

RESEARCH ARTICLE

Development and characterization of a novel anti-reflux artificial lacrimal duct via three-dimensional printing for lacrimal system reconstruction

Bingran Dong^{1†}, Fang Bai^{1†}, Hai Tao^{1*} , and Sha Huang^{2*}

¹Senior Department of Ophthalmology, Third Medical Center of Chinese PLA General Hospital, Beijing, China

²Research Center for Tissue Repair and Regeneration affiliated to the Medical Innovation Research Department, PLA General Hospital and PLA Medical College, Beijing, China

Abstract

Severe lacrimal duct defects, including congenital absence and traumatic obliteration, remain formidable clinical challenges. Current therapeutic strategies, such as Jones tube prosthesis implantation, are severely limited by poor biocompatibility, susceptibility to displacement, and retrograde infections caused by fluid reflux. To address these limitations, we developed a novel anti-reflux artificial lacrimal duct using three-dimensional (3D) printing that integrates biomimetic hydrodynamic design with a biochemically optimized microenvironment. A meticulously engineered photopolymerizable composite hydrogel—comprising 15% polyethylene glycol diacrylate (PEGDA), 3% acrylamide, and 5% gelatin methacryloyl (GelMA) (60% degree of substitution)—was formulated as a high-strength hydrogel ink. Using 3D printing, we precisely fabricated an artificial lacrimal duct with a biomimetic unidirectional valve that simulates the natural Hasner valve. Guided by proteomic screening, the luminal surface of the construct was subsequently functionalized with a young donor-derived decellularized extracellular matrix (dECM) coating, onto which epidermal stem cells (ESCs) were seeded. The structural PEGDA/polyacrylamide network endowed the construct with superior printability and the requisite mechanical resilience to withstand valve fatigue. Hydrodynamic characterizations demonstrated that the 3D-printed valve effectively prevented fluid reflux while maintaining normal drainage, providing a robust physical barrier against retrograde infections. Biologically, the incorporation of GelMA-60 into the bulk scaffold and the young dECM coating at the luminal interface established a rejuvenated biochemical niche that exhibited excellent biocompatibility and successfully induced the targeted differentiation of ESCs into the lacrimal epithelial lineage. By synergizing physical anti-reflux hydrodynamics with omics-driven microenvironment rejuvenation, this composite 3D-printed construct enables superior tissue integration and infection resistance, presenting a highly promising tissue-engineered alternative for lacrimal system reconstruction.

Keywords: Three-dimensional printing; Tissue-engineered lacrimal duct; Anti-reflux valve; Microenvironment rejuvenation; Proteomics-guided selection; Epithelial differentiation

[†]These authors contributed equally to this work

*Corresponding authors:

Hai Tao
(taohaiwj@sina.com);
Sha Huang
(stellarahuang@sina.com)

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1. Introduction

Severe structural destruction of the lacrimal drainage system, particularly resulting from congenital aplasia and severe traumatic obliteration, represents a formidable clinical challenge in ophthalmology.¹⁻³ Current global therapeutic strategies primarily include conjunctivodacryocystorhinostomy,⁴ autologous tissue transplantation,⁵ and Jones tube implantation;⁶ however, their long-term clinical efficacies remain highly unsatisfactory.⁷⁻⁸ Specifically, conventional conjunctivodacryocystorhinostomy is associated with a low surgical success rate and suboptimal post-operative tear drainage. Meanwhile, autologous tissue reconstruction frequently fails due to local fibrotic hyperplasia that re-obstructs the newly formed tract; moreover, grafted tissues lack both the intrinsic elasticity of native ducts and the essential mechanical scaffolding required to maintain long-term luminal patency.¹ Furthermore, while Jones tube implantation is widely employed, this inert, rigid prosthesis induces persistent local inflammatory irritation and a prominent foreign body sensation.⁹⁻¹¹ Its lumen is frequently occluded by ocular and nasal secretions, demanding rigorous maintenance that is often poorly tolerated by patients. Compounding these issues is the absence of an intrinsic hydrodynamic regulatory mechanism.¹² Unlike the natural Hasner valve, these conventional valveless conduits permit retrograde fluid migration during high-pressure events (e.g., sneezing or forceful nose-blowing).¹³ Given that the targeted patients typically lack a functional lacrimal sac, retrograde reflux directly reaches the ocular surface, frequently leading to ascending bacterial infections and secondary bacterial conjunctivitis.¹⁴⁻¹⁵ Consequently, there is an urgent clinical demand for a paradigm shift from passive, complication-prone tubes to dynamically functional, tissue-integrating artificial lacrimal ducts.

The biological bottleneck in artificial duct integration is the failure to establish a continuous, functional epithelial barrier, which often leads to fibrotic obliteration.¹⁶⁻¹⁷ Epidermal stem cells (ESCs) possess immense potential for epithelial regeneration; however, their targeted lineage specification requires precise microenvironmental cues.¹⁸⁻

²⁰ Recent clinical and omics-based insights suggest that host “microenvironment aging” severely impairs local regenerative capacity. During aging or chronic inflammation, the natural extracellular matrix (ECM) loses crucial basement membrane-associated and pro-adhesive proteins, creating a fibrotic and hostile niche.²¹⁻

²³ We hypothesize that a proteome-guided, youth-derived decellularized ECM (dECM) can successfully replicate a

pro-regenerative biochemical niche.²⁴⁻²⁵ By harnessing this rejuvenated microenvironment, it is possible to provide the essential bio-instructive signals necessary to direct ESCs toward a functional lacrimal epithelial lineage, thereby ensuring long-term mucosal integration and immune evasion.²⁶⁻²⁷

Beyond biological integration, addressing the hydrodynamic flaw requires precise engineering of an anti-reflux valve, a significant biofabrication challenge. Traditional soft natural hydrogels lack the intrinsic mechanical resilience to withstand sustained, repetitive hydrodynamic impacts without fatigue failure or structural collapse.²⁸⁻³⁰ To overcome this, advanced three-dimensional (3D) printing technologies coupled with tailored composite hydrogel inks are required. Indeed, recent comprehensive reviews have emphasized the critical role of dynamic hydrogel formulations and precise mechanical cues in 3D printing to ensure both structural fidelity and guided cellular responses.³¹⁻³² Digital light processing (DLP) 3D printing, renowned for its ultra-high spatial resolution and rapid photopolymerization, offers an ideal platform for fabricating complex, microscale intraluminal architectures.³³⁻³⁵ By formulating a semi-synthetic composite hydrogel—comprising polyethylene glycol diacrylate (PEGDA) and acrylamide (AAM) for robust mechanical toughness and fatigue resistance, alongside gelatin methacryloyl (GelMA) for foundational biocompatibility (the PAG hydrogel formulation)—it becomes possible to physically actualize a highly resilient, flexible biomimetic valve capable of withstanding extreme physiological pressures.³⁶

To resolve the longstanding biomechanical and biological bottlenecks of lacrimal reconstruction, we propose a modular biofabrication strategy to engineer a novel, biomimetic anti-reflux artificial lacrimal duct. Our central hypothesis is that the synergy of physical hydrodynamic regulation and omics-driven biochemical induction can orchestrate the regeneration of a fully functional lacrimal microenvironment. Specifically, a high-strength PAG scaffold featuring a DLP-printed unidirectional Hasner-like valve is fabricated to physically block retrograde infections. Simultaneously, the luminal surface is functionalized with a youth-derived dECM coating laden with *in vitro* pre-induced ESCs to biologically drive *in situ* mucosal integration. In this study, we systematically evaluate this dual-action construct through computational fluid dynamics (CFD) simulations, rigorous *in vitro* benchtop testing, and comprehensive *in vivo* assessments in a rabbit defect model, aiming to provide a next-generation tissue-engineered solution for lacrimal system reconstruction.

2. Materials and methods

2.1. Proteomics analysis

2.1.1. Harvesting of rabbit lacrimal ducts

All the animal experiments were performed according to the guidelines established by the Institutional Animal Care and Use Committee of the Chinese PLA General Hospital and approved by the Medical Ethics Committee of the Third Medical Center of the Chinese PLA General Hospital (Approval No. 2020-002). Male New Zealand White rabbits ($n = 20$), categorized into a young group ($n = 10$; aged 2 weeks; weighing 300 ± 50 g) and an old group ($n = 10$; aged 1 year; weighing $2,000 \pm 100$ g), were purchased from Beijing TongHe Litai Biotechnology Co. Ltd (China).

Following euthanasia and standard aseptic preparation, anatomical dissection was performed under an ophthalmic operating microscope. Membranous portions of the lacrimal duct were aseptically harvested using standard surgical techniques. Periductal adventitial tissues were meticulously removed, and specimens were subsequently stored at 4°C in phosphate-buffered saline (PBS) supplemented with 1% antibiotics and antifungals. To minimize tissue autolysis, the entire harvesting process was strictly completed within 20 min per animal.

2.1.2. Vacuum-assisted decellularization

Membranous portions of the lacrimal duct were decellularized in vacuum tubing connected to a negative-pressure pump (Taizhou Fujiwa Tools Co., Ltd., China), maintaining a vacuum of -0.96 MPa. All procedures performed were conducted on an orbital shaker at 60 rpm while maintaining continuous vacuum. The protocol began with incubation in 1% (v/v) Triton X-100 (Biofroxx, Germany) at 37°C for 12 h. This was followed by three rinses in sterile distilled water for 30 min each and an extended incubation in sterile distilled water at 4°C for 24 h to thoroughly remove the detergent. Subsequently, enzymatic digestion was performed in a 1 M sodium chloride solution containing DNase (2 kU/mL; Sigma, United States) and RNase (4 U/mL; Biofroxx, Germany) at 37°C for 24 h to remove residual nucleic acids. Finally, the resulting decellularized membranous portions of the lacrimal duct were stored in PBS at 4°C , supplemented with 1% antibiotic-antimycotic solution until further use.

2.1.3. Protein extraction and digestion

Decellularized lacrimal ducts from the young (Y) and old (O) groups ($n \geq 3$ per group) were snap-frozen in liquid nitrogen and stored at -80°C prior to analysis. Samples were lysed in 8 M urea buffer supplemented with protease

and phosphatase inhibitors, then sonicated and centrifuged to collect the protein supernatant. Protein concentration was determined using a bicinchoninic acid assay. For digestion, protein aliquots were first reduced with 10 mM dithiothreitol at 37°C for 1 h, then alkylated with 20 mM iodoacetamide at room temperature in the dark for 30 min. The urea concentration was subsequently diluted to ≤ 1.5 M, and proteins were digested overnight at 37°C using sequencing-grade trypsin at an enzyme-to-substrate ratio of 1:50 (w/w). The resulting peptides were desalted using C18 solid-phase extraction cartridges.

2.1.4. Nanoscale liquid chromatography coupled to tandem mass spectrometry data acquisition

Desalted peptides were reconstituted in 0.1% formic acid and spiked with Indexed retention time standards before analysis by nano-liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS) on an Orbitrap platform (Thermo Fisher Scientific, United States). Peptides were separated on a C18 analytical column ($75\ \mu\text{m} \times 25\ \text{cm}$) over a 60–120-min gradient. Data were acquired in both data-dependent and data-independent (DIA) acquisition modes. To monitor system performance and inter-batch variation, a pooled quality control sample was injected periodically throughout the analytical sequence.

2.1.5. Data processing and bioinformatic analysis

The DIA files were analyzed using DIA-NN (version 1.8.1). For protein identification and quantification, both peptide- and protein-level false discovery rates were kept $< 1\%$. Only proteins quantified by two or more unique peptides were retained for downstream analysis, and the data were normalized using locally estimated scatterplot smoothing and/or internal standards. Identified proteins were mapped to UniProt and Entrez Gene databases. Gene Ontology (GO) biological process enrichment analysis was performed using clusterProfiler in R (version 4.3+), based on a hypergeometric test with Benjamini–Hochberg correction. GO terms with a q -value < 0.05 were considered significantly enriched. Pathway concordance between the two tissue types was inferred when shared core GO terms were both significant and the relative difference in their enrichment strength (e.g., GeneRatio or $-\log_{10}[q]$) was $\leq 20\%$. This empirically determined threshold was selected to achieve an optimal balance between filtering out background noise (reducing false positives) and retaining biologically meaningful pathway regulations (minimizing false negatives). The raw mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium.

2.2. Digital light processing-based three-dimensional printing and luminal functionalization of the biomimetic lacrimal duct

The structural geometry of the biomimetic lacrimal duct, featuring a unidirectional Hasner valve with an inclined projection angle of 42°, was mathematically defined using computer-aided design software (SolidWorks; version 2020). To formulate the structural hydrogel (PAG), 15% (w/v) PEGDA, 3% (w/v) acrylamide (AAM), and 5% (w/v) GelMA were dissolved in PBS, supplemented with 0.25% (w/v) lithium phenyl-2,4,6-trimethylbenzoylphosphine as the photoinitiator. These specific concentrations were optimized based on preliminary screenings to balance printability and mechanical properties. Specifically, lower concentrations of PEGDA and AAM resulted in poor structural fidelity and inadequate mechanical strength for suturing, whereas higher concentrations of GelMA increased the viscosity to a level that compromised printing resolution.

The tubular constructs were initially fabricated without cells utilizing high-resolution DLP equipment (EFL-BP8601Pro, EFL, China) with an optical resolution of 25 µm. Post-printing, the scaffolds were rigorously washed in sterile PBS for 48 h to ensure the complete extraction of any unreacted, potentially cytotoxic monomers. For subsequent luminal functionalization, a bio-instructive, cell-laden pre-gel solution—comprising 5% GelMA, 5 mg/mL young dECM, and the 28-day *in vitro*-induced ESCs—was uniformly injected to coat the inner luminal surface, effectively seeding the cells onto the pre-fabricated constructs. This cellularized epithelial layer was then firmly adhered and stabilized via a secondary ultraviolet (UV) crosslinking step at 405 nm for 45 s. To confirm that secondary UV exposure did not compromise cell viability, we performed Live/Dead staining immediately after crosslinking, which showed that over 90% of the cells remained viable. Finally, the printability and macroscopic shape fidelity of the fabricated constructs were quantitatively assessed using digital calipers.

2.3. Comprehensive biomechanical, hydrodynamic, and biological characterization

2.3.1. Mechanical fatigue and hydrodynamic stability testing

To comprehensively evaluate the long-term durability of the 3D-printed artificial lacrimal duct, fatigue resistance was assessed at both the bulk material and functional device levels.

For bulk material cyclic compression, uniaxial cyclic compression tests were performed to evaluate the intrinsic resilience of the PAG composite hydrogel. Cylindrical

specimens were placed in a universal mechanical testing machine and subjected to 1,000 continuous compression cycles at a strain of 20% and a displacement rate of 5 mm/min. The Young's modulus was calculated from the linear elastic region (5–15% strain) of the stress–strain curves before the 1st cycle and immediately after the 1,000th cycle to quantify any mechanical degradation.

To evaluate the dynamic structural stability of the unidirectional leaflet under repetitive physiological fluidic stress, a custom pulsatile flow circuit was utilized. The 3D-printed valved ducts were subjected to 1,000 continuous opening-and-closing cycles driven by a programmable syringe pump, simulating the alternating hydrodynamic forces of tear drainage and transient retrograde impulses. Following the cyclic fluidic fatigue protocol, the ducts were re-evaluated on the hydrodynamic testing platform to measure their post-fatigue “reverse burst point”—defined as the critical pressure threshold at which retrograde fluid leakage was first detected (flow rate > 0) and orthograde flow resistance. Crucially, the high reverse burst pressure of approximately 90 mmHg was stably maintained even after the 1,000-cycle fatigue test. The retention of this high reverse burst pressure (~90 mmHg) without leaflet avulsion or prolapse served as the definitive indicator of the valve's functional durability.

2.3.2. Fluid–structure interaction computational modeling

To simulate the dynamic mechanical response of the unidirectional valve under retrograde pressure, CFD coupled with a fluid–structure interaction model was executed using ANSYS Fluent 2022 (ANSYS, Inc., United States). The physical properties of the PAG hydrogel and water (density: 998 kg/m³, dynamic viscosity: 0.001 Pa·s) were input as material parameters. A retrograde boundary condition was set at the distal orifice to simulate the transient high-pressure impulse of forceful nose-blowing. The fluid–structure interaction solver iteratively calculated the elastic deformation of the flexible leaflet and its impact on the fluid domain. Velocity vector fields, static pressure contours, and velocity magnitude profiles were extracted to visually validate the hermetic sealing mechanism.

2.3.3. *In vitro* hydrodynamic benchtop evaluation

A custom-built hydrodynamic microfluidic testing platform was assembled to empirically measure the orthograde and retrograde flow characteristics. The 3D-printed valved and straight (control) ducts were secured within a sample clamp. A high-precision syringe pump (Harvard Apparatus, United States) infused PBS into the system at pre-calibrated incremental flow rates. Two inline digital pressure transducers (P1 and P2) were

positioned at the inlet and outlet to continuously log the pressure gradient. Under orthograde testing, flow was directed from the proximal to the distal end. Conversely, for retrograde testing (anti-reflux validation), flow was directed against the valve, and the “reverse burst point” was strictly recorded.

2.3.4. Quantitative cell viability assay

The biocompatibility and pro-proliferative capacity of the bio-coatings were quantified using a Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan). ESCs (Cybercell Biotech, China) were seeded onto pure GelMA and dECM + GelMA hydrogel discs at a density of 1×10^4 cells/well. At culture days 1, 3, 5, and 7, the CCK-8 working solution was added and incubated at 37 °C for two hours. The absorbance at 450 nm was measured using a microplate reader (Multiskan, Thermo Fisher, United States). Relative cell viability was normalized to the absorbance value of the respective groups on day 1 (set as 100%). All experiments were performed with three biological replicates and three technical replicates.

2.4. Cell isolation, culture, and directed differentiation

2.4.1. Isolation and culture of rabbit epidermal stem cells

Full-thickness skin (1–2 cm²) from the dorsum or ear margin of 2–4-week-old New Zealand White rabbits was aseptically excised and rinsed in PBS containing 1% antibiotic/antimycotic. With the dermis facing downward, the tissue was incubated in Dispase II (2.4 U/mL) overnight at 4 °C or for 1–2 h at 37 °C to separate the epidermis. The epidermis was then minced and digested with 0.05% trypsin–ethylenediaminetetraacetic acid at 37 °C for 5–10 min. Digestion was neutralized with medium containing 10% FBS, the suspension was passed through a 70 µm cell strainer, and cells were collected by centrifugation at 300–400× *g* for five minutes. Pelleted cells were resuspended in a low-calcium keratinocyte culture medium (Dulbecco's Modified Eagle Medium:F12 = 3:1) supplemented with B27, epidermal growth factor (EGF; 10 ng/mL), basic fibroblast growth factor (10 ng/mL), insulin (5 µg/mL), hydrocortisone (0.5 µg/mL), norepinephrine (0.1 µM), antibiotics, and $\text{Ca}^{2+} \leq 0.09$ mM. Cells were seeded at $1\text{--}2 \times 10^4$ cells/cm² onto plates coated with type I collagen or Matrigel and maintained at 37 °C with 5% CO₂. The medium was changed at 24 h to remove nonadherent cells and subsequently refreshed every 48 h. At 70–80% confluence, selective detachment via brief, gentle trypsinization (1–2 min) was used for passaging at a 1:3–1:4 split ratio.

2.4.2. Construction of age-specific three-dimensional microenvironments and staged lacrimal epithelial induction

We established a 3D induction system to evaluate the age-specific microenvironmental effects on guiding rabbit ESCs toward a lacrimal duct epithelial lineage. First, decellularized lacrimal duct extracellular matrix homogenates from young (Y) and old (O) rabbits were generated separately via mechanical milling, DNase/RNase digestion, and iterative washing. Residual DNA content was verified to be <50 ng/mg (dry weight), followed by sterile filtration through a 0.22 µm membrane.

To construct the 3D culture platforms, the Y-dECM and O-dECM homogenates were blended with GelMA (1 mg/mL), respectively. Consequently, three experimental groups were established: the Y-dECM hydrogel, the O-dECM hydrogel, and a pure composite hydrogel (Control) serving as the blank control. These precisely engineered hydrogels successfully recapitulate the requisite mechanical and adhesive cues of the native duct. The induction protocol for these three groups proceeded in defined phases.

To facilitate differentiation of seeded ESCs toward a functional lacrimal epithelial lineage within the 3D tubular microenvironment, a meticulously staged 28-day induction regimen was implemented:

- (i) Stage I: Cell adaptation and priming (Days –2 to 0). Following cell encapsulation onto the luminal dECM/GelMA coating, initial survival and homogeneous cellular spreading were promoted by incubating the constructs for 48 h in a calcium-depleted basal medium ($\text{Ca}^{2+} \leq 0.09$ mM). This priming broth was enriched with 10 ng/mL EGF and 10 µM of the ROCK inhibitor Y27632.
- (ii) Stage II: Lacrimal lineage commitment (Days 0–5). To initiate specific progenitor differentiation while avoiding premature differentiation, the low-calcium environment was maintained. The biochemical niche was modified by supplementing the medium with 20 ng/mL bone morphogenetic protein 4 (BMP4), 10 ng/mL fibroblast growth factor 10 (FGF10), and 10 ng/mL EGF. To ensure the stability of the FGF/BMP pathways, 1 µg/mL heparin was introduced. Furthermore, hyperkeratinization was effectively suppressed, and BMP signaling was finely modulated through intermittent administration of 50 ng/mL Noggin.
- (iii) Stage III: Epithelial and phenotypic maturation (Days 5–16). To drive tight junction assembly and mucin secretion, physiological calcium concentrations were progressively restored to 0.9–1.2 mM. Concurrently, growth factor levels were adjusted, titrating EGF

down to 5 ng/mL and setting BMP4 at 10 ng/mL. A secretory luminal phenotype was robustly amplified by introducing 50 nM all-trans retinoic acid (RA) alongside cAMP pathway activators (either 5 μ M forskolin or 100 μ M 8-Br-cAMP). To accelerate basement membrane deposition without impeding the overall epithelialization process, a brief pulse of transforming growth factor beta 1 (TGF- β 1; 0.1–0.5 ng/mL) was administered for a restricted ~48-h window.

- (iv) Stage IV: Functional barrier consolidation (Days 16–28). Ultimate functionalization was achieved by shifting the bioengineered ducts to an air–liquid interface (ALI) system. To mimic the physiological osmolarity of natural tears, the constructs underwent transient daily osmotic stress (320–340 mOsm for 2–4 h) modulated by mannitol or minimal sodium chloride. During this terminal phase, TGF- β and the ROCK inhibitor were withdrawn to consolidate barrier integrity and epithelial polarity, while basal levels of EGF (2–5 ng/mL) and BMP4 (5–10 ng/mL) were maintained.

Overall, this temporally calibrated strategy synergizes biochemical signaling (EGF/BMP4 coordination, targeted TGF- β /BMP antagonism, cAMP amplification) with biophysical cues (ALI and osmotic conditioning) to orchestrate highly efficient lacrimal epithelialization upon the biomimetic inner surface.

Upon completion of the 28-day regimen, the *in vitro*-induced ESCs (now exhibiting a mature lacrimal epithelial phenotype) were carefully harvested. To construct the final biomimetic artificial duct, these pre-induced cells were re-suspended in a luminal bio-coating solution comprising GelMA and the respective dECM homogenate. This cell-laden mixture was uniformly applied as a functional coating onto the inner surface of the 3D-printed PAG scaffold. Crucially, a secondary UV photo-crosslinking step was executed to firmly adhere and stabilize this induced epithelial layer onto the luminal wall. This modular assembly successfully established a highly biomimetic and structurally integrated artificial lacrimal duct, perfectly tailored for subsequent *in vivo* implantation.

2.4.3. Phenotypic characterization and gene expression analysis

Upon completion of the 28-day induction protocol, phenotypic and transcriptomic evaluations were independently performed on the constructs from all three experimental groups (Y-dECM, O-dECM, and Control). To assess specific protein expression, the samples were subjected to immunofluorescence staining (LSM 900, Carl Zeiss, Germany). Primary antibodies targeting epithelial

and barrier markers, including mouse anti-cytokeratin 18 (CK18; ab32118, Abcam, United Kingdom; 1:200), mouse anti-mucin 1 (MUC1; NBP1-60046SS, Novus Biologicals, United States; 1:200), and rat anti-zona occludens-1 (ZO-1; 61-7300, Invitrogen, United States; 1:500), were utilized for visualization.

For transcriptomic quantification, total RNA was extracted from the cell-laden constructs using TRIzol reagent, followed by DNase I digestion to eliminate genomic DNA contamination. RNA yield and purity were verified spectrophotometrically via a NanoDrop instrument, confirming acceptable A260/280 (1.9–2.1) and A260/230 (>1.8) ratios. Subsequently, 1 μ g of the purified RNA was reverse-transcribed into complementary DNA employing a blend of random hexamers and oligo(dT) primers. To ensure amplification specificity, exon junction-spanning primer pairs (amplicon size 80–200 bp; annealing temperature $\approx$ 60 $^{\circ}$ C) were deployed as follows:

- | | |
|-----------------------------|----------|
| (i) <i>AQP5</i> : | Forward: |
| 5'-AGTGGGGAAGAGCAGGTAGA-3'; | reverse: |
| 5'-TCTCCATTCTGCAAATCGCG-3' | |
| (ii) <i>KRT5</i> (CK5): | Forward: |
| 5'-ATCTCCTCGTACTGGGCCTT-3'; | reverse: |
| 5'-CAAATCGACCCCAACATCCA-3' | |
| (iii) <i>KRT8</i> (CK8): | Forward: |
| 5'-CGGAGATCTCGGTCTTCGTG-3'; | reverse: |
| 5'-CCTGGAGCAGCAGACAAGA-3' | |

Real-time polymerase chain reaction (PCR) assays were performed utilizing a SYBR Green-based master mix. The thermocycling program consisted of an initial denaturation phase at 95 $^{\circ}$ C for 2.5 min, followed by 40 amplification cycles (95 $^{\circ}$ C for 15 s; 60 $^{\circ}$ C for 40 s). A terminal melt-curve stage was integrated to corroborate product specificity. In strict accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines, all conditions were assessed with a minimum of three biological and three technical replicates. The relative mRNA levels of *AQP5*, *KRT5*, and *KRT8* were determined using the $2^{-\Delta\Delta C_t}$ method, with *GAPDH* as the endogenous reference gene for normalization.

2.4.4. Assessment of epithelial barrier integrity via transepithelial electrical resistance measurement

To quantitatively evaluate the dynamic development of epithelial barrier function and the maturation of tight junctions, transepithelial electrical resistance (TEER) was continuously monitored over a 21-day culture period. While the overall epithelial induction protocol spanned 28 days, the initial 7 days were dedicated to cell attachment, spreading, and early lineage commitment, during which stable tight junctions had not yet formed, and TEER values

were negligible. Therefore, continuous TEER monitoring commenced on day 7 and was recorded every other day for the subsequent 21 days (up to day 28) to specifically capture the maturation phase of the barrier. Measurements were performed across the cell-laden experimental groups (young dECM and aged dECM) and a Control group. Specifically, the Control group consisted of the corresponding cell-free hydrogel scaffolds, serving as the baseline reference for the intrinsic electrical resistance of the biomaterials.

Measurements were conducted utilizing an epithelial volt-ohm meter (EVOM3, World Precision Instruments, United States) equipped with sterilized chopstick electrodes. Prior to each recording, the constructs were transiently equilibrated to 37 °C to ensure stable and reproducible electrical conductivity. The electrodes were positioned vertically to simultaneously immerse them in the apical (luminal) and basolateral compartments, strictly avoiding any physical disturbance of the luminal surface.

Raw resistance values (Ω) were recorded in triplicate for each sample. The final TEER values were normalized to the effective growth surface area (A , in cm^2) of the construct using **Equation 1**:

$$\text{TEER}(\Omega \cdot \text{cm}^2) = R_{\text{measured}} \times A \quad (1)$$

Data were acquired from at least three independent biological replicates per group.

2.4.5. Cell viability and ultrastructural morphological analysis

To assess 3D cellular viability and spatial distribution, the engineered constructs were incubated with a Live/Dead staining solution (2 μM Calcein AM and 4 μM EthD-1) in the dark for 45 min at 37 °C. Fluorescence signals were captured utilizing a confocal laser scanning microscope (LSM 900, Carl Zeiss, Germany). Serial Z-stack optical sections were acquired across the luminal interface and processed into 3D volumetric projections to visualize the cellular network. For ultrastructural characterization, parallel samples were fixed overnight in 2.5% (v/v) glutaraldehyde at 4 °C. The constructs were then sequentially dehydrated through a graded ethanol series and dried using hexamethyldisilazane. Following gold sputter-coating, the cellular adhesion morphology and surface topography were examined using a field-emission scanning electron microscope (Regulus 8100, Hitachi, Japan). Micrographs were systematically acquired at an accelerating voltage of 5.0 kV across magnifications ranging from 150 $\times$ to 3,500 $\times$.

2.5. In vivo rabbit lacrimal duct reconstruction model

2.5.1. Animals and grouping

Thirty male New Zealand White rabbits (2.5–3.0 kg, 3–4 months old) were obtained from the Beijing TongHe Lita Biotechnology Co. Ltd. The study protocol was approved by the Institutional Animal Care and Use Committee. The animals were randomly divided into five groups ($n = 6$ per group):

- (i) Group 1 (Sham control): Surgical exposure of the lacrimal duct followed by immediate closure, serving as a baseline for surgical trauma and natural healing.
- (ii) Group 2 (Defect model): A 10-mm segment of the membranous duct was completely excised to create a full-thickness defect. No scaffold or stent was implanted into the gap.
- (iii) Group 3 (Jones tube): The defect was bridged with a medical-grade Jones tube, which represents the current clinical gold standard and serves as the positive control.
- (iv) Group 4 (Bio-Straight): The defect was bridged using a 3D-printed straight tubular scaffold (fabricated from the PAG composite hydrogel), synergistically integrated with the young dECM luminal coating and ESCs.
- (v) Group 5 (Bio-Valve): The defect was bridged using a 3D-printed tubular scaffold featuring an internal anti-reflux unidirectional valve (fabricated from the PAG composite hydrogel), synergistically integrated with the young dECM luminal coating and ESCs.

2.5.2. Surgical procedures

General anesthesia was induced via marginal ear vein injection of 3% pentobarbital sodium (30 mg/kg), and local anesthesia was supplemented with 2% lidocaine. A curvilinear incision was made along the infraorbital rim to meticulously expose the underlying membranous duct.

In Group 1 (Sham), the duct was exposed but left completely intact. In Groups 2, 3, 4, and 5, a 10-mm segment of the membranous duct was surgically excised to create a critical-sized defect. For Group 2 (Model), the created defect was left untreated.

For Group 3 (Jones tube), a standard medical-grade Jones tube was implanted to bridge the defect and connect the two severed ends. To prevent clinical migration, the prosthesis was securely anchored to the surrounding connective tissues using 6–0 non-absorbable sutures.

For Groups 4 (Bio-Straight) and 5 (Bio-Valve), the 3D-printed PAG composite hydrogel scaffolds (15% PEGDA, 3% acrylamide, and 5% GelMA) loaded with

young dECM and *in vitro*-induced ESCs—configured as the straight and valved ducts, respectively—were directly implanted to bridge the defect.

Crucially, for Group 5 (Bio-Valve), the construct was strictly oriented to ensure the internal unidirectional valve opened towards the distal duct. The proximal and distal ends of the constructs were meticulously sutured to the host lacrimal duct stumps using 8–0 absorbable sutures to achieve a watertight end-to-end anastomosis, maintaining structural integrity and luminal patency without the need for internal stents.

2.6. Clinical functional evaluation

To comprehensively evaluate the functional restoration of the reconstructed lacrimal duct, clinical assessments including epiphora grading, the fluorescein dye disappearance test (FDDT), and lacrimal irrigation were performed.

2.6.1. Epiphora grading

The severity of epiphora was assessed longitudinally at pre-operation and at 1, 2, 4, and 8 weeks post-operation. A blinded observer graded the tearing severity using a standardized 5-point scale: 0: No epiphora; 1: Occasional tearing (requires wiping < 2 times/day); 2: Mild tearing (requires wiping 2–4 times/day); 3: Moderate tearing (requires wiping 5–10 times/day); and 4: Severe tearing (continuous overflow, periocular hair loss, or dermatitis). Changes in symptom scores were recorded to generate the tearing score curve.

2.6.2. Fluorescein dye disappearance test

To quantitatively assess the physiological patency and “lacrimal pump” efficacy of the bioengineered ducts, the FDDT was conducted. Briefly, a 20 μ L drop of 2% sodium fluorescein solution was administered into the conjunctival sac of the surgical eye. The ocular surface was subsequently examined under cobalt blue illumination, and the exact duration necessary for the complete clearance of the fluorescent dye from the tear meniscus was documented in minutes. For statistical calculations, instances where dye retention exceeded 20 min were capped at 20 min.

2.6.3. Lacrimal irrigation and patency rate

Lacrimal irrigation was performed to quantitatively assess the drainage function. A fixed volume of physiological saline V_{injected} was injected into the lacrimal punctum using a blunt cannula. Any fluid regurgitating from the punctum was collected and measured $V_{\text{regurgitated}}$. The average patency rate for each group was calculated based on the volume ratio using Equation 2:

$$\text{Patency Rate} = \frac{1}{N} \sum_{i=1}^N \frac{V_{\text{injected}} - V_{\text{regurgitated}}}{V_{\text{injected}}} \times 100\% \quad (2)$$

where represents the volume of saline injected into the lacrimal duct; represents the volume of saline that regurgitated; and is the total number of rabbits in the group.

2.7. Imaging assessment

2.7.1. Computed tomography dacryocystography

To longitudinally evaluate the structural integrity, luminal patency, and specific anti-reflux functionality of the reconstructed tracts *in vivo*, high-resolution contrast-enhanced computed tomography (CT) was performed at 2 and 8 weeks post-operation. To precisely outline the grafted segments in the clinical control cohort, the implanted silicone tubes (Group 3) were equipped with a built-in radiopaque strip. The imaging protocols were strategically tailored as follows.

High-resolution contrast-enhanced CT was performed at 2 and 8 weeks post-operation to assess ductal integrity, patency, and anti-reflux function. Group 3 silicone tubes included a radiopaque strip for imaging detection. The protocols were as follows. At both 2 and 8 weeks post-operation, Groups 1 (Sham), 2 (Model), and 3 (Jones Tube) exclusively underwent standard orthograde contrast imaging. Under general anesthesia, an iodine-based contrast agent (Ioversol) was gently infused downward into the lacrimal system via the punctum to assess physiological patency, pathological obliteration, or clinical prosthesis drainage. Similarly, at 2 weeks post-operation, Groups 4 (Bio-Straight) and 5 (Bio-Valve) also received orthograde injections to confirm the early-stage continuity and baseline patency of the bioengineered conduits.

At 8 weeks post-operation, a retrograde contrast injection was performed in Groups 4 and 5 to evaluate anti-reflux function. Ioversol was infused retrogradely from the distal orifice at a calibrated pressure of ~ 70 mmHg (9.3 kPa), precisely simulating the peak hydrodynamic stress of forceful nose-blowing (a rigorous clinical “worst-case” scenario). This comparative stress test directly visualized the Group 5 valve’s efficacy in blocking retrograde fluid migration, in contrast to the expected backflow in the Group 4 straight scaffold.

Following the respective contrast administrations, the animals underwent high-resolution CT scanning (SOMATOM Force, Siemens Healthineers, Germany). The acquired tomographic datasets were imported into imaging software to generate detailed 3D reconstructions, enabling macroscopic assessment of scaffold positioning,

ductal patency, and the critical unidirectional blocking capability.

2.7.2. Lacrimal endoscopy

To directly visualize the internal luminal surface, mucosal integration, and structural patency of the reconstructed ducts *in vivo*, lacrimal endoscopy was performed. In strict accordance with the longitudinal observation design established for CT dacryocystography, endoscopic evaluations were conducted precisely at 2 and 8 weeks post-operation. Under general anesthesia, a flexible lacrimal endoscope was carefully introduced into the lacrimal passage to inspect the mucosal healing status and any signs of obstruction or inflammation.

2.7.3. Hematoxylin and eosin staining

The harvested bioengineered constructs and tissue samples were fixed in 4% paraformaldehyde for 24 h, dehydrated through a graded series of ethanol, cleared in xylene, and embedded in paraffin. Sectioning was performed at a thickness of 5 μ m using a microtome. After deparaffinization and rehydration, the sections were stained with hematoxylin solution for five minutes, then rinsed in running tap water. The sections were then differentiated with 1% acid alcohol, blued in tap water, and counterstained with eosin solution for two minutes. Finally, the sections were dehydrated, cleared in xylene, and mounted with a synthetic resin. Histological evaluation was performed using an optical microscope (DM2500, Leica Microsystems, Germany).

2.8. Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD) and analyzed using GraphPad Prism 7.0. Statistical significance was assessed using twotailed Student's *t*-tests or oneway analysis of variance in the Statistical Package for Social Sciences 24.0, as appropriate. A *p*-value < 0.05 was considered statistically significant.

3. Results

3.1. Validation of young lacrimal decellularized extracellular matrix as an ideal bioactive niche for bioinks through proteomic analysis

To elucidate the molecular basis of the “microenvironment aging” hypothesis and justify the selection of young donor tissue for hydrogel formulation, comprehensive label-free quantitative proteomics was performed to compare the decellularized lacrimal duct matrix from young (Y) and old (O) rabbits.

Hierarchical clustering of the proteomic profiles revealed a striking, distinct segregation between the

young and old dECM groups, underscoring substantial age-related alterations in the matrix composition (Figure 1A). The volcano plot further illustrated the distribution of differentially expressed proteins (DEPs) (Figure 1B). Strikingly, the young dECM was significantly enriched in crucial structural and pro-regenerative matrix proteins. Notably, periostin (POSTN), laminin subunit alpha-2 (LAMA2), and prolyl 4-hydroxylase subunits (P4HA1, P4HA2, P3H1—essential enzymes for collagen stabilization and ECM remodeling) were highly upregulated in the young group. Conversely, the old dECM exhibited an upregulation of aging-associated, fibrotic, and inflammatory markers, including vimentin, Fibronectin, and complement cascade components (C5, C9).

To further decode the functional implications of these DEPs, GO enrichment analysis was conducted. Cellular component analysis confirmed that the major variations were intrinsically matrix-driven, with profound enrichments in the “extracellular space,” “basement membrane,” “collagen trimer,” and “laminin-3 complex” (Figure 1D). Correspondingly, biological process analysis showed that the upregulated proteins in the young dECM were highly involved in matrix assembly and cellular interactions, particularly enriched in pathways such as “protein hydroxylation,” “hyaluronan metabolic process,” and “regulation of cell adhesion” (Figure 1C).

Finally, advanced functional clustering and chord diagram analyses (Figure 1E and F) visually corroborated that these youth-associated matrix proteins do not function in isolation but instead form a highly interconnected functional network that governs extracellular structural organization. Taken together, this omics-driven evidence strongly supports the hypothesis that the young dECM uniquely preserves a highly adhesive, basement membrane-like biochemical niche, making it an ideal bioactive additive for 3D printing to instruct targeted epithelial differentiation.

3.2. Biofabrication, mechanical resilience, and hydrodynamic validation of the three-dimensional bioprinted biomimetic anti-reflux artificial lacrimal duct

3.2.1. High-fidelity biofabrication and mechanical fatigue resistance

To physically actualize the intricate biomimetic hydrodynamic design, a high-resolution DLP-based 3D printing system was employed (Figure 2A and B). Renowned for its rapid, spatially controlled photopolymerization capabilities, the DLP technology ensured that the specific geometric parameters and precise projection angles of the unidirectional Hasner valve, as

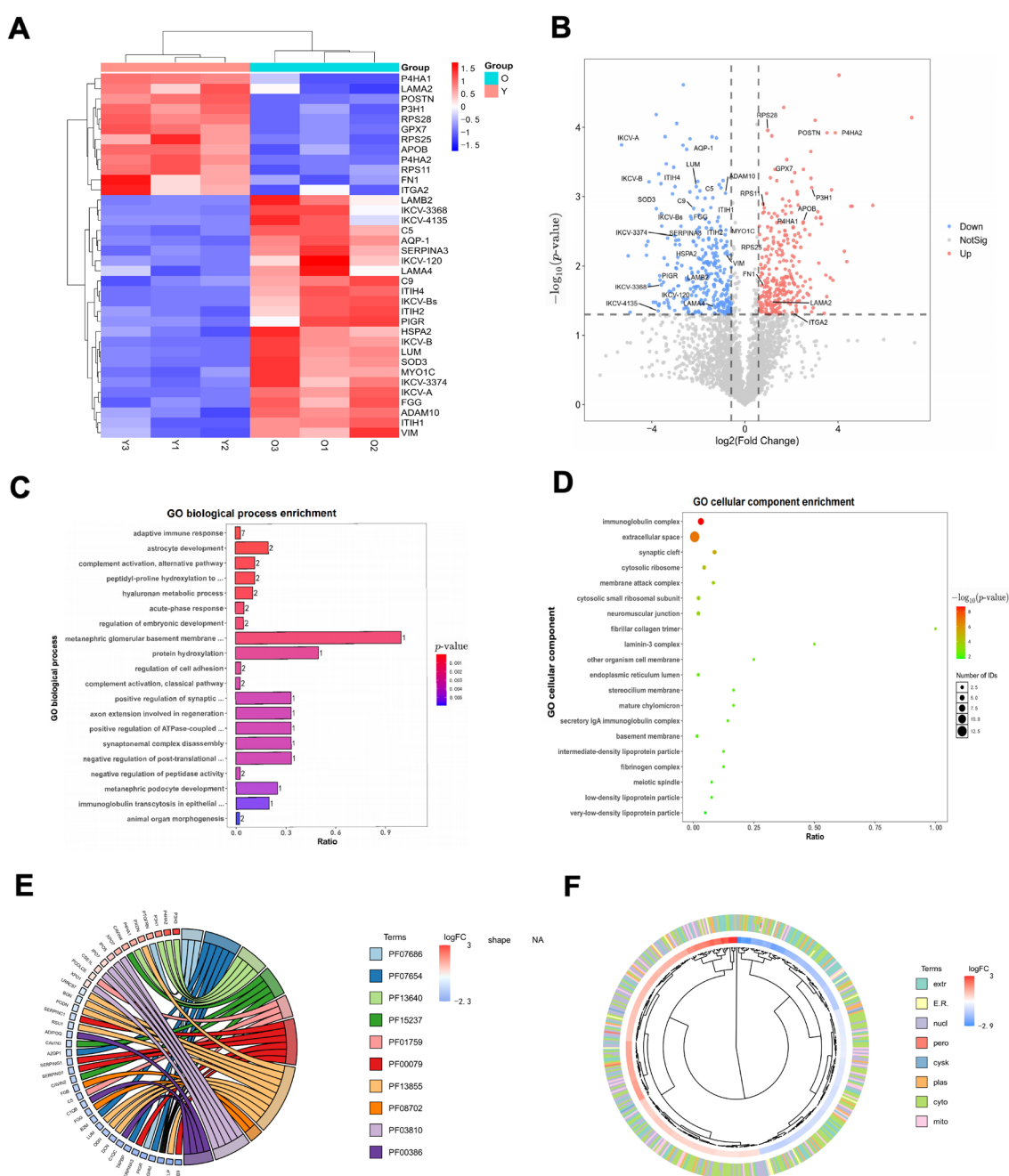


Figure 1. Quantitative proteomic profiling of age-specific decellularized lacrimal duct matrices reveals a pro-regenerative microenvironmental signature in young donors. (A) Hierarchical clustering heatmap delineating the distinct global proteomic landscapes of the young (Y) and old (O) decellularized extracellular matrix (dECM) groups ($n = 3$ per group). Relative protein abundances are depicted by a continuous color gradient from blue (low) to red (high). (B) Volcano plot visualizing the global distribution of differentially expressed proteins (DEPs). The young dECM is profoundly enriched with core structural and pro-regenerative matrix proteins (red nodes, highlighting POSTN, LAMA2, and P4HA1), in stark contrast to the upregulation of age-associated and inflammatory markers in the old dECM (blue nodes). (C) Bar graph of the top enriched Gene Ontology (GO) biological process terms. The analysis underscores a targeted enrichment in pathways critical for tissue reconstruction, notably the regulation of cell adhesion and protein hydroxylation. (D) Bubble chart mapping the significantly enriched GO cellular component terms, confirming the compositional dominance of extracellular space, basement membrane, and collagen trimer elements in the juvenile niche. Node size is proportional to the protein count, with the color spectrum reflecting statistical significance ($-\log_{10} p$ -value). (E) Chord diagram delineating the intricate network connectivity between specific high-abundance DEPs and their corresponding functional GO clusters. (F) Circular dendrogram displaying the hierarchical functional clustering and correlative fold-change profiles of the targeted protein modules.

defined by the computer-aided design model (Figure 2D), were faithfully translated into the physical construct. Macroscopic evaluations (Figure 2D and E) and digital caliper measurements (Figure 2F) confirmed that the printed PAG composite hydrogel possessed exceptional printability and shape fidelity. Utilizing the DLP modality, the hydrogel maintained a precise tubular geometry and a highly patent internal lumen without swelling-induced structural collapse. Furthermore, to endure the dynamic physiological stresses within the craniomaxillofacial region, mechanical fatigue resistance is paramount. Following 1,000 cyclic compressions, the Young's modulus of both the bare PEGDA–PAAm and the dECM–GelMA-coated scaffolds exhibited no significant degradation (Figure 2H), demonstrating the remarkable mechanical robustness and structural stability necessary for long-term *in vivo* implantation.

3.2.2. Theoretical and empirical validation of anti-reflux hydrodynamics

The functional core of the engineered duct lies in its unidirectional fluidic regulation. CFD was initially utilized to model the dynamic response of the flexible valve under retrograde high-pressure impulses. The velocity vector field (Figure 2I) revealed a transient fluid micro-jet during the initial closure phase. However, as retrograde pressure escalated, the flexible leaflet underwent profound elastic deformation, ultimately forming an absolute watertight seal against the luminal wall. This mechanical occlusion successfully truncated pressure transmission (Figure 2J) and reduced the proximal fluid velocity to absolute zero (Figure 2K).

To empirically validate these theoretical simulations, the printed constructs were evaluated using a custom-built *in vitro* hydrodynamic testing platform (Figure 2G). Crucially, the experimental flow rate–pressure curves (Figure 2L) perfectly corroborated the CFD predictions. Under orthograde conditions (positive axis), the valved duct exhibited linear, unobstructed fluid drainage, virtually identical to that of the straight control duct, confirming that the biomimetic valve imposes no pathological resistance to natural tear flow. Conversely, under retrograde conditions (negative axis), the valved duct completely blocked fluid migration until subjected to a massive “reverse burst point” of approximately 90 mmHg. Importantly, this robust reverse burst pressure of ~90 mmHg was maintained even after the constructs underwent the 1,000-cycle fluidic fatigue test. This sustained sealing capacity safely exceeds the physiological peak pressures generated during forceful nose-blowing (~70 mmHg), definitively proving its long-term mechanical stability and clinical anti-reflux efficacy.

3.2.3. Biocompatibility of the functional luminal coating

Finally, to ensure that the robust biomechanical scaffold could support cellular activity, *in vitro* cell viability was quantitatively assessed (Figure 2M). While the structural PAG scaffold provides mechanical integrity, the functional dECM + GelMA bio-coating significantly enhances cellular proliferation. Over a 7-day culture period, the dECM + GelMA group exhibited significantly higher cell viability than the GelMA-only control at days 5 and 7 ($***p < 0.0001$). This confirms the successful establishment of a highly biocompatible, pro-regenerative biochemical niche on the stable physical scaffold.

3.3. Promotion of lacrimal epithelial lineage commitment and functional barrier assembly *in vitro* by young decellularized extracellular matrix

To evaluate the bio-inductive capabilities and biocompatibility of the dECM-functionalized scaffolds, we subjected the seeded ESCs to a comprehensive *in vitro* assessment. First, protein-level phenotypic transformations were visualized via immunofluorescence (Figure 3A). After a 28-day induction, ESCs cultured on the young dECM coating exhibited robust and uniform expression of the epithelial structural marker CK18 and the mucin-secreting marker MUC1, in stark contrast to the negligible expression in the uninduced Control and the faint signals in the old dECM group. Additionally, the young dECM group displayed a continuous, membrane-localized distribution of the tight junction protein ZO-1, indicating advanced barrier assembly.

These protein-level observations were firmly corroborated by transcriptomic analysis via quantitative PCR (Figure 3B–D). The young dECM microenvironment significantly upregulated the mRNA expression of the mature luminal epithelial marker CK8 (Figure 3B) and the critical water channel AQP5 (Figure 3D) ($****p < 0.0001$) compared to both the old dECM and Control cohorts. Concurrently, the basal progenitor marker CK5 was effectively downregulated in the young dECM group (Figure 3C), confirming successful, targeted lineage commitment from stemness to a mature secretory epithelial phenotype.

The functional consequence of this ZO-1 tight junction assembly was quantified longitudinally by TEER measurements over a 21-day period (days 7 to 28 of the overall induction phase, specifically capturing barrier maturation) (Figure 3E). While the Control remained at baseline and the old dECM group plateaued at a moderate level (~450 $\Omega \cdot \text{cm}^2$), the young dECM group demonstrated a rapid, sustained exponential elevation in electrical

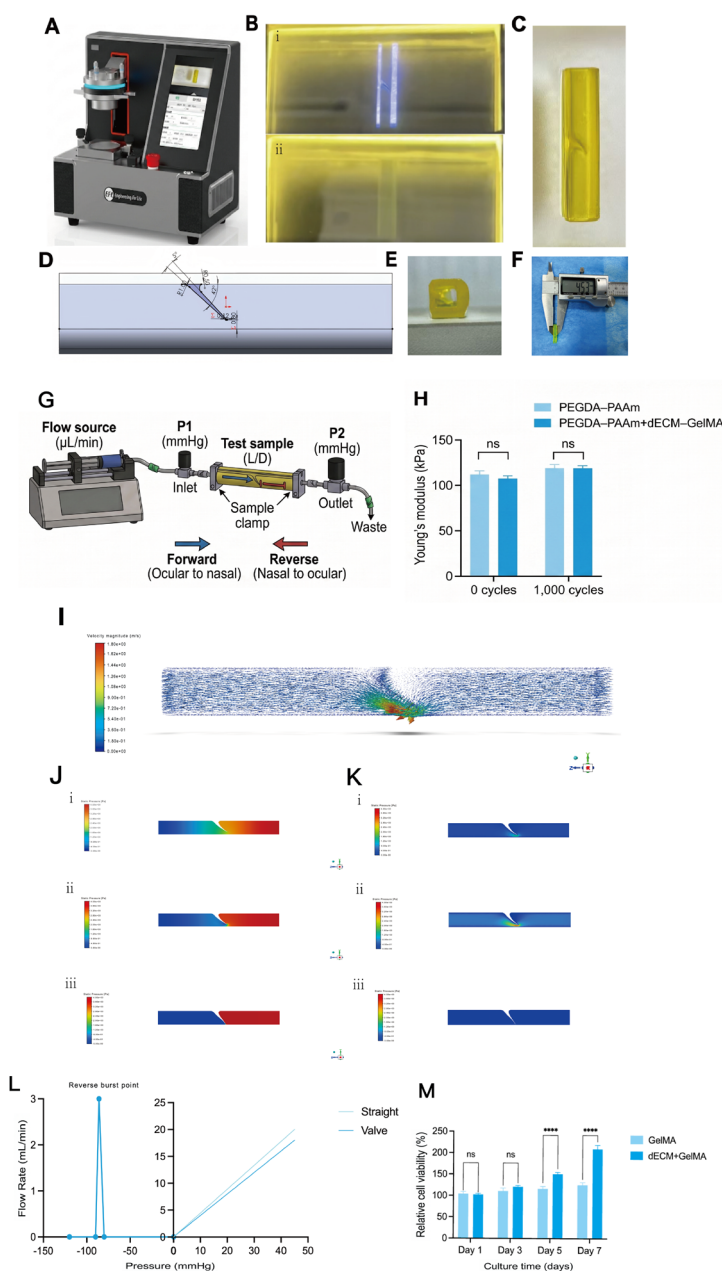


Figure 2. Biofabrication, biomechanical characterization, and hydrodynamic validation of the three-dimensional (3D)-printed biomimetic anti-reflux artificial lacrimal duct. (A) The high-resolution 3D printing system utilized for scaffold fabrication. (B) Real-time optical captures of the continuous photopolymerization process (i, ii) generating the tubular constructs. Scale bar: 10 cm. (C, E, F) Macroscopic photographs detailing the side view (C) and cross-sectional luminal view (E) of the printed hydrogel duct, alongside digital caliper measurements (F) confirming excellent shape fidelity and structural precision. Scale bar: 5 cm. For scale reference, the 3D-printed ducts shown in (B), (C), and (E) possess an outer diameter of 4.5 mm, an inner diameter of 4 mm, and a total length of approximately 4.5 mm (as confirmed in F). (D) Computer-aided design (CAD) schematic defining the exact geometric parameters and projection angles of the biomimetic Hasner valve. (G) Schematic illustration of the custom-built *in vitro* hydrodynamic testing platform used to assess orthograde drainage and retrograde sealing capacity. (H) Young's modulus of the PEGDA-PAAm and PEGDA-PAAm + dECM-GelMA scaffolds before (0 cycle) and after 1,000 cyclic compressions. Ns: not significant. (I–K) Computational fluid dynamics (CFD) simulations under retrograde flow conditions, including the velocity vector plot (I), sequential static pressure contours (J; i–iii), and velocity magnitude contours (K; i–iii). (L) Experimental flow rate–pressure curves for the straight and valved ducts under orthograde (positive axis) and retrograde (negative axis) conditions. (M) Relative cell viability of the GelMA and dECM + GelMA groups over a 7-day culture period. Data are presented as mean $\pm$ standard deviation. **** $p < 0.0001$.

Abbreviations: dECM: Decellularized extracellular matrix; GelMA: Gelatin methacryloyl; PAAm: Polyacrylamide; PEGDA: Poly(ethylene glycol) diacrylate.

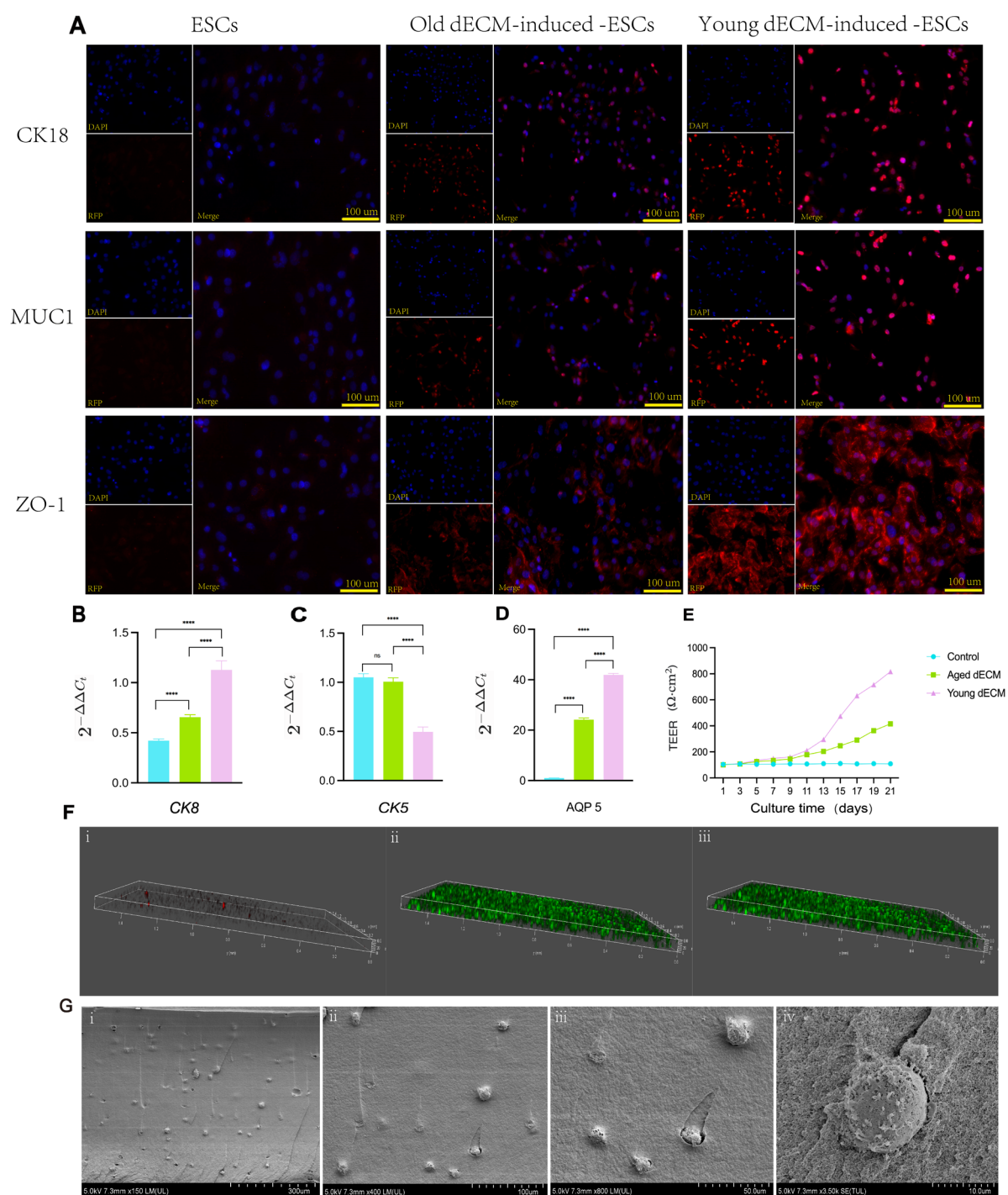


Figure 3. *In vitro* evaluation of lacrimal epithelial differentiation, barrier function, and cellular morphology on the dECM-functionalized scaffolds. (A) Immunofluorescence staining for CK18, MUC1, and ZO-1 in ESCs (Control), old dECM-induced ESCs, and young dECM-induced ESCs after 28 days of induction. Scale bar: 100 μm ; magnification: 200 $\times$. (B–D) Relative mRNA expression levels of CK8 (B), CK5 (C), and AQP5 (D) determined by quantitative real-time PCR. **** $p < 0.0001$; ns: not significant. (E) Transepithelial electrical resistance (TEER) values of the constructs recorded over a 21-day culture period. (F) 3D confocal volumetric reconstructions of Live/Dead staining (green: viable cells; red: dead cells) on the scaffolds at day 7. (G) Scanning electron microscopy images showing the surface topography and cellular ultrastructure of the bioengineered lumen at varying magnifications. Scale bars: 10 μm , 50 μm , 100 μm , 300 μm ; magnifications: 150 $\times$ (i), 400 $\times$ (ii), 800 $\times$ (iii), and 3,500 $\times$ (iv).

Abbreviations: dECM: Decellularized extracellular matrix; ESCs: Epidermal stem cells; MUC1: Mucin 1; PCR: Polymerase chain reaction; TEER: Transepithelial electrical resistance; ZO-1: Zonula occludens-1.

resistance. By day 21 of monitoring, the TEER value reached approximately 800–900 $\Omega\cdot\text{cm}^2$. This measurement significantly surpasses the physiological baseline of typical conjunctival epithelia ($\sim 200\text{--}300\ \Omega\cdot\text{cm}^2$) and firmly aligns with the highly impermeable barrier standards of mature corneal epithelia (typically $>500\ \Omega\cdot\text{cm}^2$)^{37,38}, thereby verifying the successful formation of a highly integral, functionally competent, watertight epithelial barrier.

To confirm the spatial viability of this newly formed epithelial layer, 3D volumetric Live/Dead confocal imaging was performed at day 7 (Figure 3F). The results revealed a highly confluent, 3D cellular network across the luminal surface with negligible dead cells (red fluorescence), indicating the excellent biocompatibility of the PAG composite scaffold. Finally, scanning electron microscopy was utilized to examine the ultrastructural morphology (Figure 3G). The micrographs demonstrated firm cellular adherence and spreading along the bio-engineered luminal surface. High-magnification views remarkably highlighted the development of dense apical microvilli and secretory-like cellular protrusions, structurally confirming the establishment of a highly polarized and functional lacrimal epithelial barrier.

3.4. Functional restoration and anatomical reconstruction in a rabbit lacrimal duct defect model

3.4.1. Macroscopic structural fidelity and clinical presentation

Prior to surgical implantation, macroscopic evaluation (Figure 4C) confirmed that the 3D-printed PAG composite scaffolds exhibited remarkable shape fidelity and precise dimensional accuracy (verified via digital calipers). Unlike conventional soft hydrogels that often suffer from severe swelling or structural collapse, the PAG scaffolds reliably maintained their pre-designed tubular geometry, thereby ensuring excellent structural integrity and handling characteristics for the subsequent delicate end-to-end anastomosis. Following successful surgical defect creation and in situ implantation (Figure 4A; 0 day), clinical outcomes were monitored longitudinally. At 4 weeks post-operation, the untreated Model group developed severe, persistent epiphora accompanied by prominent purulent discharge, confirming the natural pathological progression of ductal obliteration. Strikingly, the clinical positive control (Jones tube) exhibited typical foreign body complications, with frequent instances of prosthesis extrusion and migration (Figure 4A; 4 weeks). In stark contrast, both the Bio-Straight and Bio-Valve cohorts maintained a healthy, unobstructed ocular surface, devoid of inflammatory exudate, and macroscopically resembled

the physiological state of the Sham group.

3.4.2. Restoration of physiological drainage function

To quantitatively assess the functional restoration of the tear drainage pathway, lacrimal syringing and the FDDT were conducted (Figure 4B). The transition of the fluorescent dye from the tear meniscus to the nasal cavity definitively confirmed the establishment of anatomical continuity. Quantitatively, the Model group exhibited complete functional failure (dye retention capped at 20 min). Both the Bio-Straight and Bio-Valve constructs enabled rapid dye clearance, with complete clearance observed within approximately 5.5 min (Figure 4D). This clearance time was significantly faster than that of the Jones tube cohort ($****p < 0.0001$) and was statistically comparable to the native Sham baseline. Consequently, the overall clinical patency rates evaluated via irrigation in both printed groups ($>95\%$) were significantly higher than those of the standard Jones Tube intervention ($*p < 0.05$) (Figure 4E), indicating efficient fluid conduction without obstruction.

3.4.3. Longitudinal resolution of tearing symptoms

The sustained therapeutic efficacy of the printed constructs was further validated through longitudinal monitoring of clinical tearing scores over an 8-week period (Figure 4F). While the Model group suffered from persistent severe epiphora (score $\sim 3.8\text{--}4.0$), the Jones tube group experienced an initial drop but fluctuated around a moderate symptomatic level (score ~ 2.0) due to localized irritation and migration. Conversely, the tearing scores in both the bio-straight and bio-valve groups showed a steady decline. By week 8, their scores approached the asymptomatic baseline (0–1), highlighting that the dECM-functionalized biomimetic microenvironment not only restores physical patency but also promotes long-term symptom resolution and harmonious tissue integration.

3.5. Radiological, endoscopic, and histological confirmation of superior bio-integration and functional remodeling

To comprehensively evaluate the long-term anatomical continuity and hydrodynamic functionality *in vivo*, high-resolution contrast-enhanced CT imaging was performed at 8 weeks post-operation (Figure 5A). Multi-planar two-dimensional cross-sections and 3D volume-rendered reconstructions revealed that the surgical defect in the Model group led to severe anatomical obliteration of the lacrimal canal. In contrast, the 3D-printed hydrogel scaffolds in the Bio-Straight and Bio-Valve groups, alongside the clinical Jones tube group, maintained excellent tubular geometry and orthograde patency without

any signs of mechanical collapse. Most importantly, the specialized retrograde stress test imaging at this 8-week endpoint provided definitive visual validation of the biomimetic design. Under simulated hydrodynamic stress, the Bio-Straight scaffold failed to prevent fluid migration, exhibiting clear backflow contrast. In striking contrast, the

unidirectional valve in the Bio-Valve construct robustly blocked retrograde flow of the contrast agent, thereby maintaining a clear proximal lumen. This radiological evidence definitively confirms the PAG composite's long-term biomechanical stability and verifies the valve's critical hydrodynamic efficacy in preventing retrograde infection

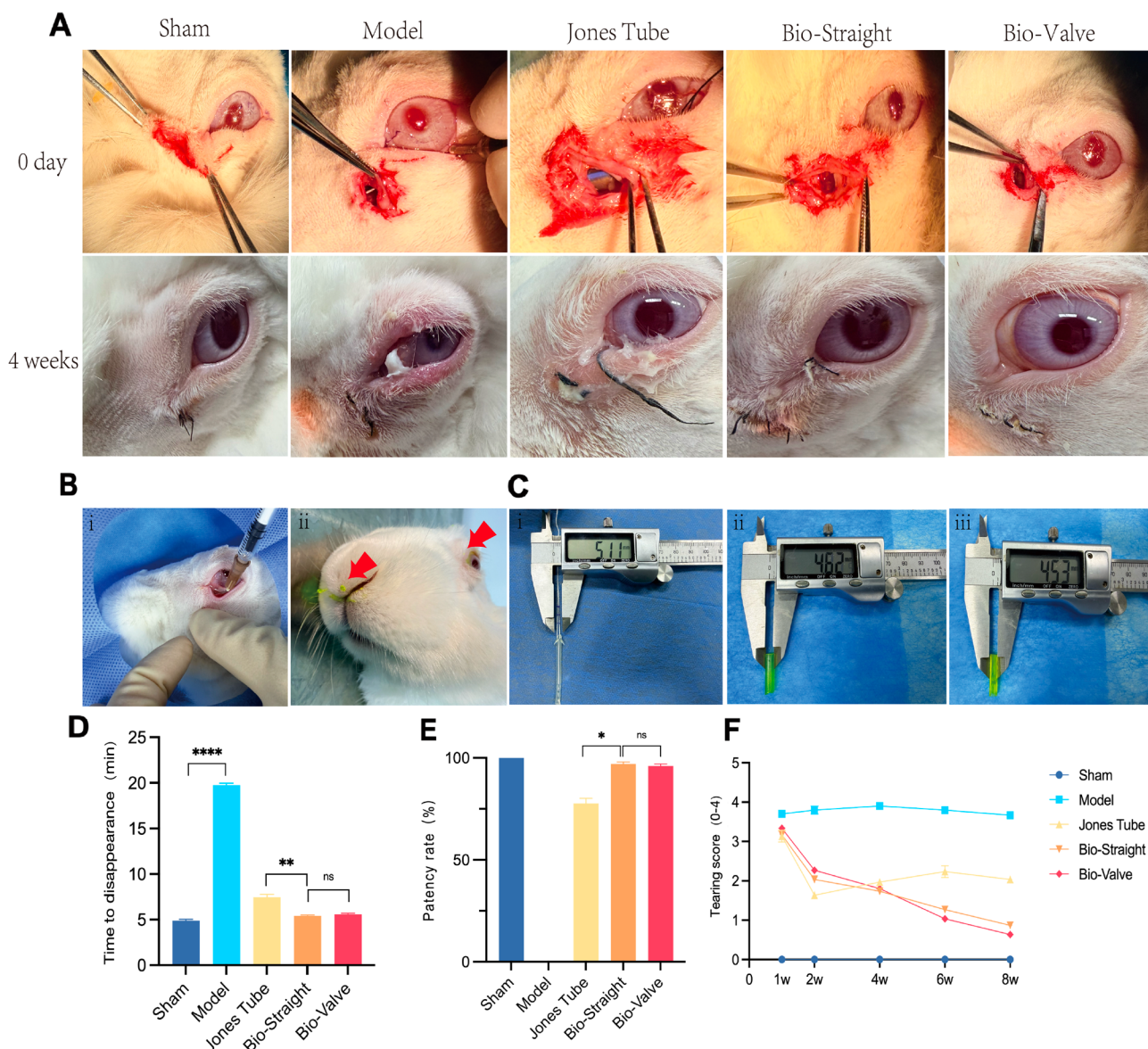


Figure 4. *In vivo* evaluation of lacrimal duct reconstruction and functional restoration in a rabbit model. (A) Representative macroscopic photographs of the surgical procedure and subsequent clinical outcomes. Top row (0 day): Surgical creation of the defect and *in situ* scaffold/prosthesis implantation across the cohorts. Bottom row (4 weeks): Macroscopic observation of the ocular surface and periocular regions in the Sham, Model, Jones tube, Bio-Straight, and Bio-Valve groups. (B) Clinical patency assessments. (i) Lacrimal syringing/irrigation procedure. (ii) Fluorescein dye disappearance test (FDDT) demonstrating the drainage pathway, with red arrows indicating the dye at the ocular tear meniscus and the nasal cavity. (C) Macroscopic explant evaluation. Digital caliper measurements of the explanted Jones tube (i), bio-Straight scaffold (ii), and bio-Valve scaffold (iii). (D) Quantitative analysis of the FDDT, measured as the time required for complete dye clearance. (E) The overall luminal patency rate evaluated via lacrimal irrigation. (F) Longitudinal monitoring of clinical tearing scores (graded 0–4) over the 8-week post-operative period. Data are presented as mean $\pm$ standard deviation. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$; ns: not significant.

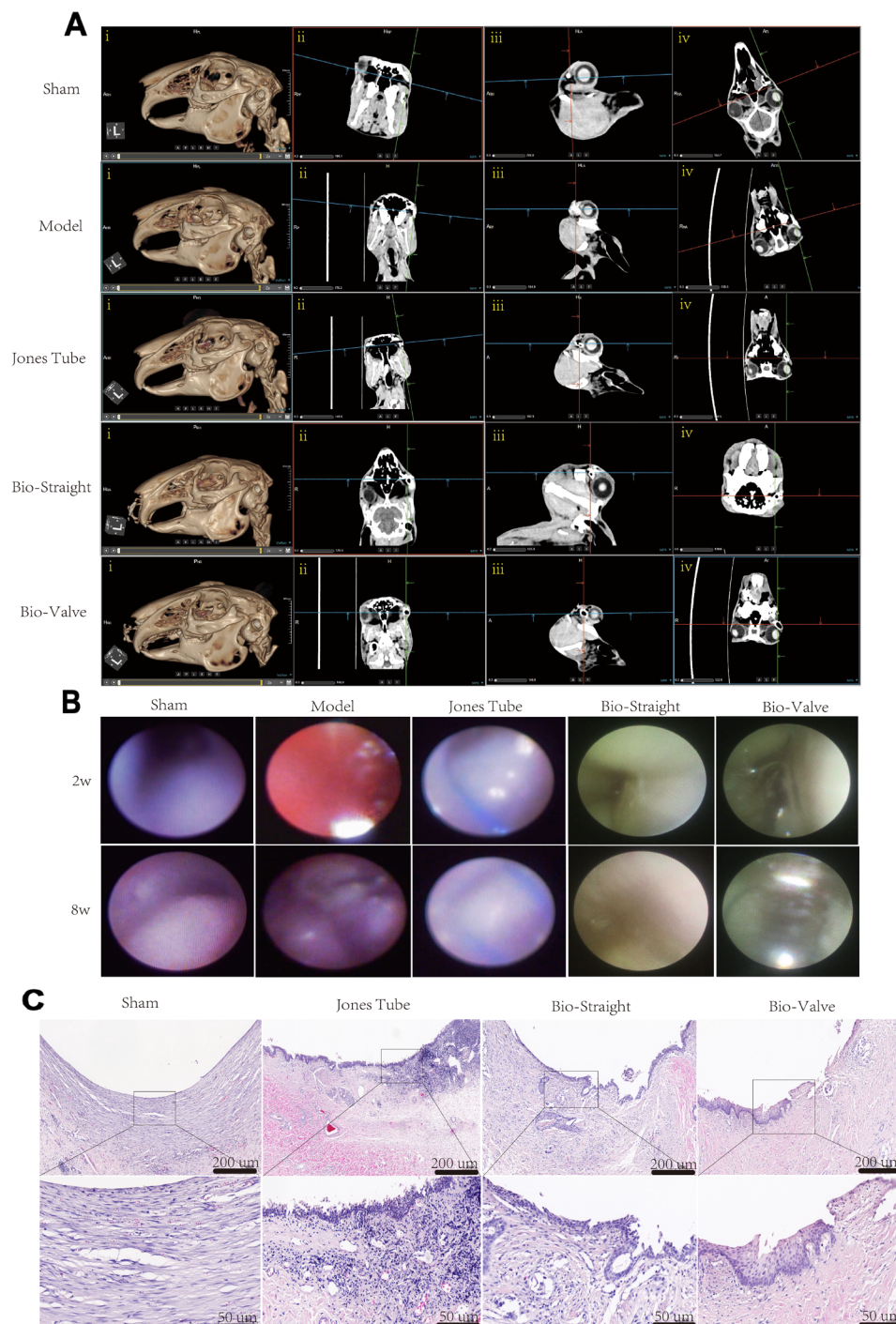


Figure 5. Radiological, endoscopic, and histological evaluation of the reconstructed lacrimal ducts *in vivo*. (A) Computed tomography (CT) imaging of the rabbit craniomaxillofacial region and lacrimal system across the five experimental cohorts. The images display (i) three-dimensional (3D) volume-rendered reconstructions alongside corresponding multi-planar 2D cross-sections in the (ii) coronal, (iii) sagittal, and (iv) axial planes to visualize the anatomical positioning and structural continuity of the defect sites and implanted constructs. (B) *In vivo* lacrimal endoscopic observations of the internal luminal mucosal surface at 1 and 4 weeks post-operation. The lacrimal endoscopic views document the macroscopic luminal patency, mucosal topography, and the presence of any intraluminal obstructions or exudates. (C) Hematoxylin and eosin (H&E) staining of the explanted lacrimal tissues (Sham, Jones tube, Bio-Straight, and Bio-Valve groups) at the end of the observation period. The top row presents low-magnification overviews of the tissue cross-sections, while the bottom row displays the corresponding high-magnification views of the boxed regions, detailing the extent of epithelial regeneration and the subepithelial inflammatory cellular infiltration. Scale bars: 50 μm , 200 μm ; magnification: 100 $\times$ (top row) and 400 $\times$ (bottom row).

pathways.

To directly visualize the dynamic process of intraluminal healing, *in vivo* lacrimal endoscopy was employed at 2 and 8 weeks post-operation (Figure 5B). The Model group exhibited progressive mucosal hyperemia and florid granulation tissue proliferation, accompanied by thick purulent exudates indicative of severe retrograde infection. Notably, visible purulent discharge was also observed on the exterior in the Jones tube group. Although the prosthesis maintained physical patency, the intraluminal view frequently revealed accumulated mucoid secretions and chronic mucosal irritation due to the lack of a physiological mucosal lining. Conversely, the bio-straight and bio-valve conduits demonstrated a highly favorable, progressively maturing luminal interface. By week 8, both 3D-printed groups presented a smooth, unobstructed, and glistening mucosal surface devoid of strictures or inflammatory exudates, macroscopically resembling the healthy physiological state of the Sham group.

These macroscopic and endoscopic observations were ultimately corroborated at the cellular level via histological examination (Figure 5C). Hematoxylin and eosin staining of the tissues surrounding the Jones tube revealed a severe foreign body reaction, characterized by dense subepithelial inflammatory cell infiltration and disorganized fibrotic scar tissue encapsulation. In stark contrast, the surrounding host tissues in the Bio-Straight and Bio-Valve groups exhibited minimal inflammatory infiltration and mild, healthy connective tissue remodeling. Most notably, a continuous, multi-layered, and well-organized epithelial lining was successfully regenerated along the bio-engineered luminal surface of both 3D-printed constructs. This successful *in vivo* re-epithelialization is directly attributable to the bio-instructive young dECM coating and the pre-induced ESCs, which synergistically suppressed foreign body inflammation and orchestrated harmonious tissue integration.

4. Discussion

Severe structural destruction of the lacrimal drainage system represents a formidable clinical challenge. In conventional clinical practice, a commonly employed surgical approach for such severe cases is Jones tube implantation. While this inert prosthesis restores initial physical patency, its rigid nature and lack of an anti-reflux mechanism frequently lead to severe complications, including prosthesis migration, foreign-body rejection, and retrograde ascending infections.^{9,14} To overcome these fundamental limitations, we developed a novel anti-reflux artificial lacrimal duct via 3D printing that seamlessly integrates biomimetic hydrodynamic design with a

biochemically optimized microenvironment. The core innovations of this construct are twofold:

- (i) Physical anti-reflux regulation: The precise 3D fabrication of a unidirectional valve to mimic the hydrodynamic function of the natural Hasner valve.
- (ii) Biochemical tissue induction: The utilization of young donor-derived dECM to replicate a youthful natural ECM niche, effectively assisting ESCs in targeted differentiation toward a lacrimal epithelial lineage.

By synergizing these dual innovations, this study successfully resolves the longstanding biomechanical and biological bottlenecks of lacrimal reconstruction.

The first pivotal breakthrough of our construct lies in the physical prevention of fluidic reflux, which is the primary culprit of post-operative secondary bacterial conjunctivitis. Replicating the delicate, dynamic movement of the natural Hasner valve requires a material that balances printability with exceptional mechanical resilience. Traditional soft hydrogels are prone to structural collapse or fatigue tearing under continuous fluidic impact.²⁸ To resolve this, we engineered the semi-synthetic PAG composite hydrogel formulation (15% PEGDA, 3% PAAm, and 5% GelMA). The incorporation of PEGDA and PAAm endowed the scaffold with remarkable mechanical toughness, maintaining intrinsic elasticity even after 1,000 consecutive compression cycles, aligning with recent advancements in tough double-network hydrogels.²⁹ Processed using high-resolution DLP 3D printing, this tailored hydrogel ink enabled high-fidelity fabrication of the inclined, flexible leaflet. Both CFD and benchtop hydrodynamic tests proved that the biomimetic valve robustly truncates retrograde pressure. Crucially, it withstood a “reverse burst point” of ~90 mmHg without prolapse, safely exceeding the peak physiological stress generated during forceful nose-blowing (~70 mmHg).³⁹ This mechanical durability successfully translated *in vivo*, where retrograde CT stress tests visually corroborated the valve’s absolute efficacy in blocking reverse fluid migration, fundamentally addressing the clinical infection pain point of conventional valveless conduits.

Beyond hydrodynamic regulation, long-term reconstructive success strictly relies on the establishment of a continuous, functional mucosal barrier to prevent luminal fibrotic obliteration. Our quantitative proteomic profiling revealed that aged dECM is fraught with pro-inflammatory and fibrotic markers. Conversely, young dECM is profoundly enriched in core adhesive and basement-membrane-associated proteins, such as POSTN, LAMA2, and P4HA1. Previous studies have established that matrix proteins such as POSTN are indispensable for epithelial proliferation and tissue regeneration.^{21,40}

Harnessing this youth-associated biochemical signature, we functionalized the luminal surface of the PAG scaffold. *In vitro* data demonstrated that this rejuvenated niche exquisitely instructed the pre-seeded ESCs to lose their basal stemness (downregulation of CK5) and acquire a mature, secretory luminal phenotype (upregulation of CK8, AQP5, and MUC1). Furthermore, the rapid increase in TEER values and ZO-1 expression confirmed the formation of a watertight barrier via tight junctions, indicating that the biological seal is as vital as the mechanical scaffold in preventing fluid leakage and bacterial infiltration.⁴¹

The synergistic efficacy of this dual-action strategy was unequivocally validated in the *in vivo* rabbit defect model. While the conventional Jones tube provoked severe foreign body reactions, dense inflammatory infiltration, and visible purulent exudation due to its inert, non-integrative nature, the anti-reflux artificial lacrimal duct in the Bio-Valve group elicited only a remarkably mild inflammatory response. Endoscopic and histological evaluations confirmed that the young dECM bio-coating effectively mitigated host immune rejection and successfully guided the *in situ* regeneration of a multi-layered, healthy epithelial lining. By simultaneously satisfying the mechanical demand for tear drainage and the biological demand for mucosal integration, the bio-valve conduit achieved an unprecedentedly high patency rate and normalized clinical tearing scores within 8 weeks, significantly outperforming the conventional surgical standard.

Despite these highly promising translational results, several limitations warrant further investigation. First, the 8-week *in vivo* observation period, while sufficient for assessing acute integration and mucosal healing, cannot fully predict the long-term degradation kinetics of the PAG composite over years. Future studies should focus on tuning the polymer degradation rate to perfectly match the pace of host tissue remodeling, and we plan to evaluate the degradation profile and metabolic toxicity of the PAG hydrogel in larger animal models (e.g., non-human primates). Second, although our construct showed minimal inflammation, a quantitative immunogenicity assessment (e.g., IL-1 β , IL-6, tumor necrosis factor alpha levels via enzyme-linked immunosorbent assay or quantitative PCR) would further strengthen the safety profile. We have initiated such cytokine profiling in an ongoing study. Third, despite the superior bioactivity of young donor-derived dECM demonstrated in this proof-of-concept study, natural tissue extracts present inherent limitations for large-scale clinical translation, including potential batch-to-batch variation, the risk of pathogen transmission, and residual immunogenicity. For future

clinical applications, rigorous quality-control standards—such as standardized decellularization validation and strict DNA quantification—will be imperative. Alternatively, employing defined recombinant human proteins, such as recombinant collagen or periostin (which was notably enriched in our proteomic analysis), could provide a more standardized, scalable, and safer bioactive coating to bypass the limitations of natural dECM. Additionally, exploring the precise mechanotransduction pathways by which the valve's dynamic fluidic movement physically influences the phenotypic maintenance of the overlying epithelial cells will deepen our understanding of functional organ 3D printing. Ultimately, this biomimetic and omics-guided biofabrication strategy holds immense clinical potential, paving the way for next-generation, dynamically functional tubular organ replacements.

5. Conclusion

In conclusion, this study presents an integrative biofabrication strategy for severe lacrimal drainage reconstruction by developing a 3D-printed, anti-reflux artificial lacrimal duct. By synergistically coupling a biomimetic hydrodynamic design with an omics-guided biochemical microenvironment, the engineered construct effectively resolves the fundamental biomechanical and biological bottlenecks of conventional inert prostheses. Mechanically, the high-fidelity unidirectional valve, fabricated from a tailored, robust PAG composite hydrogel formulation, resiliently truncates retrograde fluid flow, thereby mitigating the clinical risk of ascending infections. Biologically, luminal functionalization with young donor-derived dECM orchestrates a regenerative niche that drives targeted epithelial differentiation and restoration of a functional mucosal barrier. Consequently, this dual-action conduit demonstrated exceptional *in vivo* patency, superior immunocompatibility, and robust *in situ* epithelialization, effectively mitigating the hallmark complications associated with conventional clinical prostheses. While future investigations into long-term degradation kinetics and mechanotransduction pathways are warranted, this integrative biomaterial platform represents a substantial leap forward in translational ophthalmology, offering a highly translatable blueprint for the next generation of dynamically functional tubular organ replacements.

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Conflict of 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.

Author contributions

Conceptualization: Bingran Dong, Hai Tao

Formal analysis: Bingran Dong

Investigation: All authors

Methodology: Bingran Dong, Fang Bai, Sha Huang

Resources: Bingran Dong, Fang Bai

Supervision: Hai Tao

Writing—original draft: Bingran Dong

Writing—review & editing: Hai Tao, Sha Huang

Ethics approval and consent to participate

All animal experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee of the Chinese PLA General Hospital and approved by the Animal Ethics Committee of the Chinese PLA General Hospital (approval no. 2020-002).

Consent for publication

Not applicable.

Availability of data

All data analyzed during this study are included in this published article.

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