

# Intracellular Delivery of Short Interfering RNA in Rat Organ of Corti Using a Cell-penetrating Peptide PepFect6

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RNA interference (RNAi) using short interfering RNA (siRNA) is an attractive therapeutic approach for treatment of dominant-negative mutations. Some rare missense dominant-negative mutations lead to congenital-hearing impairments. A variety of viral vectors have been tested with variable efficacy for modulating gene expression in inner ear. However, there is concern regarding their safety for clinical use. Here, we report a novel cell-penetrating peptide (CPP)-based nonviral approach for delivering siRNA into inner ear tissue using organotypic cultures as model system. PepFect6 (PF6), a variant of stearyl-TP10, was specially designed for improved delivery of siRNA by facilitating endosomal release. We show that PF6 was internalized by all cells without inducing cytotoxicity in cochlear cultures. PF6/siRNA nanoparticles lead to knockdown of target genes, a housekeeping gene and supporting cell-specific *connexin 26*. Interestingly, application of PF6/*connexin 26* siRNA exhibited knockdown of both *connexin 26* and *30* mRNA and their absence led to impaired intercellular communication as demonstrated by reduced transfer of calcein among the PF6/*connexin 26*-siRNA-treated cells. Thus, we conclude that PF6 is an efficient nonviral vector for delivery of siRNA, which can be applied as a tool for the development of siRNA-based therapeutic applications for hearing impairments.

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## Introduction

RNA interference (RNAi) is a post-transcriptional process in which double-stranded short interfering RNA (siRNA) triggers a sequence-specific suppression of the homologous gene.<sup>1,2</sup> RNAi offers a great potential for altering a disease phenotype of various genetic disorders. Despite quite efficient and reliable gene silencing by siRNA, its applications are limited, due to poor cellular uptake of unaided siRNA and inadequate *in vivo* bioavailability. To circumvent these limitations, appropriate systems that enable safe and efficient delivery of siRNA are required for further expansion of the therapeutic applications of RNAi. Multiple delivery strategies have been developed and improved upon for both viral and nonviral carriers.<sup>3–5</sup> Nonviral carriers are popular alternatives to the viral vectors due to the toxicity associated with the latter. Among nonviral vectors, cell-penetrating peptides (CPPs) have been extensively used for the delivery of nucleic acids and other bioactive molecules both *in vitro* and *in vivo*.<sup>6–9</sup> CPPs are short cationic and/or amphipathic peptides. They gain access to the cell interior mainly by endocytosis, and thus have the capacity to promote intracellular delivery of conjugated bioactive cargo.<sup>10–13</sup> However, the endosomal entrapment of CPP-oligonucleotide nanoparticles is the major disadvantage associated with their applications.<sup>14</sup> Modification of the existing CPPs has helped to overcome the endosomal entrapment.<sup>9,15,16</sup> Recently, by introducing trifluoromethyl quinoline moieties to stearyl-TP10,<sup>17</sup> we have developed a novel CPP called PepFect6 (PF6) which exhibits increased release of

siRNA from endosomes.<sup>6</sup> In particular, PF6 displayed higher RNAi in comparison with stearyl-TP10 and other commonly used lipid-based transfection agents. Further, the systemic delivery of PF6/siRNA led to knockdown of the target gene without inducing inflammatory response or tissue damage in rats. Despite these advantages, the potency of such peptides has not been investigated for applications into the inner ear.

Hearing loss is one of the most frequently inherited sensory disorders in humans.<sup>18</sup> Nearly 130 loci have been described in nonsyndromic hearing loss and about 50 related genes have been mapped so far.<sup>19</sup> The most common genetic variations associated with congenital autosomal-dominant hearing loss are mutations in gap junction protein  $\beta$  (*gjb*) 2 and 6, encode *connexin* (Cx) 26 and 30, respectively. Several rare missense gain-of-function mutations in *gjb* have been reported in several populations.<sup>20–22</sup> In the cochlea, Cx26 and Cx30 are mainly expressed in nonsensory supporting cells and connective tissue.<sup>23</sup> Interestingly, absence of one *connexin* leads to a coordinated downregulation of the expression of the other in the cochlear supporting cells.<sup>24,25</sup> Missense mutations in Cx26 result in impaired assembly of gap junctions which leads to reduced intercellular communication and subsequently hearing loss.<sup>26–28</sup> A potential therapeutic option for such deafness would be suppression of mutated alleles by RNAi.<sup>29,30</sup> Using organotypic cultures, we investigated the feasibility of PF6 as a siRNA delivery vector for the inner ear applications. These cultures have a systematic arrangement of physiologically connected hair and supporting cells and offer a good test models before *in vivo* applications.<sup>31,32</sup> This study confirmed

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the cellular internalization of PF6/oligonucleotide nanoparticles in the cochlear cultures. Analysis of cultures showed that PF6 induces no damage to the hair cells in the cochlea. As a proof of principle, the potential of these nanoparticles was validated by RNAi of a housekeeping gene hypoxanthine phosphoribosyl transferase1 (HPRT1) and Cx26. Moreover, the PF6/siRNA-mediated gene silencing of Cx26 was translated to reduced functionality of gap junction channels in the supporting cells. Taken together, our results show that PF6 is an interesting and effective noncytotoxic, nonviral, peptide-based carrier of siRNA in the inner ear tissue. It opens an intriguing prospect for the future siRNA therapeutics for the inner ear dysfunctions.

## Results

### Cellular internalization of PF6 in the cochlea

The efficiency of PF6 as a gene vector for the inner ear was tested using organotypic cultures of the cochlea which contain the sensory hair cells and supporting cells. The cultures were incubated with noncovalently complexed Cy5-conjugated random-sequenced oligonucleotides with PF6. Cy5 fluorescence of internalized PF6/ON-Cy5 was seen in all the cells in live cultures after 6 and 24 hours using epifluorescence microscopy (data not shown). Further, the internalization of PF6/oligonucleotide nanoparticles was confirmed in fixed tissue. The cochlear cultures were incubated with biotin-labeled siRNA designed against the housekeeping gene HPRT1, with or without PF6, for 24 hours. The HPRT1-siRNA alone displayed negligible uptake levels (**Figure 1a**) while PF6/HPRT-siRNA biotin labeling was detected in all the cells with an intensive staining in the inner hair cells (**Figure 1b**). Nevertheless, these techniques do not discriminate between the cell surface bound and internalized nanoparticles. Thus, the uptake of nanoparticles was further analyzed in the live cultures incubated with PF6/oligonucleotide-Cy5 using confocal microscopy. Cy5 labeling was detected in both hair cells and supporting cells after 24 hours (**Figure 1c**). The strongest intracellular fluorescence intensity was detected in the apical region of the inner hair cells (**Figure 1d**).

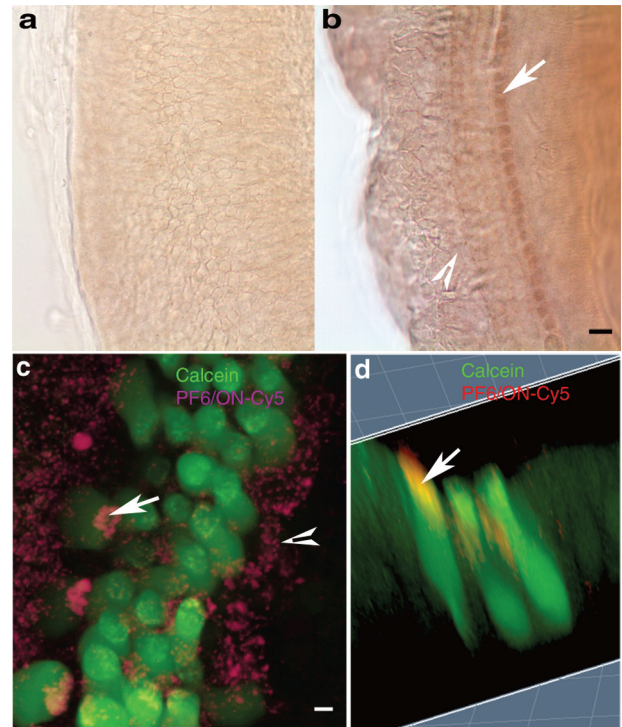
### Cytotoxicity of PF6 in the cochlea

The sensory hair cells of cochlea are orderly arranged in three parallel rows of outer hair cells and a single row of inner hair cells along the spiral-shaped cochlea. Thus, hair cells can easily be counted and any significant loss of sensory cells can readily be detected. The hair cells of the cochlea are very sensitive to a range of drugs.<sup>33</sup> To determine whether PF6 induces cytotoxicity in the cochlea, the organotypic cultures were incubated with 2 and 4  $\mu\text{mol/l}$  PF6 for 72 hours. Since, the sensitivity of inner hair and outer hair cells is variable from base to apex in the cochlea,<sup>33</sup> the cell counting was performed in all the rows separately in apical, middle, and basal segments of cochlear cultures. No damage to the hair cell or their stereocilia bundles was observed after application of PF6 (**Figure 2a**). The mean hair cell counts ( $\pm$  SD) in control cultures showed  $\sim$ 9 inner hair cells and 12 outer hair cells in each row per 100  $\mu\text{m}$  and this did not differ from the cultures incubated with 2 or 4  $\mu\text{mol/l}$  of PF6 (**Figure 2b**). These results

suggest that PF6 has no cytotoxic influence on the hair cells in cochlear cultures.

### Knockdown of a housekeeping gene using PF6/siRNA nanoparticles

The biological activity of PF6-assisted siRNA delivery was validated using a siRNA targeting the housekeeping gene HPRT1. The RNAi response was measured by reverse transcription-quantitative PCR (RT-qPCR) on the RNA extracted from the cochlear cultures. Based on our previous data from the cell lines,<sup>6</sup> the nanoparticles were formulated at two molar ratios, the ratio between HPRT1-siRNA and PF6 being either 1:20 or 1:40. The organotypic cultures were incubated with 100 and 200 nmol/l siRNA (final concentrations) for 24 hours. Subsequent RT-qPCR analysis revealed that HPRT1 mRNA was substantially reduced by PF6/HPRT1-siRNA as compared with HPRT1-siRNA alone (**Figure 3a**,  $P < 0.001$ ) when delivered in the molar ratio of 1:20 with PF6. At a molar ratio of 1:40, there was no significant downregulation of HPRT1 mRNA levels (data not



**Figure 1** PF6-mediated internalization of oligonucleotides (ON) in the organ of Corti. (a,b) Light micrograph of biotin labeling of the organotypic cultures after 1 day of incubation with (a) HPRT1-siRNA alone and (b) PF6/HPRT1-siRNA. The arrows indicate the internalized siRNA-biotin in the hair cells (HC). (c) Fluorescence images of the cellular uptake of PF6/ON-Cy5 in the cochlear cultures. After 1 day of incubation with the nanoparticles, live cultures were stained with calcein (green) and imaged for Cy5. Both HC (stained with calcein) and supporting cells (SC) internalized the ON-Cy5. Arrows indicate the internalized ON-Cy5 in the HC and arrow head in SC. (d) A longitudinal cross-section through the inner HC confirms the cellular uptake in the apical region (arrow) (Cy5 changed to pseudo red color for better visualization). Bar = 5  $\mu\text{m}$  in b and c. HPRT, hypoxanthine phosphoribosyl transferase; siRNA, short interfering RNA.

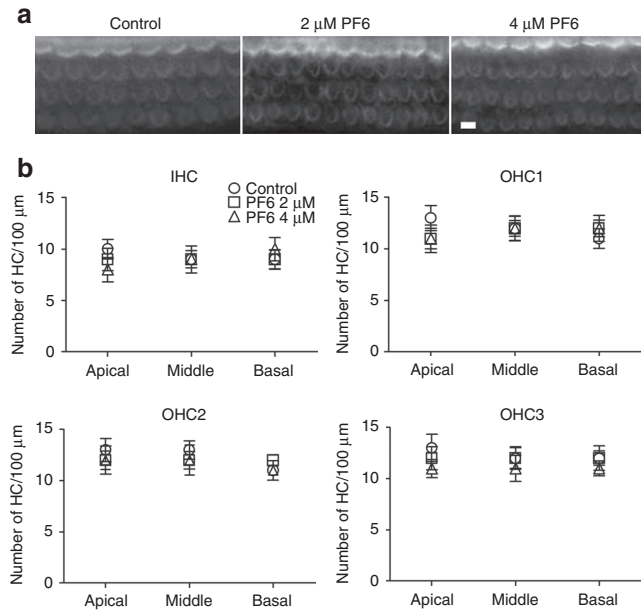
shown). The molar ratio of siRNA to PF6 was thus chosen to be 1:20 in the subsequent studies.

### PF6/Cx26-siRNA-mediated knockdown of Cx26 and Cx30

Having demonstrated a successful noncytotoxic delivery of PF6-assisted siRNA to the cochlear cells and significant diminution in the levels of a housekeeping gene, it was

attempted to inhibit the expression of a functionally relevant molecule in the inner ear. The gap junction protein Cx26, is an ideal candidate as it plays a significant role in intercellular communication between the supporting cells in the cochlea. The organotypic cultures were incubated with the PF6/Cx26-siRNA nanoparticles at two different concentrations, 50 and 100 nmol/l. Control-siRNA, a random nontargeting sequence, complexed with PF6 and Cx26-siRNA alone were included as controls. Cx26 mRNA levels were assessed using RT-qPCR after 24 and 72 hours following the incubation. The RT-qPCR data revealed that the PF6/Cx26-siRNA significantly suppressed the expression of Cx26 mRNA at both concentrations and both timepoints as compared with PF6/control-siRNA (Figure 2b,  $P < 0.05$  and  $P < 0.001$ ). Downregulation was more pronounced at 100 nmol/l PF6/Cx26-siRNA. Incubation of the cultures with Cx26-siRNA alone, exhibited no statistically significant alteration in the expression of Cx26 mRNA as compared with PF6/control-siRNA (Figure 2b).

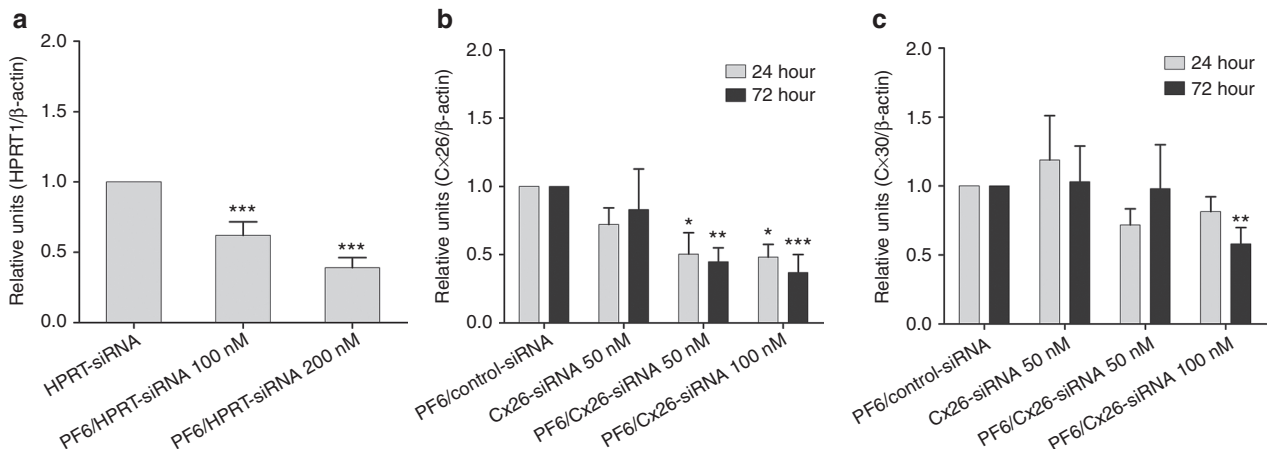
To investigate the accompanying effects of Cx26 knockdown on Cx30, the levels of Cx30 mRNA were analyzed in these cochlear cultures. The RT-qPCR results demonstrated that after 72 hours of incubation, the mRNA of Cx30 was significantly downregulated by PF6/Cx26-siRNA in a concentration of 100 nmol/l as compared with PF6/control-siRNA (Figure 3c,  $P < 0.01$ ). At 24 hours, although downregulation of Cx26 mRNA was observed (Figure 3b), no statistically significant changes in the expression of Cx30 mRNA levels were seen at either concentration (Figure 3c).



**Figure 2 PF6 induced no hair cell (HC) loss in the cochlear cultures.** (a) Representative images of cochlear organotypic culture stained with phalloidin illustrating the arrangement of single row of inner HC (IHC) and three rows of outer HC (OHC) from the cultures incubated with 2 or 4 μmol/l PF6 for 72 hours. (b) No differences in mean densities ( $\pm$  SD) of IHCs and OHCs per 100 μm of the apical, middle, and basal segments of cochlea were observed under different conditions. Bar = 10 μm.

### Reduced functionality of gap junction channels by PF6/Cx26-siRNA

The potency of PF6 to deliver siRNA into the cells was further investigated by analyzing the functionality of the gap junction channels in the cochlea incubated with Cx26-siRNA with or without PF6. The functionality of the gap junction channels was



**Figure 3 PF6-mediated silencing of the target HPRT1 and Cx26 genes in the cochlear cultures.** (a) Efficiency of PF6 was analyzed by comparing the HPRT1 RNAi responses of PF6/HPRT1-siRNA and HPRT1-siRNA alone in the cultures by RT-qPCR following 24 hours of incubation ( $n = 4$ ). (b,c) The cultures were incubated either with Cx26-siRNA alone (50 nmol/l), or PF6/control-siRNA (50 nmol/l) or PF6/Cx26-siRNA nanoparticles in two different concentrations (50 and 100 nmol/l). Quantitative analysis of transcript levels of Cx26 and Cx30, relative to  $\beta$ -actin was determined by RT-qPCR and compared with PF6/control-siRNA cultures after 24 ( $n = 6$ ) and 72 ( $n = 4$ ) hours.  $\beta$ -Actin was used as internal standard. Error bars indicate mean  $\pm$  SEM, \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$  (Mann-Whitney U-test). HPRT, hypoxanthine phosphoribosyl transferase; RNAi, RNA interference; RT-qPCR, reverse transcription-quantitative PCR; siRNA, short interfering RNA.

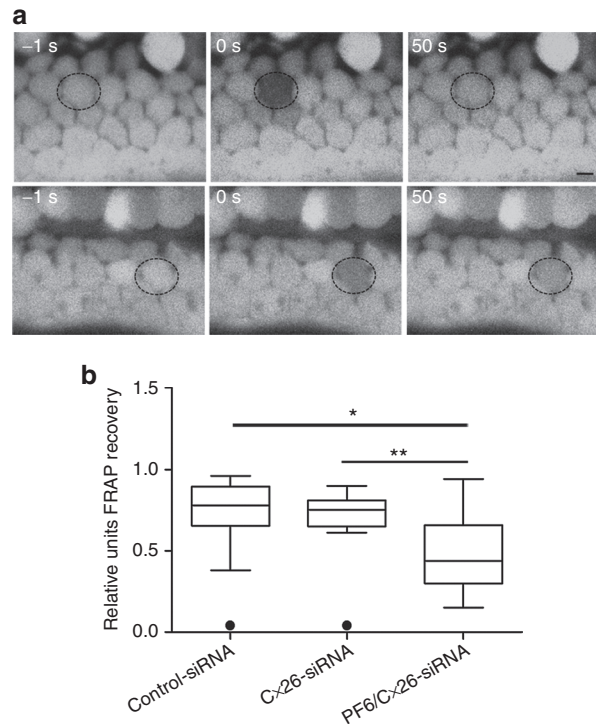
assessed by measuring the diffusion and influx of a fluorescent dye calcein between neighboring cells after photobleaching. In the cochlea, Cx26 and Cx30 are expressed strongly in the supporting cells close to the outer hair cells called outer sulcus cells.<sup>23,34</sup> Hence, fluorescence recovery after photobleaching (FRAP) was measured in these cells. The cochlear cultures were randomly divided in three groups and incubated with 50 nmol/l Cx26-siRNA alone or PF6/control-siRNA and 100 nmol/l PF6/Cx26-siRNA. After 24 and 72 hours of incubation, FRAP experiments were performed in single-blinded approach on these cultures. No differences were observed in the recovery patterns of the outer sulcus cells from the three conditions after 24 hours following incubation with the nanoparticles (data not shown). Interestingly, after 72 hours post-incubation, the FRAP experiments showed that the fluorescence intensity recovered nearly to a prebleaching levels in cells incubated with Cx26-siRNA alone, while the cells incubated with PF6/Cx26-siRNA exhibited a reduced recovery of fluorescence (Figure 4a). The quantitative measurements of the FRAP revealed a significantly slower recovery of fluorescence in cells incubated with PF6/Cx26-siRNA as compared with both Cx26-siRNA alone and PF6/control-siRNA cultures (Figure 4b,  $P < 0.001$  and  $P < 0.05$ ). These findings suggest that downregulation of both Cx26 and Cx30 subsequently led to the impaired intercellular dye transfer in the outer sulcus cells in PF6/Cx26-siRNA cultures after 72 hours.

## Discussion

The development of CPPs has opened new avenues for delivering therapeutic agents both *in vitro* and *in vivo*. The results presented here demonstrate the application of a new, improved CPP, PF6, for transducing siRNA into the tissue of the inner ear. The study showed that PF6/siRNA nanoparticles (i) were internalized by the hair cells and supporting cells in the organotypic cultures without inducing damage to the hair cells, (ii) led to a significant knockdown of target gene mRNA, and (iii) reduced functionality of the target gene.

Previous studies have shown the internalization of CPP/siRNA nanoparticles by cells in monolayers. Although the *in vivo* fluorescence imaging of labeled CPP and downregulation of the target genes are convincing,<sup>6,7</sup> a direct evidence of cellular uptake of the nanoparticles is lacking. To the best of our knowledge, this is the first study to show uptake of the CPP/siRNA nanoparticles in a three-dimensional organotypic culture from the inner ear. Most CPPs, including PF6, use endocytotic pathways to gain access to the interior of cells.<sup>35,36</sup> In line with previous studies, we observed the endocytic internalization of the nanoparticles, as judged by the extensive vesicular distribution. Furthermore, localization of internalized nanoparticles was observed in the apical part of hair cells, where active endocytosis is known to occur.<sup>37</sup>

Recently, a CPP called polyarginine has been reported to deliver enhanced green fluorescent protein (GFP) into the mouse otocyst, an anlage of the inner ear at embryonic day 9.5.<sup>38</sup> Importantly, the early exposure to this CPP did not lead to any functional abnormality in the mature cochlea. This suggests CPP is safe for *in vivo* application even during early development. However, the polyarginine-assisted enhanced GFP signal was detected only transiently into the developing



**Figure 4 Reduced gap junctional communication in PF6/Cx26-siRNA-treated cochlear cultures.** (a) Representative images of the Cx26-siRNA-treated supporting cells (upper panel) and PF6/Cx26-siRNA-treated supporting cells (lower panel) before (-1 second), immediately (0 second), and 50 seconds after photobleaching. The bleached cell (marked with a circle) recovered after 50 seconds in the Cx26-siRNA-treated cultures, while the PF6/Cx26-siRNA-treated cells showed reduced recovery. Bar = 5  $\mu$ m. (b) The quantitative measurement of the fluorescence recovery revealed significantly reduced recovery of PF6/Cx26-siRNA-treated cells in comparison with the cells from the cochlear cultures treated with Cx26-siRNA alone or PF6/control-siRNA for 72 hours. \*\* $P < 0.01$ , \* $P < 0.05$  (Mann-Whitney U-test). FRAP, fluorescence recovery after photobleaching; siRNA, short interfering RNA.

otocyst. Such polyarginine peptides are naive CPPs which get entrapped and degraded in lysosomes.<sup>39</sup> Chemical modifications like addition of cholesterol or stearyl groups to various peptides including polyarginines have been shown to improve the half-life and functionality of the cargo.<sup>9,17</sup> In the current study, we tested the application of PF6 that carries stearyl and trimethylquinoline moieties and therefore, exhibits improved endosomal release of the nanoparticles as compared with the parent peptide stearyl-TP10. PF6 forms stable noncovalently linked nanoparticles with siRNAs by a coincubation method, at different molar ratios of siRNA/PF6.<sup>6</sup> Further, nanoparticles with different molar ratios (1:10 to 1:40) led to downregulation of luciferase expression in different cell lines. As a proof of principle, we chose a widely expressing housekeeping gene HPRT1 as the target gene. We observed a significant downregulation of HPRT1 with 1:20 molar ratio of siRNA:PF6, although 1:40 elicited a HPRT1 RNAi response, however, it was not significant in our organotypic culture model. Moreover, it is well established that high transfection levels are commonly associated with cellular toxicity. Toxic effects have been previously reported for high concentrations

of the parent peptide non-stearylated TP10.<sup>40</sup> In the cochlea, hair cells are the most sensitive structures and we thus checked for the PF6-induced damage or cell death in our cultures. No cytotoxic effects or loss of cells were observed in any segment of cochlea when PF6 was applied to the organotypic cultures.

We have demonstrated that the application of PF6/Cx26-siRNA nanoparticles suppressed the expression of Cx26 mRNA. In addition, a secondary effect of downregulation of Cx26 expression was observed on Cx30 mRNA with a time lag. These findings are in accordance with the report of Ortolano and co-workers<sup>25</sup> who demonstrated a coordinated regulation between the two connexins at expression and functional levels in knockout mice. In contrast to the previously reported knockdown of NADPH oxidase (NOX) 3 and signal transducer and activator of transcription-1 (STAT1) genes by unaided siRNA in the inner ear,<sup>41,42</sup> we observed no significant alteration in expression of Cx26 mRNA by siRNA alone in our culture system. NOX3 and STAT1 are predominantly expressed by the outer hair cells.<sup>41,42</sup> The observed differences could be attributed to the fast and higher rates of endocytosis and diffusion in the outer hair cells compared with that of supporting cells.<sup>43–45</sup> Further, naked siRNA are known to frequently induce inflammatory responses *in vivo* via toll-like receptors<sup>46</sup> and PF6 effectively shields the siRNA resulting in negligible immunogenic responses.<sup>6</sup>

In accordance to the previous studies, absence of connexins leads to impaired intercellular communication in the cochlea of knockout mice. Focal and selective knockdown of Cx26 with PF6/Cx26-siRNA nanoparticles resulted in reduced functionality of the gap junction channels and the biochemical coupling of the supporting cells in the cochlear cultures. The cochlear gap junction channels consist of hetero and homomeric assemblies of Cx26 and 30 subunits.<sup>47,48</sup> Although, heteromeric channels exhibit fast intercellular communication, homomeric gap junctions composed of either Cx26 or Cx30, are sufficient to maintain a slow functionality.<sup>48</sup> This most likely explains why reduced functionality of gap junction channels was observed at 72 hours after application but not at 24 hours when only Cx26 expression was downregulated.

Previously, Maeda and co-workers<sup>29</sup> have demonstrated a lipocomplex-mediated Cx26-siRNA application into the mouse inner ear. However, the target gene in this study was not endogenous mouse Cx26 but a human-specific Cx26 which carried a R75W mutation. These authors showed that ectopically introduced human Cx26 (R75W)-GFP results in higher hearing threshold in the animals and the co-application of Cx26-siRNA with Cx26 (R75W)-GFP subsided these effects. The ectopically introduced Cx26 (R75W)-GFP was reported to be expressed in both the hair cells and supporting cells in the inner ear.<sup>49</sup> However, specific effects on supporting cell function after endogenous Cx26 RNAi were not investigated. Although, the current *in vitro* studies demonstrate that PF6-assisted siRNA delivery downregulates connexins both at mRNA and protein level, as suggested by reduced functionality, future experiments are required to study the potentials of PF6 as a carrier for siRNA *in vivo* with long-term application. Further, studies showing knockdown of dominant negative-mutated allele expression by application of PF6/siRNA and improvement of hearing would be of great interest.

In conclusion, a PF6 peptide-based approach can successfully be applied to deliver siRNA into the inner ear tissue. Taken together, our data suggest that PF6 is an efficient carrier of siRNA that leads to silencing target gene expression and function without inducing cytotoxicity. *In vivo* allele-specific RNAi with long-term availability of siRNAs may become a potential therapeutic strategy to preserve hearing in specific types of hereditary deafness.

## Materials and Methods

**Cochlear organotypic culture.** All animal experiments followed the national approved protocol for care and use of animals in Sweden (N 537/11). Cochleae were dissected from P2-3 Sprague-Dawley rat pups in ice-cold Dulbecco's phosphate-buffered saline (PBS) buffer supplemented with 1.65% glucose (Gibco, Invitrogen, Paisley, UK) and placed on coverslips coated with Cell-Tak (135 µg/ml; Becton Dickinson, Bedford, MA). Cultures were incubated in media containing Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 1% N1 (Sigma, St Louis, MO), 1.65% glucose, and 1% penicillin (Gibco, Invitrogen). For hair cell counting, explants were divided into apical, middle, and basal segments which were cultured separately. Cultures were maintained at 37 °C and 5% CO<sub>2</sub>. On the following day, nanoparticles or siRNA were added to the cultures and incubated further for 24 or 72 hours.

**PF6 and siRNA delivery.** Cy5-conjugated oligonucleotides (CCUCUUACCUCAGUUACA) were synthesized and purified at GE Healthcare, Uppsala, Sweden. Control-siRNA was purchased from Santa Cruz Biotechnology, Santa Cruz, CA (sc-37007), Germany. Other siRNA were synthesized and purified at Biospring, Frankfurt, Germany. Sequence of siRNAs: HPRT1 (biotin conjugated) sense 5'-GCCAGACUUUGUUGGAUUUGAA ATT and antisense 5'-AAUUUCAAUCCAACAAGUCUGG CUU,<sup>49</sup> Cx26 (NM\_001004099) sense 5'-AAGUUAUGAA GGGAGAGAUATT-Cy5/biotin and antisense 5'-UAUCUCUC CCUUCAUGAACUU.<sup>29</sup> PF6 was synthesized and purified as described earlier.<sup>6</sup> To prepare the nanoparticles, PF6 (100 µmol/l stock solution) was mixed with siRNA (10 µmol/l stock solution) in RNase free water and incubated for 30 minutes at room temperature. The nanoparticles were then diluted in culture media to achieve a final siRNA concentration of either 50 or 100 nmol/l. Cultures were incubated with the nanoparticles or siRNA only for 24 or 72 hours.

**Analysis of intracellular peptide distribution in organotypic cultures.** Confocal laser scanning microscopy was performed using an upright LSM 510 laser scanning microscope (Carl Zeiss, Göttingen, Germany) equipped with a Plan-Apochromat 40X objective. One-day-old cochlear cultures were incubated in medium containing the PF6/oligonucleotide-Cy5 nanoparticles for 24 hours. The tissue was rinsed with medium and further incubated with medium containing 2.5 µmol/l calcein (Molecular Probes, Invitrogen, Eugene, OR) for 10 minutes at 37 °C. The cultures were then rinsed twice with warm PBS and images were acquired immediately. The Cy5-labeled nanoparticles were detected by 650 nm wavelength. Calcein fluorescence was excited at 488 nm. All

measurements of PF6/oligonucleotide-Cy5 uptake were performed with living, non-fixed cultures. The acquired images were analyzed using ImageJ (<http://rsb.info.nih.gov/ij/>) and Imaris software (Bitplane AG, Zurich, Switzerland).

After 24 hours, the cultures incubated with 100 nmol/l HPRT1-siRNA-biotin or PF6/HPRT1-siRNA-biotin were fixed with 4% paraformaldehyde for 1 hour, rinsed three times with PBS, and permeabilized using 0.5% Triton-X in PBS. Then cultures were incubated for 1 hour in ABC (Vector Laboratories, Burlingame, CA). A color reaction was developed using diaminobenzidine and H<sub>2</sub>O<sub>2</sub>. After washing, the cultures were mounted on slides and observed under microscope.

**Histology and hair cell counting.** For hair cell counting, the apical, middle, and basal part of the cultures were treated with 2 and 4  $\mu$ mol/l PF6 diluted in H<sub>2</sub>O for 72 hours and H<sub>2</sub>O alone treated cultures were used as control. To label the hair cells, after fixation the cultures were immersed for 40 minutes in TRITC-conjugated phalloidin (1:200; Sigma) in PBS and rinsed three times in PBS. Specimens were examined with a fluorescence microscope (Zeiss Axio Scope; Carl Zeiss, Jena, Germany) and photomicrographs were obtained from representative regions of the apical, middle, and basal turn cultures. The stereocilia bundles and circumferential actin ring surrounding the cuticular plate of inner and outer hair cells were clearly visible in the TRITC-phalloidin-stained specimens, allowing for the quantification of hair cells. Cells in which the stereocilia bundle and cuticular plate was completely absent were counted as missing. To quantify hair cell numbers in control and treated specimens, the number of hair cells was counted in three separate segments (each 100  $\mu$ m) from each specimen. A mean value was computed for each specimen and typically 5–9 specimens were evaluated for each condition. Mann–Whitney U-tests were applied to determine the statistically significant differences among different groups.

**RNA extraction and reverse transcription.** Total RNA from cultured cochlear tissue was isolated using the TRIzol Reagent (Invitrogen) and purified using RNeasy Minelute kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation. The RNA was treated with DNase I (Qiagen). Amounts of RNA were quantified using Quant-iT RNA Assay Kit (Invitrogen) according to the manufacturer's protocol. Typically, 30 ng of total RNA was subjected to reverse transcription in a 20  $\mu$ l reaction. Real-time quantitative PCR was performed using Sybr green Real time PCR kit (Qiagen) in Light cycler (Roche, Mannheim, Germany). An aliquot of 1 ml of the 1:10 diluted transcribed product was added to a 20  $\mu$ l PCR reaction mixture. The primer pairs used were as following,  $\beta$ -actin forward: GCTACAGCTTACCACCACA, reverse: GCCATCTCTTGCTCGAAGTC; HPRT forward: AATTATGGACAGGACTGAACGTC, reverse: CGTGGGGTCTTTTCAC-CAGCAAG; Cx26 forward: TCTGGCTCACTGTCCTCTTC, reverse: ATGATCAGCTGCAGAGCCCA; Cx30 forward: GGC-CGAGTTGTGTTACCTGCT, reverse: TCTCTTTCAGGGCAT-GGTTGG. All the primer pairs were checked for sequence similarity in rat and size of PCR product was confirmed by gel electrophoresis. Differences between groups were examined using nonparametric one-way analysis of variance (Kruskal–Wallis) test and Mann–Whitney U-test. Statistical significance was assigned to *P* values of <0.05.

**FRAP assay.** The cochlear cultures were incubated with 2.5  $\mu$ mol/l calcein (Invitrogen) in medium supplemented for 10 minutes at 37 °C. Thereafter, the cultures were thoroughly washed with warm PBS and imaged immediately at room temperature. Calcein fluorescence was excited at 488 nm using a confocal laser scanning microscope equipped with a 40x water immersion objective (Carl Zeiss). Images of the size of 256  $\times$  256 pixels were acquired every second for 150 seconds. In all experiments, a cell surrounded from all sides by other cells was selected and photobleached (100% laser power of 488 nm) between image 15 and 16. The mean fluorescence intensity of the bleached region was corrected for the background variation. Cells which had bleaching efficiencies larger than 20% were analyzed. Since calcein fluorescence is affected by changes in the calcium concentration, we excluded cells from our analysis, which showed a calcium response larger than 5% (see **Supplementary Figure S1**). We analyzed 15 cells from cultures incubated with PF6/Cx26-siRNA, 17 cells from cultures incubated with Cx26-siRNA alone, and 14 cells from cultures incubated with PF6/control-siRNA for 72 hours. The cells were selected from five to six different cultures per condition from four independent culture preparations. Further, 15 cells from cultures incubated with PF6/Cx26-siRNA, 14 cells from cultures incubated with Cx26-siRNA, and 17 cells from cultures incubated with PF6/control-siRNA was analyzed after 24 hours of incubation. Cells were selected from five to six different cultures from four independent culture preparations. Mann–Whitney U-test was applied to determine the statistical significance and was assigned to *P* values of <0.05.

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### Supplementary Material

**Figure S1.** FRAP image series were analyzed to exclude calcium responses, which interfere with the FRAP measurement.

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