CORRIGENDUM

Development 140, 479 (2013) doi:10.1242/dev.092486

Macrophage migration inhibitory factor acts as a neurotrophin in the developing inner ear

Lisa M. Bank, Lynne M. Bianchi, Fumi Ebisu, Dov Lerman-Sinkoff, Elizabeth C. Smiley, Yu-chi Shen, Poornapriya Ramamurthy, Deborah L. Thompson, Therese M. Roth, Christine R. Beck, Matthew Flynn, Ryan S. Teller, Luming Feng, G. Nicholas Llewellyn, Brandon Holmes, Cyrrene Sharples, Jaeda Coutinho-Budd, Stephanie A. Linn, Andrew P. Chervenak, David F. Dolan, Jennifer Benson, Ariane Kanicki, Catherine A. Martin, Richard Altschuler, Alisa E. Koch, Ethan M. Jewett, John A. Germiller and Kate F. Barald

There was an error published in *Development* **139**, 4666-4674.

The name of author Alisa E. Koch was spelled incorrectly. The corrected author list appears above.

The authors apologise to readers for this mistake.

Development 139, 4666-4674 (2012) doi:10.1242/dev.066647

Macrophage migration inhibitory factor acts as a neurotrophin in the developing inner ear

Lisa M. Bank[1,4,*,‡], Lynne M. Bianchi[1,2,*], Fumi Ebisu[1,§,*], Dov Lerman-Sinkoff[1,3,¶], Elizabeth C. Smiley[1], Yu-chi Shen[1], Poornapriya Ramamurthy[1,3], Deborah L. Thompson[1,3,#], Therese M. Roth[1,], Christine R. Beck[1,4,‡‡], Matthew Flynn[1,§§], Ryan S. Teller[1,¶¶], Luming Feng[1,##], G. Nicholas Llewellyn[1,4,***], Brandon Holmes[2,‡‡‡], Cyrrene Sharples[2,§§§], Jaeda Coutinho-Budd[2,¶¶¶], Stephanie A. Linn[1,4], Andrew P. Chervenak[1,4], David F. Dolan[5], Jennifer Benson[5], Ariane Kanicki[5], Catherine A. Martin[5], Richard Altschuler[5], Alicia E. Koch[6], Ethan M. Jewett[7,###], John A. Germiller[1,8] and Kate F. Barald[1,2,4,****]**

SUMMARY

This study is the first to demonstrate that macrophage migration inhibitory factor (MIF), an immune system 'inflammatory' cytokine that is released by the developing otocyst, plays a role in regulating early innervation of the mouse and chick inner ear. We demonstrate that MIF is a major bioactive component of the previously uncharacterized otocyst-derived factor, which directs initial neurite outgrowth from the statoacoustic ganglion (SAG) to the developing inner ear. Recombinant MIF acts as a neurotrophin in promoting both SAG directional neurite outgrowth and neuronal survival and is expressed in both the developing and mature inner ear of chick and mouse. A MIF receptor, CD74, is found on both embryonic SAG neurons and adult mouse spiral ganglion neurons. *Mif* knockout mice are hearing impaired and demonstrate altered innervation to the organ of Corti, as well as fewer sensory hair cells. Furthermore, mouse embryonic stem cells become neuron-like when exposed to picomolar levels of MIF, suggesting the general importance of this cytokine in neural development.

KEY WORDS: Directional neurite outgrowth, Neurodevelopment, Neuroimmuno axis, Neurotrophin

INTRODUCTION

Over the last 20 years, a number of investigators have demonstrated interactions between the immune and neural systems during normal organismal development (Patterson, 1994; Armstrong et al., 2003; Siemion et al., 2005; Wrona, 2006). Our laboratories have been investigating proteins that regulate innervation during the earliest stages of inner ear development (reviewed by Barald and Kelley, 2004; Gerlach et al., 2000), and our recent work highlights the importance of chemokines and cytokines in the early stage peripheral auditory system (Bianchi et al., 2005; Holmes et al., 2011; Shen et al., 2012).

We began investigating the neurotrophic role of immune system chemokines and cytokines during the earliest stages of neuronal innervation, using the ear as a model system (Bianchi and Cohan, 1991; Barald et al., 1997; Germiller et al., 2004; Bianchi et al., 2005; Holmes et al., 2011; Shen et al., 2012). In earlier reports, we used

[1]Department of Cell and Developmental Biology, University of Michigan Medical School, 3728 BSRB 109, Zina Pitcher Place, Ann Arbor, MI 48109-2200, USA. [2]Neuroscience Department, Oberlin College, Science Center A243, 119 Woodland Avenue, Oberlin, OH 44074, USA. [3]Department of Biomedical Engineering, College of Engineering, 2200 Bonisteel Boulevard, University of Michigan, Ann Arbor, MI 48109-2099, USA. [4]Cellular and Molecular Biology Graduate Program, University of Michigan Medical School, 1150 W. Medical Center Drive, Ann Arbor, MI 48109, USA. [5]Department of Otolaryngology, MSRB3-Room 9200B, 1150 W. Medical Center Drive, Ann Arbor, MI 48109-0600, USA. [6]Department of Internal Medicine Division of Rheumatology, University of Michigan Medical School, 4045 BSRB, 109 Zina Pitcher Place, Ann Arbor, MI 48109-2200, USA. [7]Department of Computational Medicine and Bioinformatics, 2017 Palmer Commons Building, 100 Washtenaw Avenue, Ann Arbor, MI 48109-2218, USA. [8]Division of Pediatric Otolaryngology, The Children's Hospital of Philadelphia Department of Otorhinolaryngology, University of Pennsylvania, Wood Center, First Floor 34th Street and Civic Center Boulevard, Philadelphia, PA 19104, USA.

*These authors contributed equally to this work
[‡]Present address: Department of Biology, 316 Mark Jefferson Hall, Eastern Michigan University, Ypsilanti, MI 48197, USA
[§]Present address: Department of Otolaryngology, Head and Neck Surgery Kyoto University Hospital/Graduate School of Medicine, Kyoto University, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan
[¶]Present address: MSTP Program, Washington University St Louis, MSTP Box 8226, 660 S. Euclid Avenue, St Louis, MO 63110-1093, USA
[#]Present address: Department of Chemistry, Eastern Michigan University, Mark Jefferson Hall, Ypsilanti, MI 48197, USA
**Present address: Life Sciences Institute, 210 Washtenaw Avenue #5183, University of Michigan, Ann Arbor, MI 48109-2216, USA
[‡‡]Present address: Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Room 604B, Houston, TX 77030, USA
[§§]Present address: The Ohio State University College of Medicine, 370 West 9th Avenue, Columbus, OH 43210, USA
[¶¶]Present address: Department of Bioengineering, University of Utah, 20 S 2030 E, Salt Lake City, UT 84112, USA
[##]Present address: Michigan State University College of Osteopathic Medicine, C110 East Fee Hall, East Lansing, MI 48824, USA
***Present address: Molecular Microbiology Immunology Department, HMR 504, and Institute for Emerging Pathogens and Immune Disease, Keck School of Medicine, USC, 2011 Zonal Avenue, Los Angeles, CA 90033, USA
[‡‡‡]Present address: Department of Neurology, Washington University in St Louis, 4566 Scott Avenue, Box 8111, St Louis, MO 63110, USA
[§§§]Present address: New England Hematology and Oncology, Vernon Cancer Center, 2014 Washington Street, Newton, MA 02462, USA
[¶¶¶]Present address: Department of Neuroscience, UNC Chapel Hill, 115 Mason Farm Road, Neuroscience Research Building 5131, University of North Carolina, Chapel Hill, NC 27599, USA
[###]Present address: Department of Biology, 339 Herrin Labs Stanford University, 371 Serra Mall, Stanford, CA 94305-5020, USA
****Author for correspondence (kfbarald@umich.edu)

Accepted 24 September 2012

gain- and loss-of-function approaches to demonstrate that the cytokine macrophage migration inhibitory factor (MIF) acts as a neurotrophic factor in the developing zebrafish inner ear (Holmes et al., 2011; Shen et al., 2012). In this report, we show that MIF plays a similar role in the chick and mouse inner ear; in culture, recombinant MIF promotes both directional neurite outgrowth and the survival of statoacoustic ganglion (SAG) neurons in both species.

Previous studies from our laboratories and others have indicated that cells in the otocyst secrete target-derived factors [otocyst-derived factor(s) (ODF)] to promote the initial outgrowth and survival of the SAG [at embryonic day (E) 4-6 in chick, E11-14 in the mouse and rat] (Bianchi and Cohan, 1991; Bianchi and Cohan, 1993; Bianchi et al., 1998; Bianchi et al., 2000). However, the identity of the ODF has remained elusive. Although growth factors such as the classical neutrophins have been shown to play a role at later developmental stages in the auditory system, the active components of ODF do not contain identified neurotrophins and have been difficult to ascertain. The small size of the otocyst makes generation of sufficient quantities of bioactive material difficult, further hampering biochemical analysis of active components.

Because so little ODF can be obtained from embryonic otocysts, we generated cell lines from the E9.5 Immortomouse otocyst (IMO) (Barald et al., 1997) that secrete a functionally bioactive ODF equivalent (Germiller et al., 2004; Bianchi et al., 2005). Using cytokine arrays on IMO-generated ODF from these cell lines we first identified the cytokine monocyte chemoattractant protein 1 (MCP1; also known as CCL2) as an active, but not the sole, component of ODF (Bianchi et al., 2005). Because MCP1 alone could not entirely replicate the activity of ODF, we investigated whether other cytokines or cytokine-interacting molecules are also present in ODF.

In this study, through 2D gel analysis and proteomic screens we identified MIF as a major component of ODF, both in abundance and activity. Subsequent in vitro experiments in the developing mouse and chick, as well as analysis of *Mif* knockout (KO) mice, demonstrated that MIF can account for the major functional activities of ODF, including promoting SAG neuron survival and directional neurite outgrowth, and is required for normal hearing and inner ear innervation patterning. We also demonstrate that residual bioactivity in chick or mouse otocyst-generated ODF that is not removed by function-blocking antibodies to MIF can be entirely eliminated by function-blocking antibodies to the cytokine MCP1, which we previously identified as a component of ODF (Bianchi et al., 2005). Together, these data and our studies of zebrafish otic development (Holmes et al., 2011; Shen et al., 2012) show that the cytokines MIF and MCP1 (Bianchi et al., 2005) play crucial roles in inner ear development, directly demonstrating another important connection between the immune and nervous systems.

MATERIALS AND METHODS

Preparation of conditioned medium containing ODF

Conditioned medium containing ODF (CMODF) was prepared from H2kbtsA58 transgenic IMO cell lines as previously described (Bianchi et al., 2005; Germiller et al., 2004), as well as from E4-6 chick and E9.5-10.5 mouse otocysts as previously described (Bianchi and Cohan, 1993; Barald et al., 1997; Germiller et al., 2004; Bianchi et al., 2005; Thompson et al., 2003).

Sandwich ELISA for MIF

Sandwich ELISA assays were performed to determine MIF and MCP1 cytokine levels in IMO 2B1DT cell line CMODF and in conditioned medium from E5 chick and E9.5-10.5 mouse otocysts, using an anti-human MIF IgG monoclonal antibody that cross-reacts with human, mouse and chick MIF (monoclonal mouse IgG1 clone #12302 MAB289, R&D Systems, Minneapolis, MN, USA). The assays were performed in triplicate at room temperature; absorbance was read at 450 nm in a Halogen BELLAPHOT plate reader (Fisher Scientific, Pittsburgh, PA, USA).

Culture and immunostaining of SAG

SAG neurite outgrowth and survival bioassays were performed as described (Bianchi et al., 2005). The effects of MIF and MIF function-blocking antibodies (and in some experiments anti-MCP1 antibodies) were also assessed in these two bioassays. Images were taken on a Leitz Diavert inverted microscope (Leica) with Nikon ACT-1 software.

Analysis of MIF bioactivity and effect of MIF function-blocking antibodies

Recombinant mouse MIF (rmMIF; 1978MF/CF, R&D Systems) or recombinant human MIF (rhMIF; R&D Systems) was added to basal medium or inactive IMO conditioned medium (e.g. from cell line 3C6) at 5 pg/ml to 500 ng/ml (mouse) or 2 ng/ml to 200 ng/ml (human) to evaluate the effects on SAG outgrowth and survival. Extensive dose-response evaluations were first performed for both neurite outgrowth and neuronal survival to determine the optimal concentrations for each bioactivity in each species (mouse, chick). Initial results demonstrated that the low and mid-range concentrations produced the most consistent outgrowth and survival, and 10 ng/ml was used in subsequent experiments. All trials were performed in triplicate, with wells assigned randomly by computer.

In the experiments shown in Fig. 1, the effects of rmMIF were compared with IMO CMODF, and in both cases a MIF function-blocking antibody was used. MIF function-blocking antibody (AB289 goat anti-mouse IgG, R&D Systems) was added to 10 ng/ml rmMIF (1978MF/CF, R&D Systems), a previously determined optimal concentration in basal medium without γ-interferon or FBS, or to 2B1DT IMO CM. As a control, similar samples were treated with an antibody to bovine serum albumen (BSA) (A4338, Sigma-Aldrich, St Louis, MO, USA).

Before use in the SAG neurite outgrowth bioassay or dissociated SAG survival assay, each sample of rmMIF or 2B1DT IMO CMODF was either (1) untreated, (2) treated with 300 ng/ml MIF function-blocking antibody (determined as the optimal concentration after testing 10-1000 ng/ml) or (3) 300 ng/ml anti-BSA antibody. Samples were incubated with protein-G beads, which were removed by spin column (IP50 Protein-G Immunoprecipitation Kit, Sigma-Aldrich). *P*-values were calculated using a one-tailed *t*-test with Bonferroni correction; 16 different comparisons were performed (statistical significance is reported in supplementary material Table S1). In experiments in which the anti-MIF-treated conditioned media were subsequently treated with MCP1 function-blocking antibodies (see Bianchi et al., 2005) (supplementary material Fig. S1), all residual bioactivity could be eliminated from either type of ODF.

Culture and immunolabeling of mouse embryonic stem cells

The D3 mouse embryonic stem cell (mESC) line (S6) was cultured as described (Roth et al., 2007; Roth et al., 2008) to assess the effects of MIF bioactivity on differentiation. Control media included 1 μg/l ciliary neurotrophic factor (CNTF) (R&D Systems), 1 μg/l nerve growth factor (NGF) (Chemicon, Temecula, CA, USA), or various concentrations (1 pg/ml to 500 ng/ml) of rmMIF. mESCs were cultured for another 5 days at 37°C, fed with differentiation media (NGF, CNTF or MIF) every other day. Similar concentrations of rhMIF or rmMIF produced similar effects on neuronal development from the mESCs (data not shown). Extensive dose-response experiments were conducted to determine the optimum concentration of rmMIF or growth factors that resulted in maximum neuronal differentiation as defined by morphology and labeling with anti-neurofilament or anti-TUJ1 (also known as TUBB3) in 3-4 days.

RT-PCR assays for *Mif* and *Cd74*

RT-PCR was used to detect expression of *Mif* and of the MIF receptor *Cd74* in embryonic and adult mouse and chick inner ear tissues. Primer sequences (5′-3′; F, forward; R, reverse) were: MIF-F, CCAGAA-CCGCAACTACAGTAAGC; MIF-R, TTGGCAGCGTTCATGTCGTA-

ATAG; CD74-F, GCTTCCGAAATCTGCCAAACC; CD74-R, AAGAGCCACTGCTTCATCCAGC.

Organotypic co-culture of postnatal organs of Corti with spiral ganglia

Inner ears were isolated from mice at postnatal day (P) 3 (Chen et al., 2009); organs of Corti (OC) and spiral ganglia (SG) were isolated from age-matched wild-type mice (Balb/c, WT) and *Mif* KO mice. The OC explants (WT or *Mif* KO) were then placed 1 cm from the SG explants (n=12 per condition). Tissues were observed daily by light microscopy for the ensuing 7 days. Phase-contrast images (40×) to produce composites of the entire field of view were taken daily beginning at 24 hours. To determine the density of contacts between SG and WT or *Mif* KO OC, composite images magnified in Adobe Photoshop to the limits of pixel resolution (n=11) were analyzed using a superimposed grid. Only clearly visible contacts (with no underlying neurite extensions) between the neurite and the surface of the OC were counted; these therefore represent a minimum number of contacts owing to the omission of overlapping contact points in many regions. No contacts were seen between the WT SG and the *Mif* KO OC at any stage or under any conditions.

Immunolabeling of inner ear tissues

Developmental expression of MIF protein was assessed in sectioned embryonic inner ears and adult inner ear tissues, as well as in whole-mounted tissues (Gerlach-Bank et al., 2002) using rabbit anti-MIF (1:500; ZMD.321, Invitrogen, Camarillo, CA, USA), goat anti-MCP1 (1:40; R&D Systems) and anti-myosin VIIa (1:200; Sigma), followed by donkey anti-rabbit Alexa Fluor 488 or donkey anti-goat Alexa Fluor 555 (both 1:200; Invitrogen) secondary antibodies and observation by confocal microscopy.

MIF expression was also examined in dissociated and reaggregated inner ear 'domes' prepared from E13/14 mouse embryonic otocysts (Bianchi et al., 2002; Bianchi et al., 2006). Dome cells were double labeled for myosin VI (a marker for sensory hair cells; 10 mg/ml; T. Hasson, UCLA) and MIF (1:3000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or for cytokeratin (a marker for supporting cells; 1:100; Sigma) and MIF. Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 594 rabbit anti-goat secondary antibodies (both 1:2000; Molecular Probes, Eugene, OR, USA) were used to detect myosin VI and MIF, respectively; and Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 555 goat anti-mouse secondary antibodies were used to detect MIF and cytokeratin, respectively.

Auditory brainstem response assessment

Auditory brainstem response (ABR) was assessed for 4-week-old WT (Balb/c) (n=5) and *Mif* KO mice (n=4) (Saul et al., 2008). Responses were collected at 12, 24 and 48 kHz for a range of stimulus levels, separated by 10 dB steps at higher intensities and 5 dB steps near threshold. Thresholds were interpolated between the lowest stimulus intensity that produced a response and the highest stimulus at which no response was observed (5 dB increments).

Design of siRNAs to *Mif* and *Cd74*

siRNAs were used to knock down mouse MIF or avian CD74 proteins by introducing a homologous double-stranded (ds) RNA. The nucleotide sequences of the dsRNA and complementary dsRNA for mouse *Mif* mRNA and avian *Cd74* mRNAs are (5′-3′): mouse *Mif* siRNA duplex, CCGCAACUACAGUAAGCUGdTdT and CAGCUUACUGUAGUUGCGGdTdT; *Mif* missense control RNA duplex, GCGCGCUUUGUAGGAUUCGdTdT and CGAAUCCUACAAAGCGCGCdTdT; avian *Cd74* siRNA duplex, GCAACAAGACUGAGGAUCAAATdTd and UUUGAUCCUCAGUCUUGUUGCTdTd; *Cd74* missense control RNA duplex, GCAAGAAGACAGAGGUUCAAATdTd and UUUGAACCUCUGUCUUGUUGCTdTd. All constructs were confirmed by enzymatic digestion (*Hin*dIII) and DNA sequence analysis (DNA Sequencing Core, University of Michigan).

Culture of cells for siRNA transfection

IMO 2B1DT cells expressing MIF and chick neuronal cells expressing CD74 (Bryan et al., 2008) were used to titrate siRNA concentrations necessary for maximal inhibition of MIF or CD74 protein, as determined by western blotting (the maximal inhibition achieved was ~70%). IMO 2B1DT cells were transfected with mouse *Mif* siRNA or its missense control and chick neuronal cells were transfected with avian *Cd74* siRNA or its missense control (Mirus Reagent, Madison, WI, USA) according to the manufacturer's protocol. Twenty-four hours post-transfection, western blotting assays (Bianchi et al., 2005) were performed to examine the effect of these siRNAs on MIF and CD74 levels. SAG bioassays (Bianchi et al., 2005) in the presence of siRNA were used to monitor any effect of *Cd74* siRNA on ODF-induced SAG neurite outgrowth and survival (supplementary material Fig. S2).

Statistical analysis

For function-blocking antibody neurite outgrowth permutation test results, pairwise differences between experimental conditions were evaluated using approximate permutation tests. In all cases, the test statistic was the difference $\overline{X}-\overline{Y}$ between the means of the two groups, $\{X_i\}_{i=1}^{m}$ and $\{Y_j\}_{j=1}^{n}$, being compared. In each test, the values $\{X_i\}_{i=1}^{m}$ and $\{Y_j\}_{j=1}^{n}$ were pooled. We then drew m values from the pool without replacement and assigned them to the first group, assigning the remaining n values to the second group. The statistic was then recomputed and the procedure was repeated. P-values were based on 10^6 independent draws and evaluations of the statistic. One-sided P-values were found by computing the fraction of replicates in which the value of the resampled statistic was at least as great as its empirically observed value. Two-sided P-values were found by computing the fraction of replicates in which the resampled statistic was at least as great in magnitude as the absolute value of its empirically observed value.

For general statistical analysis of data in other experiments, a one-tail t-test was used. $P<0.05$ was considered significant in all data.

RESULTS

MIF is a bioactive protein that is present in ODF

IMO cell lines, chick (E4-5) and mouse (E11) otocysts were used as sources of ODF bioactivity. Initially, serum-free conditioned medium (CM) from IMO cell lines that promoted both robust outgrowth and significant survival of SAG neurons (bioactive CM) was compared with CM from IMO cell lines that did not promote outgrowth or survival and with serum-free control basal medium alone (inactive or basal medium). Two-dimensional gel analysis and a subsequent proteomic screen (MALDI/TOF/TOF spectroscopy; Proteomics Core, University of Michigan) were used to identify components of the biologically active samples of ODF.

MIF was found in all the bioactive cell lines (e.g. cell line 2B1DT), but not in inactive IMO cell line (3C6) CM or the basal cell-free and serum-free CM samples. Similar findings were obtained in analyses of CMODF from E5 chick and E11 mouse otocysts. Following the identification of MIF and verification that MCP1 was also present in ODF by proteomic analysis, sandwich ELISA assays were performed to quantify these proteins from IMO CMODF and chick and mouse otocyst ODF. MIF was found at 2.40±0.69 ng/ml (n=6) in IMO CMODF, 1.21±0.84 ng/ml (n=46) in chick ODF and 1.32±0.61 ng/ml (n=16) in mouse ODF.

Proteomic analysis of the ODF samples also confirmed the presence of MCP1, which we had previously detected in ODF using a cytokine protein array that did not include MIF (Bianchi et al., 2005). ELISA assays were used to document the presence of MCP1 at 1.22±0.06 ng/ml (n=6) in the IMO cell lines, 1.26±0.22 ng/ml (n=6) in chick ODF and 1.18±0.17 ng/ml (n=14) in mouse ODF.

Antibodies to MIF block ODF bioactivity

IMO CMODF and chick and mouse otocyst ODF produce similar effects on neurite outgrowth from SAG explants in vitro. To determine whether MIF alone mediates either SAG neurite outgrowth or SAG neuronal survival, recombinant MIF (rMIF) was

tested in mouse and chick in extensive dose-response SAG neurite outgrowth and survival assays. Concentrations of MIF tested varied in the pg/ml to μg/ml ranges, all of which elicited outgrowth and promoted survival. The optimal concentration was determined to be close to the physiological concentration of MIF found in embryos (see above) and in ODF; therefore, recombinant mouse MIF (rmMIF) was used at 10 ng/ml. To determine whether MIF could account for the bioactivity found in ODF, ODF pretreated either with MIF function-blocking antibody or anti-BSA antibody and then activity depleted with protein-G beads, or untreated ODF was added to cultures of explanted SAG. Neurite outgrowth was measured at 24 hours (Fig. 1). Untreated IMO-derived ODF (CM from IMO cell line 2B1DT; Fig. 1D) produced a neurite outgrowth score of 4.1 (Thompson et al., 2003; Germiller et al., 2004). The average neurite outgrowth score in 10 ng/ml rmMIF was 4.2 (Fig. 1D) (Bianchi et al., 2005).

SAG cultured in rMIF pretreated with anti-MIF antibody followed by protein-G beads to remove MIF activity displayed little or no neurite outgrowth (average neurite outgrowth score of 0.2; Fig. 1D), demonstrating a direct effect of MIF on SAG neurite outgrowth. Pretreatment of rMIF with anti-BSA did not diminish the neurite outgrowth produced by rMIF (average neurite outgrowth score of 4.0). However, although pretreatment of chick ODF with the same MIF function-blocking antibody also diminished the effect of chick ODF on neurite outgrowth, the average neurite outgrowth score was 1.1 (Fig. 1D), suggesting that additional outgrowth-promoting factors are present in ODF, such as MCP1, which was found in our earlier study (Bianchi et al., 2005). This indeed proved to be the case. When the anti-MIF-depleted CM from IMO-generated ODF and the CM from E5 chick-generated ODF were subsequently treated with the same MCP1 function-blocking antibody that we used in our earlier study (Bianchi et al., 2005), no significant neurite outgrowth was seen. However, subsequent treatment of the anti-MIF-treated CM with anti-BSA had no effect on the CM activities (supplementary material Fig. S1).

To assess the effects of rMIF on neuronal survival, dissociated SAG neurons were exposed to either IMO ODF or rmMIF, each with and without MIF function-blocking antibody pretreatment. Anti-BSA was again used as the control and did not affect neuronal survival promoted by ODF from any source. Under normal culture conditions, SAG treated with IMO CMODF or 10 ng/ml rMIF had 67% (n=31) or 77% (n=17) of neurons surviving at 24 hours, respectively (Fig. 1E). However, addition of anti-MIF antibody to either rmMIF or ODF significantly reduced SAG neuronal survival. Only 4% of neurons survived with anti-MIF-treated rmMIF (Fig. 1E), whereas 17% of SAG neurons survived when cultured in anti-MIF-treated ODF. Again, this residual survival-promoting activity in ODF is eliminated by subsequent treatment of anti-MIF-treated CM with the same MCP1 function-blocking antibody that we used in our earlier study (Bianchi et al., 2005) (supplementary material Fig. S1). No significant neuronal survival was noted in these 'twice-treated' CM; again, subsequent treatment with anti-BSA antibody had no effect on the anti-MIF-treated CM. Therefore, MIF and MCP1 can together account for essentially all the bioactive neuronal survival and outgrowth functions of IMO-

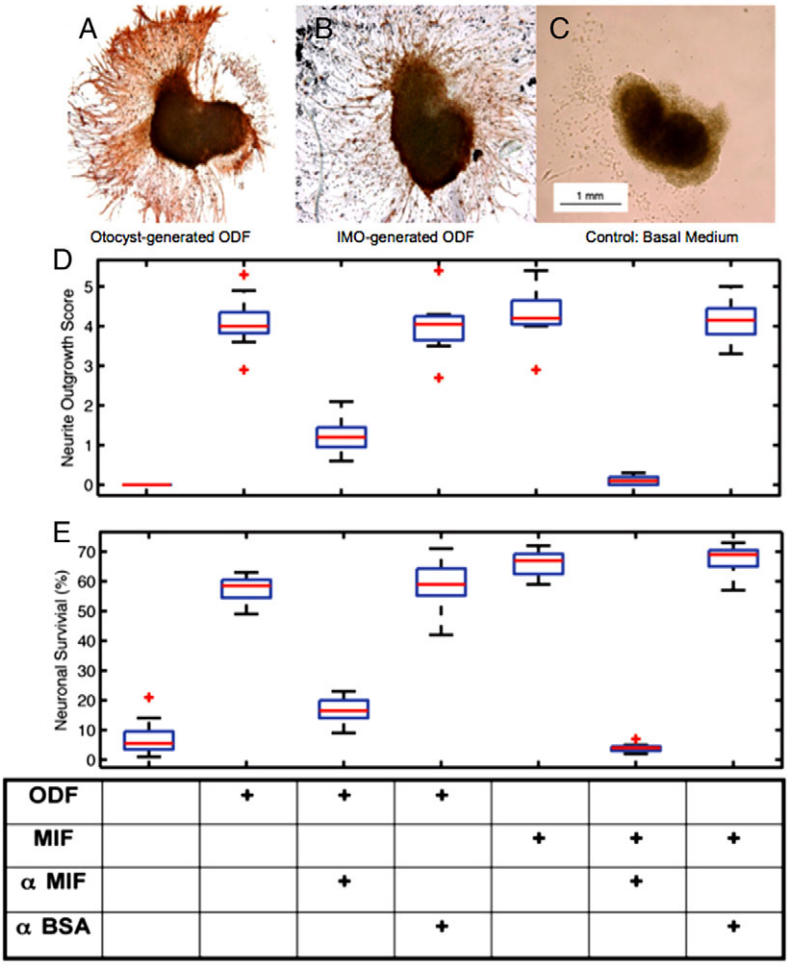

Fig. 1. Effect of treating MIF or ODF used in assays of SAG neurite outgrowth and neuron survival with MIF function-blocking antibody. (**A-C**) SAG neurite outgrowth is abundant after 24 hours of exposure to Immortomouse otocyst (IMO)-generated ODF (A) or to E5 chick otocyst-generated ODF (B). No neurite outgrowth is seen in basal serum-free medium (C). (**D**) Similar neurite outgrowth was promoted by IMO-generated ODF and 10 ng/ml recombinant MIF (rMIF), but not by control medium (measured on a scale of 1-5; the chart at the bottom indicates treatment conditions for both D and E). Pretreatment of rMIF with a MIF function-blocking antibody (α MIF) substantially inhibited the neurite outgrowth-promoting activity of MIF, whereas pretreatment with anti-BSA antibody (α BSA) did not. (**E**) Neuronal survival. The anti-MIF antibody treatment reduced neuronal survival to background levels when added to rMIF, but not to ODF, indicating that there is residual neuron survival-promoting activity that is not attributable to MIF. Neurite outgrowth: ODF alone, n=15 replicates; ODF + anti-MIF, n=7; ODF + anti-BSA, n=8; MIF alone, n=8; MIF + anti-MIF, n=10; MIF + anti-BSA, n=8. Neuronal survival: ODF alone, n=12; ODF + anti-MIF, n=8; ODF + anti-BSA, n=7; MIF alone, n=9; MIF + anti-MIF, n=8; MIF + anti-BSA, n=8. See supplementary material Table S1 for statistical significance.

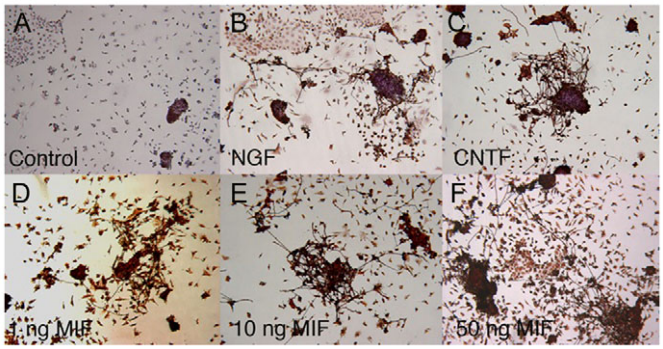

Fig. 2. mESCs treated with MIF take on a neuron-like morphology. (**A**) D3 mESCs grown in control cultures with no growth factors added did not become neuron-like. (**B**,**C**) By contrast, D3 mESCs treated with the growth factors NGF (B) or CNTF (C) produced neuron-like cells, with neurofilament-positive processes. (**D-F**) Recombinant mouse MIF (rmMIF) at 1 ng/ml (D), 10 ng/ml (E) and 50 ng/ml (F) also produced neuron-like cells with elaborate neurofilament-positive processes seen as early as 48 hours in culture. Both lower (1 pg/ml) and higher (500 ng/ml) concentrations of rmMIF were also effective (not shown). Phase contrast image (magnification 4×).

and otocyst-generated ODF. There are trace amounts of other cytokines in ODF (Bianchi et al., 2005) but they do not appear to contribute significantly to its bioactivity, although synergies among these cytokines have not been investigated extensively.

Furthermore, treatment of chick SAG explants or dissociated SAG with siRNA to the MIF receptor *Cd74* also completely inhibited SAG neurite outgrowth in response to either MIF or ODF and significantly reduced neuronal survival in the presence of MIF or ODF (see supplementary material Fig. S2).

Neuronal differentiation of mouse embryonic stem cells is promoted by MIF

To further examine the capacity of MIF to impact neuronal development, we tested the ability of rmMIF to induce a neuronal phenotype in D3 mouse embryonic stem cells (mESCs) (Doetschman et al., 1985). Concentrations of rmMIF ranging from 1 pg/ml to 500 ng/ml caused a substantial population of mESCs to become neuron-like after 48 hours in culture. Extensive dose-response analyses were performed as illustrated in Fig. 2. The cells treated with rmMIF developed long neurofilament-positive neurites (Fig. 2D-F). By contrast, D3 cells remained proliferative in the absence of rmMIF (Fig. 2A). Control cultures of D3 cells treated with the growth factors/neurotrophins NGF (Fig. 2B) or CNTF (Fig. 2C) similarly took on a neuron-like morphology after 48 hours, as described in our previous studies (Roth et al., 2007; Roth et al., 2008).

Our recent studies of the role of MIF in zebrafish support the idea that MIF might be involved in neuronal development outside the auditory system. Antisense oligonucleotide morpholinos (MOs) to *mif* and *mif-like* (*ddt* – Zebrafish Information Network) injected into either the 1- to 8-cell zebrafish embryo (Shen et al., 2012) or directly into the nascent inner ear at 20 hours postfertilization (hpf) (Holmes et al., 2011) had substantial inhibitory effects on neurite extension in other head ganglia, reducing the size of the zn-5 antibody-stained (zn-5 labels neurons in fish) SAG and other head ganglia by 50% or more (Shen et al., 2012); capped *mif* and *mif-like* RNAs rescued this loss. Thus, MIF is able to influence neural development in addition to its effects on SAG neurons.

MIF expression in the mouse medial embryonic otocyst wall and in supporting cells

To further explore the role of MIF in the developing inner ear, immunohistochemical analyses of inner ear tissues from embryonic stages to postnatal stages were undertaken to map protein expression patterns. At the early embryonic stage E9.5, MIF was expressed in the medial otocyst closest to the hindbrain (Fig. 3) and in the ventral half to two-thirds of the hindbrain. At later embryonic stages, MIF was broadly distributed in the epithelia of the cochlea and vestibular regions, although individual cell populations could not be discerned (supplementary material Fig. S3; E15.5, *n*=5).

To determine the cellular distribution of MIF at the stages of ODF activity, we used two complementary approaches. In addition to labeling sections of developing embryonic mouse otocysts and hindbrain with antibodies to MIF (Fig. 3), we also labeled cultures of re-associated embryonic inner ear cells (Bianchi et al., 2002; Bianchi et al., 2006), a model that we have found useful for distinguishing developing hair cells (HCs) from supporting cells (SCs) (supplementary material Fig. S4). In these cultures, inner ear cell 'domes' are produced from dissociated cells from isolated inner ears, which reaggregate with a central mass of HCs (myosin VIIa positive; supplementary material Fig. S4A) resting upon an SC layer (which is cytokeratin positive). MIF was localized to the underlying SC layer, but not the HC layer of these cultured inner ear domes. Cytokeratin expression colocalized with MIF in the SCs (supplementary material Fig. S4B), whereas the central regions of the domes (above the focal plane) containing HCs were not labeled for MIF (supplementary material Fig. S4B). Thus, MIF is localized to developing SC regions in embryonic inner ear tissues, where it might act as a general attractant for SAG neurons early in their development.

To test whether MIF expression persists in mature inner ear tissues, 4-week-old Balb/c (WT) mice (*n*=4) were examined. MIF was expressed in SCs that cup the inner and outer HCs (IHC and OHC, respectively) (Fig. 4). MIF expression was also detected in the stria vascularis, spiral limbus and spiral ligament (Fig. 4).

The cytokine MCP1 was detected in the IHCs and OHCs of the organ of Corti (OC), as well as in the stria vascularis (Fig. 4). Overlapping staining of MCP1 and MIF (Fig. 4, yellow) was

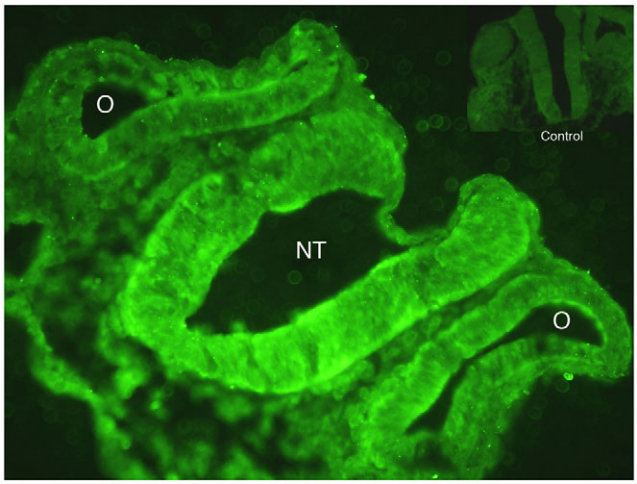

Fig. 3. MIF protein is widely expressed in E9.5 mouse otocysts and hindbrain. MIF labeling in hindbrain (NT) is ventral and that in the otocysts (O) is medial on the hindbrain side. Epithelial cells lining the otocyst, particularly at the dorsal edge, are labeled, as are epithelial cells on the external medial side of the otocyst and cells in the otocyst medial wall. Inset shows a control without primary antibody.

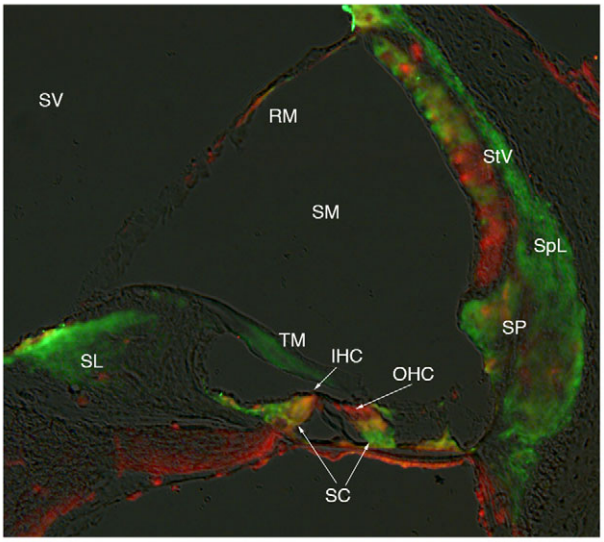

Fig. 4. Expression of MIF and MCP1 proteins in the cochlea of WT Balb/c mice. Cryosections of Balb/c cochleae were labeled for MIF (green) and MCP1 (red). MIF is expressed in SL, SpL, StV, SP and SCs. MCP1 expression is found in IHCs and OHCs, as well as in StV and basement membranes. The region of colocalization (yellow) indicates the portion of the SCs that directly contact and cup and the IHC and OHC. IHC, inner hair cell; OHC, outer hair cell; RM, Reissner's membrane; SM, scala media; SV, scala vestibuli; SL, spiral ligament; SpL, spiral limbus; SP, spiral prominences; SC, supporting cell; StV, stria vascularis; TM, tectorial membrane. Magnification 20×.

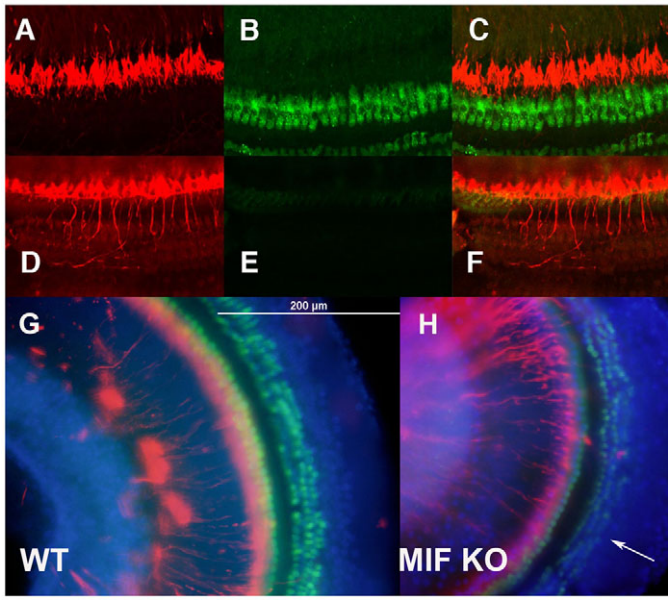

Fig. 5. Characterization of *Mif* KO embryonic and adult inner ear structures. (**A-F**) Epifluorescence of organs of Corti (OC) labeled for neurofilament (a neuronal marker, red) and myosin VIIa (an HC marker, green) in WT (Balb/c) (A-C) and *Mif* KO (D-F) mice. (A-C) Both IHCs and OHCs in the cochlea are phenotypically normal in the OC in WT mice. (D-F) There is a lower neuron density (D,F) and areas of missing HCs (E,F) in the *Mif* KO OC. Myosin labeling is considerably reduced in the three rows of outer HCs as well as in the one row of inner HCs in the KO animal (E,F). (**G**,**H**) Low-magnification view of the basal 48 kHz region of the cochlea of WT (G) and *Mif* KO (H) animals. Note that, as in B and C, there is evidence of green fluorescence (myosin VIIa labeling) in the WT OC and also some evidence of labeling in the row of IHCs in the *Mif* KO animal; myosin VIIa labeling is not apparent in the three rows of WT OHCs (G), where the DAPI labeling (nuclear marker, blue) becomes apparent. Neurofilament is in red. Both missing HCs and loss of myosin VIIa label are apparent in the *Mif* KO animal (H). Arrow (H) indicates an area with large numbers of missing HCs.

visible in the regions where the SCs cup the HC. The expression of these two cytokines in mature inner ear tissues might suggest roles in maintaining sensory epithelia or neural innervation.

MIF receptor (CD74) expression in developing SAG and adult spiral ganglia neurons

To test for a signaling pathway that might be used by inner ear-derived MIF, we assayed for the presence of the known MIF receptor CD74 in SAG or inner ear epithelial regions. RT-PCR was used to examine *Cd74* mRNA expression in E14 mouse SAG neurons and otocysts, as well as in adult mouse spiral ganglia (SG) neurons and adult mouse sensory epithelium (vestibular ampulla and combined cochlear/vestibular regions) (supplementary material Fig. S5).

Cd74 mRNA was expressed in the embryonic SAG and embryonic otocysts (supplementary material Fig. S5A, lanes 2-3). *Cd74* was also expressed in the adult ear (supplementary material Fig. S5A), including SG neurons (Fig. 5A), as well as the adult sensory epithelium in the cochlea (supplementary material Fig. S5B). The presence of CD74 on SAG neurons supports the existence of a signaling pathway by which MIF influences SAG survival and outgrowth. The distribution of CD74 in sensory epithelial regions and adult SG neurons further supports diverse roles for MIF in inner ear development. The zebrafish equivalents of CD74, Iclp1 and Iclp2 (now called Cd74a and Cd74b), have been found on SAG neurons in zebrafish (Shen et al., 2012).

Characterization of the *Mif* KO mouse

Significant hearing loss in *Mif* KO mice spans all frequency ranges tested

The in vitro and immunohistochemical data strongly suggest that MIF plays an important role in the development of the SAG and, perhaps, other inner ear structures, as in the zebrafish inner ear (Holmes et al., 2011; Shen et al., 2012).

To determine if MIF is necessary for inner ear development or function in vivo, we analyzed the hearing thresholds and inner ear structures of *Mif* KO mice, which have previously been used to study the role of MIF in the immune system (Bozza et al., 1999; Leech et al., 2003).

Auditory brainstem response (ABR) measurements were taken at 4 weeks of age, the earliest age for ABR testing, from WT and *Mif* KO mice (Table 1). *Mif* KO mice had statistically significant elevated thresholds at all frequencies compared with WT mice. Hearing loss in *Mif* KO mice was clearly evident at 24 kHz and even more pronounced at 48 kHz.

Altered innervation of the sensory epithelium in mice lacking MIF

To identify morphological changes in inner ear structures of *Mif* KO mice, neural innervation patterns and cochlear HC patterning were examined histologically in whole-mount cochlear tissues from 4-week-old WT and *Mif* KO mice, the age when hearing loss was documented in the same animals. Fig. 5 shows an analysis of neurofilament and myosin VIIa (a sensory HC marker) expression in WT and *Mif* KO mice in the basal high-frequency (48 kHz)

Table 1. ABR thresholds in WT and *Mif* KO mice at 4 weeks

Frequency (kHz)	Genotype	dB SPL (mean±s.d.)
12	WT (*n*=5)	28±3
	Mif KO (*n*=4)	36±2
24	WT (*n*=5)	24±2
	Mif KO (*n*=4)	43±2
48	WT (*n*=5)	29±14
	Mif KO (*n*=4)	58±2

Mif KO mice have statistically elevated thresholds compared with WT (Balb/c) mice in all regions of the cochlea, but the most dramatic hearing loss is seen at 48 kHz in the high-frequency-detecting basal region of the cochlea.
SPL, sound pressure level.

region. Although both IHCs and OHCs were phenotypically normal and present in the OC of WT mice, with dense nerve fiber innervation to the HC regions (Fig. 5A-C), nerve fiber density was decreased throughout all cochlear regions in *Mif* KO mice, with some areas demonstrating dramatic decreases. Additionally, OHCs were frequently absent in the *Mif* KO OC, suggesting that normal expression of MIF might also be needed for normal OHC patterning (Fig. 5D-F,H).

Neurites emerging from WT SG explants make contact with OC explants from WT but not *Mif* KO mice

The decreased nerve fiber density in *Mif* KO mice supports the hypothesis that MIF is necessary for innervation of the developing inner ear. To explore innervation patterning in WT and *Mif* KO mice, we examined interactions between P3 WT SG neurons (from SG explants) with either WT or *Mif* KO OC explanted at P3, over a 7-day period in co-culture.

Within 24 hours, neurites extended from the SG explants and advanced towards and made contact with the WT OC (*n*=19). In those cultures in which individual contacts could be visualized, the average number of contacts at 24 hours was 130±10 (±s.e.m., *n*=3). Neurite contacts persisted and grew more profuse in WT co-cultures over the 7 days of the experiment, by the end of which the contacts became too numerous to discern individually (Fig. 6).

By contrast, OC from *Mif* KO mice did not attract WT SG neurites. Only very short, radially distributed processes extended from these SG, but none of these processes ever made contact with the *Mif* KO OC, even after 7 days in culture (*n*=17). These results support the hypothesis that the developing inner ear sensory epithelium releases MIF to attract emerging neurites and to support the process of neural innervation. This also raises the question of whether MIF might have a therapeutic application in instigating regeneration events, such as the regrowth of SG neuronal processes to the OC in cases of injury or disease.

DISCUSSION

'Classical' nerve growth factors such as the neurotrophins, fibroblast growth factors (FGFs) and glial cell line derived neurotrophic factor (GDNF) have been found to play specific, stage-dependent roles in inner ear development (reviewed by Bianchi and Fuchs, 2010; Sánchez-Calderon et al., 2007). However, none of these previously identified factors was found to influence early stages of SAG development, at the time of initial neurite outgrowth (Bianchi and Cohan, 1993); nor were they found in proteomic analyses of ODF from any source (chick, mouse, IMO cell lines).

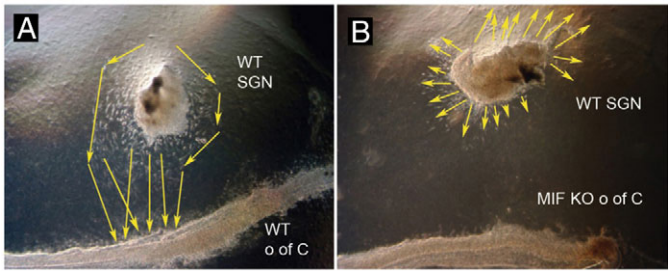

Fig. 6. Neurites from WT SG extend towards WT but not towards *Mif* KO OC. Spiral ganglia (SG) from WT (Balb/c) mice were excised at P3 and placed in organ culture with OC from either WT (A) or *Mif* KO (B) P3 mice. (**A**) After 5 days in culture, there was robust and directional neurite outgrowth from the SG towards the WT OC. Note that the trajectory of the neuronal processes is always towards the WT OC (yellow arrows). In every co-culture (*n*=19), neurites from the SG made contact with the explanted WT OC. (**B**) By contrast, when the WT SG was placed in culture with the *Mif* KO OC, neurites extended radially and uniformly for only short distances. None of the neurites made contact with the *Mif* KO OC in any experiment (*n*=17). o of C, organ of Corti; SGN, spiral ganglion neurons from SG explant.

Despite numerous studies and a variety of experimental approaches in many model systems, the composition of ODF has remained unclear for many years. Only recently have our data suggested a role for immune system cytokines (Bianchi et al., 2005; Holmes et al., 2011; Shen et al., 2012).

In the present study, using various complementary methods we demonstrate that the cytokine MIF, which is expressed in inner ear SCs, is present in mammalian and chick ODF, that it influences SAG neurite outgrowth and neuronal survival at the stage of initial neurite outgrowth, and is necessary for the development of normal hearing in mice.

Proteomic analyses demonstrate that MIF is present in the CM from both IMO and otocyst CMODF. It is also presumably released by the WT mouse OC, in contrast to the *Mif* KO OC (Fig. 6). Although IMO cell line or otocyst CMODF was able to promote outgrowth and survival of SAG neurons, bioactivity was largely lost when ODF was treated with anti-MIF antibodies. However, the bioactivity of ODF appears to rely on the combined cytokine activities of MIF and MCP1; residual bioactivity in anti-MIF-treated ODF was further decreased – essentially removed altogether – when subsequently treated with anti-MCP1 antibody (supplementary material Fig. S1). Although there are other cytokines in ODF (Bianchi et al., 2005), including RANTES (also known as CCL5) and TNF-α, they appear to play a minimal role in its bioactivity. Future studies of synergies among various cytokines should provide additional understanding of how cytokines interact to regulate inner ear development. Several lines of evidence indicate that cytokines are potentially interactive, even synergistic at specific concentrations (Wu and Bradshaw, 1996; Kavanaugh, 2002). This is supported, for example, by our observation that MIF and MCP1 interact in a yeast two-hybrid study (data not shown).

The results of the current study are also consistent with the findings of our recent studies in zebrafish demonstrating that inactivating MIF, either by creating MIF morphants at the 1- to 8-cell stage (Shen et al., 2012), by injecting the MOs directly into the developing inner ear at 20 hpf (Holmes et al., 2011), or by chemically inhibiting the action of MIF protein with a specific inhibitor, resulted in decreased survival of SAG neurons (Holmes

et al., 2011; Shen et al., 2012). Furthermore, the size of the SAG was at least partially restored when the 1- to 8-cell stage embryos were injected concomitantly with capped *Mif* RNA (*mif* and *mif-like* in combination – there are two *Mif* genes in zebrafish), further supporting a direct role for MIF in mediating SAG survival (Shen et al., 2012).

In both the current study of mouse and chick and in the previous analysis of zebrafish, the MIF receptor CD74 (Iclp1 and Iclp2 in zebrafish) was found on SAG neurons. Although it is not the only receptor for MIF (Roger et al., 2001), CD74 is the best-characterized MIF receptor in the immune system. Blocking SAG CD74 with siRNA reduced both neurite outgrowth and neuronal survival (supplementary material Fig. S2). Its presence on SAG neurons supports the involvement of a MIF signaling pathway in neuronal survival. MIF appears to support SAG survival by inhibiting p53-dependent apoptosis in the immune system (Leech et al., 2003), as we demonstrated in zebrafish SAG (Shen et al., 2012).

The present study demonstrated a role for MIF not only in vitro but also in vivo, as shown by analysis of the *Mif* KO mouse. *Mif* KO mice exhibited hearing loss across all frequencies, particularly in higher frequency (48 kHz) regions of the cochlea, providing clear evidence of the functional necessity for MIF in inner ear development. The *Mif* KO mice also had HC losses, particularly in the 48 kHz region of the cochlea (Fig. 5), as well as a prominent decrease in the density of nerve fiber innervation in the high-frequency region of the cochlea by 4 weeks of age.

MIF protein is expressed in both embryonic and adult inner ear, particularly in the SCs in the mature inner ear. Similar patterns of expression were noted in zebrafish (Shen et al., 2012). This localization of MIF to SCs rather than the sensory HCs suggests that MIF might play a role as a general guidance factor, attracting SAG neurites to the area of the sensory epithelium.

The attractant properties of MIF were observed in cultures of postnatal mouse SG neurons that exhibited directional neurite outgrowth towards the WT, but not the *Mif* KO, OC. The lack of growth towards tissue lacking MIF further supports the hypothesis that a MIF source (presumably SCs) acts to attract SG nerve fibers.

The observation that MIF is expressed in SCs that underlie sensory HCs and that significant numbers of HCs are missing in *Mif* KO mice, particularly in high-frequency regions, might also suggest that MIF expression is required to maintain sensory HCs or perhaps the relationship between HCs and SCs. HCs are also missing in *Mcp1* KO animals (data not shown), again with the greatest loss from the high-frequency region of the cochlea.

The present study also demonstrates that, whereas MIF is expressed in SCs rather than HCs, MCP1 is localized in the sensory HCs themselves (Fig. 4). Although our previous work demonstrated that MCP1 was necessary, but not sufficient, for SAG outgrowth (Bianchi et al., 2005), its presence in HCs provides additional evidence that it too is likely to play a vital role in mediating inner ear development and innervation. Further evidence that MCP1 is an important contributor to ODF was provided by the second series of experiments using function-blocking antibodies, in which we demonstrated that residual ODF bioactivity after anti-MIF antibody treatment was lost upon addition of function-blocking antibodies to MCP1 (supplementary material Fig. S1).

Our finding that immune system 'inflammatory' cytokines such as MIF and MCP1 behave as neurotrophic factors in early inner ear development further suggests that, evolutionarily and developmentally, such cytokines are multifaceted and pleiotropic. It is also clear that a requirement for MIF in development is not limited to the inner ear. For example, the present study showed that mESCs take on neuronal characteristics in the presence of MIF, and that this neuronal induction is similar to the neuronal phenotypes induced by neural growth factors NGF and CNTF, as our previous studies demonstrate (Roth et al., 2007; Roth et al., 2008). Thus, MIF might play multiple roles throughout the developing nervous system, particularly at early stages of development (Suzuki et al., 2004). Such a role is supported by evidence from several earlier studies. For example, cytokines, including MIF, have been found in the developing CNS, eye and inner ear of *Xenopus* (Suzuki et al., 2004) and mouse (Kobayashi et al., 1999) and in the zebrafish eye (Ito et al., 2008) and ear (Holmes et al., 2011; Shen et al., 2012). MOs injected into one-cell *Xenopus* embryos resulted in the absence of a neuraxis (Suzuki et al., 2004), a phenotype that was rescued by concomitant injection of *Mif* RNAs. Although such a drastic effect is not seen in zebrafish treated simultaneously with the two (*mif* and *mif-like*) MOs (Shen et al., 2012), considerable cell death was evident in both the developing otocyst and the CNS; this phenotype could be rescued by injection of capped *mif* and *mif-like* RNAs (Shen et al., 2012). MIF also appears to be crucial in regulating cell death in the CNS (Chalimoniuk et al., 2006).

Other cytokines/chemokines appear to regulate axonal outgrowth by inhibiting the repellent effects of molecules such as semaphorins and Slit2 (Chalasani et al., 2003). Additionally, some cytokines/chemokines are essential players in neuronal repair processes after axotomy in both the peripheral nervous system and CNS (Deverman and Patterson, 2009, Bajetto et al., 2001; Koda et al., 2004). Thus, cytokines/chemokines have multiple roles throughout various developmental stages of nervous system development and repair.

Additional cytokines might be important for inner ear development. For example, RANTES/CCL1 (Bolin et al., 1998) and tumor necrosis factor (TNF) (Bianchi et al., 2005), as well as the receptors CCR1 (for RANTES) and CXCR4/2 (for MIF) (Bernhagen et al., 2007) and TNFR1/2 (also known as TNFRSF1A/B) (Zou et al., 2005), are found in the inner ear. Another MIF receptor, CD44, is expressed in pillar cells (Hertzano et al., 2010) in developing and adult inner ear tissues. The function of these various cytokines and cytokine-driven signaling systems has yet to be fully explored, and any potential interactions between them still need to be addressed. Determining the exact mechanisms by which cytokines/chemokines and their receptors regulate inner ear development and innervation might find applications in cochlear repair or regeneration (Nishihira, 2000; Morand, 2005), given that MIF receptors (CD74) are found on adult SG neurons.

Acknowledgements

We thank Kristina M. Brooks and Sarah Tomkovich for technical assistance.

Funding

This work was supported by grants from the National Institutes of Health (NIH) [NIH/NINDCD 2RO1DC04184-04, 3R01DC004184-08W12 and R01 DC006436-04A2 to K.F.B.; NIH T32 HG00040 9 (Genome Sciences) to E.M.J.; NIH T32 DE007057 (TEAM) to S.A.L.; NIH/NIDCD P30 DC05188 to D.F.D.; NIH 5T32EB005582-05 (Microfluidics in Biotechnology) to P.R.; NIH T90DK070071 (Regenerative Sciences Training Grant) and NIH T32DC000011 (Hearing, Balance, and Chemical Senses Training Grant) to A.P.C.; NIH AR048267 and NIH AI040987 to A.E.K.]; National Science Foundation (NSF) [IOS 0930096 to K.F.B.; DB-0114831, OKUM to L.M.B.; NSF Graduate Research Fellowship 2004016780 to T.M.R.]; the Deafness Research Foundation (DRF) (to Y.S. and D.L.T.); and the Veterans Administration Research Service, Frederick G. L. Huetwell and William D. Robinson M.D. Professorship (A.E.K.). Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.066647/-/DC1

References

Armstrong, B. D., Hu, Z., Abad, C., Yamamoto, M., Rodriguez, W. I., Cheng, J., Tam, J., Gomariz, R. P., Patterson, P. H. and Waschek, J. A. (2003). Lymphocyte regulation of neuropeptide gene expression after neuronal injury. *J. Neurosci. Res.* **74**, 240-247.
Bajetto, A., Bonavia, R., Barbero, S., Florio, T. and Schettini, G. (2001). Chemokines and their receptors in the central nervous system. *Front. Neuroendocrinol.* **22**, 147-184.
Barald, K. F. and Kelley, M. W. (2004). From placode to polarization: new tunes in inner ear development. *Development* **131**, 4119-4130.
Barald, K. F., Lindberg, K. H., Hardiman, K., Kavka, A. I., Lewis, J. E., Victor, J. C., Gardner, C. A. and Poniatowski, A. (1997). Immortalized cell lines from embryonic avian and murine otocysts: tools for molecular studies of the developing inner ear. *Int. J. Dev. Neurosci.* **15**, 523-540.
Bernhagen, J., Krohn, R., Lue, H., Gregory, J. L., Zernecke, A., Koenen, R. R., Dewor, M., Georgiev, I., Schober, A., Leng, L. et al. (2007). MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. *Nat. Med.* **13**, 587-596.
Bianchi, E., Denti, S., Granata, A., Bossi, G., Geginat, J., Villa, A., Rogge, L. and Pardi, R. (2000). Integrin LFA-1 interacts with the transcriptional co-activator JAB1 to modulate AP-1 activity. *Nature* **404**, 617-621.
Bianchi, L. M. and Cohan, C. S. (1991). Developmental regulation of a neurite-promoting factor influencing statoacoustic neurons. *Brain Res. Dev. Brain Res.* **64**, 167-174.
Bianchi, L. M. and Cohan, C. S. (1993). Effects of the neurotrophins and CNTF on developing statoacoustic neurons: comparison with an otocyst-derived factor. *Dev. Biol.* **159**, 353-365.
Bianchi, L. and Fuchs, P. (2010). Development of the inner ear. In *The Oxford Handbook of Auditory Science* (ed. P. Fuchs), pp. 329-353. New York, NY: Oxford University Press.
Bianchi, L. M., Dolnick, R., Medd, A. and Cohan, C. S. (1998). Developmental changes in growth factors released by the embryonic inner ear. *Exp. Neurol.* **150**, 98-106.
Bianchi, L. M., Person, A. L. and Penney, E. B. (2002). Embryonic inner ear cells reaggregate into specific patterns in vitro. *J. Assoc. Res. Otolaryngol.* **3**, 418-429.
Bianchi, L. M., Daruwalla, Z., Roth, T. M., Attia, N. P., Lukacs, N. W., Richards, A. L., White, I. O., Allen, S. J. and Barald, K. F. (2005). Immortalized mouse inner ear cell lines demonstrate a role for chemokines in promoting the growth of developing statoacoustic ganglion neurons. *J. Assoc. Res. Otolaryngol.* **6**, 355-367.
Bianchi, L. M., Huri, D. and White, I. O. (2006). Embryonic inner ear cells use migratory mechanisms to establish cell patterns in vitro. *J. Neurosci. Res.* **83**, 191-198.
Bolin, L. M., Murray, R., Lukacs, N. W., Strieter, R. M., Kunkel, S. L., Schall, T. J. and Bacon, K. B. (1998). Primary sensory neurons migrate in response to the chemokine RANTES. *J. Neuroimmunol.* **81**, 49-57.
Bozza, M., Satoskar, A. R., Lin, G., Lu, B., Humbles, A. A., Gerard, C. and David, J. R. (1999). Targeted disruption of migration inhibitory factor gene reveals its critical role in sepsis. *J. Exp. Med.* **189**, 341-346.
Bryan, K. J., Zhu, X., Harris, P. L., Perry, G., Castellani, R. J., Smith, M. A. and Casadesus, G. (2008). Expression of CD74 is increased in neurofibrillary tangles in Alzheimer's disease. *Mol. Neurodegener.* **3**, 13.
Chalasani, S. H., Baribaud, F., Coughlan, C. M., Sunshine, M. J., Lee, V. M., Doms, R. W., Littman, D. R. and Raper, J. A. (2003). The chemokine stromal cell-derived factor-1 promotes the survival of embryonic retinal ganglion cells. *J. Neurosci.* **23**, 4601-4612.
Chalimoniuk, M., King-Pospisil, K., Metz, C. N. and Toborek, M. (2006). Macrophage migration inhibitory factor induces cell death and decreases neuronal nitric oxide expression in spinal cord neurons. *Neuroscience* **139**, 1117-1128.
Chen, F. Q., Schacht, J. and Sha, S. H. (2009). Aminoglycoside-induced histone deacetylation and hair cell death in the mouse cochlea. *J. Neurochem.* **108**, 1226-1236.
Deverman, B. E. and Patterson, P. H. (2009). Cytokines and CNS development. *Neuron* **64**, 61-78.
Doetschman, T. C., Eistetter, H., Katz, M., Schmidt, W. and Kemler, R. (1985). The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* **87**, 27-45.
Gerlach, L. M., Hutson, M. R., Germiller, J. A., Nguyen-Luu, D., Victor, J. C. and Barald, K. F. (2000). Addition of the BMP4 antagonist, noggin, disrupts avian inner ear development. *Development* **127**, 45-54.
Gerlach-Bank, L. M., Ellis, A. D., Noonen, B. and Barald, K. F. (2002). Cloning and expression analysis of the chick DAN gene, an antagonist of the BMP family of growth factors. *Dev. Dyn.* **224**, 109-115.
Germiller, J. A., Smiley, E. C., Ellis, A. D., Hoff, J. S., Deshmukh, I., Allen, S. J. and Barald, K. F. (2004). Molecular characterization of conditionally immortalized cell lines derived from mouse early embryonic inner ear. *Dev. Dyn.* **231**, 815-827.
Hertzano, R., Puligilla, C., Chan, S. L., Timothy, C., Depireux, D. A., Ahmed, Z., Wolf, J., Eisenman, D. J., Friedman, T. B., Riazuddin, S. et al. (2010). CD44 is a marker for the outer pillar cells in the early postnatal mouse inner ear. *J. Assoc. Res. Otolaryngol.* **11**, 407-418.
Holmes, K. E., Wyatt, M. J., Shen, Y. C., Thompson, D. A. and Barald, K. F. (2011). Direct delivery of MIF morpholinos into the zebrafish otocyst by injection and electroporation affects inner ear development. *J. Vis. Exp.* **7**, 2466.
Ito, K., Yoshiura, Y., Ototake, M. and Nakanishi, T. (2008). Macrophage migration inhibitory factor (MIF) is essential for development of zebrafish, Danio rerio. *Dev. Comp. Immunol.* **32**, 664-672.
Kavanaugh, A. (2002). Combination cytokine therapy: the next generation of rheumatoid arthritis therapy? *Arthritis Rheum.* **47**, 87-92.
Kobayashi, S., Satomura, K., Levsky, J. M., Sreenath, T., Wistow, G. J., Semba, I., Shum, L., Slavkin, H. C. and Kulkarni, A. B. (1999). Expression pattern of macrophage migration inhibitory factor during embryogenesis. *Mech. Dev.* **84**, 153-156.
Koda, M., Nishio, Y., Hashimoto, M., Kamada, T., Koshizuka, S., Yoshinaga, K., Onodera, S., Nishihira, J., Moriya, H. and Yamazaki, M. (2004). Up-regulation of macrophage migration-inhibitory factor expression after compression-induced spinal cord injury in rats. *Acta Neuropathol.* **108**, 31-36.
Leech, M., Lacey, D., Xue, J. R., Santos, L., Hutchinson, P., Wolvetang, E., David, J. R., Bucala, R. and Morand, E. F. (2003). Regulation of p53 by macrophage migration inhibitory factor in inflammatory arthritis. *Arthritis Rheum.* **48**, 1881-1889.
Morand, E. F. (2005). New therapeutic target in inflammatory disease: macrophage migration inhibitory factor. *Intern. Med. J.* **35**, 419-426.
Nishihira, J. (2000). Macrophage migration inhibitory factor (MIF): its essential role in the immune system and cell growth. *J. Interferon Cytokine Res.* **20**, 751-762.
Patterson, P. H. (1994). Leukemia inhibitory factor, a cytokine at the interface between neurobiology and immunology. *Proc. Natl. Acad. Sci. USA* **91**, 7833-7835.
Roger, T., David, J., Glauser, M. P. and Calandra, T. (2001). MIF regulates innate immune responses through modulation of Toll-like receptor 4. *Nature* **414**, 920-924.
Roth, T. M., Ramamurthy, P., Ebisu, F., Lisak, R. P., Bealmear, B. M. and Barald, K. F. (2007). A mouse embryonic stem cell model of Schwann cell differentiation for studies of the role of neurofibromatosis type 1 in Schwann cell development and tumor formation. *Glia* **55**, 1123-1133.
Roth, T. M., Ramamurthy, P., Muir, D., Wallace, M., Zhu, Y., Chang, L. and Barald, K. F. (2008). Influence of hormones and hormone metabolites on the growth of Schwann cells derived from mouse embryonic stem cells and on tumor cell lines expressing variable levels of neurofibromin. *Dev. Dyn.* **237**, 513-524.
Sánchez-Calderon, H., Milo, M., León, Y. and Varela-Nieto, I. (2007). A network of growth and transcription factors controls neuronal differentiation and survival in the developing ear. *Int. J. Dev. Biol.* **51**, 557-570.
Saul, S. M., Brzezinski, J. A., 4th, Altschuler, R. A., Shore, S. E., Rudolph, D. D., Kabara, L. L., Halsey, K. E., Hufnagel, R. B., Zhou, J., Dolan, D. F. et al. (2008). Math5 expression and function in the central auditory system. *Mol. Cell. Neurosci.* **37**, 153-169.
Shen, Y. C., Thompson, D. L., Kuah, M. K., Wong, K. L., Wu, K. L., Linn, S. A., Jewett, E. M., Shu-Chien, A. C. and Barald, K. F. (2012). The cytokine macrophage migration inhibitory factor (MIF) acts as a neurotrophin in the developing inner ear of the zebrafish, Danio rerio. *Dev. Biol.* **363**, 84-94.
Siemion, I. Z., Kluczyk, A. and Cebrat, M. (2005). The peptide molecular links between the central nervous and the immune systems. *Amino Acids* **29**, 161-176.
Suzuki, M., Takamura, Y., Maéno, M., Tochinai, S., Iyaguchi, D., Tanaka, I., Nishihira, J. and Ishibashi, T. (2004). Xenopus laevis macrophage migration inhibitory factor is essential for axis formation and neural development. *J. Biol. Chem.* **279**, 21406-21414.
Thompson, D. L., Gerlach-Bank, L. M., Barald, K. F. and Koenig, R. J. (2003). Retinoic acid repression of bone morphogenetic protein 4 in inner ear development. *Mol. Cell. Biol.* **23**, 2277-2286.
Wrona, D. (2006). Neural-immune interactions: an integrative view of the bidirectional relationship between the brain and immune systems. *J. Neuroimmunol.* **172**, 38-58.
Wu, Y. Y. and Bradshaw, R. A. (1996). Synergistic induction of neurite outgrowth by nerve growth factor or epidermal growth factor and interleukin-6 in PC12 cells. *J. Biol. Chem.* **271**, 13033-13039.
Zou, J., Pyykkö, I., Sutinen, P. and Toppila, E. (2005). Vibration induced hearing loss in guinea pig cochlea: expression of TNF-alpha and VEGF. *Hear. Res.* **202**, 13-20.

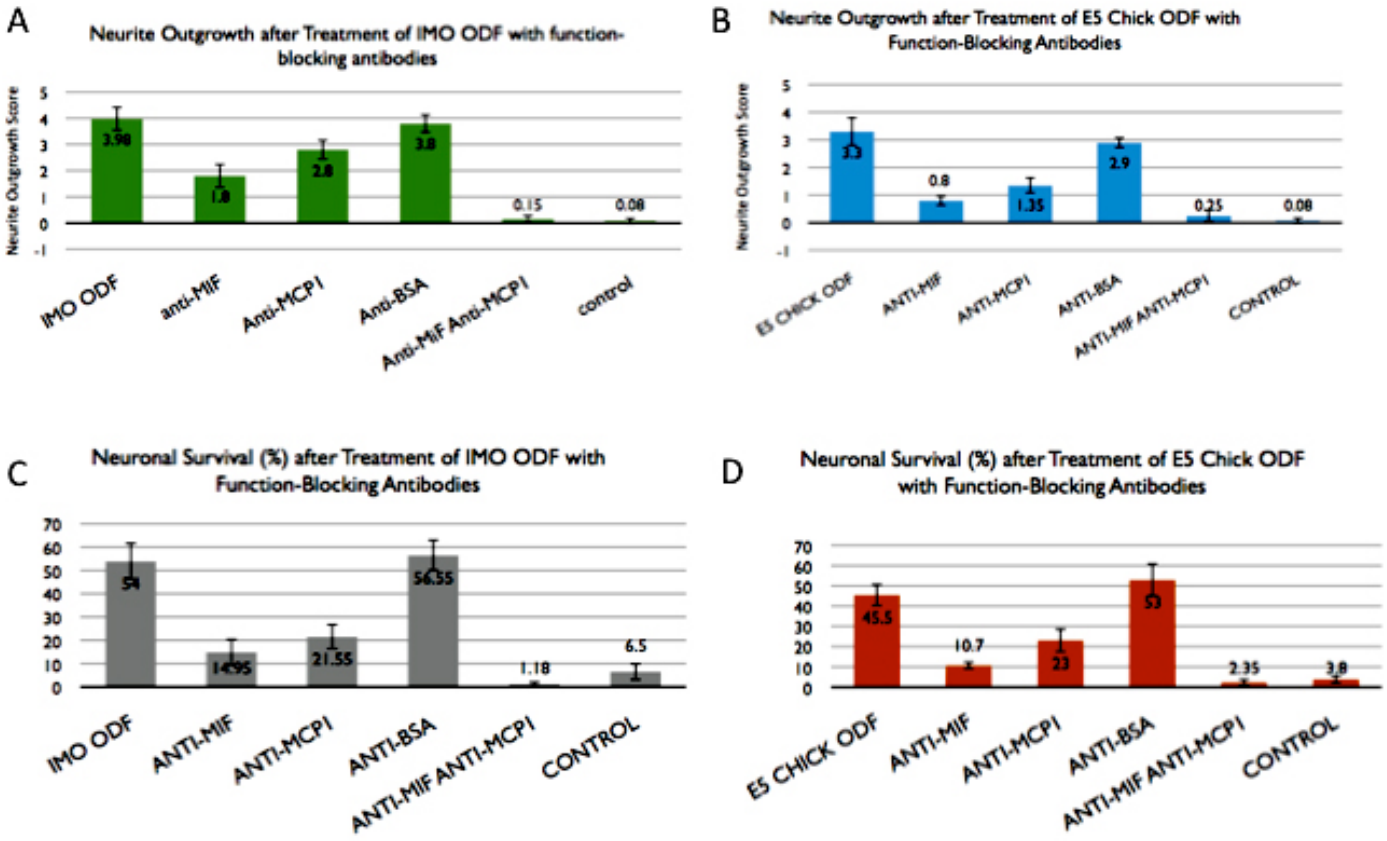

Fig. S1. Sequentially applied function-blocking antibodies to MIF and MCP1 can block the bioactivity of IMO- or chick E5 otocyst-generated ODF. (**A-D**) The MIF function-blocking antibody was added to IMO-generated and chick E5 otocyst-generated ODF to block SAG neurite outgrowth and survival. The MCP1 function-blocking antibody used in our previous work (Bianchi et al., 2005) that partially blocked ODF bioactivity in IMO- and E5 chick-generated ODF was then applied to the anti-MIF-treated CM. The bioactivity of ODF was lost with this sequential antibody treatment.

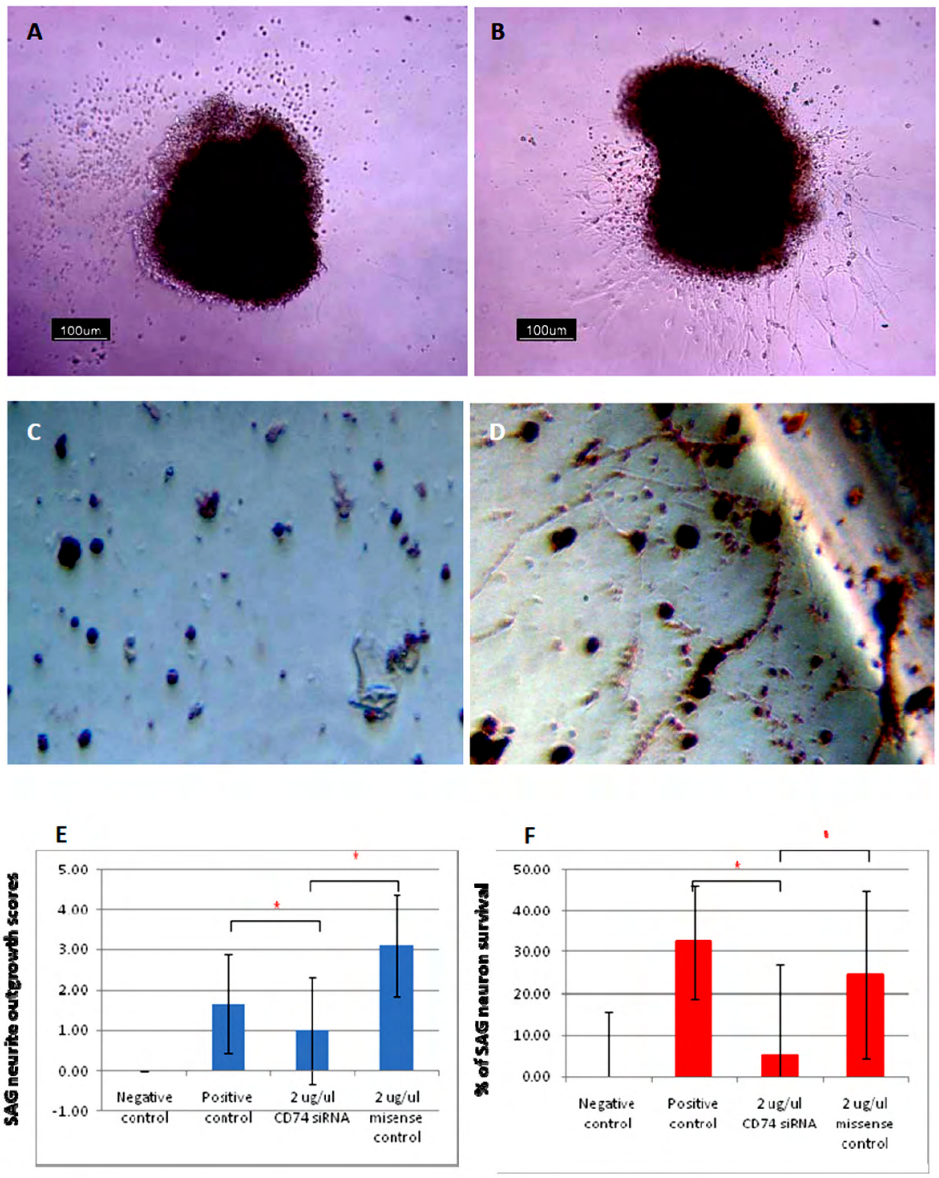

Fig. S2. Avian *Cd74* siRNA reduces SAG neurite outgrowth and survival. Neuronal bioassays using E5 chick whole SAG and dissociated SAG from the same stage embryos exposed to CM generated from E4 chick otocysts (otocyst-generated ODF) to determine the effect of blocking CD74 with siRNA. (**A**) SAG explants treated with otocyst-generated ODF in the presence of 2.0 μg/μl avian *Cd74* siRNA show little SAG neurite outgrowth. (**B**) By contrast, SAG explants treated with otocyst-generated ODF in the presence of 2.0 μg/μl missense control show SAG neurite outgrowth. (**C**,**D**) Dissociated SAG neurons treated with otocyst-generated ODF in the presence of 2.0 μg/μl avian *Cd74* siRNA demonstrate lower cell survival (C) compared with dissociated SAG neurons treated with otocyst-generated ODF in the presence of 2.0 μg/μl missense control (D). (**E**,**F**) Statistical analysis demonstrates that 2 μg/μl siRNA significantly reduced both SAG neurite outgrowth (E) and survival (F) compared with its missense control ($P<0.05$). Serum- and γ-interferon-free basal medium is used as negative control and otocyst-generated ODF is used as positive control. $n=9$, SAG neurite outgrowth; $n=12$, dissociated SAG neuronal survival.

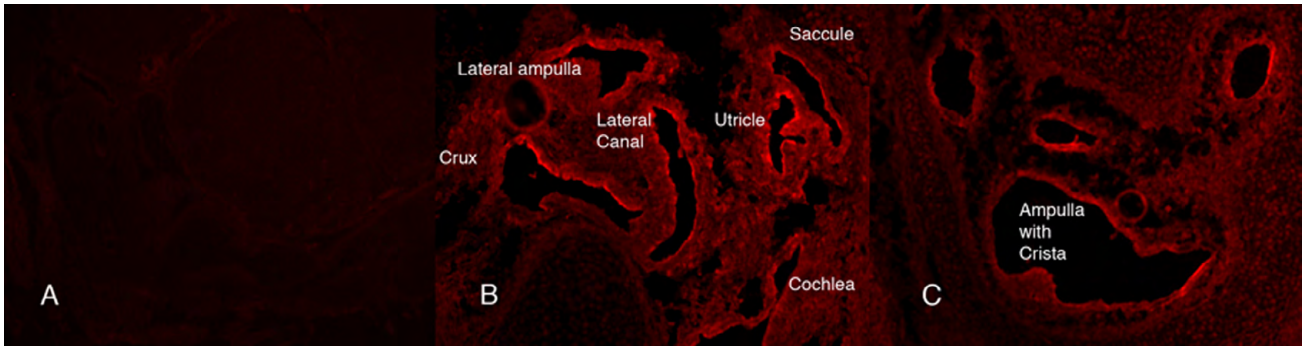

Fig. S3. MIF protein expression in the E15.5 mouse inner ear. (**A**) Control of secondary antibody alone. No background labeling is seen. (**B**,**C**) MIF expression is detected in the developing inner ear, including the cochlea, utricle, saccule, lateral canal, lateral ampulla and the crux. Magnification: 20×.

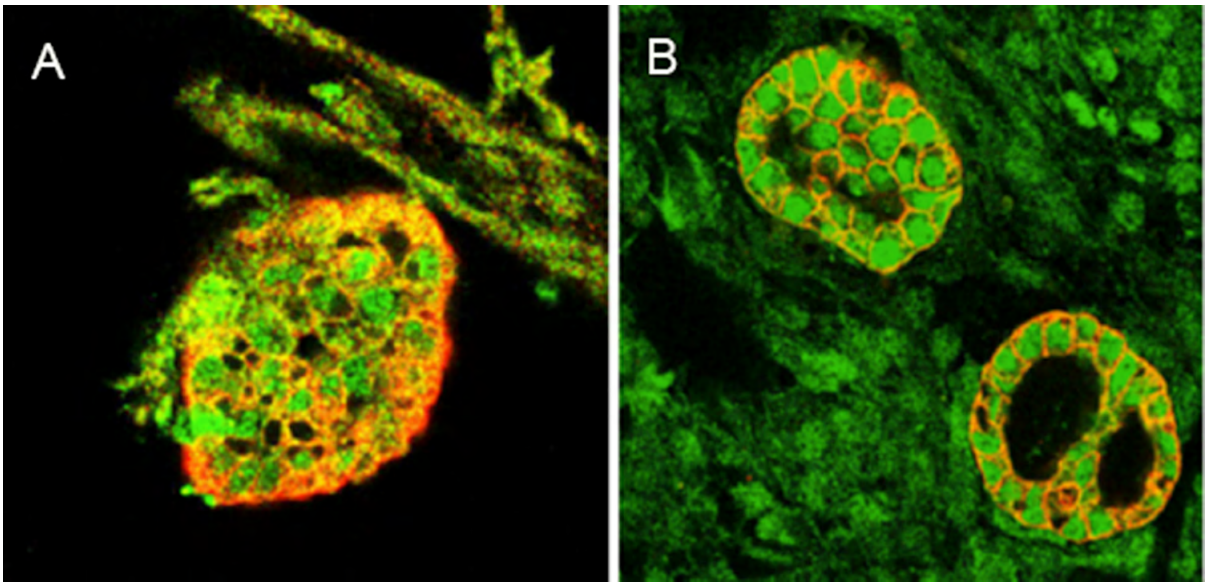

Fig. S4. Re-aggregated embryonic inner ear cells show that MIF is localized to supporting cells. (**A**) Double-labeled fluorescent immunocytochemistry shows myosin VIIa-positive hair cells primarily in the central region of the domes (green), whereas MIF (red) is detected in the surrounding (and underlying) supporting cell layer (20×). (**B**) Supporting cell external membranes are labeled with cytokeratin (red) in the underlying layer of the domes, while MIF expression (green) is detected in the cytoplasmic region of these same cells (20×). The overlying hair cells (above the focal plane) are not immunoreactive for either cytokeratin or MIF.

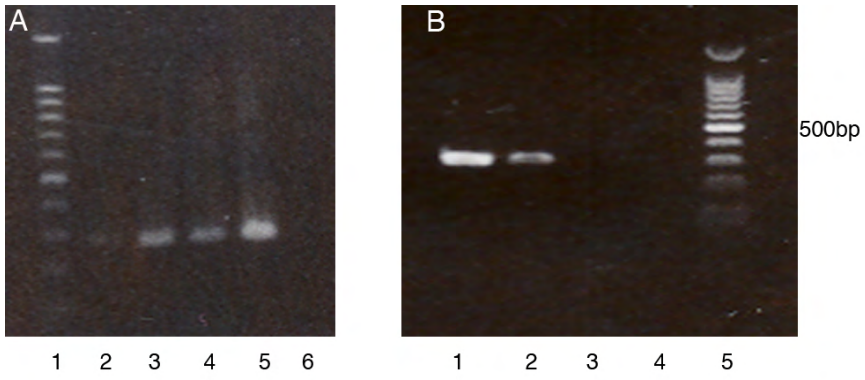

Fig. S5. Expression of *Cd74* mRNA in embryonic and adult inner ear. (**A**) Expression of *Cd74* mRNA is present in the E14 and adult WT (Balb/c) mouse SAG and otocyst. Lane 1, 100 bp ladder (NEB); lane 2, E14 SAG; lane 3, E14 embryonic ear; lane 4, adult ampulla; lane 5, adult inner ear; lane 6, blank. (**B**) Expression of *Cd74* in the adult SG (lane 1) and the sensory epithelium (lane 2). Lane 3, water control; lane 4, blank; lane 5, 100 bp DNA ladder. CD74 is also expressed at the protein level in both the embryonic SAG and postnatal spiral ganglion, as detected by western blotting (not shown).

Table S1. Summary data for the function-blocking antibody experiments

	Control	ODF	MIF	MIF+αMIF	MIF+αBSA	ODF+αMIF	ODF+αBSA
Control		NC/ $P<10^{-5}$	NC/ $P<10^{-5}$	NC/NS	NC/$P<10^{-5}$	NC/$P<10^{-2}$	NC/$P<10^{-5}$
ODF			NS/ $P<10^{-2}$	NC/NC	NC/NC	$P<10^{-4}$/ $P<10^{-3}$	NS/NS
MIF				$P<10^{-3}$/ $P<10^{-3}$	NS/NS	NC/NC	NC/NC

Function-blocking antibody effects on neurite outgrowth of whole statoacoustic ganglia (red) or on dissociated statoacoustic ganglion neuron survival (blue), showing statistical comparisons among conditions. NS, not significant; NC, no comparison was performed.