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Mouse Embryonic Fibroblasts-Derived Extracellular Matrix Facilitates Expansion of Inner Ear-Derived Cells

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Abstract

Objective: Previous reports showed that mouse embryonic fibroblasts (MEFs) could support pluripotent stem cell self-renewal and maintain their pluripotency. The goal of this study was to reveal whether the decellularized extracellular matrix derived from MEFs (MEF-ECM) is beneficial to promote the proliferation of inner ear-derived cells.

Materials and Methods: In this experimental study, we prepared a cell-free MEF-ECM through decellularization. Scanning electron microscope (SEM) and immunofluorescent staining were conducted for phenotype characterization. Organs of Corti were dissected from postnatal day 2 and the inner ear-derived cells were obtained. The identification of inner ear-derived cells was conducted by using reverse transcription-polymerase chain reaction (RT-PCR). Cell counting kit-8 (CCK-8) was used to evaluate the proliferation capability of inner ear-derived cells cultured on the MEF-ECM and tissue culture plate (TCP).

Results: The MEF-ECM was clearly observed after decellularization via SEM, and the immunofluorescence staining results revealed that MEF-ECM was composed of three proteins, including collagen I, fibronectin and laminin. Most importantly, the results of CCK-8 showed that compared with TCP, MEF-ECM could effectively facilitate the proliferation of inner ear-derived cells.

Conclusion: The discovery of the potential of MEF-ECM in promoting inner ear-derived cell proliferation indicates that the decellularized matrix microenvironment may play a vital role in keeping proliferation ability of these cells. Our findings indicate that the use of MEF-ECM may serve as a novel approach for expanding inner ear-derived cells and potentially facilitating the clinical application of inner ear-derived cells for hearing loss in the future.

Keywords: Decellularized Extracellular Matrix, Fibroblasts, Hearing Loss, Organ of Corti

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Introduction

Compared to non-mammals, hair cells (HCs) and spiral ganglion neurons (SGNs) don't have a regenerative ability in the mammalian cochlea following loss (1, 2). Recent studies have demonstrated that stem cells derived from organ of Corti, nominated inner ear-derived cells (2), possess the capability to proliferate and differentiate into new HCs and SGNs, indicating that inner ear-derived cells are promising cell source for HC regeneration (3). However, as one ages, the number of inner ear-derived cells decreases, thus rendering them unable to fully replenish lost HCs and SGNs (4). In addition, the limited proliferation ability of inner ear-derived cells currently hinders their potential application in the treatment of sensorineural hearing loss (SNHL) (5). Currently, there has been no successful method developed to efficiently increase the number of inner ear-derived cells (6). Strategies aimed at enhancing the proliferation of inner ear-derived cells are likely to be advantageous for the treatment of SNHL.

It is currently understood that the cultivation of pluripotent stem cells, such as embryonic stem cells (ESC) and induced pluripotent stem cells (iPSCs), necessitates a distinct microenvironment or niche (7). At the present time, mouse embryonic fibroblasts (MEFs) have been utilized as feeder cells for expanding pluripotent stem cells due to its capacity to sustain self-renewal and retain pluripotency (8). The use of MEFs was motivated by their potential to generate and release various biomolecules including but not limited to extracellular matrix (ECM), leukemia inhibitory factor (LIF), fibroblast growth factor (FGF), and bone morphogenetic protein (BMP) (9). In addition, ECM has a central role in establishing an environment that is conducive to tissue-specific cell functions and in the case of stem cells, this environment is the stem cell niche, where ECM signals participate in cell fate decisions (10, 11). It is possible that a lack of suitable ECM could be a contributing factor to the decline in the multipotency of stem cells observed in the course of cultivating inner ear-derived cells in a typical tissue culture environment (12).

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In this study, a cell-free MEF-ECM through decellularization was prepared. The characterization of MEF-ECM was conducted by using scanning electron microscope (SEM) and immunofluorescent staining. Cell counting kit-8 (CCK-8) was used to evaluate proliferation capability of inner ear-derived cells cultured on the MEF-ECM and tissue culture plate (TCP). The objective of this research was to evaluate the feasibility of obtaining MEF-ECM method, and determine the influences of MEF-ECM on the proliferation of inner ear-derived cells. This exploration holds significant promise in creating a favorable microenvironment to facilitate the expansion of inner ear-derived cells.

Materials and Methods

Otosphere harvest

In this experimental study, the sensory cochlear sensory epithelia were extracted from postnatal day 2 Institution of Cancer Research (ICR) mice (Shanghai SlAC Laboratory Animal Co., Ltd, Shanghai, China). The tissue samples underwent a 15-minute exposure to 0.125% trypsin (Invitrogen, USA) at 37˚C, followed by blocking with a trypsin inhibitor and DNase I solution (Invitrogen, USA). The pellets were suspended in Dulbecco's modified Eagle medium/F12 (1:1) (DMEM/F12, Invitrogen, USA) supplemented with N2 and B27 (Sigma, USA), 10 ng/mL basic FGF (bFGF, Wako, Japan), 20 ng/mL epidermal growth factor (EGF, Sigma, USA) and 50 ng/mL ampicillin (Sigma, USA). The suspension was filtered using a 70 μm cell strainer (BD Bioscience, San jose, CA) and subsequently transferred into a 6-well cell culture plate. Following a 2-day culturing period, the cell suspension was transferred into new cell culture plate that allowed to expand. Subsequently, the otospheres were identified after 5-6 days of culturing. Before visual inspection under an inverted microscope (Nikon Eclipse 80i, Japan), the otospheres were cultured in 6-well cell culture plates with DMEM/F12 containing N2, B27, 10 ng/mL bFGF, 20 ng/mL EGF, 50 ng/mL ampicillin and 10% fetal bovine serum (FBS, Gibco BRL, Life technologies, USA) for 5 days. Animal experiments were performed according to the NIH Guide for the Care and Use of Laboratory Animals, with the approval of the Animal Ethics Committee of Donghua University (DHUEC-NSFC-2019-01).

Reverse transcription and polymerase chain reaction analyses

Total RNA derived from adherent inner ear-derived cells at passages (P) 1, 2, 3 and 4 were extracted with Trizol reagent (TaKaRa, Japan). The integrity of the RNA samples was evaluated by the A260/A280 ratio. Four μg of RNA was successfully transcribed into cDNA utilizing the Prime Script RT reagent kit (TaKaRa, Japan) followed by PCR using ProFlex™ PCR (Thermo) to detect the gene expression. The expression of stem cell markers (*Sox2* and *Nestin*), early inner ear stem cell markers (*Bmp7*and *P27^{kip1}*) and mature HC markers (*Espin* and *Myosin VIIA*) was detected. The organ of Corti-derived atmosphere's from postnatal day 2 mice were utilized as a positive control. The primer sequences presented in Table 1 have been designed and synthesized by Sangon Biotech Co., Ltd (Shanghai, China).

Table 1: Primers for reverse transcription-polymerase chain reaction (RT-PCR)

Genes	Primer sequence (5′-3′)
Sox2	F: TAG AGCTAGACTCCGGGCGATGA
	R: TTGCCTTAAACAAGACCACGAAA
Nestin	F: GATCGCTCAGATCCTGGAAG
	R: GATCGCTCAGATCCTGGAAG
Bmp7	F: TCTTCCACCCTCGATACCAC
	R: GCTGTCCAGCAAGAAGAGGT
Myosin VIIA	F: CACTGGACATGATTGCCAAC
	R: ATTCCAAACTGGGTCTCGTG
P27kip1	F: ATTGGGTCTCAGGCAAACTC
	R: TTCTGTTCTGTTGGCCCTTT
Espin	F: ACCTACGTACGGTGCAAACC
	R: AGTGACTGGAGGAGCAGGAG
Gapdh	F: GGGTGTGAACCACGAGAAAT
	R: ACAGTCTTCTGGGTGGCAGT

Preparation of the extracellular matrix derived from mouse embryonic fibroblasts

MEFs were isolated and cultured according to previous research (13). In brief, embryos were harvested from female ICR mice 10.5 days after the appearance of the copulation plug. MEFs were extracted from embryos and seeded in TCP with DMEM (Gibco, USA) supplemented with 10% FBS, 1% penicillin-streptomycin (Gibco, USA) and 2 mM L-glutamine (Gibco, USA). The MEF-ECM was obtained by the method previously described with minor modifications (14). Briefly, the MEFs at P3 were subjected to a 6-day culture. Subsequently, the culture medium was removed, and the cells were exposed to a solution containing 0.25% Triton X-100 (Sigma, USA) and 20 mM NH_4OH (Sigma, USA) at 37˚C for a duration of 5 minutes. Finally, the MEF-ECM underwent incubation with 100 U/mL DNase I (Invitrogen, USA) for 10 minutes. Subsequently, rinsing with phosphate buffered saline (PBS) was carried out thrice, following which the sample was stored at 4˚C to facilitate further study.

Morphology of the extracellular matrix derived from mouse embryonic fibroblasts

The morphology of MEF-ECM was observed by the method previously described with minor modifications

(15). The specimens were fixed with 2.5% glutaraldehyde (MP Biomedicals, Irvine, CA, USA) for 30 minutes at 4˚C. After that, all samples were dehydrated with 60, 70, 80, 90 and 100% absolute ethanol for 20 minutes each time and air dried. The dried samples were fixed on the sample stage of the SEM using conductive adhesive and subsequently sputter-coated in gold for 90 s. Imaging was obtained by using a SEM (S-520; Hitachi, Japan) with a voltage of 10-15 kV.

Immunofluorescent staining

Immunofluorescent staining was conducted to evaluate the component of MEF-ECM. The MEFs and MEF-ECM were firmly fixed with a 4% paraformaldehyde solution for 15 minutes at room temperature. To facilitate permeability, the specimens were treated with a 0.1% Triton X-100 solution for 10 minutes. Subsequently, the specimens were blocked using a 1% bovine serum albumin (BSA, Sigma, USA) solution for the next 30 minutes. Next, primary antibodies including rabbit anti-laminin (1:100, Invitrogen, USA), collagen I (1:300, BD Biosciences, USA) and fibronectin (1:200, BD Biosciences, USA) were utilized to incubate MEF-ECM samples overnight at 4˚C. The MEF-ECM specimens underwent incubation with Alexa Fluor-488 conjugated goat anti-rabbit (1:300, Invitrogen, USA) in a dimly lit environment for an hour at ambient temperature. The MEF samples were incubated with Alexa Fluor-568 conjugated phalloidin (1:1000, Cell Signaling Technology, USA) for 30 min at dark. Subsequent to the completion of the staining process, 4,6-diamidino-2-phenylindole (DAPI, Invitrogen, USA) was utilized to counterstain all samples within 10 minutes, after which images were obtained under an inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Proliferation assays and cell propagation

After culturing on the MEF-ECM and TCP for 1, 4, and 7 days, the morphology of inner ear-derived cells was observed by an inverted phase contrast microscope (Nikon, Japan). For proliferation assays, the CCK-8 (Sigma, USA) was employed to detect the proliferation of inner ear-derived cells at P4 and P8 cultivated on the MEF-ECM and TCP. The inner ear-derived cells at a density of 1×10^4 cells per well were seeded on the MEF-ECM and TCP with DMEM/F12 supplemented with 10% FBS and 50 ng/mL ampicillin at 37˚C under 5% CO_2 atmosphere. After culturing for 1, 4 and 7 days, CCK-8 working solution was introduced into the wells. After incubation for a duration of 2 hours at 37˚C, optical density (OD) values were taken at 450 nm by using a plate reader (Thermo Scientific, Waltham, MA, USA).

Statistical analysis

The data were presented as mean values ± standard deviation (SD) with the number of independent experiments (n=3). The statistical significance between groups was analyzed by One-way ANOVA followed by Tukey's test. The analysis was performed through the utilization of the SPSS 13.0 software (SPSS Inc., Chicago, IL). $P<0.05$ was considered statistically significant.

Results

Morphology of mouse embryonic fibroblasts and extracellular matrix derived from mouse embryonic fibroblasts

In this study, MEF-derived ECM was obtained through decellularization. As shown in Figure 1A, mouse embryonic fibroblasts in a polygonal shape were cultured in TCP. After decellularization, the cellular structure was removed entirely (Fig.1A´). SEM images showed the micromorphology of the MEFs before decellularization and the MEF-ECM after decellularization (Fig.1B´). As shown in Figure 1B, the MEFs exhibited a polygonal morphology demonstrating strong adhesion. Notably, the SEM images revealed the presence of a three-dimensional (3D) cellular structure. Following decellularization, the MEF-ECM exhibited robust binding to the TCP substrate and maintained its 3D structure.

Immunofluorescence staining of MEF-ECM

In this study, the MEF-ECM was obtained by using modified method of decellularization. The morphology of MEFs cultured on TCP before decellularization (Fig.2A) and after decellularization (Fig.2B) identified by cytoskeleton/nuclear staining. The MEFs appeared polygonal shape and attached well before decellularization and cytoskeleton of MEFs was removed entirely after decellularization. The results indicated that various significant protein constituents, including laminin (Fig.2C, D), fibronectin (Fig.2E, F) and collagen I (Fig.2G, H), were found to be present in the cell-deposited matrix before decellularization and after decellularization. Significantly, the observed high fluorescence intensity of laminin and collagen I indicated a high abundance of these proteins within the MEF-ECM. However, fibronectin was found to be expressed at a comparatively lower level.

Identification of inner ear-derived cells

As shown in Figure 3A and B, otospheres were produced from isolating cochlear sensory epithelia and transited to an adherent condition to promote attachment. To determine whether the adherent otospheres exhibit properties of stem cells, an examination was conducted to evaluate gene expression of various markers, including stem cell markers (*Nestin*, *Sox2*), inner ear progenitor makers (*Bmp7, P27^{kip1}*) and HC makers (*Myosin VIIA, Espin*). As shown in Figure 3C, the expression of *Nestin* and *Sox2* was observed in adherent cells. Nevertheless, the expressions of *Bmp7* and *P27^{kip1}* were detected in the otospheres group and no *Bmp7* expression was detected throughout the propagation process, while *Myosin VIIA,* expression was detected during subsequent adherent culture stages.

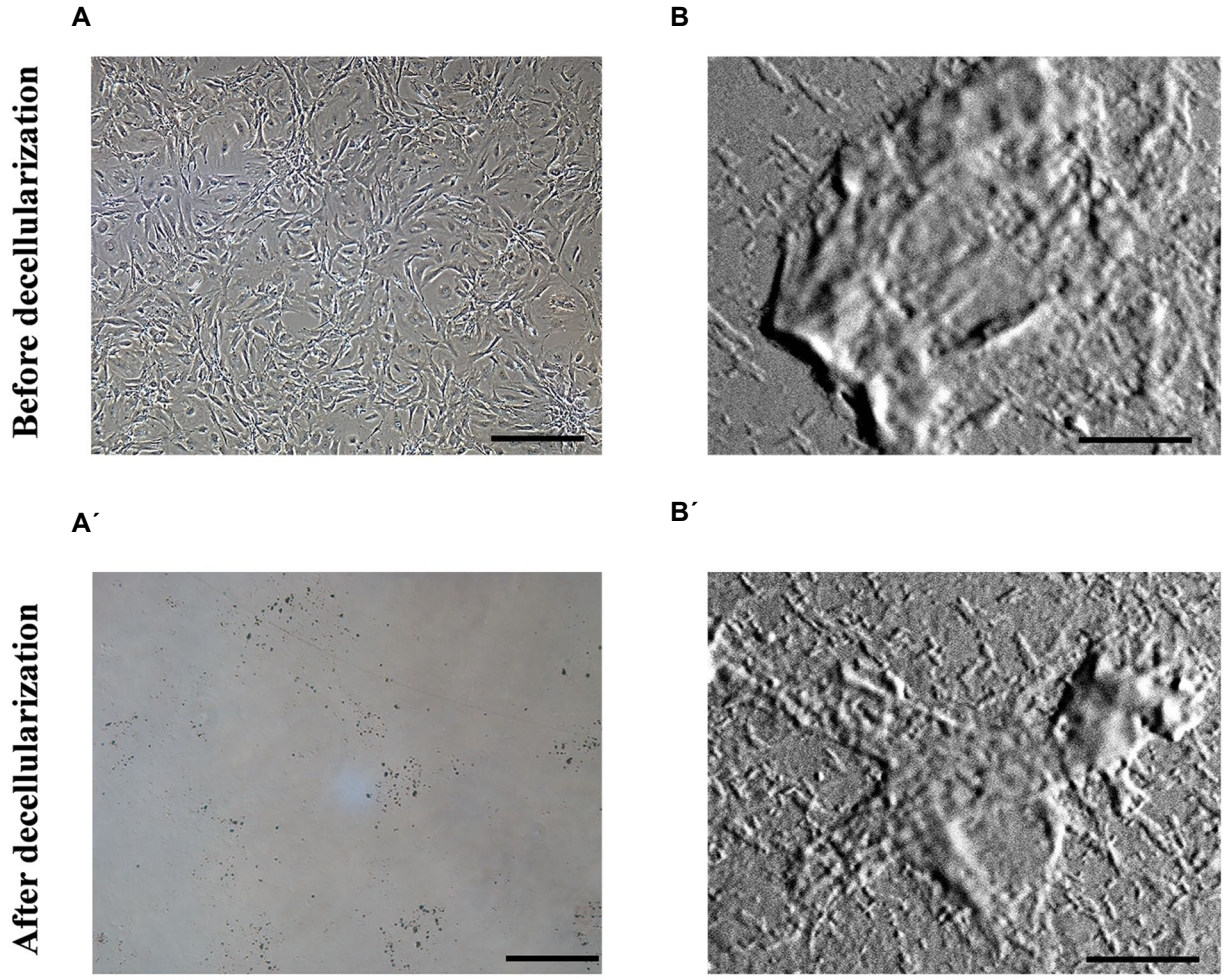

Fig.1: Morphology of MEF-ECM. Phase contrast microscope images of MEFs **A.** Before and **A´.** After decellularization. SEM images of the MEFs **B.** Before decellularization and **B´**. SEM images of MEF-ECM after decellularization (scale bar: A, A´: 500 µm; B, B´: 20 µm). MEF-ECM; Extracellular matrix derived from mouse embryonic fibroblasts and SEM; Scanning electron microscope.

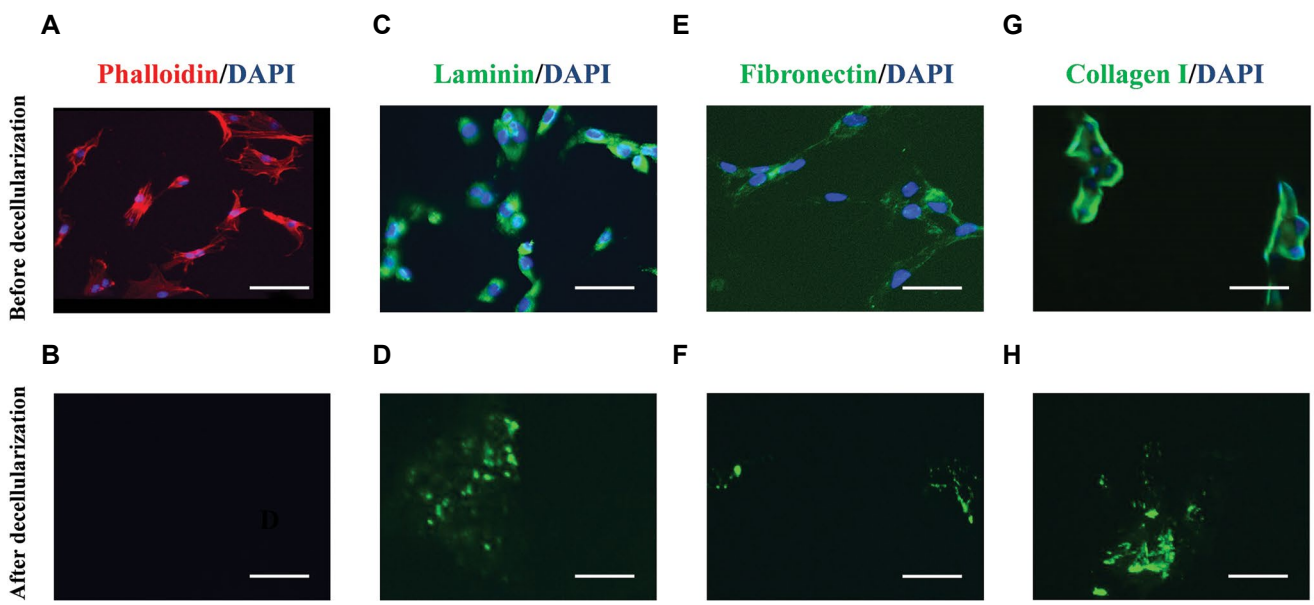

Fig.2: Immunofluorescent staining of MEF-ECM. The MEF-derived ECM was examined before (upper panels) and after (lower panels) decellularization. Phalloidin/DAPI staining of MEF-ECM **A.** Before decellularization and **B.** After decellularization. Immunofluorescence staining of matrix proteins: **C, D.** Laminin, **E, F.** Fibronectin, **G, H.** Collagen I in MEFs and MEF-ECM (scale bar: 100 µm). MEF-ECM; extracellular matrix derived from mouse embryonic fibroblasts.

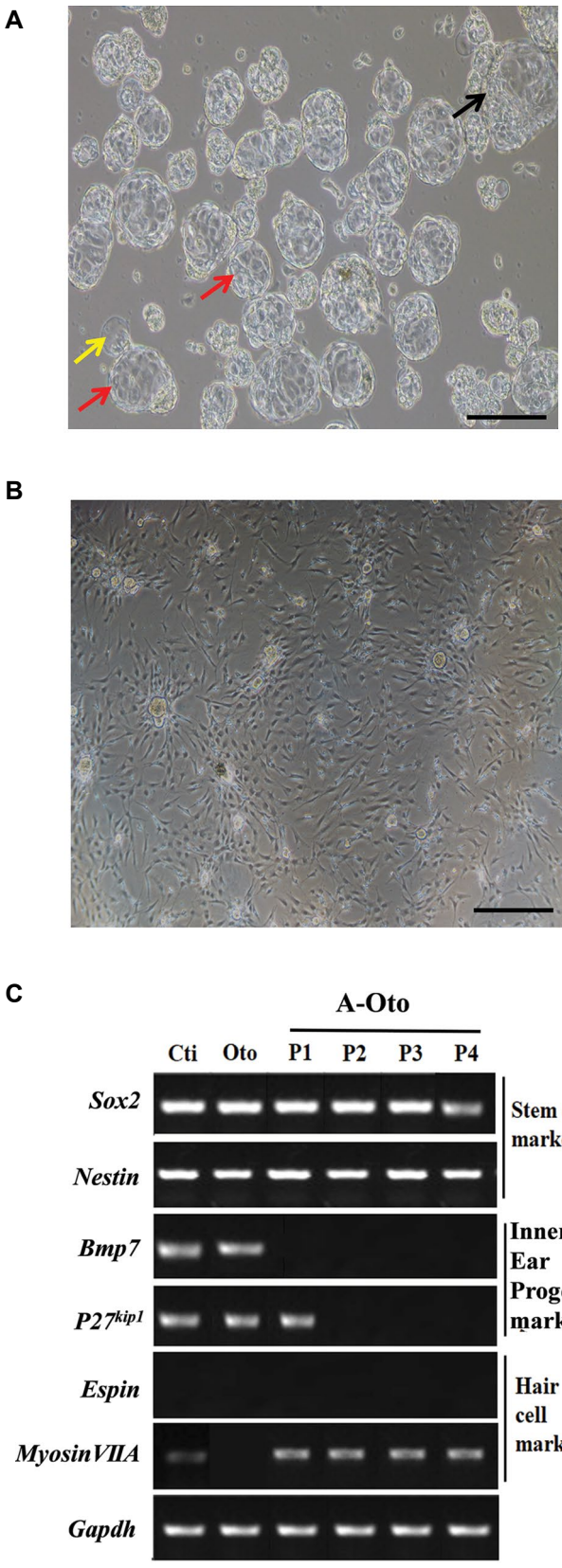

Fig.3: Generation and characterization of adherent otospheres. **A.** Phase contrast images of suspension otospheres, three kinds of sphere morphologies, including solid (red arrow), transitional (black arrow), and hollow spheres (yellow arrow) could be viewed. **B.** Morphology of P4 adherent otospheres. **C.** Reverse transcription-polymerase chain reaction (RT-PCR) analyses of the expression of the stem cell markers, inner ear progenitor cell markers and hair cell makers in adherent otospheres at different passage. Cti; Organ of Corti, Oto; Otospheres, A-Oto; Adherent otospheres; P1; Passaged 1, P2; Passaged 2, P3; Passaged 3, P4; Passaged 4 (scale bar: 100 µm).

The MEF-ECM promotes proliferation of inner ear-derived cells

To assess the impact of MEF-ECM on the proliferation of inner ear-derived cells, cells at passages 4 and 8 were cultured on the MEF-ECM and cells at passage 4 cultured on the TCP. The proliferation ability of inner ear-derived cells was determined by CCK-8 assay. As shown in Figure 4A, a consistent increase in the number of inner ear-derived cells was observed throughout the *in vitro* cultivation period of 1 to 7 days. Of noteworthy significance is the observed augmentation of cell density cultivated on MEF-ECM, as a result of cell-matrix interactions. In addition, inner ear-derived cells showed proliferation in all substances during the initial seven days of observation. On the 7th day, the proliferation of P4 cells cultured on the MEF-ECM was significant difference compared with TCP group and P8-MEF-ECM group. Overally, the proliferation ability of P4 cells cultured on the MEF-ECM was stronger compared with P8 cells (Fig.4B).

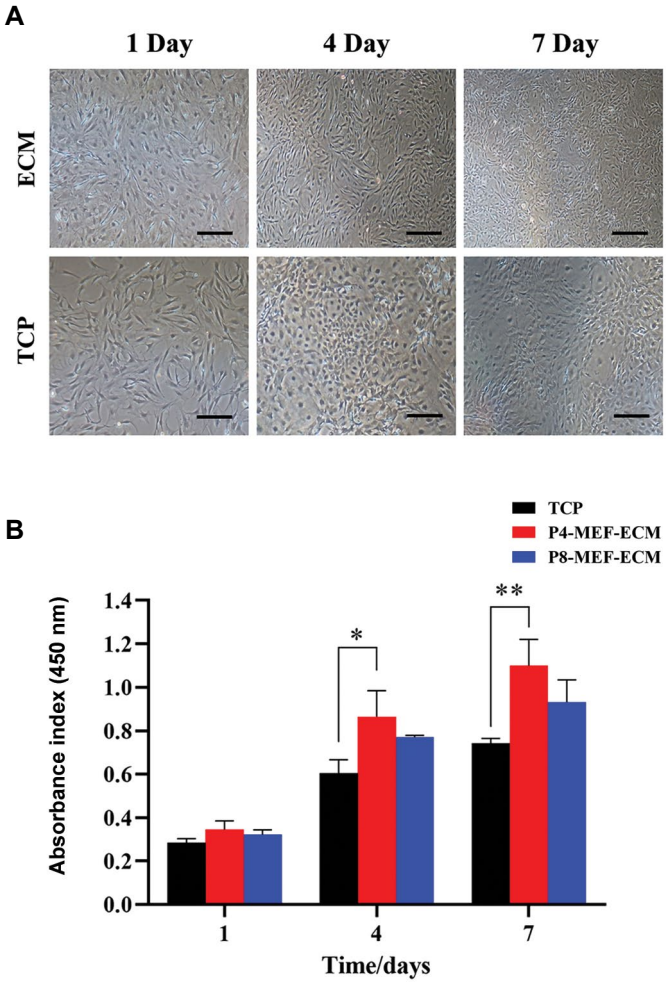

Fig.4: Proliferation of inner ear-derived cells were influenced by the substrates. **A.** Phase contrast microscope images of P4 inner ear-derived cells cultured on uncoated TCP and MEF-ECM. **B.** The CCK-8 assay for evaluating the viability of inner ear-derived cells cultured on different substrates. The results showed MEF-ECM obviously promoted the cell proliferation compared with TCP. P4-MEF-ECM: P4-adherent cells cultured on the MEF-ECM; P8-MEF-ECM: P8-adherent cells cultured on the MEF-ECM (scale bar: 500 µm). *; P<0.05, **; P<0.01, TCP; Tissue culture plate, and MEF-ECM; Extracellular matrix derived from mouse embryonic fibroblasts.

Discussion

Cochleae that are formed during earlier stages of development have the potential to contain multipotent stem cells with the ability to self-renew and differentiate into various cell types (16, 17). Previous study has revealed round spherical mass of cells that can be extracted from the basilar membrane of postnatal mice, and such cells have been nominated as inner ear-derived cells (18). Under suspension culture condition, these stem cells transform into spherical structures which possess the capability of proliferating and differentiating into HCs (19). Meanwhile, the number of cochlear stem cells decreases dramatically within the first postnatal days (20) severely hindering endogenous stem cell application in treating hearing loss. So far, there have been no reports of effective proliferation *in vitro* with inner ear-derived cells (21). In our previous study, we have conducted successful investigations into the manipulation of inner ear-derived cells behavior through the use of adherent culture techniques for otospheres (19). In this study, we have successfully produced an *in vitro* model of adherent otospheres by using the adherent culture. The expression of stem cell markers (*Sox2* and *Nestin*) was observed in the cells derived from adherent otospheres and no expression of HC marker (*Espin*) observed, which proved that the cells derived from adherent otospheres were the inner ear-derived cells (22). However, the expression of *Myosin VIIA* was observed in the cultured cells after subsequent passages. This might have been due to partial differentiation of cells derived from adherent otospheres after subsequent passages and further studies will be needed to analyze these phenomena. Our RT-PCR analysis of *Espin* as a HC marker for actin filament cross-links in stereocilia is in full accordance with previous studies (23, 24). However, the existence of heterogeneous cell population may result in a decline in purity of stem cells affecting the experiment result directly.

Previous studies have demonstrated that ECM has the potential to serve as an efficient expansion system for the production of great amount of the cells with high quality cells for cartilage tissue engineering and regeneration (25). Recently, several investigators have demonstrated that the molecular components of the cochlear bio-matrix significantly influence the modulation of cell proliferation, survival, and migration (26, 27). These findings indicate that effective ECM could simulate stem cell microenvironment while simultaneously resulting in a substantial yield of high quality adult stem cells *in vitro* (26). ESCs and iPSCs are usually cultured with feeder cells to preserve their pluripotent differentiation and proliferation capabilities (28). So far, MEFs are commonly used as feeder cells to facilitate the establishment and preservation of pluripotent/multipotent stem cells (29). Essential growth factors and cytokines secreted by MEFs have been identified as crucial elements in sustaining the pluripotent state of stem cells (30).

In this study, the application of optimized decellularization treatments yielded an ECM derived from MEF providing an important microenvironment for promoting the proliferation of inner ear-derived cells. Our results indicated that the main constituents of the MEF-ECM, including collagen I, fibronectin, and laminin, were successfully detected following the decellularization, which supported the previous report (31). The high fluorescence intensity of laminin and collagen I indicated a high abundance of these proteins within the MEF-ECM. However, it was observed that the fluorescence intensity of fibronectin was relatively low. These findings also supported previous report (32). In this study, the adherent atmospheres in P4 and P8 had a better cellular growth behavior than the adherent atmospheres in P1 after the culture of otospheres. Therefore, we chose the adherent otospheres in P4 and P8 with a more stable and uniform state through observation. We found that MEF-ECM obviously promoted the inner ear stem cell proliferation, supported the hypothesis that the bioactive components of MEF-ECM provided an important microenvironment to promote cell proliferation. In addition, the adherent cells in P4 cultured on the MEF-ECM had a higher proliferative ability compared with the adherent cells in P8. This is probably due to younger cells in P4 and the viability of P4 adherent cells is higher compared with the P8 adherent cells. Recent researches showed that ECM influenced the fate and promoted the expansion of inner ear-derived cells (33). It has been suggested that the MEF-ECM could effectively promote proliferation and adhesion of human umbilical vein endothelial cells (HUVEC) (34). Similar outcomes have been observed in the cultivation of inner ear-derived cells. However, the lack of characterization of expanded cells results in a direction of uncertainty of the differentiation of the expanded cells. Further studies would be needed to find if expanded cells could differentiate into HCs or other auditory cells. In addition, the underlying mechanisms of how MEF-ECM contributes to the modulation of the biological function of inner ear-derived cells remains a topic of debate. In mammals, ECM is composed of approximately 300 proteins, including collagen subunits, proteoglycans, and glycoproteins (14). It is challenging to predict the potential impact on inner ear-derived cells due to the complex interplay among ECM constituents. Several studies have shown that ECM plays a crucial role in determining cell fate during the early stages of embryonic development, that is also instrumental in upholding the pluripotency of inner cell mass cells, as occurs in the case of TGF-β (35, 36). It has been determined that bFGF is a crucial

growth factor that is naturally produced by feeder cells utilized in the cultivation of hESC (37, 38). Besides, proteins or short peptide sequences extracted from intact MEF-ECM, exhibiting distinct interactions with cell receptors, have the potential to promote inner ear stem cell proliferation (39). Thus, combination of the growth factors and/or proteins within MEF-ECM could be useful substrates to enhance the capability of *ex vivo* expansion of inner ear-derived cells and to advance their effectiveness in regenerating HCs. However, additional research is required for the elucidation of the processes by which the MEF-ECM modulates the proliferation of stem cells.

Conclusion

The MEF-ECM containing multiple bioactive proteins was extracted successfully using decellularization. Furthermore, our research highlighted the capacity of utilizing MEF-ECM for the expansion of inner ear-derived cells *in vitro*, which significantly augmented the proliferative rate of inner ear-derived cells. Future research should prioritize the illustration and characterization of the mechanism of action of specific bioactive components in MEF-ECM. The MEF-ECM could promote the proliferation of inner ear-derived cells expected to achieve auditory cell regeneration and hearing restoration in the future.

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Authors' Contributions

X.L., J.W.; Contributed to the conception and design of the experiments. J.Z., L.L.; Carried out the experiments, perform the statistic analyse and wrote the first draft of the manuscript. J.Z., Y.L.; Sample preparation, analysed the experimental results and revised the manuscript. X.L., J.W.; Re-analysed all the experimental results and statistical data, edited and revised the manuscript. All authors read and approved the final manuscript.

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