EarSkin and EarCartilage—combining bioengineered human skin with bioprinted cartilage for ear reconstruction

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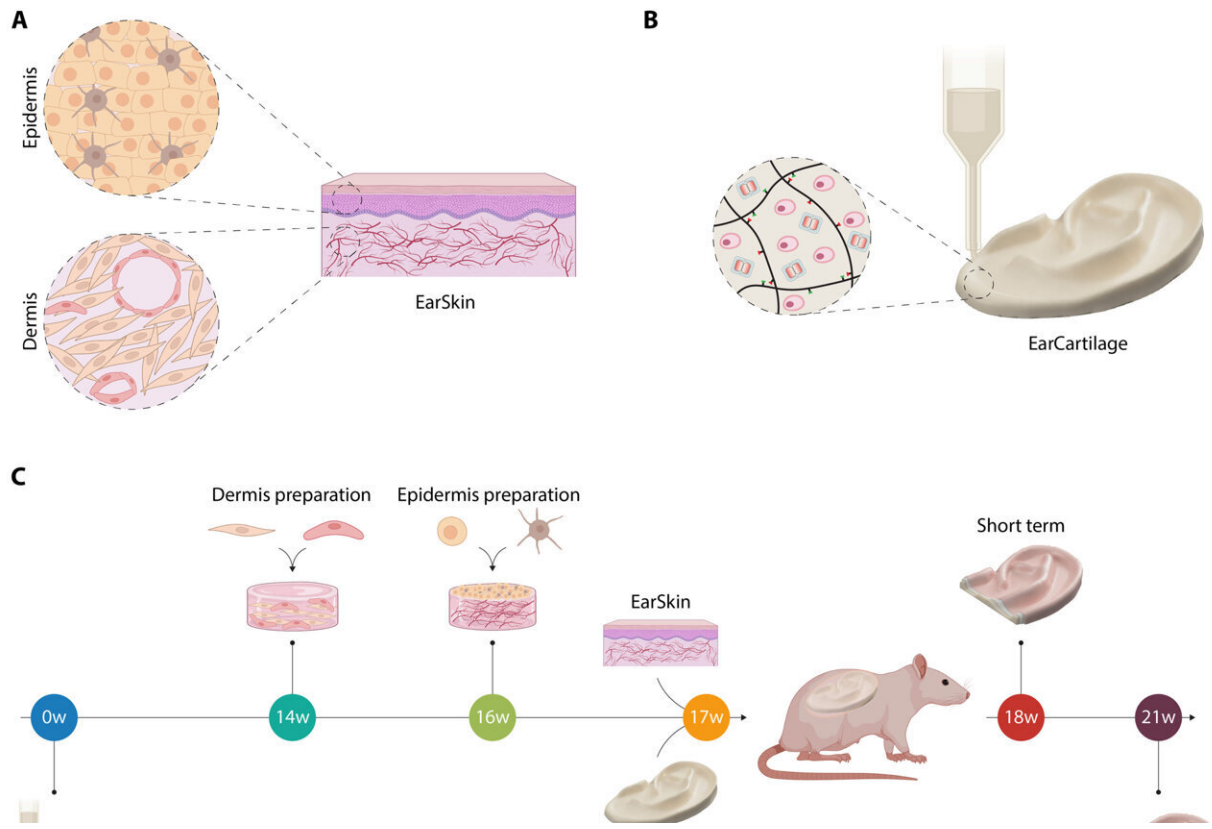


Schematic of the combination of EarCartilage and EarSkin. (A) EarSkin was prepared by creating a dermal layer fabricated out of human fibroblasts and human dermal microvascular endothelial cells (HDMECs) in a collagen I hydrogel. Once matured, an epidermal layer of human keratinocytes and melanocytes was seeded on top of the dermal part. (B) EarCartilage was fabricated using a hyaluronan transglutaminase (HATG)–based bioink together

with primary human auricular chondrocytes. Postprinting constructs can be enzymatically crosslinked using calcium-triggered enzymatic crosslinking of factor XIII (30). (C) Experimental timeline of EarCartilage and EarSkin in vivo. EarCartilage was matured for 17 weeks before implantation. EarSkin was created by combining fibroblasts and HDMECs in a collagen I hydrogel and matured for two weeks before seeding keratinocytes and melanocytes on top and culturing it for an additional week. Constructs were then implanted together in vivo and analyzed after one (short term) and four (long term) weeks. w, weeks. (D) Human EarSkin (A) and human EarCartilage (B) were combined in vivo in an immunocompromized rat model. A subcutaneous pocket below the panniculus carnosus was created along the dorsal midline into which EarCartilage was transplanted. The panniculus carnosus was used to cover the EarCartilage framework and to provide rapid nourishment to EarCartilage and EarSkin. EarSkin was then transplanted on top of the panniculus carnosus. Credit: *Science Advances*, DOI: 10.1126/sciadv.adh1890

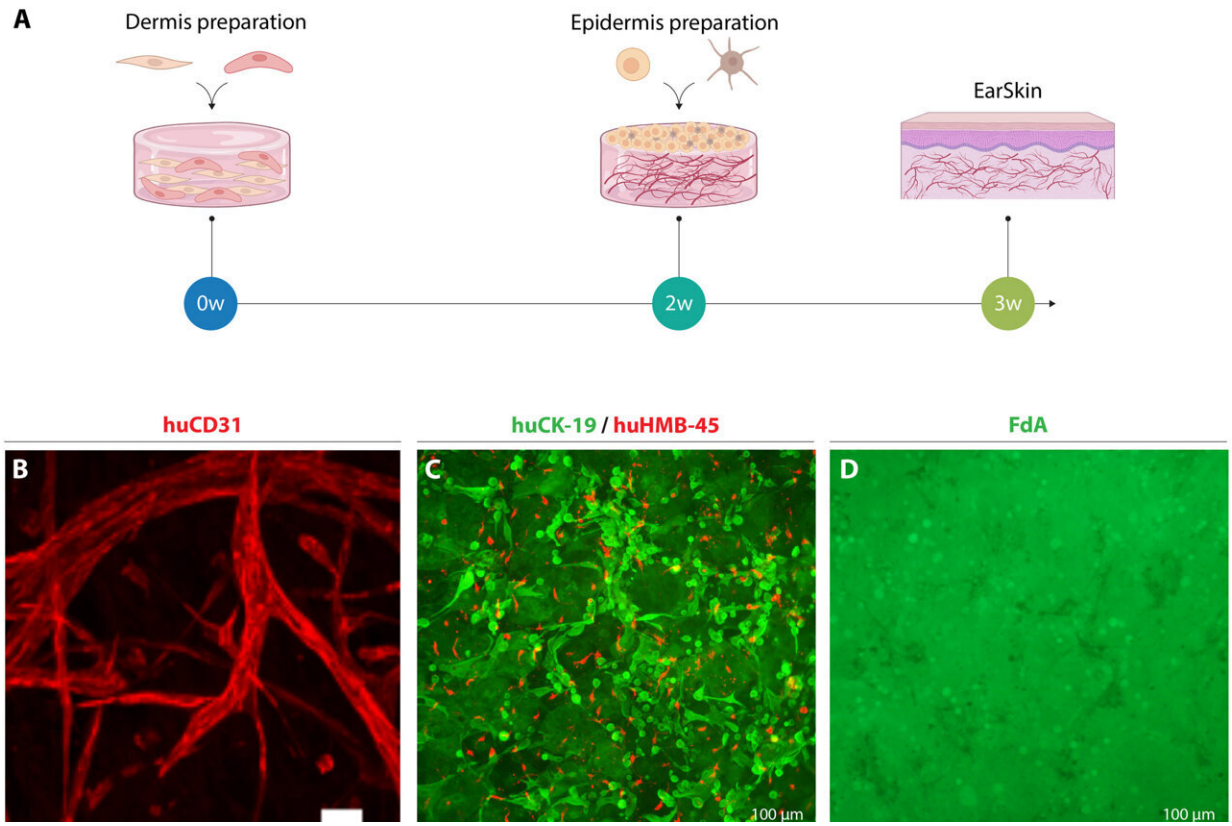
Microtia is a congenital disorder that can occur as a malformation of the external ear in children. In a new study published in *Science Advances*, Dominika Zielinska and a research team in tissue biological research, tissue engineering, polymer technologies and biofabrication at the University of Children's Hospital Zurich, ETH Zurich, Switzerland and the U.S., developed a tissue-engineered treatment approach by using bioprinted autologous auricular cartilage construct as ear cartilage, combined with a bioengineered human pigmented and prevascularized ear skin substrate already tested in immunocompromised rats.

The team confirmed the capacity of the human engineered blood capillaries of EarSkin to connect to the recipient's vasculature within a week for rapid blood perfusion and epidermal maturation. The bioengineered EarSkin demonstrated a stratified epidermis with mature keratinocytes and melanocytes, where the latter resided within the basal layer of the epidermis to efficiently restore the skin color.

Additional in vivo tests showed favorable mechanical stability of the ear cartilage accompanied with improved extracellular matrix deposition. The combined trademarks of Earskin and EarCartilage represented a new treatment approach to microtia, to overcome limits and improve the aesthetic outcomes of microtia reconstruction.

Characterizing microtia for EarSkin and EarCartilage tissue engineered grafts

During congenital conditions, microtia can be characterized by the abnormal development of the external ear at the fetal stage, where it ranges from minor deformities to a complete lack of the auricle. Microtia affects a broad population of children worldwide, affecting their psychosocial well-being, while treatment strategies only become available by the age of 10. The gold standard to treat microtia is autologous costal cartilage reconstruction, where the costal cartilage can be harvested from the ribcage to carve out an auricular graft framework.



Development of EarSkin in vitro. (A) Illustration of the preparation of human prevascularized dermo-epidermal skin substitutes—EarSkin. Fibroblasts and HDMECs were combined with a collagen I hydrogel to create a dermal prevascularized layer and cultured for two weeks in vitro. At two weeks, keratinocytes and melanocytes were seeded on top of the dermal layer to create the epidermal layer. (B) Representative confocal image of whole-mount immunofluorescence staining for human-specific CD31 (red) showing the formation of branching capillaries by HDMECs. Scale bar, 100 µm. n = 6. (C) Immunofluorescent overlay image of whole-mount staining for human cytokeratin 19 (CK19) (green) and human HMB-45 (red) demonstrating the uniform distribution of melanocytes (red) within the keratinocytes (green) one week after seeding. Scale bars, 100 µm. n = 6. (D) Fluorescein diacetate (FdA) staining (green) to assess cell viability, the presence of a continuous epidermal layer formation, and keratinocyte coverage on top of EarSkin one week after seeding. Scale bars, 100 µm. n = 6. Credit: *Science Advances*, DOI: 10.1126/sciadv.adh1890

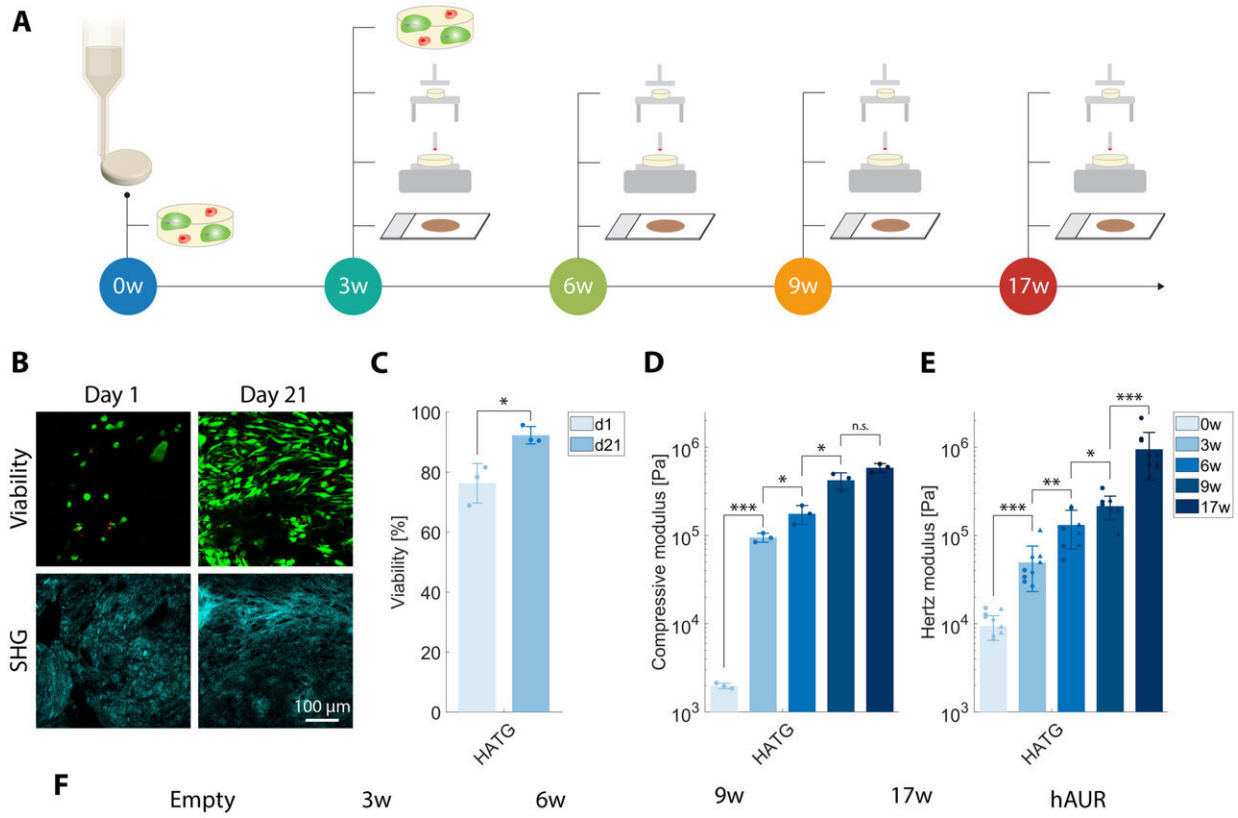
This construct is then implanted under the skin of the skull to elevate the auricle in a second stage in a highly complex plastic surgery procedure. The intervention required harvesting a sufficient amount of costal cartilage and is therefore usually delayed until children are 10 years of age.

While scaffold implantation with porous high-density polyethylene can circumvent costal cartilage harvesting, the implants suffered from higher complication rates at the site. In this work, the team combined human pigmented and prevascularized skin substitute (Earskin) with bioprinted cartilage (EarCartilage) as a first attempt to use two tissue-engineered grafts to treat microtia patients without complications.

Evaluating the EarSkin in the lab and developing bioprinted human cartilage in vitro

To develop the bioengineered EarSkin, the team incorporated a dermo-epidermal skin graft of human origin based on a collagen type I hydrogel with a prevascularized dermal layer and a pigmented dermis on top. The team co-cultured human dermal microvascular endothelial cells with human fibroblasts in the dermis by forming branching capillaries arranged into a vascular plexus within two weeks of culture in vitro. The capillary number and capillary length increased significantly in time.

The tissue maintained a complex extracellular matrix composition where the cartilage withstood significant loads. The team tested the maturation of cartilage constructs after bioprinting the cellular disks and studying their mechanical properties to show the extracellular matrix of tissue engineered constructs owing to the mature elastic network of the human auricular cartilage.

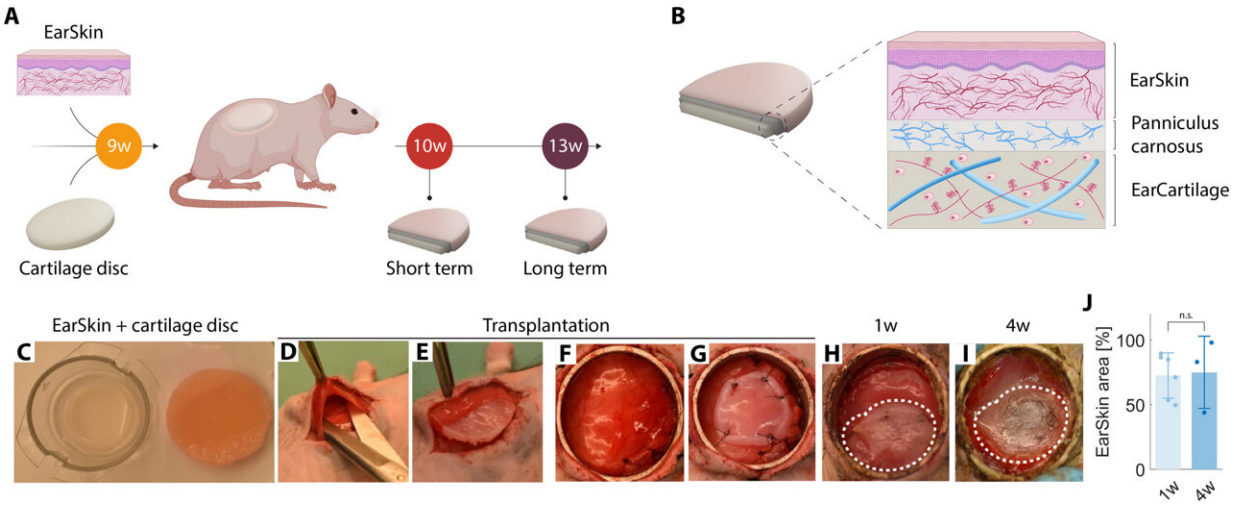


Development of cartilage in vitro. (A) Illustration of the evaluation of the development of bioprinted cartilage discs. Disks were printed using a HATG-based bioink together with primary human auricular chondrocytes (hAUR). At days one and 21, cell viability was analyzed. After 0 (crosslinked samples containing 15 million hAUR ml^{-1}, one day after bioprinting), three, six, nine, and 17 weeks, biomechanical and histological properties were evaluated. (B) Cell viability (green: live, red: dead) and second harmonic generation (SHG) after one and 21 days. Scale bar, 100 µm. n = 3. (C) Cell viability calculated from cell viability images. Viable (green) and dead (red) cells were counted, and cell viability was calculated as the total number of viable cells divided by the total number of (viable + dead) cells. n = 3. (D) Development of the compressive modulus and (E) Hertz modulus of disks over time. n = 3. (F) Histology of glycosaminoglycan (Safranin O), collagen I, collagen II, and elastin deposition over time. Scale bars, 200 µm (close-up) and 2 mm (full view). n = 3. *P Science Advances, DOI: 10.1126/sciadv.adh1890

Combining the EarSkin with cartilage disks in vivo

The scientists developed an in vivo rat model to mimic the combined engineered skin and cartilage to treat microtia. They established the model by performing a first experiment by implanting disks under the panniculus carnosus, used clinically in ear reconstitution and transplanted with prevascularized dermo-epidermal skin grafts on top. The team regulated the wound healing process after implantation weekly and documented the process photographically to detail macroscopic skin maturation.

Then using histological studies, the researchers assessed the EarSkin panniculus carnosus cartilage disks, and noted the presence of human capillaries in the dermal layer of EarSkin with encompassed fibroblasts while the cartilage disks remained avascular. Then by using histology and immunohistology studies they confirmed tissue maturation of the cartilaginous disks. The outcomes emphasized the significance of the maturation process during the culture timeframe.



EarSkin and cartilaginous disks in vivo. (A) Experimental design of the combination of EarSkin and cartilage disks in vivo. Cartilage disks were printed

and matured for nine weeks in vitro. EarSkin was cultured for three weeks in vitro to obtain a prevascularized dermo-epidermal skin graft. Cartilage disks were implanted under the panniculus carnosus, on top of which EarSkin was implanted. After one and four weeks in vivo, constructs were excised and analyzed. (B) Schematic of the surgical procedure with the panniculus carnosus placed between the cartilaginous disks and EarSkin. (C to I) Macroscopic images presenting the surgical procedure (C to G) and wound control at week one (H) and week four (I). (C) A cell culture insert with EarSkin and cartilage disk next to it, photographed directly before transplantation, (D) rat skin and panniculus carnosus wound opening by incision, the panniculus carnosus is held by forceps, (E) cartilage disk inserted underneath panniculus carnosus, (F) insertion of the metal ring protecting the transplanted EarSkin from the overgrowth of rat skin, (G) stitching EarSkin on top of the panniculus carnosus, and (H) wound control at week 1 and (I) week 4. (J) Planimetric analysis of EarSkin coverage of the cartilaginous disks after one and four weeks in vivo. The value 100% corresponds to the original size of the skin substitute at the time of transplantation. n = 6 (1 week), n = 3 (4 weeks). *P Science Advances, DOI: 10.1126/sciadv.adh1890

Combining EarSkin with EarCartilage in an animal model

Zielinska and colleagues used the EarCartilage as a close-to-physiological 3D topography for the panniculus carnosus and EarSkin. They combined the two biologically engineered structures and assessed their functionality. After transplantation, they continued the wound healing process and studied EarSkin maturation.

Before implanting the construct, the EarCartilage displayed a flexible and elastic behavior with a compressive modulus. By one to four weeks in vivo, the team preserved the morphology of the EarCartilage alongside its structure, where the auricular constructs remained significantly softer. Using histological and immunohistochemistry outcomes, they confirmed the deposition of collagen and elastin variants.

Outlook

In this way, Dominika Zielinska and team successfully combined two tissue-engineered grafts created in the lab in the form of a tissue engineered human skin graft (EarSkin) and bioprinted human cartilage graft (EarCartilage). The approach presented an innovative and promising treatment strategy for patients born with microtia, and the work can overcome costal cartilage harvesting to facilitate the intervention at an earlier age as well as provide a personalized aesthetic surgical option for microtia based on patient-derived cells.

During in vivo experiments in an animal model, the team used the panniculus carnosus; a highly vascularized muscle layer as an equivalent to the human temporal fascia flap.

The scientists observed a remodeling process of the transplanted human vasculature in vivo with human engineered capillary networks, as early as four weeks after transplantation. The EarSkin–EarCartilage constructs were stable in vivo with shape retention throughout implantation, demonstrating the successful combination of two tissue-engineered constructs of 3D cartilage and skin as a personalized treatment strategy for childhood microtia.

More information: Dominika Zielinska et al, Combining bioengineered human skin with bioprinted cartilage for ear reconstruction, *Science Advances* (2023). DOI: 10.1126/sciadv.adh1890

Benjamin P. Cohen et al, Tissue engineering the human auricle by auricular chondrocyte-mesenchymal stem cell co-implantation, *PLOS ONE* (2018). DOI: 10.1371/journal.pone.0202356