ORIGINAL ARTICLE

Repair of Tympanic Membrane Perforations with Customized Bioprinted Ear Grafts Using Chinchilla Models

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The goal of this work is to develop an innovative method that combines bioprinting and endoscopic imaging to repair tympanic membrane perforations (TMPs). TMPs are a serious health issue because they can lead to both conductive hearing loss and repeated otitis media. TMPs occur in 3–5% of cases after ear tube placement, as well as in cases of acute otitis media (the second most common infection in pediatrics), chronic otitis media with or without cholesteatoma, or as a result of barotrauma to the ear. About 55,000 tympanoplasties, the surgery performed to reconstruct TMPs, are performed every year, and the commonly used cartilage grafting technique has a success rate between 43% and 100%. This wide variability in successful tympanoplasty indicates that the current approach relies heavily on the skill of the surgeon to carve the shield graft into the shape of the TMP, which can be extremely difficult because of the perforation's irregular shape. To this end, we hypothesized that patient specific acellular grafts can be bioprinted to repair TMPs. *In vitro* data demonstrated that our approach resulted in excellent wound healing responses (e.g., cell invasion and proliferations) using our bioprinted gelatin methacrylate constructs. Based on these results, we then bioprinted customized acellular grafts to treat TMP based on endoscopic imaging of the perforation and demonstrated improved TMP healing in a chinchilla study. These ear graft techniques could transform clinical practice by eliminating the need for hand-carved grafts. To our knowledge, this is the first proof of concept of using bioprinting and endoscopic imaging to fabricate customized grafts to treat tissue perforations. This technology could be transferred to other medical pathologies and be used to rapidly scan internal organs such as intestines for microperforations, brain covering (Dura mater) for determination of sites of potential cerebrospinal fluid leaks, and vascular systems to determine arterial wall damage before aneurysm rupture in strokes.

Keywords: 3D printing, bioprinting, tympanic membrane, tissue engineering and regenerative medicine, cell migration and invasion

Introduction

TYMPANIC MEMBRANE PERFORATIONS (TMPs) represent a serious ear condition that can lead to conductive hearing loss, chronic infections, and in some cases irreversible damage to the eardrum. TMPs often occur in conjunction with other pathologies like otitis media, cholesteatoma, and barotrauma to the ear.¹ In addition, TMPs can be socially debilitating for patients, as hearing loss hampers effective communication in learning environments and during social gatherings.² Current treatments for TMPs involve dry ear treatments, ototopical

drops, and surgical reconstruction of the eardrum, known as tympanoplasty. In this procedure, the perforated eardrum is covered with Gelfilm or Gelfoam, fascia, fat, or a polymeric material. Yet, these grafts are mechanically unstable and often fail to incorporate into the tympanic membrane.³ Another tympanoplasty technique involves autologous graft of cartilage harvested from the outer ear, the tragus, or the conchal bowl.⁴ Cartilage grafting technique has demonstrated excellent mechanical stability, yet the graft must be carved free hand to the appropriate size and shape, which can be extremely difficult with irregularly shaped TMPs.⁵ The reported success rate for

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the 55,000 tympanoplasties performed yearly in the United States varies dramatically from 43% to 100% and is critically dependent on the surgeons' skills and experience.^{5–8} This huge inconsistency in tympanoplasty repair success rate underlines the need for new techniques to carefully implant the graft *in situ*, which must precisely fit into the defect with negligible overlap onto the native tympanic membrane.⁷

Bioprinting provides a direct answer to these shortcomings, allowing the fabrication of scaffolds with high spatiotemporal control. In particular, extrusion-based bioprinting enables the deposition of cells, biomaterials, and biochemical cues to build complex natural tissues and organs with microscale resolution.^{9,10} Gelatin methacrylate (GelMA), a thermo-responsive hydrogel, is a highly biocompatible and biodegradable material that can be used for cell encapsulation, making it an ideal candidate for bioprinting.⁹ Gelatin exhibits protective effects for its cargos and can be engineered for sustained protein release by tuning its electrostatic charges at physiological conditions.^{11,12}

In this work, we designed customized GelMA implants to foster tympanic membrane regeneration, a process analogous to wound healing.¹³ In wound healing, the initial wound clotting and clearance of pathogens from the wound bed release various growth factors that promote cell recruitment, cell proliferation, and synthesis of extracellular matrix proteins such as fibronectin (FN), hyaluronan, collagen, and proteoglycans.^{14,15} Among the growth factors secreted, epidermal growth factor (EGF) is a powerful chemoattractant for fibroblast, a central player in wound healing, and therefore it has been proposed as a therapeutic agent in tympanic membrane repair.¹⁴ However, EGF is ineffective in chronic lesions like TMPs because it

rapidly degrades at the wound site.¹⁴ The possibility to select a biomaterial able to protect and stabilize EGF would be especially attractive in TMPs, where the implanted graft can be leveraged for sustained release of EGF.

To this end, we hypothesized that patient-specific ear grafts for TMP repair can be fabricated by utilizing three-dimensional (3D) bioprinting and endoscopic imaging (Fig. 1). We further postulate that the bioprinted ear graft can be loaded with EGF, which will foster cell invasion from the host tissue toward the scaffold and ultimately regenerate TMPs *in vivo*. We tested our hypothesis through three steps. The first step was to design and bioprint ear grafts with a “butterfly” structure that can lock on the edge of the perforated eardrum to provide enhanced mechanical stability (Fig. 1). Mechanical testing was performed to demonstrate that the butterfly structure stabilized the bioprinted ear graft upon implantation. The second step was to determine the wound healing response of the ear graft *in vitro* by assessing the effect of EGF on the invasion and proliferation of fibroblasts.¹³ Bioprinted invasion models were fabricated to simulate the invasion of hosts' cells from the perforation into the implanted grafts. Based on these results, in the third step, we tested the wound-healing efficacy of the ear grafts *in vivo* using a chinchilla model. After endoscopic imaging of the TMP, we bioprinted and implanted patient-specific ear grafts. Repair of perforated tympanic membranes was assessed through microcomputed tomography (micro-CT) and histological observations.

The bioprinting of EGF-loaded customized ear grafts could improve the success rate of current tympanoplasty techniques, as surgeons would no longer need to hand carve grafts into the irregular shapes of TMPs and the implant

FIG. 1. Concept of automated bioprinting of tissue engineered grafts for tympanic membrane repair (ear graft). (a–c) Image acquisition CAD model development. The four steps in the process are (a) capture the image of the perforation, (b) outline the defect on a computer, (c) develop a CAD model based on the outline, and (d) design and printing of ear graft. We designed a “butterfly” structure around the ear grafts so they can be inserted and fill the perforation without any patches/sutures/glues. We designed the ear graft to have three parts that include a support structure (blue), made out of gelatin, that will be washed away and create the butterfly structure that would clamp on the tympanic membrane. Scale bars in panel D=5 mm. CAD, computer-aided design.

itself can foster healing of TMPs through EGF delivery. While the present work focuses on tympanic membrane repair, the same approach can be extended to other medical pathologies that involve the repair of tissue perforations, such as intestinal microperforations, arterial wall damages, and cerebrospinal fluid leakage from dura mater, among the others.

Materials and Methods

Cell culture

Murine fibroblast cell line NIH/3T3 was purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (ATCC) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Thermo Fisher Scientific) and 1% penicillin/streptomycin (Thermo Fisher Scientific).

GelMA synthesis

GelMA was selected as the bioink for the ear graft fabrication due to its high biocompatibility and extensive use in bioprinting. GelMA was synthesized as previously described.^{9,11,12,16} Briefly, Type A porcine skin gelatin (300 bloom; Sigma-Aldrich) was dissolved at 10% (w/v) into phosphate-buffered saline (PBS; Thermo Fisher Scientific) at 50°C for 20 min. Methacrylic anhydride (MA; Sigma-

Aldrich) was added dropwise to gelatin solution under vigorous stirring for 1 h (0.6 g MA per gram of gelatin). The mixture was then diluted twice the volume by adding PBS to stop the reaction and was centrifuged at 2000 g for 2 min. The supernatant was collected and dialyzed against deionized water using dialysis cassettes (10 kDa molecular weight cut-off; Thermo Fisher Scientific) for 48 h at 50°C to remove the excess salts and acids. The dialyzed GelMA was then lyophilized and stored at minus 80°C.

3D bioprinting

All scaffolds in this study were fabricated with a commercial bioprinter (3D-Bioplotter; EnvisionTEC). Lyophilized GelMA was dissolved at 10% (w/v) in PBS at 50°C for 10 min. Photoinitiator 2-hydroxy-1-(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone (Irgacure 2959; BASF) was added to the GelMA solution at a concentration of 0.1% (w/v) at 50°C for 15 min. The prepolymer solution was then loaded into a syringe barrel and allowed to equilibrate for 15 min at 22°C. Cells and/or growth factors of interest were added at this step, and the prepolymer solution was further incubated for 20 min before bioprinting. FN was added (50 µg/mL) in all bioprinting work presented herein to enhance cell adhesion; 3D printed ear grafts were finally photocrosslinked with UV light (0.09 mW/cm²)

FIG. 2. Mechanical testing of ear graft. Using an Instron Material Testing Machine, we demonstrated that the butterfly structure has an impact on the stress and strain behavior of the graft and resulted in higher ultimate stress. **(a)** Stress and strain response of ear graft to compression with and without butterfly structures. **(b)** Ultimate stress of bioprinted GelMA constructs, with and without butterfly structure ($n = 6$ in each group). The significantly higher ultimate stress in 3D printed GelMA group with butterfly structure suggests enhanced structural stability. $*p < 0.05$. **(c)** Overall setup of the mechanical testing. The ear graft is placed on top of a hole with defined dimension, which is a model for a defect in the tympanic membrane. A rod that has the same diameter as the size of the perforation is attached to the Instron machine, and a compression test was performed. **(c1–2)** Due to the butterfly structure, we observed double peaks in the stress curves (1–2 in **a**) that correspond to the stress when the graft is being inserted (**c1**) and removed (**c2**) from its intended position. **(c3)** In contrast, the 3D printed GelMA constructs that do not have butterfly structure have single peak (3 in panel **a**). 3D, three-dimensional; GelMA, gelatin methacrylate.

for 1 min. For cell-laden ear grafts, a calcein-AM/ethidium homodimer, LIVE/DEAD assay (Thermo Fisher Scientific) was used to assess cell viability within printed constructs as per manufacturer's instructions.

Mechanical testing

We tested the mechanical stability of the implanted ear graft in its intended position based on a standard test method for penetration resistance of flexible barrier films (ASTM F1305-16) with modifications. Briefly, we developed a perforation model composed of a white polymeric frame supporting a polycarbonate sheet with 4 mm diameter holes to model the perforated tympanic membrane (TM). Ear grafts were placed on top of the perforation model and compressed with a Instron Material Testing Machine (Instron 5585; Instron) at a rate of 25 mm/min to simulate the stress/strain response of the ear graft during insertion/removal in the injured TM (Fig. 2).

In vitro fibroblast invasion, proliferation, and matrix metalloproteinase secretion

Before bioprinting, cells were lifted with 0.25% trypsin-EDTA (Thermo Fisher Scientific), counted with a hemo-

cytometer, and added to the GelMA prepolymer solution (2×10^6 cells/mL) together with different levels of EGF (0–10 μ M). To assess cell invasion within the ear graft, an essential prerequisite for scaffold integration and TM repair *in vivo*, cylindrical GelMA scaffolds were bioprinted with three distinct zones (Fig. 3a): (1) an outer cell-laden GelMA annulus (2×10^6 cells/mL) containing FN (50 μ g/mL), an (2) inner zone containing FN (50 μ g/mL), and (3) a GelMA core loaded with EGF (0–10 μ M), which serves as chemoattractant point source. These bioprinted cell-laden GelMA discs (diameter: 1 cm; height: 2 mm) were cultured for 7 days in six-well plates with 1 mL of complete medium with reduced FBS (5% v/v) that is replaced every 2 days. Images were taken on day 2 and 7 with a conventional fluorescent microscope (ApoTome; Zeiss). The distances between the edge of the bioprinted constructs and the invasion front were measured using ImageJ and averaged over time to obtain invasion rates.

On day 7, proliferation of bioprinted fibroblasts with various loading levels of EGF was evaluated using XTT assay following manufacturer's instructions (Trevigen). At the same time, the conditioned medium of the bioprinted GelMA samples was collected and tested for matrix metalloproteinase (MMP) secretion with the MMP Activity

FIG. 3. The wound healing effect of EGF and GelMA using bioprinted cell-laden GelMA constructs *in vitro*. To simulate the *in vivo* response when the acellular ear grafts are implanted in our chinchilla models, we bioprinted cell-laden GelMA constructs to prove that the host cells can invade from the periphery toward the center of the ear graft to integrate and grow with the host. **(a)** Overall model design and representative microscopy images at day 2 and 8 showing invasion of murine fibroblast toward EGF (scale bars = 100 μ m). The dotted orange lines in panel **a** demonstrate the approximate position of the invasion front in day 2. The red arrows indicate the direction of cell invasion. **(b)** Fluorescent LIVE/DEAD stain images taken at day 8 indicating excellent viability of 3T3 (Scale bars = 200 μ m). Our results show that EGF leads to **(c)** higher invasion rates, **(d)** increased MMP secretion, **(e)** promotes proliferation of fibroblasts in a dosage-dependent manner ($n = 6$). * indicates significant differences between groups ($p < 0.05$). EGF, epidermal growth factor; FN, fibronectin; MMP, matrix metalloproteinase.

Assay following manufacturer’s instructions (Abcam). Both XTT and MMP secretion levels were normalized to acellular bioprinted GelMA constructs.

Animal surgery and in vivo TMP repair

All studies were approved by the MedStar Health Research Institute IACUC and were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals*. Ten female chinchillas (*Chinchilla lanigera*) with a minimum weight of 450 g were used for these studies after an acclimation period of at least 7 days (Moulton Chinchilla Ranch). Animals were housed in compatible pairs in Allentown Euro rabbit cage and rack system (Allentown caging) on a 12-h light/12-h dark cycle with controlled temperature and humidity room. Chinchillas were fed a pellet chinchilla diet (Mazuri #5MO1; PMI Nutrition International) and offered fresh Western Timothy hay (Oxbow) daily. Dust baths (You and Me; Petco) were offered for 2 h weekly, excluding the initial perforation week. Chinchillas also received raisins (Sun-Maid, Kingsburg, CA) one or two a day as a treat.

Animals were identified with marker numbers on outer ear. Animals were initially anesthetized with 3–5% isoflurane (Henry Schein, Dublin, OH), in an induction chamber (E-Z Anesthesia) then transferred to a nose cone and maintained with 1–3% isoflurane for the duration of the procedure. Heart rate and oxygen saturation were monitored throughout the procedure with a Nonin PalmSAT® 2500. Each ear of chin-

chilla was assigned to one of four groups: (1) no perforation ($n=3$); (2) Perforation with no graft ($n=4$); (3) Perforation grafted without EGF ($n=4$); and (4) Perforation grafted with EGF (5 μ M, $n=6$). All ears were confirmed healthy before perforation. A small perforation was made in the tympanic membrane of one ear of each animal under endoscopic guidance. Animals were allowed to recover from anesthesia and heal for 3 weeks. Each session was video recorded along with a scale near the perforation using a frame grabber software application (Epiphan Capture Tool, Epiphan Systems, Inc.).

The recordings of the tympanic membranes were used to develop computer-aided design (CAD) models using SolidWorks with precise x-y dimensions. The height of the ear grafts was kept at 1.2 mm throughout the study. Based on the CAD models, customized 3D ear grafts were bioprinted (3D-Bioplotter; EnvisionTEC). Animals were anesthetized as before 7 days after the perforation and fitted with one of the implants. All chinchillas were euthanized on day 28 following final binary assessments of the tympanic membrane through visual inspection (completely healed vs. unhealed). Examples of healed and unhealed tympanic membranes are shown in Figure 4e and f. Euthanasia was achieved through pentobarbital (Fatal-Plus) overdose 85–150 mg/kg.

Micro-CT and histological observations

Three weeks after treatment with bioprinted ear grafts, animal underwent euthanasia and skulls were fixed in 10%

FIG. 4. Wound healing capacity of ear graft *in vivo*. To test the wound healing capacity of ear graft, we implanted ear grafts with or without EGF in chinchilla models. Briefly, perforations are made on day 1 and the geometry of the defects is measured to develop CAD models. Customized grafts are bioprinted and implanted on day 7. The tympanic membranes of the chinchillas are inspected and euthanized on day 21. **(a–d)** Graft Implantation. **(a)** First, the surgeon inspects the tympanic membrane using an endoscope. **(b)** Using an endoscopic camera, the surgeon locates tympanic membrane perforation. **(c)** The customized graft is then pushed down through the ear speculum using ear delicate forceps and finally **(d)** implanted. **(e)** Example of unhealed membrane perforation on day 21. **(f)** Example of healed tympanic membrane on day 21. Scale bars = 1 mm.

formalin (Fisher Scientific). Micro-CT (Nikon XTH 225 ST PE1621 EHS) was performed to characterize the dimensions and integrity of tympanic membrane region in control and treated animals before any physical alteration. Postprocessing of the raw CT data included reconstruction using Nikon Metrology CT-Pro software and visualization by VGStudio MAX 3.0. Each sample was examined for any gross abnormalities, and the skull was divided into right and left portions and then transected at the posterior orbit, leaving two cubes containing the temporal bullae/middle ear. Right and left sides of each skull were inked individually to maintain gross/microscopic orientation. Each cube was subsequently decalcified in EDTA. Once sufficiently decalcified, the cube was processed and infiltrated in a graded series of acetone and Spurr resin. Areas of interest were cut on a rotary microtome and affixed to charged glass slides, with each receiving hematoxylin and eosin stain. Stained slides are observed under an optical microscope.

Statistical analysis

Data are expressed as average $\pm$ standard deviation. Statistical analysis was performed in MATLAB[®] using a one factor analysis of variance test followed by multiple comparison tests. In all analyses a significance level of 95% was chosen, and differences between samples were considered significant when $p < 0.05$.

Results

Design and fabrication of ear grafts

The overall concept and design of the ear graft system are illustrated in Figure 1. First, an endoscopic camera is used to image the perforation in the tympanic membrane (Fig. 1a). Based on the image, it is possible to outline precisely the shape of the perforation using PC-based software and, thus, generate a CAD model (Fig. 1b, c). Finally, the CAD model is used to bioprint patient-specific ear grafts using an extrusion-based bioprinter. For surgical simplicity, the ear graft was designed with supportive “butterfly” structures such that it can be press fit within the TMP without the need for additional surgical tools (e.g., patches, sutures, glues) (Fig. 1c). As shown in Figure 1d, the ear graft is designed in three parts that include: (L1) a bottom butterfly supporting a structure modeled after the tissue defect (both made of GelMA), (L2) a temporary support structure made of gelatin illustrated in blue, and (L3) a top butterfly made of GelMA. These three parts will be assembled and bioprinted into a single ear graft. The resulting ear graft is then photocrosslinked and finally incubated in warm PBS at 37°C. Since L2 is made out of gelatin (which do not photocrosslink and is unstable at 37°C), it will be washed away (Fig. 1d).

To test the mechanical stability of the ear graft during implant in the perforated TM, we determined the amount of stress involved in inserting/removing the graft from its intended location. Ear grafts without butterfly supports show an increasing stress with increasing strain. An abrupt collapse in the stress within the scaffold is observed only when the graft has been completely pushed throughout the defect model (3 in Fig. 2a, c). Conversely, the presence of butterfly supports results in a bimodal stress versus strain curve (1–2 in Fig. 2a, c). In this scenario, the stress withstood by the scaffold increases with

strain and a rapid drop in stress occurs when the ear graft with butterfly structure is locked into the perforation model, which corresponds to the first peak. Then the stress starts to increase again with strain and drop when the graft has been completely pushed out of the defect model, which results in another peak due to the presence of the top butterfly support. In addition, the presence of butterfly supports results in a significantly higher ultimate stress tolerated by the ear grafts (5.03 ± 2.27 and 2.66 ± 0.95 kPa for ear grafts with and without butterfly supports, respectively), as shown in Figure 2b.

In vitro fibroblast proliferation and ear graft invasion

Since cell invasion and proliferation are critical in the healing of TMP, we investigated fibroblast invasion and proliferation within the bioprinted ear graft in response to different chemotactic gradients of EGF (Fig. 3). Fluorescent microscopy shows excellent cell viability within the bioprinted ear graft after 7 days, where most of the fibroblast stained positive for marker of live cells calcein-AM (green) and negative for the apoptotic marker ethidium homodimer (red) in all experimental groups (Fig. 3b). Quantitative analysis of fibroblast invasion rate is shown in Figure 3c. The results indicate that EGF-loaded ear grafts promoted fibroblast invasion rate in a dose-dependent manner, where positive/negative signs indicate invasion toward/away from the center, respectively. To invade the ear graft, cells are required to remodel GelMA by secreting MMPs, among the other enzymes. We found that MMP secretion increased in ear grafts with an EGF 5 μ M compared to blank ear graft (Fig. 3d). Yet, a 10 μ M concentration of EGF did not affect significantly MMP activity compared to the other experimental groups tested. The presence of EGF also promoted fibroblast proliferation, analogous to invasion rate, in a dose-dependent manner (Fig. 3e).

In vivo tympanic membrane repair

Once we determined that the bioprinted ear grafts support fibroblast proliferation and invasion, we moved to an *in vivo* study using chinchilla animal models. Bioprinted ear grafts (with or without EGF at a loading level of 5 μ M) were implanted 6 days after TMPs were created. Endoscopy was first used to locate the TMP (Fig. 4a, b), and subsequently, the ear graft was implanted *in situ* through the ear speculum (Fig. 4c, d). Three weeks after implantation, visual inspection through endoscopy indicated limited healing of TMPs left untreated (Fig. 4e), with only one animal out of four showing spontaneous wound healing. However, the presence of an ear graft without any growth factors resulted in three out of four animals presenting full closure of the TMP (Fig. 4f). *In vivo* repair of the tympanic membrane further improved when using EGF-loaded ear grafts compared to blank ear grafts, with all animals displaying complete healing of the TMP.

These findings were further corroborated by micro-CT and histological analyses. We successfully utilized micro-CT to measure the dimension of the tympanic membrane region and demonstrate the differences between healed and unhealed TMPs (Fig. 5a, b). Histological analysis showed that grafted TMs were overall well healed (Fig. 5c), with areas within the membrane that were focally thicker compared to the untreated control animal. Ongoing remodeling was observed in the GelMA/growth factor group, with focal chondral observed in a single specimen. The GelMA material seems to be completely

FIG. 5. Micro-CT and histological analysis of the regenerated tympanic membrane. Micro-CT results in cross-sectional view of (a) healed TM (solid green line, 3.95 mm) and (b) unhealed TM with perforation remaining (dotted green line, 1.61 mm); Micro-CT enables clinicians/researchers to determine the clinical outcome of tympanoplasty. (c) Representative histological images of grafted tympanic membrane perforation that result in complete healing with hematoxylin and eosin staining (scale bar = 500 μ m). (d) High magnification of the mid-section of the tympanic membrane showing extensive cell infiltration with some GelMA material remaining (black arrow, scale bar = 20 μ m). micro-CT, microcomputed tomography; TM, tympanic membrane.

integrated within the host (Fig. 5d), with minimal residual remnants of the GelMA graft present in one animal, associated with few mononuclear cells (Fig. 5c).

Discussion

The goal of this work is to establish an innovative approach of combining endoscopic imaging and 3D bioprinting to fabricate patient-specific grafts to treat TMPs. Although various surgical techniques have been developed to repair TMP, the commonly used cartilage grafting techniques have a reported success rate between 43% and 100%.^{5–8} The wide variability in success rates indicates that the current approach relies heavily on the skill of the surgeon. Therefore, we believe that precise customized grafts could improve the consistency of the procedure. As such, we hypothesized that patient-specific ear grafts for TMP repair can be fabricated by utilizing 3D bioprinting and endoscopic imaging. We tested the hypothesis through three steps: (1) To bioprint grafts for tympanic membrane repair that can be press fitted in place without using sutures and/or surgical glues; (2) To determine the wound-healing effect of ear graft loaded with EGF *in vitro* by evaluating the invasive and proliferative response of fibroblasts; and (3) To test the healing capability of ear graft using a chinchilla model.

First, we had to develop techniques to fabricate grafts that can be press fitted into perforations without using sutures and/or surgical glues. We hypothesized that patient-specific grafts that have a “butterfly structure” along the periphery will provide enhanced mechanical stability when implanted. As such, we designed our ear grafts to have three layers: the bottom, middle, and top layers with the identical shape to that of the TMP and assumed an overall height of 1.2 mm (Fig. 1d). The area of the middle layer, which is the layer that

is in direct contact with the wound, is set to be the same as that of the TMP. The areas of top and bottom layer expanded uniformly along the periphery by 500 μ m and were located above and below the tympanic membrane to “clamp on” the tympanic membrane. Because the implant expands from swelling, we expect the ear graft to seal the TMP and hence act as a bridge to be invaded and integrated with the host.

In practice, we used GelMA to bioprint the bulk of the ear graft and used porcine gelatin to build the temporary support structure that was washed away to create the spacing between the top and bottom layer. Even though gelatin-based materials have been widely used (e.g., Gelfoam[®]), their application in bioprinting has never been explored in TM repair.¹³ To our knowledge, this is the first time that a support structure is utilized for 3D printing hydrogel and this technique could enable fabricating more complex hydrogel-based tissue grafts/models. As we are using bioprinting as our fabrication method, the dependency on the surgeon’s ability to manually carve out grafts is eliminated which would increase both the consistency and overall success rates of tympanoplasty.

Once we successfully incorporated gelatin as the support structure to our existing bioprinting platform, we wanted to test the feasibility of implanting bioprinted grafts by press fitting and verifying that the butterfly structure enhances the stability of the implanted grafts. We developed a novel technique to quantify the amount of force necessary to insert and remove a bioprinted graft from a perforation based on an existing material testing method (ASTM F1305-16). We first bioprinted a fixture with perforations with defined geometry and then placed the bioprinted grafts on top of the perforation. We selected polycarbonate as our material to model TMPs because it is significantly stiffer compared with the tympanic membrane. Therefore, if we can successfully insert

the bioprinted ear grafts into a polycarbonate sheet without damages, we should have no issues inserting the ear grafts into the tympanic membrane. As shown in the stress and strain curve, we observe a double peak in grafts that had a butterfly structure (red in Fig. 2a). In contrast, we only see a single peak in grafts without a butterfly structure (blue in Fig. 2a). We believe that the two peaks observed in constructs with a butterfly structure are from when the graft is compressed into place and when the graft is removed (Fig. 2c). The grafts with butterfly structure also result in significantly higher ultimate stress than those without, which validates that the butterfly structure enhances the mechanical stability of the implanted graft (Fig. 2b).

After successfully bioprinting GelMA samples to seal TMP models with good mechanical stability, we wanted to ensure that the bioprinted grafts would allow for cell invasion and proliferation from the host upon implantation, which are key cellular processes in wound healing.^{14,15,17} As a proof of concept, we selected the EGF. Even though EGF is a well-characterized growth factor in the field of wound healing, the effects of EGF on the invasion rates and proliferation of bioprinted fibroblasts have not been reported.^{9,13,18} We bioprinted GelMA constructs with patterned biomaterials to simulate the invasion of cells from the surrounding tissue of the host, which are expected to occur when we implant the acellular EGF-loaded ear graft *in vivo*. The invasion models are circular discs with different biomaterials in the radial direction starting from the center: (1) EGF source; (2) GelMA-FN; and (3) An outer layer of fibroblasts (Fig. 3a). These fibroblasts act as the invading cells from the surrounding tissue toward the EGF source, simulating wound closure.

We have determined, for the first time, a positive correlation between invasion rates of bioprinted fibroblasts and EGF where higher EGF concentrations (0, 5, 10 μ M) lead to significantly higher invasion rates *in vitro* (-10.6 ± 7.38 , 50.95 ± 9.97 , 78.76 ± 11.1 μ m/day) (Fig. 3c). The positive/negative sign for invasion rates indicates invasion toward/away from the center of the wound closure model, respectively. The negative invasion rates observed in GelMA constructs without EGF are likely due to cell invasion toward the surrounding media where there is a higher concentration of nutrients and growth factors (e.g., glucose and/or FBS).

The increased MMP secretions of murine fibroblast in response of EGF suggest that the enhanced invasion rates may be driven by increased MMP activities (Fig. 3d). MMP is critical in the process of cellular invasion because it enables cells to break down their surrounding matrix and gain motility. The increased MMP activities due to increased EGF dosage validate published work that showed MMP as a downstream effector of EGFR, the receptor that EGF acts upon.^{19,20} EGF also enhances fibroblast cell proliferation in a dosage-dependent manner which agrees with previous findings¹⁴ (Fig. 3e). These *in vitro* simulations of invasion and proliferation of the host's cells were achieved by the key advantage of extrusion-based bioprinting that enables the spatial control of different biomaterials (e.g., ECM, growth factor, and cells) in addition to controlling the macroscopic geometry of the construct.

Once we successfully determined that the bioprinted ear grafts support cell growth and invasion, we moved to *in vivo* testing using chinchilla models ($n = 14$). Perforations with

random geometries were created on day 1 and imaged to create CAD files by SolidWorks. Our current software only captures the x and y dimension because that is where the significant patient-to-patient variation occurs. In this work, we assumed a graft thickness of 0.4 mm, which is within the range of optimal thickness graft for TMPs that results in reasonable mechanical stability and low acoustic transfer loss of TMPs.²¹ Based on these CAD files, ear grafts were printed on day 6 and implanted on day 7. The loading level of EGF was selected to be 5 μ M based on the *in vitro* results that indicate enhanced invasion, proliferation, and MMP secretion compared to plain GelMA control. Our results indicated that ear grafts loaded with EGF lead to the best clinical outcome (100% of tympanic membranes healed, $n = 6$).

Qualitatively, no significant differences in the native tympanic membrane and healed tympanic membrane after treatment with ear graft were found from micro-CT scans of fixed chinchilla skulls. We have successfully visualized and measured the size of the TMP with submillimeter precision using micro-CT and these measurements are within our expectation (Fig. 5a, b, green lines). This is the first time that micro-CT, a simple and noninvasive diagnostic tool, has been utilized to evaluate the clinical outcome of tympanoplasty with detailed measurements. Histological results indicate excellent integration of the bioprinted grafts with the host with cell infiltration throughout the implanted grafts in both with and without EGF (Fig. 5c).

These *in vivo* results suggest that these customized ear grafts may be a safe and effective approach to treat TMPs. The healing rate of grafted TMP without EGF (75%) suggests that we may not need to load any growth factors in ear grafts, which would simplify the clinical translation process. Future study should further compare the healing efficacy of tympanoplasty based on grafts that are manually carved and grafts that are bioprinted and confirm the acoustic function of the healed tympanic membrane. Even though the present work centers around EGF and fibroblast invasion in the context of tympanic membrane repair, the same method can be broadly applied in many other medical pathologies such as in the vascular system to determine arterial wall damage before aneurysm rupture in strokes.

Conclusion

We have successfully developed a method to fabricate patient-specific grafts to repair TMPs using bioprinting and endoscopic imaging. Through our innovative technique, we demonstrated improved mechanical stability of ear grafts by the presence of butterfly structure features without any surgical glues and/or sutures. *In vitro* results demonstrate excellent wound healing responses using bioprinted fibroblasts. Based on these promising results, we performed animal studies and successfully bioprinted customized ear grafts based on endoscopic imaging of the TMPs and implanted them in chinchillas and demonstrated improved TMP healing. These ear graft techniques could transform current clinical practice by improving the success of the current cartridge-based tympanoplasty techniques, as surgeons no longer need to hand carve grafts. To our knowledge, this is the first proof of concept of using bioprinting to fabricate customized grafts to treat tissue perforations, which can be broadly applied in

many other medical pathologies such as in the vascular systems to determine arterial wall damage before aneurysm rupture in strokes.

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Disclosure Statement

No competing financial interests exist.

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