Abstract:
Spiral ligament fibrocytes of the cochlea play homeostatic roles in hearing and their degeneration contributes to hearing loss. Culturing fibrocytes in vitro provides a way to evaluate their functional characteristics and study possible therapies for hearing loss. We investigated whether in vivo characteristics of fibrocytes could be recapitulated in vitro by modifying the culture substrates and carried out proof of concept studies for potential transplantation of culture cells into the inner ear. Fibrocytes cultured from 4-5-week old CD/1 mice were grown on 2D substrates coated with collagen I, II, V or IX and, after harvesting, onto or into 3D substrates (hydrogels) of collagen I alone or mixed collagen I and II at a 1:1 ratio. We also assessed magnetic nanoparticle (MNP) uptake. Cell counts, immunohistochemical and ultrastructural studies showed that fibrocytes grown on 2D substrates proliferated, formed both small spindle-shaped and large flat cells that avidly took up MNPs. Of the different collagen coatings, only collagen II had an effect, causing a reduced size of the larger cells. On hydrogels, the cells were plump/rounded with extended processes, resembling native cells. They formed networks over the surface and became incorporated into the gel. In all culture formats, the majority co-expressed caldesmon, aquaporin 1, S-100 and sodium potassium ATPase, indicating a mixed or uncharacterised phenotype. Time-course experiments showed a decrease to ~50% of the starting population by 4d after seeding on collagen I hydrogels, but better survival (~60%) was found on collagen I + II gels, whilst TEM revealed the presence of apoptotic cells. Cells grown within gels additionally showed necrosis. These results demonstrate that fibrocytes grown in 3D recapitulate in vivo morphology of native fibrocytes, but have poorer survival, compared with 2D. Therefore hydrogel cultures could be used to study fibrocyte function and might also offer avenues for cell-replacement therapies, but need more optimization for therapeutic use. Fibrocyte function could be modified using MNPs in combination, for example, with gene transfection.
Response to Reviewers:

We thank the reviewers for their comments and hope our responses are acceptable. We perhaps did not make it sufficiently clear in the manuscript that this is essentially a proof of concept paper in which we have evaluated different options for cell culture of fibrocytes with an ultimate view to providing more natural conditions for growing the cells and potential for transplant. This we have now done. We should also reiterate that this is work that has been taking place over 10+ years and through several iterations, all of which we contend contain valuable information.

In the modified manuscript which we have copied in below, modifications in response are identified by a comment box which specifies the point made by the reviewer. For each change we have added a comment box which identifies the point made by the reviewer. Below we also describe our response to the reviewers, indicating where changes have been made or where we did not feel any change was needed.

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"(1) the phenotype of cells grown on 3D substrates may resemble more closely native cochlear fibrocytes and they may thus be more useful for study as transplantable cells; and (2) that collagen II containing substrates and gels would recapitulate more closely the native environment and promote better growth." While this topic is of interest, I think the manuscript needs more details for the reader unfamiliar with cochlear fibrocytes and their importance to hearing and will need significant revision for consideration.

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We have added a brief summary about transcriptional characteristics and functions to the introduction and suggested other approaches to characterising the cells.

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We have removed this suggestion as we acknowledge it is speculative.

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13) In Figure 7, Fig7b, remove lower case "a" from legend.

Done.

14) In Figure 7e legend and figure, “Occasionally, cells expressing Aqp1 but not S-100 can be found (arrows)”. This is unclear (are they cells?) and the cells look like they might be double labeled.

We agree there is weak S-100 labeling and have clarified this in the manuscript.

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We meant the arrow to demonstrate the plumper cells. This has been clarified in the legend.

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We have now clarified what the asterisks show in the legend.

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The asterisk was there but perhaps hard to see. We have enhanced it.

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We have described the lysosome better in the figure legend and added db to the image and legend.

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We have corrected the figure.
amount of work completed and the very nice images, however, I have several major concerns that I have listed below.

1) There is a distinct lack of controls for the immunofluorescent and immunogold labelling experiments. Antibodies are often unpredictable and non-specific, therefore, the paper needs negative and positive controls for fluorescence images. For example, the antibodies should be tested on sections of lateral wall from the same mouse strain, and negative controls to show antibody specificity. All the fluorescence images show positive staining for every antibody used. It’s hard to determine if these proteins are actually being expressed by all the cells or whether there is a lack of specificity/too much background? Western blot or RT-PCR could also provide more quantitative data.

We have performed negative controls on many samples using these antibodies but failed to mention that. We have now added the point (Methods, section 2.6). Regarding selectivity of the antibodies, all of those used here (including in images in Fig. 2) have also been used at the immunofluorescence and immunogold EM level in the native cochlea (published in Mahendrasingam et al., 2011b) and been shown to have distinct quantitative distributions across the different fibrocyte types. These are also from the same lots (as the work started in the late 2000s). We thus have used a panel of antibodies already demonstrated to show clear specificity to the different types, with the exception of the inability to distinguish type II and type V fibrocytes which are also very similar ultrastructurally. This implies that the labelling in culture is faithfully showing that the expression and distribution have lost their cellular distinctiveness in most cases. We have clarified this in the methods and results.

2) For figure 7, the authors state that “a few small cells were negative for S-100 whilst still expressing aquaporin 1” (I think on P.19, line 5 - there are no page numbers!), however the arrows are pointing to cells that appear both red (aqp1) and green (S-100) positive.

We agree that there is weak labelling of S-100 in these cells and have amended the text accordingly in the results and figure legend.

3) Is there a reason why the cells in FOV are different for panel a and b in Figure 10? The total number of cells is 3-4 times more in panel A than the total in panel B, for Day 1. Were the same number of cells seeded?

No specific reason as seeding density will be similar – when cells are dropped onto the gels however, we have noted that the number that settle on the surface can vary.

4) Figure 11a - there is no control for the immunogold labelling. A collagen I labelled image is needed to compare and show that this isn't just some background staining, or gold particles sticking to the collagen matrix.

We have omitted primary antibodies in these experiments (as noted above) and have not observed gold within the gels. We don’t anticipate that collagen I labelling is particularly informative; we know the protein is present as it is visible in the EM and the labelling would be everywhere. The main aim of this part was to show that collagen II was not lost from the gel when the two collagens were polymerised. We have now added an image taken with post-embedding immunogold TEM to show that even labelling is detected, and as it is on the surface of the section, gold labelling is unlikely to be trapped within a sticky gel.

5) The significant changes referred to in Figure 11b are pretty minimal. Overall, adding collagen II didn’t make that much of a difference. What were the issues with making collagen II gels? Is this a common problem?

Nevertheless the difference was significant. In terms of the gel composition, in our hands, the collagen II did not polymerise. Other authors also report that collagen II alone does not form good gels, and have mixed it with collagen I. We have added a citation to this (Velasquez et al).

6) It seems to me that a major flaw of this study (unless I have misunderstood the methodology) is the use of pipette tips and capillary tubes to solidify the gels containing fibrocytes and then extruding them into fixative for analysis after 24 hr incubations. Firstly, the enclosed environment in the tips and capillaries would limit the amount of
gas and media infiltration to the cells (how were these gels incubated?), which would likely lead to cell stress and possible death. Secondly, extruding the gels after they have set using pipette tips or capillaries could cause severe adverse effects on the cells. In particular, the Wagner study referenced in the paper states that there are several forces to which damage can occur to cells, such as shearing and stretching. Both would be a major concern to me, especially when extruding already set gels from pipette tips (which are substantially narrower at the tip, thus causing a huge pressure change). Therefore, it's not surprising that the cells were less healthy, with evidence of apoptosis and necrosis. The Kelly et al. study the author's referenced in the paper showed good viability and interconnected fibrocyte cell networks in collagen I gels; however the authors here suggest viability was a problem when attempting the same method. The major difference I assume is how the gels were made and incubated. It is my opinion that if the authors are trying to devise a strategy for cell transplant/therapy, then they need to inject the cell-matrices before they set. For example, the Wagner study use pluronic F-127 poloxamer to seed cells at cold temperatures (which keeps it in the fluid state) and then inject this into animals where it polymerizes and forms a gel at warmer temperatures. Thus, avoiding the problems of shear force and mechanical disruption to pre-formed matrices.

Although I think the authors need to rethink the idea of using these preformed scaffolds and methods for cochlear therapies, I will note that the scaffolds/matrices represent excellent tools for studying primary cochlear fibrocyte biology and function in vitro. We acknowledge these limitations but still feel that the attempt to produce injectable scaffolds was worth attempting and the low success rate will be informative to the readers who may wish to investigate such a system.

Minor edit:
1) Typo line 7/8, page 13 "assed" should be "assessed"?
This has been corrected.
Dear Editor

I am writing to ask you to reconsider our revised manuscript entitled ‘The effects of substrate composition and topography on the characteristics and growth of cell cultures of cochlear fibrocytes’ for publication in Hearing Research. We have addressed all the reviewers’ comments to the best of our ability and hope our responses are satisfactory.

Yours sincerely

David N Furness
Emeritus Professor of Cellular Neuroscience
https://www.keele.ac.uk/lifesci/people/davefurness/
School of Life Sciences, Keele University
Response to reviewers

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5) The significant changes referred to in Figure 11b are pretty minimal. Overall, adding collagen II didn’t make that much of a difference. What were the issues with making collagen II gels? Is this a common problem?

Nevertheless the difference was significant. In terms of the gel composition, in our hands, the collagen II did not polymerise. Other authors also report that collagen II alone does not form good gels, and have mixed it with collagen I. We have added a citation to this (Velasquez et al).

6) It seems to me that a major flaw of this study (unless I have misunderstood the methodology) is the use of pipette tips and capillary tubes to solidify the gels containing fibrocytes and then extruding them into fixative for analysis after 24 hr incubations. Firstly, the enclosed environment in the tips and capillaries would limit the amount of gas and media infiltration to the cells (how were these gels incubated?), which would likely lead to cell stress and possible death. Secondly, extruding the gels after they have set using pipette tips or capillaries could cause severe adverse effects on the cells. In particular, the Wagner study referenced in the paper states that there are several forces to which damage can occur to cells, such as shearing and stretching. Both would be a major concern to me, especially when extruding already set gels from pipette tips (which are substantially narrower at the tip, thus causing a huge pressure change). Therefore, it’s not surprising that the cells were less healthy, with evidence of apoptosis and necrosis. The Kelly et al. study the author’s referenced in the paper showed good viability and interconnected fibrocyte cell networks in collagen I gels; however the authors here suggest viability was a problem when attempting the same method. The major difference I assume is how the gels were made and incubated. It is my opinion that if the authors are trying to devise a strategy for cell transplant/therapy, then they need to inject the cell-matrices before they set. For example, the Wagner study use pluronic F-127 poloxamer to seed cells at cold temperatures (which keeps it in the fluid state) and then inject this into animals where it polymerizes and forms a gel at warmer temperatures. Thus, avoiding the problems of shear force and mechanical disruption to pre-formed matrices.

Although I think the authors need to rethink the idea of using these preformed scaffolds and methods for cochlear therapies, I will note that the scaffolds/matrices represent excellent tools for studying primary cochlear fibrocyte biology and function in vitro.

We acknowledge these limitations but still feel that the attempt to produce injectable scaffolds was worth attempting and the low success rate will be informative to the readers who may wish to investigate such a system.
Minor edit:

1) Typo line 7/8, page 13 "assed" should be "assessed"?
This has been corrected.

The effects of substrate composition and topography on the characteristics and growth of cell cultures of cochlear fibrocytes.
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Abstract

Spiral ligament fibrocytes of the cochlea play homeostatic roles in hearing and their degeneration contributes to hearing loss. Culturing fibrocytes in vitro provides a way to evaluate their functional characteristics and study possible therapies for hearing loss. We investigated whether in vivo characteristics of fibrocytes could be recapitulated in vitro by modifying the culture substrates and carried out proof of concept studies for potential transplantation of culture cells into the inner ear. Fibrocytes cultured from 4-5-week old CD/1 mice were grown on 2D substrates coated with collagen I, II, V or IX and, after harvesting, onto or into 3D substrates (hydrogels) of collagen I alone or mixed collagen I and II at a 1:1 ratio. We also assessed magnetic nanoparticle (MNP) uptake. Cell counts, immunohistochemical and ultrastructural studies showed that fibrocytes grown on 2D substrates proliferated, formed both small spindle-shaped and large flat cells that avidly took up MNPs. Of the different collagen coatings, only collagen II had an effect, causing a reduced size of the larger cells. On hydrogels, the cells were plump/rounded with extended processes, resembling native cells. They formed networks over the surface and became incorporated into the gel. In all culture formats, the majority co-expressed caldesmon, aquaporin 1, S-100 and sodium potassium ATPase, indicating a mixed or uncharacterised phenotype. Time-course experiments showed a decrease to ~50% of the starting population by 4d after seeding on collagen I hydrogels, but better survival (~60%) was found on collagen I + II gels, whilst TEM revealed the presence of apoptotic cells. Cells grown within gels additionally showed necrosis. These results demonstrate that fibrocytes grown in 3D recapitulate in vivo morphology of native fibrocytes, but have poorer survival, compared with 2D. Therefore hydrogel cultures could be used to study fibrocyte function and might also offer avenues for cell-replacement therapies, but need more optimization for therapeutic use. Fibrocyte function could be modified using MNPs in combination, for example, with gene transfection.

Keywords: fibrocyte replacement therapy; hydrogels; metabolic hearing loss; 2D vs 3D cell cultures
1. Introduction

Cochlear fibrocytes are primarily found in the spiral ligament where they play a critical role in cochlear homeostasis and, together with the stria vascularis, in generating the endocochlear potential (EP) (see reviews by Wangemann, 2006, Furness, 2019). Loss of the EP reduces the ‘driving force’ that makes the transduction mechanism of the sensory hair cells in the organ of Corti highly sensitive to stimulation. Deterioration of EP and subsequent lateral wall degeneration may reflect specific patterns of hearing impairment, such as those associated with metabolic hearing loss (Lang et al., 2010; Dubno et al., 2013; Saremi and Stenfelt, 2013).

There are five main types of fibrocyte, Types I – V which differ in location in the spiral ligament and in morphology (Spicer and Schulte, 1996; Furness et al 2009). They are connected together by gap junctions in a syncytial network and are thought to perform roles in ion transfer (in generating the EP), water balance, inflammation and basilar membrane tensioning (see review by Furness, 2019). They are located within a matrix comprised of collagens, the two main types being type II collagen (Slepecky et al., 1992; Buckiova et al., 2006) and type IX collagen (Usami et al, 2008). Fibrocyte development and differentiation appears to involve Wnt signalling (Bohnenpoll et al., 2014) and transcription factors SOX9 (Trowe et al., 2010), Tbox18 (Trowe et al., 2008), both regulators of Wnt signalling, and Brn4/ Pou3f4 (Phippard et al., 1999) a member of the POU family of proteins. Fibrocyte degeneration has been reported to underlie deafness in human DFN3, caused by mutations in Pou3f4 (Minowa et al., 1999) and in some strains of mice (Hequembourg and Liberman 2001; Mahendrasingam et al., 2011a). It is possible that fibrocyte degeneration leads to not only a loss of EP but also that breakdown of potassium recycling might cause hair cell death and thereby permanent hearing loss. At present, however, the precise pathology underlying the metabolic deafness patterns, and whether they can be prevented or alleviated by therapeutic methods, is relatively unexplored (Furness, 2019).
As an approach to studying fibrocytes, we have been developing fibrocyte culture techniques. These offer several possibilities including: evaluating the growth of these cells, establishing potential growth enhancing agents for therapeutic use, studying their physiology and toxicity, genetic modification and as a source of transplantable cells (Furness 2019). In previous studies, fibrocytes have been mostly cultured on flat surfaces coated in collagen I and in collagen I matrices (i.e. in two and three dimensions respectively), the former generating cells expressing markers for several types of fibrocytes (Gratton et al, 1996; Suko et al., 2000; Sun et al., 2012) and the latter for Type III (Kelly et al 2012). However, morphologically, the cells present in 2D culture do not resemble native fibrocytes closely, and may not offer an ideal method for obtaining cells with useful characteristics for understanding their function or as therapeutic agents, whilst cells cultured in collagen matrices (hydrogels) have been primarily investigated for their contractile properties (Kelly et al., 2012).

We hypothesise that: (i) the phenotype of cells grown on 3D substrates may resemble more closely native cochlear fibrocytes and they may thus be more useful for study and as transplantable cells; and (ii) specifically that collagen II containing substrates and gels would recapitulate more closely the native environment and promote better growth. Therefore, the aim of the present work was to evaluate the growth of fibrocytes, starting with their derivation from ligaments in vitro and subsequent growth on different collagen substrates and matrices. We have accordingly grown fibrocytes on collagen hydrogels and compared them with cells derived using 2D culturing on different collagen coated coverslips, by evaluating their morphology and ultrastructure, growth, and expression of a variety of fibrocyte markers. Cells were also acquired from suspensions inside a collagen I matrix within a custom-made injection device as a proof-of-concept approach to determine how well they could integrate into gels and to evaluate a possible method of transferring.
the cell-laden matrices. Finally, we prepared samples of immature cochleae (CD/1, P14) for comparison of aspects of the ultrastructure of the culture cells.

2. Materials and Methods

2.1. Animals

CD/1 mice were used in this study. The mice were bred and maintained in the Biomedical Services Unit of the University of Keele according to the 1986 Animals (Scientific Procedures) Act under regulatory control of the UK Home Office and following project approval from the local ethical committee. All terminations were carried out under Schedule 1 of the act.

2.2. Preparation of primary fibrocyte cultures

Primary cultures were derived from spiral ligament according to the methods of Gratton et al (1996) with minor modifications (Suko et al., 2000). 6-well plates were first coated with collagen I by incubating each well in 2 mL of an aqueous 0.01% collagen I (Sigma-aldrich, UK) solution for 5 h, aspiration of the solution and drying overnight at 37°C in an incubator.

Male and female mice of 4 – 5 w old were anaesthetised using 0.1 mL sodium pentobarbitone (Vetalar, UK), decapitated and the bullae removed. Cochleae were excised and opened in ice-cold culture medium, MEM-α (Invitrogen; catalogue number: 22561) containing 1 x Antibiotic-Antimycotic (Sigma-aldrich, UK). Aseptically dissected 2-3 pieces of lateral wall were placed strial side down in one drop of culture medium MEM-α per well, supplemented with 10% foetal calf serum (Invitrogen), 1 x Antibiotic-Antimycotic, and 1% ITS-G supplement (Invitrogen) on collagen type I (Sigma; product number: C3867) coated 30 mm wells, cover-slipped and left overnight in the
incubator (37°C: 5% CO₂, 95% air). One cochlea was sufficient for two wells. Once the ligaments were adherent, the coverslip was removed, culture medium was added and the segments were incubated for 2-3 weeks (culture medium changed every 3-4 days) forming primary cultures. When the primary cultures became near-confluent (2-3 weeks), the lateral wall segments were removed and the cells were rinsed with sterile phosphate buffered saline (PBS, pH 7.4) followed by trypsinization (0.025 %-0.05 % trypsin/ethylenediamine tetra acetic acid (EDTA)) and centrifugation (at 1200 rpm for 5 min). The pellet was then re-suspended and diluted in culture medium and reseeded for secondary culturing.

After reaching confluence, cells were collected for passaging onto collagen I coated T25 flasks (Corning, Sigma-Aldrich, Poole, Dorset UK). The cells were detached from the wells by incubation for up to 20 mins using 0.05% trypsin solution in PBS, culture medium added and centrifugation performed to concentrate them. A small volume of concentrated cells was then seeded directly into the T25 flasks and culture medium with supplements was added. They were maintained as above for approximately two weeks until confluence was reached. Finally, cells were harvested by trypsinisation and either used for further experiments or frozen with 10% dimethylsulfoxide (Sigma-Aldrich, UK) in liquid nitrogen for storage.

2.3 Preparation of substrates and seeding of cells

Substrates took the form mainly of either collagen coated coverslips or collagen containing gels, although one set of 2D cultures was also grown on collagen I coated Aclar sheets (Agar Scientific, UK) to enable examination by transmission electron microscopy (TEM).
To test the effects of different substrate types on growth of the cultures on flat substrates, coverslips were incubated with 0.01% purified collagen I, II, V (Sigma-Aldrich, UK) or IX (Chondrex Inc, WA, USA) for up to 5 h and then allowed to dry overnight at 37°C.

From the 2D data and the known distributions of different collagens in the cochlea (Slepecky et al., 1992), we decided to focus on collagen I and II for the production of matrices in which the fibrocytes could be grown. Although collagen I is not expressed in the ligament, because it has been used as a substrate in all previous fibrocyte culture work, it was used as a control substrate. In addition, collagen II is difficult to polymerise into useable gels on its own, as others have also found, but can be mixed with collagen I (Vasquez-Portalati et al., 2016). The gel compositions made were: 3 mg/ml of collagen I alone and 2 mg/mL each of collagen I + II. Stock collagen solutions were mixed to the appropriate final concentrations very gently on ice and polymerised by addition of 4N NaOH. 250-400 μl volumes were placed into wells and incubated at 37°C until they solidified.

Fibrocytes obtained either from fresh cultures or re-derived from frozen stocks were seeded on top of the collagen gels and either directly into wells or onto coverslips at a density of 5000 - 50000 (depending on cell harvesting) per cm², allowed to settle and grown for varying times. The culture medium was changed every two to three days.

To mix cell suspensions and incorporate them into a collagen I matrix within a custom made injection device, a simple device was constructed from a 1 mL syringe attached either to a disposable 200 μL pipette tip (pipette gels), with a 50 mm length and approximate tip aperture of 0.5 mm, or by attaching tubing to the pipette tip into the distal end of which was inserted a Clark glass capillary (Harvard Apparatus, Cambridge, UK) with diameter 0.94 mm and length 100 mm (tube...
Cells were incorporated into these gels in two ways: (i) unpolymerised gel solution was taken up first into the pipette tip or capillary tube followed by cell suspensions in medium described above, in a ratio of 2:1 so that they mixed gently within the pipette tip or capillary tube; (ii) cells were premixed in equal volumes of double-concentration collagen and cell suspension and then taken up into the pipette tip or capillary tube.

2.4. Magnetic nanoparticle uptake

We assessed whether fibrocytes in culture could take up magnetic nanoparticles (MNPs), a useful vector for intracellular delivery of a variety of molecules, such as gene constructs. MNPs (SPHERO Fluorescent Carboxyl Magnetic Particles, Nile Red, 0.20-0.39 μm #FCM-02556-2) were added to the culture medium applied to established 2-D cell cultures for either 24 or 48 h at a concentration of 20 μg/mL. Cells were then immunolabelled with S-100 and examined in a confocal microscope. We also harvested the MNP labelled cells for incorporation into 3D gels (pre-mixed method), and fixed for immunocytochemistry (see below).

2.5 Analysis of cultures

To identify which cells were exiting the ligament to generate the cultures, and the mode of cell exit, attached ligaments were observed live by light microscopy (LM) or placed on Aclar sheets (Agar Scientific, Stansted, UK) and then fixed at 4 and 7 days for LM and TEM examination (see electron microscopy section below for fixation method).

For evaluation of the effect of substrate on the growing fibrocytes, collagen coated coverslip cultures were fixed using 4% paraformaldehyde in 0.1 M sodium phosphate buffer (NaPi) for investigation of the expression of immunocytochemical markers, to verify the nature of cultures for
comparison with previous studies. Once the coverslips had all reached confluence, cell size distribution was measured using ImageJ (Abramoff et al., 2004) on the immunocytochemically labelled coverslips from images of 5 randomly taken field of views. The data were assessed for normality using an Anderson-Darling test (FOV) and as they were non-parametric, statistically analysed using the Wilcoxon Sign Rank test (available online at https://www.socscistatistics.com/tests/signedranks/). A second experiment using the same method was performed to ensure reproducibility. These could not be pooled as the data were collected on separate days.

Analysis of cell survival on gels was performed using phase contrast microscopy. The number of cells on each gel was assessed by daily analysis of 10 FOV in four replicates using the same magnification and in the approximate middle of the gels (a dark mark was placed underneath the area for evaluation). Cell counts were thus given as mean number per FOV. The data were pooled across replicates and then evaluated for normality. As many of the population data sets were found not to be normally distributed, comparisons were made using the non-parametric Wilcoxon Sign Rank test between the samples of the pooled data.

Finally, for gels with cells prepared in pipette tips or capillary tubes, after 24h incubation these gels were extruded by gentle pressure on the syringe into fixative for immunocytochemistry and electron microscopy evaluation of cell morphology as described below.

2.6 Immunocytochemistry

To determine the immunophenotype and where required the size and shape of cells grown in different culture conditions, we performed immunocytochemistry with known fibrocyte markers
(see for example Mahendrasingam et al., 2011b). Coverslips and gels were fixed using 4% freshly dissolved paraformaldehyde in NaPi for 1 h. They were stored in 1/10th diluted fixative in the same buffer until used for labelling. Cell morphology was evaluated using anti-tubulin conjugated to FITC or phalloidin-FITC (Sigma-Aldrich, Poole, UK, both 1:100 dilution), a marker for actin. Previously used antibodies to Na,K,ATPase α1 (Abcam AB33133, 1:100), caldesmon (Acris antibodies Gmbh, Germany S783, 1:100), aquaporin 1 (AB3272 Millipore-Chemicon, Temecula, USA, 1:100) and S-100 (52644 Sigma-Aldrich, 1:100) were employed to distinguish potential fibrocyte types. All of the antibodies used here have previously been used for immunofluorescence and immunogold EM in the native cochlea, even from the same specific tubes (Mahendrasingam et al., 2011) and been shown to produce distinct quantitative distributions across the different fibrocyte types. We also performed negative controls which showed that background labelling caused by secondary antibodies, was virtually non-existent. Since there have also been reports of substantial number of resident macrophages in the SL (e.g., Wiu et al, 2018), we also assessed culture cells for macrophages using the same marker in that study, IBA1 (PA527436, Invitrogen, 1:100). Cultures were washed in phosphate buffered saline (PBS), permeabilised with 0.5% Triton-X-100 in PBS, blocked in 10% goat serum in PBS (GS-PBS) and incubated with phallodin-FITC or anti-tubulin-FITC and/or primary antibodies diluted in 1% GS-PBS. After washing, primary antibodies were visualised using appropriate secondary antibodies (Alexa-fluor 488 and/or Alexa fluor 568; Molecular Probes/Thermofisher) by incubation at 1:50 dilution in GS-PBS for 1 – 2 h and then imaged using a BioRad MRC1024 microscope in confocal mode.

2.7 Substrate evaluation by immunoelectron microscopy

To confirm that the mixed gels (collagen I + II) used in this study did contain collagen II within them, because of difficulties polymerising this protein, some were fixed in 4% w/v paraformaldehyde in NaPi for immunocytochemistry at the electron microscopic level.
For scanning electron microscopy (SEM) immunogold, the fixed gels were labelled with anti-collagen II (ab53047, Abcam, Cambridge, UK) as follows: they were incubated sequentially in tris buffered saline (TBS) containing 10% v/v goat serum (TBS-GS), 1:100 anti-collagen II in TBS-1% GS, washed in TBS and then in 15 nm gold-conjugated goat anti rabbit secondary antibody diluted 1:20, washed in TBS and distilled H₂O. They were postfixed in 2.5% glutaraldehyde in sodium cacodylate buffer/2 mM CaCl₂ (SCB) and made electrically conductive using the OTOTO technique where sequential incubations in alternating solutions of 1% osmium tetroxide (OsO₄) (1 h) in SCB and saturated aq thiocarbohydrazide (20 min), repeated once and then with a final OsO₄ incubation, were used to impregnate the samples with osmium (for details see Furness and Hackney 1985). These samples were dehydrated and critical point dried for examination in a Hitachi S4500 field emission SEM at 15 kV using a Deben backscattered electron detector.

In order to reduce the chances that the labelling was due to trapping of antibodies and gold particles in the sticky gels, we also performed post-embedding immunogold labelling for collagen II in the mixed collagen gels for examination by TEM. Gels were dehydrated through an ethanol series and placed in pure LR White for four changes. The resin was then polymerised in a gelatin capsule at 50°C overnight and 70 nm ultrathin sections were cut, placed on nickel grids and then labelled in sequential drops of solution placed on parafilm strips in a moist chamber as follows: blocked in TBS-GS (30 min), 1:100 anti-collagen II in TBS-1% GS (overnight at 4°C), washed in TBS and then in 15 nm gold-conjugated goat anti rabbit secondary antibody diluted 1:20, washed in TBS and distilled H₂O. They were examined in a JEOL 1230 TEM at 100 kV and digital images were captured using a Megaview III digital camera system.
2.8 Culture evaluation by electron microscopy

For SEM evaluation of the cultures, the majority were fixed in 2.5% glutaraldehyde in SCB for 2 h. Some of these samples were then prepared using the OTOTO technique described above.

Some 13 d gel cultures that had previously been prepared and observed for immunocytochemistry were dehydrated through an ethanol series and then dried using the hexamethyldisilazane method (Bray et al., 1993). The cultures were taken from 100% ethanol to a 1:1 mix of ethanol:hexamethyldisilazane (1 h), then pure WA 98052 (1 h). The samples were left in a fume hood until the solvent had evaporated completely, mounted on specimen stubs using silver colloid paint and sputter coated with gold, before examination in the FESEM.

For sectioning for light microscopy and TEM, Aclar sheets or gels loaded with cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer/2 mM CaCl$_2$ (SCB) for 2 h followed by 1% OsO$_4$ in SCB for 1 h, washed, dehydrated and embedded in Spurr resin using our conventional procedure (Furness and Hackney, 1985). Semithin sections for light microscopy were cut at 1–2 μm, stained with 0.1% toluidine blue and examined in a Lietz Dialux EB20 microscope. Images were captured using an Infinity camera supplied by DeltaPix. Ultrathin sections were cut onto 200 mesh thin bar copper grids, stained with 2% ethanolic uranyl acetate (20 min) and 2% lead citrate (5 – 7 min), allowed to dry and examined as described above in a JEOL 1230 TEM.

2.9 Preparation of immature CD/1 mice cochleae for ultrastructural comparison with culture cells

Some observations of the culture cells suggested potentially immature features may be present. For comparison with culture cells we therefore fixed postnatal day 14 (P14) cochleae by intralabyrinthine perfusion with glutaraldehyde and osmium fixative solutions as described,
decalcified in 5 mM EDTA for 48 h, and embedded in Spurr so as to cut ultrathin sections containing spiral ligament which were stained and examined, all as above.

3. Results

3.1 Initial development of cultures

We used LM and TEM to examine the ligaments after placing the coverslips on them and then subsequently after coverslip removal, during establishment of the cultures. The cells appeared to crawl out from the ligaments over a period of up to 7 days, when the ligament remnants were usually removed. By LM, the first sign of cells were single long, thin individual cells or chains of cells appearing to exit the ligament along the edge where type III fibrocytes would normally be present (Fig 1a, b). Cells then appeared around the ligament in small patches that gradually enlarged (Fig. 1c, d). They exhibited variable morphology (Fig. 1c, inset) including triangular shaped bodies with thin processes. In semi-thin sectional views, the residual ligament still contained cells at 4 d after ligament placement (Fig 1e), but these had mostly gone by 7 d leaving gaps, except for root cells which still appeared to be present (Fig. 1f). The exiting cells stretched down to the substrate (Fig 1e).

By TEM the presence of degenerating cells and gaps in the ligament became apparent, leaving behind an extensive extracellular matrix network in different ligament locations (Fig. 1g - j). Exiting cells became extremely thin and grew over each other forming contacts at various places (Fig. 1k).

3.2 Characteristics of fibrocytes grown in 2D cultures on collagen coated coverslips

Experiments using type I collagen coatings on coverslips, as in previous studies, resulted in areas of confluent cells that expressed fibrocyte markers, using the same antibodies we had previously used (Mahendrasingam et al., 2011b) in the native cochlea (Fig. 2a - e). For example, the cells were positive for Na,K,ATPase, S-100, caldesmon (Fig. 2c-d) and aquaporin 1 (Fig. 2e) representing a
mixed fibrocyte phenotype. We failed to detect any labelling for the macrophage marker, IBA1 (data not shown).

Cell morphology was varied but with areas of uniform morphology: One type could be categorized into small cells and a second type into substantially larger cells (Fig. 2d). In these areas cells were often elongated with a central bulge, presumed to be an area of cytoplasm containing the nucleus (Fig 2a, c-e). These central areas were rich in Na,K,ATPase, S-100 and aquaporin 1. In a few cells, caldesmon (Fig. 2c) and aquaporin 1 (Fig. 2e), both primarily a Type III fibrocyte marker (Mahendrasingam et al., 2011) were expressed strongly and filled the whole cell. With those exceptions, caldesmon was primarily localized to the cell edge. Actin, used as a marker for cell morphology, was concentrated at the center, confirming a cytoplasmic bulge there, and around the edge.

In non-confluent regions, cells were larger and appeared more spread out, with processes pointing in several directions (Fig. 2b). They were also positive for the same markers as above, and with similar distributions of these proteins with the exception of caldesmon and actin, both of which showed groups of parallel cables throughout the cell and around the nuclear area, which were confirmed as regular cytoskeletal cables in SEM images (Fig. 3).

When cells were grown in 2D on collagen I, II, V or IX substrates and labelled for S-100 and tubulin, no qualitative morphological differences were detected between substrates (Fig. 4). The two populations in the confluent areas noted above were present on all. These experiments also suggested that the smaller spindle shaped cells were Na,K,ATPase rich whilst the larger cell population were well demarcated from the former and also expressed S-100 (Figs. 2, 4).
Measurements of cell area in a typical experiment with five random sample areas showed some significant differences between large cells on the different substrates, such that the areas differed significantly between Type I and Type II substrates (Wilcoxon Sign Rank test, p = 0.015, n = 5). This was replicated in two experiments. In one experiment, we also found a significant difference between large cell areas in Type II and Type IX (Wilcoxon Sign Rank Test, p = 0.02, n = 5). No other differences were detected between each of these two populations on the different substrates (Fig. 4).

Using SEM on 2D collagen I grown cultures confirmed that the cells were flat in the confluent patches (Fig. 5a) and splayed out in non-confluent areas, with fine cell processes extending along the substrate (Fig. 5b, c). In both areas there was a shallow central bulge likely representing the cytoplasmic region containing the nucleus observed by LM. Overlying the other cells were occasional singlets and doublets of more globular appearing cells (Fig. 5a, d), the latter potentially undergoing cell division. In addition, at the edges of the patches of confluent cells were large single cells with multiform shapes. These data correlated well with the cultures observed by light microscopy, and further demonstrated that the central area was a shallow bulge. We examined 2D collagen II, V and IX substrates as before, but found no differences (data not shown).

TEM examination of these cultures confirmed that the central bulge contained a regular, elliptical nucleus and showed fine extensions representing the flattened cell body (Fig. 6a-c). Within the central bulge in particular, normal-appearing mitochondria (Fig 6a, d) and both flattened and expanded areas of rough endoplasmic reticulum were present (Fig. 6b, e). Similar areas of expanded rough endoplasmic reticulum were also found in immature fibrocytes from P14 cochleae of each type. As an example, we illustrate type III and type I cells (Fig. 6f). Cells in the cultures also often associated with each other at overlap points by adherens junctions (Fig. 6a, inset). Many of the cells commented [r14]: Reviewer 1, minor 8.
possessed fine processes of varying dimensions (Fig. 6d), some of which surrounded circular gaps (Fig. 6d), reminiscent of type III cells in the cochlea (Mahendrasingam et al, 2011b).

3.3 Characteristics of fibrocytes grown in 3D cultures on and in collagen gels

In order to examine the effect of growing cells onto a 3D, potentially transplantable matrix, we opted in our first experiments to seed cells directly onto pre-formed collagen I hydrogels at the higher seeding densities (~50,000 per mL) to maximize initial population size and increase the probability of success. Gels were therefore polymerized in 24-well plates and cell suspensions applied to the upper surface. The cells usually adhered successfully but, instead of becoming flattened out, as happened with the collagen coated coverslips, the cells formed clusters and networks (Fig. 7a), with groups of more plump and rounded cells linked together by elongated cells (Fig. 7b).

To reveal how these clusters and networks formed when the cells were seeded onto gels, time lapse imaging was also performed (Fig. 7c). This showed that the cells, once settled, were initially round. They then began to clump together within 20 mins, being relatively mobile, whilst extending processes that communicated with each other until by 30 mins they appeared like networks on the surface of the gel.

Immunocytochemical experiments showed that most of the cells expressed the same markers as found in the 2D cultures (Fig. 7d, e). Most cells co-expressed Na,K,ATPase, S-100 and aquaporin1. However, a few small cells had low S-100 labelling whilst still strongly expressing aquaporin 1 (Fig. 7e). We did not label for caldesmon as it is similar to aquaporin 1 as a marker, reflecting primarily a Type III phenotype in adults (Mahendrasingam et al. 2011b).
Morphological and ultrastructural analysis was also performed on cells grown on the collagen I hydrogels for 24 h or 48 h by SEM (Fig. 8) and TEM (Fig. 9). SEM performed on 48 h cultures confirmed that most cells were typically plump rather than flattened, unlike 2D culture cells, and they formed clumps inter connected by processes (Fig. 8a, b), as observed over the first 30 – 40 mins in our video analysis; unconnected individual cells could also be observed. Cells also had regions of multiple processes and folds and some had many fine surface protrusions, whereas in others the surfaces appeared smooth (Fig. 8b). Cells also showed numerous pores in their surfaces. The cells showing extension across the gels (Fig. 8c) could project over the top of other cells (Fig. 8d), consistent with the motility observed in the video analysis and the clumping/network forming activity. Processes appeared also to be burying themselves into the collagen matrix (Fig. 8e, f). Over longer periods (up to 13 d), although survival was poor, the cells became increasingly integrated into the collagen substrate (Fig. 8g, h). (The images shown are from the 13 d cell-survival experiment described in the section below and were the only samples included that were prepared using the hexamethyldisilazane method.)

When clumps of cells were examined by TEM, it could be seen that the cell membranes formed close associations with each other. In comparison with 2D culture cells, nuclear morphology was more varied, nuclei having irregular outlines, as is found in native type III cells especially. The cells showed overlapping processes (Fig. 9a). Fine cell processes and folds could be found in greater quantities but in small clumps (Fig. 9b), as observed using SEM. Adherens junctions and areas of close membrane contact were also detected as with 2D cultures (Fig. 9c). Mitochondrial and RER morphology was similar to 2D grown culture cells, with sometimes dilated RER cisternae, though to a lesser extent (Fig. 9d, e) than that observed in some 2D culture cells. Mitochondria generally appeared to be in good condition. However, there was greater evidence of cell damage and disturbance noted, on 3D compared with 2D cultures, consistent with poorer survival of cells on gels. Some evidence of
apoptotic bodies was noted in the clumps of cells, implying cell death was occurring; necrotic cells, however, were not detected.

3.4 Growth of cells on type I collagen gels

Preliminary experiments suggested that the longevity of cells on these 3D substrates was poor. Over several experiments, it appeared that cells death occurred within the first few days after seeding with the majority surviving between 2 – 4 days. They appeared in several categories according to morphology: clumps of cells (where individual cells could not be counted), individual rounded cells, individual elongated cells with processes and cells that were difficult to classify. After 13 days, a period normally sufficient to generate a large quantity of confluent cells in wells or on coverslips, only a small number of residual cells could be found (Fig. 10a). We did not count clumps of cells in these preliminary experiments. The preliminary experiments suggested that the main cell death occurred before five days, with very few remaining after longer periods.

To examine cell survival on hydrogels more closely we repeated the cell growth study over five days, by analyzing four replicates and generating 10 images by random selection at approximately the same time each day in the same area. We classified cells according to morphology: clumps of cells (where individual cells could not be counted), individual rounded cells, individual elongated cells with processes and cells that were difficult to classify. It was evident that good survival in round cells and clumps occurred only over the first 24 h, with reduction in both resulting in a loss of cells to be about half the initial cell number in total over the remaining five days (Fig. 10b). The elongated and unclassified cells did not show dramatic changes over the period, but were themselves only a small proportion of all cells present. The loss of clumps may correspond with the presence of apoptotic
cells observed by TEM. We did not test whether any proliferation was taking place in these cultures because of the low survival rate.

3.5 Varying the substrate for enhanced growth of 3D cultures

As the previous experiments showed that cell survival on collagen I hydrogels was low, we attempted to grow cells on different types of 3D collagen matrix. However, it was difficult to form complete gels of type II collagen alone consistently, so we chose to make mixed collagen I and collagen II hydrogels. We compared the growth of the cells on two different types of gel (in mg/mL): collagen I only (3+0), collagen I + II (2+2).

Because of the difficulties in polymerizing collagen II alone, we first confirmed that the collagen II was present and distributed evenly throughout these gels. Therefore, some 1:1 mixed gels were prepared for SEM and immunogold labelling was performed (Fig. 11a). Preliminary SEM had shown collagen I to be present as typical large fibers, so labelling was performed for collagen II only: gold particle labelling for collagen II was seen to be distributed within the gels, implying that they had formed a properly mixed gel.

We then seeded cells onto the collagen mixed gels as before. Cell morphology was similar initially to that observed for collagen I alone (data not shown). We then evaluated cell survival rate. In two experiments each with four replicates, survival appeared to be improved by addition of collagen II to collagen I. Starting with a cell density that was not significantly different on day 1, at days 2, 3, 4 and 5 there was significantly better survival on mixed gels versus collagen I alone, however, the cell density in all cases still decreased over a period of 4 d (Fig. 11b).
3.6 Incorporation of cells inside gels

We also evaluated incorporation of cells inside gels. To achieve this, we used two methods: the first was to pre-mix the cells and draw them up into either a 200 \( \mu \text{L} \) pipette tip or into a 120 \( \mu \text{m} \) external diameter/94 \( \mu \text{m} \) internal diameter capillary attached to tubing and drawn up with a 1 mL syringe.

The second method used was to draw up a 100 \( \mu \text{L} \) volume of gel mixture (4 mg/mL collagen I) followed by 50 \( \mu \text{L} \) volume of cell suspension (5000 – 10000 cells per mL) into a 200 \( \mu \text{L} \) pipette tip using a 1 mL syringe. Cells were allowed to survive for 24 h after being incorporated by these methods.

In all the methods of mixing the cells and gels, there was successful incorporation. In the case where cells were pre-mixed before take up, gels stained by phalloidin showed cells spread relatively evenly within the gel (Fig. 12a). In the second (drawing two separate solutions: gels and cells, into a pipette or capillary), the gels tended to have hollow centers with the cells often lining the hollow, more rarely spread within the gel (Fig. 12b). The gels could be readily expelled from the pipette tip or capillary, suggesting the possibility of using these simple devices as a delivery method into the target.

Cell morphology could be visualized by LM in the gels prepared for electron microscopy by virtue of the dense colouration produced by osmium fixation. In both cases, cells exhibited multiple, 3D morphologies, from round to elongate and with variable numbers of processes extending from them in three dimensions, which were especially clear in the osmium fixed samples (Fig. 12b, c).

The cells in gels fixed with glutaraldehyde and osmium were amenable to ultrastructural analysis by TEM but not SEM (because the cells were internalized). By TEM, cells could be seen integrating into
the gels and interacting with the collagen fibers. They contained typical cell organelles in relatively large numbers, and often the surface was irregular (Fig. 12c-e). Nuclei tended to be irregular and had invaginations, unlike 2D cultured fibrocytes, and the cytoplasm contained unusual round vacuolar structures, dense bodies, more extensive endoplasmic reticulum cisternae and surface blebs (Fig. 12d, e, f) than fibrocytes in mouse tissue. They also contained lysosome-like vacuoles (Fig. 12f). Folds in the surface took various forms, including long slender folds reminiscent of type II, III and V fibrocytes (Fig. 12g). Some cells in the gels appeared to be undergoing necrosis (Fig. 12e), unlike the gels where cells were seeded onto them. This suggests either poorer conditions for survival or trapping of cell debris inside the gel. The cells in the gels were not readily amenable to counting because the depth of the gel and the center hollow, together with folds in the gels, made random sampling of known volumes difficult to achieve.

3.7 Magnetic nanoparticle uptake

For transfection of cells pre-transplantation or identification and potential manipulation of cells within cochleae post-transplantation, uptake of magnetic nanoparticles (MNPs) was evaluated. MNPs can be detected by light or electron microscopy and functionalized with gene constructs and have been used in other culture systems (see Pickard et al. 2011).

To determine whether fibrocytes in vitro would also be amenable to this kind of approach, we incubated 2D cultures with media containing magnetic nanoparticles for 24 h – 48 h. Over the longer time period, uptake appeared to be close to 100% as cells that did not contain them were not detected (Fig. 13a). Cells that were harvested after MNP uptake were also then incorporated into gels using the pipette technique described above. The cells retained the MNPs within the gels, thus confirming their potential use for marking or modifying cells in constructs (Fig. 13b).
4. Discussion

The aim of the present study was to determine how cochlear fibrocyte cultures form and whether they are subject to modulation by substrate specific cues, especially in 3D. This was done because there has been a growing awareness that 2D cultures often do not generate good morphological or functional models of cells in their native tissues, ascribed to the lack of environmental cues such as 3D organization and substrate composition (see review by Knight and Przyborski 2015). Thus data obtained using 2D cultures in other systems have often been called into question, for example in drug treatments for cancer cells (Aggarwal et al., 2009).

The use of fibrocyte cultures is uncommon, but potential of such cultures as an approach to study fibrocytes is considerable (Furness 2019). We compared the characteristics of cultured fibrocytes using morphological, and immunocytochemical techniques to determine what effect different substrates have and specifically whether (i) the phenotype of cells grown on 3D substrates resembles more closely native cochlear fibrocytes making them more useful for study and as transplantable cells; and (ii) specifically that collagen II containing substrates and gels would recapitulate more closely the native environment and promote better growth.

4.1 Formation of the cultures

Our data suggest that cells primarily exit from the type III fibrocyte region, whilst deeper cells probably degenerate in situ. Thus, amongst others, we labelled for aquaporin 1 since it is known to be exclusive to type III cells in the ligament (Mahendrasingam et al., 2011b). However we found that although the majority of cells did express aquaporin 1, they also expressed other markers not characteristic of type III cells such as NaKATPase and S-100. Unequivocal labeling for a macrophage
marker was not detected in the cultures. As discussed below, these findings suggest a non-specific fibrocyte phenotype within the cultures.

4.2 The effect of 2D substrate type

Unlike previous reports, the cells grown on the 2D coverslip surfaces were commonly of mixed morphology: large and flat cells were found with patches of smaller, spindle-shaped cells more often reported in other studies. The two different morphologies showed similar mixed marker protein expression although larger cells had more obvious actin, presumably to support the larger cell bodies. The variations in shape likely depend on the state of confluence and age of the cultures, as well as physical factors such as stiffness and substrate composition (Xu et al., 2020). Indeed, the cells were similar on Aclar sheets and on the different types of collagen coated coverslips.

In previous studies, NaKATPase negative type I cells, (Gratton et al., 1996; Suko et al. 2000) or type IV cells (Qu et al., 2006) were present. The differences between our studies and these may reflect differences in culture conditions, although we followed the same methods, or more recent antibodies/marker combinations. This begs the question, therefore, whether the immunophenotype is sufficient to characterize fibrocyte cultures. Alternative approaches might be to assess expression patterns with qPCR and western blotting (Ng et al., 2017).

Ultrastructurally, the cells in 2D culture appeared flattened with a central cytoplasmic bulge, comparable between all three types of microscopy used. Cultures of other cell types often show this (possibly default) morphology, for example HCC1954 cancer cells (Breslin and O’Driscoll, 2016). The fibrocyte culture cells also formed adherens junctions and close contact points, and had fine folds extending from their surfaces. Mitochondria were normal, which is of interest because in the 3-4
weeks CD/1 mice from which these cultures were derived, mitochondrial damage is apparent in the fibrocytes (Mahendrasingam et al., 2011a). The RER had distended cisternae, similar to those seen in P14 fibrocytes and in other developing cells (Bielek, 2005). These data suggest potentially a mixed or immature phenotype undergoing a high level of protein synthesis, as might be expected in a growth phase.

The ECM of the spiral ligament contains collagen II in abundance (Slepecky et al. 1992; Buckiova et al 2006). We therefore explored collagen substrates other than type I, the substrate of choice in most previous studies, in an attempt to modify the phenotype of the cells. Using collagen II coating on the coverslips made a small but significant difference to the size of the larger population, although not detectably to the smaller one. Thus, in terms of hypothesis 2, we did find that collagen II coating altered the phenotype to a degree, by reducing the size of the larger cells. This might suggest an effect on how the cells grow or spread on this surface.

4.3 The effect of 3D substrate type

Cells grown on 3D hydrogels made of collagen I, compared with all collagen coated coverslips, have plump cell bodies that extended slender processes, which is a more fibrocyte-like morphology. In other respects, when comparing the cells grown on Aclar sheets with those in hydrogels, features were similar with distended RER, normal mitochondria and very fine processes. The fine processes, or folds, are characteristic especially of type II and type V fibrocytes (Furness et al., 2009). Marker labelling showed no substantive differences compared with 2D cultures. The morphological but not immunocytochemical data thus suggest that hypothesis 1 is partly correct and that it may be possible to direct fibrocyte morphology to a more native state in the cultures by manipulating the topography of the substrate.
On these gels, the cells appeared to contact each other, rapidly forming networks held together by intercellular adherens junctions, potentially with the ability to replicate gap junction networks seen in the native spiral ligament (see Jagger and Forge, 2006). These networks involved clumping of cells within which some cell death occurred. We observed apoptosis here, but not necrosis, probably because the debris of the latter was washed away during processing. The speed with which this occurred would suggest that the morphological changes were driven by contact with the different substrate rather than other changes such as cell death. The extensions from the cells may form towards other cells either randomly, or there may be trophic factors released from some cells that attract them. Over longer periods, the cells became integrated into the gel surface where extracellular matrix fibers were present often over the processes and, less frequently, over the entire cell. It is most probable that the cells buried themselves (processes first) as they grew into the gels, although an alternative is that the fibers seen covering them were secreted by the cells.

Overall, the results suggest that: (i) cells grown on hydrogels provide potentially more fibrocyte-like cells and an alternative way to investigate fibrocyte characteristics e.g. to test the role of fibrocytes in potassium handling, or for genetic manipulation and modification; and (ii) that it may be possible to replicate native fibrocyte networks in a three-dimensional culture. They may also be more effective for testing potential therapeutic agents for use to stimulate endogenous cell repair and regrowth. The fact that these cells readily take up MNPs (at least in 2D cultures) and retain them when cultured into 3D provides a means of transfecting them and thereby altering their gene expression.
4.4 The development of potentially transplantable matrices of fibrocyte cultures – proof of concept.

Although speculative, the future goals of these experiments are to evaluate how fibrocyte culture cells might re-establish themselves either in vivo into the ligament, or to develop a biomimetic, transplantable cell-laden construct that could be attached to it. With regards to the former, the cellular integration into the hydrogel bodes well for the possibility of recapitulating the original lateral wall either within a matrix, or by injecting cells into an existing region of extracellular matrix where they may reintegrate. Indeed studies of the degeneration of the spiral ligament in CD/1 mice have shown that as cells in the ligament die, they leave behind a cavity into which replacement cells might migrate within the extracellular matrix (Mahendrasingam et al., 2011a). These cavities were also observed here, as the cells exited the ligaments. In either case, these possibilities may help in preventing the progression of metabolic and subsequent sensorineural hearing loss that is seen in some animal models of age-related hearing loss (Shone et al., 1991; Hequembourg and Liberman 2001; Mahendrasingam et al., 2011a).

The only other study we are aware of where fibrocytes have been cultured in a collagen matrix is that of Kelly et al (2012) where the authors reported they had primarily type III fibrocytes with contractile properties. However, they derived their cells from a more restricted region of the ligament than has been done here, and furthermore the cells were mixed first with the unpolymerised collagen rather than seeded directly onto polymerized collagen.

The poor longer term survival of the surface gel cultures on collagen I gel suggests gel composition was sub-optimal. The composition of matrices can be critical in terms of supporting specific mesenchymal cell types compared with others (Hayrapetyan et al., 2016). Because (a) collagen II
coated coverslips appeared to influence the cell growth, at least in terms of size, and (b) collagen II is the dominant component of the spiral ligament ECM, we compared collagen type I and type II containing gels, the latter mixed with collagen I. The results here suggest that cells survive better with collagen II present, but not very substantially, perhaps by 10%, suggesting hypothesis two is correct to some degree. It has also been shown for adipose mesenchymal stem cells that making hydrogels of type I and type II collagen mixtures can enhance cell growth and the expression of phenotypic markers (Tao et al., 2016). Our data are consistent with these studies insofar as better survival was noted in the presence of collagen II but other combinations of matrix proteins may be needed to improve survival further.

We attempted another strategy by mixing cells within collagen I the matrices, as was done by Kelly et al. (2012). We used two different methods to mix cells either before incorporation into a small gel space or after, by mixing through a syringe or capillary which could potentially be used to inject the matrix. Both produced viable cells, with morphologies ranging from rounded to highly fibrocytic with multiple processes, but there was evidence of apoptosis and necrosis occurring, that suggested viability was also a problem in these gels.

The use of hydrogels offers several possibilities in terms of both research and clinical approaches. They could be used in co-culturing, for example with other tissues of the cochlea, to evaluate the protective capacity of fibrocytes. There is a possibility that gels could be transplanted into the cochlea using the simple devices here (i.e. via a cochlear implant surgical approach) to replace or add to dwindling fibrocyte populations in the native ligament, as suggested by Wagner et al (2014). Appropriately shaped and sized replacement ligaments could be produced in vitro for example through molding or bio-printing a hydrogel containing cells (Lee and Yeong, 2016).
4.5 Conclusions

In summary, we are able to generate cells of distinct morphology on collagen based hydrogels as opposed to flat surfaces, the former appearing to be more like native fibrocytes. In both cases, however, the type of fibrocyte generated was indistinct – markers gave no clear characterization – and appeared as a mixed or immature phenotype. Although, survival was poor on hydrogels, it was better when type II collagen was included. Further optimization of these gels may therefore enable them to become useful for studying fibrocytes and as biomimetic transplants.

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Figure 1. The initial development of the cultures from ligaments. a, b). LM after 48 h following placement of ligaments in wells and covering with coverslips. Cells are exiting both singly and in chains (*) from the margins of the ligament pieces along the side normally populated by type III cells. c, d) Cells subsequently formed a patch around the ligament remnant that expanded over 4 – 7 days; arrows indicate the ligament remnant. Inset shows the morphology of cells at 4 d at higher magnification. e, f) Ligaments seeded onto Aclar sheets, embedded for semi-thin and ultrathin sections for LM and TEM confirmed there were gaps in the type III area at 4 days (e, *) whilst by day 7, most of the fibrocytes were missing from the ligament, leaving behind root cells (f; large arrows) and blood vessels. Below the ligament at 7 days, cells can be seen in transit, with extremely thin processes extending down from the tissue (f, small arrow); arrowhead in ‘f’ indicates the substrate (Aclar sheet) with flattened cells on it. g – j) TEM showing degeneration of fibrocytes (arrows) within several regions of the ligament at 4 days, in places leaving behind holes but intact extracellular matrix (ecm) where they were once located. k). Exiting cells (*) appear to grow over each other (left inset, arrow), as suggested by the chains in (a) and (b) and to be coupled together by adherens junctions (k, right inset, arrowhead). Scale bars: a = 750 μm; b = 400 μm; c, d = 1 mm (inset = 100 μm); e, f = 50 μm; g – j =20 μm; k = 20 μm (inset = 500 nm).

Figure 2. 2D cell culture of cochlear fibrocytes on collagen I coated coverslips. a) Phase contrast LM of a confluent region of culture cells. The cells are mostly spindle shaped, although some are round (*) and possess a central bulge representing the nucleus and small area of cytoplasm (arrow). b) Non-confluent region of cells where they are flattened and extend processes along the surrounding surfaces. The central bulge is also evident (arrow). c) Immunocytochemical labelling for three typical fibrocyte markers, NaKATPase (NaK), S.100 and caldesmon (Cal). All three markers are expressed at varying levels in most cells, but some show particularly strong labelling for all three and appear
white in the merged image. d) Cells of varying sizes, but generally appearing to form groups of small and large cells, both expressing S-100, especially in the central bulge (*). Large cells also have significant actin content, in places concentrated in a peripheral ring especially in the large cells (arrow) e) Co-labelling for caldesmon (Cal) and aquaporin 1 (Aqp) shows the presence of both proteins in all cells, but with especially strong labelling for the latter in some (*). The caldesmon parallels actin in forming peripheral rings (arrow). Scale bars = 100 μm.

Figure 3. a) The distribution of caldesmon in 2D culture cells resembles that of actin, forming peripheral rings and stripes or cables through the cytoplasm, (arrows). b) higher magnification showing the parallel stripes suggesting an association with actin cables. c) SEM of very thin 2D culture cell. d) higher magnification showing cable like structures in the cytoplasm (c) similar to the actin and caldesmon cables. Scale bars: a = 100 μm; b = 50 μm; c = 60 μm; d = 6 μm.

Figure 4. a) – d) Images of cultures grown on four different collagen substrates and labelled for S-100 (red) and tubulin (green): a) collagen I, b) collagen II, c) collagen V and d) collagen IX. Two populations of cells are visible on each coverslip and no differences can be distinguished qualitatively. e) Measurements of mean cell area in arbitrary units from 5 randomly selected areas in each population. No differences can be seen in the small cell populations between collagens, but the large cell populations are smaller on collagen II than the others, the difference being significant (p < 0.05; Wilcoxon Signed Rank Test) between collagen II and collagen I, and collagen II and collagen IX. Scale bar = 100 μm.
Figure 5. SEM of the 2D cultures grown on collagen I coated coverslips showing that the fibrocytes have a monolayer appearance. a) Confluent region of cells, showing the flattened cell morphology with a central bulge (*). Round cells lie on top of the flattened cells (e.g. arrows). b) Region at the edge of confluency where cells appear to be migrating. They have a central bulge (*) and flattened processes apparently stretching outwards (arrowhead). c) Detail at the edge of a more rounded cell showing fine filopodia (arrows) and finer threadlike processes (arrowhead). d) Pair of adjacent round cells (*) that may be offspring of a dividing cell. e) Cell apparently undergoing cell division. Scale bars a = 100 μm; b = 50 μm; c = 6 μm; d = 20 μm; e = 10 μm.

Figure 6. TEM of the 2D cultures grown on collagen I coated Aclar sheets. a) Micrograph of the central region of a small, rounded cell, showing it contains the nucleus (nu) and the typical cytoplasmic components such as mitochondria (arrowheads). An adherens junction (arrow) can be seen where two cells lie close to each other (inset). b) The region of a the central bulge of a larger rounded cell compared with that shown in (a) illustrating similar features, but with a fine process extending from it (large arrow) and tiny processes (arrowheads); RER (small arrows). c) A large flattened cell showing the very thin cytoplasmic areas around the central bulge containing an ovoid nucleus. d) Rounded cell showing some extensive thin processes (arrow) and mitochondria in apparently good condition (arrowhead). e) Central bulge of a cell showing extended and dilated RER (arrows). f) Cells from a P14 cochlea spiral ligament showing type III (darker cell) and type I (lighter cell) both containing dilated RER (arrows). Scale bars = 2 μm.

Figure 7. Phase contrast and immunofluorescence microscopy of 3D cultures on collagen I hydrogels. a) When seeded on top of the gel, after 2 d the cells have formed into groups, clumps and networks across the gel. b) At higher magnification clumps of cells can be seen to be connected across the gel by the elongated processes of individual cells. c) Frames taken from a time-lapse video of the cells
starting immediately after the seeding. Cells are initially round, but over time develop extensions projecting along the gel and move together to form clumps. Some of the processes contact other cells and clumps, so that a network is established between 30–40 mins after seeding. d) and e) immunocytochemical labelling for aquaporin 1 (Aqp1) and NaKATPase, and for Aqp1 and S-100 show that most cells express the same markers as in 2D cultures. Occasionally, cells expressing Aqp1 but only weak S-100 can be found (arrows). Scale bars = 100 μm.

Figure 8. SEM of cultures grown on the 3D collagen I hydrogel at 48 h. a) Micrograph showing the plumper morphology of the cells (cf Fig. 5a, arrow) and the network formed between them. The cells show both plumper/rounder (r) and elongate (e) morphology, and processes extending across the fibrous surface of the gel. b) Example of a clump of cells. The clumps appear quite tightly bound together. Cells in the clumps can have a smooth surface (s) or may be more decorated with fine surface processes (fsp). c) In several places the processes of the cells interact in various ways by lateral contact (e.g. arrow). d) Cell processes also cross over other cells (arrow). Many cells also show surface pores (inset, box enlarged in main picture). e) The processes can show expanded ends interacting with the collagen (arrow). f) Enlargement of the expanded process in e. Its appearance suggests partial penetration into the gel. g) a rounded cell with a long process, showing partial penetration into the gel. h) After 13 days in vitro, some cells are significantly integrated into the gel, with fibers covering them. Scale bars: a = 50 μm; b = 20 μm; c = 30 μm; d = 10 μm (inset = 1 μm); e = 20 μm; f = 5 μm; g, h = 10 μm.

Figure 9. TEM of the cells seeded onto hydrogels (48 h after seeding). a) A clump of cells in which at least three cells can be defined by the presence of their nuclei (1–3). The tightly packed cells have irregular nuclei and the cytoplasm of some cells contains dense bodies that have the appearance of apoptotic bodies (ap). Processes from two cells (P(a) and P(b)) exiting the clump overlap each other.
closely. The fibers of the gel can be seen underneath the clump. b) Detail of cells with fine processes (P(f)) that resemble those of native type II and type V fibrocytes. c) Detail of two cells contacting each other via adherens junctions (a) and close contact points (arrow). d) Detail of the cytoplasm showing normal mitochondria, lipid-like droplets and cisternae of RER, the latter distended (*) in places. e) Developing type III fibrocyte from a P14 CD/1 mouse cochlea. The RER in the cytoplasm shows similarly distended cisternae (*) to that shown in (d). Scale bars: a = 5 μm; b = 2 μm; c – e = 1 μm.

Figure 10. Histograms showing the survival of different morphological subtypes of culture cells on the collagen I hydrogels. ‘Round’ cells have no processes; ‘process’ cells have elongated projections from the cell body; ‘unclassified’ had indeterminate shapes. a) Example of a preliminary experiment over 13 days revealed a relatively rapid fall off in cell numbers of different morphological subtypes. (This sample is also included because SEM images from it shown in Fig. 8). b) Detailed analysis of four replicates showing cell survival over a period of 5 days. The numbers of clumps has been included. Clumps and rounded cells both decline whereas process cells do not show much change. Overall, the total number of cells declines by close to 50%.

Figure 11. a) Backscattered SEM of a collagen I +II mixed hydrogel labelled for collagen II. Large fibers representing collagen I and gold label (visible as white dots – arrow shows an example) were distributed throughout the gel showing incorporation of both collagens. b) Postembedding immunogold labelling of a type I + II collagen gel labelled for collagen II. This shows that the collagen is II is evenly spread and as the labelling is on the section surface it is unlikely simply to be trapped within the collagen gel. A large striped fiber of collagen I is also visible. c. Histogram showing the effect of collagen II incorporation into the hydrogels on cell survival. The cell numbers on collagen I and collagen I+II gels fall over the first 2 d, but the fall is smaller for collagen I+II gels. Thereafter,
collagen II containing gels show significantly higher survival than collagen I gels alone. Significant differences (p < 0.05, n = 4; Wilcoxon Sign Rank Test) in cell density were detected on each subsequent day.

Figure 12. Light and electron microscopic assessment of cells incorporated into gels by mixing. a) Gel formed with premixed cells and extruded from a pipette tip, fixed and labelled for actin. Cells can be seen throughout the gel and to be variable in morphology. b) Gel extruded from a pipette tip after cells were taken up after the gel solution, fixed and embedded in resin for electron microscopy. The gel tends to be hollow (h) and cells dispersed primarily along the edges of the hollow. They form rounded, elongated and fibrous morphologies, with the position of several cell processes. c) Example of cell morphology in the extruded gel include round cells and cells with multiple processes (arrow). d) TEM of a cell in the gel shown in b. The cell has an irregular nucleus, and the cytoplasm contains dense potentially apoptotic bodies (ap), and uncharacterized clear vacuoles (v), amongst the normal organelles. e) Example of another rounded cell with multiple bleb like processes (arrows), and cells that have lost their integrity and appear to be undergoing necrosis (nec). f) Cytoplasm in these cells appeared to have normal organelles such as mitochondria (m) and RER, but in addition there were lysosome like vacuoles (arrow points to the circular membrane) and dense bodies (db). g) Some cells were decorated with fine processes (fsp). nu = nucleus. Scale bars: a = 200 μm; b = 100 μm; c = 50 μm; d = 5 μm; e = 10 μm; f, g = 1 μm.

Figure 13. Magnetic nanoparticle uptake in 2D and harvested cells in 3D cultures. a) Virtually 100% of the cells in the 2D monolayer cultures contained MNP after 24 or 48 h (shown) incubation. The particles (e.g. indicated by *, orange against the green S-100 labelling of the cells) tended to clump around the nuclei (nu). The cut plane (below) represents an orthogonal slice through the stack of confocal images and shows that MNPs are definitely cytoplasmic and not lying on the surface. b)
after pre-incubation with MNPs in 2D culture, followed by harvesting and mixing into gels using the pipette method, MNPs were still clearly observed within the cells in the gels (orange particles denoted), showing robust uptake. Scale bars = 50 μm.
Highlights

- Cochlear fibrocytes grow effectively in 2D culture on four different collagen substrates.
- 3D hydrogel cultures show lower survival on collagen I hydrogels compared to 2D cultures, but was improved by the addition of type II collagen.
- 3D cultures generate cells of distinct morphology appearing to be more like native fibrocytes.
- In both 2D and 3D culture, marker labelling revealed fibrocytes of mixed, potentially immature phenotype.
- Further optimization of these gels may enable them to become useful for studying fibrocytes and as biomimetic transplants.
Figure 10

(a) Bar graph showing the number of cells per field of view (FOV) over different days in culture. The y-axis represents the number of cells/FOV, and the x-axis represents the days in culture.

(b) Bar graph showing the number of cells per FOV over different days in culture. The y-axis represents the number of cells/FOV, and the x-axis represents the days in culture.
Figure 11
The authors of this manuscript declare that they have no conflict of interest.

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The effects of substrate composition and topography on the characteristics and growth of cell cultures of cochlear fibrocytes.

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Abstract

Spiral ligament fibrocytes of the cochlea play homeostatic roles in hearing and their degeneration contributes to hearing loss. Culturing fibrocytes in vitro provides a way to evaluate their functional characteristics and study possible therapies for hearing loss. We investigated whether in vivo characteristics of fibrocytes could be recapitulated in vitro by modifying the culture substrates and carried out proof of concept studies for potential transplantation of culture cells into the inner ear. Fibrocytes cultured from 4-5-week old CD/1 mice were grown on 2D substrates coated with collagen I, II, V or IX and, after harvesting, onto or into 3D substrates (hydrogels) of collagen I alone or mixed collagen I and II at a 1:1 ratio. We also assessed magnetic nanoparticle (MNP) uptake. Cell counts, immunohistochemical and ultrastructural studies showed that fibrocytes grown on 2D substrates proliferated, formed both small spindle-shaped and large flat cells that avidly took up MNPs. Of the different collagen coatings, only collagen II had an effect, causing a reduced size of the larger cells. On hydrogels, the cells were plump/rounded with extended processes, resembling native cells. They formed networks over the surface and became incorporated into the gel. In all culture formats, the majority co-expressed caldesmon, aquaporin 1, S-100 and sodium potassium ATPase, indicating a mixed or uncharacterised phenotype. Time-course experiments showed a decrease to ~50% of the starting population by 4d after seeding on collagen I hydrogels, but better survival (~60%) was found on collagen I + II gels, whilst TEM revealed the presence of apoptotic cells. Cells grown within gels additionally showed necrosis. These results demonstrate that fibrocytes grown in 3D recapitulate in vivo morphology of native fibrocytes, but have poorer survival, compared with 2D. Therefore hydrogel cultures could be used to study fibrocyte function and might also offer avenues for cell-replacement therapies, but need more optimization for therapeutic use. Fibrocyte function could be modified using MNPs in combination, for example, with gene transfection.
Keywords: fibrocyte replacement therapy; hydrogels; metabolic hearing loss; 2D vs 3D cell cultures
1. Introduction

Cochlear fibrocytes are primarily found in the spiral ligament where they play a critical role in cochlear homeostasis and, together with the *stria vascularis*, in generating the endocochlear potential (EP) (see reviews by Wangemann, 2006, Furness, 2019). Loss of the EP reduces the ‘driving force’ that makes the transduction mechanism of the sensory hair cells in the organ of Corti highly sensitive to stimulation. Deterioration of EP and subsequent lateral wall degeneration may reflect specific patterns of hearing impairment, such as those associated with metabolic hearing loss (Lang et al., 2010; Dubno et al., 2013; Saremi and Stenfelt, 2013).

There are five main types of fibrocyte, Types I – V which differ in location in the spiral ligament and in morphology (Spicer and Schulte, 1996; Furness et al 2009). They are connected together by gap junctions in a syncytial network and are thought to perform roles in ion transfer (in generating the EP), water balance, inflammation and basilar membrane tensioning (see review by Furness, 2019). They are located within a matrix comprised of collagens, the two main types being type II collagen (Slepecky et al., 1992; Buckiova et al., 2006) and type IX collagen (Usami et al, 2008). Fibrocyte development and differentiation appears to involve Wnt signalling (Bohnenpoll et al., 2014) and transcription factors SOX9 (Trowe et al., 2010), Tbox18 (Trowe et al., 2008), both regulators of Wnt signalling, and Brn4/ Pou3f4 (Phippard et al., 1999) a member of the POU family of proteins. Fibrocyte degeneration has been reported to underlie deafness in human DFN3, caused by mutations in Pou3f4 (Minowa et al., 1999) and in some strains of mice (Hequembourg and Liberman 2001; Mahendrasingam et al., 2011a). It is possible that fibrocyte degeneration leads to not only a loss of EP but also that breakdown of potassium recycling might cause hair cell death and thereby permanent hearing loss. At present, however, the precise pathology underlying the
metabolic deafness patterns, and whether they can be prevented or alleviated by therapeutic methods, is relatively unexplored (Furness, 2019).

As an approach to studying fibrocytes, we have been developing fibrocyte culture techniques. These offer several possibilities including: evaluating the growth of these cells, establishing potential growth enhancing agents for therapeutic use, studying their physiology and toxicity, genetic modification and as a source of transplantable cells (Furness 2019). In previous studies, fibrocytes have been mostly cultured on flat surfaces coated in collagen I and in collagen I matrices (i.e. in two and three dimensions respectively), the former generating cells expressing markers for several types of fibrocytes (Gratton et al, 1996; Suko et al., 2000; Sun et al., 2012) and the latter for Type III (Kelly et al 2012). However, morphologically, the cells present in 2D culture do not resemble native fibrocytes closely, and may not offer an ideal method for obtaining cells with useful characteristics for understanding their function or as therapeutic agents, whilst cells cultured in collagen matrices (hydrogels) have been primarily investigated for their contractile properties (Kelly et al., 2012)

We hypothesise that: (i) the phenotype of cells grown on 3D substrates may resemble more closely native cochlear fibrocytes and they may thus be more useful for study and as transplantable cells; and (ii) specifically that collagen II containing substrates and gels would recapitulate more closely the native environment and promote better growth. Therefore, the aim of the present work was to evaluate the growth of fibrocytes, starting with their derivation from ligaments in vitro and subsequent growth on different collagen substrates and matrices. We have accordingly grown fibrocytes on collagen hydrogels and compared them with cells derived using 2D culturing on different collagen coated coverslips, by evaluating their morphology and ultrastructure, growth, and expression of a variety of fibrocyte markers. Cells
were also acquired from suspensions inside a collagen I matrix within a custom-made injection device as a proof-of-concept approach to determine how well they could integrate into gels and to evaluate a possible method of transferring the cell-laden matrices. Finally, we prepared samples of immature cochleae (CD/1, P14) for comparison of aspects of the ultrastructure of the culture cells.

2. Materials and Methods

2.1. Animals

CD/1 mice were used in this study. The mice were bred and maintained in the Biomedical Services Unit of the University of Keele according to the 1986 Animals (Scientific Procedures) Act under regulatory control of the UK Home Office and following project approval from the local ethical committee. All terminations were carried out under Schedule 1 of the act.

2.2. Preparation of primary fibrocyte cultures

Primary cultures were derived from spiral ligament according to the methods of Gratton et al (1996) with minor modifications (Suko et al., 2000). 6-well plates were first coated with collagen I by incubating each well in 2 mL of an aqueous 0.01% collagen I (Sigma-aldrich, UK) solution for 5 h, aspiration of the solution and drying overnight at 37°C in an incubator.

Male and female mice of 4 – 5 w old were anaesthetised using 0.1 mL sodium pentobarbitone (Vetalar, UK), decapitated and the bullae removed. Cochleae were excised and opened in ice-cold culture medium, MEM-α (Invitrogen; catalogue number: 22561) containing 1 x Antibiotic-Antimycotic (Sigma-aldrich, UK). Aseptically dissected 2-3 pieces of lateral wall
were placed strial side down in one drop of culture medium MEM-\(\alpha\) per well, supplemented with 10\% foetal calf serum (Invitrogen), 1 x Antibiotic-Antimycotic, and 1\% ITS-G supplement (Invitrogen) on collagen type I (Sigma; product number: C3867) coated 30 mm wells, cover-slipped and left overnight in the incubator (37\(^\circ\)C: 5\% CO\(_2\), 95\% air). One cochlea was sufficient for two wells. Once the ligaments were adherent, the coverslip was removed, culture medium was added and the segments were incubated for 2-3 weeks (culture medium changed every 3-4 days) forming primary cultures. When the primary cultures became near-confluent (2-3 weeks), the lateral wall segments were removed and the cells were rinsed with sterile phosphate buffered saline (PBS, pH 7.4) followed by trypsinization [0.025 \%-0.05 \% trypsin/ethylene diamine tetra acetic acid (EDTA)] and centrifugation (at 1200 rpm for 5 min). The pellet was then re-suspended and diluted in culture medium and reseeded for secondary culturing.

After reaching confluence, cells were collected for passaging onto collagen I coated T25 flasks (Corning, Sigma-Aldrich, Poole, Dorset UK). The cells were detached from the wells by incubation for up to 20 mins using 0.05\% trypsin solution in PBS, culture medium added and centrifugation performed to concentrate them. A small volume of concentrated cells was then seeded directly into the T25 flasks and culture medium with supplements was added. They were maintained as above for approximately two weeks until confluence was reached. Finally, cells were harvested by trypsinisation and either used for further experiments or frozen with 10\% dimethylsulfoxide (Sigma-aldrich, UK) in liquid nitrogen for storage.

2.3 Preparation of substrates and seeding of cells
Substrates took the form mainly of either collagen coated coverslips or collagen containing gels, although one set of 2D cultures was also grown on collagen I coated Aclar sheets (Agar Scientific, UK) to enable examination by transmission electron microscopy (TEM).

To test the effects of different substrate types on growth of the cultures on flat substrates, coverslips were incubated with 0.01% purified collagen I, II, V (Sigma-Aldrich, UK) or IX (Chondrex Inc, WA, USA) for up to 5 h and then allowed to dry overnight at 37°C.

From the 2D data and the known distributions of different collagens in the cochlea (Slepecky et al., 1992), we decided to focus on collagen I and II for the production of matrices in which the fibrocytes could be grown. Although collagen I is not expressed in the ligament, because it has been used as a substrate in all previous fibrocyte culture work, it was used as a control substrate. In addition, collagen II is difficult to polymerise into useable gels on its own, as others have also found, but can be mixed with collagen I (Vasquez-Portalati et al., 2016). The gel compositions made were: 3 mg/ml of collagen I alone and 2 mg mL each of collagen I + II. Stock collagen solutions were mixed to the appropriate final concentrations very gently on ice and polymerised by addition of 4N NaOH. 250 - 400 µl volumes were placed into wells and incubated at 37°C until they solidified.

Fibrocytes obtained either from fresh cultures or re-derived from frozen stocks were seeded on top of the collagen gels and either directly into wells or onto coverslips at a density of 5000 - 50000 (depending on cell harvesting) per cm², allowed to settle and grown for varying times. The culture medium was changed every two to three days.
To mix cell suspensions and incorporate them into a collagen I matrix within a custom made injection device, a simple device was constructed from a 1 mL syringe attached either to a disposable 200 µL pipette tip (pipette gels), with a 50 mm length and approximate tip aperture of 0.5 mm, or by attaching tubing to the pipette tip into the distal end of which was inserted a Clark glass capillary (Harvard Apparatus, Cambridge, UK) with diameter 0.94 mm and length 100 mm (tube gels). Cells were incorporated into these gels in two ways: (i) unpolymerised gel solution was taken up first into the pipette tip or capillary tube followed by cell suspensions in medium described above, in a ratio of 2:1 so that they mixed gently within the pipette tip or capillary tube; (ii) cells were premixed in equal volumes of double-concentration collagen and cell suspension and then taken up into the pipette tip or capillary tube.

2.4. Magnetic nanoparticle uptake

We assessed whether fibrocytes in culture could take up magnetic nanoparticles (MNPs), a useful vector for intracellular delivery of a variety of molecules, such as gene constructs. MNPs (SPHERO Fluorescent Carboxyl Magnetic Particles, Nile Red, 0.20-0.39 µm #FCM-02556-2) were added to the culture medium applied to established 2-D cell cultures for either 24 or 48 h at a concentration of 20 µg/mL. Cells were then immunolabelled with S-100 and examined in a confocal microscope. We also harvested the MNP labelled cells for incorporation into 3D gels (pre-mixed method), and fixed for immunocytochemistry (see below).

2.5 Analysis of cultures

To identify which cells were exiting the ligament to generate the cultures, and the mode of cell exit, attached ligaments were observed live by light microscopy (LM) or placed on Aclar
sheets (Agar Scientific, Stansted, UK) and then fixed at 4 and 7 days for LM and TEM examination (see electron microscopy section below for fixation method).

For evaluation of the effect of substrate on the growing fibrocytes, collagen coated coverslip cultures were fixed using 4% paraformaldehyde in 0.1 M sodium phosphate buffer (NaPi) for investigation of the expression of immunocytochemical markers, to verify the nature of cultures for comparison with previous studies. Once the coverslips had all reached confluence, cell size distribution was measured using ImageJ (Abramoff et al., 2004) on the immunocytochemically labelled coverslips from images of 5 randomly taken field of views. The data were assessed for normality using an Anderson-Darling test (FOV) and as they were non-parametric, statistically analysed using the Wilcoxon Sign Rank test (available online at https://www.socscistatistics.com/tests/signedranks/). A second experiment using the same method was performed to ensure reproducibility. These could not be pooled as the data were collected on separate days.

Analysis of cell survival on gels was performed using phase contrast microscopy. The number of cells on each gel was assessed by daily analysis of 10 FOV in four replicates using the same magnification and in the approximate middle of the gels (a dark mark was placed underneath the area for evaluation). Cell counts were thus given as mean number per FOV. The data were pooled across replicates and then evaluated for normality. As many of the population data sets were found not to be normally distributed, comparisons were made using the non-parametric Wilcoxon Sign Rank test between the samples of the pooled data.

Finally, for gels with cells prepared in pipette tips or capillary tubes, after 24h incubation these gels were extruded by gentle pressure on the syringe into fixative for
immunocytochemistry and electron microscopy evaluation of cell morphology as described below.

2.6 Immunocytochemistry

To determine the immunophenotype and where required the size and shape of cells grown in different culture conditions, we performed immunocytochemistry with known fibrocyte markers (see for example Mahendrasingam et al., 2011b). Coverslips and gels were fixed using 4% freshly dissolved paraformaldehyde in NaPi for 1 h. They were stored in 1/10th diluted fixative in the same buffer until used for labelling. Cell morphology was evaluated using anti-tubulin conjugated to FITC or phalloidin-FITC (Sigma-Aldrich, Poole, UK, both 1:100 dilution), a marker for actin. Previously used antibodies to Na,K,ATPase α1 (Abcam AB33133, 1:100), caldesmon (Acris antibodies GmbH, Germany S783, 1:100), aquaporin 1 (AB3272 Millipore-Chemicon, Temecula, USA, 1:100) and S-100 (S2644 Sigma-Aldrich, 1:100) were employed to distinguish potential fibrocyte type. All of the antibodies used here have previously been used for immunofluorescence and immunogold EM in the native cochlea, even from the same specific tubes (Mahendrasingam et al., 2011) and been shown to produce distinct quantitative distributions across the different fibrocyte types. We also performed negative controls which showed that background labelling caused by secondary antibodies, was virtually non-existent. Since there have also been reports of substantial number of resident macrophages in the SL (e.g., Wiu et al, 2018), we also assessed culture cells for macrophages using the same marker in that study, IBA1 (PA527436, Invitrogen, 1:100). Cultures were washed in phosphate buffered saline (PBS), permeabilised with 0.5% Triton-X-100 in PBS, blocked in 10% goat serum in PBS (GS-PBS) and incubated with phallodin-FITC or anti-tubulin-FITC and/or primary antibodies diluted in 1% GS-PBS. After washing, primary antibodies were visualised using appropriate secondary antibodies (Alexa-
fluor 488 and/or Alexa fluor 568; Molecular Probes/Thermofisher) by incubation at 1:50 dilution in GS-PBS for 1 – 2 h and then imaged using a BioRad MRC1024 microscope in confocal mode.

2.7 Substrate evaluation by immunoelectron microscopy

To confirm that the mixed gels (collagen I + II) used in this study did contain collagen II within them, because of difficulties polymerising this protein, some were fixed in 4% w/v paraformaldehyde in NaPi for immunocytochemistry at the electron microscopic level.

For scanning electron microscopy (SEM) immunogold, the fixed gels were labelled with anti-collagen II (ab53047, Abcam, Cambridge, UK) as follows: they were incubated sequentially in tris buffered saline (TBS) containing 10% v/v goat serum (TBS-GS), 1:100 anti-collagen II in TBS-1% GS, washed in TBS and then in 15 nm gold-conjugated goat anti rabbit secondary antibody diluted 1:20, washed in TBS and distilled H$_2$O. They were postfixed in 2.5% glutaraldehyde in sodium cacodylate buffer/2 mM CaCl$_2$ (SCB) and made electrically conductive using the OTOTO technique where sequential incubations in alternating solutions of 1% osmium tetroxide (OsO$_4$) (1 h) in SCB and saturated aq thiocarbohydrazide (20 min), repeated once and then with a final OsO$_4$ incubation, were used to impregnate the samples with osmium (for details see Furness and Hackney 1985). These samples were dehydrated and critical point dried for examination in a Hitachi S4500 field emission SEM at 15 kV using a Deben backscattered electron detector.

In order to reduce the chances that the labelling was due to trapping of antibodies and gold particles in the sticky gels, we also performed post-embedding immunogold labelling for collagen II in the mixed collagen gels for examination by TEM. Gels were dehydrated
through an ethanol series and placed in pure LR White for four changes. The resin was then polymerised in a gelatin capsule at 50°C overnight and 70 nm ultrathin sections were cut, placed on nickel grids and then labelled in sequential drops of solution placed on parafilm strips in a moist chamber as follows: blocked in TBS-GS (30 min), 1:100 anti-collagen II in TBS-1% GS (overnight at 4°C), washed in TBS and then in 15 nm gold-conjugated goat anti rabbit secondary antibody diluted 1:20, washed in TBS and distilled H2O. They were examined in a JEOL 1230 TEM at 100 kV and digital images were captured using a Megaview III digital camera system.

2.8 Culture evaluation by electron microscopy

For SEM evaluation of the cultures, the majority were fixed in 2.5% glutaraldehyde in SCB for 2 h. Some of these samples were then prepared using the OTOTO technique described above.

Some 13 d gel cultures that had previously been prepared and observed for immunocytochemistry were dehydrated through an ethanol series and then dried using the hexamethyldisilazane method (Bray et al., 1993). The cultures were taken from 100% ethanol to a 1:1 mix of ethanol: hexamethyldisilazane (1 h), then pure WA 98052 (1 h). The samples were left in a fume hood until the solvent had evaporated completely, mounted on specimen stubs using silver colloid paint and sputter coated with gold, before examination in the FESEM.

For sectioning for light microscopy and TEM, Aclar sheets or gels loaded with cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer/2 mM CaCl₂ (SCB) for 2 h followed by 1% OsO₄ in SCB for 1 h, washed, dehydrated and embedded in Spurr resin using
our conventional procedure (Furness and Hackney, 1985). Semithin sections for light microscopy were cut at 1-2 μm, stained with 0.1% toluidine blue and examined in a Lietz Dialux EB20 microscope. Images were captured using an Infinity camera supplied by DeltaPix. Ultrathin sections were cut onto 200 mesh thin bar copper grids, stained with 2% ethanolic uranyl acetate (20 min) and 2% lead citrate (5 – 7 min), allowed to dry and examined as described above in a JEOL 1230 TEM.

2.9 Preparation of immature CD/1 mice cochleae for ultrastructural comparison with culture cells

Some observations of the culture cells suggested potentially immature features may be present. For comparison with culture cells we therefore fixed postnatal day 14 (P14) cochleae by intralabyrinthine perfusion with glutaraldehyde and osmium fixative solutions as described, decalcified in 5 mM EDTA for 48 h, and embedded in Spurr so as to cut ultrathin sections containing spiral ligament which were stained and examined, all as above.

3. Results

3.1 Initial development of cultures

We used LM and TEM to examine the ligaments after placing the coverslips on them and then subsequently after coverslip removal, during establishment of the cultures. The cells appeared to crawl out from the ligaments over a period of up to 7 days, when the ligament remnants were usually removed. By LM, the first sign of cells were single long, thin individual cells or chains of cells appearing to exit the ligament along the edge where type III fibrocytes would normally be present (Fig 1a, b). Cells then appeared around the ligament in small patches that gradually enlarged (Fig. 1c, d). They exhibited variable morphology (Fig. 1c, inset) including triangular shaped bodies with thin processes. In semi-thin sectional views, the residual
ligament still contained cells at 4 d after ligament placement (Fig 1e), but these had mostly gone by 7 d leaving gaps, except for root cells which still appeared to be present (Fig. 1f). The exiting cells stretched down to the substrate (Fig 1e). By TEM the presence of degenerating cells and gaps in the ligament became apparent, leaving behind an extensive extracellular matrix network in different ligament locations (Fig. 1g – j). Exiting cells became extremely thin and grew over each other forming contacts at various places (Fig. 1k).

3.2 Characteristics of fibrocytes grown in 2D cultures on collagen coated coverslips

Experiments using type I collagen coatings on coverslips, as in previous studies, resulted in areas of confluent cells that expressed fibrocyte markers, using the same antibodies we had previously used (Mahendrasingam et al., 2011b) in the native cochlea (Fig. 2a - e). For example, the cells were positive for Na,K,ATPase, S-100, caldesmon (Fig. 2c-d) and aquaporin 1 (Fig. 2e) representing a mixed fibrocyte phenotype. We failed to detect any labelling for the macrophage marker, IBA1 (data not shown).

Cell morphology was varied but with areas of uniform morphology: One type could be categorized into small cells and a second type into substantially larger cells (Fig. 2d). In these areas cells were often elongated with a central bulge, presumed to be an area of cytoplasm containing the nucleus (Fig 2a, c-e). These central areas were rich in Na,K,ATPase, S-100 and aquaporin 1. In a few cells, caldesmon (Fig. 2c) and aquaporin 1 (Fig. 2e), both primarily a Type III fibrocyte marker (Mahendrasingam et al., 2011) were expressed strongly and filled the whole cell. With those exceptions, caldesmon was primarily localized to the cell edge. Actin, used as a marker for cell morphology, was concentrated at the center, confirming a cytoplasmic bulge there, and around the edge.
In non-confluent regions, cells were larger and appeared more spread out, with processes pointing in several directions (Fig. 2b). They were also positive for the same markers as above, and with similar distributions of these proteins with the exception of caldesmon and actin, both of which showed groups of parallel cables throughout the cell and around the nuclear area, which were confirmed as regular cytoskeletal cables in SEM images (Fig. 3).

When cells were grown in 2D on collagen I, II, V or IX substrates and labelled for S-100 and tubulin, no qualitative morphological differences were detected between substrates (Fig. 4). The two populations in the confluent areas noted above were present on all. These experiments also suggested that the smaller spindle shaped cells were Na,K,ATPase rich whilst the larger cell population were well demarcated from the former and also expressed S-100 (Figs. 2, 4). Measurements of cell area in a typical experiment with five random sample areas showed some significant differences between large cells on the different substrates, such that the areas differed significantly between Type I and Type II substrates (Wilcoxon Sign Rank test, $p = 0.015$, $n = 5$). This was replicated in two experiments. In one experiment, we also found a significant difference between large cell areas in Type II and Type IX (Wilcoxon Sign Rank Test, $p = 0.02$, $n = 5$). No other differences were detected between each of these two populations on the different substrates (Fig. 4).

Using SEM on 2D collagen I grown cultures confirmed that the cells were flat in the confluent patches (Fig. 5a) and splayed out in non-confluent areas, with fine cell processes extending along the substrate (Fig. 5b, c). In both areas there was a shallow central bulge likely representing the cytoplasmic region containing the nucleus observed by LM. Overlying the other cells were occasional singlets and doublets of more globular appearing cells (Fig. 5a, d), the latter potentially undergoing cell division. In addition, at the edges of the patches of
confluent cells were large single cells with multiform shapes. These data correlated well with the cultures observed by light microscopy, and further demonstrated that the central area was a shallow bulge. We examined 2D collagen II, V and IX substrates as before, but found no differences (data not shown).

TEM examination of these cultures confirmed that the central bulge contained a regular, elliptical nucleus and showed fine extensions representing the flattened cell body (Fig. 6a-c). Within the central bulge in particular, normal-appearing mitochondria (Fig 6a, d) and both flattened and expanded areas of rough endoplasmic reticulum were present (Fig. 6b, e). Similar areas of expanded rough endoplasmic reticulum were also found in immature fibrocytes from P14 cochleae of each type. As an example, we illustrate type III and type I cells (Fig. 6f). Cells in the cultures also often associated with each other at overlap points by adherens junctions (Fig. 6a, inset). Many of the cells possessed fine processes of varying dimensions (Fig.6d), some of which surrounded circular gaps (Fig. 6d), reminiscent of type III cells in the cochlea (Mahendrasingam et al, 2011b).

3.3 Characteristics of fibrocytes grown in 3D cultures on and in collagen gels

In order to examine the effect of growing cells onto a 3D, potentially transplantable matrix, we opted in our first experiments to seed cells directly onto pre-formed collagen I hydrogels at the higher seeding densities (~50,000 per mL) to maximize initial population size and increase the probability of success. Gels were therefore polymerized in 24-well plates and cell suspensions applied to the upper surface. The cells usually adhered successfully but, instead of becoming flattened out, as happened with the collagen coated coverslips, the cells formed clusters and networks (Fig. 7a), with groups of more plump and rounded cells linked together by elongated cells (Fig. 7b).
To reveal how these clusters and networks formed when the cells were seeded onto gels, time lapse imaging was also performed (Fig. 7c). This showed that the cells, once settled, were initially round. They then began to clump together within 20 mins, being relatively mobile, whilst extending processes that communicated with each other until by 30 mins they appeared like networks on the surface of the gel.

Immunocytochemical experiments showed that most of the cells expressed the same markers as found in the 2D cultures (Fig. 7d, e). Most cells co-expressed Na,K,ATPase, S-100 and aquaporin1. However, a few small cells had low S-100 labelling whilst still strongly expressing aquaporin 1 (Fig. 7e). We did not label for caldesmon as it is similar to aquaporin 1 as a marker, reflecting primarily a Type III phenotype in adults (Mahendrasingam et al. 2011b).

Morphological and ultrastructural analysis was also performed on cells grown on the collagen I hydrogels for 24 h or 48 h by SEM (Fig. 8) and TEM (Fig. 9). SEM performed on 48 h cultures confirmed that most cells were typically plump rather than flattened, unlike 2D culture cells, and they formed clumps inter connected by processes (Fig. 8a, b), as observed over the first 30 – 40 mins in our video analysis; unconnected individual cells could also be observed. Cells also had regions of multiple processes and folds and some had many fine surface protrusions, whereas in others the surfaces appeared smooth (Fig. 8b). Cells also showed numerous pores in their surfaces. The cells showing extension across the gels (Fig. 8c) could project over the top of other cells (Fig. 8d), consistent with the motility observed in the video analysis and the clumping/network forming activity. Processes appeared also to be burying themselves into the collagen matrix (Fig. 8e, f). Over longer periods (up to 13 d), although survival was poor, the cells became increasingly integrated into the collagen substrate (Fig. 8g, h). (The images shown are from the 13 d cell-survival experiment
described in the section below and were the only samples included that were prepared using the hexamethyldisilazane method.

When clumps of cells were examined by TEM, it could be seen that the cell membranes formed close associations with each other. In comparison with 2D culture cells, nuclear morphology was more varied, nuclei having irregular outlines, as is found in native type III cells especially. The cells showed overlapping processes (Fig. 9a). Fine cell processes and folds could be found in greater quantities but in small clumps (Fig. 9b), as observed using SEM. Adherens junctions and areas of close membrane contact were also detected as with 2D cultures (Fig. 9c). Mitochondrial and RER morphology was similar to 2D grown culture cells, with sometimes dilated RER cisternae, though to a lesser extent (Fig. 9d, e) than that observed in some 2D culture cells. Mitochondria generally appeared to be in good condition. However, there was greater evidence of cell damage and disturbance noted, on 3D compared with 2D cultures, consistent with poorer survival of cells on gels. Some evidence of apoptotic bodies was noted in the clumps of cells, implying cell death was occurring; necrotic cells, however, were not detected.

3.4 Growth of cells on type I collagen gels

Preliminary experiments suggested that the longevity of cells on these 3D substrates was poor. Over several experiments, it appeared that cells death occurred within the first few days after seeding with the majority surviving between 2 – 4 days. They appeared in several categories according to morphology: clumps of cells (where individual cells could not be counted), individual rounded cells, individual elongated cells with processes and cells that were difficult to classify. After 13 days, a period normally sufficient to generate a large quantity of confluent cells in wells or on coverslips, only a small number of residual cells could be found (Fig. 10a). We did not count clumps of cells in these preliminary experiments.
The preliminary experiments suggested that the main cell death occurred before five days, with very few remaining after longer periods.

To examine cell survival on hydrogels more closely we repeated the cell growth study over five days, by analyzing four replicates and generating 10 images by random selection at approximately the same time each day in the same area. We classified cells according to morphology: clumps of cells (where individual cells could not be counted), individual rounded cells, individual elongated cells with processes and cells that were difficult to classify. It was evident that good survival in round cells and clumps occurred only over the first 24 h, with reduction in both resulting in a loss of cells to be about half the initial cell number in total over the remaining five days (Fig. 10b). The elongated and unclassified cells did not show dramatic changes over the period, but were themselves only a small proportion of all cells present. The loss of clumps may correspond with the presence of apoptotic cells observed by TEM. We did not test whether any proliferation was taking place in these cultures because of the low survival rate.

3.5 Varying the substrate for enhanced growth of 3D cultures

As the previous experiments showed that cell survival on collagen I hydrogels was low, we attempted to grow cells on different types of 3D collagen matrix. However, it was difficult to form complete gels of type II collagen alone consistently, so we chose to make mixed collagen I and collagen II hydrogels. We compared the growth of the cells on two different types of gel (in mg/mL): collagen I only (3+0), collagen I + II (2+2).

Because of the difficulties in polymerizing collagen II alone, we first confirmed that the collagen II was present and distributed evenly throughout these gels. Therefore, some 1:1
mixed gels were prepared for SEM and immunogold labelling was performed (Fig. 11a). Preliminary SEM had shown collagen I to be present as typical large fibers, so labelling was performed for collagen II only: gold particle labelling for collagen II was seen to be distributed within the gels, implying that they had formed a properly mixed gel.

We then seeded cells onto the collagen mixed gels as before. Cell morphology was similar initially to that observed for collagen I alone (data not shown). We then evaluated cell survival rate. In two experiments each with four replicates, survival appeared to be improved by addition of collagen II to collagen I. Starting with a cell density that was not significantly different on day 1, at days 2, 3, 4 and 5 there was significantly better survival on mixed gels versus collagen I alone. However, the cell density in all cases still decreased over a period of 4 d (Fig. 11b).

### 3.6 Incorporation of cells inside gels

We also evaluated incorporation of cells inside gels. To achieve this, we used two methods: the first was to pre-mix the cells and draw them up into either a 200 μL pipette tip or into a 120 μm external diameter/94 μm internal diameter capillary attached to tubing and drawn up with a 1 mL syringe. The second method used was to draw up a 100 μL volume of gel mixture (4 mg/mL collagen I) followed by 50 μL volume of cell suspension (5000 – 10000 cells per mL) into a 200 μL pipette tip using a 1 mL syringe. Cells were allowed to survive for 24 h after being incorporated by these methods.

In all the methods of mixing the cells and gels, there was successful incorporation. In the case where cells were pre-mixed before take up, gels stained by phalloidin showed cells spread relatively evenly within the gel (Fig. 12a). In the second (drawing two separate solutions: gels
and cells, into a pipette or capillary), the gels tended to have hollow centers with the cells often lining the hollow, more rarely spread within the gel (Fig. 12b). The gels could be readily expelled from the pipette tip or capillary, suggesting the possibility of using these simple devices as a delivery method into the target.

Cell morphology could be visualized by LM in the gels prepared for electron microscopy by virtue of the dense colouration produced by osmium fixation. In both cases, cells exhibited multiple, 3D morphologies, from round to elongate and with variable numbers of processes extending from them in three dimensions, which were especially clear in the osmium fixed samples (Fig. 12b, c).

The cells in gels fixed with glutaraldehyde and osmium were amenable to ultrastructural analysis by TEM but not SEM (because the cells were internalized). By TEM, cells could be seen integrating into the gels and interacting with the collagen fibers. They contained typical cell organelles in relatively large numbers, and often the surface was irregular (Fig. 12c - e). Nuclei tended to be irregular and had invaginations, unlike 2D cultured fibrocytes, and the cytoplasm contained unusual round vacuolar structures, dense bodies, more extensive endoplasmic reticulum cisternae and surface blebs (Fig. 12d, e, f) than fibrocytes in mouse tissue. They also contained lysosome-like vacuoles (Fig. 12f). Folds in the surface took various forms, including long slender folds reminiscent of type II, III and V fibrocytes (Fig. 12g). Some cells in the gels appeared to be undergoing necrosis (Fig. 12e), unlike the gels where cells were seeded onto them. This suggests either poorer conditions for survival or trapping of cell debris inside the gel. The cells in the gels were not readily amenable to counting because the depth of the gel and the center hollow, together with folds in the gels, made random sampling of known volumes difficult to achieve.
3.7 Magnetic nanoparticle uptake

For transfection of cells pre-transplantation or identification and potential manipulation of cells within cochleae post-transplantation, uptake of magnetic nanoparticles (MNPs) was evaluated. MNPs can be detected by light or electron microscopy and functionalized with gene constructs and have been used in other culture systems (see Pickard et al. 2011).

To determine whether fibrocytes in vitro would also be amenable to this kind of approach, we incubated 2D cultures with media containing magnetic nanoparticles for 24 h – 48 h. Over the longer time period, uptake appeared to be close to 100% as cells that did not contain them were not detected (Fig. 13a). Cells that were harvested after MNP uptake were also then incorporated into gels using the pipette technique described above. The cells retained the MNPs within the gels, thus confirming their potential use for marking or modifying cells in constructs (Fig. 13b).

4. Discussion

The aim of the present study was to determine how cochlear fibrocyte cultures form and whether they are subject to modulation by substrate specific cues, especially in 3D. This was done because there has been a growing awareness that 2D cultures often do not generate good morphological or functional models of cells in their native tissues, ascribed to the lack of environmental cues such as 3D organization and substrate composition (see review by Knight and Przyborski 2015). Thus data obtained using 2D cultures in other systems have often been called into question, for example in drug treatments for cancer cells (Aggarwal et al., 2009).
The use of fibrocyte cultures is uncommon, but potential of such cultures as an approach to study fibrocytes is considerable (Furness 2019). We compared the characteristics of cultured fibrocytes using morphological, and immunocytochemical techniques to determine what effect different substrates have and specifically whether (i) the phenotype of cells grown on 3D substrates resembles more closely native cochlear fibrocytes making them more useful for study and as transplantable cells; and (ii) specifically that collagen II containing substrates and gels would recapitulate more closely the native environment and promote better growth.

4.1 Formation of the cultures

Our data suggest that cells primarily exit from the type III fibrocyte region, whilst deeper cells probably degenerate in situ. Thus, amongst others, we labelled for aquaporin 1 since it is known to be exclusive to type III cells in the ligament (Mahendrasingam et al., 2011b). However we found that although the majority of cells did express aquaporin 1, they also expressed other markers not characteristic of type III cells such as NaKATPase and S-100. Unequivocal labeling for a macrophage marker was not detected in the cultures. As discussed below, these findings suggest a non-specific fibrocyte phenotype within the cultures.

4.2 The effect of 2D substrate type

Unlike previous reports, the cells grown on the 2D coverslip surfaces were commonly of mixed morphology: large and flat cells were found with patches of smaller, spindle-shaped cells more often reported in other studies. The two different morphologies showed similar mixed marker protein expression although larger cells had more obvious actin, presumably to support the larger cell bodies. The variations in shape likely depend on the state of confluence and age of the cultures, as well as physical factors such as stiffness and substrate composition.
(Xu et al., 2020). Indeed, the cells were similar on Aclar sheets and on the different types of collagen coated coverslips.

In previous studies, NaKATPase negative type I cells, (Gratton et al., 1996; Suko et al. 2000) or type IV cells (Qu et al., 2006) were present. The differences between our studies and these may reflect differences in culture conditions, although we followed the same methods, or more recent antibodies/marker combinations. This begs the question, therefore, whether the immunophenotype is sufficient to characterize fibrocyte cultures. Alternative approaches might be to assess expression patterns with qPCR and western blotting (Ng et al., 2017).

Ultrastructurally, the cells in 2D culture appeared flattened with a central cytoplasmic bulge, comparable between all three types of microscopy used. Cultures of other cell types often show this (possibly default) morphology, for example HCC1954 cancer cells (Breslin and O’Driscoll, 2016). The fibrocyte culture cells also formed adherens junctions and close contact points, and had fine folds extending from their surfaces. Mitochondria were normal, which is of interest because in the 3-4 weeks CD/1 mice from which these cultures were derived, mitochondrial damage is apparent in the fibrocytes (Mahendrasingam et al., 2011a). The RER had distended cisternae, similar to those seen in P14 fibrocytes and in other developing cells (Bielek, 2005). These data suggest potentially a mixed or immature phenotype undergoing a high level of protein synthesis, as might be expected in a growth phase.

The ECM of the spiral ligament contains collagen II in abundance (Slepecky et al. 1992; Buckiova et al 2006). We therefore explored collagen substrates other than type I, the substrate of choice in most previous studies, in an attempt to modify the phenotype of the
cells. Using collagen II coating on the coverslips made a small but significant difference to the size of the larger population, although not detectably to the smaller one. Thus, in terms of hypothesis 2, we did find that collagen II coating altered the phenotype to a degree, by reducing the size of the larger cells. This might suggest an effect on how the cells grow or spread on this surface.

4.3 The effect of 3D substrate type

Cells grown on 3D hydrogels made of collagen I, compared with all collagen coated coverslips, have plump cell bodies that extended slender processes, which is a more fibrocyte-like morphology. In other respects, when comparing the cells grown on Aclar sheets with those in hydrogels, features were similar with distended RER, normal mitochondria and very fine processes. The fine processes, or folds, are characteristic especially of type II and type V fibrocytes (Furness et al., 2009). Marker labelling showed no substantive differences compared with 2D cultures. The morphological but not immunocytochemical data thus suggest that hypothesis 1 is partly correct and that it may be possible to direct fibrocyte morphology to a more native state in the cultures by manipulating the topography of the substrate.

On these gels, the cells appeared to contact each other, rapidly forming networks held together by intercellular adherens junctions, potentially with the ability to replicate gap junction networks seen in the native spiral ligament (see Jagger and Forge, 2006). These networks involved clumping of cells within which some cell death occurred. We observed apoptosis here, but not necrosis, probably because the debris of the latter was washed away during processing. The speed with which this occurred would suggest that the morphological changes were driven by contact with the different substrate rather than other changes such as
cell death. The extensions from the cells may form towards other cells either randomly, or there may be trophic factors released from some cells that attract them. Over longer periods, the cells became integrated into the gel surface where extracellular matrix fibers were present often over the processes and, less frequently, over the entire cell. It is most probable that the cells buried themselves (processes first) as they grew into the gels, although an alternative is that the fibers seen covering them were secreted by the cells.

Overall, the results suggest that: (i) cells grown on hydrogels provide potentially more fibrocyte-like cells and an alternative way to investigate fibrocyte characteristics e.g. to test the role of fibrocytes in potassium handling, or for genetic manipulation and modification; and (ii) that it may be possible to replicate native fibrocyte networks in a three-dimensional culture. They may also be more effective for testing potential therapeutics agents for use to stimulate endogenous cell repair and regrowth. The fact that these cells readily take up MNPs (at least in 2D cultures) and retain them when cultured into 3D provides a means of transfecting them and thereby altering their gene expression.

4.4 The development of potentially transplantable matrices of fibrocyte cultures – proof of concept.

Although speculative, the future goals of these experiments are to evaluate how fibrocyte culture cells might re-establish themselves either in vivo into the ligament, or to develop a biomimetic, transplantable cell-laden construct that could be attached to it. With regards to the former, the cellular integration into the hydrogel bodes well for the possibility of recapitulating the original lateral wall either within a matrix, or by injecting cells into an existing region of extracellular matrix where they may reintegrate. Indeed studies of the degeneration of the spiral ligament in CD/1 mice have shown that as cells in the ligament die,
they leave behind a cavity into which replacement cells might migrate within the extracellular matrix (Mahendrasingam et al., 2011a). These cavities were also observed here, as the cells exited the ligaments. In either case, these possibilities may help in preventing the progression of metabolic and subsequent sensorineural hearing loss that is seen in some animal models of age-related hearing loss (Shone et al., 1991; Hequembourg and Liberman 2001; Mahendrasingam et al., 2011a).

The only other study we are aware of where fibrocytes have been cultured in a collagen matrix is that of Kelly et al (2012) where the authors reported they had primarily type III fibrocytes with contractile properties. However, they derived their cells from a more restricted region of the ligament than has been done here, and furthermore the cells were mixed first with the unpolymerised collagen rather than seeded directly onto polymerized collagen.

The poor longer term survival of the surface gel cultures on collagen I gel suggests gel composition was sub-optimal. The composition of matrices can be critical in terms of supporting specific mesenchymal cell types compared with others (Hayrapetyan et al., 2016). Because (a) collagen II coated coverslips appeared to influence the cell growth, at least in terms of size, and (b) collagen II is the dominant component of the spiral ligament ECM, we compared collagen type I and type II containing gels, the latter mixed with collagen I. The results here suggest that cells survive better with collagen II present, but not very substantially, perhaps by 10%, suggesting hypothesis two is correct to some degree. It has also been shown for adipose mesenchymal stem cells that making hydrogels of type I and type II collagen mixtures can enhance cell growth and the expression of phenotypic markers (Tao et al., 2016). Our data are consistent with these studies insofar as better survival was noted in
the presence of collagen II but other combinations of matrix proteins may be needed to improve survival further.

We attempted another strategy by mixing cells within collagen I the matrices, as was done by Kelly et al. (2012). We used two different methods to mix cells either before incorporation into a small gel space or after, by mixing through a syringe or capillary which could potentially be used to inject the matrix. Both produced viable cells, with morphologies ranging from rounded to highly fibrocytic with multiple processes, but there was evidence of apoptosis and necrosis occurring, that suggested viability was also a problem in these gels.

The use of hydrogels offers several possibilities in terms of both research and clinical approaches. They could be used in co-culturing, for example with other tissues of the cochlea, to evaluate the protective capacity of fibrocytes. There is a possibility that gels could be transplanted into the cochlea using the simple devices here (i.e. via a cochlear implant surgical approach) to replace or add to dwindling fibrocyte populations in the native ligament, as suggested by Wagner et al (2014). Appropriately shaped and sized replacement ligaments could be produced in vitro for example through molding or bio-printing a hydrogel containing cells (Lee and Yeong, 2016).

4.5 Conclusions

In summary, we are able to generate cells of distinct morphology on collagen based hydrogels as opposed to flat surfaces, the former appearing to be more like native fibrocytes. In both cases, however, the type of fibrocyte generated was indistinct – markers gave no clear characterization – and appeared as a mixed or immature phenotype. Although, survival was poor on hydrogels, it was better when type II collagen was included. Further optimization of
these gels may therefore enable them to become useful for studying fibrocytes and as biomimetic transplants.

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Figure legends

Figure 1. The initial development of the cultures from ligaments. a, b). LM after 48 h following placement of ligaments in wells and covering with coverslips. Cells are exiting both singly and in chains (*) from the margins of the ligament pieces along the side normally populated by type III cells. c, d) Cells subsequently formed a patch around the ligament remnant that expanded over 4 – 7 days; arrows indicate the ligament remnant. Inset shows the morphology of cells at 4 d at higher magnification. e, f) Ligaments seeded onto Aclar sheets, embedded for semi-thin and ultrathin sections for LM and TEM confirmed there were gaps in the type III area at 4 days (e, *) whilst by day 7, most of the fibrocytes were missing from the ligament, leaving behind root cells (f; large arrows) and blood vessels. Below the ligament at 7 days, cells can be seen in transit, with extremely thin processes extending down from the tissue (f, small arrow); arrowhead in ‘f’ indicates the substrate (Aclar sheet) with flattened cells on it. g – j) TEM showing degeneration of fibrocytes (arrows) within several regions of the ligament at 4 days, in places leaving behind holes but intact extracellular matrix (ecm) where they were once located. k). Exiting cells (*) appear to grow over each other (left inset, arrow), as suggested by the chains in (a) and (b) and to be coupled together by adherens junctions (k, right inset, arrowhead). Scale bars: a = 750 μm; b = 400 μm; c, d = 1 mm (inset = 100 μm); e, f = 50 μm; g – j =20 μm; k = 20 μm (inset = 500 nm).

Figure 2. 2D cell culture of cochlear fibrocytes on collagen I coated coverslips. a) Phase contrast LM of a confluent region of culture cells. The cells are mostly spindle shaped, although some are round (*) and possess a central bulge representing the nucleus and small area of cytoplasm (arrow). b) Non-confluent region of cells where they are flattened and extend processes along the surrounding surfaces. The central bulge is also evident (arrow). c) Immunocytochemical labelling for three typical fibrocyte markers, NaKATPase (NaK), S.100
and caldesmon (Cal). All three markers are expressed at varying levels in most cells, but some show particularly strong labelling for all three and appear white in the merged image. d) Cells of varying sizes, but generally appearing to form groups of small and large cells, both expressing S-100, especially in the central bulge (*). Large cells also have significant actin content, in places concentrated in a peripheral ring especially in the large cells (arrow) e) Co-labelling for caldesmon (Cal) and aquaporin 1 (Aqp) shows the presence of both proteins in all cells, but with especially strong labelling for the latter in some (*). The caldesmon parallels actin in forming peripheral rings (arrow). Scale bars = 100 μm.

Figure 3. a) The distribution of caldesmon in 2D culture cells resembles that of actin, forming peripheral rings and stripes or cables through the cytoplasm, (arrows). b) higher magnification showing the parallel stripes suggesting an association with actin cables. c) SEM of very thin 2D culture cell. d) higher magnification showing cable like structures in the cytoplasm (c) similar to the actin and caldesmon cables. Scale bars: a = 100 μm; b = 50 μm; c = 60 μm; d = 6 μm.

Figure 4. a) – d) Images of cultures grown on four different collagen substrates and labelled for S-100 (red) and tubulin (green): a) collagen I, b) collagen II, c) collagen V and d) collagen IX. Two populations of cells are visible on each coverslip and no differences can be distinguished qualitatively. e) Measurements of mean cell area in arbitrary units from 5 randomly selected areas in each population. No differences can be seen in the small cell populations between collagens, but the large cell populations are smaller on collagen II than the others, the difference being significant (p < 0.05; Wilcoxon Signed Rank Test) between collagen II and collagen I, and collagen II and collagen IX. Scale bar = 100 μm.
Figure 5. SEM of the 2D cultures grown on collagen I coated coverslips showing that the fibrocytes have a monolayer appearance. a) Confluent region of cells, showing the flattened cell morphology with a central bulge (*). Round cells lie on top of the flattened cells (e.g. arrows). b) Region at the edge of confluency where cells appear to be migrating. They have a central bulge (*) and flattened processes apparently stretching outwards (arrowhead). c) Detail at the edge of a more rounded cell showing fine filopodia (arrows) and finer threadlike processes (arrowhead). d) Pair of adjacent round cells (*) that may be offspring of a dividing cell. e) Cell apparently undergoing cell division. Scale bars a = 100 μm; b = 50 μm; c = 6 μm; d = 20 μm; e = 10 μm.

Figure 6. TEM of the 2D cultures grown on collagen I coated Aclar sheets. a) Micrograph of the central region of a small, rounded cell, showing it contains the nucleus (nu) and the typical cytoplasmic components such as mitochondria (arrowheads). An adherens junction (arrow) can be seen where two cells lie close to each other (inset). b) The region of a the central bulge of a larger rounded cell compared with that shown in (a) illustrating similar features, but with a fine process extending from it (large arrow) and tiny processes (arrowheads); RER (small arrows). c) A large flattened cell showing the very thin cytoplasmic areas around the central bulge containing an ovoid nucleus. d) Rounded cell showing some extensive thin processes (arrow) and mitochondria in apparently good condition (arrowhead). e) Central bulge of a cell showing extended and dilated RER (arrows). f) Cells from a P14 cochlea spiral ligament showing type III (darker cell) and type I (lighter cell) both containing dilated RER (arrows). Scale bars = 2 μm.

Figure 7. Phase contrast and immunofluorescence microscopy of 3D cultures on collagen I hydrogels. a) When seeded on top of the gel, after 2 d the cells have formed into groups,
clumps and networks across the gel. b) At higher magnification clumps of cells can be seen to be connected across the gel by the elongated processes of individual cells. c) Frames taken from a time-lapse video of the cells starting immediately after the seeding. Cells are initially round, but over time develop extensions projecting along the gel and move together to form clumps. Some of the processes contact other cells and clumps, so that a network is established between 30 – 40 mins after seeding. d) and e) immunocytochemical labelling for aquaporin 1 (Aqp1) and NaKATPase, and for Aqp1 and S-100 show that most cells express the same markers as in 2D cultures. Occasionally, cells expressing Aqp1 but only weak S-100 can be found (arrows). Scale bars = 100 μm.

Figure 8. SEM of cultures grown on the 3D collagen I hydrogel at 48 h. a) Micrograph showing the plumper morphology of the cells (cf Fig. 5a, arrow) and the network formed between them. The cells show both plumper/rounder (r) and elongate (e) morphology, and processes extending across the fibrous surface of the gel. b) Example of a clump of cells. The clumps appear quite tightly bound together. Cells in the clumps can have a smooth surface (s) or may be more decorated with fine surface processes (fsp). c) In several places the processes of the cells interact in various ways by lateral contact (e.g. arrow). d) Cell processes also cross over other cells (arrow). Many cells also show surface pores (inset, box enlarged in main picture). e) The processes can show expanded ends interacting with the collagen (arrow). f) Enlargement of the expanded process in e. Its appearance suggests partial penetration into the gel. g) a rounded cell with a long process, showing partial penetration into the gel. h) After 13 days in vitro, some cells are significantly integrated into the gel, with fibers covering them. Scale bars: a = 50 μm; b = 20 μm; c = 30 μm; d = 10 μm (inset = 1 μm); e = 20 μm; f = 5 μm; g, h = 10 μm.
Figure 9. TEM of the cells seeded onto hydrogels (48 h after seeding). a) A clump of cells in which at least three cells can be defined by the presence of their nuclei (1 – 3). The tightly packed cells have irregular nuclei and the cytoplasm of some cells contains dense bodies that have the appearance of apoptotic bodies (ap). Processes from two cells (P(a) and P(b)) exiting the clump overlap each other closely. The fibers of the gel can be seen underneath the clump. b) Detail of cells with fine processes (P(f)) that resemble those of native type II and type V fibrocytes. c) Detail of two cells contacting each other via adherens junctions (a) and close contact points (arrow). d) Detail of the cytoplasm showing normal mitochondria, lipid-like droplets and cisternae of RER, the latter distended (*) in places. e) Developing type III fibrocyte from a P14 CD/1 mouse cochlea. The RER in the cytoplasm shows similarly distended cisternae (*) to that shown in (d). Scale bars: a = 5 μm; b = 2 μm; c – e = 1 μm.

Figure 10. Histograms showing the survival of different morphological subtypes of culture cells on the collagen I hydrogels. ‘Round’ cells have no processes; ‘process’ cells have elongated projections from the cell body; ‘unclassified’ had indeterminate shapes. a) Example of a preliminary experiment over 13 days revealed a relatively rapid fall off in cell numbers of different morphological subtypes. (This sample is also included because SEM images from it shown in Fig. 8). b) Detailed analysis of four replicates showing cell survival over a period of 5 days. The numbers of clumps has been included. Clumps and rounded cells both decline whereas process cells do not show much change. Overall, the total number of cells declines by close to 50%.

Figure 11. a) Backscattered SEM of a collagen I +II mixed hydrogel labelled for collagen II. Large fibers representing collagen I and gold label (visible as white dots – arrow shows an example) were distributed throughout the gel showing incorporation of both collagens. b)
Postembedding immunogold labelling of a type I + II collagen gel labelled for collagen II. This shows that the collagen is II is evenly spread and as the labelling is on the section surface it is unlikely simply to be trapped within the collagen gel. A large striped fiber of collagen I is also visible. c. Histogram showing the effect of collagen II incorporation into the hydrogels on cell survival. The cell numbers on collagen I and collagen I+II gels fall over the first 2 d, but the fall is smaller for collagen I+II gels. Thereafter, collagen II containing gels show significantly higher survival than collagen I gels alone. Significant differences (p < 0.05, n = 4; Wilcoxon Sign Rank Test) in cell density were detected on each subsequent day.

Figure 12. Light and electron microscopic assessment of cells incorporated into gels by mixing. a) Gel formed with premixed cells and extruded from a pipette tip, fixed and labelled for actin. Cells can be seen throughout the gel and to be variable in morphology. b) Gel extruded from a pipette tip after cells were taken up after the gel solution, fixed and embedded in resin for electron microscopy. The gel tends to be hollow (h) and cells dispersed primarily along the edges of the hollow. They form rounded, elongated and fibrous morphologies, with the position of several cell processes. c) Example of cell morphology in the extruded gel include round cells and cells with multiple processes (arrow). d) TEM of a cell in the gel shown in b. The cell has an irregular nucleus, and the cytoplasm contains dense potentially apoptotic bodies (ap), and uncharacterized clear vacuoles (v), amongst the normal organelles. e) Example of another rounded cell with multiple bleb like processes (arrows), and cells that have lost their integrity and appear to be undergoing necrosis (nec). f) Cytoplasm in these cells appeared to have normal organelles such as mitochondria (m) and RER, but in addition there were lysosome like vacuoles (arrow points to the circular membrane) and dense bodies (db). g) Some cells were decorated with fine processes (fsp). nu = nucleus. Scale bars: a = 200 μm; b = 100 μm; c = 50 μm; d = 5 μm; e = 10 μm; f, g = 1 μm.
Figure 13. Magnetic nanoparticle uptake in 2D and harvested cells in 3D cultures. a) Virtually 100% of the cells in the 2D monolayer cultures contained MNP after 24 or 48 h (shown) incubation. The particles (e.g. indicated by *, orange against the green S-100 labelling of the cells) tended to clump around the nuclei (nu). The cut plane (below) represents an orthogonal slice through the stack of confocal images and shows that MNPs are definitely cytoplasmic and not lying on the surface. b) after pre-incubation with MNPs in 2D culture, followed by harvesting and mixing into gels using the pipette method, MNPs were still clearly observed within the cells in the gels (orange particles denoted*), showing robust uptake. Scale bars = 50 µm.
The effects of substrate composition and topography on the characteristics and growth of cell cultures of cochlear fibrocytes.

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