Supplementary data

Repopulation of decellularized retinas with hiPSC-derived retinal pigment epithelial and ocular progenitor cells shows cell engraftment, organization and differentiation

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METHODS:

Animals and transplantation

All procedures were approved by the Animal Care and Use Committee of the IDIBELL and were performed in accordance with the tenets of the European Community (86/609/CEE) and the Association for Research in Vision and Ophthalmology. CD-1 mice (males; 6 weeks old; n = 3 mice for each group) were purchased from Charles River Laboratories (Germany). Animals were kept on a 12 h light/dark cycle with *ad libitum* food and water.

Mice were anesthetized using 2% Isoflurane / 1% O_2 and maintained with a rat nasal mask. The mouse legs hairs were shaved and the underlying skin cleaned. To implant the RPE-repopulated mouse and porcine ECM, a 10 mm incision were made on each mouse leg and one scaffold implanted subcutaneously in the quadriceps femoris muscle. The incisions were then sutured using monofilament polypropylene 6–0. After 2 and 4 weeks, animals were sacrificed and both quadriceps were collected for histology analysis.

Table S1: List of antibodies

Antibody	Supplier and reference	Specie	Dilution
Bestrophin-1	Santa Cruz Biotechnology, sc-32792	Mouse	1:25
Chondroitin sulfate	Sigma, C8035	Mouse	1:200
CHX10	Abcam, ab16141	Sheep	1:100
CD3	Santa Cruz Biotechnology, sc20047	Mouse	1:50
CD68	Santa Cruz Biotechnology, sc20060	Mouse	1:50
Collagen I	Abcam, ab34710	Rabbit	1:50
Collagen IV	Sigma, C1926	Mouse	1:50
CRX	Abnova, H00001406-M02	Mouse	1:50
Cytokeratin 19	Genetex, GTX112666	Rabbit	1:100
Cytokeratin 3	Millipore, CBL218	Mouse	1:100
Cytokeratin 5	Abcam, ab24647-50	Rabbit	1:100
Fibronectin	Sigma, F0791	Mouse	1:100
GFAP	Dako, Z0334	Rabbit	1:1000
GS	BD Transduction Laboratories, 610517	Mouse	1:100
Ki67	Abcam, AB16667	Rabbit	1:100
Ku80	Cell Signaling, 2180	Rabbit	1:200
Laminin	Sigma, L9393	Rabbit	1:25
MITF	Santa Cruz Biotechnology, sc-56725	Mouse	1:25
Mucin-1	Genetex, GTX100459	Rabbit	1:50
Na+/K+-ATPase	Millipore, 05-369	Mouse	1:50
NANOG	R&D Systems, AF1997	Goat	1:25
NRL	R&D Systems, AF2945	Goat	1:25
OCT4	Santa Cruz, sc-5279	Mouse	1:25
OPN1SW	Santa Cruz Biotechnology, sc-30021	Rabbit	1:50
P63	Abcam, ab735	Mouse	1:50
PAX6	Covance, PRB278P	Rabbit	1:100
RAX	Abcam, ab23340	Rabbit	1:50
Recoverin	Millipore, AB5585	Rabbit	1:500
Rhodopsin	Sigma, O4886	Mouse	1:500
RPE65	Santa Cruz Biotechnology, sc-73616	Mouse	1:100
SSEA3	Iowa, MC-631	Rat	1:2
SSEA4	Iowa, MC-813-70	Mouse	1:2
SOX2	Santa Cruz Biotechnology, sc-17320	Goat	1:25
Synaptophysin	Millipore, MAB329-C	Mouse	1:100
TRA-1-60	Chemicon, MAB4360	Mouse	1:100
TRA-1-81	Chemicon, MAB4381	Mouse	1:100
Tuj1	Covance, MMS-435P	Mouse	1:1000
Vimentin	Cell signaling kit Arigobio, SQab1721	Rabbit	1:100
ZO-1	Millipore, ab2272	Rabbit	1:100
γ-crystallin	Santa Cruz Biotechnology, sc-22415	Goat	1:50

Table S2: List of primers used in RT-qPCR

Gene	Forward	Reverse
AQP1	ACCTCCTGGCTATTGACTACA	CCCTTCTATTTGGGCTTCATCT
CK12	AGCAGAATCGGAAGGACGCTG	ACCTCGCTCTTGCTGGACTGAAA
CK19	ACAGCCACTACTACACGACC	CCTGTTCCGTCTCAAACTTGGT
CK3	ACGTGACTACCAGGAGCTGATG	ATGCTGACAGCACTCGGACACT
COL8A1	CCTGGGTCAGCAAGTACCTC	TTGTTCCCCTCGTAAACTGG
CRX	TCCAGGGTTCAGGTTTGGTT	CATCTGTGGAGGGTCTTGGG
GAPDH	CCTGCACCACCAACTGCTTAG	TGGCATGGACTGTGGTCATG
MITF	GTGCCAACTTCTTTCATCA	ACCTAAACCGTCCATTCA
Na^+/K^+- ATPase	CAGGGCAGTGTTTCAGGCTAA	TCGACGATTTTGGCGTATCTT
OCT3/4	GTTCTTCATTCACTAAGGAAGG	CAAGAGCATCATTGAACTTCAC
P63	GAAAACAATGCCCAGACTCAATTT	TCTGCGCGTGGTCTGTGTTAT
PAX6	TCTAATCGAAGGGCCAAATG	TGTGAGGGCTGTGTCTGTTC
PEDF	AGATCTCAGCTGCAAGATTGCCCA	ATGAATGAACTCGGAGGTGAGGCT
RECOVERIN	TCTACGACGTGGACGGTAACG	CGTCCTCGGGAGTGATCATT
RHODOPSIN	GGGAGAACCATGCCATCAT	TCGTCTCCGTCTTGGA
SIX6	ACGGCGAACAGAAGACACAC	TGCTGGAGTCTGTTCTTGGCT

Table S3: Count distribution of matrisome related genes from mouse and porcine acellular retinal ECM (arECM) total proteomes. Core matrisome and matrisome-associated main categories and respective subcategories from 'The Matrisome Project' were considered [1]. These genes were identified from an initial list of 3,833 and 928 unique genes from mouse and porcine acellular retinas, respectively.

arECM	Core-Matrisome			Matrisome-Associated			Total
	Collagens	ECM Glycoproteins	Proteoglycans	ECM-Regulators	ECM-affiliated proteins	Secreted Factors	
Mouse	12	38	6	10	20	7	93
Porcine	16	52	7	13	15	13	116

Table S4. Percentage of total proteome and matrisome-related proteins with any GO term annotation for mouse and porcine acellular retinal ECM (arECM). The three GO ontology domains are shown separately. Values are computed over the number of proteins in the corresponding set, total proteome or matrisome-related. In the case of Sus scrofa, 99% of proteins with no GO term annotation were retrieved from the TrEMBL database, independently of the ontology.

arECM	Total Proteome proteins 3837 unique proteins (mouse)) 2612 unique proteins (pig))			Matrisome-related proteins 93 unique proteins (mouse) 265 unique proteins (pig)		
	Biological process	Molecular Function	Cellular Component	Biological Process	Molecular Function	Cellular Component
Mouse	97.9%	97.1%	98.3%	100%	97.8%	100%
Porcine	31.7%	31.7%	32.1%	45.3%	40.0%	43.0%

Table S5. Number of unique mapped GO terms for mouse and porcine acellular retinal ECM (arECM). The three GO ontologies domains are shown separately. Unique mapped GO terms do not include the ancestor terms. The GO annotations from the corresponding total proteome lists are considered. Percentage in “In common” terms refer to the number of GO terms found in pig.

arECM	GO Ontology Domain		
	Biological Process	**Molecular Function**	**Cellular Component**
Mouse	7,213	2,344	1,243
Porcine	2,527	729	547
In common	2,394 (94.7%)	680 (93.3%)	535 (97.8%)

Table S6. Number of overrepresented GO terms and KEGG Pathways considering those genes derived from total proteome and matrisome-related proteins. The reduced list of overrepresented GO terms is considered. Obtained quantities for mouse and porcine arECMs are shown. 'In common' row refers to the GO terms in common between both organisms.

arECM	Total Proteome				Matrisome-related			
	GO BP terms	GO MF terms	GO CC terms	KEGG pathways	GO BP terms	GO MF terms	GO CC terms	KEGG pathways
Mouse	370	140	115	121	49	21	9	13
Porcine	38	19	42	85	22	10	6	15
In common	7	7	15	65	2	8	3	12

SUPPLEMENTARY FIGURES

FIGURE S1

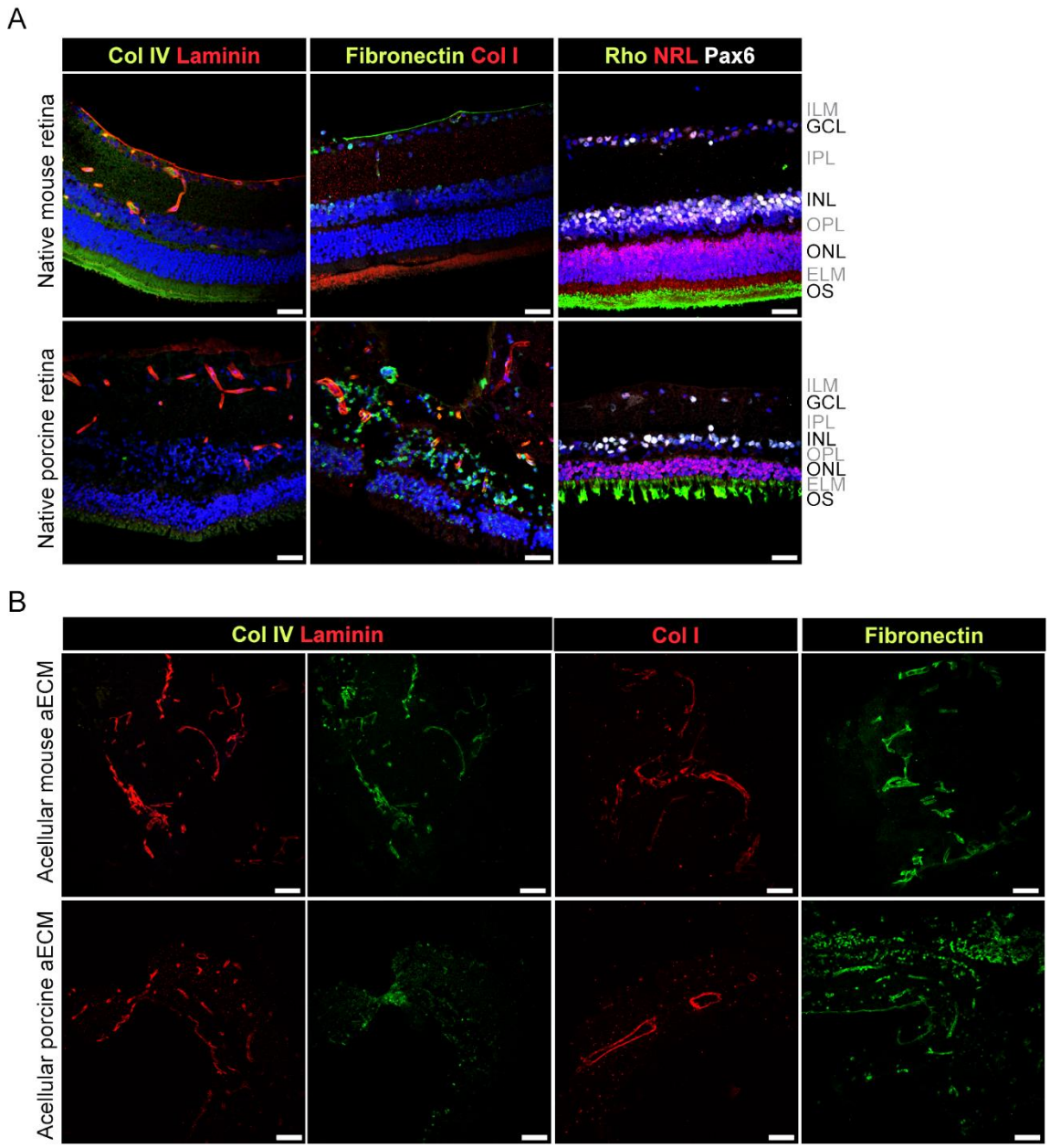

Figure S1: **Distribution of ECM proteins in native and decellularized retinas**. A) Paraffin sections of isolated mouse and porcine native retinas stained with extracellular matrix proteins Collagen IV (Col IV), Laminin, Collagen I (Col I) and fibronectin, and retinal markers rhodopsin (Rho) and NRL for photoreceptors and Pax6 for INL and GCL. Laminin and fibronectin stained ILM and retinal vascular basement membranes whereas Col I and IV also stained ELM. Scale bars, 50 µm. Nuclei are stained with DAPI. B) Paraffin sections of decellularized (acellular) mouse and porcine retinas stained with extracellular matrix proteins Collagen IV (Col IV), Laminin, Collagen I (Col I) and fibronectin. Scale bars, 50 µm. ECM, extracellular matrix; ELM, external limiting membrane; GCL, ganglion cell layer; ILM, inner limiting membrane; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segments.

FIGURE S2

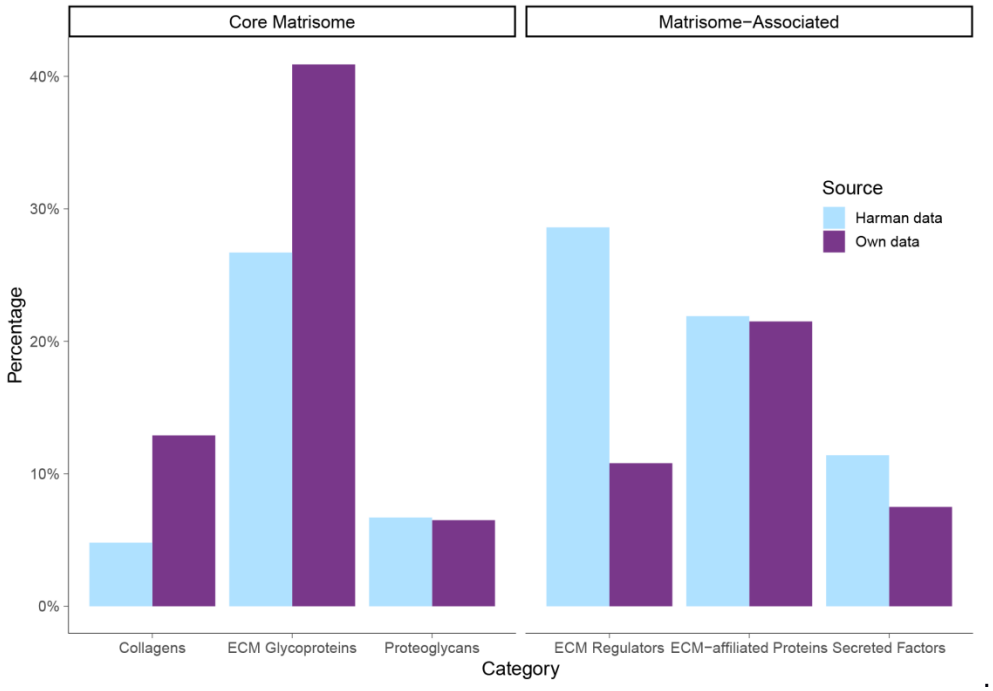

Figure S2. Percent distribution of mouse acellular retinal matrisome-related genes compared with mouse native retinal total proteome described by the Harman *et al*., dataset [2]**.** Core matrisome and matrisome-associated main categories and respective subcategories from 'The Matrisome Project' were considered [1]. Percentages are computed with regard to 93 (own data) and 105 genes [2].

FIGURE S3

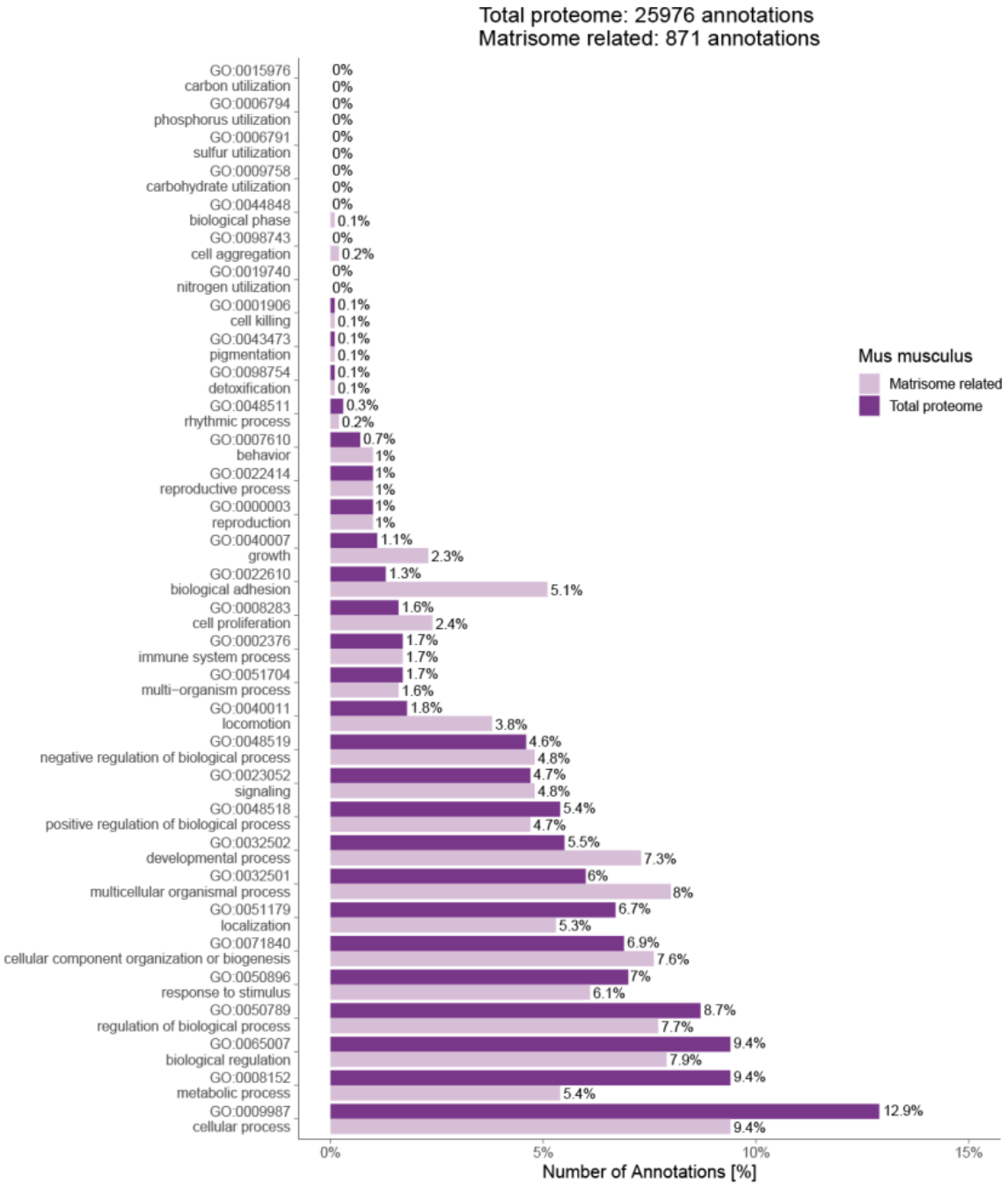

Figure S3. GO Biological Process distribution among total proteome and matrisome-related proteins from mouse acellular retinas. GO terms are sorted by the total proteome percentage.

FIGURE S4

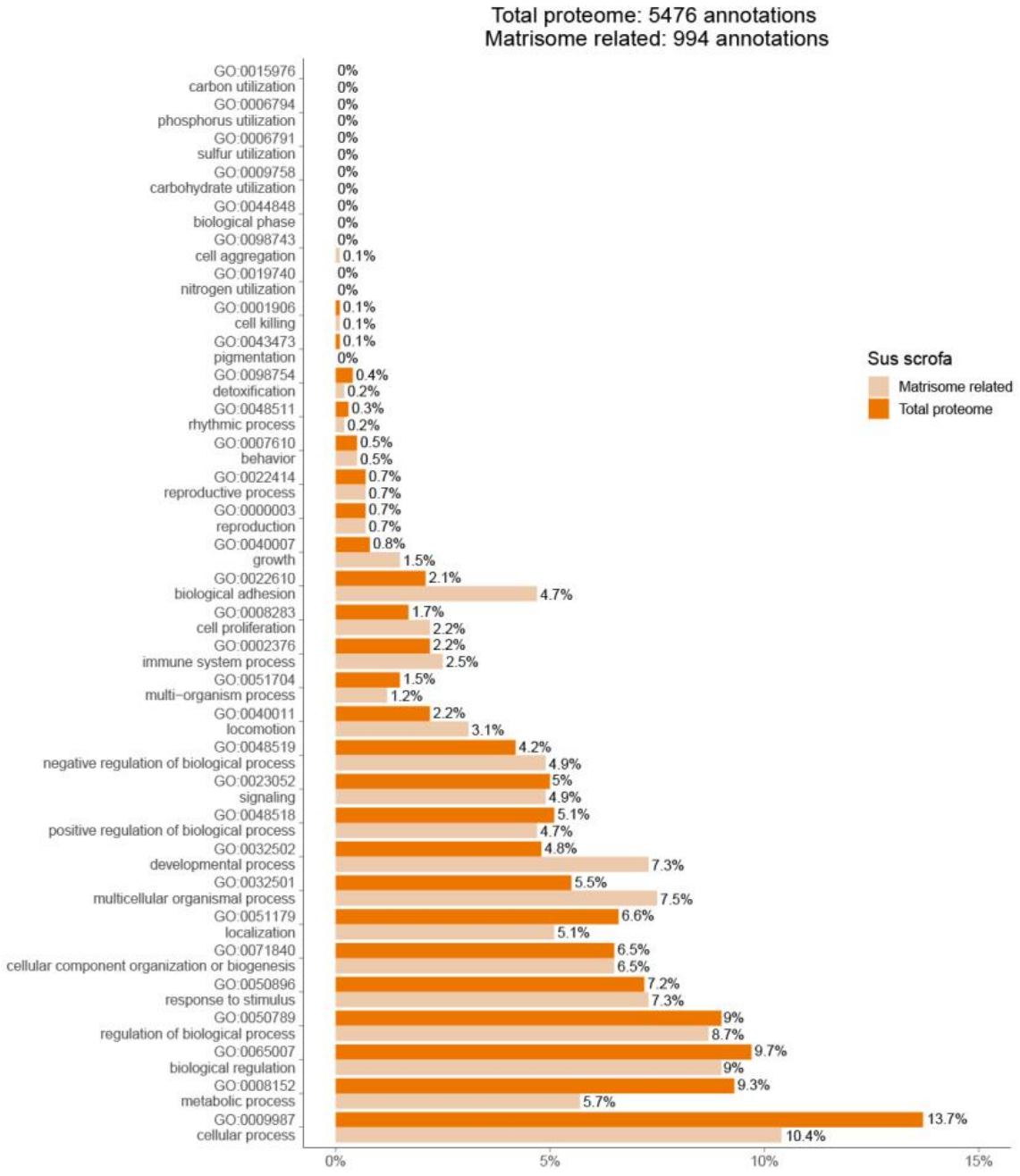

Figure S4. GO Biological Process distribution among total proteome and matrisome-related proteins from porcine acellular retinas. GO terms are sorted by the total proteome percentage from mouse acellular retinas (Suppl. Fig. S3).

FIGURE S5

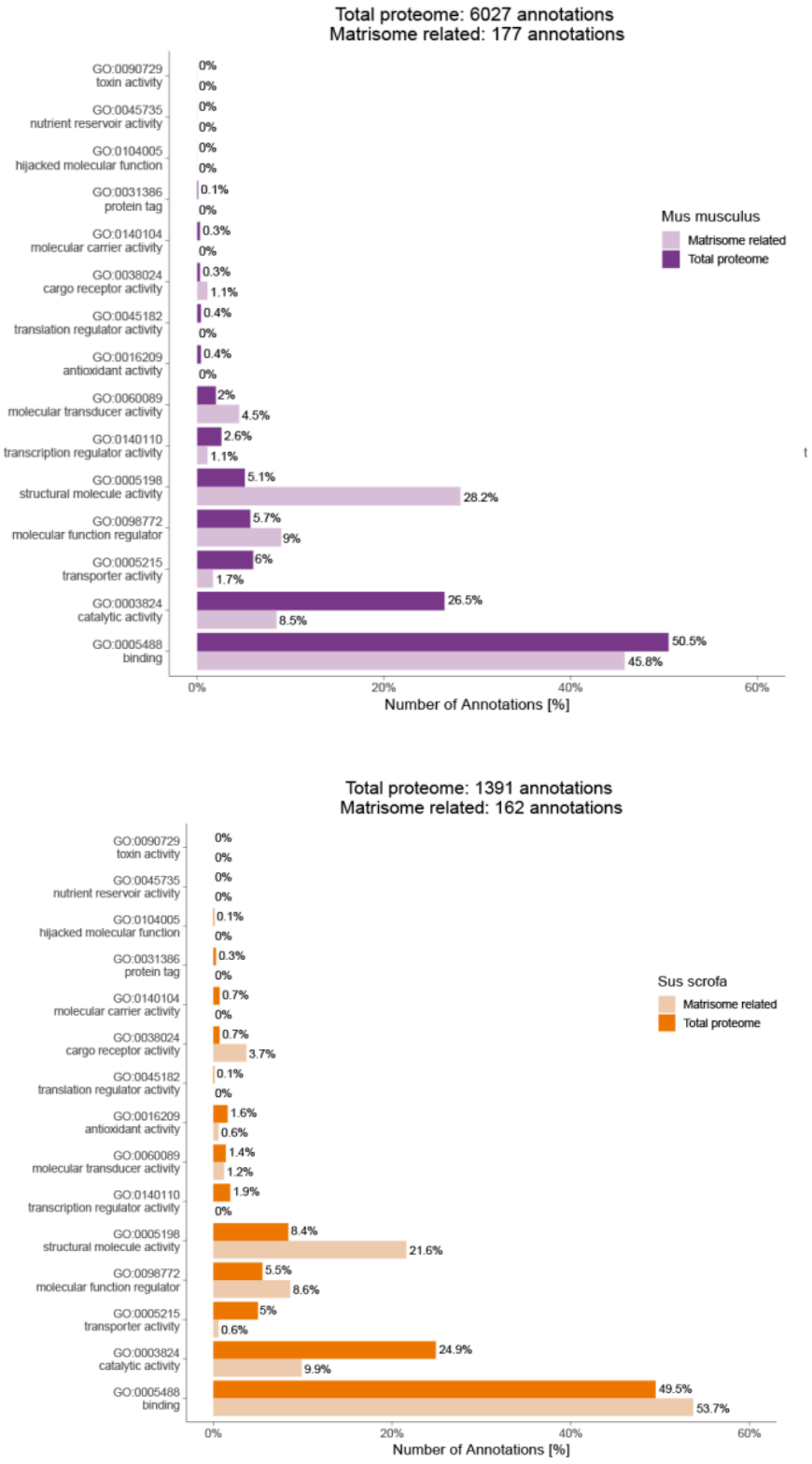

Figure S5. GO Molecular Function distribution among total proteome and matrisome-related proteins from mouse and porcine acellular retinas. Upper figure: Mouse. Lower figure: Pig. GO terms are sorted by the mouse total proteome percentage

FIGURE S6

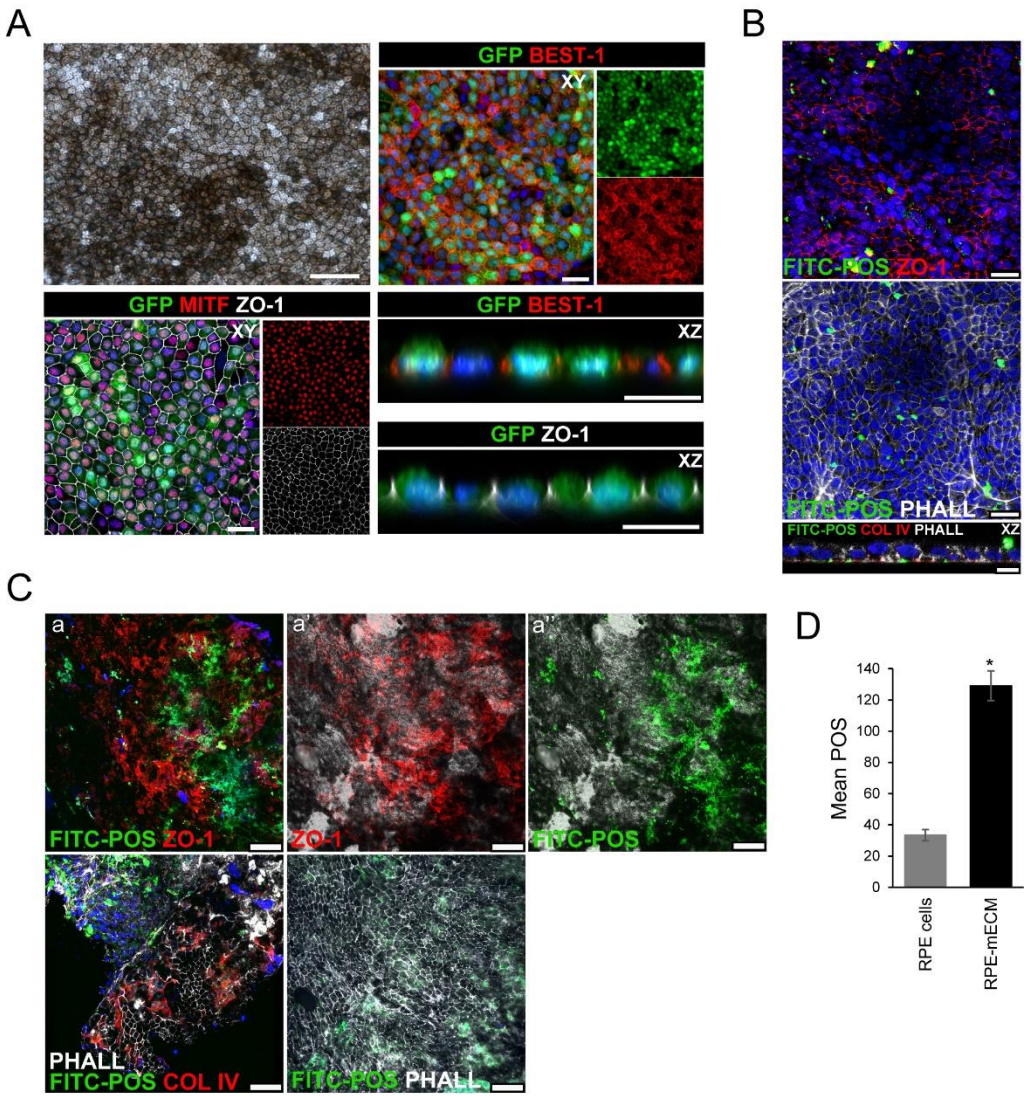

Figure S6: Characterization of retinal pigment epithelial cells from FiPS-4F-7 hiPSC line and phagocytosis of photoreceptor outer segment by both RPE cells and RPE-repopulated mouse ECM. A) Phase contrast image showing cobblestone pigmented hiPSC-derived RPE cells in culture. Representative xy and xz confocal images showing RPE cells expressing GFP, and RPE specific markers bestrophin 1 (BEST-1), MITF and zonula occludens 1 (ZO-1). Scale bars: 50 µm in phase contrast image; 25 µm in xy images;10 µm in xz images. B.C) Z-stack fluorescent images with cross-section side view showed the location of internalized FITC-POS in RPE cells (B) and RPE-repopulated mECM (C) at day 30. Cells were stained with specific antibodies: ZO-1, Alexa 647-conjugated phalloidin (PHALL) and collagen IV (COL IV). (a' and a'') Bright field merged images with ZO-1 (red) and FITC-POS (green) from (a) showing pigmentation of RPE-repopulated mECM. Scale bars: (B) 25 µm in xy; 10 µm in xz; (C) 50 µm. Nuclei are stained with DAPI (blue). D) The number of internalized FITC-POS by RPE cells and RPE-repopulated mECM per field view (382 µm × 382 µm; bars: mean + SD; n = 3). Single asterisk (*) indicated p value < 0.05 calculated by unpaired Student's t test. Abbreviations: hiPSC, human induced pluripotent stem cells; GFP, green fluorescent protein; RPE, retinal pigment epithelium

FIGURE S7

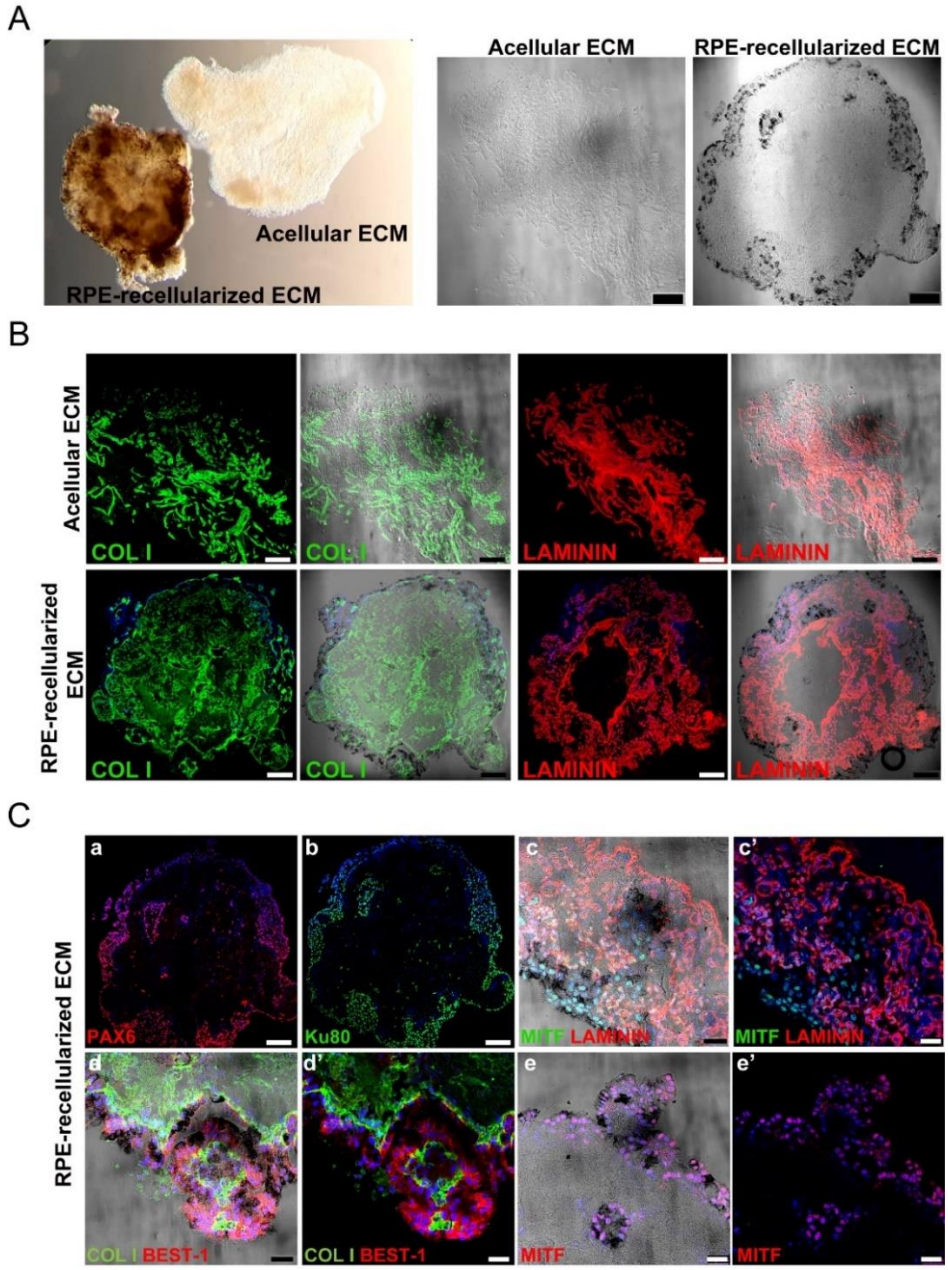

Figure S7: Recellularization of acellular retinal ECM with RPE cells. A) Bright field images showing the repopulation of arECM with RPE cells. Left panel: images of the whole arECM (control) and the pigmented RPE-repopulated ECM. Right panels: paraffin section corresponding to arECM and RPE-repopulated ECM. Scale bars: 100 µm in RPE-repopulated ECM; 50 µm in arECM. **B)** Paraffin section of arECM and RPE-repopulated mouse retinas at DIV 50 immunolabelled with ECM protein markers collagen type I (COL I) and laminin. Bright field merges show pigmented RPE cells mainly at the surface of repopulated ECM. Scale bars: 100 µm. **C)** Paraffin section of RPE-repopulated mouse retinas at day 50 immunolabelled with RPE markers MITF, BEST-1, PAX6 and ZO-1; ECM markers COL I and laminin; and human marker Ku80. Scale bars: 100 µm in a,b; 25 µm in c-e'. Nuclei are stained with DAPI (blue). Abbreviations: arECM, acellular retinal extracellular matrix; DIV, days in vitro; RPE, retinal pigment epithelium

FIGURE S8

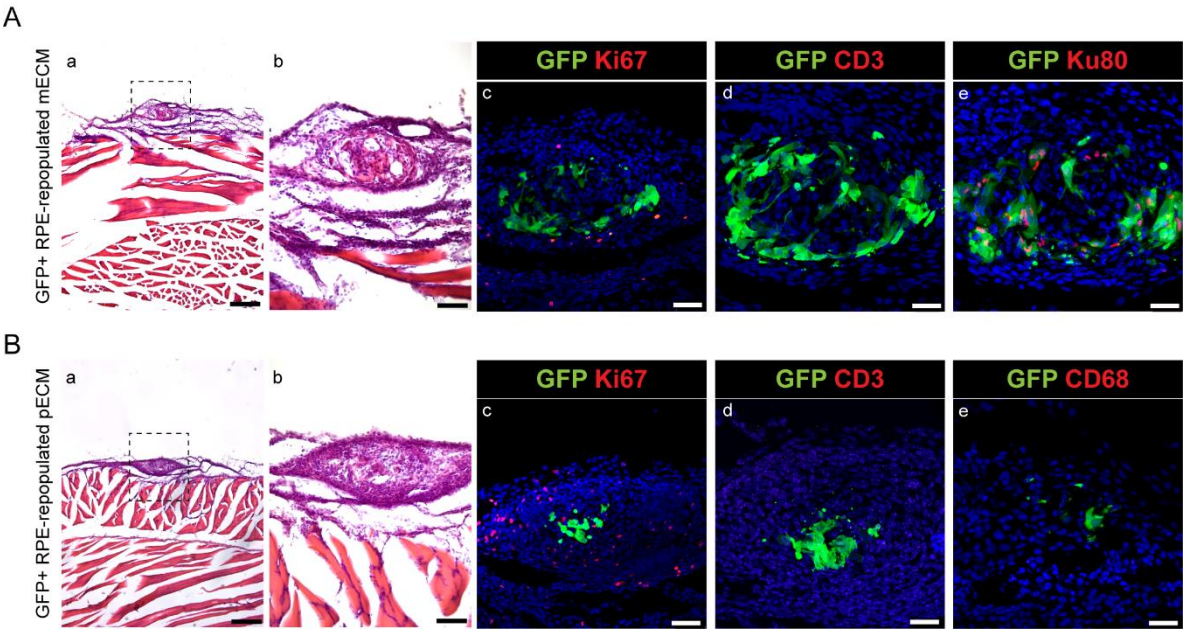

Figure S8: Immunogenic properties of GFP+ RPE cell-repopulated mouse and porcine ECM. Histology from 14-days subcutaneous transplantation of mouse (A) and porcine (B) repopulated retinal ECM with hiPSC-derived RPE expressing GFP (expressing the human marker Ku80+) in non-immunosuppressed CD-1 mice. Dashed squares in a indicate magnification in b. Immunolabeled images showed that graft areas were infiltrated by host cells which were proliferative (Ki67) but negative for CD3 or CD68. Nuclei were stained with DAPI. Scale bars: 250 in a; 100 in b; 50 µm in c; 25µm in d,e.

FIGURE S9

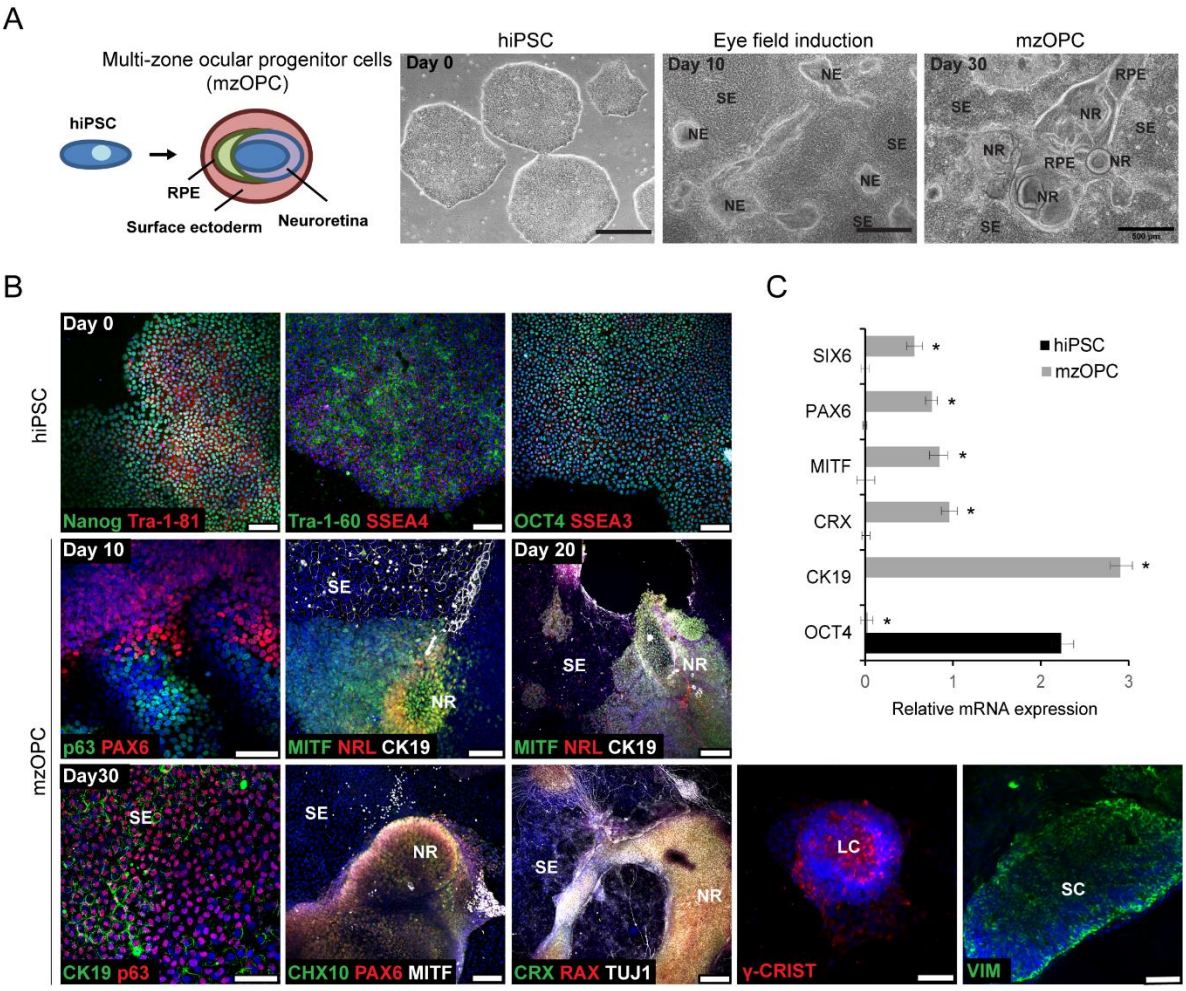

Figure S9: Generation of multi-zone ocular progenitor cells from FiPS-4F-7 hiPSC line. A) Schematic illustration of differentiation of human induced pluripotent stem cells (hiPSC) towards multi-zone ocular progenitor cells (mzOPC). Phase contrast images of hiPSC at day 0 showing compact colonies. The mzOPC at day 10 and 30 of differentiation showing different ocular lineages: neuroectoderm (NE), neuroretina (NR), retinal epithelium (RPE), and surface ectoderm (SE). Scale bars: 500 µm. **B)** Immunocytochemistry of hiPSC at day 0 stained with pluripotent-specific markers NANOG, OCT4, SSEA3, SSEA4, Tra-1-81 and Tra 1-61. Immunostaining of mzOPC at days 10, 20 and 30 of differentiation stained with antibodies against PAX6 (ectodermal marker), MITF (RPE marker), NRL, RAX CHX10, and TUJ1 (NE/NR markers), p63, CK19 (SE markers), γ-crystallin (lens cells; LC) and vimentin (VIM; stromal cell marker; SC). Nuclei are stained with DAPI. Scale bars: 50 µm. **C)** Relative gene expression detected by RT-qPCR in hiPSC and mzOPC at day 30 of eye-field transcription factors *PAX6, SIX6;* the photoreceptor marker *CRX*; the SE marker *CK19*; the RPE-specific marker *MITF;* and the pluripotency marker *OCT4.* Values are normalized to *GAPDH*. Data presented as mean ± SD (n=3). Values indicated with stars are significantly different from those in hiPSCs (Student's t-test; **p*<0.01).

FIGURE S10

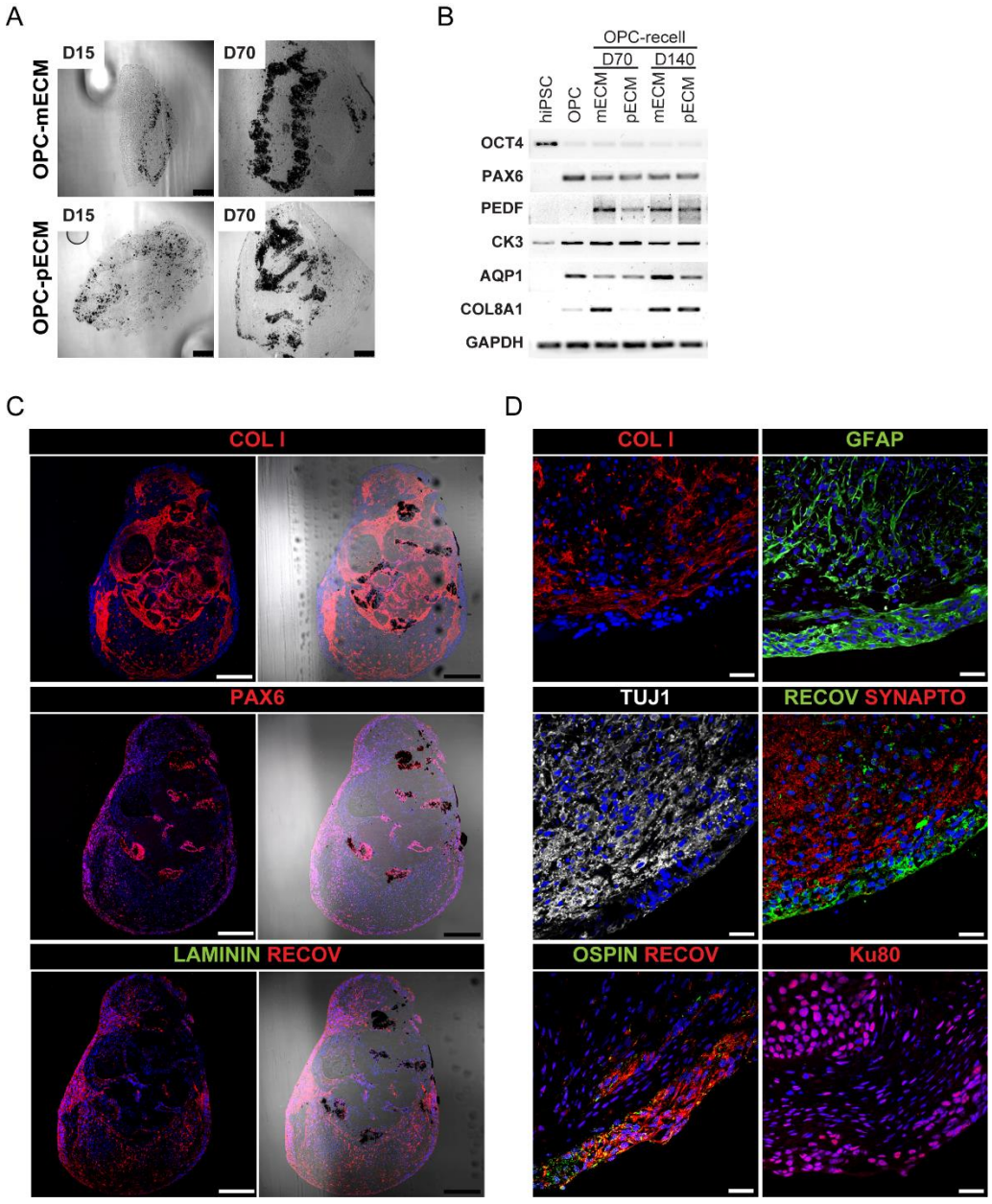

Figure S10: OPC-repopulated mouse and porcine acellular retinal ECMs. A) Bright field images of paraffin sections of repopulated mouse and porcine arECM at days 15 and 70 showing the central pigmented cell rings that increased in size and pigmentation over time. Scale bar: 100 µm. **B**) PCR analysis of specific eye-field (*PAX6*), corneal (*CK3, AQP1, COL8A1*), retinal pigment epithelium (*PEDF*), and pluriontecy (*OCT4*) gene expression levels in samples from human induced pluripotent stem cells (hiPSC), ocular progenitor cells (OPC) at day 30 of differentiation, and OPC-repopulated mouse (mECM) or porcine (pECM) at days 70 and 140 in culture. Expression of *GAPDH* was used as internal control. **C**) Immunohistochemical and bright field merged images of OPC-repopulated mouse ECM labelled with ECM marker collagen I (COL I); neuroectodermal marker PAX6; and photoreceptor marker recoverin (RECOV). Scale bars: 250 µm. **D)** Enlarged confocal images of OPC-repopulated mouse arECM labelled with ECM marker COL I; neuroretinal markers GFAP, TUJ1, synaptophysin (SYNAPTO) and glutamine synthetase (GS); photoreceptor markers RECOV and opsin; and human nuclear marker Ku80. Scale bars: 25 µm. Nuclei are stained in DAPI.

FIGURE S11

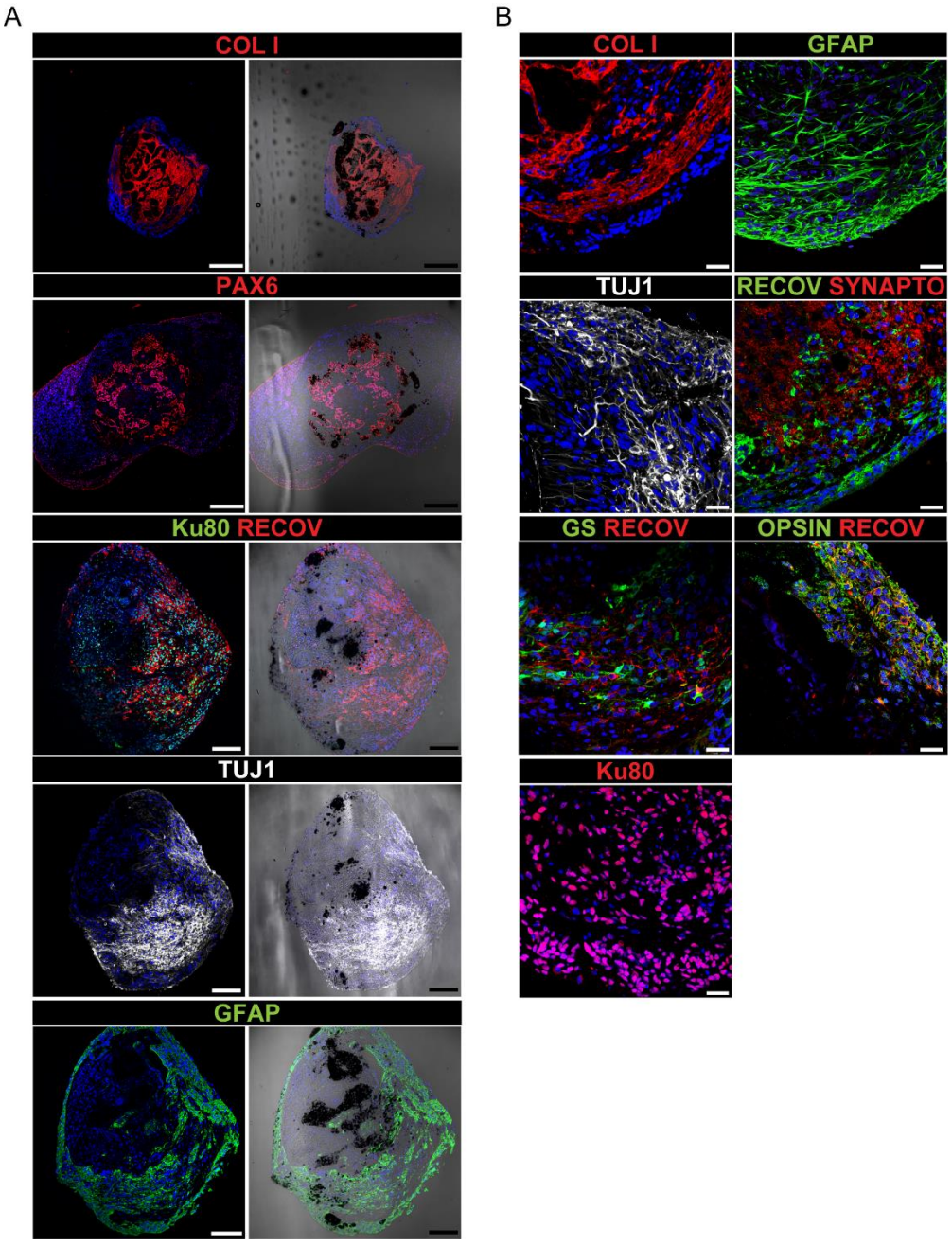

Figure S11: OPC-repopulated porcine acellular retinal ECMs. A) Immunohistochemical and bright field merged images of OPC-repopulated porcine arECM labelled with ECM marker collagen I (COL I); neuroectodermal marker PAX6; neuroretinal markers glial fibrillary acidic protein (GFAP), neuron-specific class III beta-tubulin (TUJ1); photoreceptor marker recoverin (RECOV); and all cells repopulating ECM are Ku80+ (human nuclei marker). Scale bars: 250 µm in COL I and PAX stainings; 100 µm in Ku80, TUJ1 and GFAP. **B**) Confocal images of OPC-repopulated porcine ECM labelled with ECM marker COL I; neuroretinal markers GFAP, TUJ1, SYNAPTO and glutamine synthetase (GS); photoreceptor markers RECOV and opsin; and human nuclear marker Ku80. Scale bars: 25 µm. Nuclei are stained in DAPI.

References

1 Naba A, Clauser KR, Hoersch S, et al. The matrisome: in silico definition and in vivo characterization by proteomics of normal and tumor extracellular matrices. Mol Cell Proteomics 2011.

2 Harman JC, Guidry JJ, Gidday JM. Comprehensive characterization of the adult ND4 Swiss Webster mouse retina: Using discovery-based mass spectrometry to decipher the total proteome and phosphoproteome. Mol Vis 2018;24:875–889.