■ REVIEW

Intervention of Spiral Ligament Fibrocytes in the Metabolic Regulation of the Inner Ear

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Maintenance of the K^+ gradient between endolymph and perilymph is essential for normal hearing and depends primarily on the activity of the stria vascularis. Abundant Na-K-ATPase in marginal strial cells provides a pumping mechanism for preserving the K^+ level of the endolymph and consequently, the endocochlear potential. Fibrocytes in the lateral wall of the cochlea supply K^+ to the strial pump, via gap junctions, by recycling back into the stria the ions that efflux from the scala media during auditory transduction. The lateral wall of the cochlea encloses 5 types of fibrocytes, differentiated by their location, structural features, and content of enzymes mediating or energizing ion transport. The disruption of the gap junction bonds by connexin mutations and other pathologies leads to an interruption of K^+ recirculation pathways. The expression of cochlin and otoraplin, proteins that participate in structural or regulatory functions in the inner ear, suggests more diversity and complexity of the mesenchymal tissues than envisioned previously. The presence of otospiralin, a novel protein found in fibrocytes of spiral limbus, spiral ligament and subepithelial regions of the vestibule, represents a critical finding since that protein has been shown to be essential for the survival of the hair cells and supporting cells of the inner ear. A more profound knowledge and understanding of the function of inner ear fibrocytes will provide a new and promising aetiopathogenic approach to the treatment of inner ear disorders.

Key words: Fibrocyte. Gap junction. K^+. Hair cell. Deafness. Connexin. Cochlin. Otospiralin. Inner ear. DFNA9.

Intervención de los fibrocitos del ligamento espiral en la regulación metabólica del oído interno

El mantenimiento de un gradiente de K^+ entre la endolinfa y la perilinfa es imprescindible para la audición normal y depende inicialmente de la actividad de la estría vascular. La presencia de abundante Na-K-ATPasa en las células marginales de la estría vascular proporciona un mecanismo de bombeo al objeto de preservar la cantidad de K^+ en la endolinfa y, consecuentemente, el potencial endococlear. Los fibrocitos de la pared lateral coclear suministran K^+ a la estría, vía *gap junctions*, mediante la recirculación hacia la estría de los iones que fluyen desde la escala media durante la transducción auditiva. La pared lateral de la cóclea contiene cinco tipos de fibrocitos, que se diferencian según su localización, sus características estructurales y su contenido de enzimas que median o facilitan la energía para el transporte iónico. La rotura de las uniones como los puentes celulares por mutaciones de conexinas y otras condiciones patológicas conduce al bloqueo de las vías de recirculación de K^+. La expresión de coclina y otorraplina, proteínas que intervienen en funciones estructurales o reguladoras del oído interno, indica una diversidad y una complejidad de los tejidos mesenquimales mayores que lo imaginado previamente. La presencia de otospiralina, una proteína novedosa encontrada en los fibrocitos del limbo espiral, el ligamento espiral y las regiones subepiteliales del vestíbulo, es un hallazgo muy importante, ya que dicha proteína se ha mostrado esencial para la supervivencia de las células ciliadas y las células de sostén del oído interno. Conocer y entender mejor la función de los fibrocitos proporcionará un nuevo y prometedor abordaje etiopatogénico para el tratamiento de las enfermedades del oído interno.

Palabras clave: Fibrocito. *Gap junction*. K^+. Célula ciliada. Sordera. Conexina. Coclina. Otospiralina. Oído interno. DFNA9

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INTRODUCTION

The cochlea contains 2 main compartments filled with fluids called endolymph and perilymph. The perilymphatic spaces (*scala vestibuli* and *scala tympani*) contain a solution which is rich in Na^+ and poor in K^+. By contrast, the ionic composition of the endolymph contains a large amount of

K^+ ions and low concentration of Na^+ ions. In addition, the endolymph has a positive potential of 80-100 mV. Traditionally it has been postulated that the *stria vascularis* produces the endolymph and endocochlear potential essential for the transduction of sound by the ciliate cells of the Corti organ. The intercellular space located in the *stria vascularis* or intrastrial space is unique because it is isolated from the perilymph and endolymph by 2 layers of cells, marginal and basal, connected by tight junctions. It has been also proposed that the ionic conditions of the intrastrial space are essential for the generation of the endocochlear potential.[1,2] Two types of cell membranes have been described as limiting the intrastrial space: the basolateral membrane of the marginal cells and the syncytium, consisting of intermediate cells, basal cells, fibrocytes of the spiral ligament and cells of the capillaries, all linked by gap junctions[2] (Figure 1).

The integrity of the intercellular junctions can be broken under many conditions, pathological or otherwise, and this interrupts ion recirculation and, consequently, the modification of the composition of endolabyrinthine fluids and the endocochlear potential. This metabolic disorder leads to a disturbance of the homeostasis of the inner ear, preventing the proper functioning of the sensory cells, the ciliate cells, in the transduction of the sound message, resulting in a hearing loss which may be reversible or not depending on the noxa involved, the time of action of the causative agent and the intensity of the metabolic alterations triggered.

The aim of the present work is to review the role of fibrocytes in the metabolic events involved in the maintenance of the homeostasis of the inner ear, as well as highlighting the role of these cells in the pathogenesis of various diseases of the cochleovestibular organ.

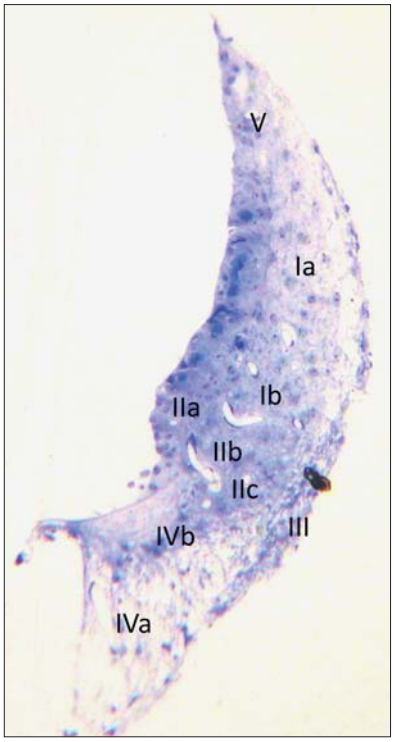

Figure 1. Semifine 0.5 Mm optical microscope slice of the lateral wall of a guinea pig cochlea, showing the location of different types of fibrocytes (toluidine blue, ×10).

RECIRCULATION OF K^+ THROUGH GAP JUNCTION SYSTEMS

When the ciliate cells are activated by mechanical stimuli, the K^+ ions of the endolymphatic space enter the cytoplasm of ciliate cells. Subsequently, these ions are expelled by the basal and lateral regions, and move back towards the endolymph.

The gap junction type of intercellular junctions have channels that allow the passage of molecules smaller than 1000 Da between the cells. These channels are closely grouped and are called connexons, which are hemichannels which join with their counterparts in the plasma membrane of adjacent cells. Each connexon is a hexamer of a protein called connexin. The location of various connexins in the cochlea of mammals has enabled the establishment of 2 independent gap junction systems, the epithelial system and the connective system. The epithelial system, composed by the interdental cells of the spiral lamina, the cells supporting the Corti organ and cells in the roots of the lower region of the spiral ligament, is involved in the recirculation of K^+ through the ciliate cells during mechanosensory transduction process. However, the connective system, composed by various fibrocytes of the spiral ligament (Figure 1) and the suprastrial region, intermediate and basal cells of the *stria vascularis*, mesenchymal cells limiting the *scala vestibuli*, the supralimbic dark cells, and the fibrocytes of the spiral lamina, is involved in the maintenance of the endolymphatic potential. At least 4 types of connexins have been described in the cochlea of mammals: connexin 26,[3] connexin 30[3] (Figure 2), connexin 31,[4] and connexion 43.[3] The mutation of connexin genes, which causes hereditary non-syndromic deafness, could be justified by the disruption of the pathways for ion recirculation of the gap junctions.

The presence of Na-K-ATPase[5] and a co-transporter of Na-K-Cl (NKCC1)[6] in the basolateral membrane of marginal cells of the *stria vascularis* and the type II fibrocytes of the spiral ligament and fibrocytes of the suprastrial region supports the assumption that the production of endolymph, the endocochlear potential generation and the recirculation of K^+ are located in these cells of the cochlear lateral wall.[7] The role of Na-K-ATPase consists in the uptake of K^+ from the intrastrial space and in maintaining a low intracellular concentration of Na^+. The transport of Na^+ from perilymph into the intrastrial space via marginal cells of the *stria vascularis* or fibrocytes of the spiral ligament, could be altered by the action of some drugs, which would allow this ion to accumulate in the intrastrial space, thus triggering a reduction of the endocochlear potential and a greater flow of Na^+ from the perilymph towards the endolymph.[8]

Other enzymes involved in the transport of ions (carbonic anhydrase and creatine kinase) have also been described in the fibrocytes of the cochlear lateral wall, which have been classified into several types (I-V) depending on their location, orientation, and immune staining.[9]

The K^+ ions, located in the perilymph, captured by type II fibrocytes of the spiral ligament, move through the narrow,

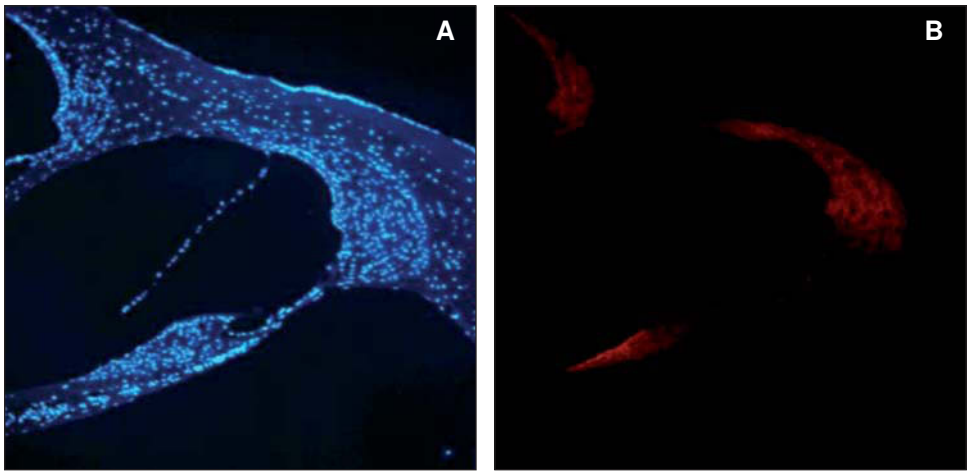

Figure 2. Expression of connexin 30 inside the cochlea of a Sprague-Dawley rat. A: the demonstration of nuclear DNA was obtained with DAPI (4,6-diamidino-2-phenylindole). B: there was an increase in the immune staining of connexin 30 (in red) in several cell populations of the spiral limbus and lateral wall (×200).

tight junction type barrier of the basal cells of the *stria vascularis* towards the intrastrial space via the gap junctions interconnecting the type II fibrocytes and the intermediate cells of the *stria vascularis*. The active uptake of K^+ by marginal cells from the intrastrial space can create a strong concentration gradient of K^+ ions between the intrastrial space and the intracellular space of the type II fibrocytes, which pulls the ions towards the intermediate cells, from where they are expelled into the intrastrial space. Once actively captured by the marginal cells, these expel them towards the endolymph, where they are available to the ciliate cells of the Corti organ.

ULTRASTRUCTURAL CHARACTERISTICS OF THE FIBROCYTES INVOLVED IN THE ION TRANSPORT

Five types of highly specialized fibrocytes have been described in the lateral wall of the cochlea, differing according to location, ultrastructure, and content of enzymes involved in ion transport (Figure 3). This could be summed up by stating that there are fibrocytes with a more structural role, types I, III and, IV, and functional fibrocytes, types II and V. Small nuances of morphological structure and location have justified the subdivision of some fibrocytes into various subpopulations (Figure 1). Thus, circumferentially oriented type III fibrocytes, which delimit the otic capsule, and spindle-shaped type IV fibrocytes, located lateral to the basilar membrane, pack the cochlear content and counteract the mechanical forces generated by sounds. Type I fibrocytes behind the *stria vascularis*, are closely packed by collagen fibres and mould the curvature of the lateral wall. Type II fibrocytes, below the *stria vascularis*, and type V fibrocytes, above the *stria vascularis*, are rich in mitochondria and have many extensions, indicating a very high metabolic activity. Fibrocytes of types I, II, and V and the intermediate and basal cells of the *stria vascularis* are interconnected by gap type junctions (Figure 4).

The presence of Na-K-ATPase in the plasma membrane of type II fibrocytes facilitates the flow of K^+ from the cells in the outer groove to the type I fibrocytes and subsequently to the marginal and basal cells of the *stria vascularis*. The large folds of the plasma membrane of type II fibrocytes increases the contact surface with the cells of the outer groove and the type I fibrocytes notably increase the availability of Na-K-ATPase. Moreover, the close contacts and gap junctions between the fibrocytes and between these cells and the marginal cells of the *stria vascularis* justify the transport of K^+ from the fibrocytes to this structure.

The type Ia fibrocytes, which contact extensively with the basal cells of the *stria vascularis* through gap junction type connections, have a membrane profile which appears to be a canalicular network, known as canalicular reticulum, which is infiltrated by numerous mitochondria. This structure allows the passage of K^+ through the cell and also the sequestering of ions from the cytosol, preventing their toxic effects, which does not happen in cells lacking this system such as those of Hensen, Claudius, and the internal groove. The presence of abundant mitochondria in proximity to the canalicular reticulum could indicate that they provide the energy required for ion transport. Some cells possess a thin Golgi apparatus, consisting of several small clusters of 5 short cisterns and/or aligned adjacent vesicles, which are located close to the canalicular reticulum.[10] The fibrocytes of type Ib are located below the Ia types and above and deep with respect to the IIb. They are different from the latter in that they have more mitochondria and a more abundant perinuclear cytosol, as well as contacting with elongated extensions of the lower pole of the type II fibrocytes.

Type IIb fibrocytes are located in the area of the spiral prominence. They exhibit structural polarity due to the existence of extensions and Na-K-ATPase in the superior pole of the elongated cell body, in close proximity to the cells of the outer groove, where they obtain the K^+ ions from the roots and release them into the inferior pole towards the type Ib fibrocytes through gap junctions. Some of the cells show a profile with an abundant canalicular reticulum and others present numerous vesicles.

The fibrocytes of types IV and V facilitate the flow of electrolytes towards the type I fibrocytes from the *scalae vestibuli* and *tympani* respectively, rather than from the organ

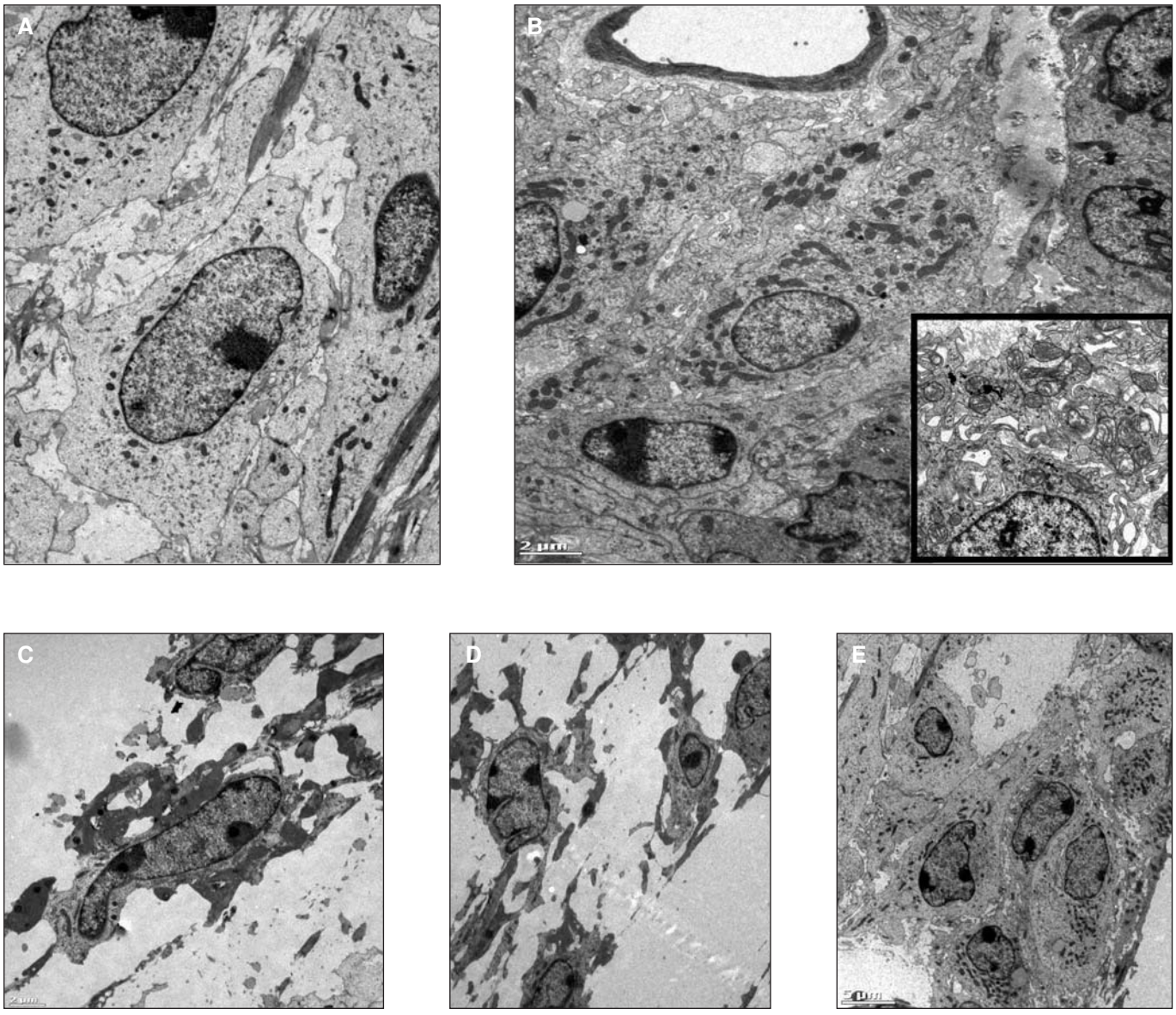

Figure 3. Photocomposition of transmission electron microscopy (TEM) of the lateral wall of a guinea pig. A: type I fibrocytes located in the middle portion of the spiral ligament; note the scarcity of mitochondria and cytoplasmic extensions (×5000). B: type II fibrocytes, with a notably large number of mitochondria and extensions (×6000); these are shown in the lower right rectangle at a greater enlargement (×12 000). C: type III fibrocytes (×6000). D: type IV fibrocytes (×4000). E: type V fibrocytes (×2500).

of Corti, and they also contain an extended canalicular reticulum.

The beams of the roots composed of the extensions of the cells of the outer groove extend into the region of the spiral prominence, populated by type IIb fibrocytes and dense stromal bands. The content of roots is highly variable regarding the number of mitochondria, canalicular reticulum and Golgi apparatus.

IMMUNE STAINING OF COCHLIN IN THE FIBROCYTES OF THE INNER EAR

Cochlear and vestibular fibrocytes are cells expressing the *COCH* gene, responsible for synthesis of the cochlin

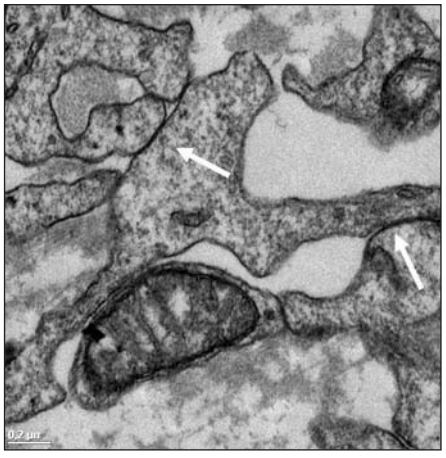

Figure 4. Gap junctions between type II fibrocytes (×60 000).

Elements Expressed in the Fibrocytes of the Inner Ear

Element	*Function*	*Disease*
Connexins 26, 30, 31, 43	Intercellular ion transport	Genetic deafness DFNA3 and DFNB1
Na-K-ATPase, Na-K-Cl co-transporter, carbonic anhydrase	Generation of the endocochlear potential, recirculation of K^+	Toxicity by cisplatin, sudden deafness, autoimmune hearing loss, presbyacusis
Pendrin	I-Cl transporter	Pendred syndrome
Otoraplin	Cochlear chondrogenesis	Structural malformation
Cochlin	Unknown	DFNA9 dominant autosomal deafness, autoimmune hearing loss, presbyacusis?, Ménière's disease?
Otospiralin	Survival of ciliate cells and support cells	Possible genetic hearing loss

protein. These cells are highly atrophied in autosomal dominant deafness (DFNA9) and present homogeneous acellular deposits in those areas where cochlin is expressed.[11] The *COCH* gene mutations have not only been implicated in DFNA9, but could also have a role in presbyacusis and in some vestibular diseases. Moreover, cochlin has been considered one of the antigens causing autoimmune deafness. A proteomic analysis has identified cochlin as one of the most abundant proteins in the inner ear.[12] The accumulation of mutated cochlin exerts a toxic effect that causes the reduction and degeneration of fibrocytes and their replacement by eosinophilic aggregates in the spiral ligament and spiral limbus,[13] at the level of the K^+ recirculation pathways from the epithelial cells of the Corti organ towards the endolymph of the middle chamber (*scala media*), implies a disruption of the integrity of the gap junction network that normally exists between these cells and is essential in the ion homeostasis necessary for a proper functioning of the ciliate cells.

THE OTOSPIRALIN PRODUCED BY THE FIBROCYTES OF THE INNER EAR IS ESSENTIAL FOR THE SURVIVAL OF THE CILIATE CELLS

In this review it has been amply shown that the proper functioning of the ciliate cells depends on the ionic composition of the endolabyrinthine fluids, endolymph and perilymph. Thus, the structures involved in ionic balance, the *stria vascularis* in the cochlea and the dark cells in the vestibule, which produce endolymph rich in K, require non-sensorial mesenchymal regions, the spiral limbus and spiral ligament in the cochlea and the stroma inferior to the sensory epithelium in the vestibule, to maintain the homeostasis of the inner ear. This assertion is supported by the expression in fibrocytes of Na-K-ATPase, carbonic anhydrases II and III,[14,15] several channels and transporters,[16,17] proteins of the extracellular matrix,[18,19] and regulatory molecules.[20] The finding that some fibrocytes also express connexins 26, 30, and 31[3,4] and the I-Cl transporter pendrin[21] is a further indication of its importance in the movement of fluids and the flow of ions in the inner ear (Table).

Other proteins like otoraplin, which could induce chondrogenesis in the cochlea during development,[22] and cochlin[11] could participate in regulatory or structural roles in the inner ear, which would suggest more diversity and complexity of the mesenchymal tissues.

However, the discovery of a hitherto unknown 6.4 kDa protein called otospiralin, secreted by the fibrocytes of the spiral limbus, the spiral ligament and the subepithelial regions of the vestibule (macula and semicircular canals), offers a valuable contribution to the knowledge of the homeostasis of ciliate cells. The transient blockade of the synthesis of otospiralin in guinea pigs produces vestibular dysfunction and irreversible deafness, justified by the degeneration of the ciliate cells.[23] This degeneration follows a pattern whereby there is greater involvement of external ciliate cells and support cells than of the internal ciliate cells. The absence of otospiralin could alter fibrocytes, induce the appearance of vacuoles and modify important factors for the ciliate cells and/or ion homeostasis. The development of an animal model presenting a deletion in the *Otos* gene, which encodes for otospiralin, produces a moderate hearing loss and a degeneration of fibrocytes II and IV, thus indicating that this moderate dysfunction may precede the onset of presbyacusis.[24]

In conclusion, the involvement of non-sensorial mesenchymal structures, in the homeostasis of the inner ear represents a significant advance in the understanding of cochlear physiology, as these cellular elements had traditionally been attributed a merely mechanical role, of structural support. Understanding the importance of the ion flows responsible for the smooth functioning of sensorial cells could allow the development of therapies specifically aimed at these metabolic abnormalities. The early implementation of these treatments, examples of which may be in sudden deafness or autoimmune hearing loss,[25] has proven very effective and it may have therapeutic targets in the fibrocytes of the lateral wall and the spiral limbus. Furthermore, these cells are the initial target of ototoxic drugs such as cisplatin.[26] Other causes of cochleovestibular dysfunction such as DFNA9, some variants of Ménière's disease and presbyacusis, may be due to the aggression suffered by the fibrocytes of the inner ear.

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