ECM regulates essential cellular processes such as adhesion, differentiation, and cell signaling. In the human adrenal gland, composed of cortex and medulla surrounded by a capsule, the ECM has not yet been directly described, although its impact on the processes of proliferation and steroidogenesis of the adrenal cortex is recognized. This study analyzes the ECM of the adult human adrenal cortex, which was separated into outer fraction (OF) and inner fraction (IF), by comparing their proteomic profiles. The study discusses the composition, spatial distribution, and relevance of differentially expressed ECM signatures of the adrenal cortex matrisome on adrenal structure and function. The findings were validated through database analysis (cross-validation), histochemical, and immunohistochemical approaches. A total of 121 ECM proteins were identified and categorized into glycoproteins, collagens, ECM regulators, proteoglycans, ECM-affiliated proteins, and secreted factors. Thirty-one ECM proteins were identified only in OF, nine only in IF, and 81 were identified in common with both fractions. Additionally, 106 ECM proteins were reported in the Human matrisome DB 2.0, and the proteins differentially expressed in OF and IF, were identified. This study provides significant insights into the composition and regulation of the ECM in the human adrenal cortex, shedding light on the adrenal microenvironment and its role in the functioning, maintenance, and renewal of the adrenal gland.

Introduction

The extracellular matrix (ECM) is a complex structure that constitutes a three-dimensional network of macromolecules [1]. This structure plays a fundamental role in filling the intercellular space of tissues, as structural support, in regulating cellular development, in cell signaling, and in responding to injuries, among other functions [2]. By creating a dynamic and highly regulated microenvironment, the ECM contributes significantly to tissue integrity and function, as well as influencing pathological processes. This intricate network is composed of various components, such as fibrous proteins, proteoglycans, and glycoproteins [3,4]. Fibrous proteins include collagen fibrils, elastin, and fibronectin. Collagen is the primary fibrous structure providing strength and support to tissues; elastin affords elasticity, while fibronectin plays a role in cell adhesion and signaling [3]. Proteoglycans constitute another complex group of macromolecules, including core proteins and chains of glycosaminoglycans (GAGs). They are essential in tissue hydration, cellular growth regulation, and signaling [4]. Additionally, glycoproteins such as laminin and tenascin are essential in cell adhesion, differentiation, and regulation of cell migration, further enriching the functional complexity of the ECM [5]. Knowledge of the composition of ECM and the ECM-cell interaction in different tissues is fundamental to unraveling the mechanisms of various cellular processes.

The human adrenal gland comprises a cortex constituted of three zones: glomerulosa (ZG), fasciculata (ZF), reticular (ZR), and a central medulla, both surrounded by a capsule (C). Understanding of the composition and function of the ECM of the adrenal cortex is still limited. Previous studies on the adrenocortical ECM of rats and cattle

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https://doi.org/10.1016/j.mbplus.2024.100158
Received 20 April 2024; Received in revised form 9 July 2024; Accepted 25 July 2024
Available online 26 July 2024

highlight its potential impact on steroidogenesis, cell proliferation, tissue structure, and remodeling [6,7]. In adult rats, ECM modulates basal and adrenocorticotropic hormone (ACTH)-induced function with fibronectin and collagens (type I, II, and IV), favoring adrenal steroid secretion while laminin inhibits steroid secretion and promotes proliferation [8,9]. In studies using primary cultures of human fetal adrenal cells, ECM proteins also modulated the profile of steroid secretion by the fetal cells and cell behaviors such as proliferation and cell death [10].

Considering the lack of in-depth studies on the composition and distribution of the ECM of the adrenal cortex and its functional relevance, this manuscript provides a comprehensive analysis of the constitution of the ECM of the adult human adrenal cortex. Through the fractionation of the adrenal gland into outer (OF=capsule + ZG cells) and inner fraction (IF=ZF+ZR cells) and the comparison of their proteomic profiles, we investigated the relevance of the differentially expressed ECM signature and ECM-related proteins in adrenal structure and function. The relevant findings were validated by database analysis (cross-validation) and histochemical and immunohistochemical approaches.

Results

Quantitative proteomic analysis of decellularized human adrenal gland

ECM of OF and IF of decellularized human adrenal glands (n = 5; 2 men and 3 women; 61.5 ± 30.9 years of age) were obtained and analyzed according to the experimental design presented in the workflow shown in Fig. 1. The five human adrenal glands were fractionated into an outer fraction (OF=capsule + ZG) and an inner fraction (IF=ZF+ZR). ECM enrichment was achieved through decellularization. Extracted proteins were prepared, purified, and analyzed using label-free quantitative Liquid Chromatography-Mass Spectrometry (LC−MS/MS)-based proteomics. Raw files were processed, and statistical analysis was performed using Perseus version 2.0.11 software. Total proteins were identified (Table S1). Considering a minimum of 60 % of valid values in at least one group, ECM proteins were classified and listed in Table S2. Protein-protein interaction networks and functional enrichment analysis were performed, and a student's *t*-test ($p < 0.05$) was applied to identify the significant regulated proteins between OF and IF fractions. ECM proteins were in silico validated using the Homo sapiens matrisome Database 2.0. Histochemistry and immunohistochemistry validated some regulated proteins in human adrenal gland sections. Principal Component Analysis (PCA) showed that the ECM proteome distinguishes between groups OF and IF (Fig. 2A). The dimensional contributions (dimensions 1 and 2) of each protein (Fig. 2B) suggest a specific regulation and signature of fraction samples. All dimensional analyses and contributions are represented in supplementary Fig. S2. A plot was generated using R software to visualize the distribution of proteins across the 10 dimensions analyzed in PCA.

A total of 1,250 proteins were identified (Table S1). Among them, 121 were categorized as ECM-proteins by Gene Ontology (Fig. 2C). Of the 121 ECM proteins, 81 are common to the OF and IF fractions, while 31 were identified only in the OF and nine only in the IF (Fig. 2D and Table S2). All raw mass spectrometry data sets are deposited in the PRIDE repository, project accession 1–20240418-203625–3275960.

The human matrisomeDB 2.0 database provides a comprehensive and organized collection of ECM components [11]. Using the Matrisome DB 2.0 database, STRING data annotations (https://string-db.org) and the Universal Protein Resource (UniProt) (https://www.uniprot.org) provided 106 cross-validated ECM proteins (Fig. 3A).

In human matrisome DB 2.0, the structural proteins of the ECM were categorized as CoreMatrisome [ECM-glycoproteins, collagens, proteoglycans] and Matrisome-Associated [ECM-affiliated proteins, ECM regulators, secreted factors] (Fig. 3B). The identified human adrenal ECM proteins were categorized into 42 glycoproteins, 22 collagens, 17 ECM-regulator proteins, 10 proteoglycans, 9 ECM-affiliated proteins, 6 secreted factors, and 15 ECM-associated proteins. Additionally, interaction networks were generated using the STRING database (https://string-db.org) based on evidence and utilizing interaction sources such as databases, text mining, experiments, co-expression, and neighborhood, with a minimum required interaction score of high confidence (0.700). The edges represent protein–protein physical and functional interactions (Fig. 3B-C).

Based on Gene Ontology (GO), 15 proteins were identified as ECM-associated (Serum amyloid P-component, Apolipoprotein A-I, Apolipoprotein A-IV, Apolipoprotein E, Calreticulin, Clusterin, Chymase, Golgi apparatus protein 1, Heterogeneous nuclear ribonucleoprotein M, Heat shock protein HSP 90-alpha, Endoplasmin, Keratin, type II cytoskeletal 1, Pyruvate kinase PKM, Prosaposin, Secreted phosphoprotein 24). These proteins were not found in the matrisome DB 2.0 human and were selected as candidates to compose the specific human adrenal matrisome (Fig. 3C). More data are needed to confirm the ECM localization of these proteins.

ECM regulation of the outer and inner fractions of the human adrenal cortex

Thirty-two ECM proteins were quantified using the label-free quantification (LFQ) method (Fig. 4A). These ECM-proteins were categorized and grouped as proteins of ECM-structural constituent (Gene Ontology GO: 0005201), implicated in cell migration (GO:0016477), cell differentiation (GO:0030154), and PI3K-Akt signaling pathway (KEGG pathways; hsa04151), according to GO analysis (Fig. 4B-E). The analysis of protein abundance differences between OF and IF shows 11 proteins are significantly upregulated in OF compared to IF: collagen I alpha 1 chain, collagen I alpha 2 chain, collagen VI alpha 1 chain, collagen VI alpha 3 chain, fibrillin-1, laminin alpha 5 chain, laminin beta 2 chain, laminin gamma 1 chain, versican, periostin, and heparan sulfate proteoglycan 2 or perlecan. In contrast, the pyruvate kinase M protein is upregulated in IF compared to OF (Fig. 5). Histochemistry for total collagen stained in blue (Masson's trichrome) and collagen I in red (Picrosirius red) showed a higher concentration of collagen fibers in the OF, with a transverse arrangement in the capsule and subcapsule (Fig. 6B and 6C, respectively). In the ZG, collagen fibers look around the rosettes, infiltrating vertically towards the ZF. Orcein staining shows the arrangement of dark brown elastic fibers in the OF, predominantly composed of elastin (Fig. 6D).

The immunohistochemistry assay confirms the higher expression of elastin and periostin in OF (Fig. 6E and F), while in Fig. 6G we observe the expression of collagen IV distributed in both fractions.

Discussion

This study aims to elucidate the composition of the ECM and its differences in the OF and IF of the human adrenal cortex through the identification and characterization of specific matrisome tissue from samples of decellularized fractions of the adrenal cortex. This is the first time a human adrenal cortex-specific matrisome was constructed by proteomic analysis.

An in-depth proteomic analysis of the human adrenal cortex was conducted based on the different cellular components and functions found in the adrenal cortex. This analysis included consideration of both external and internal microenvironments, as demonstrated through spectral decomposition graphs and dimensional contributions. This approach not only enables the identification of the protein composition in each region but also facilitates the understanding of the spatial distribution of proteins and their regulatory levels within each microenvironment.

A total of 106 ECM proteins were identified, 32 of which were identified and quantified by LFQ after applying the filtering criteria. GO enrichment analyses revealed that clusters of these proteins are associated with cell migration, cell differentiation, and the PI3K-Akt signaling

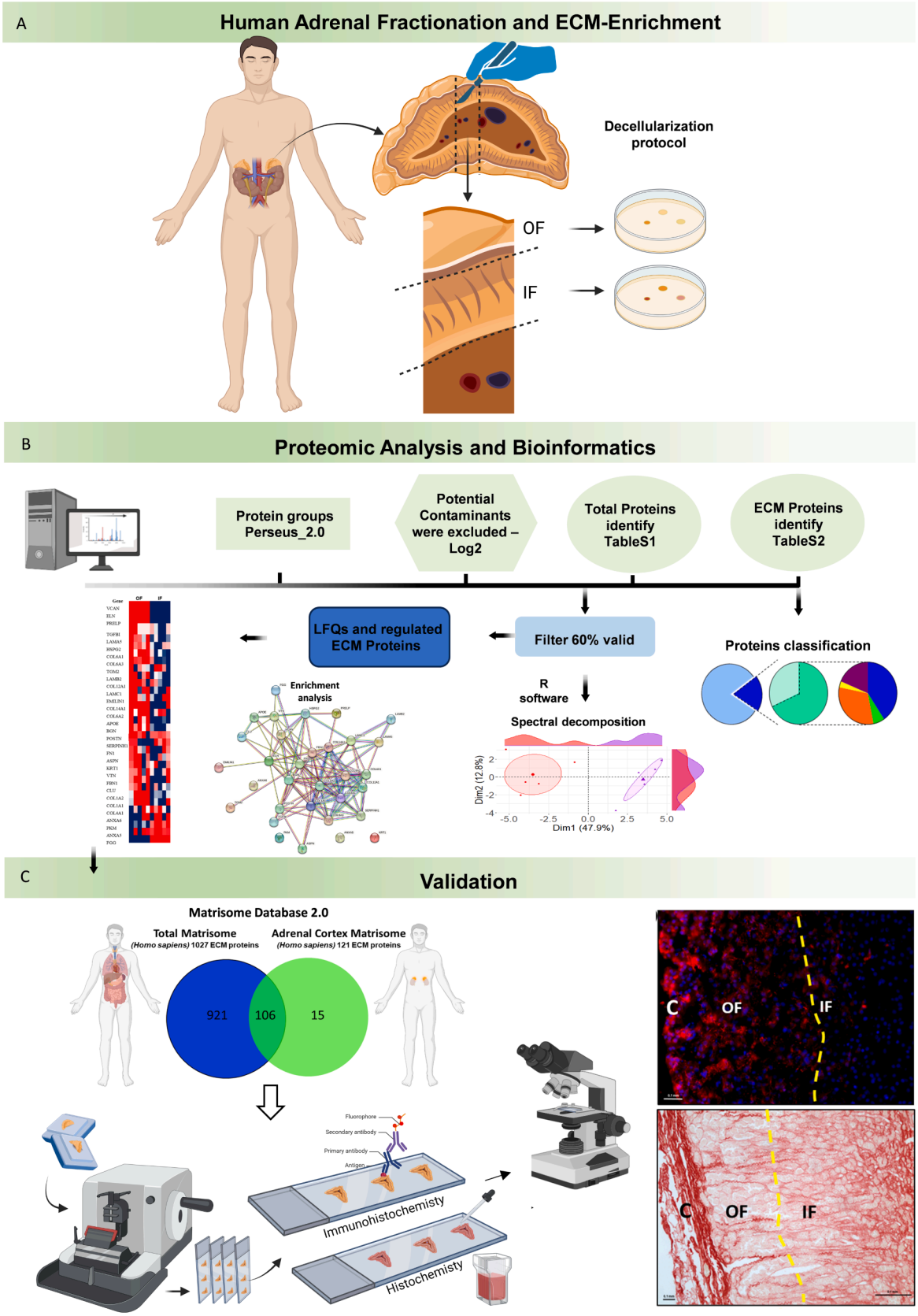

Fig. 1. Schematic overview of the methodology of the different study phases. A) Collection and fractionation of human adrenal glands. Obtaining outer fraction (OF) and inner fraction (IF) from an adrenal pool for each sample; n = 5 samples decellularization for the extracellular matrix (ECM) enrichment and sample preparation for proteomic analysis. B) ECM protein quantification through a high-resolution proteome-wide quantification Liquid Chromatography-Mass Spectrometry (LC–MS/MS) and bioinformatic analysis; C) Validation by using matrisome database, histochemistry, and immunohistochemistry of proteins differentially expressed identified in OF and IF samples.

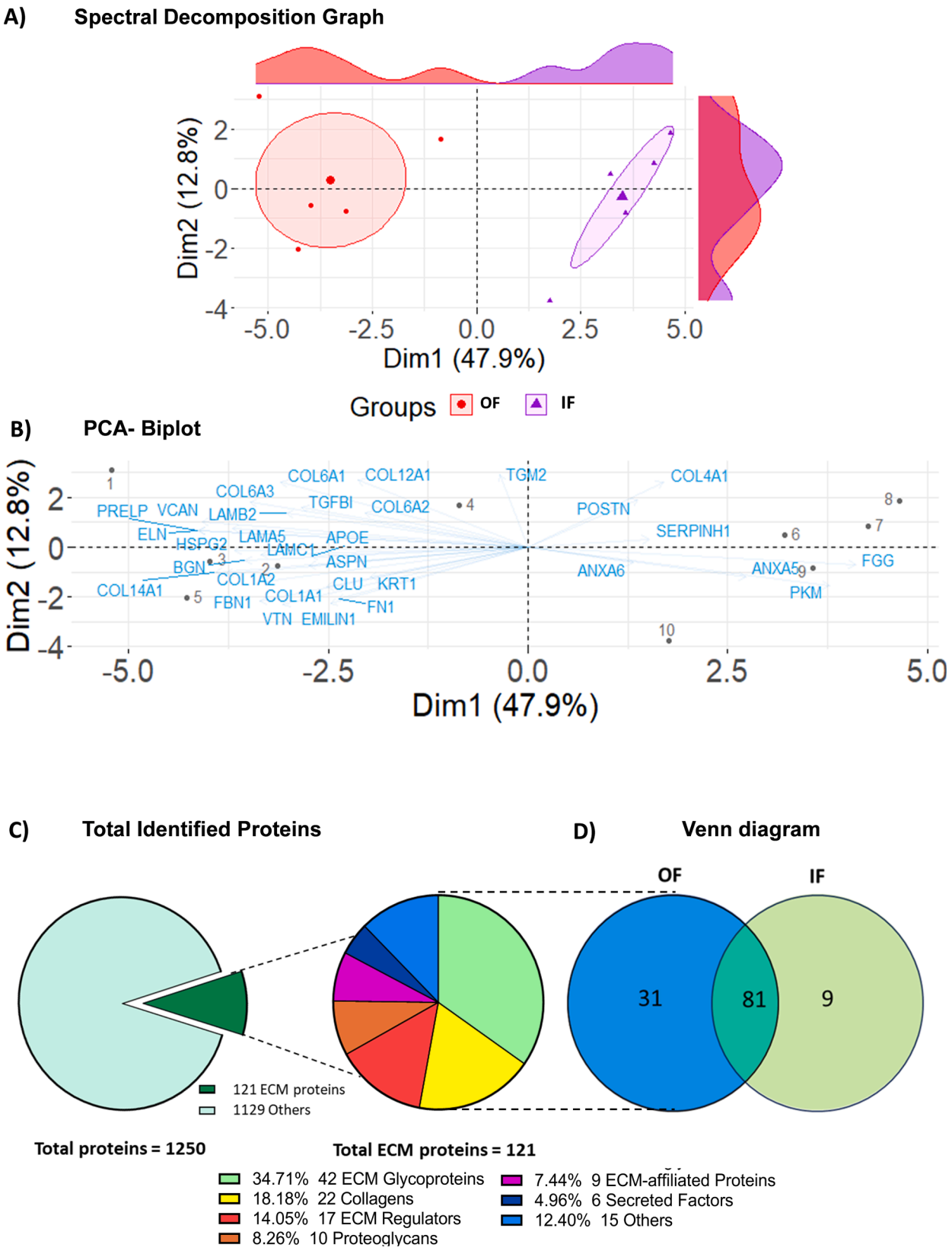

Fig. 2. Proteomic analysis of human adrenal samples and identification of adrenal cortex matrisome. A) Principal Component Analysis (PCA) with spectral decomposition highlights the power of extracellular matrix (ECM) protein stratification to distinguish between the outer fraction (OF) and inner fraction (IF) samples; B) PCA-Biplot with dimensional contributions (dimensions 1 and 2) of each protein; C) Total and ECM-proteins identified and categorized according to human matrisome 2.0 database; D) ECM-proteins common between OF and IF fractions, identified only in the OF or IF.

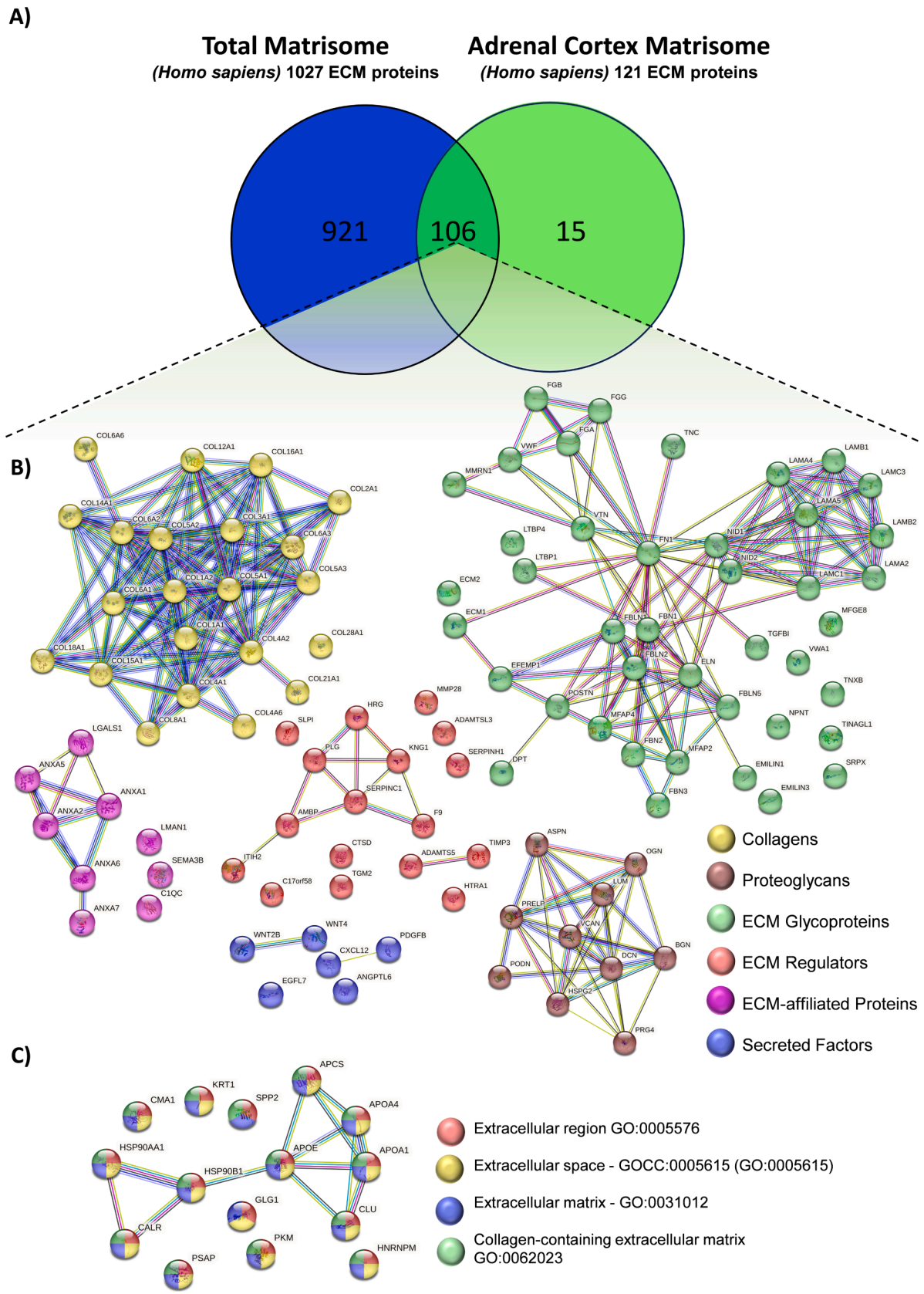

Fig. 3. Cross-validation of adrenal ECM-proteins in human matrisome 2.0 database. A) ECM-proteins cross-validated in human matrisome 2.0 database; B) Protein-protein interaction network, constructed by the String Consortium tool using Cytoscape software (https://string-db.org), according to the classification of each protein; C) Proteins not identified in the human matrisome and selected to be included in the human adrenal cortex matrisome. Edges represent protein–protein physical and functional interactions. Known interactions [from curated databases (cyan blue), experimentally determined (purple)]; Predicted interactions [from gene neighborhood (green), gene fusions (red), gene co-occurrence (blue)]; and others [co-expression (black) and protein homology (light blue)].

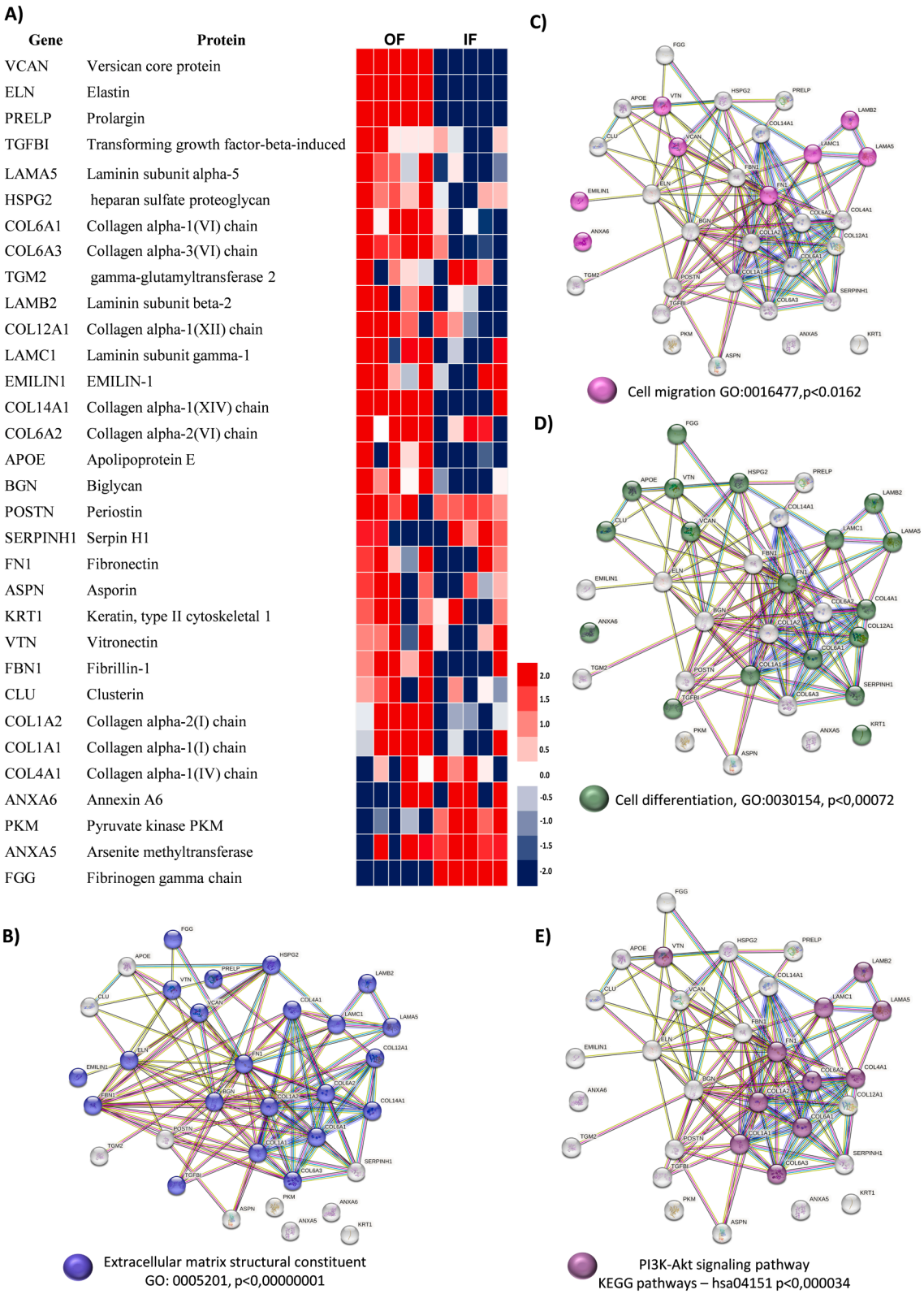

Fig. 4. ECM-proteins differentially expressed in the outer and inner fractions of the human adrenal cortex. A) Heatmap of the differentially expressed ECM proteins identified in outer (OF) and inner fractions (IF) of the adrenal cortex. Comparisons by the z-score of the label-free protein quantification (LFQ) intensity values, where red and blue represent the upregulated and downregulated proteins, respectively; Protein-protein interaction network, constructed by the String Consortium tool using Cytoscape software (https://string-db.org), according to the classification of each protein B) extracellular matrix structural constituent (GO: 0005201); C) related to cell migration (GO:0016477); D) related to cellular differentiation (GO:0030154) and E) PI3K-Akt signaling pathway (KEGG pathways-hsa04151). Edges represent protein–protein physical and functional interactions. Known interactions [from curated databases (cyan blue), experimentally determined (purple)]; Predicted interactions [from gene neighborhood (green), gene fusions (red), gene co-occurrence (blue)]; and others [co-expression (black) and protein homology (light blue)].

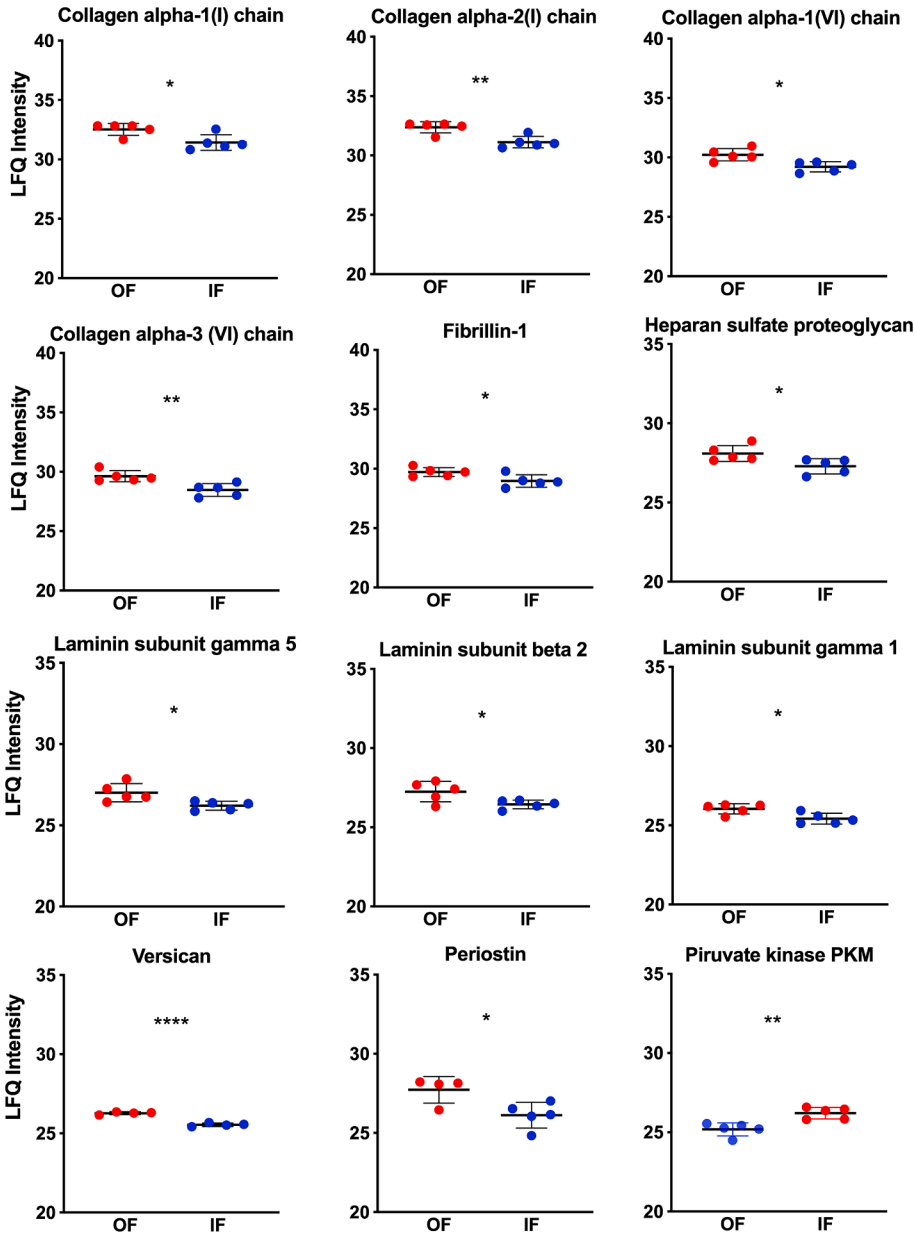

Fig. 5. Up-regulated ECM proteins in fractions of the human adrenal cortex. Proteins were identified as differentially regulated in the outer fraction OF (red) and the inner fraction IF (blue) by proteomic analysis. Student test: *$p \leq 0.05$, ** $p \leq 0.01$, ****$p \leq 0.0001$. The dots represent each sample's label-free protein quantification (LFQ) value, and the horizontal line represents the mean LFQ value ± the standard deviation (SD).

pathway.

The maintenance and renewal of the adult adrenal cortex are currently understood through two cellular processes: the centripetal migration and the zonal renewal. During these processes, both the subcapsular and the glomerulosa cells participate in normal cellular renewal [12–14]. The adrenal gland employs multiple cell populations from the capsule and subcapsule (GLI1 + and WT1 + stem cells) and ZG (SHH+and Wnt-responsive cells) with differing renewal capacities, enabling the gland to maintain high plasticity and adaptability to both physiological and pathological conditions [15]. However, little is known about the influence of the extracellular microenvironment on these processes and the specific tissue role of the ECM in maintaining cells.

Early studies in the human fetal adrenal gland have shown that ECM components and integrins follow a zonal distribution in the adrenal cortex [16]. *In vitro* studies on primary adrenal cultures grown on different substrates suggest that different ECM components affect cellular responses to ACTH. In human fetal adrenal glands, collagen IV is expressed throughout the adrenal gland, and *in vitro* studies on primary adrenal cultures show that collagen IV promotes hydroxy-delta-5-steroid dehydrogenase (*HSD3B2)* expression and cortisol production [10].

Here, proteomic analysis of adult adrenal glands identified eight collagen chains from five types of collagens: collagen I alpha 1 and 2 chains, collagen IV alpha 1 chain, collagen VI alpha 1, 2 e 3 chains, collagen XII alpha 1, and collagen XIV alpha 1. Of these, four are upregulated in OF collagen I alpha 1 chain and 2 and collagen VI alpha 1 and 3 chains, whose functions may be compatible with the cell renewal played by the adrenal cortex OF.

Collagen I alpha 1 and 2 chains are fibrillar collagens that provide mechanical resistance and tissue stability. They interact with collagens IV, V, VI, XII, and XIV, as well as fibronectin and proteoglycans, regulating cell proliferation and differentiation [17]. Collagen VI exerts mechanical roles and specific cytoprotective functions, such as inhibiting apoptosis and oxidative damage, while also regulating cell

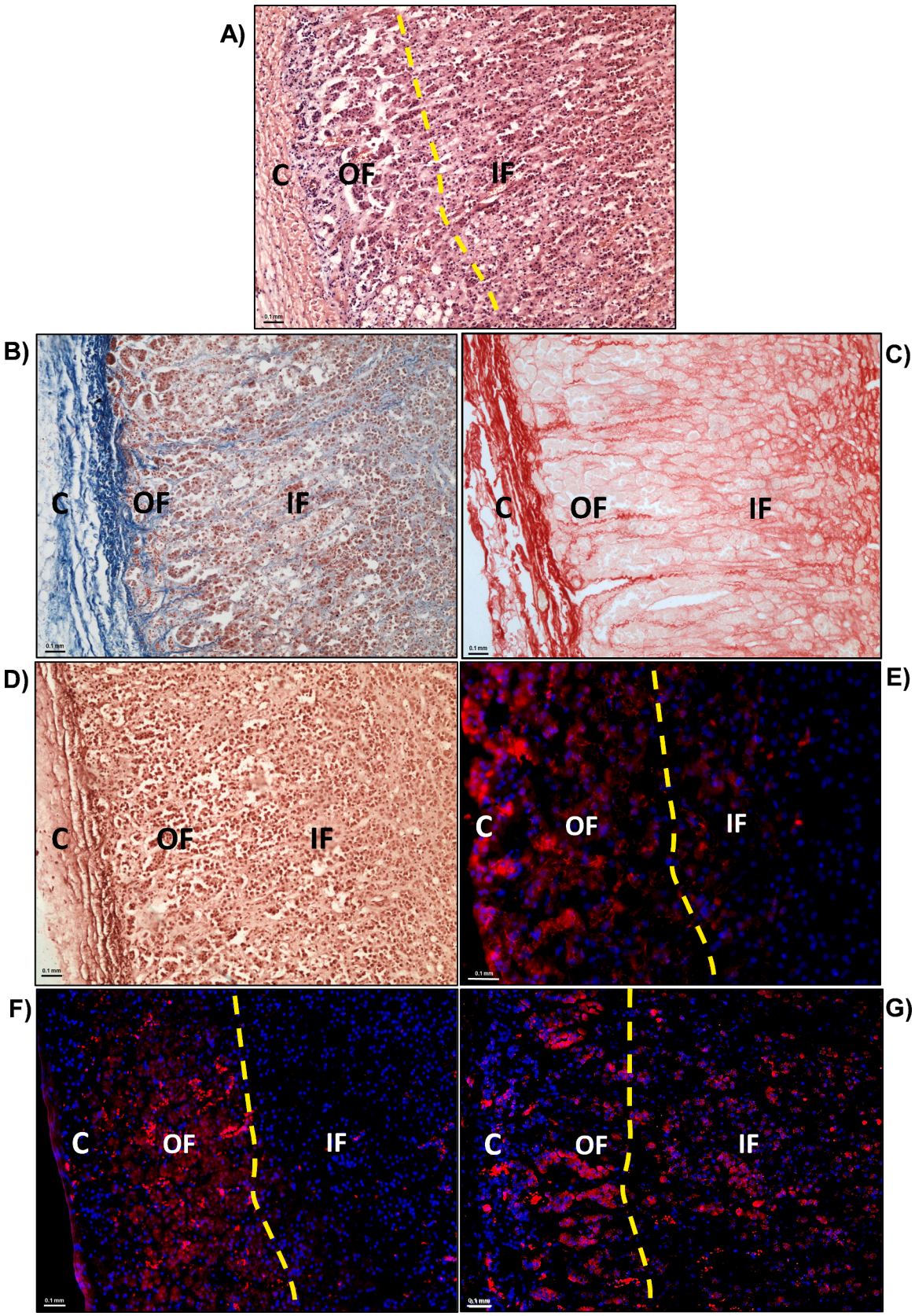

Fig. 6. Validation of ECM protein expression in human adrenal cortex. A) Hematoxylin and eosin staining; B) Collagens, Masson's trichrome staining; C) Collagen I, Picrosirius red staining; D) Elastin fibers, Orcein staining; E) Elastin in red and in blue DAPI (4′,6-diamidino-2-phenylindole), immunofluorescence; F) Collagen IV in red, Immunofluorescence; G) Periostin in red Immunofluorescence. C-capsule, OF-outer fraction, IF-inner fraction. The dashed yellow line determines the probable limit between OF and IF. Microscopy images obtained on a Nikon microscope, using the Microlucida Explorer software (MBF Bioscience),

differentiation and autophagic mechanisms. Additionally, it is involved in maintaining cellular pluripotency [18].

Laminins and fibronectin showed a zonal distribution in the human fetal adrenal cortex [16]. Laminins are glycoproteins found in the basement membrane (BM) and have a fundamental role in cell adhesion [19]. In a study using immunofluorescence in the human adult adrenal cortex, laminin-α2, laminin-α5, and laminin-γ1 were found in epithelial basement membranes (BM) in all cortical zones, laminin-α4 in vessels, laminin-β1 in the outer zone, and laminin-β2 in the two inner zones of the cortex, respectively [20]. In primary cultures from human fetal adrenals, laminin exerts an inhibitory effect on ACTH-induced steroidogenesis and a stimulating effect on cell proliferation [10]. Also, in rat primary culture of ZG and ZF cells, laminin-coated plates promote proliferation and inhibition of steroid secretion [9]. Therefore, laminin has been proposed to influence the function of the human adrenal cortex. All the laminin chains identified in the proteomic analyses of the human adrenal cortex, laminin-α5, laminin-β2, and laminin-γ1 chain, are more expressed in the OF, the proliferative niche of adrenocortical renewal.

Another ECM component that is more expressed in OF is the proteoglycan versican (VCAN). Versican is a large chondroitin sulfate/dermatan sulfate proteoglycan belonging to the aggrecan/lectican family. It is secreted and incorporated into the ECM and serves as a structural macromolecule in some tissues such as epithelia, loose connective tissue, blood vessels, and neural tissues [21]. Versican has been immunolocalized to elastic fibers and interacts with fibrillin-1 [22], which is also upregulated in human adrenal OF.

Fibrillin-1 is a glycoprotein secreted and incorporated into the ECM network. It interacts with fibrillin-2 and other ECM proteins such as fibulin-2, −4, and −5, microfibril-associated glycoprotein (MAGP)-1, and the proteoglycans aggrecan and versican [22–27]. Fibrillin-1-containing microfibrils act as a scaffold for the deposition of elastin, whose initial monomer depends on interaction with itself in addition to fibronectin and the heparan sulfate proteoglycan perlecan. Perlecan, a component of the basement membrane, is thought to be essential in stabilizing the ECM [28]. Perlecan and fibrillin-1 are more expressed in the OF, providing peculiar characteristics to this outermost fraction of the adrenal cortex.

Periostin, also identified as an ECM protein upregulated in OF, regulates cell adhesion, cell differentiation, and organization of ECM. Periostin has been demonstrated to act as a scaffold for assembling ECM proteins like collagen I, fibronectin, tenascin C, and laminin-δ2. This scaffold function is possible due to the multidomain structure of periostin, which allows interacting proteins in a large complex, contributing to the mechanical resistance of tissues [29]. An interaction between periostin and transforming growth factor beta-induced protein is also identified in the ECM-human adrenal cortex matrisome and found essential for the proper secretion of periostin/TGFBI hetero-multimer [30].

There are also interesting reports of periostin function in stem cells. Periostin has been shown to maintain hematopoietic stem cells in a quiescent state [31]. It is also reported that periostin is associated with a matricellular protein, CCN3 (NOV), suggesting an intrinsic activity of periostin together with CCN3 for maintaining hematopoietic stemness [32]. Furthermore, periostin is overexpressed and secreted by mesenchymal stem cells to support tendon formation [33]. As periostin is more expressed in the OF, adrenocortical stem cell niche, it would be interesting to hypothesize and test the involvement of periostin in the maintenance of adrenocortical stemness in OF.

Pyruvate kinase PKM was identified in the human adrenal cortex matrisome as a component located in collagen-containing ECM and in the extracellular region by Gene Ontology (GO:0005576; 0005615; 0031012; 0062023). Based on the GO annotations, PKM could play a role in extracellular signaling and/or regulation and be classified as a secreted factor in the ECM. It was the only ECM-constituent protein identified as upregulated in the IF of the adrenal cortex compared to the OF. Pyruvate kinase catalyzes the final rate-limiting step of glycolysis, transferring the phosphoryl group from phosphoenolpyruvate to ADP, generating ATP. In mammals, four isozymes of pyruvate kinase (L, R, M1, and M2) are encoded by two genes, PKLR and PKM. PKM gene generates M1 and M2 isozymes by differential mRNA splicing [34]. PKM1 is expressed in most adult tissues, and it is constitutively active. In contrast, PKM2 is highly expressed during embryonic development and could be reactivated in tissue regeneration and tumor development, suggesting it is critical for actively proliferating cells [35,36]. It is also important to note that PKM2 is highly expressed and secreted in lung cancer cells, and secreted PKM2 facilitates tumor metastasis [37]. An interesting approach would be to identify the predominant PKM isoforms in the adrenal cortex and whether this kinase has any role in adrenocortical tumors.

The study acknowledges several limitations, such as a limited sample size of only five donors, which restricts the depth and breadth of the analysis due to the significant biological variability of human tissues. Obtaining human adrenal gland samples is challenging due to ethical and practical constraints. Despite meticulous decellularization protocols, cellular remnants can interfere with proteomic analysis, leading to background noise, masking low-abundance proteins, protein loss, and modifications that affect accurate protein composition assessment. In addition, the sample preparation protocol does not reverse enzymatically induced or spontaneously occurring crosslinks. Therefore, any change in the state of the protein can be interpreted as a modification in its expression. This makes identifying and characterizing proteins challenging. The proteomic techniques may have limited sensitivity to low-abundance proteins and need to be expanded to quantify proteins with a wide range of abundances accurately. The complexity of analyzing proteomic data tissues requires integrating it with other datasets (genomic, transcriptomic, and functional analyses) to gain a comprehensive understanding of tissue composition and function, aiding in the validation of proteomic data and understanding of the ECM in human adrenal glands.

Conclusion

For the first time, we described a specific matrisome of the human adrenal cortex, demonstrating the composition, distribution, and regulation of the ECM in distinct functional niches of the adrenal cortex, namely the OF and IF. We have also identified several components of the ECM, including some that have not been previously described, which may be involved in the various processes of renewal and maintenance of the adrenal cortex (Fig. 7). These findings may contribute to understanding the microenvironment of the adrenal cortex, its morphology, and its function in health and disease.

Experimental procedures

Human adrenal glands

Human adrenal glands (n = 5) were obtained from cadavers from the Death Verification Service of the Capital of the University of São Paulo with approval from the Ethics Committee of the Institute of Biomedical Sciences and the National Research Ethics Council (Comissão Nacional de Ética em Pesquisa – CONEP) with n° 6.524.377. The donors were two men and three women, with a mean age of 61.5 ± 30.9 years. The adrenal glands were collected 13.5 ± 2.9 h after death, which showed a size and weight of 4.6 ± 0.9 g and 6.1 ± 0.7 cm, respectively. After removal, the glands were transported on dry ice and then frozen at −80 °C.

Obtaining the outer and inner fractions of the human adrenal gland

After semi-thawing the adrenal gland, it was positioned on a frozen surface and sectioned in half in the sagittal plane. Sections of 3 mm

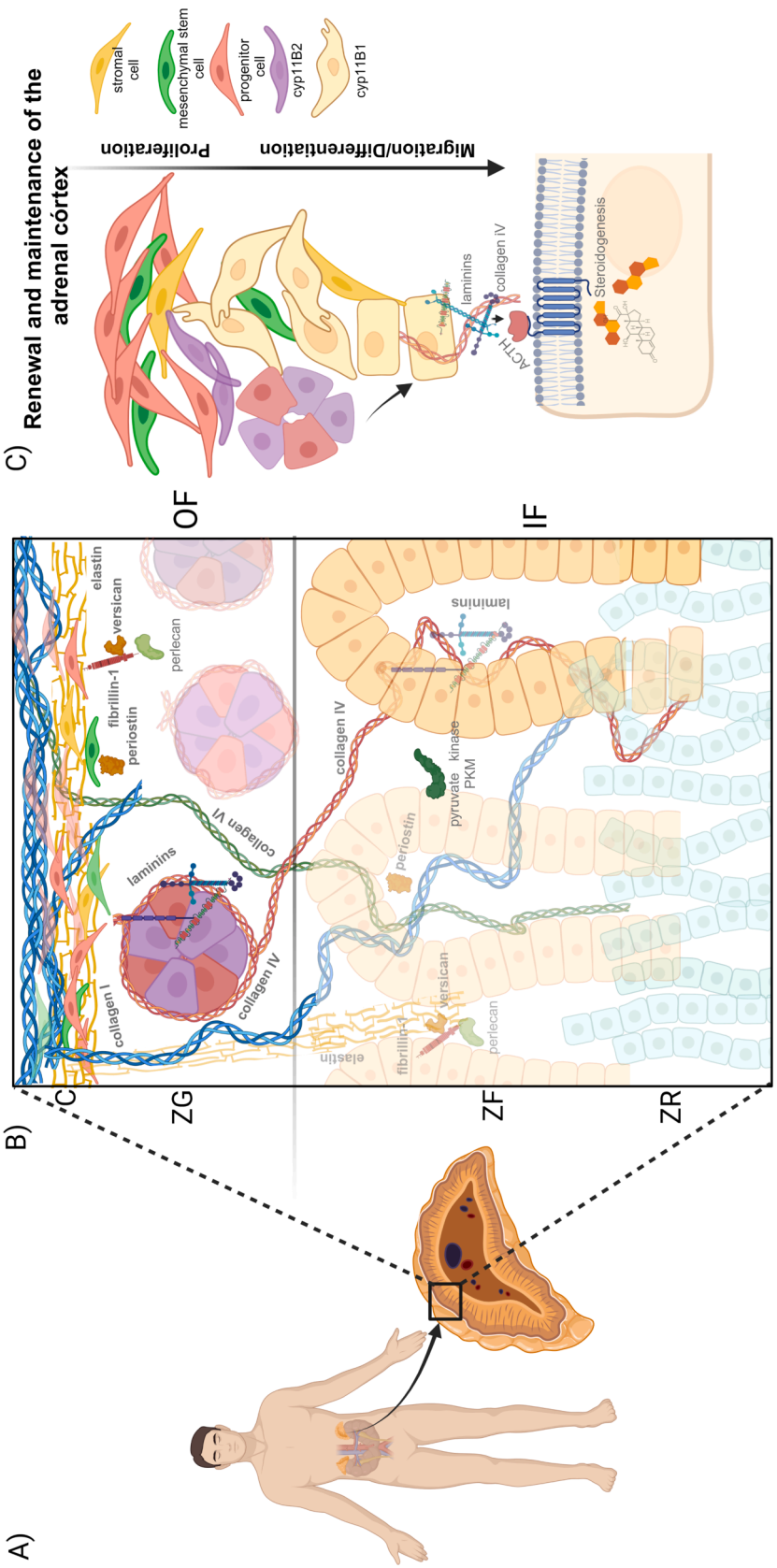

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Fig. 7. Illustrative model representing the specific human adrenal cortex matrisome. A) Separation of the adrenal cortex into outer fraction (OF) composed of capsule/subcapsular and glomerulosa cells, and inner fraction (IF) composed of fasciculata cells; B) Expansion of OF and IF to observe extracellular matrix (ECM) elements more expressed in OF. In OF, Collagen I, Collagen VI, Fibrillin-1, Heparan sulfate proteoglycan 2, Versican, Elastin may be related to mechanical resistance; Periostin, Collagen VI, Fibrillin-1, Laminins related to proliferation and differentiation; Periostin, Collagen VI, Collagen IV, Fibrillin-1, Perlecan, Laminins related to adhesion/migration, while Periostin, Collagen VI may be related to maintenance and renewal of the adrenal cortex cells. In IF, particular attention must be given to the upregulation of Pyruvate kinase M, whose function requires further investigation; C) Cellular types are involved in different cellular processes in the adrenal cortex. Collagen IV, when associated with laminins, seems to be related to Adrenocorticotropic hormone (ACTH)-induced cell proliferation and steroidogenesis. Created with BioRender.com, copyright registered. ZG-zone glomerulosa; ZF-zone fasciculata; ZR-zone reticular.

thickness were performed in the same plane, exposing all regions that comprise the gland. Each sample was gently decapsulated to separate the capsule and the ZG, thus forming the OF, while the ZF and ZR gave rise to the IF, which was separated from the medulla (M). The fractionated samples were frozen at −80 °C for at least 24 h before decellularization.

ECM enrichment through decellularization of adrenal fractions

The adrenal fractions OF and IF were incubated in 1 % Triton-X (Sigma-Aldrich, St Louis, MO; 10 mL/cm3) for 2h30min at room temperature (RT) under mild agitation, followed by incubation in 4.0 % sodium deoxycholate (Sigma-Aldrich) for 3 h. After, the samples were incubated at 4 °C in 1.0 % sodium deoxycholate overnight and incubated for another 3 h at RT in 4.0 % sodium deoxycholate. All the steps were followed by washes of 15 min with sterile water. The material was stored at −80 °C. The remaining intact cells were verified by DNA quantification using Nanodrop (Thermo Fisher Scientific) and considered adequate at a concentration < 50 ng/µL (Fig. S1A). Decellularized samples of adrenal and control were stained with 4 mg/mL 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, MA, USA) for 5 min and analyzed under a fluorescence microscope (Nikon) (Fig. S1B).

Sample preparation for proteomic analysis

ECM protein samples were resuspended in 1 % sodium deoxycholate in 1 mM dithiothreitol (DTT) containing 5 % of protease inhibitors and incubated for 30 min at 56 °C until complete solubilization. The samples were sonicated using a probe tip sonicator thrice on ice at 40 % power for 30 s each. Extracted proteins were reduced with 10 mM DTT for 45 min at 56 °C, alkylated in 40 mM iodoacetamide for 30 min in the dark, and then digested with trypsin 2 % (w/w) overnight at 37 °C. Samples were acidified with 1 % trifluoroacetic acid (TFA) to stop trypsin digestion and centrifuged at 10,000 g for 10 min to remove insoluble material. The supernatant was collected and desalted with Stagetips prepared with a 3 M Empore C18 disk (Sigma-Aldrich) inserted in a P200 tip. The peptide samples were dried in a speed vacuum concentrator (Labconco, Kansas City, MO, USA).

Liquid chromatography coupled to tandem mass spectrometry analysis

Peptide samples were resuspended in 0.1 % formic acid (FA) and analyzed on the Orbitrap Exploris 480 mass spectrometry (MS) (Thermo Fisher Scientific, Bremen, Germany) in tandem with a nano-scale liquid chromatograph (nano-LC) nLC Vanquish Neo (Thermo Fisher Scientific). The MS is coupled to an Easy Flex source with a commercial nanoLC Acclaim PepMap NEO analytical column of dimensions 150 mm × 75 µm, packed with 2 µm C18 resin, and a nanoLC trap column Acclaim PepMap 100 C18 of dimensions 20 mm × 75 µm and particle diameter 3 µm (Thermo Fisher Scientific). 0.5 µg of peptide extract was injected and eluted from the column with a gradient of 5–40 % solvent B (90 % acetonitrile, 0.1 % formic acid) over 100 min at a flow rate of 200 nL/min. The electrospray source was operated at 2.2 kV. The MS is coupled to FAIMS (Field Asymmetric Ion Mobility Spectrometry) module, allowing ion separation in the gas phase based on their different mobility characteristics under the influence of high and low electric fields. The peptide mixture was analyzed by acquiring spectra in full MS mode with a resolution of 120,000 for MS1 determination, with an automatic injection time in the range of 375 to 1500 *m/z*. The 20 most intense peaks were automatically selected through Data-Dependent Acquisition (DDA) for subsequent MS/MS spectra acquisition using two compensation voltages configured in the FAIMS system: −45 V and −60 V, with a resolution of 15,000, a maximum injection time of 40 ms, a range of 120 to 2,000 *m/z*, and a dynamic exclusion of 45sec.

Data and bioinformatics analysis

Raw files were processed with the MaxQuant software version 2.1.4 using the Andromeda search engine against *Homo sapiens* databases (82,518 entries, downloaded from Uniprot.org in January 2024). Database searches were performed with the precursor mass tolerance of 10 ppm, product ion mass tolerance of ± 0.6 Da, and trypsin cleavage with two missed cleavages allowed. However, carbamidomethylation of cysteine (57.021 Da) was set as a fixed modification, and oxidation of methionine (15.994 Da) and protein N-terminal acetylation (42.010 Da) have been selected as variable modifications. All identifications were filtered to achieve a protein peptide, and Peptide-Spectrum Matches (PSMs) false discovery rate (FDR) of less than 1 %, and a minimum of one unique peptide was required for protein identification. Potential common contaminants, proteins identified in the decoy database, and proteins only identified by site were excluded. Protein quantification was based on the MaxQuant label-free algorithm using unique and razor peptides and the matching-between-run feature selection. Statistical analysis was performed using Perseus version 2.0.11 software (https://maxquant.net/perseus/). The raw data was initially transformed using a logarithmic base 2 transformation. This was followed by quantile normalization using the 'normalize.quantiles' method from the R package preprocessCore [38]. Based on the LFQ intensity, data was log2 transformed considering a minimum of 60 % of valid values in at least one group. A Student's T-test ($p < 0.05$) was applied to identify the significant proteins between OF and IF fractions. The total identified and regulated proteins were compared with the Homo sapiens matrisome accessed in January 2024 [39]. The proteins were annotated using UniProtKB codes (https://www.uniprot.org/) and Gene Ontology AmiGO2 (https://amigo.geneontology.org/amigo/landing). Protein-protein interaction networks and functional enrichment analysis were performed using the STRING version 12.0 platform (https://string-db.org/) with the minimum required interaction score of High confidence 0.700. A summary was generated using the BioRender software (https://www.biorender.com/), accessed in March 2024.

Histochemistry and immunohistochemistry

Human adrenal glands were fixed in 4 % paraformaldehyde (Merck, Darmstadt, Germany), dehydrated, and embedded in paraffin. Histological sections of 8 µm were performed using a conventional microtome, submitted to a deparaffinization and rehydration protocol followed by hematoxylin and eosin staining (H&E), Masson's Trichrome stain and Orcein stain. For histochemistry staining of collagen type 1, the sections were incubated in a 0,1% Sirius Red solution in an aqueous picric acid solution for 40 min, washed in water, and mounted in Permount mounting medium (Fisher Chemical). The sections were analyzed under a light microscope (Nikon®) using the Microlucida Explorer software (MBF Bioscience, VT, USA). For immunofluorescence

detection, sections were immersed in citrate buffer (pH 6.0) at 96 °C for 20 min, followed by washing in phosphate buffer solution (PBS) with 0.025 % Triton (PBST) for antigen retrieval. The sections were blocked with serum anti-goat for 1 h and further incubated at 4 °C overnight with antibody anti-elastin [(1:50), Abcam, ab21610]; anti-Periostin [(1:50); Abcam, ab92460]; anti-collagen IV [(1:50), Abcam, ab6586]. Secondary antibodies Alexa Fluor 594 anti-rabbit IgG fluorescent antibody (1:2000; Jackson ImmunoResearch Laboratories, PA, USA) was used for 1 h followed by DAPI for nuclear staining (50 ng/ul for 5 min). The sections were mounted in VECTASHIELD® PLUS Antifade Media (H-1900, Vector Laboratories Burlingame, CA, USA) and analyzed in a fluorescence microscope using ZEN lite software (Zeiss Microscopy).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9 and R version 4.3.3. Results are expressed as mean ± SD, and statistical significance was considered for $p < 0,05$.

Funding

JLK, TSS, and HSO, are the recipient of a scholarship from São Paulo State Research Foundation-FAPESP (n° 2021/04770-1; n° 2022/11567-0, n° 2023/11931-7); CFPL, GP, and LKI received funding from FAPESP (n° 2020/02988-7; 2020/04923-0; 2013/07467-1; 2018/18257-1, 2018/15549-1, 2020/04923-0).

CRediT authorship contribution statement

Jean Lucas Kremer: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Henrique Sanchez Ortega:** Methodology. **Talita Souza-Siqueira:** Writing – original draft, Formal analysis, Data curation. **Claudia Blanes Angeli:** Methodology. **Leo Kei Iwai:** Methodology, Formal analysis. **Giuseppe Palmisano:** Methodology, Formal analysis. **Claudimara Ferini Pacicco Lotfi:** Writing – review & editing, Writing – original draft, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

We thank Kelly Patricia N. Borges, Marta Maria da Silva Righetti, and Ismael Feitosa Lima for general technical assistance.

Author contributions

JLK, HSO, CBA, LKI, and GP performed the experiments; JLK and TSS conducted the data analyses. JLK wrote the manuscript. CFPL idealized the study and wrote the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mbplus.2024.100158.

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