Research Paper

In Vitro Regeneration of Patient-specific Ear-shaped Cartilage and Its First Clinical Application for Auricular Reconstruction

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\textbf{A B S T R A C T}

Microtia is a congenital external ear malformation that can seriously influence the psychological and physiological well-being of affected children. The successful regeneration of human ear-shaped cartilage using a tissue engineering approach in a nude mouse represents a promising approach for auricular reconstruction. However, owing to technical issues in cell source, shape control, mechanical strength, biosafety, and long-term stability of the regenerated cartilage, human tissue engineered ear-shaped cartilage is yet to be applied clinically. Using expanded microtia chondrocytes, compound biodegradable scaffold, and in vitro culture technique, we engineered patient-specific ear-shaped cartilage in vitro. Moreover, the cartilage was used for auricle reconstruction of five microtia patients and achieved satisfactory aesthetic outcome with mature cartilage formation during 2.5 years follow-up in the first conducted case. Different surgical procedures were also employed to find the optimal approach for handling tissue engineered grafts. In conclusion, the results represent a significant breakthrough in clinical translation of tissue engineered human ear-shaped cartilage given the established engineering approach in a nude mouse represents a promising approach for auricular reconstruction. However, owing to technical issues in cell source, shape control, mechanical strength, biosafety, and long-term stability of the regenerated cartilage, human tissue engineered ear-shaped cartilage is yet to be applied clinically. Using expanded microtia chondrocytes, compound biodegradable scaffold, and in vitro culture technique, we engineered patient-specific ear-shaped cartilage in vitro. Moreover, the cartilage was used for auricle reconstruction of five microtia patients and achieved satisfactory aesthetic outcome with mature cartilage formation during 2.5 years follow-up in the first conducted case. Different surgical procedures were also employed to find the optimal approach for handling tissue engineered grafts. In conclusion, the results represent a significant breakthrough in clinical translation of tissue engineered human ear-shaped cartilage given the established vitro engineering technique and suitable surgical procedure. This study was registered in Chinese Clinical Trial Registry (ChiCTR-ICN-14005469).

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1. Introduction

Microtia is a congenital malformation of the external ear, with a varied regional prevalence rate of 0.83 to 17.4 per 10,000 births worldwide, and higher prevalence rates in Hispanics and Asians (Bly et al., 2016; Luquetti et al., 2011; Paput et al., 2012). The auricle is an important identifying feature of human face, and hence its deformity has a profound effect on self-confidence and psychological development in the afflicted children. Current cosmetic procedures of treating microtia mainly include the use of auricular prosthesis, implantation of non-absorbable auricular frame materials or an autologous rib cartilage framework (Bly et al., 2016; Jessop et al., 2016; Wiggenhauser et al., 2017). Non-absorbable frames, such as silastic or high-density polyethylene (Medpor®), generate an excellent ear shape without donor site morbidity, but they lack bioactivity and can lead to extrusion and infections. Autologous rib cartilage transplantation is the current gold-standard treatment for microtia, but harvesting rib cartilage inevitably leads to donor site injury, and replicating the complex 3D ear structure is hard to achieve using surgeons’ hand skill, which is highly
in vitro engineered human ear-shaped cartilage for human auricular reconstruction.

2. Materials and Methods

2.1. Patient Information and Study Design

This study was approved by the Ethical Committee of Plastic Surgery Hospital, Chinese Academy of Medical Sciences, and was registered in Chinese Clinical Trial Registry (ChiCTR, one of primary registers of the WHO International Clinical Trial Registry Platform (ICTRP, http://www.who.int/ictrp/network/chictr2/en/). The registration number for this study is ChiCTR-INC-14005469). Seed cell expansion and manufacturing of the scaffold were conducted according to the defined standard operating procedures (SOP) and established quality management system approved by National Institutes for Food and Drug Control (identification number: SH201300928 for microtia chondrocytes, and QH201300641 for the scaffold). The manufacturing facilities were approved by Shanghai Food and Drug Packaging Material Control Center (identification number: 20130281).

Five patients (male or female, between 6 and 10 years old) with unilateral microtia were recruited. Table 1 lists the full inclusion and exclusion criteria. Three surgical methods for implanting engineered ear cartilages were respectively adopted according to each patient’s physical condition. Table 2 lists the indications and detailed procedures of these three surgical methods. The patients will be followed up intermittently up to 5 years. The primary outcome parameters are the shape, size, and cranio-auricular angle of the reconstructed auricle, which are expected to match those of the contralateral ear. The secondary outcome parameters are the quality of cartilage formation and mechanical property of the reconstructed auricle. The purpose and detailed procedures of this study were explained to the patients and their parents, to whom written informed consent was provided. The current study provides detailed description of a comprehensive case (case 1), a 6-year-old female child, who was the first to receive the tissue engineered ear graft treatment and was followed up for 2.5 years. Preliminary data on the other four on-going cases were also included, and full reporting will be expected after follow up data are completely collected.

A flow chart of the manufacturing and surgical procedure of the first conducted case is shown in Fig. 1. Briefly, CT scanning and 3D reconstruction were applied to obtain a digital image of the patient’s healthy contralateral ear (Fig. 1A). A digital mirror-image was created to guide patient’s ear reconstruction (Fig. 1B), and a corresponding resin model was generated through 3D-printing (Fig. 1C). This resin ear model was used to cast a pair of negative molds (Fig. 1D), in which biodegradable materials made from polyglycolic acid (PGA, Mw = ~30,000, provided by National Tissue Engineering Center of China, Shanghai, China), polyactic acid (PLA, Mn = ~10,000, Sigma-Aldrich, St. Louis, Mississippi, USA), and polycaprolactone (PCL, Mn = ~60,000, Purac Biochem, the Netherlands) were processed into the ear scaffold with the same shape as the resin positive model (Fig. 1E). At the first stage of surgery, microtia cartilage was harvested for chondrocyte isolation (Fig. 1F1) and a tissue expander was implanted for skin expansion (Fig. 1F2). Patients with low skin tension in the retro-auricular region (such as case 4, which is rare for Asian children) may not require skin expansion. During the period of skin expansion, the isolated microtia chondrocytes (MCs) were expanded (Fig. 1G) and seeded onto the ear scaffold for in vitro cartilage engineering (Fig. 1H). After 12 weeks, when a sufficiently-sized skin flap was achieved via tissue expansion and the ear-shaped cartilage was also generated in vitro, the second stage surgery was conducted to implant the engineered ear cartilage into the expanded skin flap for auricular reconstruction (Fig. 1I). Post-
Table 2
Three methods applied for auricular reconstruction using the tissue engineered ear graft

| Method 1 | Method 2 | Method 3 (the Nagata approach) |
|----------|----------|-------------------------------|
| Indication: High skin tension in the retroauricular region (common in Asian people); Severe hemifacial microsomia | Indication: High skin tension in the retroauricular region (common in Asian people); Unwilling to leave scar by skin harvest | Indication: Low skin tension in the retroauricular region |
| Skin expansion: Yes; Split skin graft transplantation: Yes; Adopted by: case 1, 2, and 5; Surgical procedures: First stage: Microtia cartilage harvest and tissue expander insertion and inflation | Skin expansion: Yes; Split skin graft transplantation: No; Adopted by: Case 3; Surgical procedures: First stage: Microtia cartilage harvest and tissue expander insertion and inflation | Skin expansion: Yes; Split skin graft transplantation: Yes; Adopted by: Case 4; Surgical procedures: First stage: Microtia cartilage harvest |
| 1. A 2.0-cm incision was created at the postauricular region; 2. The microtia cartilage was harvested through the incision, and a 80 mL kidney-shaped expander was inserted subcutaneously; 3. The expander was infused intermittently with 0.9% saline solution commencing at the 8th day postoperatively and continued every other day until the volume reached approximately 140 mL and the surface area of the expanded flap reached 9 × 6 cm²; 4. Static expansion was kept until the end of total tissue expansion duration (3 months). Second stage: Auricular reconstruction with tissue engineered ear framework | 1. A C-shaped fascia flap was dissected from the retroauricular region superficial to the periosteum; 2. The engineered ear framework was inserted between the fascial flap and the expanded skin flap; 3. The fascial flap was sutured to the external ear helix; 4. The expanded skin flap was draped over the anterior aspect in a tension-free way and then covered tautly to the framework by means of vacuum drainage | 1. The microtia cartilage was harvested and a 80 mL kidney-shaped expander was inserted between the subfascial layer of the non-hair bearing area and the subcutaneous layer of the scalp; 2. The expander was infused intermittently with 4–7 mL 0.9% saline solution commencing at the 7th day postoperatively and continued every other day until the volume reached approximately 140 mL; 3. Static expansion was kept until the end of total tissue expansion duration (3 months). Second stage: Auricular reconstruction with tissue engineered ear framework |
| Removal of the skin expander: Same as method 1 | Insertion of the ear framework beneath the expanded fascial skin flap: An additional horizontal incision was created across the previous incision. The engineered ear framework was inserted into the envelope through the crossing incisions. The expanded flap covered the entire framework. | Removal of the skin expander: The expander was removed through the same incision created in the first stage surgery. The thickened, edge part of the expander capsule was dissected to loosen the flap. The capsule of the pedicle side was usually reserved. |
| Vacuum drainage: Two negative-pressure drainage tubes were placed in the reconstructed ear, one beneath the anterior side of the flap, the other beneath the posterior side. The outline of the reconstructed ear was clear immediately after suction was applied. | Incision closure: The detached scalp of the mastoid region and the incisions were closed using Z-plasty with two-layer suturing. The suction system was removed 7 days after surgery. | A 2.0-cm incision was created at the mastoid region; 2. The microtia cartilage was harvested and a 80 mL kidney-shaped expander was inserted between the subfascial layer of the non-hair bearing area and the subcutaneous layer of the scalp; 3. Static expansion was kept until the end of total tissue expansion duration (3 months). Second stage: Auricular reconstruction with tissue engineered ear framework |
| Lobule transposition: An anterior and a posterior skin flap from the lobule were created. The posterior flap remains attached to the mastoid skin flap and the anteriorly based tragal flap was used to surface the tragus. A "lazy W-flap" was created by the margins of the mastoid and posterior lobule flap and the middle limbs of the “W” would eventually meet helping form the intertragal notch. This “W-flap” and the anterior lobule flap became transposed in a reciprocal manner resembling a Z-plasty. Vascularity of the W flap was increased by maintaining a subcutaneous pedicle in the floor of the conchal bowl. The above described incisions provided access for creation of the subcutaneous pocket. | Insertion of the ear framework: The engineered ear framework was introduced into the subcutaneous pocket, a suction drain was positioned beneath the framework, and the flaps were secured over this framework with bolsters. Drains were removed in 48–72 h, and bolsters were removed in 2 weeks. | A C-shaped graft engineered using the same approach as the engineered ear framework was “banked” in the subcutaneous layer of the right side abdomen for use in the third stage. |
| 1. Lobule transposition: An anterior and a posterior skin flap from the lobule were created. The posterior flap remains attached to the mastoid skin flap and the anteriorly based tragal flap was used to surface the tragus. A "lazy W-flap" was created by the margins of the mastoid and posterior lobule flap and the middle limbs of the “W” would eventually meet helping form the intertragal notch. This “W-flap” and the anterior lobule flap became transposed in a reciprocal manner resembling a Z-plasty. Vascularity of the W flap was increased by maintaining a subcutaneous pedicle in the floor of the conchal bowl. The above described incisions provided access for creation of the subcutaneous pocket. | 1. A C-shaped graft engineered using the same approach as the engineered ear framework was “banked” in the subcutaneous layer of the right side abdomen for use in the third stage. | 2. Insertion of the ear framework: The engineered ear framework was introduced into the subcutaneous pocket, a suction drain was positioned beneath the framework, and the flaps were secured over this framework with bolsters. Drains were removed in 48–72 h, and bolsters were removed in 2 weeks. |
| Second stage: Auricular reconstruction with tissue engineered ear framework | Third stage: Elevation of the reconstructed ear | Third stage: Elevation of the reconstructed ear |
| 1. Lobule transposition: An anterior and a posterior skin flap from the lobule were created. The posterior flap remains attached to the mastoid skin flap and the anteriorly based tragal flap was used to surface the tragus. A "lazy W-flap" was created by the margins of the mastoid and posterior lobule flap and the middle limbs of the “W” would eventually meet helping form the intertragal notch. This “W-flap” and the anterior lobule flap became transposed in a reciprocal manner resembling a Z-plasty. Vascularity of the W flap was increased by maintaining a subcutaneous pedicle in the floor of the conchal bowl. The above described incisions provided access for creation of the subcutaneous pocket. | 1. Six months after the second stage, the ear framework was elevated from the mastoid skin by dissection into the postauricular sulcus. The postauricular skin was then undermined and advanced into the area of the postauricular sulcus allowing adequate projection of the ear. | 1. Six months after the second stage, the ear framework was elevated from the mastoid skin by dissection into the postauricular sulcus. The postauricular skin was then undermined and advanced into the area of the postauricular sulcus allowing adequate projection of the ear. |
| The previously banked C-shaped graft was retrieved, which was then placed under the neoauricle (between the framework and mastoid), and secured by suture. The graft was then covered with a well vascularized tissue to allow for overlying skin graft adherence and prevention of infection or extrusion. A temporoparietal fascia flap was raised and advanced forward to cover the posterior aspect of the neoauricle and the C-shaped graft. | 2. Insertion of the ear framework: The engineered ear framework was introduced into the subcutaneous pocket, a suction drain was positioned beneath the framework, and the flaps were secured over this framework with bolsters. Drains were removed in 48–72 h, and bolsters were removed in 2 weeks. | 2. Insertion of the ear framework: The engineered ear framework was introduced into the subcutaneous pocket, a suction drain was positioned beneath the framework, and the flaps were secured over this framework with bolsters. Drains were removed in 48–72 h, and bolsters were removed in 2 weeks. |
| A split thickness skin graft was harvested from the groin area to cover the remaining exposed temporoparietal fascia flap. A bolster was then secured into the sulcus with suture. The bolster was kept in place for 1 week. | (Refer to Nagata, 1993 and Shokri and White, 2017 for detailed descriptions) | (Refer to Nagata, 1993 and Shokri and White, 2017 for detailed descriptions) |
implantation follow-up assessments were conducted at different
time intervals to evaluate the clinical outcomes (Fig. 1).

2.2. Harvest of Microtia Cartilage and Skin Flap Expansion

For the first conducted case (case 1), a 2.0-cm incision was made at
the postauricular region, into which a kidney-shaped expander was
inserted subcutaneously. Meanwhile, microtia cartilage (approximately
1.5 × 1.5 cm²) was harvested and delivered to the lab which is of good
manufacturing procedure (GMP) level for human chondrocyte isolation.
The expander was intermittently infused with 0.9% saline solution com-
mencing at the 8th day postoperatively. The infusion was continued
every other day until the volume reached approximately 140 mL and
the surface area of the expanded \textit{flap} reached 9 × 6 cm². The skin
\textit{flap} was then kept in a state of static expansion until the end of total tissue
expansion duration (3 months). Reviewers may refer to Jiang et al.,
2008 for more detailed description. Procedures for the other two
methods are listed in Table 2.

2.3. Isolation and Expansion of MCs

The harvested microtia cartilage was carefully dissected to remove
fibrous tissue and perichondrium, then fragmented into 1 mm³ pieces,
washed in phosphate buffered saline (PBS) solution containing
100 U/mL penicillin and 100 μg/mL streptomycin, and digested with
0.3% collagenase NB4 (Worthington Biochemical Corp., Freehold, New
Jersey, USA) for 8 h at 37 °C. The isolated cells were cultured and ex-
panded in Dulbecco’s modified Eagle’s medium (DMEM, Gibco BRL,
Grand Island, New York, USA) containing 5.0 ng/mL basic fibroblast
growth factor (bFGF, R&D Systems Inc., Minneapolis, USA) and 10%
fetal bovine serum (FBS, Hyclone, Logan, Utah, USA). Cells in passage 2
were used for \textit{in vitro} cartilage engineering.

2.4. Manufacture of Ear-shaped Scaffold

The ear mold was created according to a previously-described meth-
method (Liu et al., 2010). Briefly, the healthy ear of the patient was scanned
by CT and the image reflected across the vertical access (with mirror
symmetry) by a computer aided design (CAD) system (3DPRO Technol-
ogy Co., Ltd, Shanghai, China). These data were used to generate the
resin ear model via 3D-printing by a computer aided manufacturing
(CAM) system (Spectrum 510, Z Corporation, Massachusetts, USA).
This 3D-printed ear model was cast by clay and silicone to produce a
set of negative molds in which biomaterial scaffolds could be molded
into the ear shape identical to the 3D-printed resin ear.

The ear scaffold used a PCL mesh as an inner core, which was wrapped
with PGA unwoven fibers and coated with PLA. To generate the inner
core, a 9 × 9 cm² PCL mesh with 3 × 3 mm² grids (~1.37 mm in thickness)
was 3D-printed. The mesh was pre-shaped by hot (55 °C) compression
molding in the negative ear molds and the resulting scaffold was
trimmed to make an ear outline. To generate the outer PGA/PLA layers,
two pieces (500 mg each) of unwoven PGA fibers were pressed into 8
× 9 cm² sponges respectively, which were then compression-molded
by the negative ear molds to generate the ear contour. The PCL inner
core was then sandwiched between the pair of PGA layers. The entire
scaffold of PCL inner core surrounded by PGA nonwoven fibers was
merged by immersion into 0.3% PLA (Sigma Aldrich, Product No.
765112) solution in dichloromethane and hot (55 °C) compression mold-
ing again till dry, which was able to partially merge the PGA nonwoven
fibers with the PCL core material. The edge of the resultant scaffold was
trimmed according to the previous 3D-printed resin ear.

2.5. Biocompatibility Evaluation of the Scaffolds

The cross section of a scaffold piece (1 × 1 cm² with a thickness of
1.5 mm) prepared using the same methods as the ear scaffold was

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Fig_1}
\caption{A flow chart for schematic illustration of auricular reconstruction based on \textit{in vitro} tissue engineered human ear-shaped cartilage.}
\end{figure}
examined by scanning electron microscopy (SEM) (Philips XL-30, Amsterdam, Netherlands) to confirm integration of the PGA fibers with the PCL core. After cell seeding, the chondrocyte-scaffold constructs were also examined by SEM at different culture time points to observe cell attachment and extracellular matrices (ECM) production on the scaffolds.

2.6. In Vitro Engineering of Ear-shaped Cartilage

Expanded MCs (passage 2) at a seeding density of $4.5 \times 10^8$ cells in 5 mL medium (90 million cells/mL) were evenly dropped onto the PGA/PLA layer of the ear-shaped scaffold, followed by 5 h incubation at 37 °C, 5% CO$_2$. The construct was then cultured in chondrogenic medium.

Fig. 2. Preparation of the patient-specific ear-shaped scaffold. The CT reconstructed 3D mirror image of the patient healthy ear is used as a shape model (A), according to which, a pair of negative molds is produced (B, C). PCL mesh is 3D-printed (D) and pre-shaped by the negative molds to generate an ear contour (E, F). Nonwoven PGA fibers are prepared as a sponge (G) and pre-shaped to a pair of ear contours (H, I). The pre-shaped PCL inner core is sandwiched between the pair of pre-shaped PGA layers (J). The whole set of scaffold is coated with PLA, pressed into the ear shape, and trimmed according to the shape of the 3D-printed ear (K, L).
composed of DMEM, 10 ng/mL transforming growth factor beta-1 (TGF-$\beta$1, R&D Systems Inc. Minneapolis, USA), 50 ng/mL insulin-like growth factor-I (IGF-I, R&D Systems Inc.), and other supplements (without any serum, all the ingredients of the medium were definable). The medium was changed every other day. The in vitro engineered cartilage framework was harvested at the 12th weeks for external ear reconstruction. Before implantation, a cartilage piece (1 × 1 cm$^2$ with a thickness of 1.5 mm) engineered with the same method as the ear graft and cultured

Fig. 3. In vitro generation and evaluation of human ear-shaped cartilage. The prepared scaffold shows clear ear features including the helix, anti-helix, triangular fossa, and cavum conchae (A). On cell seeding, the scaffold quickly absorbs the cell suspension (B). After 12 weeks of in vitro culture, the cell-scaffold construct forms a neo-cartilage tissue with the original shape (C). Laser scanning confirms that the shape of the regenerated ear cartilage reaches more than 90% similarity compared with the original scaffold (D–F). SEM confirms good cell compatibility of both the PGA/PLA fibers and PCL core, and ECM coverage of the whole scaffold at week 4 of in vitro culture (G–H). Mechanical testing confirms that the PCL core significantly enhances the mechanical strength of both the scaffold and in vitro engineered cartilage (I). (The laser scanning data were collected from another patient (Case 4); corresponding gross samples are shown in Fig. 9).

Fig. 4. Cross section and its histological characterization of the in vitro tissue engineered cartilage. The cross section of the engineered cartilage showing a chimeric structure with outer semi-transparent cartilage-like tissue, inner non-transparent PCL grids, and incompact tissue between the PCL bars (A). The outer cartilaginous regions (red frames) showing abundant lacuna structures (B, C) with strong positive SO (D) and collagen II (E) staining. The inner PCL regions (yellow and green frames) corresponding to PCL grid bars contain evenly distributed chondrocytes (F–H, L–N). The areas between the grid bars (blue frames) reveal incompact tissue with weak positive SO and collagen II staining (B, I–K). Yellow arrows indicate residual PGA fibers. Black windows indicate the magnified area.
in the same condition was used for histological and immunohistochemical examinations as a quality control sample. When the control sample was confirmed for obvious cartilage formation and free of contamination, the corresponding ear graft was then released for clinical use.

2.7. Shape Analysis of In Vitro Engineered Human Ear-shaped Cartilage

Surface image data were collected from both the ear-shaped scaffold (before cell seeding) and the ear-shaped cartilage framework (before in vivo implantation) by a 3D laser scanning system, in accordance with previously established methods (Liu et al., 2010). The 3D data of the ear framework were compared to those of the ear scaffold with an accepted variation in voxels smaller than 1.5 mm for evaluating the shape similarity level between the ear framework and original scaffold (Liu et al., 2010).

2.8. Implantation of Ear Cartilage Framework

The surgical techniques required for implantation of the engineered ear framework were identical to those established for the autologous rib...
cartilage framework. For case 1 (Method 1), after removing the skin expander, the skin flap and a random retroauricular neurovascular fascia flap (about 7.0 × 4.5 cm²) were prepared. During the procedure, care must be taken to avoid vascular compromise of the expanded flap, and a C-shaped fascia flap was dissected from the retroauricular region superficial to the periosteum. The in vitro engineered ear framework was then inserted between the fascial flap and the expanded skin flap. The fascial flap was sutured to the external ear helix to cover the posterior aspect of the ear framework. The expanded skin flap was draped over the anterior aspect in a tension-free way and then covered tautly to the framework by means of vacuum drainage. Finally, a split-thickness skin graft, which was harvested from the groin region, was sutured onto the mastoid region to cover the posterior auricular fascial flap. Long sutures were tied over a bolster to tamponade the graft to the recipient bed. A head dressing was applied to protect the engineered ear and all the incisions. Sutures were removed at day 10 post-operation. The main surgical procedures for case 1 were demonstrated in Supplemental Video 1. The above method (method 1) was also applied on cases 2 and 5. Method 2 for case 3 and method 3 for case 4 are listed in Table 2.

2.9. Post-Implantation Follow-Up Assessments

After suture removal, the reconstructed auricle was photographed at 1, 2, 3, 6, 9, 12, 18, 24, and 30 months post-surgery to record swelling, inflammation signs, and shape recovery. Subsequent surgeries were
conducted for removing the pedicle of skin flap at 6 months and repairing scar at 18 months, which allowed for tissue biopsies of the implanted ear framework. Both biopsied samples were subjected to histological and immunohistochemical examination to evaluate the state of cartilage formation in vivo. Magnetic resonance imaging (MRI) was performed using a 1.5 T MR System at different time points post-implantation to trace the state of cartilage regeneration and PCL core degradation in the implanted ear framework.

2.10. Histological and Immunohistochemical Analysis

Samples from the in vitro engineered cartilage piece and in vivo biopsied tissue were frozen in liquid nitrogen for cryosection or fixed in 4% paraformaldehyde for 24 h prior to embedding in paraffin. The samples were sectioned into 5-μm slices, mounted on glass slides, and stained with hematoxylin and eosin (HE) or Safranin-O/Fast Green (SO/FG) using previously established protocols (Zhang and Spector, 2009). Detection of elastin was performed using a modified Verhoeff van Gieson (EvG) elastic stain kit (Sigma-Aldrich, St. Louis, Missouri, USA) following the manufacturer’s instructions.

Expression of type II collagen was detected using mouse anti-human type II collagen monoclonal antibody (1:200 in PBS, Santa Cruz, California, USA), followed by incubation of horseradish peroxidase (HRP)-conjugated anti-mouse antibody (1:200 in PBS, Santa Cruz), and color development with diaminobenzidine tetrahydrochloride (DAB, Santa Cruz) (Yan et al., 2009).

2.11. Molecular Weight Assay of PCL

Analyses of number-average molecular weight (Mn) and weight-average molecular weight (Mw) of PCL were conducted by size exclusion chromatography (SEC) at different stages, including raw PCL, PCL core (after 3D-printing and hot compression molding), pre-implantation (after in vitro culture for 3 months), and post-implantation (after in vivo implantation for 18 months), the sample under evaluation was part of the biopsy taken from case 1 after in vivo implantation for 18 months. The PCL degradation profile was revealed according to changes in Mn, Mw, and Mw/Mn (polydispersity) values at different stages.

2.12. Statistical Analysis

All results are expressed as mean ± s.d. Differences among/between experimental groups were analyzed using one-way ANOVA/Student t-test. A value of p < 0.05 was considered as statistically significant.

3. Results

3.1. Preparation of the Ear Scaffold

The ear scaffold was produced by the processes depicted in Fig. 2. Using these methods, the ear scaffold had a sandwiched structure and
presented the mirror image of the patient’s normal ear with detailed structures including the helix, anti-helix, triangular fossa, and cavum conchae (Fig. 2K, L). The cross-section of a scaffold piece, produced using the same method as the ear scaffold, presented a chimeric structure with outer PGA fibers and inner PCL grid bars (Supplemental Fig. 1A). It is worth noting that the PCL bars themselves also showed a chimeric structure, where PGA fibers were evenly embedded within the PCL matrix (Supplemental Fig. 1B–F), indicating that the hot compression process was able to partially merge the PGA fibers with the PCL core.

3.2. In Vitro Engineering of Ear-shaped Cartilage Framework

In our cell expansion conditions, MCs underwent robust proliferation (Supplemental Fig. 2) and the expanded cells showed good chondrogenic function after being seeded onto PGA/PLA scaffold and in vitro cultured for 12 weeks (Supplemental Fig. 3). Approximately $4.5 \times 10^6$ MCs were originally isolated from the microtia cartilage. These cells were expanded to about $4.5 \times 10^8$ cells at passage 2. After being seeded onto the ear scaffold, the cell suspension was quickly absorbed and distributed throughout the whole scaffold (Fig. 3A–B, G). After 12 weeks of in vitro culture, a cartilage-like framework was formed and the original ear shape was largely retained (Fig. 3C) which was also supported by the histological examination of similarly engineered cartilage peace (Fig. 4). Laser scanning analysis showed that the shape of the regenerated ear at 12 weeks attained >90% similarity compared with the shape of the original scaffold (Fig. 3D–F), indicating that accurate shape control of the in vitro engineered cartilage could be achieved by controlling the shape of its scaffold.

Scanning electron microscopy examination revealed that cells were evenly distributed among the PGA fibers and PCL core, and gradually produced ECM to cover the whole scaffold with increased in vitro culture time (Fig. 3G, H; Supplemental Fig. 4). No obvious cell detachment was observed during in vitro culture, implying good cell affinity to both PGA fibers and PCL core. It is worth noticing that the mechanical strength of the in vitro engineered constructs was significantly enhanced by the PCL core and reached a 4-fold increase compared with that of the constructs without the PCL core (Fig. 3I), which contributed to the shape maintenance after in vivo implantation.

The cross section of a regenerated cartilage piece engineered in the same way as the ear framework (a quality control sample) showed a chimera structure, in which the outer cartilaginous tissue surrounded and covered the inner PCL grids, with incompact tissue observed between the grid bars (Fig. 4A). Histological examination further confirmed typical cartilage features at the outer layer, with abundant lacuna structures, glycosaminoglycan (GAG) deposition, and collagen II expression (Fig. 4B–E). In the central part, un-degraded PCL regions (corresponding to the PCL bars) and incompact tissue between bars were observed (Fig. 4F–N). It is worth noticing that high numbers of chondrocytes were observed in the PCL bar regions with a pore...
3.2. Transformation of the PCL Bar Regions

Transformation of the PCL bar regions, from PGA fiber/PCL chimeric structures (Supplemental Fig. 1) to porous chondrocyte/PCL chimeric structures (Fig. 4F–H, L–N), implied that the pore structure of the PCL bars resulted from PGA degradation during in vitro culture, thus providing a path for cell infiltration. In the region between the grid bars, consistent with the gross view, incompact tissue with the un-degraded PGA residues was observed (Fig. 4I–K). Importantly, after 3 months of in vitro culture, the PGA fibers were mostly degraded with abundant cartilage ECM formation (especially those in the outer layer) (Fig. 4A–E). This occurrence would likely reduce direct exposure of the existing PGA residues to the immune system, and therefore alleviate the host responses towards the engineered graft, as we have previously demonstrated (Luo et al., 2009; Liu et al., 2017).

3.3. Implantation of the Ear Framework

After 12 weeks of skin expansion, a kidney-shaped skin capsule was formed and abundant capillaries were distinctly observed in the expanded skin (Fig. 5A). After removing the expander and stripping the fibrous capsule, an expanded skin flap and a post-auricular neurovascular fascia flap were prepared (Fig. 5B, C). The in vitro engineered ear framework was then inserted between the fascial flap and the expanded skin flap, and fixed by suturing the fascial flap to the helix (Fig. 5D, E). The expanded skin flap was used to cover the frontal surface of the ear framework, which was tightly attached to the flap under negative pressure of the suction drain and comprised detailed structures of auricular helix, anti-helix, triangular fossa, and cavum conchae (Fig. 5F–H). The main surgical procedures were demonstrated in Supplemental Video 1 and the ear framework displayed a cartilage-like appearance with mechanical properties strong enough for surgical handling.

3.4. Post-Implantation Follow-Up of Auricular Outline

Within the first two weeks, the reconstructed ear showed obvious edema with a blurring shape (data not shown). After two weeks, the edema slowly reduced and the shape of the reconstructed ear, as well as the color of the covered skin, gradually recovered (Fig. 6). Within 6 months post-implantation, only the basic ear contour was observed, while key auricular structures, such as helix, triangular fossa, anti-helix, and cavum conchae, became gradually distinct after 9 months (Fig. 6). It was worth noticing that at 12 months, the reconstructed auricle presented high stiffness and low flexibility (Supplemental Video 2), whereas at 24 months, an obvious improvement in flexibility with more distinct structures were achieved (Supplemental Video 3; Fig. 6).

The molecular weight assay of the PCL core revealed a decline trend in both Mn and Mw at different time points, indicating slow degradation of the PCL (Supplemental Fig. 5). These results were further supported by the gradual decline in MRI signals in the PCL core regions with increased post-implantation time. In particular, a significant decrease was observed at 24 months post-implantation (Fig. 6, red arrowed), which provided a reasonable explanation for improved flexibility at 2 years.
3.5. Histological Examinations of In Vivo Regenerated Cartilage

During the procedure of skin flap pedicle removal at 6 months post-implantation, a biopsy was taken from the tragus region of the ear framework (Fig. 7A). The biopsied tissue showed an ivory-white cartilaginous appearance (Fig. 7B). Histological examinations revealed typical cartilage formation with abundant lacuna structures, GAG deposition, and strong collagen II expression (Fig. 7C–E, G–I), similar to the native ear cartilage (Fig. 7K–M). Particularly, the expression of elastin (indicated by EvG staining) was also detected in the regenerated cartilage (at a slightly lower level than that in native cartilage) (Fig. 7F, J, N), indicating the formation of elastic cartilage. No PCL core was observed in this sample as a result of the biopsy location. At 18 months post-implantation, further scar revision procedure was performed, which allowed a very small biopsy to be obtained from the cavum concha region of the ear framework. Histological examinations showed that a chimeric structure with both undegraded PCL (Fig. 8, red frames) and cartilage-like tissue characterized by abundant lacuna structures and positive staining of SO/FG and collagen II (Fig. 8, blue frames). Most importantly, consistent with the histological findings before implantation, scattered cells were still detected in the PCL regions (Fig. 8, red frames), indicating good cell compatibility of the PCL core.

3.6. Post-Implantation Follow-Up of Four Other Cases

Four other cases, in which patients had received an engineered ear transplant and were being followed-up, revealed similar in vitro and clinical outcomes corresponding to the first patient (Fig. 9). Among the total five cases, four cases showed obvious cartilage formation after 6 months post implantation. Summary for all five patients is provided in Table 3. Owing to the short follow-up period, full examination data will be provided in future. Note that one original case failed to show up for all of the post-surgery follow-ups after the second stage surgery and therefore was removed from the current trial. An additional case (case 5 in Fig. 9) was thus enrolled instead of the original one to make up five cases in total.

4. Discussion

Tissue engineered auricle is a promising alternative to current ear reconstructive options, but its clinical translation is yet to be accomplished. In the current study, cartilage frameworks with patient-specific ear shapes and proper mechanical strength were successfully engineered in vitro. Using these engineered ear frameworks, we performed external ear reconstruction on 5 patients and achieved satisfactory therapeutic outcome as revealed during 2.5 years’ follow-up so far. The achievement of this clinical translation should be attributed to the integration and innovation of several strategies, including using expanded MCs as seed cell source, in vitro culture to alleviate the host response towards the implanted graft, CAD-CAM for patient-specific cartilage shape control, and PCL inner core for long-term shape maintenance.

Microtia chondrocytes have been proposed as a promising cell source due to their abilities to form elastic cartilage and since they can be isolated from the patient’s microtic ear without injuring healthy cartilage (Ishak et al., 2015; Kamil et al., 2004; Kobayashi et al., 2011; Nakao et al., 2017). In the current study, the patient’s MCs that were expanded in a condition containing bFGF demonstrated the abilities of robust proliferation, 3D cartilage generation, and stable subcutaneous cartilage formation, which confirmed their candidacy as a practical cell source to engineer auricular cartilage for clinical application.

Polyactic acid (PLA) coated PGA, as the traditional synthetic scaffold employed by many pioneered tissue engineering studies, is especially suitable for engineering cartilage with complex shape due to its controllable mechanical properties and shape processing (Cao et al., 1997; Mooney et al., 1996). However, PGA/PLA could induce significant host response once implanted in immunocompetent mammals, leading to the failure of cartilage formation (Ceonzo et al., 2006; Haisch, 2010; Rotter et al., 2005). In the current study, an in vitro engineering strategy

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### Table 3

Summary of the total five cases.

| Age | Gender | Diagnosis (Weerda’s classification) | Surgical method for graft implantation | Follow-up period | Outcome |
|-----|--------|-------------------------------------|----------------------------------------|-----------------|---------|
| 6   | F      | Unilateral grade III microtia, right side. | Method 1 | 2.5 years | • Obvious cartilage formation before implantation  
• Shape, size and craniaoauricular angle match the contralateral normal ear  
• Landmark structures are distinct  
• No sign of absorption or extrusion |
| 9   | F      | Grade III microtia on the right side; grade I microtia on the left side. | Method 1 | 1.5 years | • Obvious cartilage formation before implantation  
• Craniaoauricular angle match the contralateral ear.  
• Helix and cavum concha are distinct, triangular fossa and anti-helix are vague.  
• No obvious cartilage formation at the biopsy site after 6 m.  
• No sign of absorption or extrusion |
| 8   | F      | Unilateral grade III microtia, right side | Method 2 | 1 year | • Obvious cartilage formation before implantation  
• Shape, size and craniaoauricular angle match the contralateral normal ear.  
• Landmark structures are distinct  
• Obvious cartilage formation at the biopsy site after 6 m.  
• Slight distortion occurred after 6 m post surgery |
| 7   | M      | Unilateral grade III microtia, right side | Method 3 | 6 months | • Obvious cartilage formation before implantation  
• Shape and size match the contralateral normal ear  
• Landmark structures are distinct  
• Obvious cartilage formation after 6 m post implantation  
• Slight distortion occurred after the second stage surgery (creation of the craniaoauricular angle)  
• No sign of absorption or extrusion |
| 7   | F      | Unilateral grade II microtia, right side | Method 1 | 2 months | • Obvious cartilage formation before implantation  
• Shape and size of craniaoauricular angle match the contralateral normal ear  
• Landmark structures are distinct  
• Obvious post-Op cartilage formation  
• No sign of absorption or extrusion |
was applied, which allowed sufficient degradation of the PGA/PLA scaffold and abundant deposition of the autologous cartilage ECM and thus greatly reduced direct exposure of PGA fibers to the immune system. This procedure can therefore alleviate the host responses as we previously demonstrated (Luo et al., 2009, 2013; Liu et al., 2017). In addition, in vitro culture also allows for quality assessments of the engineered cartilage before transplantation.

Generally, in vitro engineered ear cartilage is usually too weak to maintain its complex 3D structures after its implantation under skin tension. In the current study, by means of combining CT scanning, CAD-CAM, and 3D-printing technologies, we were able to generate a PCL inner core supported PGA/PLA scaffold that not only replicated the patient-specific ear structures, but also provided mechanical support for shape maintenance of the patient-specific cartilage regeneration in vitro and in vivo.

The advantages of using mechanically stiff material as an inner stent to support the ear shape of the engineered cartilage has been described by previous researchers (Bichara et al., 2014). However, most of them used non-degradable materials (such as titanium wire), which is likely to be extruded after a certain time period. The current study used PCL as the inner core, which can be biodegraded by hydrolysis of its ester linkages in a slow manner so that the engineered cartilage may have sufficient time to mature and gain mechanical properties while gradually replacing the degrading inner core (Yan et al., 2009). According to literature, complete degradation of PCL in vivo requires 2–4 years (Höglund et al., 2007; Hutmacher et al., 2001; Oh et al., 2011; Sun et al., 2006; Tuba et al., 2014; Woodruff and Hutmacher, 2010). As this particular case has been followed up for 2.5 years and plus 3 months of in vitro culture, the PCL frame has already undergone over 2 years’ degradation, by which time a significant portion of PCL has degraded as confirmed by MRI examination, whereas the engineered ear cartilage remained able to retain its original 3D shape supported with significantly decreased stiffness and increased elasticity, indicating that it was the regenerated cartilage rather than the PCL core that maintained the ear shape. Meanwhile, we have previously shown that in vivo regenerated cartilage itself without PCL frame had mechanical properties similar to those of native cartilage (Yan et al., 2009). In the current study, histological examination of the biopsied samples revealed formation of mature in vivo cartilage at 6 m and 18 m post-op (Figs. 7 and 8). Obviously, the mechanical support contributed by the engineered cartilage should not be denied. Moreover, the low melting point (63 °C) of PCL allowed a small portion of PGA without PCL framework to surgical trauma again shortly after its implantation, which may aggravate the inflammatory reaction caused by the previous surgery and lead to long lasting edema which may compromise the ear shape. Therefore, the selection of an optimal surgical procedure for handling and implanting the engineered ear graft is extremely important. Besides, the in vitro engineered ear graft (neocartilage) was more delicate and fragile than the graft carved from the fully developed rib cartilage, and the acute inflammatory trauma environment as well as the excessive handling during surgery may reduce the viability of the resident chondrocytes of the engineered ear graft, thus, hindering the subsequent chondrogenesis and shape maintenance after implantation. Moreover, developing more mature cartilaginous grafts to ease the surgical handling is also important for the widespread application of engineered ear graft in future.

In summary, we were able to successfully design, fabricate, and regenerate patient-specific external ears. The first clinical study of translating the well-known human-ear-shaped cartilage from nude mouse to human may represent a follow-up significant achievement in the field of tissue engineering after its original experimental study (Cao et al., 1997). Nevertheless, further efforts remain necessary to eventually translate this prototype work into routine clinical practices. In the future, long-term (up to 5 years) follow-up of the cartilage properties and clinical outcomes after complete degradation of the PCL inner core will be essential. In addition, further optimization and standardization in scaffold fabrication, cell expansion, in vitro cartilage engineering, surgical procedures, as well as multi-center clinical trials would also be the targets for the future investigations. 3D bioprinting (print with cells) for direct fabrication of ear-shaped cartilage may also be a future direction (Kang et al., 2016).

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**Conflicts of Interest**

We declare that we have no conflicts of interest.

**Author Contributions**

GZ was the principal investigator of the grant supporting the current scientific work, conceived the overall technical procedures and participated in surgical design with detailed supervision particularly on scaffold design, manufacture, and in vitro cartilage engineering; GZ also significantly contributed to the preparation of this report by initial draft, data collection and interpretation, and figure preparation. HJ was responsible for the surgical design and performed main surgery and peri-operative care. ZY performed most of the primary technical work, including scaffold preparation, cell isolation and culture, peri-operative care. ZY performed most of the primary technical work, conceived the overall technical procedures and participated in surgical design, manufacture, and in vitro cartilage reconstruction, examinations for cartilage formation, and primary data collection. YL was responsible for the technical design, developed the key techniques for shape control, ear scaffold manufacture, massive expansion of microtia chondrocytes, and in vitro regeneration of ear-shaped-cartilage based on these cells. YL also significantly contributed to the manuscript drafting, data interpretation, and figure preparation. QZ, CZ, BY, JZ, XZ, and HS participated in the surgical design and were responsible for the post-operative care and data collection from the five patients. DL and AH helped to conduct the primary experiment. ZZ, WZ, and WL participated in overall design and manuscript preparation, and WL advised overall manuscript structure, edited and finalized the report. YC was the principal investigator on the grant supporting the preliminary and current work, proposed, supervised, and participated in the study. YC was the principal investigator on the grant supporting the preliminary and current work, proposed, supervised, and participated in the study.

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