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SHORT COMMUNICATION

Repair of traumatized mammalian hair cells via sea anemone repair proteins

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ABSTRACT

Mammalian hair cells possess only a limited ability to repair damage after trauma. In contrast, sea anemones show a marked capability to repair damaged hair bundles by means of secreted repair proteins (RPs). Previously, it was found that recovery of traumatized hair cells in blind cavefish was enhanced by anemone-derived RPs; therefore, the ability of anemone RPs to assist recovery of damaged hair cells in mammals was tested here. After a 1 h incubation in RP-enriched culture media, uptake of FM1-43 by experimentally traumatized murine cochlear hair cells was restored to levels comparable to those exhibited by healthy controls. In addition, RP-treated explants had significantly more normally structured hair bundles than time-matched traumatized control explants. Collectively, these results indicate that anemone-derived RPs assist in restoring normal function and structure of experimentally traumatized hair cells of the mouse cochlea.

KEY WORDS: Cochlea, Deafness, Hair cell evolution, Mouse, Outer hair cells

INTRODUCTION

Hair cells are sensory cells equipped with an apical hair bundle mechanoreceptor that transduces a signal when deflected in the appropriate direction. The evolutionary origin of vertebrate hair cells is debatable (Coffin et al., 2004). One possibility is that hair cells arose in common ancestors of vertebrates and invertebrates. In vertebrates, hair bundles consist of stereocilia, each of which contains a core of cross-linked, actin filaments. Stereocilia are graded in length across the hair bundle (Hudspeth, 1985). In ascidians, sensory hair cells of the coronal organ feature hair bundles consisting of actin-based stereocilia that are graded in length across the hair bundle (Burighel et al., 2011).

Similarly, on tentacles of sea anemones, sensory hair bundles consist of actin-based stereocilia (Watson et al., 1997). Signal transduction in anemone hair bundles, like that of their counterparts in vertebrates, is abolished by aminoglycosides (Watson et al., 1997). Furthermore, in both vertebrate and anemone hair bundles, pairs of stereocilia are joined together by extracellular linkages, including 'tip links' that interconnect the tip of shorter stereocilia to the side of the adjacent, taller stereocilia. Tip links consist, in part, of cadherin-23 in both vertebrate and anemone hair bundles (Siemens et al., 2004; Watson et al., 2008). Tip links are essential to mechanotransduction. The structural integrity of tip links is disrupted after overstimulating hair cells or after immersing hair cells in calcium-depleted buffers (Pickles et al., 1987; Assad et al., 1991; Zhao et al., 1996; Clark and Pickles, 1996).

In humans, overstimulating hair cells can result in noise-induced hearing loss. Structural aberrations to hair cells after noise trauma include a loss of tip links, disarrayed stereocilia, and even a complete loss of hair bundles (Saunders et al., 1985; Pickles et al., 1987; Clark and Pickles, 1996). In vertebrates, noise trauma can lethally or sub-lethally damage hair cells because of structural damage, oxidative stress and excitotoxicity (Cheng et al., 2005; Hakuba et al., 2000; Henderson et al., 2006). Excitotoxicity damages afferent synapses, rendering hair cells incapable of communicating signals. Synaptopathy is a growing field of study in which treatments are being sought to restore synapses to hair cells after trauma (Kujawa and Liberman, 2015).

The repair of sub-lethally damaged hair cells ranges from replacing lost tip links while restoring order to the splayed stereocilia, to replacing the lost hair bundle. The replacement of damaged or lost tip links is known to occur in birds and mammals (Zhao et al., 1996; Jia et al., 2009; Indzhykulian et al., 2013) within 24 h of trauma. Hair cells in lower vertebrates and in mammalian vestibular sensory epithelia can survive the loss of the hair bundle and spontaneously develop a new hair bundle within a few days to a week of trauma (Baird et al., 1996; Gale et al., 2002; Zheng et al., 1999). Early reports indicated that damaged cochlear hair cells of mammals can likewise regrow hair bundles (Sobkowicz et al., 1992, 1996), but more recent studies have been unable to confirm this finding (Jia et al., 2009). Interestingly, in guinea pigs, noise-damaged hair cells can be induced to regrow stereocilia after forced expression of the transcription factor *Atoh1* (Yang et al., 2012).

Once lost, hair cells in adult mammals are not thought to be replaced. In contrast, in other classes of vertebrate animals, lost hair cells are replaced by mitosis of supporting cells to form new supporting cells and replacement hair cells (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). Alternatively, new hair cells can arise directly from transdifferentiation of supporting cells (Baird et al., 1996). An active field of research is aimed at identifying the means by which key genes might be activated in supporting cells (or perhaps latent stem cells) in the mammalian cochlea so that these cells re-enter the cell cycle and/or transdifferentiate into hair cells. A detailed discussion of this fascinating research appears in several recent reviews (Rubel et al., 2013; Burns and Corwin, 2013; Oesterle, 2013).

How do hair cells in invertebrates fare after trauma? In anemones, hair bundles are severely traumatized after a 1 h immersion in calcium-depleted seawater (Watson et al., 1997, 1998). However, even severely traumatized hair cells recover within 4 h, in part because of specific, secreted proteins named 'repair proteins' (RPs) (Watson et al., 1998). The RPs can be isolated and then exogenously supplied to shorten the time course of functional recovery of

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Received 30 November 2015; Accepted 18 May 2016

Journal of Experimental Biology (2016) 219, 2265-2270 doi:10.1242/jeb.135459

anemone hair cells from 4 h to 8 min (Watson et al., 1998). Similarly, exogenously supplied anemone RPs assist recovery of traumatized lateral line hair cells in blind cavefish such that functional recovery is shortened from 9 days to ≤1.3 h (Repass and Watson, 2001; Berg and Watson, 2002). The present study tested whether anemone RPs can assist damaged hair cells in mammals to recover from trauma. The question is intriguing because the answer may help to reveal the extent of the similarity between anemone and vertebrate hair bundles.

MATERIALS AND METHODS

Tissue culture of the organ of Corti

All animal handling procedures were approved by the Institutional Animal Care and Use Committee of the University of Louisiana at Lafayette. Organ of Corti explants were dissected from 3–5 day old CD1 mouse pups (Charles River Laboratories, MA, USA) in Hepes-buffered Hank's balanced salt solution (HBHBSS; Life Technologies, CA, USA). The organ of Corti explants were placed on laminin-coated coverslips (Carolina Biological, NC, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with Hepes, 7% fetal bovine serum (FBS) and 10 µg ml^{-1} ampicillin (Life Technologies) at 37°C and 5% CO_2 (Parker et al., 2010). Explants were cultured for at least 1 h before the experiment was initiated. Calcium-depleted HBHBSS was prepared with 1× calcium/magnesium-free HBSS (Life Technologies) supplemented with 10 mmol l^{-1} Hepes (pH 7.2) and 8 mmol l^{-1} EGTA (final concentrations; Sigma-Aldrich, MO, USA). Explants were incubated in HBHBSS (healthy controls) or in calcium-depleted HBHBSS (traumatized controls and RP-treated explants) for 15 min at room temperature (RT) followed by either immediate processing or processing after a 1 h period. During this 1 h period, RP-treated explants were incubated in culture media (1 ml) enriched with RPs eluted from four specific bands purified from two blue-native PAGE gels (Life Technologies) corresponding to RPs (Tang and Watson, 2015). Healthy controls and traumatized controls were cultured in media enriched with gel pieces eluted from protein-free blue-native PAGE gels for 1 h.

FM1-43 uptake assay

Explants were immersed in 5 µmol l^{-1} FM1-43FX (freshly prepared for each experiment) for 10 or 30 s followed by three rinses in phosphate-buffered saline (PBS). Stained explants were fixed in 4% paraformaldehyde in PBS for 10 min at RT. Fixed explants were washed in PBS twice before imaging using epifluorescence microscopy (model RP011-T, LOMO America, IL, USA) using a 20× objective (Plan Achromat, NA=0.45). Images were captured with an STL-11000M SBIG cooled CCD camera (SBIG, CA, USA) operated using Maxim-DL software (Diffraction Limited, ON, Canada). Three to four images were taken step-wise around the middle turn of each explant. In each image, the mean fluorescence intensity was measured in gray values from a randomly chosen area that encompassed 15 outer hair cells (OHCs) using ImageJ software (National Institutes of Health, MD, USA). Fluorescence intensity data were averaged across the three to four micrographs taken of each cochlear explant such that a single mean fluorescence intensity value was obtained for each explant. Data were analyzed using a factorial ANOVA to determine the effect of treatment and litter (each treatment included several litters). Fisher's *post hoc* LSD tests were used to test for significant differences ($P<0.05$) between treatments with STATISTICA (StatSoft, Inc., OK, USA).

Hair bundle structure and OHC scores

Explants were fixed in 4% paraformaldehyde in PBS for 2 h followed by two 5 min washes in PBS. They were permeabilized using 0.2% Triton X-100 in PBS for 5 min and washed three times in PBS for 5 min each before immersion in 20 µmol l^{-1} TRITC-phalloidin (Sigma) for 1 h in the dark at RT. Explants were washed three times in PBS for 5 min each and mounted in ProLong Gold antifade reagent (Life Technologies). Specimens were imaged with epifluorescence microscopy using a 100× oil immersion objective (Plan Fluorite, NA=1.30, LOMO). We semi-quantified differences in hair bundle morphology and abundance by scoring hair bundles along a single row of OHCs along linear transects of 50 µm. Each hair bundle was assigned a value of 1.0 if it was V-shaped and 0.5 if it was disorganized; a value of 0.0 was assigned if no phalloidin-stained hair bundle was apparent. Using a blind design, an evaluator selected a region in the image having the most well-structured hair bundles per 50 µm transect. Thus, there was a unidirectional bias applied across the treatments to select normally structured hair bundles. Scores were summed for OHCs along the 50 µm transect to give a single value for each explant. Data were analyzed using a one-way ANOVA, with Fisher's *post hoc* LSD tests ($P<0.05$) to test for significant differences among treatments.

RESULTS AND DISCUSSION

Restoration of dye uptake after 1 h incubation in anemone RP-enriched culture media

This study focused on OHCs of the cochlea. Uptake of FM1-43 into hair cells via mechanotransduction channels is commonly used to assess hair cell function (Gale et al., 2001). Control and experimental explants of the murine organ of Corti were treated as described in Materials and methods and then immersed in FM1-43 for 10 s (Fig. 1). Representative images are shown for healthy controls (Fig. 1A), traumatized controls allowed to spontaneously recover in culture media for 1 h (Fig. 1B) and explants that were traumatized and then allowed to recover for 1 h in culture media enriched with RPs (Fig. 1C). Fluorescence intensity was assayed from digital micrographs. A factorial ANOVA confirmed the effect of treatment ($P=0.003$) and littermates ($P=0.004$) on FM1-43 fluorescence within OHCs. If traumatized controls were tested immediately after trauma, dye uptake was significantly less than in time-matched, healthy controls ($P=0.040$; Fig. 1D), indicating that loss of function occurred rapidly. If traumatized controls were allowed to spontaneously recover from trauma for 1 h before they were tested, dye uptake was again significantly less than in time-matched healthy controls ($P=0.001$; Fig. 1D). Interestingly, if traumatized explants were incubated for 1 h in RPs and then tested, dye uptake was statistically comparable to that of time-matched, healthy controls ($P=0.966$), despite having been traumatized (Fig. 1D). As expected, the RP-treated explants had significantly greater dye uptake than time-matched, traumatized controls ($P=0.002$; Fig. 1D).

Because limiting FM1-43 exposure to 10 s imposes technical challenges associated with rapidly moving specimens between solutions that may inadvertently mechanically damage delicate hair cells, experiments similar to those described above were performed, but with 30 s periods of FM1-43 immersion. Again, traumatized controls had significantly less dye uptake than time-matched, healthy controls (Fig. 1E, $P=0.002$). Explants that were traumatized and then incubated in RP for 1 h had uptake of dye comparable to time-matched, healthy controls ($P=0.498$) and significantly greater than in time-matched, traumatized controls ($P=0.010$; Fig. 1E).

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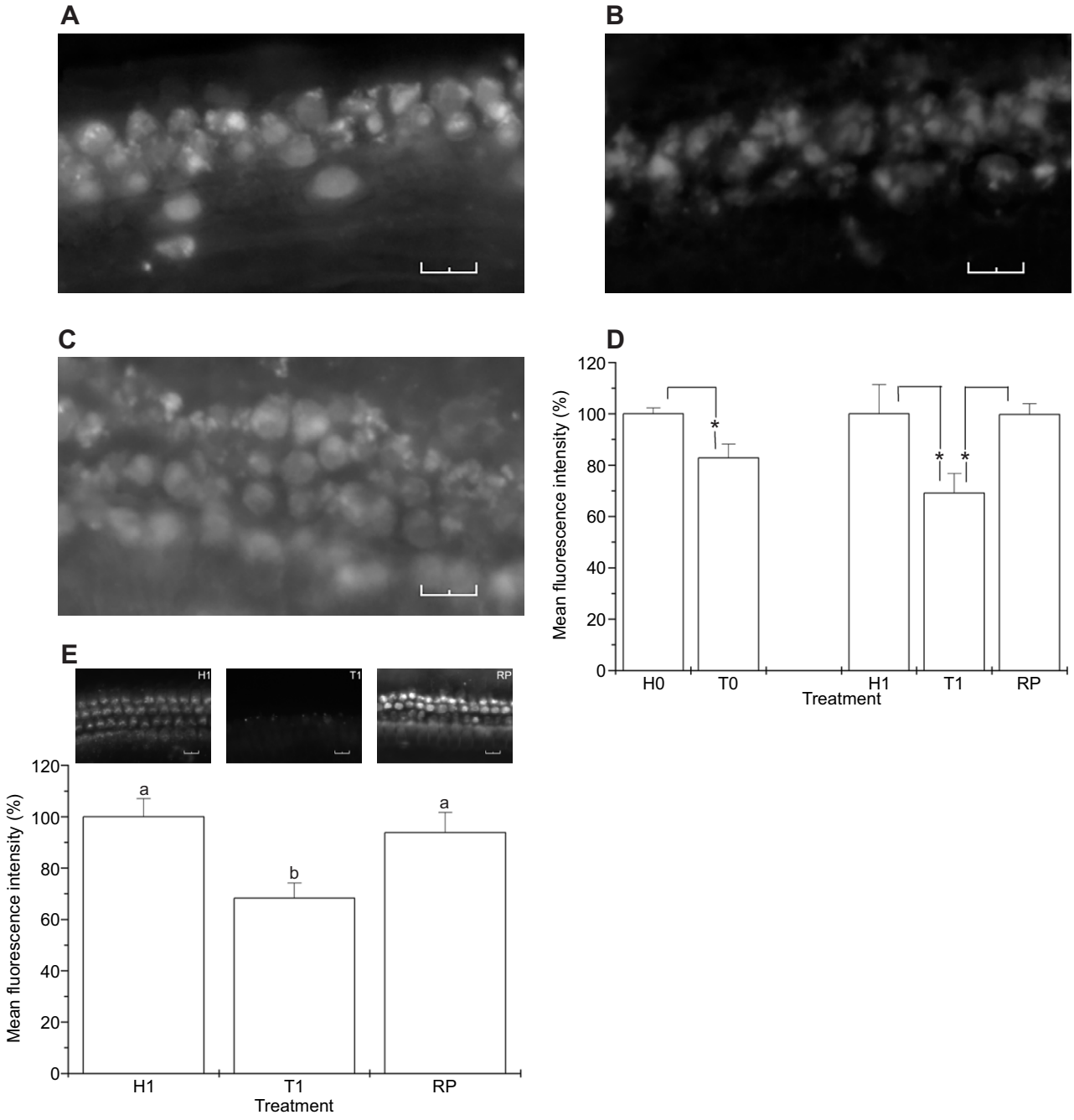

Fig. 1. Effects of anemone repair proteins (RPs) on FM1-43 uptake in murine outer hair cells after incubation in calcium-depleted culture media. Specimens were traumatized by a 15 min immersion in calcium-depleted culture media (or not, for healthy controls) and then assayed immediately (time-zero traumatized controls, T0) or allowed to recover for 1 h in calcium-containing culture media alone (1 h traumatized controls, T1) or in the presence of anemone RPs (RP). At this point, specimens were briefly incubated in FM1-43 to label functional hair cells. Representative images of: (A) a healthy control organ of Corti after 1 h culture in calcium-containing culture media (H1), (B) a traumatized control after trauma followed by 1 h recovery in calcium-containing culture media alone (T1) and (C) an experimental organ of Corti after trauma followed by 1 h recovery in calcium-containing culture media enriched with anemone RPs (RP). Scale bars: 10 μm. For presentation of A–C, contrast was linearly stretched in the original 16-bit images between the minimum gray value (5250) and maximum gray value (25,000) and then converted to 8-bit images. For the quantitative data in D, raw images were analyzed without any image processing. (D) Percentage of mean (+s.e.m.) FM1-43 fluorescence intensity (after a10 s incubation in FM1-43) relative to healthy controls at the same time point. Asterisks indicate a significant difference (Fisher's LSD $P<0.05$). For healthy control explants, there were $N=3$ replicates at $t=0$ (H0) and $N=5$ replicates at $t=1$ h (H1). For traumatized control explants, there were $N=2$ replicates at $t=0$ (T0) and $N=4$ replicates at $t=1$ h (T1). For RP-treated explants, there were $N=5$ replicates (RP). (E) Percentage of mean (+s.e.m.) FM1-43 fluorescence intensity (after a 30 s incubation in FM1-43) relative to time-matched, healthy controls. Data are shown for healthy controls ($N=15$ replicates, H1), traumatized controls ($N=14$ replicates, T1) and RP-treated explants ($N=11$ replicates, RP). Individual data points were derived by sampling several groups of 15 outer hair cells (OHCs) from an area of the middle turn (see Materials and methods). Gray values were obtained from raw images without any image processing. Error bars indicate s.e.m. calculated from replicate experiments. Lowercase letters positioned above the bars indicate statistically significant groups identified by ANOVA followed by Fisher's LSD *post hoc* tests ($P<0.05$). Representative images of explants from each treatment are shown above the bars; contrast was linearly stretched in the original 16-bit images from the minimum gray value (4000) to the maximum gray value (65,000) and then converted to 8-bit images. Scale bars: 10 μm.

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Representative images of the explants are shown above each bar in Fig. 1E.

Restoration of normal morphology in hair bundles after 1 h incubation in anemone RP-enriched culture media

Whereas healthy controls featured well-organized, V-shaped hair bundles (Fig. 2A), specimens immersed in calcium-depleted media showed somewhat disorganized hair bundles (Fig. 2B,C). However, incubation in RPs after trauma often led to a partial recovery of normal structure (Fig. 2D). In traumatized controls processed immediately after trauma, the hair bundle score did not differ significantly from that of time-matched, healthy controls (P=0.880; Fig. 2E). Thus, the immediate effects of immersion in calcium-depleted buffers on the structure of hair bundles were modest. In traumatized controls allowed to spontaneously recover for 1 h after trauma, the hair bundle score was significantly decreased relative to that of time-matched, healthy controls (P=0.002; Fig. 2E), suggesting that the full extent of damage requires some time after

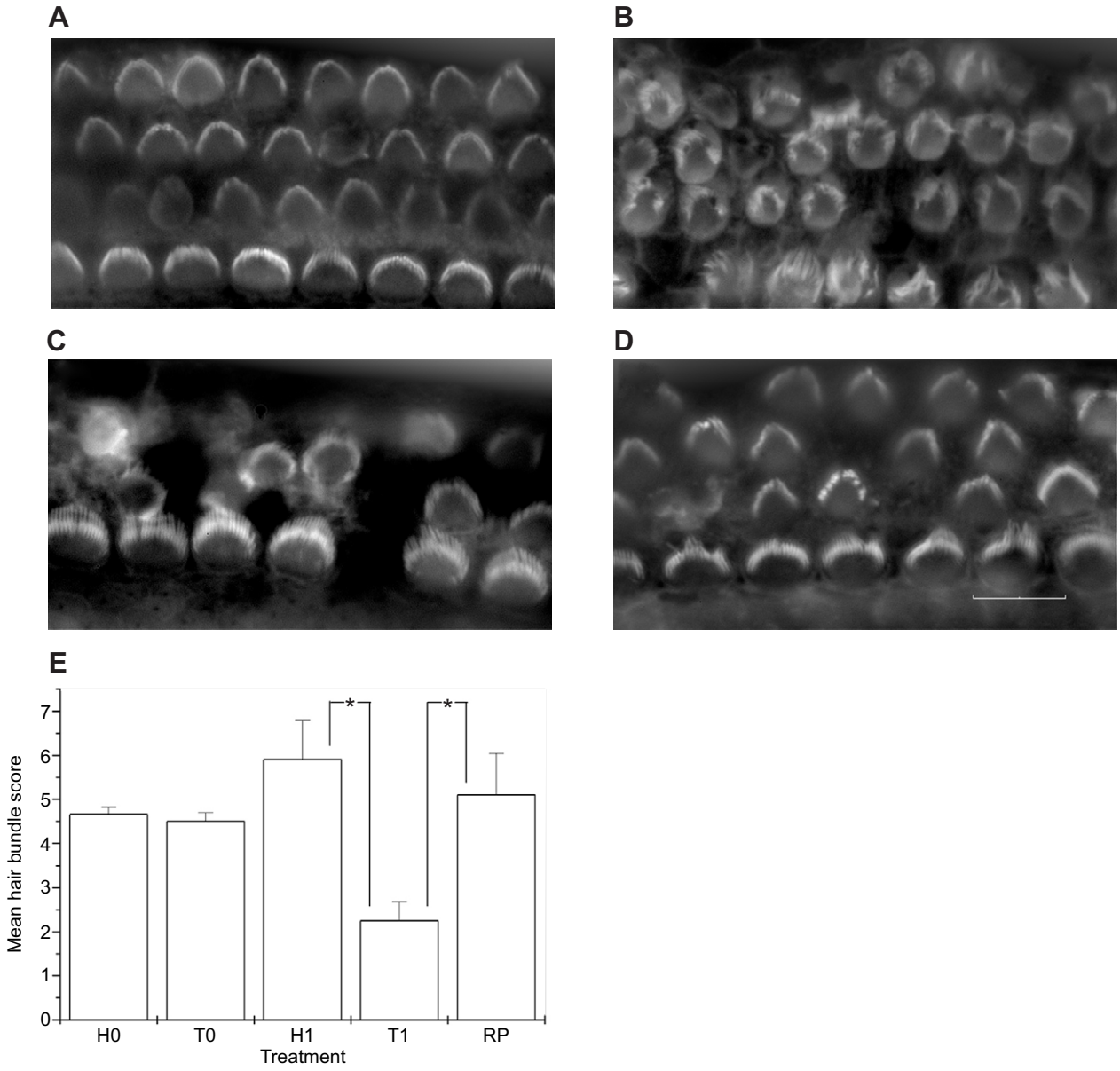

Fig. 2. Effects of anemone RPs on morphology of hair bundles of OHCs. Specimens were treated as described in the legend for Fig. 1 and then fixed and processed for F-actin cytochemistry using a fluorescent derivative of phalloidin. Representative images of: (A) a healthy control incubated for 1 h in calcium-containing culture media (H1), (B) a traumatized control fixed immediately after trauma (T0), (C) a traumatized control fixed after a 1 h recovery period in calcium-containing culture media (T1), (D) and an experimental explant fixed after trauma and 1 h recovery in calcium-containing culture media enriched with repair proteins (RP). Scale bars: 10 μm. For presentation, images were Lucy–Richardson deconvolved, and then subjected to background subtraction, a sharpening filter and contrast enhancement. Image manipulations were identical across treatments. (E) Average score for explants in which OHCs were scored for morphology of hair bundles, if present. Those OHCs having V-shaped hair bundles were scored as 1.0, those having disorganized hair bundles were scored as 0.5 and those OHCs missing hair bundles were scored as 0.0. These values were summed across a 50 μm transect for each explant. Data indicate the mean (+s.e.m.) hair bundle score for healthy control explants at t=0 (H0, N=3 replicates) and t=1 h (H1, N=5 replicates); traumatized control explants at t=0 (T0, N=4 replicates) and t=1 h (T1, N=4 replicates); and RP-treated explants (RP, N=5 replicates). Asterisks indicate statistically significant differences between specific groups identified after ANOVA followed by Fisher's LSD *post hoc* test (P<0.05).

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trauma to fully develop. In contrast, explants incubated in RPs for 1 h after trauma had a significantly higher hair bundle score than time-matched traumatized controls (P=0.013; Fig. 2E). Thus, it appears that RPs stabilized the damaged hair bundles and even helped to restore order to them. How do anemone RPs assist murine hair bundles to recover?

Homologs of RPs from sea anemones are present in the murine proteome

Recently, several polypeptide constituents of RPs utilized by the anemone *Nematostella vectensis* were identified using mass spectrometry (Tang and Watson, 2015). Secreted proteasomes and HSP70 chaperones were identified in the RP suite and, furthermore, experimentally implicated in the repair of traumatized hair cells (Tang and Watson, 2015). To determine the degree to which RPs are evolutionarily conserved, anemone polypeptides were aligned (BLASTp) to the *Mus musculus* proteome, and homologs were identified for all 37 polypeptides identified in RP samples (Table S1). Sixteen out of 37 anemone polypeptides yielded E (expectation)-values of 0, and the highest E-value of all was 10^{-15}. Such low E-values indicate a high degree of homology between the anemone and mouse polypeptides. Most of the anemone polypeptides were greater than 60% identical to mouse homologs at the amino acid level.

Conclusions

The homology between anemone RP polypeptides and mouse polypeptides can perhaps help to explain the biological activity of exogenously supplied anemone RPs on traumatized murine OHCs. Taken together, the results of this study are consistent with the possibility that anemone repair proteins assist in restoring normal structure and mechanotransduction to experimentally traumatized murine OHCs. Such a recovery likely involves repositioning stereocilia and replacing or reattaching linkages, including tip links. The subcellular processes by which order is restored to the disorganized hair bundles are undoubtedly complex. In anemone hair bundles, trauma results in a decrease in F-actin levels in stereocilia, followed by a recovery of F-actin levels at the conclusion of repair (Watson and Mire, 2001). In birds, a reversible 'softening' of hair bundles follows overstimulation in hair cells (Duncan and Saunders, 2000). Variable levels of F-actin were observed in stereocilia of mammalian cochlea after noise exposure (Avinash et al., 1993; Hu and Henderson, 1997), raising the possibility that depolymerization and re-polymerization of F-actin might also occur in recovering stereocilia of noise-damaged hair cells of the inner ear. The results of this study suggest that at least some of the subcellular mechanisms by which damaged hair bundles are repaired were conserved in evolution.

Acknowledgements

We thank Drs A. B. Mayfield for comments on early drafts of the manuscript, G. I. Frolenkov for advice on culturing explants, and P. Mire for scoring hair cells.

Competing interests

The authors declare no competing or financial interests.

Author contributions

All authors had full access to all data in the study and take responsibility for the integrity of the data. P.-C.T. performed experiments, and acquired and analyzed the data. P.-C.T. and G.M.W. designed the study, interpreted the data and were involved in manuscript preparation. K.M.S. assisted in culturing explants of the organ of Corti.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.135459.supplemental

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