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Research Paper

Microenvironmental support for cell delivery to the inner ear

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ABSTRACT

Transplantation of mesenchymal stromal cells (MSC) presents a promising approach not only for the replacement of lost or degenerated cells in diseased organs but also for local drug delivery. It can potentially be used to enhance the safety and efficacy of inner ear surgeries such as cochlear implantation. Options for enhancing the effects of MSC therapy include modulating cell behaviour with customized bio-matrixes or modulating their behaviour by *ex vivo* transfection of the cells with a variety of genes. In this study, we demonstrate that MSC delivered to the inner ear of guinea pigs or to decellularized cochleae preferentially bind to areas of high heparin concentration. This presents an opportunity for modulating cell behaviour *ex vivo*. We evaluated the effect of carboxymethylglucose sulfate (Cacicol®), a heparan sulfate analogue on spiral ganglion cells and MSC and demonstrated support of neuronal survival and support of stem cell proliferation.

1. Introduction

1.1. Cell transplantation to the inner ear

Cell transplantation presents a promising approach not only for the replacement of lost or degenerated cells in diseased organs but also for local drug delivery. Transplanted cells may release a variety of factors such as cytokines or growth factors for the support of the organ, supporting its cells and their functional integrity. Several types and sources of cells have been considered for transplantation. Genetically engineered fibroblasts and stem cells have been used for growth factor delivery to the brain (Makar et al., 2014). Haematopoietic stem and progenitor cells are attractive vectors for gene delivery since they are easily obtained from the recipient and allow autologous transplantation (Bordignon and Roncarolo, 2002). Alternate strategies include using embryonic stem cells (ESC) and mesenchymal stromal cells (MSC) derived from various sources such as bone marrow, adipose tissue or umbilical cord.

Cell transplantation to the inner ear has been addressed experimentally in many studies (reviewed in (Warnecke et al., 2017)). Depending on the therapeutic purpose, cells need to reach structures in both, the perilymphatic and endolymphatic space, which can potentially negatively impact cell survival. Mesenchymal stromal cells are multipotent cells that can be isolated from a variety of adult tissues. Most intensively investigated are MSC derived from bone marrow. Their low immunogenicity has made them attractive for clinical application (Lee et al., 2016). Intravenous administration of MSC resulted in recruitment and engraftment into the inner ear to various degrees (Dai et al., 2010; Choi et al., 2012). In addition, local administration of MSC to the inner ear leads to the regeneration of the fibrocytes in the lateral wall and enhanced functional recovery of the cochlea (Kamiya et al., 2007). Engraftment and differentiation of MSC to fibrocytes was shown preferentially in paediatric animal models rather than in adults after perilymphatic administration of the cells (Kasagi et al., 2013). Most of their actions are believed to be due to their secretome (D'souza et al., 2015; Tran and Damaser, 2014), and this has been also demonstrated recently for the treatment of the inner ear (Yoo et al., 2015). Given that many of the beneficial effects of these cells are related to production of growth factors and miRNAs, a novel functional description for MSC has been proposed: "*medicinal signalling cells*" emphasizing their paracrine effects (Girolamo et al.,

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2013). There is increasing evidence that other populations of the bone marrow such as hematopoietic stem cells may be involved in cochlear repair (Tan et al., 2008; Yoshida et al., 2007), especially the repair of the lateral wall.

1.2. Matrix support of transplanted cells

The extracellular matrix (ECM) provides structure, mechanical integrity and biochemical activity in a three dimensional complex (Guvendiren and Burdick, 2013). In addition, it hosts soluble signalling molecules including growth factors (Guvendiren and Burdick, 2013). In a diseased organ, the native ECM is altered and does not offer physiological support. Thus, cells transplanted to a diseased organ can potentially sense and response to such deviations. The ECM of the cochlear microenvironment offers important cues for stem cell behaviour and differentiation (Mellott et al., 2017). As has been recently demonstrated for the brain (Kabu et al., 2015), cochlear insult may also create a hostile microenvironment that could affect survival and integration of transplanted cells. Pharmacological agents that mimic the effects of the ECM could therefore be utilized to support not only transplanted but also residual cochlear-residing cells.

Heparan sulfate is a multi-disaccharide-chain located in the ECM. In interaction with various other proteoglycans, it forms the structure of the ECM and induces signal transduction (Häcker et al., 2005). Heparan sulfate can bind various growth factors and thereby enabling a change to their active state (Okolicsanyi et al., 2014). Heparan binding growth factors (HBGF) are known to support neural survival (Yu et al., 2011). Regenerating agents (RGTA) are heparan sulfate analogues that substitute the natural proteoglycans as they mimic their abilities with a minimized vulnerability to proteolysis making them ideal for translational research (Rouet et al., 2007). In addition, RGTA provide growth factors, cytokines, interleukins, colony stimulating factors, chemokines and neurotrophic factors (Barritault and Caruelle, 2006). After cell death, a liberation of glycanases and proteases occurs that degrades heparan sulfate. This leads to a release of the cytokines, especially the heparan binding growth factors (HBGF), which are then soluble and therefor unprotected from proteolysis. RGTA rebind the HBGF and thus protect them from proteolysis. Therefore, they preserve the natural signal cascade and as a result support tissue regeneration. Previous studies demonstrated their promising implementation in wound healing including chronic corneal (Kymionis et al., 2014) and dermal ulcers (Groah et al., 2011).

The aim of the present study was to evaluate the migration of MSC *in vivo* and evaluate potential matrix factors that impacted transplanted MSC. Heparin and heparan sulfate proteoglycans have been shown to affect MSC growth and differentiation (Ling et al., 2016; Titmarsh et al., 2017). Heparan sulphate is present in the lateral wall of the cochlea (Cosgrove et al., 1996), where prior studies have demonstrated engraftment of stem cells (Lang et al., 2006). In the following study, the question whether the cochlea and especially the scala media provides adequate scaffold for integration of MSC was investigated in an animal model using cell transplantation to the scala tympani of guinea pigs. In addition, engraftment of MSC after their perfusion into decellularized cochlea scaffolds was investigated in order to identify putative binding preferences based on the presented extracellular matrix of the endolymphatic or perilymphatic space. Finally, carboxymethylglucose sulfate (Cacicol®), a heparan sulfate analogue and RGTA, was tested *in vitro* for its ability to function as a supportive biomimetic matrix for spiral ganglion neurons and for MSC.

2. Materials and methods

2.1. Ethical statement

Human bone marrow was obtained during routine orthopaedic procedures from otherwise healthy donors after approval of the institutional ethical committee of Hannover Medical School as described previously (Roger et al., 2016). Written informed consent was obtained from all donors (Roger et al., 2016). All personal information apart from age and gender was deleted. The use of the cells for research purpose was approved by the Ethics Committee of Hannover Medical School (2080–2013). For the experiments with the decellularized cochleae, MSC were isolated from human umbilical cords according to the protocols approved by the University of Kansas Human Subjects Committee (KU-Lawrence IRB approval #15402). The *in vivo* study on guinea pigs was permitted by the local government (LAVES, registration no. 05/1053) and was conducted in accordance with the German “Law on Protecting Animals”.

For mouse studies, C57BL/6 female mice (~6 weeks old) were purchased from Charles River and euthanized according to approved IACUC protocol (ACUP #2014–2234) at the University of Kansas Medical Center (KUMC).

Spiral ganglion cells (SGC) were dissected from neonatal Sprague-Dawley rats of both sexes (P3-5). This procedure was approved by the Institutional Animal Care and Research Advisory Committee and by the local state authorities. The study was conducted in accordance with the German ‘Law on Protecting Animals’ and with the European Communities Council Directive 86/609/EEC for the protection of animals used for experimental purposes. The euthanasia for the *in vitro* experiments is registered (no.: 2013/44) with the local authorities (Zentrales Tierlaboratorium, Laboratory Animal Science, Hannover Medical School, including an institutional animal care and use committee) and is reported on a regular basis as demanded by law. For exclusive euthanasia of animals for tissue analysis in research, no further approval is needed if no other treatment is applied beforehand (German Animal Welfare Act, §4). The rats (breeding stock was supplied by Charles River (Charles River, Wilmington, USA)) were bred and born for research study purposes.

2.2. Isolation and cultivation of human MSC

Human MSC were prepared from fresh bone marrow aspirates by density gradient centrifugation and subsequent plastic adhesion of mononuclear cells as described previously (Schäck et al., 2013). Heparin-containing bone marrow diluted with phosphate buffered saline (PBS) was layered onto a Biocoll density gradient (Biochrom AG, Berlin, Germany) and centrifuged for 30 min at 500 g. The mononuclear cell fractions at the interface was obtained and seeded in cell culture flasks in MSC medium at 37 °C with 5% CO_2 at 85% humidity (DMEM FG 0415; Biochrom, Berlin, Germany supplemented with 10% v/v fetal bovine serum; Thermo Fisher Scientific, Schwerte, Germany, 20 mM HEPES, 1% 100 U/ml/100 g/ml penicillin/streptomycin; Biochrom AG, Berlin, Germany and 2 ng/mL human recombinant FGF2; PeproTech, Hamburg, Germany). The cells were passaged at a density of around 70% by the use of 0.025% Trypsin-EDTA solution (Biochrom AG, Berlin, Germany) and seeded at a density of 2*103 cells per cm (Bordignon and Roncarolo, 2002) for transfection. Plastic adhesion and expression of surface markers such as CD73, CD105, and CD90 were used as criteria to define MSC. Analysis of presumptive osteogenic or adipogenic differentiation and stemness of cultured MSC was randomly performed using

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quantitative RT-PCR for genes involved in osteogenic and adipogenic differentiation: *ADIPOQ* (Hs00605917_m1), *CHRDL1* (Hs01035484_m1), *ALPL* (Hs00758162_m1), *BGLAP* (Hs01587814_g1), *FABP4* (Hs00609791_m1) and *PPARg* (Hs01115513_m1) and for the chemokine *CCL7* (Hs00171147_m1) as described previously (Roger et al., 2016). All probes and quantitative RT-PCR reagents were purchased from life technologies.

2.3. Transfection of human MSCs

The construction, generation and titration of the lentiviral vectors to produce GFP as a marker gene have been described in detail previously (Wissel et al., 2008). One day following seeding of cells, the lentiviral pLOX system was used for genetically modifying MSC to produce GFP. A co-infection of a MIK- virus and GFP- lentivirus was performed at a concentration of 1:10 and 8 mg/mL polybrene. After 24 h, gene expression was induced by adding 1 mg/mL doxycycline (Dox, hydroxy-doxycycline [Sigma, Taufkirchen, Germany]).) GFP expression was detected by fluorescence microscopy (Olympus IX81, Olympus Deutschland GmbH, Hamburg, Germany; excitation 490 nm/emission 520 nm) 5 days after transfection. The cells were harvested by trypsination (5 g/l Trypsin; 2 g/l EDTA in 10× PBS) and passaged 4 times before used for transplantation into guinea pigs.

2.4. In vivo delivery of GFP-labelled MSC to the inner ear

A total of n = 14 normal hearing guinea pigs (Charles River WIGA, Sulzfeld, Germany) of both sexes (250–450 g) were transplanted with human mesenchymal stromal cells transfected with lentivirus expressing green fluorescent protein (GFP) to produce GFP expressing MSCs. The cells were allowed to integrate into the host tissue for either 48 h (n = 4) or 7 days (n = 2) prior to termination of the experiment and the engraftment of the cells was investigated in whole mount preparation of the cochlea. For investigations using embedding of the cochlea in paraffin and sectioning, cells were also allowed to integrate into the host tissue for either 48 h (n = 4) or 7 days (n = 4). All animal procedures were performed under general anaesthesia with xylazine (10 mg/kg, i.m.) and ketamine (40 mg/kg, i.m.). In addition, local prilocaine anaesthesia was used for the comfort of the animals. For stem cell transplantation, a unilateral postauricular incision was made, the musculature was prepared to caudal, the bulla was opened to expose the middle ear cavity and the cochlea was microscopically visualised (OPMI-6-M, Carl Zeiss AG, Oberkochen, Germany). The scala tympani was accessed by drilling a hole into the basal turn of the cochlear near the round window. This opening was used for the transplantation of the stem cell solution using a small cannula (5 μL suspension containing 1×10^4 cells/μL). Carboxylate cement (Durelon, ESPE Dental AG, Seefeld, Germany) was used for the sealing of the cochleostomy and the bulla defect. Wound closure was obtained using two layered single-knot sutures. Following implantation, cyclosporin A (5.6 mg/kg body weight) was intraperitoneally administered for immune suppression. All animals were anaesthetised and euthanized by transcardiac perfusion with 200 mL phosphate buffered saline (PBS) and 200 mL 4% paraformaldehyde (PFA). Temporal bones were harvested for histological analysis. The presence of the transplanted cells was investigated by fluorescence microscopy due to GFP (green fluorescent protein) expression. In six cochleae, native preparation in PBS following transcardiac perfusion was performed. Eight cochleae were fixed with 4% PFA for 12 h at 4 °C and decalcified in 20% EDTA/PBS for approximately 4 weeks at 37 °C. After dehydration in an ascending concentration of ethanol solutions, (70–100% ethanol), the cochleae were embedded in paraffin and serially sectioned at 5 mm (Thermo Shandon Finesse 315, Astmore, UK) in a midmodiolar plane. Every section was mounted on glass slides (Menzel-Glas, Super-Frost Plus, Braunschweig, Germany), rehydrated with ethanol and water and stained with haematoxylin/eosin (Merck, Darmstadt, Germany). The remaining specimens were prepared as fresh whole mount tissue after fixation.

2.5. Decellularized cochlea experiments

C57BL/6 female mice (~6 weeks old) were euthanized according to approved IACUC protocol (ACUP #2014–2234) at the University of Kansas Medical Center (KUMC). Cochleae were isolated, decellularized and decalcified according to previous published protocols. Briefly, mice were decapitated and the auditory complexes (cochleae and vestibular organs) were removed via blunt dissection. The stapes was removed and the oval window membrane was punctured on each cochlea. A fresh 28.5 gauge Ultra-Fine insulin needle (BD Biosciences, San Jose, CA) was used to perfuse each cochlea with 3.5 mL of 2% penicillin-streptomycin in HBSS over 2 min in a glass scintillation vial. An additional 0.5 mL of antibiotic solution was added to each scintillation vial, and auditory complexes were agitated on a rocker at 10 rpm for 24 h. Cochleae were washed with PBS, then decellularized by perfusing 100 μL of 1% sodium dodecyl sulfate (SDS) (Life Technologies, Grand Island, NY) in deionized (DI) water through each cochlea. Cochleae were soaked in decellularization solution for 24 h while agitated on a rocker at 10 rpm. The decellularization process was repeated each day for three days. Cochleae were rinsed in PBS (Life Technologies) three times then decalcified by perfusing 100 μL of 10% ethylenediaminetetraacetic acid (EDTA) in DI (Life Technologies, Grand Island, NY) water through each cochlea. Cochleae were soaked in the same decalcification solution for 24 h while agitated on a rocker at 10 rpm. The decalcification process was repeated each day for three days, then cochleae were washed in PBS three times. Cochleae were soaked in 10% antibiotic-antimycotic in HBSS (Life Technologies) over night at 4 °C.

Human MSC were isolated from umbilical cords according to the protocols approved by the University of Kansas Human Subjects Committee (KU-Lawrence IRB approval #15402). All cords (n = 3) were obtained from males born at full term under normal conditions. Human MSC were cultured in mesenchymal stromal cell grown medium (MSCGM™) (Lonza Group Ltd., Basel, Switzerland) and expanded to passage 5 for experiments.

Decellularized cochleae were pre-soaked in MSCGM at 37 °C for 1 h. A total of 100,000 MSC in 100 μL of MSCGM was perfused through each cochlea. Cochleae were cultured in 6-well plates in containing 2.5 mL of MSCGM with media changes occurring every 48 h. After 7 days of culture, cochleae were washed in HBSS, and fixed overnight in 4% paraformaldehyde in HBSS (VWR, Randor, PA) at 4 °C. Cochleae were dehydrated with a series of increasing ethanol-HBSS solutions, cleared in xylene, and embedded in paraffin. Samples were sectioned at a thickness of 10 μm using a microtome (Leica, Buffalo Grove, IL) and mounted on SuperFrost glass slides (Thermo Fisher, Waltham, MA). Afterward, samples were deparaffinised and were gently rehydrated over 30 min in a series of decreasing ethanol-HBSS solutions. Samples were washed in HBSS twice and either stained with haematoxylin and eosin or stained with DAPI. Whole-mount samples were imaged via an Olympus IX81/31 spinning disc confocal microscope (Olympus America, Center Valley, PA) at a magnification of 20×. A montage of neighbouring fields of view was stitched together using the acquisition software Slidebook (Intellent Imaging Innovations (3i), Denver, CO).

A sub-set of MSC were divided into two groups: (1) unmodified MSC (no transfection) and (2) HATH1-transfected MSC.

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Transfection with 0.5 μg HATH1 plasmid DNA was performed according to previous published protocols using a 4D Nucleofector (Lonza Group Ltd.) at a ratio of 100,000 cells per transfection (Mellott et al., 2015). Cells were cultured for seven days in a 2D 6-well plate (BD Biosciences) on decellularized cochlea sections. Cells were imaged at 7 days following transfection and seeding (Mellott et al., 2015).

2.6. Immunohistochemistry

After paraffin embedding, samples were deparaffinized in two washes of xylene, and gently rehydrated over 30 min with a series of decreasing ethanol solutions in PBS. Samples were incubated in 3% hydrogen peroxide in PBS for 10 min at room temperature to block endogenous peroxidase activity. Afterward, samples were washed twice for 5 min each with PBS. Samples were incubated twice with 0.2% Tween 20 (Sigma-Aldrich, St. Louis, MO) in PBS for 10 min each. Afterward, samples were washed with PBS twice. Samples were blocked for 1 h with primary blocking solution (6% bovine serum albumin (BSA) (Fisher Scientific, Pittsburgh, PA), 10% normal donkey serum (NDS) (Life Technologies) in PBS). Afterward, samples were incubated overnight at 4 °C with a dilution of 1:100 primary antibody (Rabbit Anti-Human MYOSIN VIIA (MYO7A) (Cat No: NBP1 84266; Novus Biologicals, Littleton, CO). The following day, samples were washed with PBS three times for 5 min each. Samples were incubated overnight with a secondary antibody conjugated to a fluorophore (Donkey Anti-Rabbit Qdot 525 (Cat. No. Q22074; Life Technologies)) at a dilution of 2:100 secondary antibody to secondary blocking solution. The following day, samples were washed three times with PBS for 5 min each, and counterstained with DAPI (Life Technologies) according to manufacturer's instructions. Afterward, glass coverslips were mounted over samples with Histomount Mounting Solution (Life Technologies) according to manufacturer's instructions.

2.7. Treatment of dissociated spiral ganglion neurons with carboxymethylglucose sulfate

After decapitation and removal of the scalp, the skull base was bisected. Under microscopic view, the membranous cochlea was removed and ganglia were collected in an Eppendorf vial filled with ice-cold HEPES buffer. After centrifugation, HEPES buffer was replaced with HBSS (Hank's balanced buffered solution, Gibco Invitrogen, Germany) containing 0.01% trypsin (Biochrom GmbH, Germany) and 0.01% DNase I (Roche, Germany) for enzymatic dissociation. This was performed using a maximum total of 30–40 ganglia/2 mL dissociation solution incubated at 37 °C for 15 min with intermediate shaking. After removing the dissociation solution, 200 μL FCS (foetal calf serum; Invitrogen, Germany) were added in order to stop enzymatic dissociation. Short spin centrifugation was performed. The supernatant was removed and the cell pellet was washed for three times and re-suspended with serum-free culture medium. The serum-free culture medium consists of Panserin 401 (Pan Biotech, Germany) supplemented with penicillin (30 U/mL, Biochrome GmbH, Germany), 1 M HEPES-buffer (25 mM; Invitrogen, Germany), glucose (40%/mL, B. Braun, Germany), insulin (4 mg/mL, Biochrome, Germany) and N2-supplement (3 μg/mL; Invitrogen, Germany).

Cells were dissociated mechanically as previously described (Kaiser et al., 2013). The cell number was determined by counting the cells in a Neubauer chamber (Brand GmbH, Germany). Dead cells were identified using trypan blue (Sigma Aldrich, Germany) and were excluded from counting. Wells of a 48 well-plate (TPP, Switzerland) were coated with poly D/L-ornithine (0.1 mg/mL; Sigma-Aldrich) and laminin (0.01 mg/mL; natural from mouse, Life Technologies, Carlsbad, USA) and were seeded with 100 μL of the dissociated spiral ganglion cell solution, containing 2×10^4 cells. Cells were treated with different amounts of carboxymethylglucose sulfate (Cacicol®; Thea Pharma GmbH, Germany) and cultured in a final volume of 200 μL for 48 h. The following concentrations of carboxymethylglucose sulfate were tested: 25 μg/mL, 10 μg/mL, 5 μg/mL, 2 μg/mL, 1 μg/mL and 0.5 μg/mL. Cells treated with brain-derived neurotrophic factor (BDNF; 50 ng/mL) served as positive control whereas cells cultured in serum-free culture medium without the addition of any growth factor served as negative control.

After 2 days at standard culture conditions (37 °C, 5% CO_2), the spiral ganglion cells were fixed with acetone/methanol (1:1; AppliChem/Merck, Darmstadt, Germany). Acetone/methanol fixed cells were stained with a mouse 200 kD neurofilament antibody (clone RT97; Leica Biosystems, Wetzlar, Germany), a secondary biotinylated anti-mouse antibody and ABC complex solution as described in detail previously using the Vectastain® Elite® ABC Kit (Wefstaedt et al., 2005). The antibody complexes were visualized by the addition of diaminobenzidine (Peroxidase Substrate Kit DAB; Vector Laboratories Inc., Burlingame, USA). Three independent experiments were performed and each condition was tested in triplets resulting in a total of N = 3; n = 3 for each condition.

2.8. Treatment of spiral ganglion micro-explants with carboxymethylglucose sulfate

After full-length preparation, each spiral ganglion was dissected in parts of about 0.2–0.4 mm. Three to four pieces were transferred in a poly D/L-ornithine (0.1 mg/mL; Sigma-Aldrich) and laminin (0.01 mg/mL; natural from mouse, Life Technologies, Carlsbad, USA) coated 24 well-plate and incubated in serum-free culture medium for 5 days (37 °C, 5% CO_2). Here, we tested again carboxymethylglucose sulfate concentrations of 25 μg/mL, 10 μg/mL, 5 μg/mL, 2 μg/mL, 1 μg/mL and 0.5 μg/mL, as well as 25 μg/mL carboxymethylglucose sulfate with BDNF (50 ng/mL). Micro-explants treated with BDNF (50 ng/mL) served as positive control and micro-explants cultured in serum-free culture medium served as negative control. After 5 days, the micro-explants were fixed with acetone/methanol. The acetone/methanol fixed explants were also labelled with the mouse 200 kD neurofilament antibody (clone RT97; Leica Biosystems, Wetzlar, Germany), the secondary biotinylated anti-mouse antibody and ABC complex solution as described in detail previously (see section above). By the addition of diaminobenzidine (Peroxidase Substrate Kit DAB; Vector Laboratories Inc., Burlingame, USA) the antibody complexes were visualized. Each condition was tested in triplets and three independent experiments were performed resulting in a total of N = 3.

2.9. RGTA as an adhesion matrix for spiral ganglion neurons

In order to adhere and survive, spiral ganglion neurons need an adhesion matrix. Therefore, experiments with dissociated spiral ganglion cells are usually performed in poly D/L-ornithine and laminin coated wells. In order to test if carboxymethylglucose sulfate provides a sufficient adhesion matrix for spiral ganglion neurons, different concentrations of carboxymethylglucose sulfate were mixed with calcium gluconate in order to create an alternative coating of the culture well plates. Uncoated wells served as control. Carboxymethylglucose sulfate (Cacicol® as RGTA; 100 μg/mL) was mixed with calcium gluconate (10% weight per volume; purchased from RegenLab) in the following concentrations: 50% calcium gluconate and 50% carboxymethylglucose sulfate (equals 50 μg/mL) as well as 33% calcium gluconate and 66% carboxymethylglucose sulfate (equals 66 μg/mL). In addition, a solution of 49.5%

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carboxymethylglucose sulfate (equals 50 μg/mL), 49.5% calcium gluconate and 1% BDNF (50 ng/mL) was tested. Furthermore, a mixture consisting of 33.3 μg/mL carboxymethylglucose sulfate and 66.6 μg/mL commercially available fibrin glue (Tisseal, Baxter) was also analyzed.

For the coating of a 24 well-plate, 100 μL of each solution was pipetted per well and incubated at 37 °C for 10 min. Subsequently, 2×10^4 of the dissociated spiral ganglion cells were seeded in each well. Cultivation was performed for 2 days. Thereafter, cells were fixed with aceton/methanol (AppliChem/Merck) 1:1 and were stained for 200 kD neurofilament as described above.

2.10. Effect of carboxymethylglucose sulfate on bone marrow-derived mesenchymal stromal cells

Mesenchymal stromal cells were isolated from human bone marrow. The donor's age was between 22 and 59 years and all were male. The cells were pooled to avoid intrapersonal differences. Mesenchymal stromal cells at passage 6 were seeded in 48 well-plates with 1×10^5 cells per well and incubated for 1 week. Culture medium was changed every 2–3 days and was supplemented with the defined concentration of carboxymethylglucose sulfate. The culture medium consists of 78 mL DMEM (Biochrome GmbH), 1 mL Penicillin (30 U/mL, Biochrome GmbH), 20 mL FCS Hyclone (Thermoscientific) and 1 mL HEPES-buffer solution (Invitrogen) for 100 mL of culture medium. We tested n = 3 series – as triplets - with a solution series of carboxymethylglucose sulfate featuring the following concentrations: 25 μg/mL, 10 μg/mL, 5 μg/mL 2 μg/mL, 1 μg/mL and 0.5 μg/mL.

The stem cells were incubated for one week with daily microscopic control and then fixed with acetone/methanol 1:1 and stained with trypan blue 10% (Biochrom AG).

2.11. Statistical analysis and bioinformatics

Statistical analysis was performed with Prism 6 (GraphPad, La Jolla). All results were.

validated by using one-way ANOVA followed by Bonferroni's multiple comparison test. P values less than 0.05 were considered to be statistically significant. All quantitative data represent the means of at least three independent approaches (N), including at least triplicates of each sample (n). Error bars in the figures indicate the standard error of the mean. Levels of significance are indicated as follows: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Interaction of heparan sulfate with key biological pathways was evaluated using Ingenuity Pathway Analysis (IPA) software (Qiagen Bioinformatics).

3. Results

3.1. Human MSC delivery to the inner ear of Guinea pigs

To examine the survival and integration of human MSC following transplantation, we performed native whole mount preparation of the cochleae immediately after as well as evaluation of paraffin embedded cochlear sections. Cochleae were examined 48 h (n = 4) and 7 d (n = 2) following transplantation in native whole mount specimens and at 48 h (n = 4) and 7 d (n = 4) following transplantation in paraffin embedded sections. Under fluorescence microscopy, cells were found throughout all cochlear compartments but appeared to concentrate in the lateral wall. Fig. 1A shows an example of integration of MSC 48 h after transplantation into the scala tympani in paraffin embedded sections of the cochlea. Cells can be identified due to the GFP signal induced by prior transfection with lentivirus. Only 2 of the six specimens evaluated 7 days after cell delivery showed the presence of GFP positive MSCs. A view of the stria vascularis obtained from fresh whole mount preparations is depicted in Fig. 1B demonstrating GFP expressing cells.

3.2. Effect of decellularized cochlear scaffolds on MSC

Mouse cochleae were decellularized, decalcified and subsequently seeded with human MSC from umbilical cords. As depicted in Fig. 2, decellularization resulted in the elimination of cells and nuclei (lack of DAPI staining) within the cochlea (Fig. 2A and B). Injection of MSC into the cochlear scaffold resulted in cells engrafting within the lateral wall and to lower degrees in the modiolus and spiral ligament (Fig. 2C; blue DAPI stained nuclei). Consistency of delivery to the cochlea was evaluated by repeated injection of a set number of MSC into the decellularized scaffold. This resulted in broad distribution of cells. After seven days of culture, a mean of 232 ± 15.4 cells was observed in each cochlea perfused with MSC, and cells engrafted within the lateral wall (Fig. 3). Cells only engrafted onto the cochlear scaffold and no cell clusters were observed within the cochlear spaces.

The impact of cochlear matrix on the phenotype of genetically modified stem cells was evaluated by injecting human MSC that had been pre-treated with transfection of hath1 and hes1/5 RNAi (Sharif et al., 2007; Parker and Cotanche, 2004) in decellularized cochleae. The pre-treatment of MSC led to a decreased number of cells engrafting within the cochlear scaffold when compared to the delivery of native untreated MSC (Fig. 4 vs. Fig. 3). The rare cells that engrafted stained positive for myosin VII or neurogenin 1 (Fig. 4) indicating a starting differentiation process. In order to investigate whether the culture conditions of 3D cochleae did influence the engraftment, the basilar membranes harbouring the organ of Corti were harvested from decellularized cochleae and placed in 96 well plates. In this this model, MSC were seeded at a higher density and were allowed to attach to the decellularized matrices for 7 days without any shaking. Transfected as well as non-transfected cells attached to the decellularized matrix (Fig. 5). Non-transfected cells did not express any hair-cell like markers after seven days, but showed a good proliferation on the matrix. Expression of sox2, an early differentiation marker, was detected in the cells that were pre-treated with hath1 (Fig. 5). Only cells that were transfected with hath1 and hes1/5 RNAi expressed both sox2 and myosin VII.

3.3. Carboxymethylglucose sulfate and spiral ganglion neuron survival

Cultures of cells isolated from the spiral ganglia of neonatal Sprague-Dawley rats were treated with different concentrations of carboxymethylglucose sulfate. Controls were either treated with BDNF-containing medium (BDNF control) or cultured in serum-free spiral ganglion cell medium without any further supplements (medium control). The spiral ganglion cell culture consists of different cells such as neurons, glial cells and fibroblasts. After a cultivation period of 48 h and a neuron-specific staining as described above, the survival of the neurons was quantified.

Cells treated with BDNF (50 ng/mL) showed a significantly increased survival of spiral ganglion neurons ($p < 0.001$) when compared to the medium control (Fig. 6). A significantly increased number of surviving spiral ganglion neurons with a mean of 38 ± 16 neurons (a more than doubled increase when compared to the medium control without supplements) was observed when the cells were treated with a concentration as low as 0.5 μg/mL carboxymethylglucose sulfate. Increasing the concentration of carboxymethylglucose sulfate led to a concentration dependent improvement of neuronal survival (up to a mean of 52 ± 17 neurons at a concentration of 25 μg/mL Cacicol®). When compared to the

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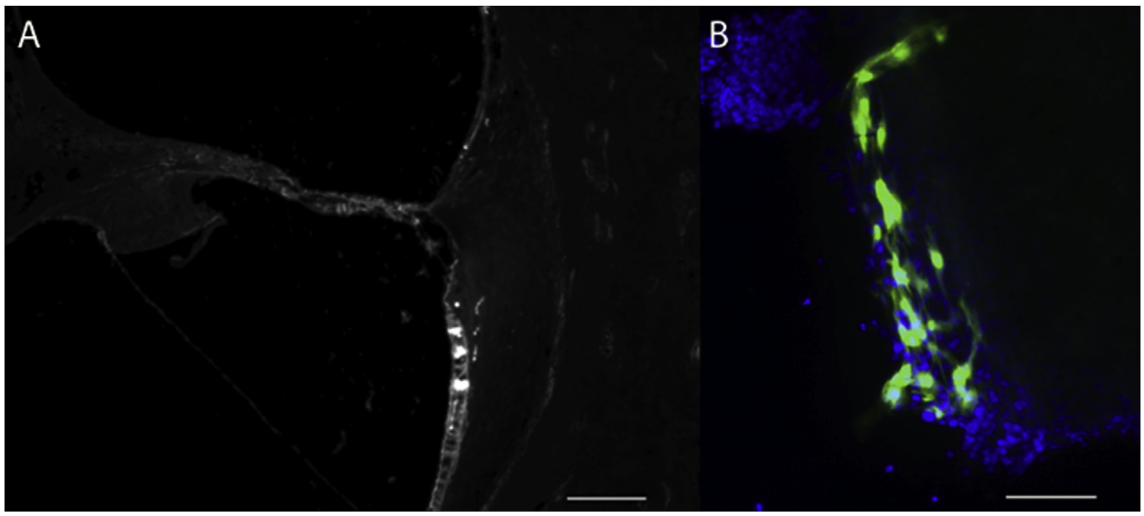

Fig. 1. Distribution of GFP labelled mesenchymal stromal cells in the guinea pig cochlea after delivery of 5×10^4 cells through a basal turn cochleostomy. GFP labelled MSC can be seen throughout the inner ear but are predominantly found in the stria vascularis. At 2 days post delivery, GFP positive cells are integrated in the stria (arrow) in a paraffin embedded and sliced cochlea (A). A high power view of the stria vascularis of a whole mount preparation after fixation at 7 days post delivery shows groups of GFP positive cells and (B). Scale bar = 100 μm.

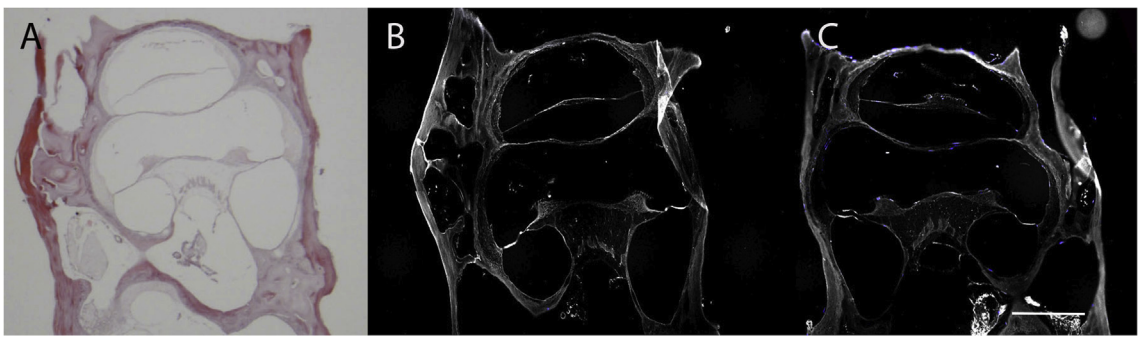

Fig. 2. Whole mount cochlea of mice after decellularization and delivery of human MSC from umbilical cords. Hematoxilin and eosin stained section of a cochlear that has undergone decelularization (A). Cell nuclei were stained with DAPI (Blue). Evaluation of fluorescent staining (405 nm) shows no remaining nuclei in the tissue (B). After delivery of human MSC cells, 242 cells with nuclei can be seen, with some cells engrafting into the lateral wall of the cochlea (C). Scale Bar = 100 μm.

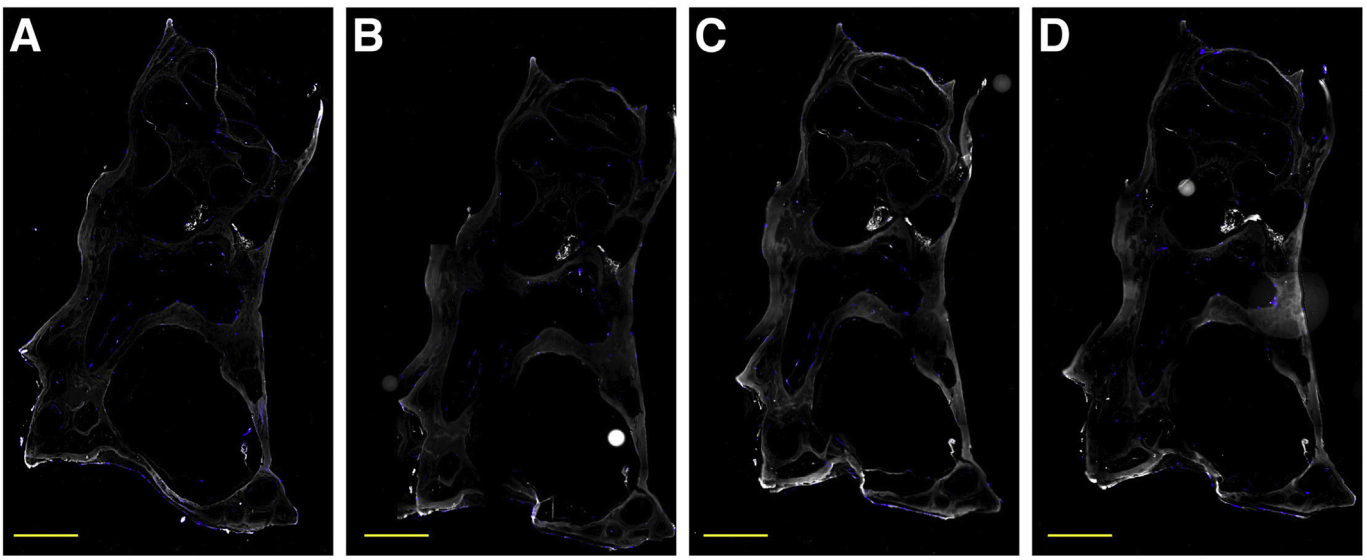

Fig. 3. Whole mount cochlea of mice comparison of implanted human MSC from umbilical cords. Human MSC were perfused through the oval window of the cochlea. Confocal image of whole-mount cochleae displayed clear identification of approximately 200 cells or more per cochlea slice. Panels A–D represent different cochleae through which MSC were perfused. A) 242 cells. B) 211 cells. C) 245 cells. D) 230 cells. Cell nuclei were stained with DAPI (Blue). Scale Bar = 100 μm.

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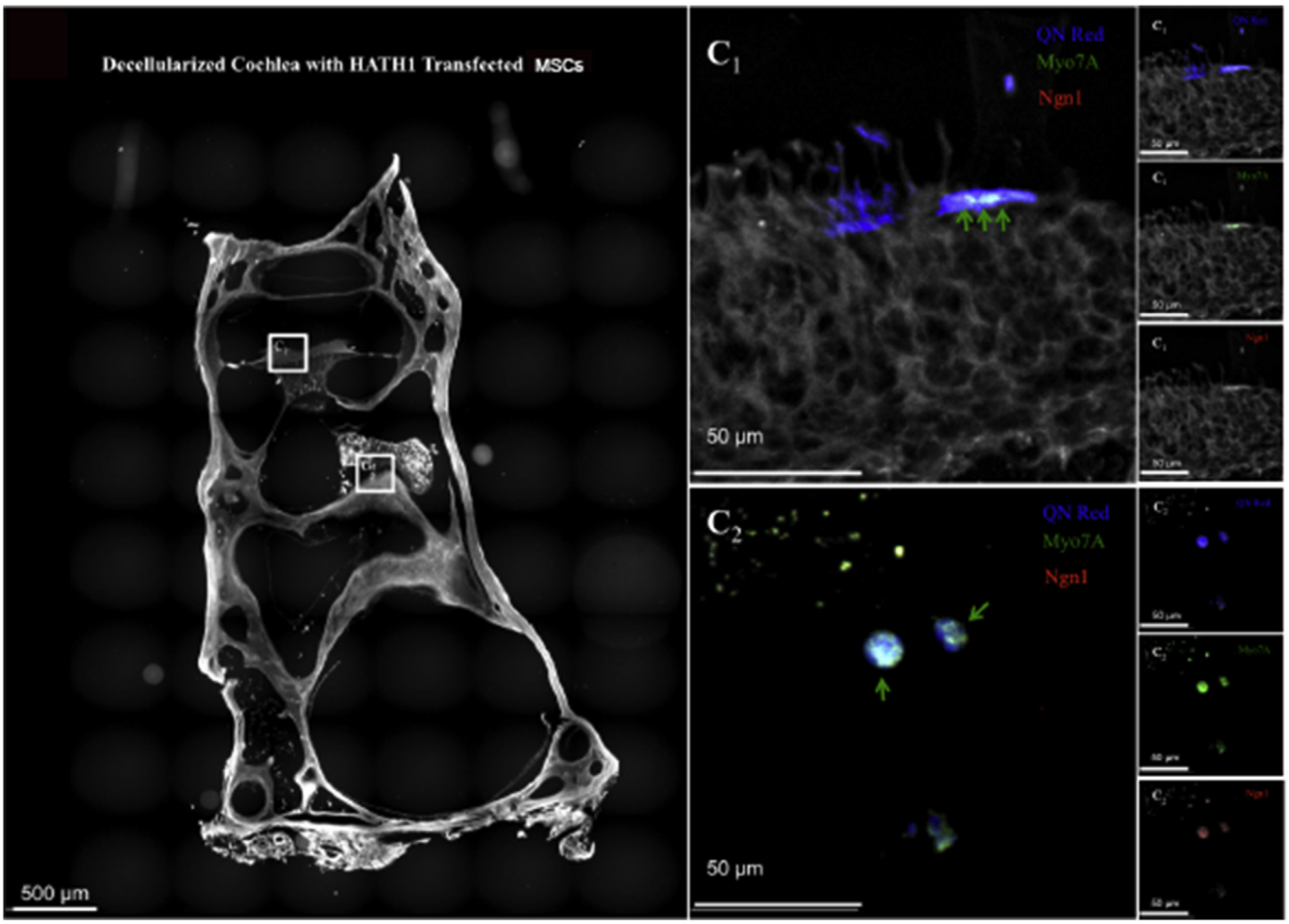

Fig. 4. Implantation of human MSC in decellularized and decalcified cochlea of mice. Cells that were transfected with HATH1 and perfused into the decellularized and decalcified cochlea (DC) were positively identified and showed positive expression of the hair cell marker, Myosin VIIA (Myo7A), and the neuronal differentiation marker, Neurogenin 1 (Ngn1) after seven days of culture. Myo7A was labelled with Qdot 525 (Green). Ngn1 was labeled with Qdot 605. Cell nuclei were stained with QNuclear Red (QN Red) (Blue). Blue arrows identify cells. Green arrows identify positive expression of Myo7A. White arrows correspond to labelled anatomical parts. MSC = human mesenchymal stromal cells. Yellow Scale Bar = 500 µm. White Scale Bar = 50 µm.

medium control, concentrations from 5 µg/mL to 25 µg/mL significantly increased neuronal survival ($p < 0.05$ to $p < 0.001$). By contrast, spiral ganglion cultures subjected to lower carboxymethylglucose sulfate concentrations (0.5 µg/mL, 1 µg/mL, 2 µg/mL) revealed no significant improvement in neuronal survival when compared to the untreated control. The highest neuronal survival, however, was observed after treatment with recombinant growth factor BDNF resulting in a mean of 82 ± 12 neurons per well ($p < 0.01$).

3.4. RGTA and spiral ganglion micro-explants

For cultivation of the micro-explants, the spiral ganglia were micro-dissected in small pieces as described above. After 5 days of incubation, all explants treated with carboxymethylglucose sulfate attached to the well plates. Of the overall 12 micro-explants without any treatment, only 4 adhered. Interestingly, 25 µg/mL, although neuroprotective on dissociated spiral ganglion neurons, did not affect any significant outgrowth of the neurites from the explants (Fig. 7A). At a concentration of 1 µg/mL carboxymethylglucose sulfate, however, the micro-explants (Fig. 7B) showed an enlarged (but not significantly) neuronal radius around the explant with more out sprouting neurites detectable after 5 days. A super-additive effect was observed after treatment of the explants with 25 µg/mL carboxymethylglucose sulfate in combination with 50 ng/mL BDNF resulting in massive neuronal outgrowth (Fig. 7C). Compared to the other tested conditions, the outgrowth was highly significant (Fig. 7E; 25 + BDNF vs all other conditions; $p < 0.0001$). Treatment with only BDNF resulted in a less pronounced neurite outgrowth from the explants (Fig. 7D), but this was significantly increased when compared to the medium control (Fig. 7E; $p < 0.01$). Quantifying the neuronal outgrowth, there was no statistically significant increase in the explants treated with the carboxymethylglucose sulfate compared to the medium control without any supplements. However, the combination of BDNF with carboxymethylglucose sulfate leads to a highly significant increase of neuronal outgrowth (Fig. 7C, E).

3.5. Carboxymethylglucose sulfate as a matrix for neuronal cells

In order to identify the use of carboxymethylglucose sulfate as matrix for neuronal adhesion, it was used as alternative to coatings with ornithine/laminin in our cell culture model. Cell adhesion and neuronal outgrowth of dissociated spiral ganglion cells was obtained for all conditions using carboxymethylglucose sulfate and calcium gluconate as coating of the cell culture well plates.

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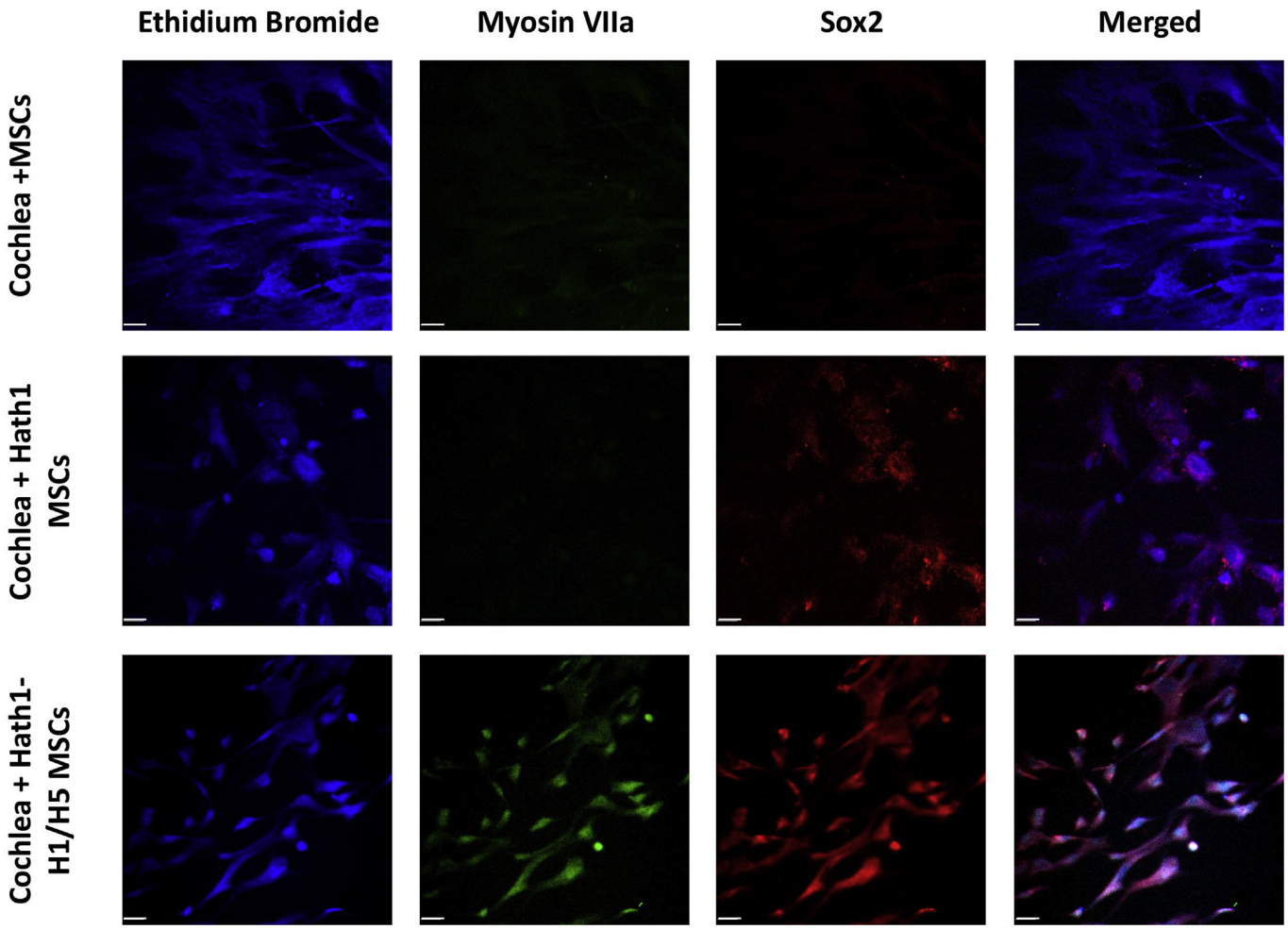

Fig. 5. Culture of transfected human MSC grown on decellularized murine cochlear tissue. Human MSC were transfected with a plasmid containing the human homolog of ATOH1 (HATH1) or a plasmid containing both ATOH1 and siRNA against HES1 and HES5 (HATH1-H1/H5). Transfected MSC were cultured in decellularized cochlear tissue from C57BL/6 mice. SOX2 was positively identified in HATH1-transfected MSC cultured on decellularized cochlear tissue. However, HATH1-H1/H5 transfected MSC showed an intense presentation of MYO7A and SOX2 when cultured in decellularized cochlear tissue four days post transfection. Cell nuclei were stained with Ethidium Bromide (Blue). Cells were immunostained for MYO7A (Green), a motor protein found on stereocilia in hair cells, and SOX2 (Red), a marker for neuronal lineage commitment usually up-regulated before hair cell differentiation. The data indicate that the cochlear tissue may have an effect on the ability of MSC to differentiate toward a hair cell phenotype. MYO7A = Myosin VIIA. Scale Bar = 20 μm.

In uncoated wells, neurons did not adhere. Coating with RGTA (Fig. 8A,B,C) allowed neurons to adhere. However, outgrowing neurites were only observed for 66% RGTA and 50% RGTA with BDNF (Fig. 8B and C). Highest adhesion and survival was detected after coating with carboxymethylglucose sulfate at a concentration of 66% (Fig. 8D). At lower concentrations of carboxymethylglucose sulfate (50%) in the coating solution, a mean of 16 ± 5 neurons still adhered (Fig. 8D). Furthermore, a coating solution containing 50% RGTA supplemented with BDNF (50 ng/mL) was tested and no additive effect of both substances (mean of 17 ± 6 neurons) was observed in terms of support of adhesion when compared to 50% RGTA without BDNF (Fig. 8D).

3.6. Effect of carboxymethylglucose sulfate on bone marrow-derived MSC

Mesenchymal stromal cells were seeded at defined densities and treated with carboxymethylglucose sulfate for 1 week. Cells located within a 1 mm^2 square were identified and quantified under light microscopy. Human MSC showed increased proliferation after treatment with carboxymethylglucose sulfate. The highest proliferation rate was obtained after treatment with RGTAs at a concentration of 10 μg/mL (238.56 cells per 1 mm (Bordignon and Roncarolo, 2002)) (Fig. 9). There was an increased proliferation of MSC due to RGTA when compared with the treatment with BDNF (196.22 cells per 1 mm (Bordignon and Roncarolo, 2002); $p < 0.001$). Spontaneous proliferation of MSC in culture medium without the addition of any factors resulted in the survival of 65.11 cells per 1 mm (Bordignon and Roncarolo, 2002) (Fig. 9). Compared to this control (medium control), any experimental condition led to an increased density of the cells per 1 mm (Bordignon and Roncarolo, 2002) ($p < 0.001$).

4. Discussion

Transplantation of cells for a sustained and local drug delivery is an emerging technology in various fields. A range of studies have shown that transplantation of stem cells into the ear results in distinct patterns of cell adhesion and engraftment (Kamiya et al., 2007; Ma et al., 2016). The results presented herein and obtained from decellularized cochlear studies and *in vivo* delivery studies suggest that attachment of stem cells occurs in distinct patterns

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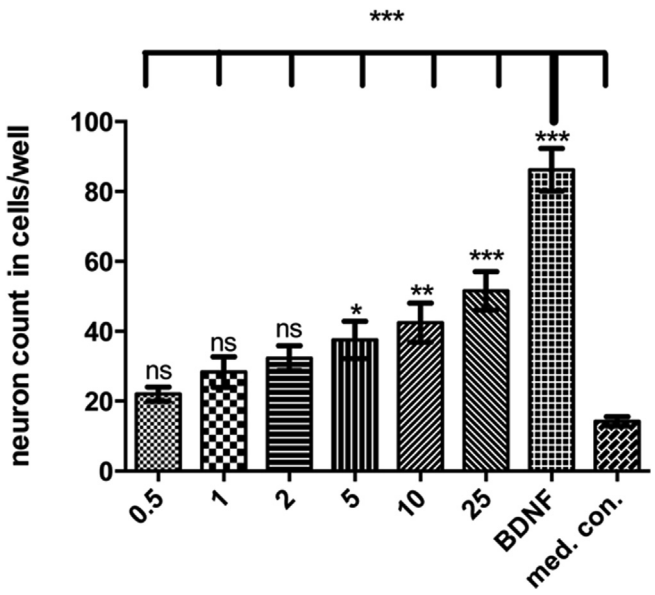

Fig. 6. Survival effect of carboxymethylglucose sulfate on dissociated spiral ganglion cells isolated from neonatal rats. Carboxymethylglucose sulfate showed a dose-dependent survival effect on spiral ganglion cells compared to cells in medium alone. Survival effects were less than that seen with BDNF supplementation.

making investigation of the microenvironment that induces this effect important. Most studies additionally note that wide migration of MSC takes place and factors such as injury of tissue and delivery approach can modulate the patterns of cell engraftment and function (Santi and Johnson, 2013; Liu et al., 2015; Bettini et al., 2017).

The importance of the components of the basement membrane and structural components of the cochlea for both development and cell transplantation has previously been recognized (Cosgrove et al., 1996). Immunohistochemical studies have shown that the basilar membrane consists mainly of fibronectin and atypical collagens. Heparin is distributed in the osseous spiral lamina, in the perineural areas and in the stria vascularis (Cosgrove et al., 1996). This suggests that different areas of the cochlea may have different substrates that could affect binding properties for delivered stem cells and potentially affect their behaviour. The important function of matrix contact in three-dimensional culture has been widely acknowledged in biomedical engineering and decellularized scaffolds as well as 3D cell cultures are being investigated for the creation of artificial organs. Since preliminary studies have shown that mesenchymal stromal cells may be used to mitigate cochlear injury, matrix factors that affect delivery of cells into the ear were investigated in the present study. Options for delivering cells include free injection into the perilymph, injection directly into Rosenthal's canal, injection into the lateral wall of the cochlea or delivery of cells on a variety of scaffolds including cochlear implants. Alternative options also include pre-treating cells with matrix or matrix like factors (RGTA) or modifying the cells being transplanted with transfection with a plasmid or viral vector. Depending on the pre-treatment, the *ex vivo* modification of cells can result in the delivery of drugs, the differentiation of cells into a neuronal-like or hair cell-like state. In this study, the impact of matrix factors was investigated on both spiral ganglion cells and mesenchymal stromal cells. Based on the obtained results, glycosaminoglycans (RGTA) could induce moderate survival effects on spinal ganglion cells in a dose-dependent fashion and were able to support neurite outgrowth and adhesion of neurons. When attempting to use stem cell therapy to replace spiral ganglion cells, combining stem cells with substances like RGTA could aid engraftment when specifically targeting the spiral ganglion.

Biological scaffolds present an interesting option for tissue engineering and offers a natural environment for cellular repopulation (Rana et al., 2017). However, this is a crucial step and even in more favourable organs such as the kidney, sufficient results on complete renal scaffold repopulation have not been reported yet (Figliuzzi et al., 2017). Using the cochlea as scaffold for repopulation experiments is a promising approach in order to gain insight into mechanisms that lead to integration and differentiation of transplanted cells. For cochlear implantation, the scaffold could be built onto the implant itself (Ceyssens et al., 2017). Alternately, pharmacological support of transplanted cells by pre-treating them with matrix or matrix like factors is another option.

During embryonic development, a variety of growth factor mediated signalling pathways are involved in the development (Lin, 2004). These signalling pathways depend on heparan sulfate. The receptor-ligand interactions are directed by modification of heparan sulfate by sulfonated heparan sulfatases. They are expressed in complex, dynamic spatial and temporal pattern in the mammalian inner ear (Freeman et al., 2015). For example, mice lacking the expression of sulfatase 1 and 2 develop supernumerary hair cells suggesting that these are important regulators. Endogenous sulfatases fine-tune cellular responses to several signalling pathways by remodelling of the heparan sulfate structure. Exogenously applied heparan sulfates could potentially be used to support cells transplanted into the inner ear. In the present study, neuronal survival could be increased by treating spiral ganglion cells with carboxymethylglucose sulfate, a heparan sulfate analogue, but this treatment also improved adhesion of neuronal cells. In addition, it seems to offer structural support also to MSC since these showed improved survival and proliferation in the presence of carboxymethylglucose sulfate. Heparan sulfate or its analogues are a binding and activation molecule for already present endogenous growth factors and could be also used to stabilize factors released by cells that are used as drug delivery systems (Fig. 10). In addition to structural support, heparan sulfate activates the Wnt/Ca^{2+} pathway in the embryonic development of vertebrates (Lin, 2004). This pathway may also be responsible for the mediation of neuronal survival and could be one of the pathways that needs further consideration in future studies. In smooth muscle cells, heparin sulfates modulate the collagen-type expression via the fibroblast growth factor (FGF) and transforming growth factor (TGF)-beta pathways (Alexakis et al., 2004). Both pathways are involved in neuronal survival of the spiral ganglion and FGF and TGF-beta could possibly also mediate the herein observed neuroprotection (Kaiser et al., 2013; Wang et al., 2009).

Treating micro-explants with a combination of BDNF and carboxymethylglucose sulfate leads to an additive effect in terms of increased neurite outgrowth. In addition, migration of cells out of an existing cell-complex was promoted due to the treatment with carboxymethylglucose sulfate. This observation underlines the basic idea of the “matrix-therapy”, i.e., preserving and restoring the natural cell environment, the extracellular matrix. Based on the results, it can be assumed that heparan sulfates stimulate cell survival and are able to restore the matrix architecture.

In combination with calcium gluconate, carboxymethylglucose sulfate provides an adequate adhesion matrix for the attachment and outgrowth of spiral ganglion neurons emphasising the need of structural support. Our results are corroborated in an experimental model of ischemic and denervated muscle, where heparan sulfates enhanced structural support and led to an increased survival of the muscle fibres (Desgranges et al., 1999). Similar results were observed in pigs where heparan sulfates were beneficial for the treatment of acute myocardial infarction (Yamauchi et al., 2000).

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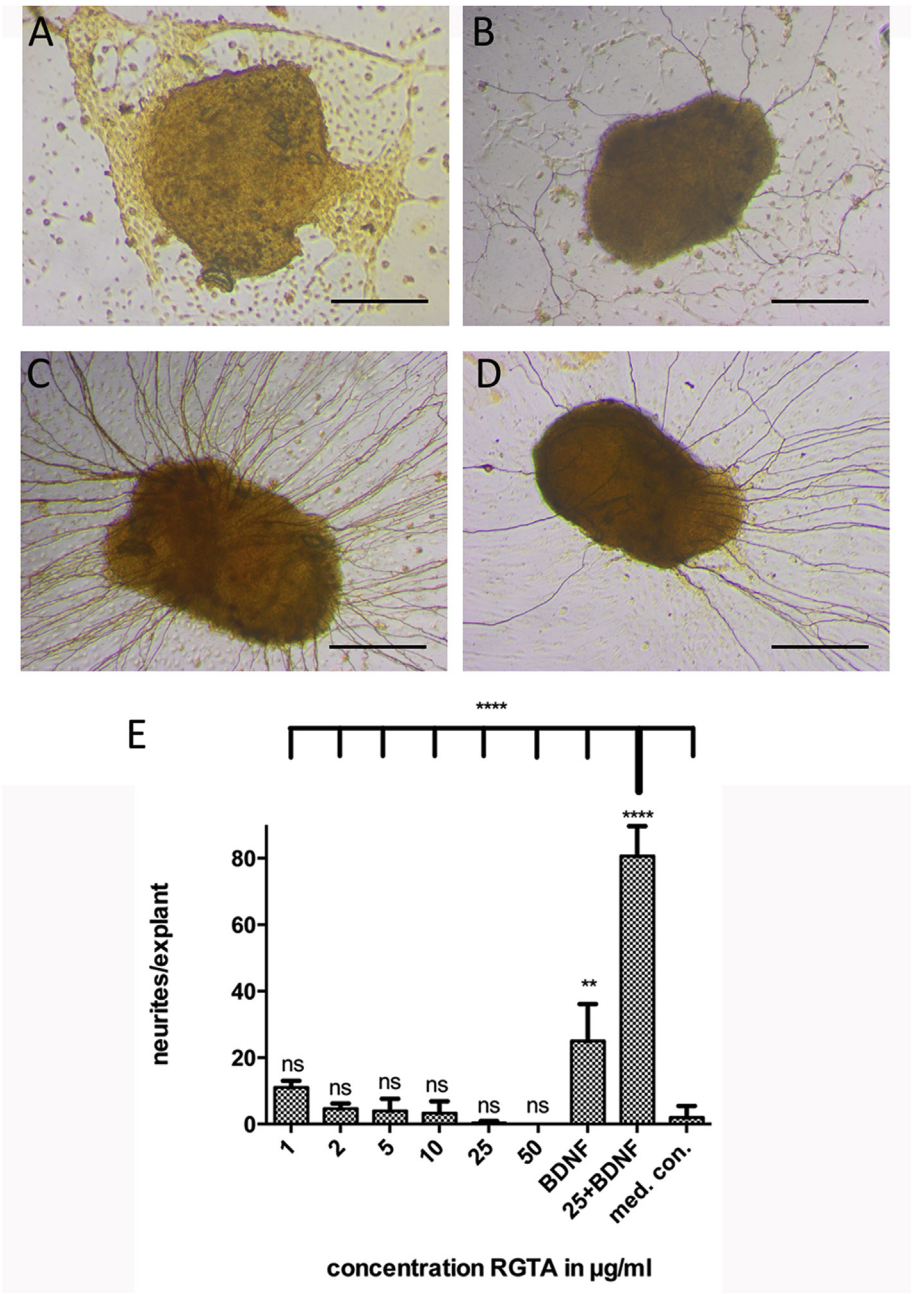

Fig. 7. Carboxymethylglucose sulfate promotes neurite outgrowth. Spiral ganglion micro explants from neonatal rats were cultured with carboxymethylglucose sulfate alone (25 µg/mL (A) and 1 µg/mL (B)), BDNF alone (50 ng/mlL; D), or in combination (C). The combination of matrix factors with BDNF yielded optimal neurite outgrowth suggesting that a combination of signals is needed. (E) Counted neurites per explant. Scale Bar = 200 µm.

They are even potent in epidermic repair after coagulation necrosis of the skin (Zakine et al., 2011; Georgoudis et al., 2015). Increased neurite outgrowth was observed when BDNF was added to the cultures containing heparin sulfate. Spiral ganglion neurons need specific coating or a special matrix in order to be cultured and maintained *in vitro*. The results of the present study indicate that heparan sulfates can function as supportive agents and may also offer structural support to neurons. This effect may be used *in vivo* in order to guide matrix remodelling towards a regenerative state despite putative damage that occurs during cochlear implantation. Incorporating heparan sulfates into decellularized cochleae prior to cell perfusion may help to validate the idea of matrix remodelling and proliferation of MSC in future experiments. In addition, heparan sulfates could be utilized to support the release and stability of growth factors applied using a cell-based therapy approach.

Recruitment and proliferation of the endogenous stem cells is another approach in order to warrant the regenerative state. In the inner ear, mesenchymal stromal cells reside and may be recruited when damage occurs. Based on the herein presented results, heparan sulfates exert proliferative effects on human mesenchymal stromal cells. In this case we can hypothesize that the contact with matrix is required for optimal growth of mesenchymal stromal cells. This may also explain why optimized binding and migration to areas that express these heparin like substances is observed. Interestingly, evaluation of mesenchymal stromal cells in

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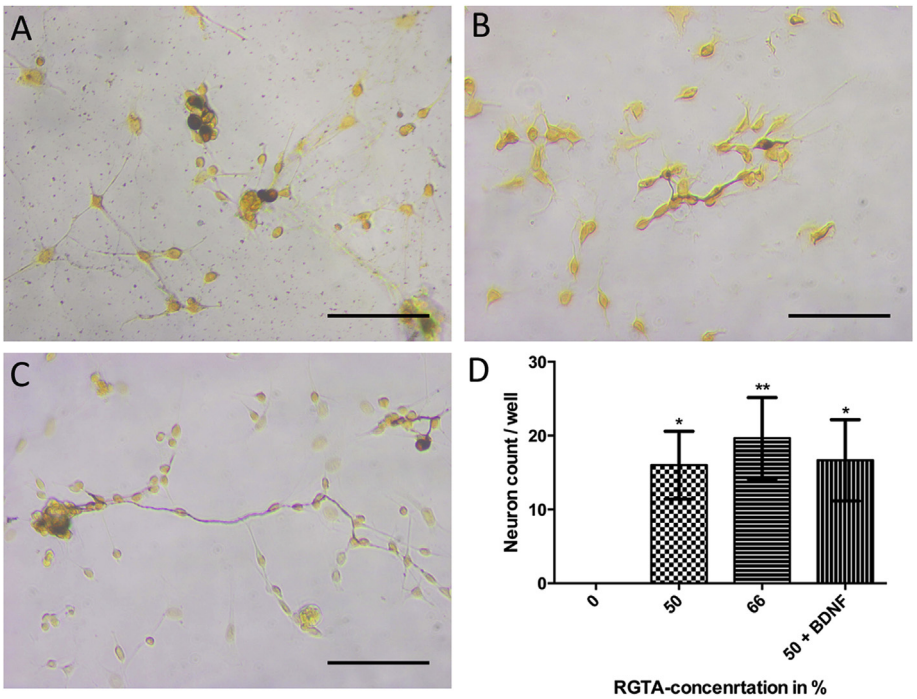

Fig. 8. Evaluation of carboxymethylglucose sulfate mediated neuronal adhesion. Neonatal rat spiral ganglion neurons were cultured in wells coated with carboxymethylglucose sulfate (A = 50% RGTA, B = 50%RGTA with BDNF, C = 66%). A) The presence of carboxymethylglucose sulfate clearly affected adhesion of neurons. Scale Bar = 200 μm.

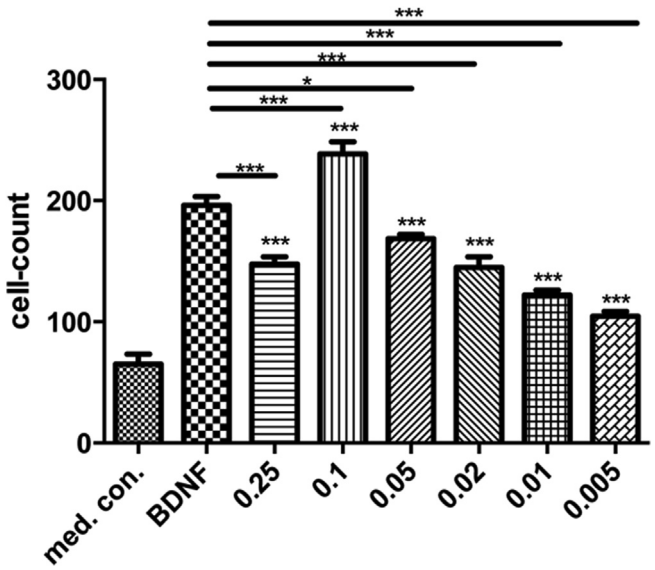

Fig. 9. Proliferation effects of carboxymethylglucose sulfate on human MSC. Both BDNF and carboxymethylglucose sulfate effected proliferation of MSC *in vitro*. Interestingly, high concentrations of carboxymethylglucose sulfate induced less proliferation than BDNF suggesting that there is an optimal dose effect.

decellularized cochlear scaffolds show distribution of the cells predominantly to areas with high levels of heparin expression. Based on these observations, we could also evaluate if heparan sulfates applied without cell therapy can mobilize inner ear resident MSC for improved (scar-less) healing after trauma to the inner ear.

Based on the herein presented results, a physiological repair of damaged structures due to support of the matrix environment is conceivable after cochlear implantation. Ophthalmic application of heparan sulfates in humans since years as well as toxicological evaluation showed no side effects at moderate doses (up to 50 mg/kg body weight per day) making translation from animal models to clinical trials less complex (Charef et al., 2007). Increased doses may be associated with side effects since they significantly prolonged the clotting time and reduced the activities of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase (Charef et al., 2007). A clear advantage of RGTA is the half-life time of the substance that is quite long compared to traditional growth factors. Therefore, heparan sulfates could be proposed as promising therapeutic agents for future bio-activated cochlear implant therapy for neuronal and structural support as demonstrated in our *in vitro* model.

When considering cell-based approaches and the transplantation of cells to the cochlea, structural support seems necessary in order to enable sufficient survival and integration of the cells into the host tissue. That the cochlear lateral wall, especially the stria vascularis and the spiral ligament, seem preferable sites for integration has been demonstrated also by other groups (Hildebrand et al., 2005; Sullivan et al., 2011). This preference could be related to the high content of heparan sulfate of these regions (Cosgrove et al., 1996). In addition to the generation of new cells by mitosis of cochlear fibrocytes around the injured area (Kamiya et al., 2007), bone marrow-derived cells have the potential to engraft into the lateral wall and differentiate into the cochlear fibrocytes (Lang et al., 2006). Also, promoting the homing of MSC, e.g., by the use of monocyte chemotactic protein (Kamiya, 2015) or deferoxamine (Peyvandi et al., 2018) may improve the engraftment of MSC to the inner ear.

Differentiation into fibrocytes was also observed after transplantation of MSC into the mouse cochlea, although this effect was dependent from the age of the recipient and engraftment occurred only in young mice (Kasagi et al., 2013). Experiments with reconstitution after lethal irradiation with bone marrow from GFP-expressing donors showed that bone marrow derived cells

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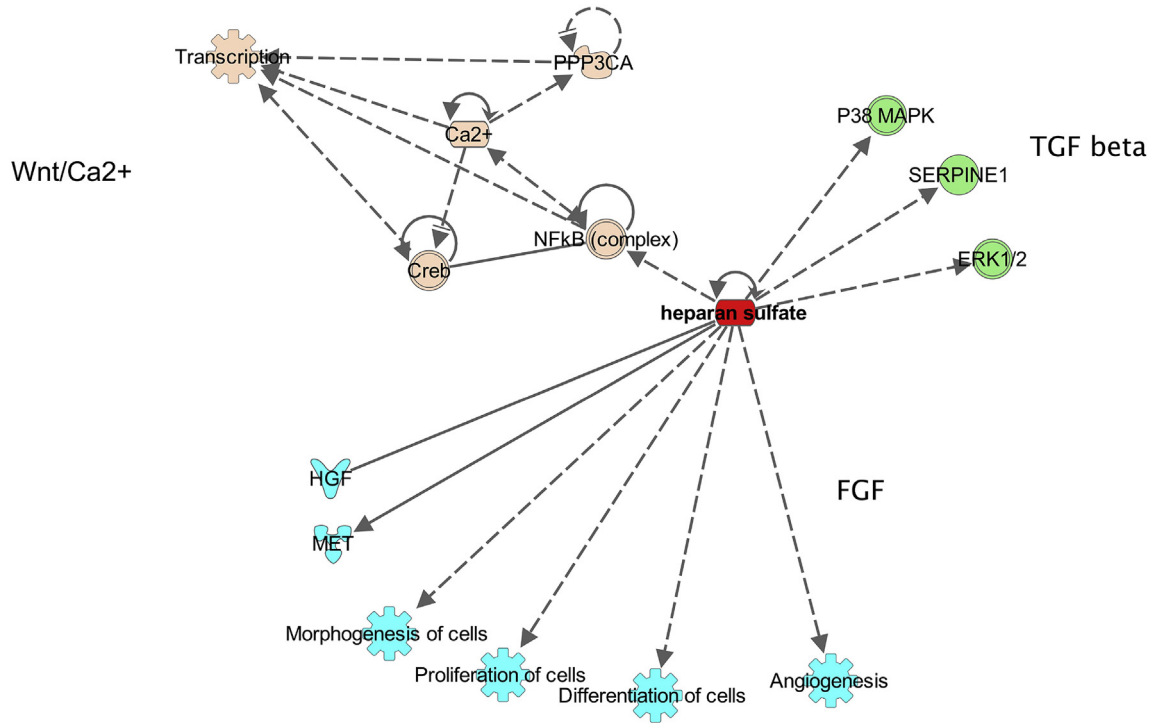

Fig. 10. Heparan sulfate (red) is an important component of the extracellular matrix and has been shown to impact a diverse series of signalling pathways involved in neuronal survival including Wnt/Ca^{2+} (tan) TGF β (green) and FGF (blue).

regularly reside in the inner ear (Tan et al., 2008) and this phenomenon has been also observed in other studies (Lang et al., 2006; Hirose et al., 2005). In an animal model of autoimmune inner ear disease, IL-4 expressing MSC were identified in the scala tympani and scala vestibuli after their transplantation to the inner ear and only few cells were found in the organ of Corti, the stria vascularis and the osseous spiral lamina (Tan et al., 2014). The variable results indicate that techniques for improving engraftment of cells after transplantation are required. Several other medicinal areas confirmed this since the efficacy of multiple transplanted cell types to various tissues in the human body was relatively poor. For example, poor long-term survival of transplanted cells has been described for the liver, muscle and heart (Fox et al., 2014). Despite the still accepted dogma of low immunogenicity, there is increasing evidence that MHC-mismatched MSC are not immune privileged (Berglund et al., 2017). Another explanation for the poor engraftment rates of MSC might be the fact that MSC loose the interaction with the ECM due to isolation and clinical application in diseased organs and undergo apoptosis (Baldari et al., 2017). Preservation of ECM-cell contact by cultivation of embryonic stem cells in matrigel showed a long-term engraftment in a murine myocardial ischemia model (He et al., 2015). In addition, natural three-dimensional ECM scaffolds for the recreation of microenvironments combined with organ-specific progenitor cells were most efficient for maintenance of immature functional stem cells (Mokhtari et al., 2018). Therefore, biological scaffolds may increase the engraftment of MSC by providing an almost physiological environment for the cells. The use of decellularized organs as scaffolds has revolutionized whole organ tissue engineering. In the present study, the use of artificial biological scaffolds increased MSC proliferation and that 3D decellularized cochlear scaffolds showed engraftment of cells into areas of high heparin content such as the stria vascularis and spiral limbus. These results encourage further research for structural support of MSC for improved engraftment and controlled differentiation. However, limitations due to the complexity of the organs and due to the prothrombotic properties of ECM compounds such as collagen occurred. In this context, coatings with heparan have been utilized in order to prevent thrombus formation (Wang et al., 2017). Thus, compounds such as heparan sulfate could not only aid by stabilization of endogenous growth factors and matrix support, but also by warranting sufficient nutrition supply due to the prevention of thrombotic events. There are some limitations associated with this work. First, different sources of the MSC were used for studies on decellularized cochleae and for the *in vivo* experiments this may influence their behaviour differently (Le Blanc and Davies, 2018). Second, the species of the animal models used were also different: mice for the decellularized cochleae and guinea pigs for the *in vivo* engraftment studies. Finally, the different experimental set-ups were performed in different centres. Despite these limitations, the present study provides unprecedented data on the support of inner ear cells and MSC by heparan sulfates. However, further investigations are required in order to elucidate the potentials of heparan sulfates in cell-based therapies and more specifically in cell-based drug delivery of the inner ear.

5. Conclusion

Matrix factors play an important role in the cell transplantation. We have shown that mesenchymal stromal cells delivered to the inner ear of guinea pigs or to decellularized cochleae preferentially bind to areas of high heparin concentration. This presents an opportunity for modulating cell behaviour *ex vivo*. We evaluated the effect of carboxymethylglucose sulfate (Cacicol®), a heparan sulfate analogue, on spiral ganglion cells and mesenchymal stromal cells and demonstrated support of neuronal survival and support of stem cell proliferation. Combining cell therapy with agents such as heparan sulfate analogues that mimic the biology of matrix scaffolds may allow us to enhance attachment and survival of cells

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introduced into the inner ear on scaffolds such as cochlear implants.

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