Tympanic membrane organ culture using cell culture well inserts engrafted with tympanic membrane tissue explants

Lawrence J. Liew^{1,2}, Richard M. Day³, and Rodney J. Dilley^{1,2,4}

¹Ear Sciences Centre, School of Surgery, University of Western Australia, Perth, Australia, ²Ear Science Institute Australia, Perth, Australia, ³Division of Medicine, University College London, UK, and ⁴Centre for Cell Therapy and Regenerative Medicine, University of Western Australia, Perth, Australia

BioTechniques 62:109–114 (March 2017) doi 10.2144/000114523

Keywords: tympanic membrane; explant culture; silk fibroin

Supplementary material for this article is available at www.BioTechniques.com/article/114523.

Tissue engineering approaches using growth factors and various materials for repairing chronic perforations of the tympanic membrane are being developed, but there are surprisingly few relevant tissue culture models available to test new treatments. Here, we present a simple three-dimensional model system based on micro-dissecting the rat tympanic membrane umbo and grafting it into the membrane of a cell culture well insert. Cell outgrowth from the graft produced sufficient cells to populate a membrane of similar surface area to the human tympanic membrane within 2 weeks. Tissue grafts from the annulus region also showed cell outgrowth but were not as productive. The umbo organoid supported substantial cell proliferation and migration under the influence of keratinocyte growth medium. Cells from umbo grafts were enzymatically harvested from the polyethylene terephthalate (PET) membrane for expansion in routine culture and cells could be harvested consecutively from the same graft over multiple cycles. We used harvested cells to test cell migration properties and to engraft a porous silk scaffold material as proof-of-principle for tissue engineering applications. This model is simple enough to be widely adopted for tympanic membrane regeneration studies and has promise as a tissue-equivalent model alternative to animal testing.

The rapid spontaneous healing of injuries to the tympanic membrane (TM) gives rise to the concept of a robust regenerative mechanism. Studies have localized areas of high cell turnover in the TM to the center of the membrane (the umbo region) and the outer rim (annulus region), which have been suggested to contain stem cells (1,2) or regenerative centers (3,4). However, there is limited understanding of the biology of the cells responsible for regeneration or their niche within the TM, and in vitro models of the TM are currently unavailable.

Tympanic membrane cell cultures have been generated (2,5,6) using trypsin digestion or TM tissue explants in conjunction with selective collagen IV adhesion in vitro. Previously, we showed that TM cells can be isolated via tissue explant culture from

humans (5); however, these methods can yield inconsistent results. An opportunity to generate consistent cell cultures arises from a unique aspect of TM biology: large numbers of cells are produced at the umbo, which then migrate en masse across the TM surface and along the ear canal to a point where they are ultimately shed externally. An ink dot placed on the keratinized surface at the TM umbo will migrate to the peripheral margin of the membrane and then along the ear canal; the pattern and rate of migration in vivo has been described for various species (7–10). This property of the TM helps maintain a clear ear canal and a minimal TM keratin burden for sensitive transmission of sound through the middle ear. This also has a profound effect on repair mechanisms, which in contrast to other skin-like tissues,

is led by keratinocyte layer responses rather than granulation tissue formation (11–13).

We have used this property of the TM epidermis to generate cell cultures from umbo organoids placed in a collecting membrane. Here, we describe our method and its use to generate a TM tissue-equivalent model as well as a tissue-engineered TM construct in vitro using state-of-the-art scaffolds.

Materials and methods

Animals

Male Sprague-Dawley rats, weighing 250–300 g, were obtained from the Animal Resources Centre (Murdoch, Western Australia, Australia). This study was approved by the University of Western

METHOD SUMMARY

Here, we present a tympanic membrane organoid explant culture using a tissue culture insert membrane that allows rapid isolation of tympanic membrane primary cells for biocompatibility testing and tissue engineering.

UVP ChemStudio touch Studio Series Imagers

An Imaging Studio Fit for Your Proteomic & Genomic Research.

- Western blots
- Multiplex fluorescent westerns
- DNA Gels
- Protein blots
- Immunoblots
- RGB
- Coomassie Blue, GelGreen, SYBR Green, other stains... and more!

Visit us & Learn More at:

**American Association for
Cancer Research**

Booth 1620

Experimental Biology

Booth 1213

<http://us.analytik-jena.com>

analytikjena

An Endress+Hauser Company

Figure 1. Tympanic membrane and grafts from its umbo and annulus used to grow cells in vitro. (A) Tympanic membrane after dissection from the tympanic ring. The annulus (AN), malleus (M), and pars tensa (PT) can be seen. Scale bar = 1 mm. (B) An umbo explant showing the remnant malleus (M) and transparent pars tensa (PT) around it. The umbo (U) is positioned under the tip of the malleus bone. Scale bar = 500 μ m. (C) The umbo explant with malleus handle inserted through a slit (black arrow heads) in the polyethylene terephthalate (PET) membrane. Scale bar = 500 μ m. (D) The annulus explant inserted through the slit in the PET membrane. Scale bar = 500 μ m. (E) Cells have migrated out from the explant and cover the PET membrane surface. Scale bar = 500 μ m. (F) Cells migrating across the surface of the PET membrane (black dashed line indicates the migration front, above the black arrows) and approaching the periphery of the insert (bottom). Scale bar = 500 μ m.

Australia Animal Ethics Committee (No. 100/1249). The experiments were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Extraction of tympanic membrane

The animals ($n = 4$) were euthanized by intraperitoneal administration of pentobarbitone (160 mg/kg). Using en bloc excision, the left and right external ears were removed at the osteocartilaginous junctions, and the tympanic bulla was isolated from each. The TM was then dissected free. The harvested TMs were rinsed in phosphate buffered saline (PBS) pH 7.2 containing 2% antibiotic-antimycotic solution (Gibco, Grand Island, NY) for 2 min prior to processing.

Enzymatic digestion

For tissue culture, freshly extracted temporal bones were rinsed thoroughly in 2 mL PBS and transferred to a 35-mm glass-bottom culture dish (Greiner Bio-One) containing 2 mL 0.25% Dispase II solution (Gibco). Following overnight incubation at 4°C, the TMs were peeled from the bony bulla and separated from the ear canal skin using forceps.

Rat TM explant culture on culture well insert membrane

A culture well insert with a polyethylene terephthalate (PET) membrane (0.4 μ m

pore size, 12-well Thincert; Greiner Bio-One, Frickenhausen Germany) was rinsed in PBS, and a 1 mm scalpel incision was made in the center. The malleus bone attached to the TM umbo was then partially inserted into the incision with the outer (lateral) surface of the umbo facing vertically and close to the level of the upper surface of the PET membrane. A length of annulus tissue was dissected from the pars tensa and similarly placed through a PET membrane incision. The inserts were then mounted into 12-well plates and filled with 1 mL keratinocyte growth media: keratinocyte serum-free medium (KSFM) supplemented media with human recombinant epidermal growth factor (EGF) a.a. 1–53, bovine pituitary extract, 10% fetal bovine serum (FBS) (Bovogen, East Keilor, Vic, Australia) and 1% penicillin-streptomycin (KSFM and the additives other than FBS were purchased from Gibco). Explant cultures were incubated at 37°C in 5% CO₂ with twice-weekly medium changes for up to 2 weeks.

Culture expansion

Cell outgrowth from the explant onto the upper surface of the PET membrane was allowed to continue until a monolayer of cells approached the periphery of the culture well insert membrane, after which the cells were passaged by enzymatic digestion into fresh culture dishes. Briefly, the cell monolayer was rinsed thoroughly with PBS and digested

with 250 μ L pre-warmed Tryple Express (Gibco) for 15 min at 37°C. Digestion was stopped by adding 2 mL KSFM containing 10% FBS. The supernatant containing the detached cells was collected and centrifuged at 600 $\times g$ for 5 min and cells were resuspended in 5 mL of medium before replating in a 25 cm² cell culture flask (Greiner Bio-One). The cells were cultured in KSFM supplemented with 10% FBS and fed with fresh medium twice weekly. Upon reaching 60%–70% confluence, cells were then passaged into fresh dishes at a 1:3 ratio. The explant and membrane construct was kept intact, and fresh culture medium was added to investigate continued outgrowth.

Immunofluorescent staining for characterization of the primary cells

Cells were cultured in 35-mm glass-bottom culture dishes (Greiner Bio-One) for 2–3 days at 37°C before staining. The cells were rinsed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min at RT. They were then incubated with ice-cold 100% methanol for 10 min at -20°C before blocking with 5% BSA in PBS for 30 min at RT. The primary antibodies used for staining were mouse anti-pan cytokeratin (Biocare, Concord, CA) and anti-vimentin (Abcam, Cambridge, UK). Following an overnight incubation at RT, the cells were rinsed 3 times with PBS containing 0.01% Tween 20 before the addition of secondary antibodies: anti-rabbit IgG Alexafluor 555 (Abcam) or anti-mouse IgG Alexafluor 488 (Molecular Probes, Eugene, OR). The cells were incubated for 1 h at RT then rinsed in PBS/0.01% Tween 20 and counterstained with 4',6-diamidino-2-phenylindole (DAPI) (0.05 mg/mL, Molecular Probes) for 20 min. The cells were mounted in PBS:glycerol (1:1) before viewing with a fluorescence microscope (Olympus, Hachioji-shi, Tokyo, Japan) with appropriate fluorescence filters.

Figure 2. Culture expansion and characterization of harvested cells.

(A) Fluorescence micrograph showing DAPI-stained cell nuclei in a 10-day umbro explant. Cells have grown out to cover the entire surface of the polyethylene terephthalate (PET) membrane. Scale bar = 1 mm. (B) Fluorescence micrograph showing DAPI-stained cell nuclei in a 10-day annulus explant with cells partially covering the surface of the PET membrane. Scale bar = 1 mm. (C) DAPI-stained cell nuclei in 10-day cell outgrowth showing mitotic bodies and dividing cells (white arrows). Scale bar = 100 μ m. (D) Rat tympanic membrane whole mount showing radial and circumferential arrangement of DAPI-stained cell nuclei. Scale bar = 200 μ m.

Epidermal cell enrichment using timed trypsinization

Homogeneous epidermal cell cultures were obtained by carefully timed trypsinization. Confluent cells grown in a 25 cm² cell culture flask were rinsed once in PBS and incubated with 2 mL pre-warmed Tryple Express. Cells were incubated at 37°C for 5–10 min, and the flask was gently tapped to detach the fibroblasts. Enzyme solution and suspended fibroblasts were discarded or cryopreserved for later use. The remaining epidermal colonies were fed with fresh KSFM containing 10% FBS and passaged as previously described upon reaching 70%–80% confluence.

Wound-healing migration assay

Epidermal cells [passage 4–5 (p4–5)] were seeded (5 $\times 10^4$ cells) into the reservoirs of a 2-well culture insert (Ibidi, Martinsried, Germany), adhered to a 35-mm glass-bottom dish, and grown overnight at 37°C. On the day of assay, the insert was removed using a pair of forceps, and the dish was briefly rinsed with PBS to remove cell debris. The dish was then filled with 1 mL KSFM containing 10% FBS and placed on a stage top incubator (Tokai Hit, Fujinomiya, Japan). Phase-contrast images were viewed and captured at 10 $\times$ objective magnification using an Olympus IX-81 microscope system at 15 min intervals over 18 h.

Tissue engineering using cells isolated from membrane cultures

TM cells at p2 were resuspended in KSFM (5 $\times 10^5$ cells/mL) and seeded onto silk fibroin-coated coverslips in a 12-well culture plate. The scaffold was produced by immersing 13 mm borosilicate coverslips in 50 mg/mL silk fibroin solution (Advanced Biomatrix, Carlsbad, CA). The coated coverslips were immediately immersed into liquid nitrogen to achieve thermally induced phase separation.

BIOSPA SYSTEM FOR AUTOMATED LIVE CELL ANALYSIS

More Than an IncuCyte®

	BioSpa™ System	IncuCyte® Zoom
Live cell imaging	✓	✓
Temperature, humidity, gas control	Included, monitored	Incubator required
Labware capacity	8	6
Objective capacity	6	1
Magnification range	1.25x to 60x	4x to 20x
Fluorescence colors available	>15	2
Z-stacking for 3D biology	✓	
Laser autofocus	✓	
Advanced cell-level analysis	✓	
Washing, media replacement	✓	
Reagent dispensing	✓	

www.biotek.com/biospasystem

Think Possible

BioTek®

www.biotek.com

The frozen coverslips were placed into an Edwards Modulyo freeze drier (Edwards Ltd, Crawley, UK) and lyophilized for 24 h. The porous silk films were sterilized in 70% ethanol overnight before conditioning for 2 h in growth medium containing 10% FBS. Once seeded with cells, scaffolds were incubated at 37°C with 5% CO₂ for 24 h, then rinsed twice in PBS, fixed in 4% paraformaldehyde for 10 min, and stained with DAPI for 20 min before visualizing with an epifluorescence microscope as described above.

Results and discussion

TM regeneration is of considerable general interest due to its robust and relatively scarless healing compared with skin; however, it is most specifically relevant in otology due to chronic failure of wound healing after TM perforation and to the serious consequence of invasive growth of TM cells into the middle ear and mastoid cholesteatoma (14,15).

There are few models for exploring the biology of TM cells (2,6), and those currently available are limited by the very small amount of tissue and cells available. One approach we explore here is to establish a cell culture model that provides access to TM cells and allows exploration of their properties in relation to growth and use for tissue engineering. A flow diagram for this method of culturing rat TM umbo explants on culture well insert membranes is shown in Supplementary Figure S1. Rat TM was dissected as a thin sheet suspended in a shell-like bony ring structure, the tympanic ring. Figure 1A shows a TM peeled whole off the bone and still attached to the malleus after overnight digestion in 0.25% Dispase II. The annulus of the membrane was retained as a prominent fibrous ring, and the thin and transparent pars tensa was intact within it. As shown in Figure 1B, the umbo could be dissected free from the whole TM and the handle of the malleus removed, leaving only a remnant at the umbo. This method of rat TM extraction is superior to *in situ* harvest or other previously described methods (2,6) as it preserves the normal anatomical relationships of TM and, more importantly, enables cell isolation from specific areas of the TM (umbo and annulus).

The excised umbo was inserted in the slit (Figure 1C, black arrow heads) of the PET membrane, and the umbo surface and pars tensa remnant were positioned on top of the membrane upper surface to create a PET membrane/explant construct that mimicked

the structural arrangement of the umbo in its native TM (Figure 1C). As a comparison, a separate explant using TM annulus tissue was also placed through a similar slit in the membrane (Figure 1D). The appearance of a culture well insert and its porous and semitransparent PET membrane is depicted in Supplementary Figure S2.

Figure 1E shows cell outgrowth from the umbo explant onto the PET membrane at 48–72 h. Cells continued to grow peripherally until confluent across the membrane surface (~3 cm²) at 12–14 days, as depicted in Figure 1F. Explant cultures fixed in 4% paraformaldehyde were stained with DAPI to visualize the presence and organization of cells. In Figure 2A, a substantial cell population could be visualized in the umbo explant and also grew as a monolayer over the entire culture well insert membrane surface, indicating that the model likely supports a strong source of cell proliferation in the explant rather than just a founding population of TM cells that migrated out over the surface. Fewer cells were observed to grow out onto the membrane from the annulus explant (Figure 2B). The TM annulus is thought to contain regenerative centers (1,16,17); however, when we attempted to graft annulus tissue

into the membrane, the grafts were not as successful. Orienting the annulus tissue in the PET membrane was not as straightforward as it was with the umbo grafts possessing the malleus handle. Outgrowth from an anchored annulus tissue explant did produce cells on the membrane, but the area covered in the same time frame was not as extensive. Nevertheless, we continue to pursue this avenue as the annulus provides more opportunity to source autograft tissue for myringoplasty in patients who may have lost their umbo regenerative centers after subtotal perforation.

Some cell nuclei on the culture well insert membrane were identified as being in mitosis, showing proliferative expansion on the membrane (Figure 2C). The cell nucleus orientation in an intact TM was tightly aligned in the radial and circumferential directions, as shown in Figure 2D (whole mount DAPI staining), but this same arrangement was not apparent on the PET membrane.

Once fully confluent, the cells were passaged into fresh culture dishes, analyzed for phenotype, and explored for tissue engineering. Passaging was performed up to 5 times from a single explant, and the same explant continued to produce cells

Figure 3. Expansion and phenotype of passaged tympanic membrane cells. (A) Cells passaged from the umbo explant cultures into plastic culture flasks. The cells were heterogeneous in morphology and at Day 1 after passaging had spindle-shaped fibroblasts mixed among cells with epithelioid phenotypes. By Day 10, the cells had proliferated in culture to form confluent monolayers, and in some areas they had developed the tightly packed cobblestone morphology of epithelial cells. Scale bar = 500 μ m. (B) Immunofluorescence staining of rat tympanic membrane (TM) primary cells. Images in the first row show staining for nuclei (DAPI), pancytokeratin (immunofluorescence), and the merged image, respectively. In the bottom row, images show staining for nuclei (DAPI) and vimentin (immunofluorescence), as well as the merged image. Scale bars = 200 μ m.

Figure 4. Cell migration of harvested cells. Passaged cells were subjected to a wound-healing assay where they were found to migrate to cover the exposed surface over 18 h. Scale bar = 200 μ m.

over 2 months. Aside from this capacity to harvest multiple cultures continuously from the same explant, TM explant cultures on well inserts were superior to conventional tissue culture plastic cultures because the porous PET membrane provides a supportive microenvironment for rapid outgrowth onto the membrane while also maintaining the 3-D structure of the malleus and umbo. It is reasonable to speculate that the device maintains an anatomy similar to the native TM through multiple cell harvesting cycles, rather than the disrupted structures used for TM explants. Moreover, the malleus handle was securely held within the PET membrane, thus eliminating the problem with conventional explant methods where tissue is prone to lift off the culture dish when cell culture medium is added.

This model has two major benefits in regard to reducing animal use: First, it allows the efficient recovery and expansion of cells from a small amount of tissue, reducing the number of animals required to create a usable culture within a short time frame. Second, creation of an epithelialized membrane may enable in vitro testing of novel materials and factors for TM perforation healing that would normally be performed in vivo (18–20). Multiple samples can be generated from each umbo and will provide a new method for short- to medium-term screening that may approach high-throughput.

An attempt to transplant an umbo from one membrane to another after cell harvesting did not produce any cell outgrowth. The explant most likely carries a durable niche for the regenerative center, and further exploration is needed to understand what might constitute a supportive environment for long-term grafts in TM tissue repair. Trans-well membranes are attractive for this purpose and the various membrane varieties available may give further opportunity to optimize aspects of the system, such as migration rate, proliferation, or differentiation of cells. The activity of various agents (ECM, growth factors) on cell prolif-

eration and migration mechanisms may be tested by coating the PET membrane with an agent, adding it to the culture medium, or even adding it to a co-culture in the underlying well for testing of paracrine activity from cells in the underlying well.

Cell phenotypes on the PET membrane of the culture well insert were difficult to ascertain by phase-contrast microscopy, where the resolution was reduced by the membrane pores. Cells grew as monolayers but phenotype was best observed after replating in plastic cell culture dishes. Figure 3A shows cells that were grown in supplemented KSFM to form clumps of

epithelial-like cells. Cells proliferated further to form confluent layers of cells, some of which developed into tightly packed cobblestone-like morphology and a fibroblast-like shape.

A 2-day-old mixed culture, grown on a glass coverslip showed sparse epithelial cells positive for pancytokeratin and a large number of vimentin positive fibroblast-like cells (Figure 3B). When confluent, the mixed population could be enzymatically sorted to generate homogenous epidermal and fibroblast cultures. As epidermal cells and fibroblasts respond to trypsin differently, a carefully timed trypsinization is easy and sufficient to enrich/isolate homogeneous cell cultures and is particularly useful when cell numbers are inadequate for FACS or magnetic sorting.

A time-lapse wound-healing migration assay was performed to assess the functionality of the epidermal cells. As depicted in Figure 4, epidermal cells isolated from rat TM migrated to close the gap created by the 2-well culture insert over 18 h. Nevertheless, rat TM epidermal cells appeared to migrate and respond to serum differently than human

DETECT CRISPR GENE EDITING EVENTS Using Agarose Gels

Streamline gene editing workflows with a simple and accurate enzymatic assay.

AccuCleave™ T7 Kit

- T7 based enzyme kit for mutation frequency detection of CRISPR events.

AccuCleave™ C2 Control DNA Kit

- Contains 2 Control DNA fragments - intact and -2 deletion fragment.

Start now at
www.aati-us.com/AccuCleave

TM keratinocytes previously isolated in our laboratory (data not shown) (5), which may be due to cross-species variation and different culture conditions.

Silk fibroin materials have effective mechanical properties for TM tissue engineering (21) and provide a suitable substrate for human TM keratinocytes (18). In order to test whether these TM cells would support a tissue-engineering approach, we cultured explant-derived cells on porous silk membrane scaffolds (Supplementary Figure S3A). Primary cultures were replated directly from the PET membrane onto silk-coated coverslips. Cells adhered and engrafted on the silk within 6 h, producing clusters and sheets of cells covering the surface and the adjacent culture dish (Supplementary Figure S3B). Nuclear staining confirmed that the adherent cells formed contiguous patches of cells and, in some areas, confluent monolayers (Supplementary Figure S3C). Over time, sheets of confluent epithelia developed on the silk scaffold to form tissue-engineered membranes.

Evidence of proliferation on the silk scaffold indicates that this mechanism of expansion happens in a short time frame. Tissue engineering will benefit from a more controlled analysis by seeding specific cell cultures rather than mixed cultures, but we have established proof-of-principle that the explant-sourced cells can be suitable for tissue engineering. These TM cells have a unique biology that cannot be simply modeled using cells from other skin sources, which don't have such robust and specific migratory behavior. A simple direct measure of a scaffold's engraftment potential might be gained by placing the PET membrane in contact with the scaffold material (20), replacing the PET membrane with scaffold material in the insert, or even, based on the cell behavior principles established here, implanting the umbo explant as a graft directly into the scaffold material and evaluating the migration of cells from the explant onto the scaffold surface. The approach may also prove useful in exploring other tissue explants with a defined stem cell niche, such as hair follicle or corneal limbus explants.

Our model shows that it is possible, using a simple culture device, to recapitulate the in vivo condition of a TM, where the umbo tissues serve as a regenerative center and the cells produced actively migrate along the lateral surface of the lamina propria to contribute to the cell populations of the membrane and ear canal. The TM organoid

or tissue-equivalent models produced may be suitable to explore the biology of TM cells and their unique migratory capacities, including mechanisms of disruption during wound healing in chronic perforations or invasiveness in cholesteatoma (22). The model is simple enough to be widely adopted for TM regeneration studies and has further promise as a tissue-equivalent model that may help to reduce the need for animal testing.

Author contributions

L.J.L. carried out the experiment and data analysis. R.J.D. and R.M.D. carried out part of the data analysis. L.J.L. and R.J.D. designed and supervised the experiments. R.M.D. prepared the silk fibroin scaffold. L.J.L., R.M.D., and R.J.D. wrote and approved the manuscript.

Acknowledgments

This work was in part funded by Garnett Passe and Rodney Williams Memorial Foundation (L.J.L., R.J.D.), National Health and Medical Research Council of Australia (R.J.D.) and University of Western Australia Centre for Cell Therapy and Regenerative Medicine (R.M.D., R.J.D.). Part of this work was undertaken at UCL/UCLH which receives funding from the UK Department of Health's NIHR as a Comprehensive Biomedical Research Centre.

Competing interests

The authors declare no competing interests.

References

- Knutsson, J., M. von Unge, and H. Rask-Andersen. 2011. Localization of progenitor/stem cells in the human tympanic membrane. *Audiol. Neurotol.* 16:263-269.
- Kim, S.W., J. Kim, H. Seonwoo, K.-J. Jang, Y.J. Kim, H.J. Lim, K.-T. Lim, C. Tian, et al. 2015. Latent progenitor cells as potential regulators for tympanic membrane regeneration. *Sci. Rep.* 5:11542.
- Santa Maria, P.L., S. Kim, Y.K. Varsak, and Y.P. Yang. 2015. Heparin Binding-Epidermal Growth Factor-Like Growth Factor for the Regeneration of Chronic Tympanic Membrane Perforations in Mice. *Tissue Eng. Part A* 21:1483-1494.
- Santa Maria, P.L., K. Weierich, S. Kim, and Y.P. Yang. 2015. Heparin binding epidermal growth factor-like growth factor heals chronic tympanic membrane perforations with advantage over fibroblast growth factor 2 and epidermal growth factor in an animal model. *Otol. Neurotol.* 36:1279-1283.
- Redmond, S.L., B. Levin, K.A. Heel, M.D. Atlas, and R.J. Marano. 2011. Phenotypic and genotypic profile of human tympanic membrane derived cultured cells. *J. Mol. Histol.* 42:15-25.
- Choi, M.Y. and K.H. Park. 2014. Establishment of endogenous human tympanic membrane-derived somatic stem cells for stem cell therapy. *In Vitro Cell. Dev. Biol. Anim.* 50:747-755.

- Alberti, P.W. 1964. Epithelial Migration on the Tympanic Membrane. *J. Laryngol. Otol.* 78:808-830.
- Lim, D.J. 1995. Structure and function of the tympanic membrane: a review. *Acta Otorhinolaryngol. Belg.* 49:101-115.
- Tabacca, N.E., L.K. Cole, A. Hillier, and P.J. Rajala-Schultz. 2011. Epithelial migration on the canine tympanic membrane. *Vet. Dermatol.* 22:502-510.
- Makino, K. and M. Amatsu. 1986. Epithelial migration on the tympanic membrane and external canal. *Arch. Otorhinolaryngol.* 243:39-42.
- von Unge, M. and M. Hultcrantz. 2011. The early events in the healing of laser-produced tympanic membrane perforation. *Acta Otolaryngol.* 131:480-487.
- Somers, T., V. Houben, G. Goovaerts, P.J. Govaerts, and F.E. Offeciers. 1997. Histology of the perforated tympanic membrane and its muco-epithelial junction. *Clin. Otolaryngol. Allied Sci.* 22:162-166.
- Mondain, M. and A. Uziel. 1995. Tympanic membrane regeneration and growth factors. *Acta Otorhinolaryngol. Belg.* 49:159-162.
- Barbara, M., S. Raffa, L. Leone, C. Murè, C. Scrofani, S. Monini, and M.R. Torrisi. 2012. Characterization of primary cultures of cholesteatoma-associated fibroblasts. *Otol. Neurotol.* 33:988-995.
- Kim, H.J., S.P. Tinling, and R.A. Chole. 2002. Expression patterns of cytokeratins in cholesteatomas: evidence of increased migration and proliferation. *J. Korean Med. Sci.* 17:381-388.
- O'Donoghue, G.M. 1983. Epithelial migration on the guinea-pig tympanic membrane: the influence of perforation and ventilating tube insertion. *Clin. Otolaryngol. Allied Sci.* 8:297-303.
- Kakoi, H. and M. Anniko. 1997. Auditory epithelial migration. 3. An immunohistologic study using anti-BrdU antibody on tympanic membrane in mouse. *Ann. Otol. Rhinol. Laryngol.* 106:414-421.
- Levin, B., S.L. Redmond, R. Rajkhowa, R.H. Eikelboom, M.D. Atlas, and R.J. Marano. 2013. Utilising silk fibroin membranes as scaffolds for the growth of tympanic membrane keratinocytes, and application to myringoplasty surgery. *J. Laryngol. Otol.* 127(Suppl 1):S13-S20.
- Shen, Y., S.L. Redmond, B.M. Teh, S. Yan, Y. Wang, L. Zhou, C.A. Budgeon, R.H. Eikelboom, et al. 2013. Scaffolds for tympanic membrane regeneration in rats. *Tissue Eng. Part A* 19:657-668.
- Hung, S.H., C.H. Su, and H. Tseng. 2016. A transwell-based cellular model for the rapid pre-evaluation of tympanic membrane repair materials. *Eur. Arch. Otorhinolaryngol.* 273:2027-2034.
- Rajkhowa, R., B. Levin, S.L. Redmond, L.H. Li, L. Wang, J.R. Kanwar, M.D. Atlas, and X. Wang. 2011. Structure and properties of biomedical films prepared from aqueous and acidic silk fibroin solutions. *J. Biomed. Mater. Res. A* 97:37-45.
- Tanaka, Y., Y. Yaguchi, K. Wada, H. Kojima, and H. Moriyama. 2005. Creation of a Cholesteatoma Model using Three-Dimensional Cultured Skin Equivalents. *Laryngoscope* 115:1421-1427.

Received 14 October 2016; accepted 18 January 2017.

Address correspondence to Rodney J Dilley, Ear Sciences Centre, School of Surgery, University of Western Australia, Perth, Australia. E-mail: rodney.dilley@earscentre.org.au

To purchase reprints of this article, contact: biotechniques@fosterprinting.com