

CAPÍTULO REIVINDICATORIO MODIFICADO
(Primer Examen de Fondo)

1. Un vector adenoviral que comprende una secuencia de ácido nucleico que comprende la secuencia de SEQ ID NO: 3.
2. Una composición que comprende el vector adenoviral de la Reivindicación 1 y un vehículo farmacéuticamente aceptable.
3. La composición de la Reivindicación 2 que comprende entre 1×10^{10} y 1×10^{13} partículas del vector adenoviral por mililitro.
4. Un vector adenoviral que comprende una secuencia de ácido nucleico que consiste en la secuencia de SEQ ID NO: 3.
5. Una composición que comprende el vector adenoviral de la Reivindicación 4 y un vehículo farmacéuticamente aceptable.
6. La composición de la Reivindicación 5 que comprende entre 1×10^{10} y 1×10^{13} partículas del vector adenoviral por mililitro.

Patient narrative. Patient 5102 is a 28 year old woman with profound bilateral hearing loss since a near drowning event at age 2. She has no other known neurological impairment. Baseline pure tone audiometry demonstrated hearing thresholds indicating profound hearing loss (Table 1). Patient 5102 had minimal residual hearing at frequencies between 125Hz and 1000Hz. She received CGF166 in February 2015 without complication. Beginning at 6 months after CGF166 administration, patient 5102 reported that she could hear sounds that she could not previously hear. These included a fire alarm, a telephone with the assistance of a hearing aid in her treated ear, and her children's voices. These changes correlated with decreased pure tone hearing thresholds for frequencies between 125Hz and 1000Hz (Table 2). For frequencies greater than 1000Hz, no hearing was observed at baseline or following CGF166 treatment. At 18 months, hearing thresholds decreased from 50dB to 30dB at 125Hz, 50dB to 40dB at 250Hz, 90dB to 65dB at 500Hz, and 110dB to 105dB at 1000Hz. 500Hz and 1000Hz are within the speech recognition frequencies of hearing. There were no significant changes in hearing thresholds in the non-treated left ear (Table 2). It is highly unlikely for a person with profound hearing loss for 26 years to have any significant improvements in hearing. This patient example demonstrates that CGF166 can improve hearing in patients with longstanding chronic hearing loss.

Table 1. Patient 5102: Right (treated) ear pure tone audiometry hearing thresholds
Hearing threshold (dB) at frequency (Hz) tested

Assessment period	125Hz	250Hz	500Hz	1000Hz
Screen	50	50	90	110
Baseline	50	50	90	110
Month 1	45	55	100	100
Month 2	45	50	90	110
Month 3	40	40	85	105
Month 4	35	40	80	110
Month 5	35	40	85	105
Month 6	35	40	85	105
Month 12	30	40	80	100
Month 18	30	40	65	105

Table 2. Patient 5102: Left (untreated) ear pure tone audiometry hearing thresholds

Assessment period	Hearing threshold (dB) at frequency (Hz) tested			
	125Hz	250Hz	500Hz	1000Hz
Screen	60	60	65	70
Baseline	60	60	65	70
Month 1	70	80	80	90
Month 2	75	80	80	90
Month 3	70	70	80	85
Month 4	70	80	80	85
Month 5	55	55	65	75
Month 6	50	50	65	70
Month 12	70	75	80	85
Month 18	60	60	65	70

Señores

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIRECCIÓN DE NUEVAS CREACIONES

E. _____ S. _____ D. _____

Re.: Expediente No. NC2017/0003318

Solicitud de Patente de Invención titulada: “VECTOR ADENOVIRAL QUE CODIFICA EL HOMOLOGO-1 ATONAL HUMANO (HATH1)”

Solicitante: GENVEC INC.

Nuestra Ref.: P2017/000053

RESPUESTA AL OFICIO No. 10254, NOTIFICADO EL 31 DE JULIO DE 2018
(PRIMER EXAMEN DE FONDO)

CARLOS R. OLARTE, mayor de edad y vecino de Bogotá, identificado tal como aparece al pie de mi firma, en mi carácter de apoderado de la sociedad solicitante, me permito manifestar lo siguiente ante el auto emanado por ese Despacho, dentro del plazo legal de prórroga previsto en el artículo 45 de la Decisión 486, en los siguientes términos:

1. a- En primer lugar, con el ánimo de brindar mayor claridad a la presente solicitud, me permito adjuntar un **Capítulo Reivindicatorio Modificado**, que reemplaza en su totalidad al Capítulo Reivindicatorio presentado el 20 de junio de 2017, junto con la respuesta al Primer Examen de Fondo. Así entonces, el Capítulo Reivindicatorio Modificado adjunto contiene los siguientes cambios:

- Se eliminan las Reivindicaciones 1 a 11;
- las nuevas Reivindicaciones 1 y 2 corresponden a las antiguas Reivindicaciones 10 y 11, respectivamente;
- las nuevas Reivindicaciones 3 y 6 están dirigidas a la concentración del vector adenoviral, lo cual encuentra soporte en el párrafo [0044] del Capítulo Descriptivo;
- las nuevas Reivindicaciones 4 y 5 están soportadas en las antiguas Reivindicaciones 10 y 11 (nuevas Reivindicaciones 1 y 2);
- la numeración de las Reivindicaciones y las dependencias se adecuaron teniendo en cuenta las Reivindicaciones eliminadas y las modificaciones realizadas

Valga anotar que las modificaciones efectuadas al Capítulo Reivindicatorio cumplen a cabalidad con lo estipulado en el Artículo 34 de la Decisión 486, ya que éstas de ninguna manera implican una ampliación de la protección correspondiente a la divulgación contenida en la solicitud original. Estas por el contrario corresponden a una limitación de la materia reclamada originalmente.

2. a- La Examinadora eleva las siguientes objeciones en relación con la concisión del Capítulo Reivindicatorio:

- la Reivindicación 1 no es concisa porque no está sustentada en la descripción ya que se refiere a un vector adenoviral que comprende cinco componentes estructurales de los cuales solamente se define por su secuencia completa de nucleótidos del componente (c). Además, expresiones tales como *“excepto que dos pares de bases se eliminan”* o *“uno o más nucleótidos endógenos de la región (...) se eliminan”* no definen apropiadamente el objeto de la protección. La Examinadora también menciona que la especificación menciona que el Capítulo Reivindicatorio solo provee soporte para el desarrollo de un vector adenoviral denominado AdGFAP.HATH1.11D (SEQ ID NO: 3), una delección de los nucleótidos 356-3510 de la región E1, una delección de los nucleótidos 28, 593-30,471 de la región E3, una delección de los nucleótidos 32, 827-35, 563 de la región E4, y una delección de los nucleótidos 10.594 y 10595 de la región VA-ARN-1;
- las Reivindicaciones 2 y 5 no son concisas ya que se refieren a delecciones de regiones específicas del genoma del adenovirus modificado, sin referir las mismas por sus posiciones exactas con base en secuencias de referencia;
- La Reivindicación 9 no es clara ni concisa porque describe un espaciador transcripcionalmente inerte (TIS) el cual no se identifica como un elemento conocido dentro del campo técnico, por otra parte parece referir a una característica obvia de un espaciador ya que por definición, corresponde a una secuencia no codificante de ADN y por lo tanto es inactiva transcripcionalmente.

b- i) En primer lugar me permito llamar la atención sobre las modificaciones realizadas en el Capítulo Reivindicatorio Modificado adjunto, como lo mencioné en el numeral 1 del presente escrito, en el cual todas las Reivindicaciones 1 a 9 fueron removidas, así todas las objeciones al respecto quedan apropiadamente superadas.

Además, la nueva Reivindicación 1 (antigua Reivindicación 10) limita drásticamente el alcance de la protección reclamada. Particularmente, la Reivindicación 1 está dirigida a un vector adenoviral que comprende una molécula de ácido nucleico de secuencia SEQ ID NO: 3. Por lo tanto, el vector adenoviral queda estructuralmente definido por sus características esenciales inequívocas. Así, en vista de ésta drástica limitación,

respetuosamente me permito señalar que las objeciones elevadas en contra de la amplitud de la materia reclamada quedan superadas puesto que las nuevas Reivindicaciones 1 a 6 están completamente soportadas en las antiguas Reivindicaciones 10 y 11, y en los Ejemplos del Capítulo Reivindicatorio, tal como lo solicitaba la Examinadora. Así, el alcance reclamado es completamente claro, limitado y conciso para cualquier persona medianamente versada en la materia puesto que los límites de la protección son evidentes.

Sin embargo, a pesar de las limitaciones y restricciones señaladas anteriormente, me permito resaltar que un inventor está en su derecho de reclamar protección para la totalidad de un concepto inventivo desarrollado, sin importar la amplitud de éste, y no únicamente a modalidades preferidas específicamente ilustradas en los ejemplos, siempre y cuando se conserve el principio de unidad de invención. Es decir, una reivindicación puede ser tan amplia como se requiera para así poder enmarcar la totalidad del concepto inventivo desarrollado, y dicha amplitud no necesariamente implica falta de claridad.

Así, en el presente caso, la materia reclamada en el Capítulo Reivindicatorio Modificado adjunto corresponde al concepto inventivo desarrollado por el inventor, y no debería existir obligación alguna de limitar drásticamente la materia reivindicada, pues esta medida reduciría injustamente el concepto inventivo a que el inventor tiene derecho. Asimismo, la posibilidad de obtener la invención en diversas modalidades es una práctica aceptada a nivel mundial en el ámbito de las patentes, ya que una patente de invención no se limita a una única modalidad, sino que cubre un concepto inventivo global que abarca diferentes modalidades (diversas materializaciones de la invención) ligadas por un efecto técnico común que finalmente delimita el alcance de la invención.

Por lo tanto, no existe requerimiento legal alguno que determine que los ejemplos deban ilustrar todos y cada uno de los vectores que se encuentran dentro del alcance de la invención, o que las reivindicaciones sólo pueden reclamar lo ilustrado en los ejemplos, ya que lo fundamental es que el Capítulo Descriptivo proporcione la información necesaria para que la persona medianamente versada en la materia pueda reproducir la invención, sin tener que acudir a experimentación adicional excesiva. Además, vale la pena recordarle a la Examinadora que la función de los Ejemplos en el Capítulo Descriptivo **es estrictamente ilustrativa** y de ninguna manera puede considerarse como el único alcance reivindicable posible. De hecho, reclamar estrictamente aquello ejemplificado en el Capítulo Descriptivo limita injustamente el alcance de la protección a la cual el inventor tiene derecho, de tal forma que para que existiera una infracción, dichos elementos específicos ilustrados en los ejemplos deberían necesariamente estar presentes en el producto o proceso infractor, lo que fácilmente podría lograrse al tratarse de elementos que pueden ser cambiados o modificados.

De esta manera, a la luz de las drásticas limitaciones, considero superadas todas las objeciones de claridad y concisión, dado que las nuevas Reivindicaciones fueron limitadas cumpliendo con los requisitos legales de claridad de tal forma que ahora se reclama una generalización razonable de lo ejemplificado en el Capítulo Descriptivo que le permite a la persona medianamente versada en la materia comprender el alcance del concepto inventivo desarrollado.

3. a- La Examinadora indica que la patente WO2004/076626 (D1) y los artículos “*Adenovector-Mediated Gene Delivery to Human Umbilical Cord Mesenchymal Stromal Cells Induces Inner Ear Cell Phenotype*”¹ (D2) y “*Adenovector-mediated hair cell regeneration is affected by promoter type*”² (D3) afectan el nivel inventivo de la invención.

i) La Examinadora sostiene que D1 es el arte previo más cercano a la Reivindicación 1 porque divulga un vector adenoviral que comprende (a) un genoma adenoviral deficiente en uno o más genes esenciales para la replicación de la región E1, E4 y opcionalmente la Región E3 que puede comprender una delección de: los nucleótidos 457-3332 de la región E1, los nucleótidos 28593-30470 de la región E3, los nucleótidos 32826-35561 de la región E4 y opcionalmente los nucleótidos 10594 y 10595 de la región VARNA1 correspondiente al genoma adenoviral del serotipo 5. Los adenovirus reclamados también comprenden una secuencia de poliadenilación del virus SV40 una secuencia de ácido nucleico que codifica un factor atonal-asociado que corresponde a la SEQ ID NO: 3 y 4. En donde el casete de expresión es insertado en la región E1 preferiblemente en orientación 3’-5’. El vector adenoviral también comprende un espaciador en la región E4 preferiblemente entre 2000 a 3000 nucleótidos de longitud entre la secuencia de poliadenilación y el promotor. Además, D1 divulga una composición que comprende el adenovirus y un vehículo farmacéuticamente aceptable.

La diferencia entre la presente invención y D1 consiste en que el vector adenoviral de la solicitud comprende la secuencia promotora de la proteína ácida fibrilar glial humana (GFAP). El efecto técnico asociado a un promotor GFAP es permitir la expresión específica del gen Hath1 (SEQ ID NO: 1) en células del cabello del vestíbulo del oído interno. Por lo tanto, el problema técnico objetivo se puede formular así: “*Cómo modificar el vector adenoviral conocido en D1 para obtener un vector de expresión específico para células del cabello del vestíbulo del oído interno*”.

Sin embargo, D2 divulga un vector adenoviral que comprende el esqueleto del genoma de Ad5 (Adf11D, AdAtoh1.11D, y Ad5.GFAP.Atoh1) en el cual se han eliminado las

¹ Keerthana Devarajan et al., “Adenovector-Mediated Gene Delivery to Human Umbilical Cord Mesenchymal Stromal Cells Induces Inner Ear Cell Phenotype,” *Cellular Reprogramming* 15, no. 1 (Febrero de 2013): 43–54, <https://doi.org/10.1089/cell.2011.0097>.

² Mark Praetorius et al., “Adenovector-Mediated Hair Cell Regeneration Is Affected by Promoter Type,” *Acta Oto-Laryngologica* 130, no. 2 (Febrero de 2010): 215–22, <https://doi.org/10.3109/00016480903019251>.

regiones E1, E3 y E4, específicamente un vector AdAtoh1.11D o Ad5.GFAP.Atoh1 en donde alguna de las regiones se ha reemplazado por un casete de expresión que comprende en el 3' terminal comprende un marco abierto de lectura, un sitio de poliadenilación y de terminación transcripcional del virus SV40 conducido por el promotor GFAP humano. D2 también divulga que el perfil de expresión mediado por el promotor GFAP dirige la expresión del gen Atoh1 a las células de soporte vestibular donde el gen GFAP se expresa constitutivamente.

La Examinadora señala que D2 incluye ensayos de transducción de células con dicho vector que resultan en cambios celulares similares a haces ciliares de las células de cabello, morfología celular que indica diferenciación a este tipo de células.

Por lo tanto, cualquier persona medianamente versada en la incluiría un promotor GFAP específico para la expresión en células del cabello del oído interno como el del vector adenoviral del documento D2, en un esqueleto de vector adenoviral Ad5 que comprende un gen Hath1 como divulgado en el documento D1, para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

ii) Adicionalmente, la Examinadora analiza el nivel inventivo de la materia reclamada en las Reivindicaciones a la luz de los documentos D1 y D3, manteniendo los mismos argumentos respecto a D1.

En conexión con D3, la Examinadora señala que el documento divulga vectores adenovirales que comprenden genomas del serotipo Ad5 con una eliminación o delección de las regiones E1/E3/E4, una secuencia de ácido nucleico atoh1 y un promotor de la proteína glial fibrilar ácida (GFAP). D3 también divulga como modificar el perfil de expresión a través del promotor para dirigir la expresión del gen atoh1 las células de soporte vestibular donde se expresa dicho gen GFAP constitutivamente, por lo que, la expresión celular específica del gen de interés puede lograrse al usar promotores tejido-específicos del oído interno; esta expresión específica confiere ventajas para su aplicación a la expresión de transgénos ya que, los promotores específicos evitarían los efectos secundarios de la entrega que suceden a veces con promotores constitutivos.

En consecuencia, la persona medianamente versada en la materia incluiría un promotor GFAP específico para la expresión en células del cabello del vestíbulo del oído interno como el del vector adenoviral del documento D3, en un esqueleto de vector adenoviral que comprende un gen Hath1 como divulgado en el documento D1, para llegar así al objeto de la Reivindicación 1 de la solicitud. Por lo cual se considera obvia.

b) En primer lugar, me permito reiterar que la materia reclamada en el Capítulo Reivindicatorio Modificado adjunto fue drásticamente limitada y corresponde

exclusivamente al vector adenoviral que comprende la secuencia de ácidos nucleicos que comprende la secuencia SEQ ID NO: 3. Así, el alcance es completamente claro, limitado y conciso.

Ahora bien, en respuesta a las objeciones de la Examinadora relacionadas con la falta de nivel inventivo, me permito realizar el análisis de nivel inventivo de acuerdo con la Guía para Examen de Solicitudes de Patente de Invención y Modelo de Utilidad de la SIC (en adelante, Guía de la SIC)³.

La Guía de la SIC señala que *“Durante el examen de nivel inventivo debe hacerse un juicio de valor y un análisis objetivo de las divulgaciones previas del estado de la técnica, no influenciados por el conocimiento que ya se tiene de la Invención que se está evaluando. Por tanto, con el fin de minimizar la subjetividad y evitar que se realice un análisis en retrospectiva (“hindsight” ó “a posteriori”), el examen debe relacionar la invención con la solución de un problema técnico, mediante el Método “problema-solución”* (subrayado fuera de texto). De acuerdo con lo anterior, en adelante se presenta el análisis a través del método problema-solución de acuerdo con las etapas establecidas por la SIC en su Guía, teniendo en cuenta el alcance de las reivindicaciones y de la materia divulgada por el arte previo citado:

A. Identificar el estado de la técnica cercano a la invención reivindicada.

En primer lugar, me permito aclarar que la Guía de la SIC establece claramente que el estado de la técnica cercano es *“un documento que se encuentra en el mismo campo técnico que la invención o trata de solucionar el mismo problema o uno relevante. Suele ser el que tiene más características en común con la invención o presenta leves modificaciones de estructura e indica la misma función, o propósito”*. La Examinadora identifica a D1 como el documento que está más cercano a la invención, así que con el único fin de hacer una comparación directa con las conclusiones a las que llega la Examinadora en su análisis, consideraré también a D1 como el arte previo más cercano a la invención.

D1 describe un vector adenoviral (a) deficiente en una o más funciones génicas esenciales para la replicación de la región E1 y la región E4, (b) que comprende un espaciador en la región E4, y (c) que comprende una secuencia de ácido nucleico que codifica un factor asociado a atonal, en el que la secuencia de ácido nucleico se expresa para producir el factor asociado a atonal tal que se genera una célula capilar.

³ Superintendencia de Industria y Comercio, Guía para Examen de Solicitudes de Patente de Invención y Modelo de Utilidad de la SIC, sección 2.13.5, páginas 131-134.

B. Determinar la diferencia entre la invención y el estado de la técnica más cercano.

A la luz de las modificaciones y limitaciones introducidas en el Capítulo Reivindicatorio Modificado, es pertinente señalar que aunque D1 describe que el adenovirus es un genoma de adenovirus deficiente en al menos una función esencial de replicación del gen de una o más regiones del genoma de adenovirus, y puede ser replicación deficiente múltiple, **no describe ni sugiere** la secuencia específica reclamada en las Reivindicaciones que comprende una proteína ácida fibrilar glial humana (GFAP), es decir, la SEQ ID NO: 3. Por lo tanto, D1 describe un adenovirus que tiene una estructura completamente diferente a la del adenovirus de la presente invención.

C. Definir el efecto técnico causado y atribuible al elemento diferencial.

En este punto, difiero nuevamente de la opinión de la Examinadora en su determinación del efecto técnico asociado a las diferencias entre D1 y la presente invención.

La Examinadora menciona que el efecto técnico logrado por la presente invención es que el promotor GFAP permite la expresión del gen Hath1 (SEQ ID NO: 1) en las células vestibulares del oído interno.

Sin embargo, la Examinadora pasó por alto que el Ejemplo 2 en el Capítulo Descriptivo de la presente invención ilustra un estudio clínico diseñado para evaluar la seguridad, la tolerabilidad y la capacidad potencial de un vector adenoviral para **mejorar la audición y la función vestibular**. Este estudio clínico se realizó con CGF116 (que corresponde al adenovirus reivindicado) y se encontró que **es seguro y bien tolerado** (párrafo [0089] del Capítulo Descriptivo). Además, se demostró una mejora en la audición en ciertos sujetos.

Además, respetuosamente me permito llamar la atención de la Examinadora sobre los datos clínicos adjuntos a esta respuesta (ANEXO 1), los cuales demuestran la mejora en la audición en un sujeto adulto que había estado sordo desde la infancia, después de administrar el adenovirus reivindicado durante 18 meses. En este tratamiento, la invención reivindicada se administró solo en el oído derecho del paciente, manteniendo el oído izquierdo sin tratar. La Tabla 2 del Anexo 1 ilustra los umbrales de audición durante el período de evaluación de 18 meses, comparados en ambos oídos. Después de 18 meses de tratamiento, el paciente experimentó una disminución en el umbral de audición en su oído derecho de 50 a 30 dB a 125 Hz, 50 a 40 dB a 250 Hz, 90 a 65 dB a 500 Hz y 110 a 105 dB a 1000 Hz. Es importante destacar que 500 Hz y 1000 Hz están dentro de las frecuencias de reconocimiento de voz de la audición. La oreja izquierda, sin tratamiento, no experimentó ningún cambio en la audición. Como se mostrará a continuación, la

efectividad de CGF166 para mejorar la audición en este entorno clínico 26 años después de la pérdida auditiva original es sorprendente a la vista de la técnica anterior.

D. Deducir el problema técnico a solucionar.

En cuanto a la formulación del problema técnico, la Guía de la SIC establece que el problema técnico debe ser formulado de la siguiente manera: “¿cómo *modificar o adaptar el estado de la técnica cercano para obtener el efecto técnico que la invención proporciona?*”⁴. Adicionalmente, de acuerdo con el Manual Andino de Patentes (MAP) “se debe definir el problema **sin incluir elementos de la solución**, porque entonces la solución sería evidente”⁵ (énfasis fuera del texto).

Dado que la Examinadora no consideró todos los efectos técnicos logrados por los péptidos reclamados, formula el problema técnico resuelto por la presente invención de manera equivocada, así: “Cómo *modificar el vector adenoviral conocido en D1 para obtener un vector de expresión específico para células del cabello del vestíbulo del oído interno*”.

No obstante, atendiendo lo estipulado por la Guía de la SIC y el MAP, considero que el problema técnico objetivo de la presente invención debería ser formulado de la siguiente manera: ¿Cómo *modificar o adaptar la técnica anterior más cercana para obtener la solución técnica proporcionada por la invención?* Además, la definición de problema técnico deberá estar basada en hechos concretos del arte previo y los resultados de la invención.⁶

Por lo tanto, de manera respetuosa discrepo con la evaluación de la Examinadora con respecto al problema abordado por la presente invención, ya que parece que se ignoran los resultados mostrados en el Capítulo Descriptivo. En consecuencia, el problema técnico abordado por la presente invención es “¿Cómo *modificar el vector adenoviral de D1 para obtener un vector adenoviral seguro y bien tolerado que mejore la audición y la función vestibular en un sujeto con pérdida auditiva a largo plazo?*”

E. Evaluar si la invención reivindicada, partiendo del estado de la técnica cercano y del problema técnico objetivo, habría sido obvia para la persona medianamente versada en la materia.

i) De acuerdo con la Guía de la SIC “esta etapa consiste en responder a la pregunta de si en el estado de la técnica en su conjunto hay un segundo documento que contiene una enseñanza que indicaría (no sólo que **podría indicar**, sino que **indicaría**)

⁴ Ibídem.

⁵ Comunidad Andina de Naciones, Manual Andino de Patentes, 2004, sección 10.2.1

⁶ Guía para Examen de Solicitudes de Patente de Invención y Modelo de Utilidad (2014), sección 2.13.5.1, páginas 133

a la persona medianamente versada en la materia, enfrentada al problema técnico, cómo modificar o adaptar el estado de la técnica más cercano para resolver el problema, de la forma reivindicada o adaptar el estado de la técnica cercano para obtener el efecto técnico que la invención proporciona?”⁷ (énfasis fuera del texto). Así, el análisis de nivel inventivo de solicitudes de patente, no se trata de encontrar en el arte previo una mera invitación a experimentar con uno o más cambios aleatorios, sino de identificar en los documentos citados una enseñanza y guía específica para hacer las modificaciones necesarias que lo lleven con certeza a la materia reclamada.

Por lo tanto, el objetivo de este paso es: (i) determinar la existencia o ausencia de enseñanzas en el arte previo citado que guiaría de manera obvia a la persona medianamente versada en la materia a la estructura particular de la invención; (ii) determinar si existe un segundo documento, proporcionando las enseñanzas faltantes en el primer documento que, una vez más, guiarían de manera obvia a la persona medianamente versada en la materia a la invención.

Como tal, el análisis del nivel inventivo no solo se basa en el hallazgo de meras invitaciones en la técnica anterior para probar modificaciones arbitrarias y aleatorias, sino también para identificar enseñanzas específicas que brindan una guía obvia sobre las modificaciones necesarias para llegar inequívocamente a la invención. Sin ninguna duda y ambigüedad.

Evidentemente, D1 no proporciona las enseñanzas y orientaciones necesarias que de manera obvia y sin ambigüedades motivarían a la persona medianamente versada en la materia a llegar a la presente invención, que comprende las características estructurales reivindicadas. En particular, y como se mencionó anteriormente, D1 no enseña ni sugiere la SEQ ID NO: 3.

Por lo tanto, la persona medianamente versada en la materia basada únicamente en las revelaciones y enseñanzas de D1 y el problema de proporcionar *un vector adenoviral seguro y bien tolerado que mejore la audición y la función vestibular en un sujeto con pérdida auditiva a largo plazo* no estaría motivado en absoluto para diseñar el ácido nucleico con la estructura de la SEQ ID NO: 3. En otras palabras, la técnica anterior no proporciona información suficiente para que la persona medianamente versada en la materia llegue de manera obvia al adenovirus de la invención.

En cuanto a D2, evalúa el potencial de las células estromales mesenquimales del cordón umbilical humano (hUCMSCs) como una posible fuente para regenerar las células ciliadas del oído interno. Particularmente, D2 divulga que la expresión de Hath1 induce la

⁷ Guía para Examen de Solicitudes de Patente de Invención y Modelo de Utilidad. (2014), Sección 2.13.5.1, páginas 13-134

diferenciación de hUCMSCs en células que se parecen morfológicamente e inmunocitoquímicamente a células internas, y describe adenovectores. Sin embargo, no logra proporcionar a la persona medianamente versado en la materia ninguna expectativa de éxito al considerar la entrega de un adenovector de expresión Hath1 al oído interno para mejorar la audición de un sujeto con pérdida auditiva a largo plazo, ya que D2 describe el uso de un adenovector para la entrega de Hath1 a células del estroma mesenquimal del cordón umbilical humano para inducir un fenotipo de células ciliadas. Las células obtenidas por este método se consideran posiblemente útiles como un tratamiento de trasplante para la pérdida auditiva (ver el párrafo final de la conclusión de la página 52), lo que es una estrategia completamente diferente para tratar la pérdida auditiva en comparación con la administración directa del vector al oído interno de la presente invención. Por lo tanto, D2 no proporciona ninguna enseñanza complementaria a las de D1, que permitan a la persona medianamente versada en la materia producir un adenovirus que comprende la SEC ID NO: 3 que podría administrarse en el oído interno para tratar la pérdida de audición.

Por otro lado, D3 divulga adenovectores que utilizan vectores en los cuales se han eliminado las regiones E1/E3 y vectores en los cuales se han eliminado las regiones E1/E4. Aunque D2 y D3 divulgan adenovirus, no describen las secuencias específicas del genoma adenoviral del serotipo 5 y el promotor de GFAP (SEQ ID NO: 1) y el espaciador en la región E4. Además, la Figura 8 de D3 muestra los recuentos de células ciliares para los cultivos de órganos maculares tratados con neomicina seguido de Hath1 (murino) administrado por adenovectores con promotores hCMV, CBA y GFAP. Los resultados muestran una recuperación de las células capilares en comparación con los cultivos tratados con neomicina sola o con el vector control inmediatamente después de la ablación de las células ciliares mediante el tratamiento con neomicina.

Posteriormente, pero antes de la fecha de prioridad de la solicitud actual, Yang y otros (ANEXO 2) describieron cómo los adenovirus recombinantes con deficiencia de replicación que expresan Hath1 murino inoculados en la cóclea de cobayas sordos con ruido, solo pueden mejorar el umbral de audición cuando se produce la inoculación “antes de la muerte de las células ciliares” después de la exposición al ruido (ver, por ejemplo, Resumen de Yang, et al), proporcionando una ventana para la intervención de aproximadamente 10 días (ver Yang et al., p.3, columna derecha, último párrafo). De hecho, la figura 7 de Yang et al., muestra que no se obtuvo una mejoría en el umbral de audición cuando se produjo la inoculación un mes después de la exposición al ruido, es decir, después de que las células ciliadas dañadas hubieran muerto, lo que llevó a los autores a concluir: *"La terapia génica basada en Hath1 fue efectiva solo cuando las células ciliadas dañadas todavía estaban presentes en el órgano de Corti"*.

Estos hallazgos fueron consistentes con aquellos previamente reportados por Izumikawa et al., en 2005 (ANEXO 3), donde el vector Ad.Atoh1 fue inoculado e la cóclea de cobayas solo 4 días después de ensordecer mediante el uso de un régimen de fármacos ototóxicas y con los informados en D3, donde se produjo la administración inmediata de adenovirus.

Por lo tanto, a la luz de Yang et al., la persona medianamente versada en la materia no habría tenido ninguna expectativa razonable de éxito al usar las enseñanzas de D3, es decir, usar adenovectores de expresión de Hath1 para el tratamiento de la pérdida auditiva en sujetos con sordera a largo plazo, como se describe en el Ejemplo 2 de la presente invención, y como se ha demostrado en los datos clínicos presentados aquí en los cuales se describe a un paciente con una pérdida auditiva de 26 años.

Teniendo en cuenta lo anterior, en este punto vale la pena señalar que el MAP establece que para que pueda negarse la existencia de nivel inventivo, es necesario no solamente que la combinación de las enseñanzas pueda hacerse, sino también que exista una sugerencia o razón tal, que lleve al técnico con conocimientos medios a combinar las enseñanzas de los documentos⁸. Además, el MAP también establece que las invenciones en el campo de la química tienen nivel inventivo cuando la materia reclamada tiene características **estructurales inesperadas** (no derivable por la persona medianamente versada en la materia) o cuando presentan un **efecto técnico inesperado o mejorado impredecible del arte previo**⁹. Por tales razones, **nada** en el arte previo sugiere ni enseña la materia de la presente invención, por lo que ésta reviste nivel inventivo sobre el arte previo citado. De manera similar, la Guía de la SIC establece que, para que una invención no resulte inventiva “*el conjunto de enseñanzas del estado de la técnica lo llevarían* (a la persona medianamente versada en la materia) **de forma inmediata a la solución propuesta por la solicitud. Si se requiere de pasos adicionales o de corroboración o de superar obstáculos revelados por las propias enseñanzas entonces el estado de la técnica no revela la solución al problema**”¹⁰ (énfasis fuera del texto).

En este caso, la persona medianamente versada en la materia, siguiendo las enseñanzas de D1, D2 y D3, no habría sido motivado para construir un adenovector con la secuencia de ácido nucleico como se establece en la SEQ ID NO: 3, ya que ninguno de estos documentos enseña o sugiere esta estructura. De manera similar, nada en ninguno de estos documentos habría motivado a la persona medianamente versada en la materia a usar estos adenovectores en la sordera a largo plazo.

ii) Salta a la vista que en el momento de la realización del examen de fondo, la Examinadora no fundamenta su análisis según lo estipulado en la Guía de la SIC

⁸ Ibídem.

⁹ Ibídem.

¹⁰ Superintendencia de Industria y comercio, *óp. cit.*, sección 2.13.2

en cuanto a la evaluación del nivel inventivo, ya que ésta debe basarse únicamente en si existen sugerencias explícitas y suficientes en el arte previo que indicarían de manera clara cómo desarrollar de manera obvia los péptidos aquí reclamados, las cuales, como se demostró anteriormente, no existen. En consecuencia, no es aceptable a quien examina o a quién concede o deniega una patente desvirtuar el nivel inventivo de la misma por desglosar sus elementos separadamente y luego buscar referencias en el estado del arte que los incluyan, como si de armar un rompecabezas con piezas aisladas, o una colcha de retazos, se tratara. En este caso particular, aunque D1 se refiere a adenovirus y contrario al análisis de la Examinadora, la única forma en que una persona medianamente versada en la materia podría obtener un vector adenoviral para mejorar la audición y la función vestibular en un sujeto con pérdida auditiva a largo plazo, no significa que tales revelaciones obviamente guiarían a dicha persona para obtener la presente invención aquí reclamada.

Sin embargo, el estudio de solicitudes de patente que conduzca a ese tipo de conclusiones anticipadas corresponde abiertamente a un análisis conocido en el ámbito de patentes como “análisis hindsight” o en retrospectiva. Para entender el análisis equivocado que realiza la Examinadora, podemos citar nuevamente el MAP, que indica claramente que “...la búsqueda de anterioridades se efectúa a posteriori, tomando como punto de partida la misma invención,...(La SIC) debe realizar el esfuerzo intelectual de colocarse en la situación que ha tenido que afrontar el técnico con conocimientos medios en la materia en un momento en que la invención no era conocida, es decir antes de la invención”¹¹ (mi subrayado). Por ende, no es apropiado anticiparse a los resultados presentados por la invención, ya que así, siempre resultará “obvio” todo objeto de una solicitud de patente, pues inevitablemente está parecerá derivada de la teoría en que se basa su desarrollo, sin dar mayor importancia al trabajo, intelecto y creatividad involucrados en la invención para llegar a la solución del problema planteado en la solicitud. En el presente caso, se evidencia claramente un enfoque de retrospectiva ya que, como se demostró anteriormente, ni D1, D2 y D3, combinados o solos, proporcionan las enseñanzas que permitirían a la persona medianamente versada en la materia llegar a la presente invención.

En este sentido, la Examinadora no reconoce el nivel inventivo de la presente invención debido a una búsqueda acomodada y subjetiva de las características esenciales reclamadas en las enseñanzas de D1, D2 y D3, sin considerar adecuadamente si el arte previo, entendido como un todo, comprende sugerencias claras y evidentes que guíen inequívocamente a la persona medianamente versada en la materia a obtener la presente invención de manera obvia.

En conclusión, a la luz de la clara evidencia en favor del nivel inventivo suministrada anteriormente la invención no habría podido ser dilucidada ni mucho menos prevista a

¹¹ Ibídem.

partir de la información aportada por los documentos citados. La persona medianamente versada en la materia no habría encontrado sugerencia o motivación alguna que la condujera directamente a desarrollar el vector adenoviral aquí reclamado. Por lo tanto, la presente invención es inventiva a la luz del arte previo citado.

4. Finalmente, si el Despacho lo considera necesario, solicito amablemente proceda a realizar un segundo examen de fondo, para lo cual adjunto el comprobante de pago de la tasa de examen correspondiente. De otra forma, considero que la presente solicitud cumple con los requisitos de patentabilidad establecidos por la Decisión 486 y por consiguiente solicito respetuosamente que el Despacho proceda con la concesión de la patente.

Atentamente,



CARLOS R. OLARTE

C.C. No. 79.782.747 de Bogotá

T.P. 74.295

Anexos:

- Capítulo Reivindicatorio Modificado;
- Comprobante de pago correspondiente a la Tasa de examen adicional.
- Anexo 1 - Datos clínicos
- Anexo 2 - Yang et al.
- Anexo 3 - Izumikawa et al.

Auditory hair cell replacement and hearing improvement by *Atoh1* gene therapy in deaf mammals

Masahiko Izumikawa^{1,2}, Ryosei Minoda^{1,3}, Kohei Kawamoto², Karen A Abrashkin¹, Donald L Swiderski¹, David F Dolan¹, Douglas E Brough⁴ & Yehoash Raphael¹

In the mammalian auditory system, sensory cell loss resulting from aging, ototoxic drugs, infections, overstimulation and other causes is irreversible and leads to permanent sensorineural hearing loss. To restore hearing, it is necessary to generate new functional hair cells. One potential way to regenerate hair cells is to induce a phenotypic transdifferentiation of nonsensory cells that remain in the deaf cochlea. Here we report that *Atoh1*, a gene also known as *Math1* encoding a basic helix-loop-helix transcription factor and key regulator of hair cell development, induces regeneration of hair cells and substantially improves hearing thresholds in the mature deaf inner ear after delivery to nonsensory cells through adenovectors. This is the first demonstration of cellular and functional repair in the organ of Corti of a mature deaf mammal. The data suggest a new therapeutic approach based on expressing crucial developmental genes for cellular and functional restoration in the damaged auditory epithelium and other sensory systems.

The most common reason for sensorineural hearing loss is degeneration of cochlear sensory (hair) cells, resulting from overstimulation, ototoxic drugs, infections, autoimmune disease or aging. Sensorineural hearing loss affects millions of people worldwide. This impairment is irreversible because lost auditory hair cells cannot be spontaneously replaced. Treatment for replacing lost hair cells is currently unavailable.

During mammalian embryogenesis, cochlear hair cells and supporting cells have common cellular precursors^{1–3}. Precursor cell differentiation is regulated by several genes; key among them is the gene *Atoh1* (also known as *Math1*), a mouse homolog of the *Drosophila* gene *atonal*, that encodes a basic helix-loop-helix transcription factor. *Atoh1* has been shown to act as a 'pro-hair cell gene' and is required for the differentiation of hair cells from multipotent progenitors^{4,5}. Experimental overexpression of *Atoh1* or *ATOH1* (the human *atonal* gene) in nonsensory cells of the normal cochlea generates new hair cells, both *in vitro*⁶ and *in vivo*⁷. Here we test the influence of *Atoh1* overexpression on hair cell regeneration and hearing restoration in the mature deaf cochlea.

Young adult guinea pigs with normal hearing were deafened by systemic administration of ototoxic drugs, resulting in complete bilateral hair cell loss in the high- and mid-frequency regions of the cochlea. In animals killed 3 d after the deafening procedure, scanning electron microscopy (SEM) analysis of the organ of Corti surface showed complete absence of hair cells (Fig. 1a). At this time point, whole mounts of the organ of Corti stained with phalloidin showed a complete lack of hair cells at the upper (luminal) surface of the epithelium (Fig. 1b). Hair cell features such as stereocilia and cuticular plate were absent, whereas pillar cells remained in the tissue (Fig. 1a,b). In normal cochleae

stained with bisbenzimidazole (Hoechst) and analyzed with fluorescence microscopy, outer hair cell (OHC) nuclei were arranged in three distinct rows (Fig. 1c) with supporting cell nuclei similarly arranged, at a lower focal plane (data not shown). OHC nuclei were absent 3 d after deafening (Fig. 1d); however, supporting cell nuclei were present at the lower level (data not shown), showing that the original OHCs were eliminated by this deafening protocol. Cochlear whole mounts stained 3 d after the ototoxic insult with antibodies to myosin VIIa, a hair cell-specific marker⁸, showed a complete lack of myosin VIIa (data not shown). Auditory brain-stem response (ABR) thresholds in all deafened animals were either unmeasurable or extremely high (>95 dB), indicative of profound deafness. The battery of post-deafening analyses we have used showed absence of stereocilia bundles and apical surfaces of hair cells with SEM, absence of actin-rich hair cell features with phalloidin staining, lack of myosin VIIa staining with immunocytochemistry and lack of nuclei at the OHC plane. These analyses were performed in cochlear turns one through three, and showed that these three turns were consistently devoid of inner hair cells (IHCs) and OHCs by day 3 after the ototoxic insult.

To deliver the *Atoh1* transgene into nonsensory cells in the deafened auditory epithelium, an adenoviral vector was infused into the left cochlea 4 d after the ototoxic lesion. Animals received adenovirus alone (Ad.empty), with a GFP cassette (Ad.GFP), with *Atoh1* (Ad.*Atoh1*) or with a dual cassette (Ad.*Atoh1*-GFP). To test the pattern of *Atoh1* expression, animals were killed 4 d after Ad.*Atoh1* inoculation and processed for *Atoh1* immunocytochemistry. We found numerous *Atoh1*-positive cells among the nonsensory cells that

¹Kresge Hearing Research Institute, Department of Otolaryngology, University of Michigan, MSRB 3, Room 9303, 1150 W. Medical Center Drive, Ann Arbor, Michigan 48109-0648, USA. ²Department of Otolaryngology, Kansai Medical University, 10-15 Fumizono-Cho, Moriguchi, Osaka 570-8506, Japan. ³Department of Otolaryngology-Head and Neck Surgery, Kumamoto University School of Medicine, 1-1-1 Honjo, Kumamoto 860-8556, Japan. ⁴Department of Vector Sciences, GenVec Inc., 65 W. Watkins Mill Road, Gaithersburg, Maryland 20879-4021, USA. Correspondence should be addressed to Y.R. (yoash@umich.edu).

Published online 13 February 2005; doi:10.1038/nm1193

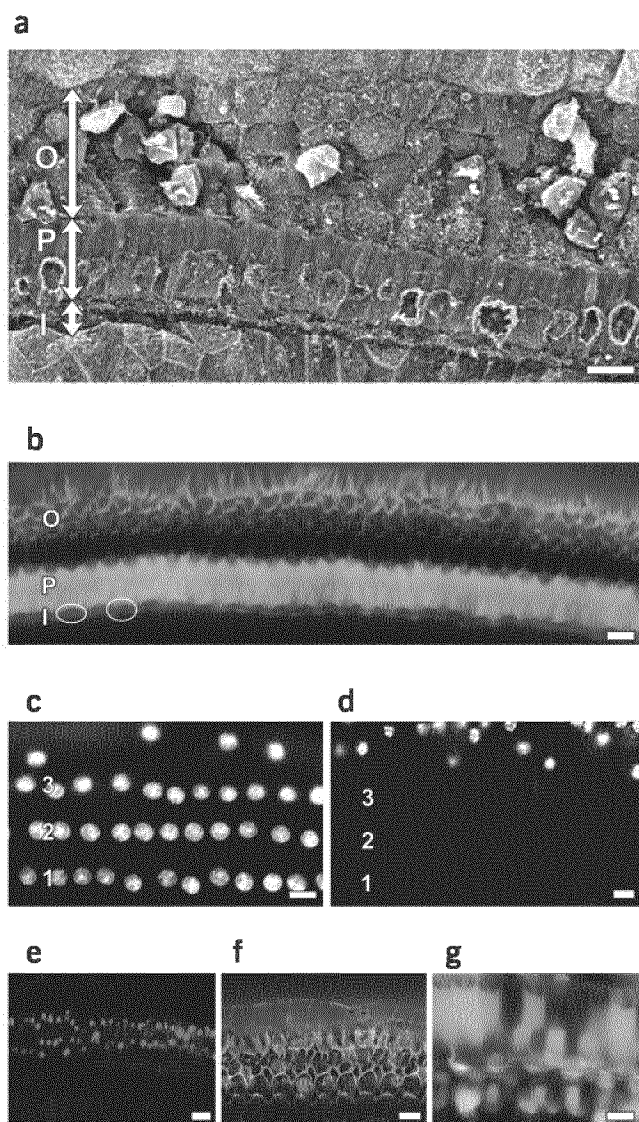


Figure 1 Hair cell elimination and *Atoh1* expression in deaf cochleae. SEM (a) or epifluorescence (b–g) showing ototoxic lesions with complete hair cell loss (a,b and d), nuclei in the organ of Corti (c,d) and *Atoh1* expression after Ad.*Atoh1* inoculation (e–g) in the second cochlear turn. (a) Complete hair-cell loss was observed 3 d after systemic (bilateral) deafening, with nonsensory cells replacing degenerated hair cells in the IHC (I) and OHC (O) region. Pillar cells (P) survive the insult. (b) Distribution of F-actin shows lack of hair cells 3 d after deafening. Ovals encircle representative sites of missing IHCs and O points to cells missing in OHC area. Pillar cells (P) survive the insult. (c) In the normal organ of Corti, Hoechst stain depicts nuclei of the three rows of OHCs. (d) All OHC nuclei are missing 3 d after an ototoxic insult. (e) Antibody to *Atoh1* is seen in nuclei of nonsensory cells that replaced hair cells (first turn) 4 d after Ad.*Atoh1* inoculation. (f) *Atoh1* (red) in nonsensory cells in second cochlear turn, with phalloidin (green) showing actin in cell-cell junctions. (g) *Atoh1* (red, nuclear stain) and GFP (green, cytoplasmic stain) are coexpressed in nonsensory cells (Deiters and pillar cells) 4 d after Ad.*Atoh1*-GFP inoculation. Additional positive nuclei are present out of the focal plane shown in e–g. Scale bars, 25 μ m in e and 10 μ m in all other micrographs.

Atoh1-treated ears was relatively normal (Fig. 2f,g) but the supporting cells that separated one hair cell from its neighbor were narrow and not well defined.

Hair cells detected in Ad.*Atoh1*-treated deafened cochleae showed normal surface morphology and orientation in the organ of Corti (Fig. 2a,f,g), suggesting that the positional cues for cellular organization remain in the mature traumatized tissue. Organization of third-row OHCs was poorer than other rows, suggesting that cues for organizing the tissue are closer to the first row of OHCs. Ectopic hair cells found outside the organ of Corti (Fig. 2a) were neither well differentiated nor correctly oriented. Based on their location in the organ of Corti and their highly polarized morphology, we speculate that pillar cells provide cues for reorganizing the auditory epithelium.

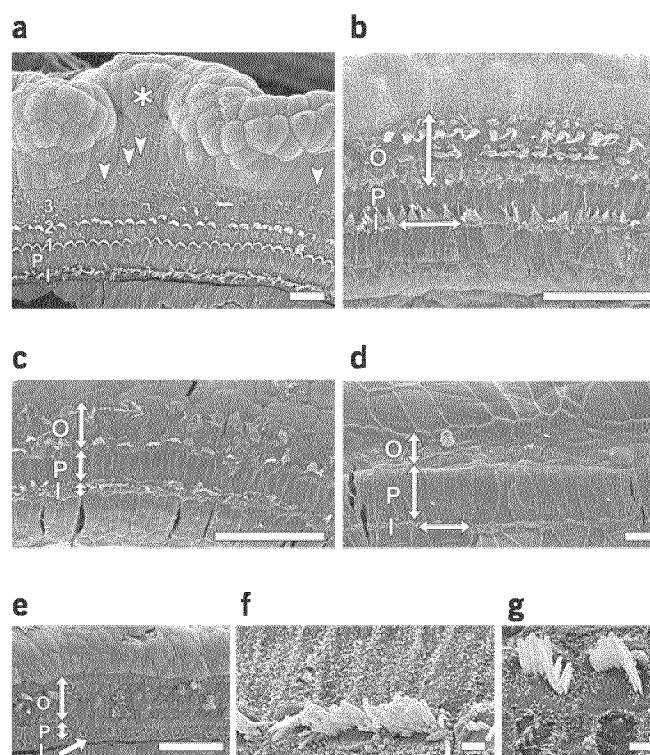
Hair cells in *Atoh1*-treated ears expressed myosin VIIa, providing further evidence for hair-cell phenotype (Fig. 3a). Ad.empty-inoculated ears (data not shown) and contralateral ears (Fig. 3b) were negative for myosin VIIa, confirming that all original hair cells have been eliminated. In the normal auditory epithelium there is a 1:1 ratio of OHCs to Deiters cells (supporting cells), with OHC nuclei positioned above the supporting-cell nuclei. In deafened ears, OHC nuclei were absent (Fig. 1d) and the total number of nuclei in this area was half that of normal ears. Two months after Ad.*Atoh1* inoculation, nuclei reappeared at a plane above the supporting cells (Fig. 3c). An average number of 1,548 nuclei was observed in the same area of *Atoh1*-treated ears, compared to an average of 830 nuclei in contralateral ears ($n = 5$ each, s.d. = 209 and 51, respectively), a statistically significant increase ($P = 0.0006$). The increase in nuclei number probably is not a direct result of *Atoh1* expression, because *Atoh1* acts as a differentiation factor. Therefore, we hypothesize that new hair cells recruit nonsensory cells from outside the organ of Corti to migrate and become supporting cells, as described *in vitro*⁹. Supporting cells may also induce proliferation of other nonsensory cells as part of a self-organizational capacity of the regenerating cochlea⁹. The *Atoh1*-induced transdifferentiation from a nonsensory to sensory phenotype can be either direct, with no mitosis, or indirect, with mitosis preceding the appearance of the hair cell features.

The numbers and appearance of hair cells were similar in Ad.*Atoh1* and Ad.*Atoh1*-GFP inoculated ears. Some Ad.*Atoh1*-GFP-inoculated left ears contained hair cells positive for GFP, establishing a direct relationship between transgene expression in surviving nonsensory cells and transdifferentiation into hair cells (Fig. 3d). Although adenoviral-mediated gene expression is not expected to last for 2 months, the GFP gene product probably persisted in transduced cells because of slow degradation.

remained in the organ of Corti (Fig. 1e,f). We observed additional *Atoh1*-positive nuclei in other focal planes (data not shown). We observed the highest efficiency of *Atoh1* transduction in the first and second cochlear turns, within the normal boundaries of the organ of Corti. We determined that in 2 mm flanking the inoculation site, an average of 865 *Atoh1*-positive cells were found in the organ of Corti 4 d after Ad.*Atoh1* inoculation ($n = 10$ ears, s.d. = 196.6). Right (contralateral) ears and left ears inoculated with Ad.empty or Ad.GFP were *Atoh1*-negative (data not shown). Deafened animals killed 4 d after inoculation with Ad.*Atoh1*-GFP showed widespread expression of both genes (Fig. 1g). These data show efficient transduction and *Atoh1* transgene expression in nonsensory cells of the deaf cochlea and absence of *Atoh1* expression in control ears.

To determine the effects of *Atoh1* overexpression in deafened ears, ABR thresholds were recorded and animals killed 4, 5 or 8 weeks after inoculation. We prepared cochleae for SEM or whole-mount fluorescence microscopy. Ad.*Atoh1*-treated cochleae showed large numbers of IHCs and OHCs 8 weeks after inoculation (Fig. 2a–c). All right ears (Fig. 2d) and all left ears treated with Ad.empty (Fig. 2e) or Ad.GFP (not shown) were devoid of hair cells. Higher-magnification SEM analysis showed that the surface morphology of the hair cells in

Figure 2 Hair cells reappear in deaf ears treated with *Ad.Atoh1*. SEM view of deafened cochleae 2 months after *Atoh1* inoculation (**a–c**), contralateral cochlea (**d**), *Ad.empty*-inoculated ear (**e**), and higher magnification of IHC (**f**) and OHC (**g**) 2 months after *Atoh1* inoculation. (**a**) The site of inoculation in the second cochlear turn (asterisk) is shown along with numerous stereocilia bundles at the normal sites of IHCs (I) and OHCs (rows 1–3). Pillar cells (P) are present between IHCs and OHCs. Ectopic bundles (arrowheads) are seen lateral to the third row of OHCs. (**b**) In some *Atoh1*-treated ears the morphology of IHC (I) and OHCs (O) is less well differentiated. (**c**) In other *Atoh1*-treated ears, hair cell reappearance is incomplete and third row OHCs are missing. (**d,e**) second cochlear turns of right (**d**, contralateral to **a**) or *Ad.empty*-inoculated left cochlea (**e**), showing complete absence of hair cells. (**f,g**) Stereocilia bundle organization is relatively normal in IHCs (**f**) and OHCs (**g**) but supporting cells between neighboring hair cells are narrow and not well defined. Scale bars, 25 μ m in **a**; 50 μ m in **b**, **c** and **e**; 10 μ m in **d** and 5 μ m in **f** and **g**.



Phalloidin epifluorescence facilitated identification of hair cells based on the presence of stereocilia (data not shown) and the distribution of actin in the cell border areas and the cuticular plate, and the absence of actin in the centrosomal region (Fig. 3d). Quantitative analysis of these phalloidin-stained cochleae 2 months after *Atoh1* treatment showed the number of new hair cells in the 2 mm of the organ of Corti flanking the inoculation site to be 256 IHCs and 691 OHCs ($n = 5$ animals, s.d. = 35 and 159 for IHCs and OHCs, respectively). The number of hair cells in the *Atoh1*-treated ears was significantly greater than contralateral ears, which were devoid of hair cells ($P < 0.0006$). The restoration of hair cells in *Atoh1*-treated ears was best near the site of inoculation (Figs. 2a and 3a,d). These data show that many new hair cells appear in the 8 weeks after *Atoh1* expression in deafened mature cochleae.

Cross-sections of *Atoh1*-treated cochleae showed that some cells in the OHC area displayed a mixed phenotype with features of both OHC and supporting cell. These mixed-phenotype cells showed a luminal projection similar to stereocilia on their apical surface and a prominent cuticular plate, yet spanned the distance from the luminal surface to the basement membrane, suggesting that they also retained several supporting-cell features (Fig. 3e). In some sections, two layers of nuclei were found above the basement membrane (Fig. 3f), in agreement with data obtained with Hoechst staining in whole mounts (Fig. 3c). IHC morphology appeared relatively normal (Fig. 3g). The presence of cells with a mixed phenotype suggests that, at least in some cases, the process of transdifferentiation can occur directly, without a preceding step of de-differentiation or mitosis. The increase in the number of nuclei compared to the contralateral ears may be a result of mitosis or of migration from adjacent regions in the epithelium.

We analyzed *Ad.Atoh1*-treated cochleae at intermediate time points between deafening and 2 months. Cochleae analyzed with SEM 4 weeks after *Ad.Atoh1* inoculation showed numerous bundles of very tall stereocilia that resembled the apical surfaces of immature hair cells (Fig. 4a). The high density of immature bundles observed at 4 weeks corroborates the high efficiency of *Atoh1* transduction shown at day 4. In the contralateral cochleae there were no surviving hair cells and no immature bundles, and the area where OHCs used to reside often appeared narrow and greatly diminished (Fig. 4b). The pattern of myosin VIIa distribution in *Atoh1*-treated cochlea analyzed 5 weeks after the inoculation of *Ad.Atoh1* delineated both IHCs and OHCs (Fig. 4c). The myosin VIIa-positive cells in the OHC area were rather disorganized, in agreement with the SEM image obtained 1 week earlier (Fig. 4a). Contralateral ears were negative for myosin VIIa (Fig. 4d).

To test the influence of *Atoh1* treatment on hearing, we measured ABR thresholds at 4, 8 and 10 weeks after *Ad.Atoh1* inoculation. Measurements at 4 weeks indicated profound deafness with thresholds

high or unmeasurable, similar to post-deafening thresholds (not shown). These data are consistent with the absence of mature hair cells at 4 weeks after *Atoh1* treatment (Fig. 4a). At 8 weeks, the average ABR thresholds in a group of five *Ad.Atoh1*-treated ears was lower (better) than contralateral ears at all frequencies, and ABR waveforms contained the normal four to five vertex-positive peaks (Fig. 5). The considerable improvement in thresholds (in some cases to near baseline) at the high frequency region of the guinea pig cochlea (4–24 kHz) is in agreement with the restoration of IHC morphology in this area¹⁰. Nevertheless, in the absence of a normal OHC population, frequency selectivity is unlikely to be normal¹¹. When present, the thresholds recorded in the contralateral (right) ears probably represent hearing from *Atoh1*-treated left ears and reflect interaural crossover¹². ABR thresholds measured in four animals at 10 weeks after *Ad.Atoh1* inoculation appeared similar to their 8-week thresholds (data not shown) suggesting that functional recovery is stable, at least up to 10 weeks. In *Ad.empty*-treated ears, no thresholds could be recorded (data not shown). These data provide the first demonstration of a therapeutic approach leading to substantial recovery of hearing in deaf mammalian ears.

Our results show generation of new hair cells and improvement of hearing in deaf animals treated with *Atoh1*. New OHCs are incompletely differentiated and unlikely to provide the functions of the active cochlear amplifier. In contrast, regenerated IHCs seem normal and the restoration of thresholds attests to their functionality. We show that differentiated cells (nonsensory cells of the auditory epithelium) can be induced to alter their phenotype by expression of the developmental gene, *Atoh1*. Together with loss-of-function and *in vitro* experiments^{4,5,9,13}, our data suggest that *Atoh1* is a master regulatory gene that is both necessary and sufficient for producing hair cells in the mammalian cochlea. The competence of mature and differentiated nonsensory cells to respond to *Atoh1* is notable because the structural and functional changes that accompany differentiation of mammalian cells are usually irreversible. Our data, therefore, sug-

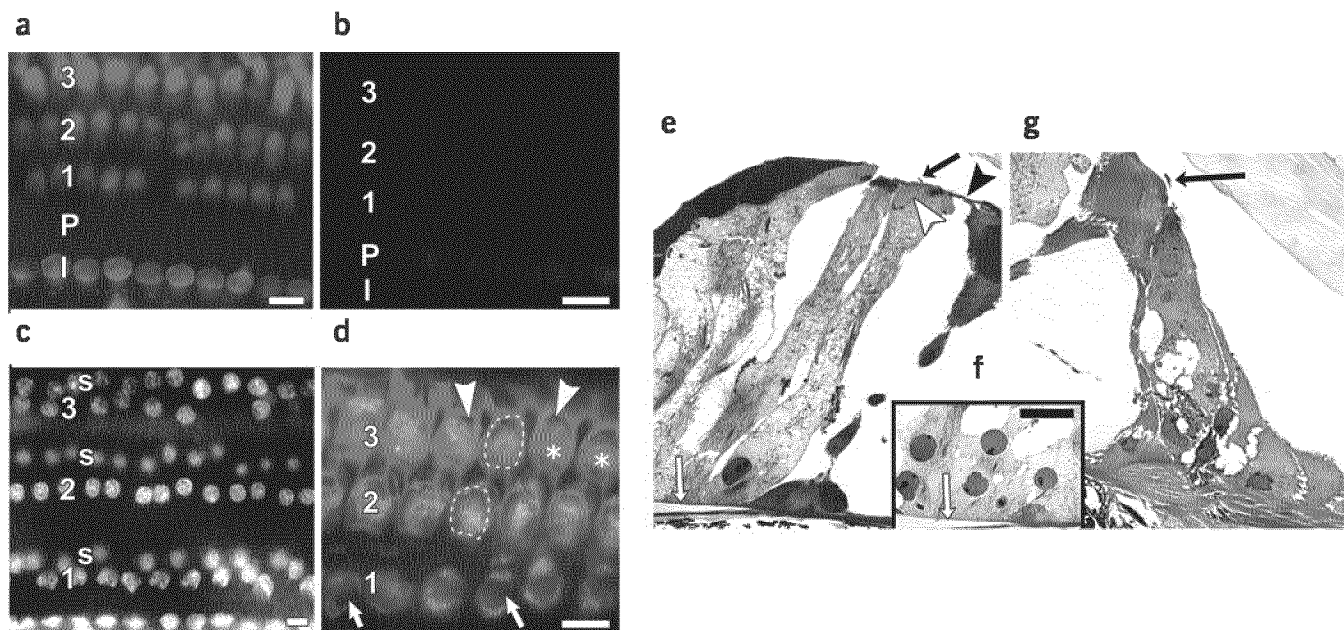


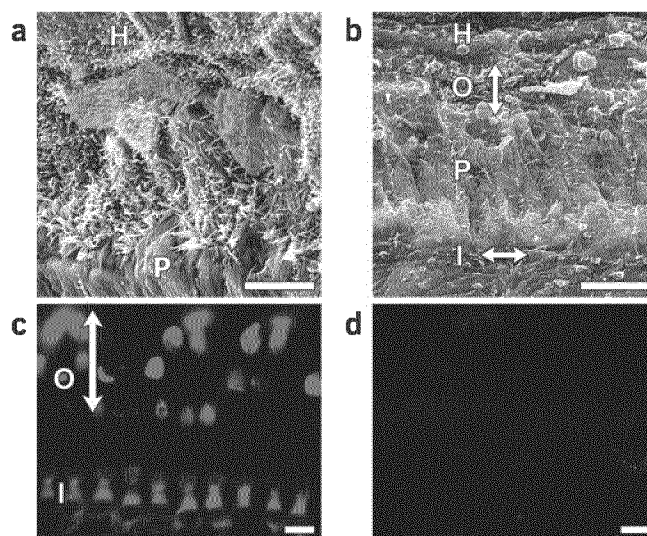
Figure 3 New hair cells and nuclei in the deafened cochlea inoculated with *Ad.Atoh1*. *Atoh1*-treated ears (**a** and **c–g**) and contralateral cochlea (**b**) processed 2 months later for myosin VIIa immunocytochemistry (**a,b**), Hoechst stain (**c**), epifluorescence for actin and GFP (**d**) or plastic sections (**e–g**). (**a**) Pillar cells (P) are flanked by IHCs (I) and OHCs (rows 1–3) positive for myosin VIIa in the second cochlear turn. (**b**) Lack of myosin VIIa-positive hair cells in the contralateral (right) ear. (**c**) Nuclei are present in rows at the supporting cell focal plane (S), and above, closer to the OHC focal plane (rows 1–3). (**d**) Two months after inoculation with *Ad.Atoh1-GFP*, actin (red), is seen in junctional complexes delineating the contour of OHCs (dotted circles) and in cuticular plates (asterisks), but not in the area of the centrosomes which is actin-free (arrowheads). GFP (green, cytoplasmic) is seen in all three rows of OHCs (rows 1–3). First row OHCs are shown in focal plane beneath apical junctions. Nuclei visible in this focal plane are unstained for GFP (arrows). (**e**) A cell in the OHC area of the second turn has a cuticular plate (white arrowhead) and a luminal projection (black arrow) protruding from the cuticular plate. The cell body extends from the luminal surface (black arrowhead) to the basement membrane (white arrow), and the nucleus is basal. (**f**) In some regions of OHC area a dual layer of nuclei can be seen above the basement membrane (arrow). (**g**) An IHC featuring a luminal projection (arrow) and normal morphology. Scale bars, 10 μ m in **a**, **b**, and **d–g**, and 5 μ m in **c**.

gest that re-expression of developmental regulatory genes in mature tissues is a potential strategy for cell replacement therapy in the cochlea and elsewhere.

The increase in number of nuclei in the sensory epithelium after *Atoh1* treatment can be explained either by cell migration from areas flanking the organ of Corti, as seen in birds¹⁴, or by a proliferative response of nonsensory cells that occurs secondary to the generation of new hair cells. *Atoh1* by itself is unlikely to induce mitosis¹³. The cochlea may also have a population of unidentified stem cells that divide after deafening, a possibility suggested by recent findings in the utricle¹⁵. It is unknown whether specific subpopulations of supporting cells are capable of (i) generating new hair cells following *Atoh1* expression (ii) migrating into the organ of Corti and/or (iii) dividing and repopulating the epithelium.

Figure 4 Cochlea analyzed with SEM at 4 weeks after *Atoh1* treatment (**a**) or myosin VIIa immunocytochemistry at 5 weeks after treatment (**c**) and the respective contralateral ears (**b** and **d**). (**a**) Several immature stereocilia bundles (some colored to highlight the morphology) are observed lateral to the pillar cells (P). The OHC region is wide and the tall surface projections cover much of the surface. (**b**) In the contralateral cochlea the distance between pillar cells (P) and Hensen cells (H) where OHCs had resided before deafening (O, vertical double-arrow) is greatly diminished. Neither OHCs nor IHCs (I and horizontal double-arrow) are present. (**c**) In a *Atoh1*-treated ear, a well-organized single row of IHCs and relatively disorganized rows of OHCs are seen with cytoplasmic staining of myosin VIIa in the second turn. (**d**) Myosin VIIa-positive cells are absent in contralateral ear. Scale bars, 5 μ m in **a** and **b** and 10 μ m in **c** and **d**.

The *Atoh1*-induced restoration of the luminal surface of the auditory epithelium generates both hair cell and supporting cell-like areas which, in some cases, resemble the normal cellular mosaic in the organ of Corti. We speculate that dual-phenotype cells contribute in part to the generation of areas with surface features of supporting cells. Newly added cells that derive from proliferation or migration probably also contribute to the restoration of the surface morphology.



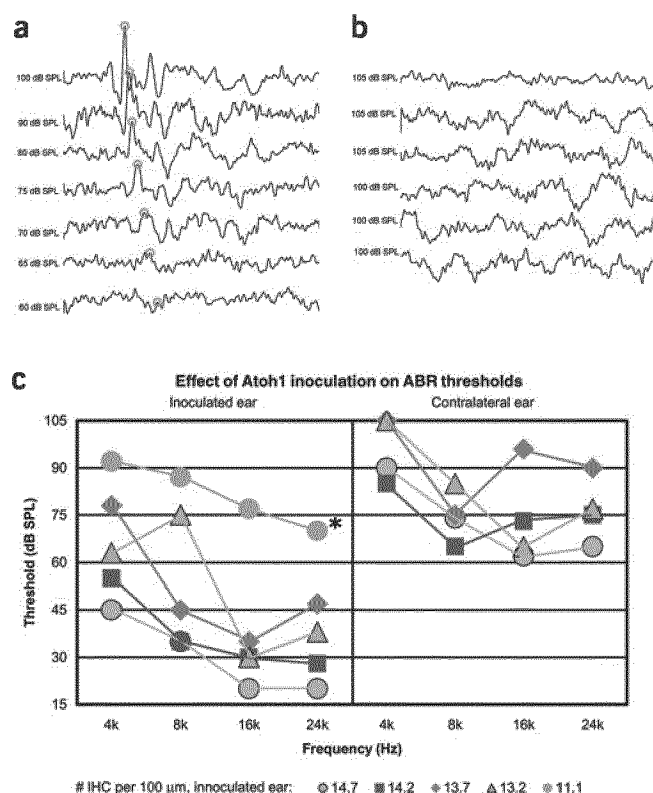


Figure 5 *Atoh1* treatment improves ABR thresholds. (a) ABR waves for Ad.*Atoh1*-treated ear represented in c by orange circle with asterisk. Waves show marked peaks in response to input as low as 65 dB sound pressure level (SPL). The waveform shows that the latency of the marked peak increases with decreasing level of the stimulus, as expected in normal ABR response²⁰. (b) In right (contralateral) ear no consistent peaks are seen. (c) Data points for thresholds at each frequency for the left (*Atoh1*-inoculated) and right (contralateral) ears of five animals. ABRs could not be obtained from the right ear of the orange animal at the limits of the sound delivery system (105 dB SPL). Average dB SPL thresholds in treated ears are significantly lower (better) than in contralateral ears at each frequency ($P < 0.004$). The number of IHCs per 100 μ m is given for each animal. Thresholds are significantly correlated with the number of IHCs in *Atoh1*-treated ears. The weakest correlation was at 4 kHz ($r^2 = 0.78$, $P < 0.05$).

(500 mg/kg, subcutaneous) followed 2 h later by ethacrynic acid (50 mg/kg, intravenous). We verified deafening by measuring ABR thresholds. This ototoxic drug regimen is designed to consistently eliminate hair cells but spare the pillar cells. Hair-cell elimination is complete in the first three cochlear turns, although a few hair cells may survive in the apical turn. We excluded animals that did not show a threshold greater than 95 dB SPL (numbers of animals given above do not include excluded animals). Viral vectors were inoculated into the left cochlea 4 d after the deafening surgery, using a procedure previously described¹⁷, except that the inoculation was into the second cochlear turn.

Adenovirus vector. Replication-deficient recombinant adenoviruses with deleted E1, E3, and E4 regions¹⁸ were Ad.*Atoh1*, Ad.*Atoh1*-GFP, Ad.empty and Ad.GFP. The *Atoh1* cDNA was obtained from H. Zoghbi (Baylor College of Medicine). The insert in Ad.*Atoh1* was driven by the human cytomegalovirus promoter and the GFP by the chicken beta-actin promoter. We used undiluted vectors at a concentration of 1×10^{12} total particles purified virus per milliliter. Viral suspensions were kept at -80°C until thawed for use.

ABR measurement. To assess auditory thresholds, we recorded ABRs at 4, 8, 16, and 24 kHz (tone bursts, 15 ms duration, 1 ms $\cos^2$ -shaped rise-fall times) as previously described¹⁹. Measured frequencies correspond to the basal and lower second turn of the guinea pig cochlea, close to the site of inoculation. When present, the thresholds recorded in the contralateral (right) ears probably represent hearing from *Atoh1*-treated left ears. For this reason, an animal with lesser threshold recovery in the left ear was selected for showing ABR waveforms in the contralateral ear in Fig. 5. Lower frequency thresholds are not reliably measurable with ABR audiometry²⁰. The researcher who measured ABRs was blinded to the identity of the animal.

Immunocytochemistry and SEM. We stained whole mounts of the auditory epithelium with antibodies to myosin VIIa or *Atoh1* as described⁷, except that tissues were double stained with phalloidin (Molecular Probes) to stain actin²¹. To augment GFP fluorescence, we stained tissues with a GFP-specific rabbit antibody (Chemicon, diluted 1:500). Antibody to myosin VIIa was purchased from the University of California San Diego. Antibody to *Atoh1* was purchased from the Tissue Culture Hybridoma Core at the University of Iowa. To stain nuclei, we used the DNA-specific label Hoechst as previously described²². The specimens were examined and photographed using a Leica DMRB epifluorescence microscope (Leica) with a CCD Cooled SPOT-RT digital camera (Diagnostic Instruments). For SEM, samples were prepared as described²³. The samples were mounted on stubs using silver paste and photographed digitally using a Philips XL30 Field Emission Gun SEM (FEI).

Plastic sections. We decalcified cochleae, embedded them in Epon as described²⁴ and sectioned (1 μ m) them with a glass knife. Sections were photographed using a Leica DMRB epifluorescence microscope and a 100 $\times$ objective lens.

Data analysis. We performed the statistical analysis of the ABR data using Excel. Conventional t-tests were performed to determine the significance of the difference between treated and nontreated ears at each tested frequency. Because we performed multiple tests, we applied sequential Bonferroni adjustment

The organization of the tissue is likely to depend on the ability of hair cells to dictate the phenotype of their neighbors and to attract supporting cells, as shown *in vitro*⁹. The ability of the tissue to respond to *Atoh1* overexpression and to reorganize the new hair cells generated by *Atoh1* may depend on the mechanism of deafening, the timing of treatment after deafening and the degree of differentiation of the surviving nonsensory cells. Better understanding of the response of supporting cells to *Atoh1* expression will also shed light on the process of post-trauma repair of the mosaic-like cytoarchitecture at the luminal surface of the auditory epithelium.

A clinical restoration of threshold sensitivity similar to that shown here in guinea pigs would be an extremely attractive therapeutic outcome for patients with profound deafness. Addition of new hair cells may also enhance the outcome of cochlear implantation, because presence of some hair cells improves implant function¹⁶. Our findings support the feasibility of genetic manipulation for cell replacement therapies based on inducing transdifferentiation of endogenous cells in the inner ear and in other systems.

METHODS

Animals. All animal experiments were approved by the University of Michigan Institutional Committee on the Use and Care of Animals and performed using accepted veterinary standards. We used young adult guinea pigs (Elm Hill Breeding Laboratory) weighing 250–400 g with normal ABR baseline thresholds of 15–50 dB sound pressure level (SPL). Animal groups were Ad.*Atoh1* ($n = 30$), Ad.empty ($n = 12$), Ad.*Atoh1*-GFP ($n = 5$), Ad.GFP ($n = 5$) and deafening alone ($n = 4$). Inner ears were analyzed using epifluorescence, SEM or plastic cross-sections of the organ of Corti. Animals that did not have a complete loss of hair cells in the three lower cochlear turns, as determined by observing the right (contralateral) ear, were excluded from the study.

Deafening surgery and viral inoculation. Guinea pigs were deafened bilaterally with a single systemic dose of kanamycin

to keep the table-wide error rate below 0.05. ABR thresholds for each animal were regressed on the number of IHC to obtain the correlation for each tested frequency. A *t*-test was performed on the 4 kHz data to determine whether the correlation was significant (slope > 0).

Evaluation of the morphology of *Atoh1*-inoculated ears 8 weeks after the inoculation showed three animals that were completely devoid of hair cells. These animals were assumed to have had failed inoculation surgeries and were excluded from these statistical analyses.

We performed quantitative analysis of whole mounts of the organ of Corti to determine the number of hair cells (phalloidin-stained cochleae) or positively stained nuclei (*Atoh1* or Hoechst stains). Nuclei counts in Hoechst-stained whole mounts were performed in the OHC area, where nuclei of hair and supporting cells can be distinguished from each other based on their organization in distinct focal planes. We counted a segment of 1 mm on each side of the inoculation site for a total of 2 mm. Conventional *t*-tests were performed to determine the significance of differences between treated and nontreated cochleae.

ACKNOWLEDGMENTS

We thank L.A. Beyer and L.L. Kabara for technical assistance. We thank P.F. Hitchcock and D.M. Martin for discussions and comments on the manuscript. This work was supported by a gift from B. and A. Hirschfeld, by GenVec and by US National Institutes of Health—National Institute on Deafness and Other Communication Disorders grants R01 DC01634, DC05401 and P30 DC05188. We thank P.J. Olynyk for graphics work on the cover illustration.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

Received 10 September 2004; accepted 25 January 2005

Published online at <http://www.nature.com/naturemedicine/>

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Regeneration of Stereocilia of Hair Cells by Forced Atoh1 Expression in the Adult Mammalian Cochlea

Shi-Ming Yang^{1*}, Wei Chen², Wei-Wei Guo¹, Shuping Jia³, Jian-He Sun¹, Hui-Zhan Liu³, Wie-Yen Young¹, David Z. Z. He^{3*}

1 Department of Otolaryngology—Head and Neck Surgery, Institute of Otolaryngology, Chinese PLA General Hospital, Beijing, China, **2** Department of Otolaryngology—Head and Neck Surgery, PLA Air Force General Hospital, Beijing, China, **3** Department of Biomedical Sciences, Creighton University School of Medicine, Omaha, Nebraska, United States of America

Abstract

The hallmark of mechanosensory hair cells is the stereocilia, where mechanical stimuli are converted into electrical signals. These delicate stereocilia are susceptible to acoustic trauma and ototoxic drugs. While hair cells in lower vertebrates and the mammalian vestibular system can spontaneously regenerate lost stereocilia, mammalian cochlear hair cells no longer retain this capability. We explored the possibility of regenerating stereocilia in the noise-deafened guinea pig cochlea by cochlear inoculation of a viral vector carrying Atoh1, a gene critical for hair cell differentiation. Exposure to simulated gunfire resulted in a 60–70 dB hearing loss and extensive damage and loss of stereocilia bundles of both inner and outer hair cells along the entire cochlear length. However, most injured hair cells remained in the organ of Corti for up to 10 days after the trauma. A viral vector carrying an EGFP-labeled Atoh1 gene was inoculated into the cochlea through the round window on the seventh day after noise exposure. Auditory brainstem response measured one month after inoculation showed that hearing thresholds were substantially improved. Scanning electron microscopy revealed that the damaged/lost stereocilia bundles were repaired or regenerated after Atoh1 treatment, suggesting that Atoh1 was able to induce repair/regeneration of the damaged or lost stereocilia. Therefore, our studies revealed a new role of Atoh1 as a gene critical for promoting repair/regeneration of stereocilia and maintaining injured hair cells in the adult mammal cochlea. Atoh1-based gene therapy, therefore, has the potential to treat noise-induced hearing loss if the treatment is carried out before hair cells die.

Citation: Yang S-M, Chen W, Guo W-W, Jia S, Sun J-H, et al. (2012) Regeneration of Stereocilia of Hair Cells by Forced Atoh1 Expression in the Adult Mammalian Cochlea. PLoS ONE 7(9): e46355. doi:10.1371/journal.pone.0046355

Editor: Li I. Zhang, University of Southern California, United States of America

Received: June 21, 2012; **Accepted:** August 29, 2012; **Published:** September 27, 2012

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Funding: This work was supported by grants from the Major State Basic Research Development Program of China (973 Program #2011CBA01000), the National Basic Research Program of China (973 Program #2012CB967900), National Hi-Tech Research and Development Program of China (863 Program #2007AA02Z150), the National Natural Science Foundation of China (#30871398, 30730040) to SMY, and by National Institutes of Health (NIH) grants R21 DC 009908 and R01 DC 004696 to DH from the National Institute on Deafness and Other Communication Disorders (NIDCD). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: yangsm301@263.net (SMY); hed@creighton.edu (DZZH)

Introduction

Noise-induced hearing loss (NIHL) is a major health problem. Acoustic trauma causes NIHL when permanent cochlear damage results from exposure to high-intensity sounds, such as explosions, gunfire, and firecrackers. NIHL is usually due to destruction of cochlear hair cells and/or damage to their hair bundles [1]. Cochlear hair cells transduce mechanical stimuli into electrical activity [2]. The hair bundle, a staircase array of stereocilia of different heights, is the site of mechanoelectrical transduction [3]. The delicate hair bundle is susceptible to both acoustic trauma and ototoxic drugs. While hair cells in lower vertebrates and mammalian vestibular systems can spontaneously regenerate lost stereocilia [4–6], mammalian cochlear hair cells no longer retain this capability [7]. The inability of stereocilia to self-repair can subsequently lead to hair cell death and permanent hearing loss. Injured neonatal gerbil hair cells can live for 10 to 12 days *in vitro* after their stereocilia are destroyed [7]. If adult cochlear hair cells can likewise survive for a number of days after loss of stereocilia due to exposure to impulsive noise or ototoxic drugs, this window of opportunity could be crucial for potentially rescuing and

repairing hair cells using therapeutic genetic or chemical interventions.

The transcription factor *atoh1* belongs to the family of basic helix-loop-helix (bHLH)-containing proteins and plays an essential role in the development of the *Drosophila* nervous system [8]. In mammals, Atoh1 (also known as *math1*) has been shown to be essential for neurogenesis in the central and peripheral nervous system [9–12], and for the formation of several non-neural cell types [13,14]. In the auditory system, Atoh1 plays a critical role for hair cell differentiation during development [15–17]. Embryonic Atoh1-null mice fail to generate cochlear and vestibular hair cells, as indicated by the absence of stereocilia and other hair cell-specific markers [15]. Furthermore, overexpression (or misexpression) of Atoh1 in *in vitro*, *in vivo*, and *in utero* cochleae results in the production of ectopic hair cells derived from non-sensory supporting cells [18–21]. These studies suggest that Atoh1 is important for hair cell genesis or apical specialization (i.e., stereocilia development and formation). We questioned whether forced expression of Atoh1 in the noise-deafened cochlea can promote repair/regeneration of the stereocilia destroyed by impulsive noise.

Materials and Methods

1. Animals

Care and use of the animals in this study was approved by the Institutional Animal Care and Use Committee of the Chinese PLA General Hospital. Healthy (250–300 g) adult albino guinea pigs of either sex were used for the experiments. Hearing in both ears was measured using auditory brainstem responses (ABR) before and seven days after noise exposure. Only those animals whose hearing thresholds were normal before exposure and significantly elevated (≥ 60 dB SPL at all frequencies tested) after noise exposure, with no obvious sign of middle ear infection and damage, were selected for further experiments.

2. Impulsive Noise Exposure

Animals were put in a specially designed cage with their heads constrained. The cage was housed inside a sound isolation room. Each animal was continuously exposed to 200 rounds of simulated gunfire inside the soundproof room. The sound source was placed 20 cm away from each ear. The peak sound pressure level measured near the ear canal was approximately 164 dB SPL (C-frequency weighting). The impulse had a duration of 10 ms. The interval between each pulse was 10 seconds.

3. Viral Construct and Inoculation

Replication-deficient recombinant adenoviruses (Ad5) with deleted E1 and E3 regions were used to construct *Ad.Atoh1-GFP* and *Ad.GFP* using the Adeno-X expression system (K1650-1, Clontech). Dr. W. Q. Gao from Genentech, San Francisco, CA, kindly provided us with the *Atoh1* plasmids. The sequence and other information about the constructs have been published elsewhere [18,20,21]. Animals were anesthetized with xylazine and ketamine, and kept warm using a 37°C warm pad. Viral vectors were inoculated into the cochlea through the round window either seven days or one month after noise exposure. 5 μ l of viral suspension with concentration of 1×10^{10} total particles of purified virus per milliliter was inoculated.

4. Immunocytochemistry and Scanning Electron Microscopy

Cochleae were perfused with 4% formaldehyde in phosphate buffered saline (PBS) and treated with 0.2% Triton X-100/PBS. Goat serum (10%) was used to block nonspecific binding. The tissue was then incubated with an anti-C-mPres antibody [22] and washed with PBS, followed by incubation with secondary antibodies. To visualize the stereocilia, F-actin was labeled with rhodamine-phalloidin, as described previously [7]. An anti-myosin 7a antibody was also used to label the hair cells as described elsewhere [21]. A sample was mounted on glass slides with antifade solution (Prolong Antifade Kit, Invitrogen, Carlsbad, CA) before imaging on an Olympus FluoView FV1000 Confocal Microscope (Olympus (China), Ltd., Beijing).

For scanning electron microscopy (SEM), the cochleae were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) containing 2 mM CaCl_2 , washed in PBS, then post-fixed for 15 minutes with 1% OsO_4 in the same buffer and washed. The tissues were dehydrated in an ethanol series, critical point dried from CO_2 , and sputter-coated with gold. The tissue was then examined using a Hitachi S-3700N scanning electron microscope.

5. ABR and Cochlear Microphonic Measurements

Details of ABR and cochlear microphonic (CM) measurements are described as previously performed [22]. During ABR and CM

measurements, guinea pigs were anesthetized with xylazine and ketamine. For ABR, needle electrodes were inserted at the vertex and pinna. ABRs were evoked with clicks and/or 5-ms tone pips (0.5 ms rise-fall, at 30/sec) with frequencies of 4, 8, 16, and 20 kHz. The response was amplified, filtered, and averaged using the Intelligent Hearing System (Intelligent Hearing System Corp., Miami, FL). Sound level was raised in 20- and/or 5-dB steps. At each level, 1,024 responses were averaged. Hearing threshold was determined by visual inspection. For CM measurement, a silver wire electrode was placed near the round window membrane. Responses to a 4-kHz tone burst were amplified, filtered, and averaged using the Intelligent Hearing System.

Results

Adult guinea pigs were exposed to 200 rounds of simulated gunfire. We measured the CM to monitor the damage to the hair cells in some animals. The CM, an electrical potential generated in the cochlear hair cells in response to acoustic stimulation, primarily reflects mechanotransduction in the stereocilia of outer hair cells [23]. The CM response before and after 1, 10, and 200 rounds of gunfire was recorded. As examples, Figure 1A shows the CM response waveforms at three high sound intensity levels before, during, and after noise exposure. As shown, the magnitude of CM responses at saturation levels was reduced by as much as 26% after exposure to just one round of gunfire. After exposures to 10 and 200 rounds of gunfire, the magnitude decreased by 66% and 97%, respectively. The magnitude-intensity (input-output) function of the CM (Fig. 1B) shows that there was at least a 60 dB elevation in sound pressure level for the same magnitude of the CM after noise exposure. The reduction represents a significant disruption of outer hair cell (OHC) mechanotransduction after noise exposure. We measured hearing thresholds (Fig. 1C) before and seven days after noise exposure, using sound-evoked ABRs [22]. ABRs, frequently used to monitor hearing loss, are electrical signals evoked from the brainstem during presentation of an acoustic signal. ABR thresholds were obtained from both ears of 19 animals at 4, 8, 16, and 20 Hz using tone pips. The means and standard deviations of the thresholds before and seven days after noise exposure are presented in Figure 1C. Consistent with CM measurements, hearing thresholds for both ears were elevated by more than 60 dB for all frequencies tested. Since the ABR thresholds were measured at seven days after noise exposure to minimize the effect of temporary threshold shift, this threshold elevation should represent the permanent hearing loss. Only those animals whose hearing thresholds were elevated by more than 60 dB were used for further experiments.

We examined the morphological integrity of the stereocilia along the entire cochlear length at seven days after noise exposure using SEM. Figure 2 (A–F) shows examples obtained from three cochlear locations. Extensive stereocilia damage and loss of both inner and outer hair cells in the first, second, and third turns were observed. Sporadic hair cell death, as indicated by scar formation (marked by arrows in Fig. 2B), was also seen. Notably, injured hair cells remained in the organ of Corti despite severe bundle damage/loss. This is indicated by the presence of rootlets of the truncated stereocilia in the reticular lamina (Fig. 2G, H) and/or by the presence of OHC somas sandwiched between reticular lamina and the Deiters' cells (Fig. 2I).

To examine the fate of hair cells after stereocilia damage/loss, prestin immunoreactivity combined with confocal microscopy was used. Prestin is the motor protein of OHCs [24] and is only expressed in the plasma membrane [25]. Therefore, prestin immunoreactivity can be used as an assay to probe the status of

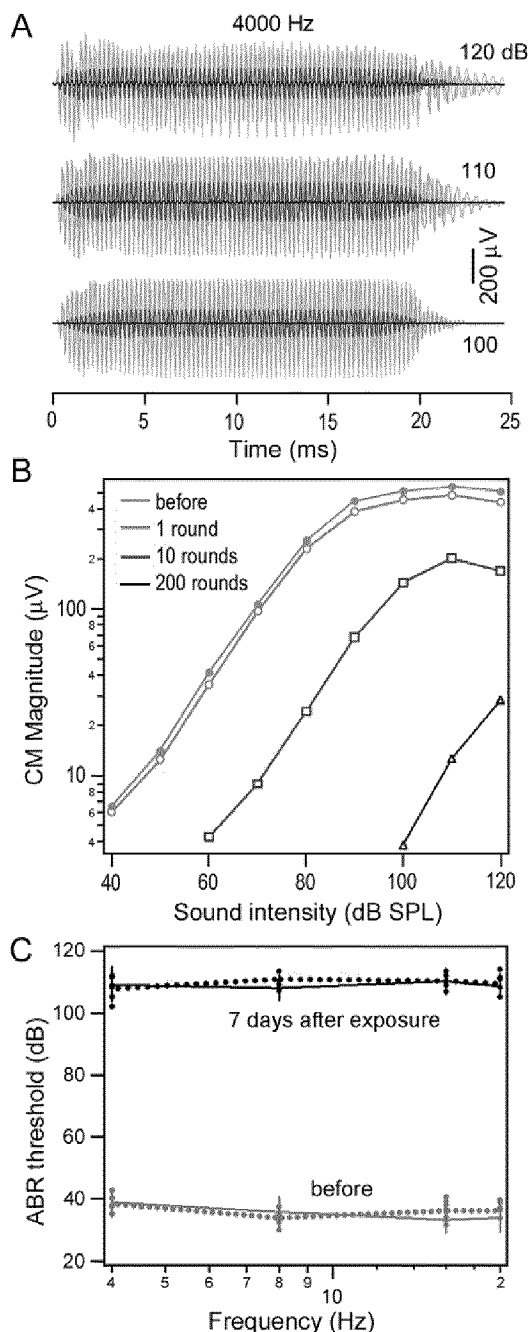


Figure 1. Hearing loss after exposure to simulated gunfire. A: Three examples of CM responses obtained before, during, and immediately after noise exposure. B: CM magnitude as a function of sound level before, during, and after noise exposure. Note the magnitude is plotted on log scale. C: Tone pip-evoked ABR thresholds (mean $\pm$ SD, $n=19$) for both ears before and seven days after noise exposure. No statistical difference (student's t-test) in thresholds at each frequency was found between left (dot lines) and right (solid lines) ears either before or after noise exposure ($p>0.05$). doi:10.1371/journal.pone.0046355.g001

OHCs. Absence of staining or presence of membrane debris would suggest cell death. To simultaneously visualize the hair bundle, rhodamine-phalloidin was used to label F-actin-containing stereocilia [7]. Figure 3A shows a composite confocal image obtained

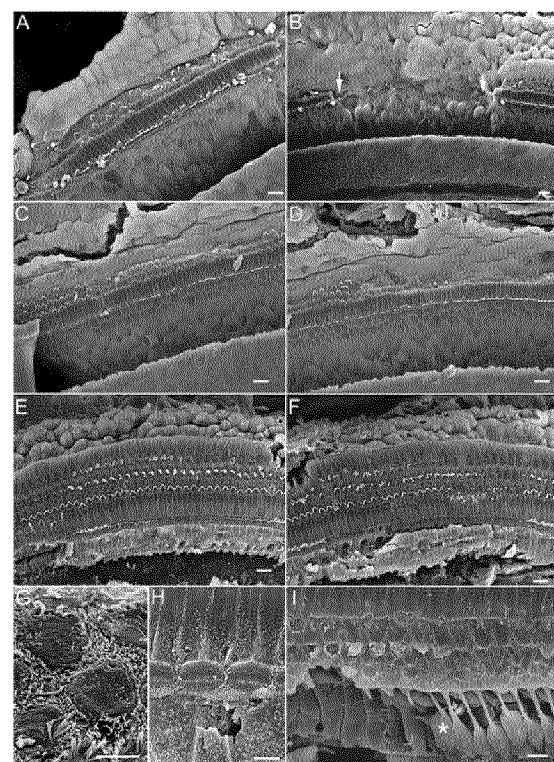


Figure 2. SEM images of the hair bundles seven days after exposure to impulsive noise. A–F: Images of hair bundle damage/loss obtained from representative locations in the basal (A,B), second (C,D), and third (E,F) turns. An area with hair cell death and scar formation is marked by arrows. G,H: High-magnification pictures of OHCs and IHCs with complete loss of stereocilia bundles. I: Image of complete loss of OHC stereocilia bundles in a second turn region. Note that the OHCs still remained in the organ of Corti despite loss of stereocilia bundles. A Deiters' cell is marked with an asterisk. Scale bars: 20 μ m (A–F), 5 μ m (G,H), and 10 μ m (I). doi:10.1371/journal.pone.0046355.g002

from a cochlea 10 days after noise exposure. As shown, the “V”-shaped OHC bundles (in red) were missing in the outmost (third) row and in some second row OHCs. However, the bundleless hair cells remained in the organ of Corti, although some sporadic hair cell loss was also observed (evidenced by empty space between prestin-labeled OHCs). Figure 3B shows a 3-D prestin-immunolabeled image (stacked along the z-axis) from a different cochlear location. This, again, shows that most OHCs survived the acoustic trauma despite sporadic OHC death (e.g., membrane debris and missing OHCs in Fig. 3B). Consistent with a previous study using the cultured organ of Corti [7], we concluded that noise-damaged OHCs with bundle damage/loss could survive up to at least 10 days after exposure.

Since the bundleless hair cells could remain in the organ of Corti for at least 10 days, this window could be crucial for intervention to repair hair bundles and to prevent the eventual death of hair cells. We explored the possibility of rescuing and repairing the injured hair cells by overexpressing Atoh1. A viral vector carrying an EGFP-labeled *Atoh1* gene was inoculated into the cochlea through the round window on the seventh day after noise exposure. We chose the left ear for viral inoculation and the right (contralateral) ear as the control in eight animals. In another six animals, viral vectors carrying only the EGFP gene were inoculated into the left cochlea (seven days after noise exposure) as

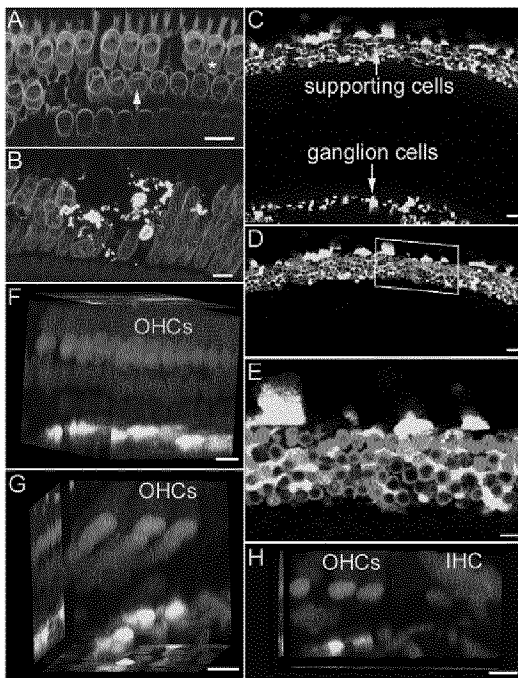


Figure 3. Confocal images of the stereocilia and prestin immunoactivity, and the pattern of Atoh1-EGFP expression in the organ of Corti. A: Stereocilia bundles in one cochlear location. The hair bundle was labeled with rhodamine-phalloidin (in red). The "V"-shaped OHC bundles were missing in the outmost (third) rows of OHCs. One bundleless OHC is indicated by an asterisk. One "V"-shaped hair bundle is marked by a yellow arrow. Prestin immunoactivity was labeled in green. Scale bar: 10 μ m. B: Z-axis stack image of prestin immunoactivity at another cochlear location. Scale bar: 10 μ m. All the images in A and B were obtained from cochleae 10 days after noise exposure. C: Confocal image of Atoh1-expression in a cochlea. D: Composite images of hair cells (labeled with myo7a antibody, in red) and EGFP-positive cells (from the same location as shown in panel C). The images were acquired seven days after Ad.Atoh1-EGFP inoculation in the noise-damaged cochlea (second turn). A magnified image of the area marked by white lines is presented in panel E. Scale bar: 20 μ m for C and D. E: High magnification image of Atoh1 and myo7a expression in the organ of Corti. Scale bar: 10 μ m. F: Confocal image obtained from optical sectioning from a basal turn location at 3 days after Atoh1 treatment. Hair cells were labeled with myo7a (in red) and the nuclei were labeled with DAPI. Most preparations examined at 3 days after transfection showed weak or no expression of EGFP in the organ of Corti. For those that expressed EGFP, the expression was in the area of Deiters' cells. G, H: Confocal image using optical sectioning from a basal turn location at 7 and 14 days after Atoh1 treatment. Scale bars (F, G, H): 10 μ m.

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an additional control. Expression of Atoh1 by the EGFP expression was examined by confocal microscopy seven days after inoculation. Figure 3C exhibits a survey confocal image obtained from the basal turn in a whole-mount preparation. As shown, intense EGFP-positive labeling was observed in the areas of the organ of Corti and spiral ganglion neurons. To determine where the forced expression of Atoh1 occurred in the organ of Corti, we used a myo7a antibody to label hair cells. Figure 3D shows that EGFP and myo7a were expressed in the different cell types in the organ of Corti. High magnification images show that EGFP expression was not in the hair cells (Fig. 3E). To further confirm that forced Atoh1 expression was not in hair cells, we took a series of optical sections to reconstruct 3-D images of the organ of Corti

at 7 days after transfection. Reconstruction from the obtained images confirmed that forced Atoh1 expression was not seen in the hair cells, but rather in the supporting cells (Fig. 3G). To determine whether forced Atoh1 expression ever occurred in the hair cells at different time points after transfection, we also examined EGFP expression at 3 (Fig. 3F) and 14 (Fig. 3H) days after Atoh1 inoculation. As shown, forced Atoh1 expression was confined to the supporting cells and was not detected in the hair cells.

The morphology of stereocilia at three cochlear locations was examined in the control (right) and Atoh1-treated (left) ears using SEM at one month after viral inoculation. In the control ears without Atoh1 inoculation after noise exposure, the reticular lamina was mostly flat with a few remaining stereocilia bundles (Fig. 4A–C). The remaining bundles had obvious signs of damage such as truncation, fusion, and folding. In contrast, most hair bundles in the Atoh1-treated ears appeared to have a normal appearance. Although hair cells with damaged or lost bundles were also sporadically observed along the entire length of the cochlea in the Atoh1-treated ears, most areas contained four rows of stereocilia (i.e., one row of IHCs and three rows of OHCs) on the reticular lamina (Fig. 4D–F). The Deiters' cells were coupled to the synaptic poles of OHCs (Fig. 4E). The presence of both OHCs and Deiters' cells suggests that the architectural relationship between OHCs and Deiters' cells was maintained in the "recovered" organ of Corti.

To quantify bundle repair/regeneration after Atoh1 treatment, we counted the total number of bundles from three cochlear locations (each 1 mm in length) from three normal (i.e., without noise exposure) cochleae as reference. Cochleae from Atoh1-treated left ears, as well as from control (right) ears, at one month after inoculation were also examined. The three sites were approximately 14.5–15.5, 10.5–11.5, and 4.0–5.0 mm from the basal end of the basilar membrane. Although the characteristic

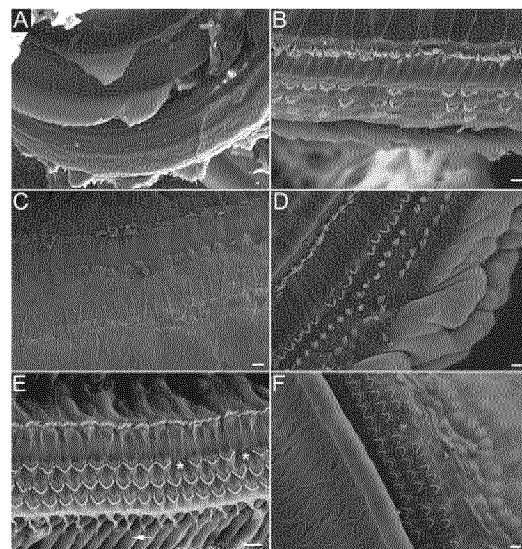


Figure 4. Representative SEM pictures obtained from different cochlear locations from Atoh1-treated and untreated (control) ears one month after viral inoculation. A–C: SEM images showing different extent and severity of damage from the control cochleae. D–F: SEM pictures of hair bundles from the Atoh1-treated ears. Missing hair bundles in panel E are marked by asterisks. The arrow indicates a Deiters' cell. Scale bars: 20 μ m (A) and 10 μ m (B to F).

doi:10.1371/journal.pone.0046355.g004

frequencies of these locations were unknown due to the lack of a place-frequency map of the guinea pig cochlea [26], we estimated the characteristic frequency of these three locations based on recordings of inner hair cell (IHC) receptor potentials near these locations. Cheatham and colleague [27,28] have determined that locations 17 (in the fourth turn), 15 (in the third turn), and 10 (in the second turn) mm from the basal end of the cochlea correspond to the characteristic frequencies of 270, 1000, and 4000 Hz, respectively. Based on this information, we estimated that the three locations we chose encompassed the areas that had the characteristic frequencies of roughly 1000, 4000, and 15,000 Hz, respectively. Three cochleae for each group of normal, Atoh1-treated, and untreated ears were used for bundle count. Status of the bundles was determined by visual inspection of the bundles under the scanning electron microscope. Hair bundles that had obvious signs of damage such as truncation (Fig. 5A), fusion (Fig. 5B), or folding were excluded in the bundle count. Although tip-links are known to be vulnerable to noise damage [29,30], tip-link integrity was not considered in the bundle count.

Figure 5 (D and E) exhibits the means and standard deviations of the total numbers of bundles of IHCs and OHCs at three cochlear locations. As shown, noise exposure caused a significant reduction in the number of the hair bundles of both inner and outer hair cells in the untreated (right) ears ($p < 0.01$ for both IHCs and OHCs at all three locations, student's *t*-test). After Atoh1 treatment, the numbers of bundles of both IHCs and OHCs were significantly greater than those of the untreated cochleae ($p < 0.01$ for all cases). However, these numbers were still significantly less than in the normal cochlea, suggesting that a large number of damaged/lost bundles were not repaired. Transfection efficacy and hair cell death likely occurred before Atoh1 treatment were responsible for the disparity seen between the normal and Atoh1-treated ears.

We measured hearing thresholds from both (Atoh1-treated and contralateral) ears from eight animals using click-evoked ABRs at two weeks, three weeks, and one and two months after viral inoculation. As an additional control, hearing thresholds were also measured from noise-deafened animals with inoculation of the EGFP vector alone in the right ear. The results are presented in Figure 6A. Atoh1-treated ears showed an approximately 35–40 dB (SPL) threshold improvement (comparing to the contralateral ears) 30 days after Atoh1 treatment. We measured tone pip-evoked ABR thresholds at 4, 8, 16, and 20 kHz at one month after viral inoculation. Significant threshold improvement was seen at all frequencies tested (Fig. 6B). In contrast, thresholds in the contralateral ears without Atoh1 treatment were only improved by about 10–15 dB. Although thresholds of the contralateral ears also recovered somewhat (Fig. 6A,B), this improvement was likely due to neural input from the cross (nerve) fibers originating from the Atoh1-treated ears [20] since the improvement disappeared when the middle ear structures in Atoh1-treated ears were intentionally damaged (in two ears, data not shown). ABR thresholds measured from noise-deafened ears with inoculation of the EGFP vector alone did not show any improvement one month after inoculation (Fig. 6B). To determine whether Atoh1 treatment was still effective after hair cell death, we introduced the *Atoh1* gene into the cochleae one month after noise exposure. None of the four animals showed any improvement in ABR thresholds examined at one month after the “delayed” treatment (Fig. 7). SEM showed that the hair cells were completely lost and the reticular lamina was flat with no signs of any normal-looking stereocilia bundles (Fig. 7). The absence of sensory epithelia and lack of improvement in hearing threshold after the delayed

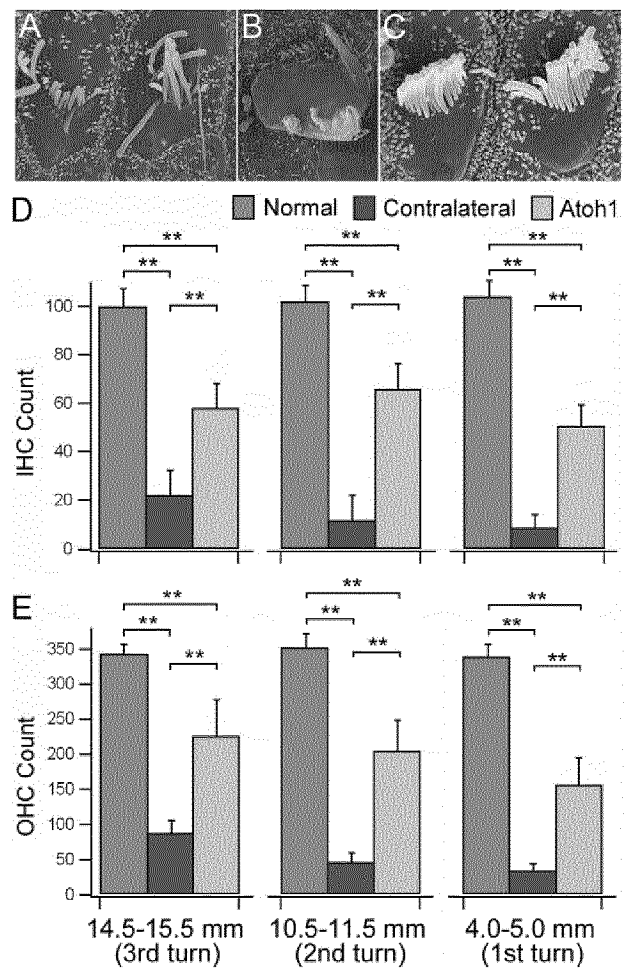


Figure 5. Stereocilia bundle counts in the normal (without noise exposure), Atoh1-treated and untreated cochleae. A–B: Examples of damaged bundles. C: Examples of normal-looking bundles. Scale bar represent 5 μm for all images in panels from A to C. D, E: Counts of IHC and OHC bundles in three different cochlear locations, each 1 mm in length. Three cochleae were used for each group (normal, Atoh1-treated, and untreated). Status of the bundles was determined by visual inspection of the bundles under scanning electron microscope. Hair bundles with no clear signs of truncation, fusion, or folding were included in the bundle count. Student's *t*-test was used for statistical analysis. Two asterisks represent statistical significance with a *p*-value less than 0.01.

doi:10.1371/journal.pone.0046355.g005

treatment suggest that the Atoh1-based gene therapy was effective only when injured hair cells were still present in the organ of Corti.

Discussion

In this study, we show for the first time that introduction of the *Atoh1* gene into a noise-damaged adult cochlea one week after noise exposure was able to substantially improve the hearing thresholds. Morphological examinations revealed that the numbers of stereocilia bundles of both IHCs and OHCs in the Atoh1-treated animals were significantly greater than those of the untreated cochleae. The re-emergence of stereocilia bundles after Atoh1 treatment suggests that Atoh1 is able to induce repair/regeneration of the damaged and/or lost stereocilia in the noise-deafened cochlea. We speculate that the new bundles originated

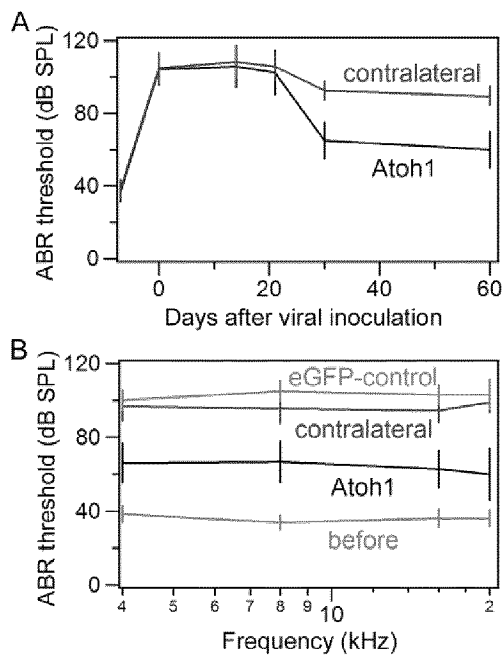


Figure 6. ABR thresholds measured from Atoh1-treated and control ears. A: ABR thresholds (means $\pm$ SD, $n=8$) were measured at different days after the viral vector carrying the *Atoh1* gene was introduced. Contralateral ears were used as control. Click was used to evoke ABRs. B: ABR thresholds (means $\pm$ SD) of the Atoh1-treated ears ($n=8$), contralateral ears ($n=8$), and EGFP vector-treated ears ($n=6$) at one month after cochlear inoculation. Tone pips with frequencies of 4, 8, 16, and 20 kHz were used to evoke ABRs. doi:10.1371/journal.pone.0046355.g006

from elongation of the truncated stereocilia, since the roots of truncated stereocilia were still present (Fig. 2G,H). It is also possible that regeneration originated from the microvilli. Gorclik et al. [31] show that microvilli may act as an elementary “building block” for the assembly of the specialized structures on the apical surface of cells. The mechanism(s) by which Atoh1 alone can promote repair/regeneration of the hair bundles with a functional mechanotransduction apparatus in both types of hair cells is unknown. It appears Atoh1 does not exert direct influence on hair cells, because forced Atoh1 expression was not detected in the hair cells (Fig. 3). However, supporting cells with forced Atoh1 expression may release diffusible factor(s) that may then influence adjacent hair cell repair.

It has been shown that the structure of the hair bundle is dynamically maintained by regulating the turnover of the actin filaments in a process described as treadmilling [32]. Recent evidence suggests that myosin-3a can facilitate hair bundle elongation by transporting espin-1 to the plus ends of actin filaments [33] and that the interaction of myosin-15a and whirlin is important for hair bundle morphogenesis and elongation [34–35]. Thus, it is possible that forced Atoh1 expression in the supporting cells may ultimately influence genes involved in hair bundle morphogenesis and elongation in the adjacent injured hair cells. Alternatively, the factor(s) released by supporting cells may stabilize the injured hair cells, allowing them to live long enough to repair themselves by upregulating genes involved in hair bundle elongation [33–35]. This notion that Atoh1 is important for maintaining hair cell viability in the adult cochlea is supported by a recent study [36]. We should point out that the molecular mechanism of how and why Atoh1 is critical for hair cell

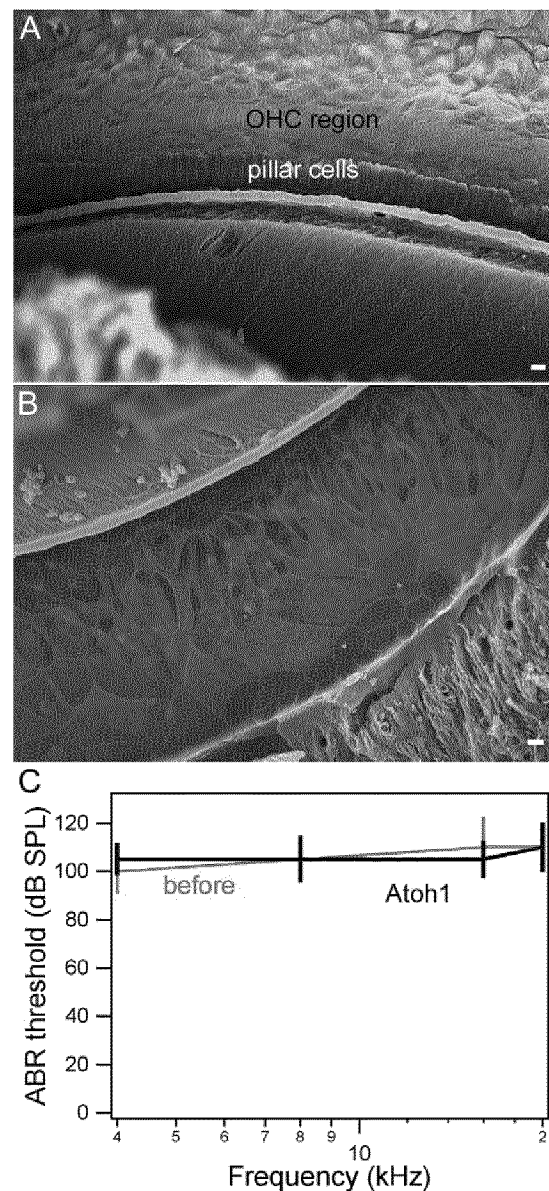


Figure 7. Representative SEM pictures and ABR thresholds obtained from cochleae treated with the Atoh1 gene one month after noise deafening. A, B: SEM images obtained from basal and middle turns. The reticular lamina was flat with no sign of stereocilia. Bars represent 10 μ m. C: ABR thresholds (means $\pm$ SD, $n=4$) measured from control and Atoh1-treated ears one month after the treatment. doi:10.1371/journal.pone.0046355.g007

differentiation is not clear, despite the fact that its critical role in hair cell morphogenesis was discovered some 13 years ago [15]. Regardless of whatever the mechanisms may be, our study reveals a role of Atoh1 that has not been revealed before. This new role in maintaining injured hair cells and subsequently promoting repair of damaged stereocilia in the adult mammalian cochlea has the potential to treat hearing loss after exposure to impulsive noise if gene therapy can be applied shortly after acoustic trauma.

It is important to distinguish between hair bundle repair/regeneration and transdifferentiation of supporting cells into hair cells after forced Atoh1 expression. Izumikawa et al. have shown

that *Atoh1* is able to induce hair cell regeneration in cochleae deafened by ototoxic drugs [20]. According to their study, the newly generated hair cells are likely formed by phenotypic conversion of supporting cells that remained in the deafened cochlea. However, there are no follow-up studies to determine how long the hair cells converted from supporting cells can live without supporting cells. Hair cells and supporting cells in the organ of Corti depend on each other; malfunction or apoptosis of one population can trigger degeneration of the other. Follow-up studies to determine whether a critical period during which the *Atoh1*-based treatment is effective are also lacking.

Our proposed mechanism for repair/regeneration is different from that of Izumikawa et al. [20]. In our model, we demonstrated that it was the regeneration/repair of the stereocilia rather than transdifferentiation of supporting cells. The strongest evidence to support this notion is based on the fact that both hair cells and supporting cells were present and that the architectural relationship between OHCs and Deiters' cells was maintained in the "recovered" cochleae after the treatment (Fig. 4E). If Deiters' cells had been converted to hair cells without mitosis, we would not have seen both supporting cells and hair cells in the *Atoh1*-treated cochlea. Furthermore, it has long been known that hair cells and supporting cells depend on each other for survival in the organ of Corti. Hair cell death can rapidly trigger signaling pathways that lead to supporting cell death [37,38]. Thus, it is inconceivable that either supporting cells or transdifferentiated hair cells could survive alone in the adult mammalian cochlea. Besides, although supporting cells have the capability for phenotypic conversion to hair cells, such capability of the organ of Corti is only demonstrated in prenatal and neonatal preparations where hair cells and supporting cells are still under development [18,21,22]. It is not clear whether supporting cells in the adult mammalian organ of Corti still retain the potential to transdifferentiate, although interdental cells may be able to convert to ectopic hair cells after *Atoh1* gene transfer [19]. Finally, regeneration of stereocilia occurred only when *Atoh1* inoculation was performed before hair cell death. *Atoh1* treatment at one month after noise exposure could no longer rescue hair cells and restore hearing.

We should point out that there are substantial differences in how the *Atoh1* gene was introduced into the cochlea and how the animals were deafened between our experiments and those of Izumikawa et al. [20]. In our study, *Atoh1* was inoculated through the round window and entered into the perilymph, which bathed both hair cells and Deiters' cells. In contrast, Izumikawa et al. inoculated the gene into the endolymph via the scala media. In our study the animals were deafened by impulsive noise, while Izumikawa et al. used ototoxic drugs to destroy hair cells. These differences may explain different underlying mechanisms for the two studies. However, it is known that individual susceptibility to ototoxic drugs varies significantly among animals (i.e., lethal

dosage for one animal may be only sublethal to another) and that sublethal dosage of ototoxic drugs often only destroys stereocilia [5,6]. Thus, we entertain the possibility that the hearing recovery observed by Izumikawa et al. could also be the result of hair bundle repair/regeneration after *Atoh1* treatment.

We are fully aware that there are other mechanical and metabolic injuries (besides stereocilia damage) to hair cells, supporting cells, ganglion neurons, the stria vascularis, and Reissner's membrane after noise trauma [39–48]. The severity and extent of damage depends on the type (continuous vs. impulsive), level, duration, and interval of the noise [40–48]. In this study, possible damage to structures other than the stereocilia was not examined since our focus was the damage and repair in the hair bundles. However, we were aware that damage to other structures may cause subsequent hair cell death and hearing loss. We also note that the total number of bundles of inner and outer hair cells in the *Atoh1*-treated ears was significantly less than that of the intact cochlea (Fig. 5D and E). The reduction in the number of functional stereocilia bundles, together with possible damage to the Reissner's membrane and stria vascularis, may explain why the hearing threshold was not fully recovered with *Atoh1* treatment. We measured ABR thresholds at 4, 8, 16, and 20 kHz, representing the locations that are most vulnerable to impulsive noise in the animal models [41,46–48]. Impulsive noise has a relatively wide spectrum and does more damage in the higher frequency region than in the lower frequency region [41,46–48]. The frequencies we have measured represent a large area in the basilar membrane, sufficient to demonstrate the damage and the subsequent recovery, and to rule out any site-specific variability.

Some evidence suggests hair cell apoptosis resulting from continuous and/or low-level noise is likely caused by metabolic dysfunction [49], while damage to the basilar membrane (and the organ of Corti) and Reissner's membrane by impulsive noise usually originates directly from mechanical trauma [41,46–48]. Thus, *Atoh1*-based gene therapy may not be suitable for the treatment of all types of noise-induced hearing loss. However, if *Atoh1* treatment can stabilize injured hair cells and directly or indirectly promote repair, such treatment may be effective for treating both noise- and drug-induced hearing loss. In the context of acoustic trauma, this could be especially important for the prevention/treatment of hearing impairment in the allied troops serving in Afghanistan, where the duration of their combat missions could be arranged based on a seven- to 10-day treatment window after exposure to impulsive weapon noise.

Author Contributions

Conceived and designed the experiments: DH SMY. Performed the experiments: SMY WC WWG SPJ JHS HZL WYY DH. Analyzed the data: WC WWG SPJ JHS HZL WYY DH. Wrote the paper: DH.

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