Original Article

DEVELOPMENT OF CYTOSKELETON IN PILLAR CELLS OF THE ORGAN OF CORTI

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Summary

We examined the morphological maturation of the outer and inner pillar cells, specialized supporting cells, in the organ of Corti. Three stages were defined, during which pillar cells exhibited different patterns in shape, cell organelle arrangement, intercellular junctions, development of secondary acentriolar microtubule organizing centre (MTOC), as well as reorganization of cytoskeleton. The most prominent changes were found occurring during the second period (7-8 day after birth), when the cells generated phalangeal processes, accompanied by translocation of the centrosomal MTOC to the phalangeal apex and rearrangement of microtubule bundles. There were indications that MTOCs acted in concert during microtubule positioning. Fibrous meshwork that was not positioned at major MTOC sites was involved in connecting a microtubule bundle to a cytoskeleton. This concentration of specialized cytoskeletal materials and junctions at the cell surface has been detected as surfoskelosome. During the development of the pillar cells the following events were registered: stage I- nucleation of microtubules; stage II – elongation of mictrotubules downwards and capture by a medial MTOC at the mid-level; stage III – translocation of mictrotubules to the basal end and extension back up to the medial MTOC, and final establishment of the two microtubule bundles.

Key words: organ of Corti, development of pillar cells, cytoskeleton, microtubule organizing centre

Introduction

Pillar cells in the mammalian organ of Corti are specialized supporting cells which surround the tunnel of Corti and provide mechanical support to neighboring cells by transmitting mechanical vibrations from the basilar membrane to hair cells. Pillar cells are characterized by their highly organized cytoskeletal complex, composed of several thousand microtubules, and a perfect organization of intercellular junctions [1, 2]. The centrosomes play an important role in the development of this microtubular cytoskeleton. They are associated with the apical portion of the plasma membrane and serve as microtubule-organizing centers (MTOCs), which control the growth of a cytoskeleton with a construction most suitable for each cell. In an artificial medium, microtubules are labile polymer structures with plus

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and minus ends, involved in a continuous process of assembling and disassembling of α- and β-tubule dimers. In the living cell, however, under the control of MTOCs, microtubules in some cytoplasmic regions initiate microtubule nucleation, and in other regions they suppress them [3]. In pillar cells, however, microtubules are characterized with a unique structure. They are composed of 15 protofilaments of tyrosinated and acetylated tubulin isoforms, which ensure stability by providing structural support for the entire construction [4-7]. The pillar cell cytoskeleton also contains cytokeratin intermediate filaments, which anchor microtubules to the cellular surface in the intercellular junctional zone. Intercellular junctions in the zone between the heads of the inner and outer pillar cells are characterized with extensively developed adherent junctions, which add mechanical strength to the construction. During the morphogenesis of the organ of Corti, this complex pillar cell cytoskeleton participates in the overall development of the organ by way of successive formation of different MTOCs, and gives asymmetric design to the inner pillar cells. These centers accomplish supracellular coordination by controlling the initiation, packing, condensation, and attachment of the pillar cells microtubular cytoskeleton to the cytoskeletal elements of neighboring cells. Thus the overall cytoskeletal construction of the organ of Corti develops to position cell populations in a perfect manner, and its mechanical characteristics effect the hearing process [8-11].

Material and methods

Thirty-two Wistar rats were used, divided into 9 age groups: new-born, 2, 4, 6, 8, 10, 12, 14 and 16 days old. The experiment was approved by the Animal Care and Use of Laboratory Animals section of the Ethical Committee of the University, based on the principles described in the Guide for the Care and Use of Laboratory Animals [15].

Transcardiac perfusion with a mixture of 2% glutaraldehyde, 1% paraformaldehyde and 0.015% $CaC1_2$, in 0.1 M cacodylate buffer (pH 7.2-7.4) was performed under anesthesia. Cochleas were removed and postfixed for 2 h in the same solution. The material was finally fixed n 1% OsO_4 for 1 h, and was then embedded in Durcupan. Ultrathin sections were double-contrasted with 4% uranylacetate and 0.1% lead citrate, and observed with a Tesla 500 BS electron microscope.

Results

In newborn rats, the inner and outer pillar cells were differentiated, but they possessed many features typical of immature cells. They were characterized with a prismatic shape with a base situated on the basilar membrane above the spiral vessel. Their tips, covered with microvilli and a primary kinocilium, reached the endolymphatic surface of the Kölliker's organ. Pillar cells, tightly squeezed against neighboring cells, sealed tiny intercellular spaces isolated from the endolymph by adhesion junctions. The cytoplasmic organization of pillar cells resulted in polar distribution of organelles. The basal end was occupied by a large amount of glycogen and an oval nucleus with smooth delineated contour and uniformly distributed fine chromatin. The apical end contained mitochondria, rough endoplasmic reticular cisternae, Golgi apparatus and vesicles. At the base of the primary kinocilium a basal body was seen, and a centriole coated by an insignificant amount of amorphous fibrillar material that connected them to the apical portion of the cytolemma. Profiles of single microtubules were detected in this pericentriolar material (Fig. 1).

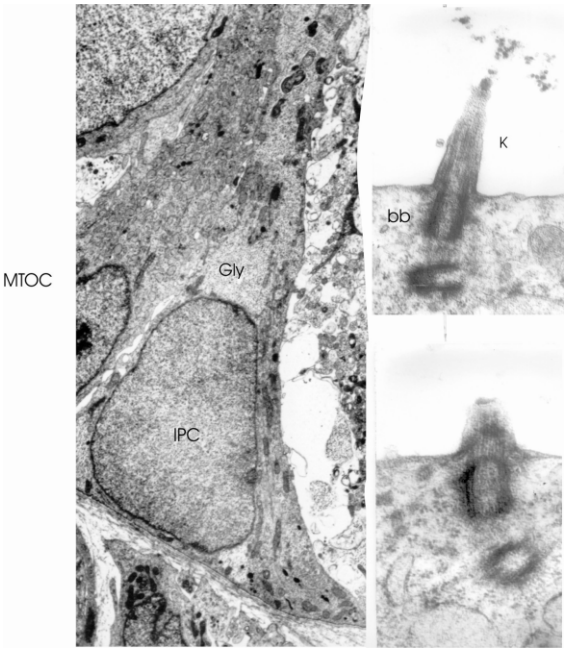

Fig. 1. Pillar cells in newborn rats (Panel A; x 2500) and primary kinocilium (Panel B; x 15000)
MTOC - microtubule-organizing center
IPC – inner pillar cell; Gly – glycogen; K – kinocilium
bb – basal body

Between postnatal days 1 and 5, the pillar cells remained roughly prismatic in shape but the lateral surface changed with the appearance of multiple villus-like protrusions. This process was accompanied by medial narrowing of cell bodies and broadening of extracellular spaces. Hence, the concavities of both the tunnel of Corti and Nuel's space were formed. Flocculent material and cytoplasmic processes were detected in the lymph filling these spaces. Opening up began from the cochlear basal turn and advanced to the apex. In most cases, kinocilium was resorbed, and the pericentriolar material in the cells increased. The released basal body transformed into a second centriole, and together with the pericentriolar material formed a common structure – centrosomal microtubule-organizing center (MTOC). Microtubules predominated among cell organelles. They were thousands in number, with one end oriented to the centrosomal cell region, while the other end did not reach the basal parts. Between day 1 and 5, the glycogen reserve progressively decreased, and then disappeared by the end of the period (Fig. 2, Fig. 3, Fig. 4).

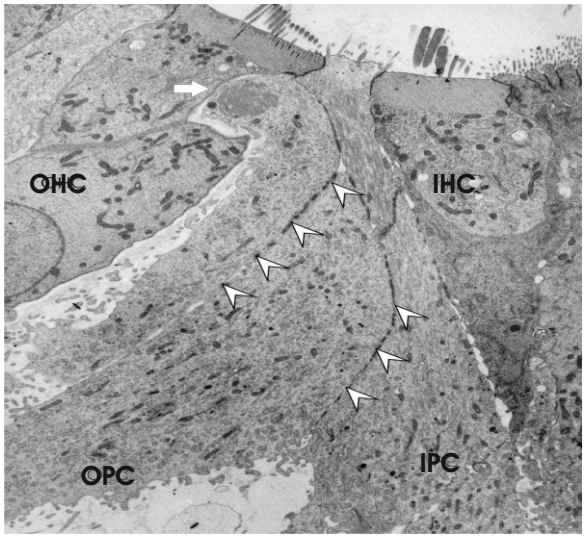

Fig. 2. Opening of both the tunnel of Corti and Nuel` space. Arrowheads point the intercellular junctions. Appearance of microvilli and microtubules. (x 2500)
OHC – outer hair cell
IHC – inner hair cell
OPC – outer pillar cell
IPC – inner pillar cell

During the period of days 6-8 after birth inner and outer pillar cells acquired an irregular asymmetric shape. The apical ends of both types of cells began to grow in a lateral direction toward

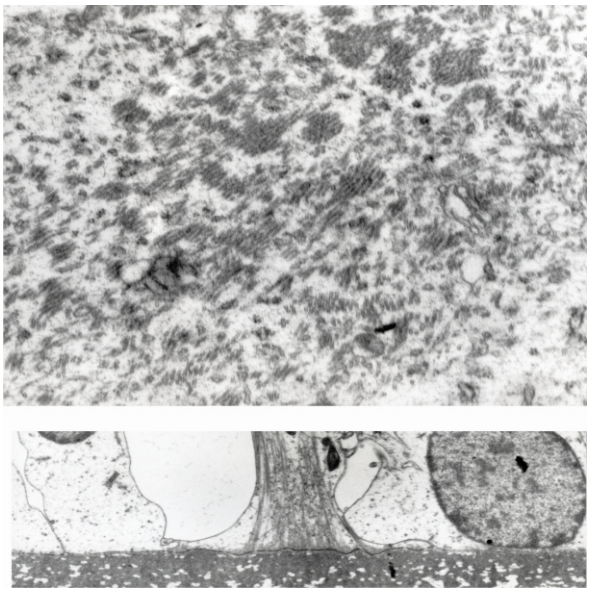

Fig. 3. Above: Irregularly orientated microtubules in the middle portion of the cell body (x 10 000).
Below: Anchored lower portion of the microtubules (x 4 800)

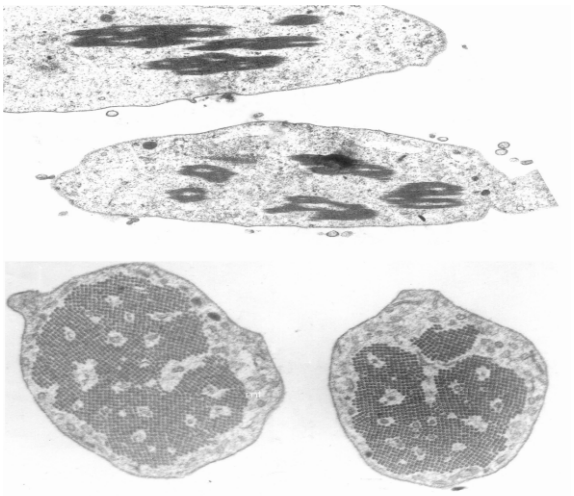

Fig. 4. Transverse section of microtubule bundles (x 10000)
Above: 4th day after birth
Below: 10th day after birth

the outer hair cells, forming phalangeal processes. The phalangeal processes of the inner pillar cells occupied the space between the cuticules of the inner and outer hair cells in the first row. They resembled quadrangular plates which widened the distance between them. Phalangeal processes of the outer pillar cells occupied the space between the cuticules of the outer hair cells in the first and second row. Membranes of contact surface areas possessed multiple adhesion junctions. Maturation of cells

in the apical region resulted in the reduction of microvilli and reorganization of the centrosomal microtubule-organizing center. In inner pillar cells, MTOC moved laterally to the apex of the growing phallangeal processes. Translocation of this center pulls the apical ends of a part of the formed microtubules. Another part of the microtubules escaped from the centrosomal MTOC and migrated to the basal portion of the plasma membrane, where they anchored their basal ends. In this way, the transcellular and basal arrays were formed. Initially, the arrays appeared loose, structured in single or grouped microtubules, and cytosol filled the space between them. The transcellular array, based along the cell inner side, was bent between the body and the phalangeal process. Its microtubules were densely packed unlike those of the basal array, which tended to progressively decrease in number in a distal direction. The basal array was shorter, loosely structured and possessing relatively fewer microtubules. It was situated laterally and parallelly to the transcellular array between the cell base and the adhesion junctions of the head. In outer pillar cells, the amorphous material around the centrioles formed two clumps – outer and inner. The outer clump occupied the space in which the phalangeal processes grew. The inner clump was located on the opposite side in the region of the head. Separation of the pericentriolar material split the microtubules into two bundles, perpendicular to each other. Microtubules in one bundle extended along the phalangeal process, parallel to the endolymphatic surface of the organ of Corti, attached to its tip near the adhesion junction, known as phalangeal. Microtubules in the other bundle were parallel to the cell apicobasal axis. This is called a pillar bundle. Initially, the phalangeal and pillar bundles were composed of a small number of microtubules arranged in groups which tended to increase in number and occupied the space between them. The stages of compactization and aggregation of microtubules were mostly notable in the middle areas of the spindles, and toward the end of the period they dispersed in a fan-shape manner (Fig. 5).

Between days 9 and 12 after birth, maturation changes in pillar cells were characterized by narrowing of the cell bodies, particularly in the middle portions. As they moved away from neighboring cells, they opened up the broad intercellular Corti's and Nuel's spaces.

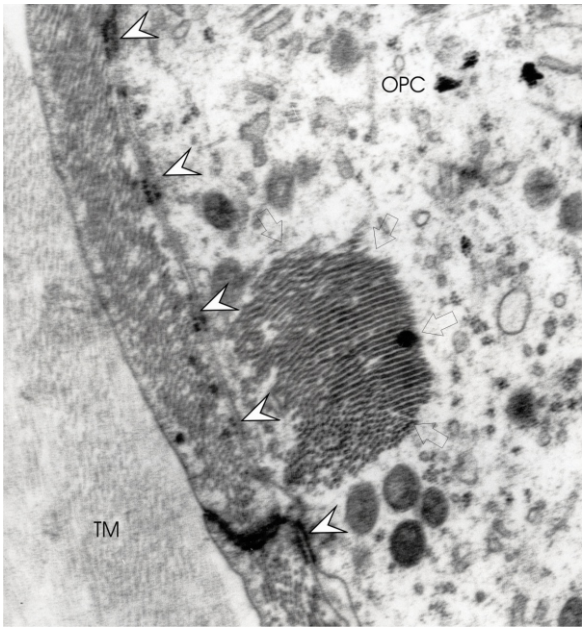

Fig. 5. Transcellular and phalangeal microtubule bundles (x 14000)
OPC – outer pillar cell
TM – tectorial membrane
Arrow heads – intercellular junctions
Arrow – phalangeal microtubule bundle

Throughout the period, cytoplasmic maturation was characterized by disappearance of both centrioles by the end of the period, and they could not be detected even in the apical portions of the cochlea. Microtubule assembly and elongation continued. The bundles seemed more compact and structured in a larger number of microtubules with their transverse profiles resembling a reticular structure. In comparison, the other cell organelles seemed smaller in size and number. A characteristic change in the pillar cells cytoplasmic organization was the development of a fibrous meshwork of specialized cytoskeletal material associated with the intercellular junctions, known as surfoskelosome. These cellular skeleton elements connected the ends of the microtubular bundles to the adhesion junctions. Surfoskelosomes had an amorphous fibrillar structure and nonhomogeneous density, identical to that of the pericentriolar material. During development, there was an increase in the distance that separated the microtubule ends and the plasma membrane, which permitted interpolation of the surfoskelosomal material between them. The amorphous material appeared as tufts which quickly increased in size and fused. Microtubule ends penetrated only the superficial, loose layers of the surfoskelosome but they were

not detected in the denser parts. Inner pillar cells formed apical, phalangeal, medial and basal surfoskelosomes. The apical surfoskelosome was attached to the junctions with inner hair cells. Inside, the ends of a few microtubules were established but it was hard to determine whether they terminated there, or just passed through. The phalangeal surfoskelosome, associated with the outer hair cell junctions, was attached to the apical end of the transcellular array. The medial surfoskelosome was connected with the adhesion junctions in the zone of the head, and the apical end of a pillar array was anchored to it. The basal surfoskelosome, also known as the basal cone, was very well expressed. It could be compared to a focal junction with a densely structured surfoskelosomal substance in the base and a loose tip. It served for attachment of the basal ends of the transcellular and pillar arrays to the basilar membrane. Outer pillar cells formed phalangeal, apical, and basal surfoskelosomes. The phalangeal surfoskelosome joined the phalangeal bundle's distal end to the plasma membrane of the phalangeal process. This bundle's other end and the pillar array were included in the apical surfoskelosome situated along the transition from the body to the phalangeal process. The basal surfoskelosome, located at the cell base, was also responsible for the attachment of the pillar array to the basilar membrane (Fig. 6, Fig. 7, Fig. 8).

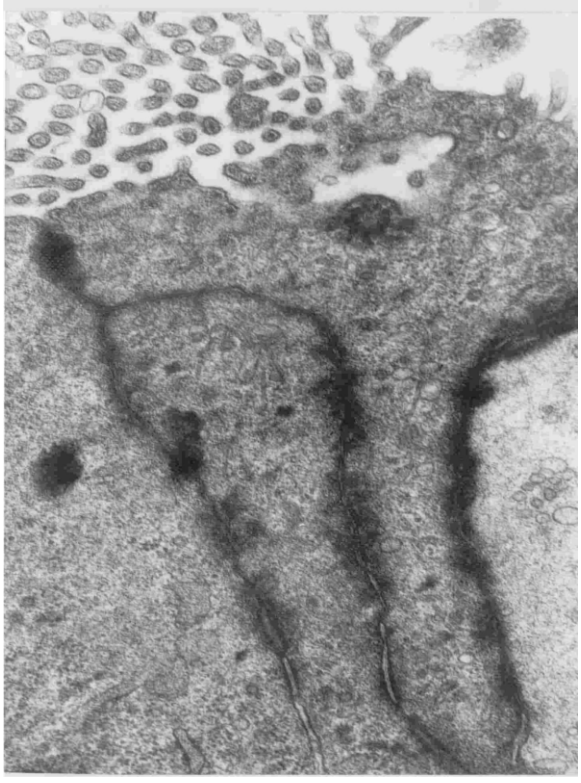

Fig. 6. Phalangeal process containing one of the migrated centrioles of existing cytocentre (x 8 600)

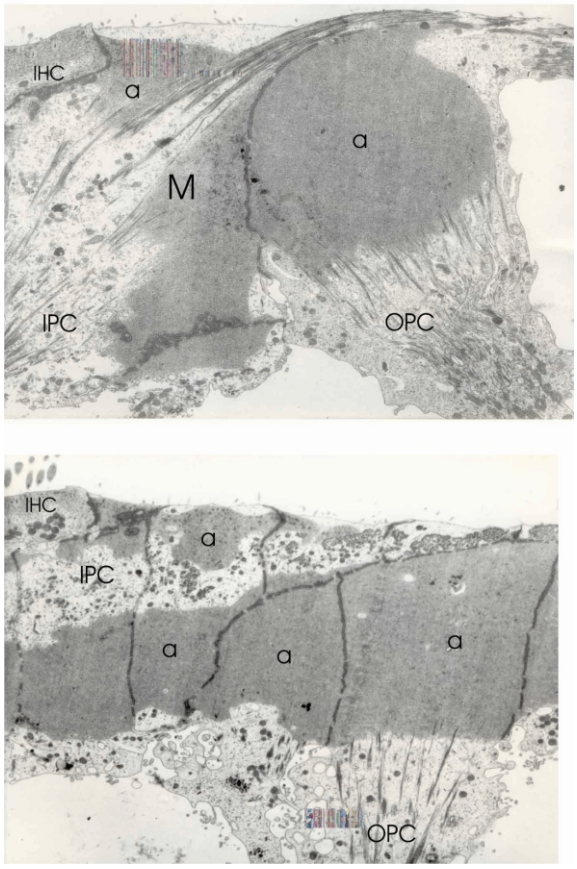

Fig. 7. Surfoskelosomes (x 2 800)
IHC – inner hair cells
M – middle surfoskelosome a (apical surfoskelosome)
IPC – inner pillar cells
OPC – outer pillar cells

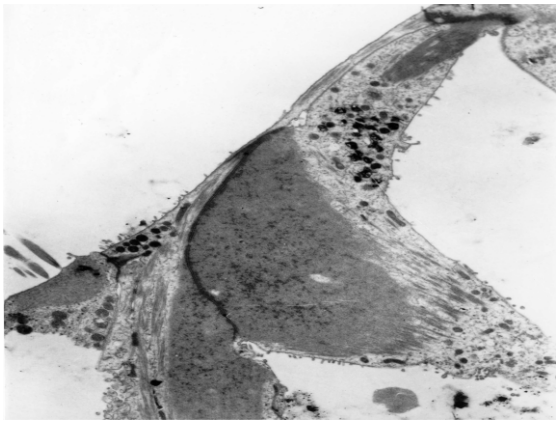

Fig. 8. Heads and phalangeal process of the pillar cells with surfoskelosome and microtubular bundles (x 4 000)

After postnatal day 12, morphological changes in the pillar cells were complete, and we believe they had completed their development.

Discussion

Maturation of pillar cells differs from that of other cells in the organ of Corti in that they undergo metamorphosis in the postnatal period, characterized by change in the shape and cell organelle arrangement. We observed three distinct stages in the morphological maturation of the pillar cells in the organ of Corti.

During the first stage (day 1-6 after birth), the cells exhibited active synthetic activity and developed an extensive microtubular cytoskeleton, the centrosome playing the most active role as the main site for microtubule nucleation. It performed MTOC functions where the most dynamic changes occurred. Up to day 6 after birth, the centrosomal MTOC consisted of centrioles orthogonally positioned to each other, enveloped by dense, partially filamentous material which bound the centrosomes to the apical plasma membrane.

Centrosomal MTOC migration from the central position near the nucleus and the formation of new links with other cell components has been observed in differentiating muscle, nerve, and epithelial cells [12-15]. In these cases of deep reorganization of the centrosomal MTOC, unforeseen mechanisms have been discovered that affect the microtubular cytoskeletal construction. The apical position of the centrosomal MTOC in pillar cells orients microtubules along the transverse cell axis, and emphasizes their polarity. Centriole formation in these cells occurs in a strange manner, contradictory to the known one. One of the centrioles originates from the already existing basal body of the primary kinocilium. Pericentriolar satellite bodies are not detected until completion of differentiation. It is believed that these bodies have relation to the intensity of microtubule nucleation, because they are always found during the interphase of dividing cells, and are absent during the mitotic phase of the cell cycle when microtubule production is most intensive [7, 16].

We observed pericentriolar satellite bodies in our specimens after postnatal day 20, when the nucleation activity was low.

Data from literature suggest that these bodies contain concentrated and relatively inactive centrosomal components that perform microtubule nucleation. Pericentriolar material plays a leading role in nucleation of microtubules and can individually organize microtubular bundles. It has an amorphous ultrastructure, and immunohistochemical methods indicate that it contains over 200 structural and regulatory proteins. Identification and specificity of these proteins are important for understanding the MTOC organization and the microtubule nucleation mechanism. The main protein, pericentrin, detected in centrosomal and non-centrosomal MTOC, participates in the assembly of α-, β-, γ-tubulins, capping-proteins and pericentrin-like material [17]. It serves as a scaffold for embedding other proteins in the MTOC structure. Pericentrin is a conservative protein which has been discovered in the centrioles of all mammals (rats, hamsters, mouse, humans, monkey) studied up to this day, as well as in the centrosomes of the drosophila and Negleria gruberi [17, 18]. Immunohistochemical tests reveal that the pericentrin model of organization is variable. It is compact at the time of the mitotic spindle, and disperses when the mitotic spindle disintegrates during telophase. Experiments with antipericentrin antibodies reveal that pericentrin has an effect on the nucleation, but not on the elongation of microtubules. Pericentrin is always found with γ-tubulin. It seems that pericentrin and γ-tubulin are required for the microtubule nucleation. Both proteins are highly conservative, located along the MTOC inner side [3, 8, 17]. Microtubules grow from the γ-tubulin rings located inside the MTOCs, which serve as nucleating sites for each individual microtubule. They assemble heterodimers of the α- and β-tubulins under the control of a capping-proteins system mediated by GTP and Mg^{++}. The microtubules thus formed have the same polarity, and their minus ends remain part of the centrosomal MTOCs. During the stage when the centrosomal MTOC of the pillar cells is active, an increase in the pericentriolar material and surplus of microtubules have been recorded [2, 19-22].

During the second stage (days 7-8 after birth) the pillar cells in the white rat cochlear drastically change in shape. They generate phalangeal processes, which increase the distance separating the inner hair cells row from the first row of the outer hair cells. Nuel's and Corti's spaces grow until they reach a size typical for the mature structure. Undoubtedly, the precise specification of these parameters determines the functional features of cochlear design. Growth and elongation of the phalangeal process are

controlled by paracrine signals from neighboring cells. Bronx waltzer mice have no inner hair cells, and even though the necessary cytoskeletal hardware is present, phalangeal processes are strongly reduced. [23, 24]. Simultaneously with the formation of phalangeal processes, there occurs a rearrangement of microtubules and formation of bundles. Translocation of the centrosomal MTOC to the phalangeal apex is an important part of this process. Movement occurs in a lateral direction at about 10-12 μm between postnatal days 6 and 8. No explanation for the lateral movement exists, but it could be assumed that in the cellular cortex of the growing phalangeal process, anchoring protein complexes (dynein, dynactin, etc.) are embedded and actively capture the plus ends of the newly formed microtubules. These anchoring protein complexes exert traction forces on the centrosomal MTOC and move it in a lateral direction, similar to fibroblasts which always have the cytocenter between the nucleus and the leading pseudopodium [2, 20, 25, 26]. Translocated centrosomal MTOC is regarded primarily as a site for attachment of the microtubular spindle to the cell surface rather than a site for nucleation of new microtubules. Immunohistochemical studies show that the accumulation of actin, intermediary fibers, and cytokeratin reorganize the centrosomal MTOC for mechanical purposes [3, 7]. The lateral translocation of the centrosomal MTOC results in the reduction of attractive forces, and splitting of part of the microtubules. At the same time, secondary acentriolar MTOCs develop that capture minus ends of the released microtubules. The consensus is that the centrosomal MTOC is a site where nucleation occurs and the entire amount of microtubules is produced. Elongation, bundle formation, and attachment of their ends are controlled by the secondary acentriolar MTOCs. The development of the secondary acentriolar MTOCs during the inner pillar maturation changes the microtubule arrangement and cell shape. It seems that secondary MTOCs act to bind microtubules by controlling their number and position by forming microtubular bundles. Despite differences in the cytoskeletal organization of the outer and inner pillar cells, it has been discovered that each type of pillar cells possesses two bundles, of which one is connected with the centrioles and the other is isolated from them. Therefore, a common cell control mechanism exists with regard to the cytoskeleton. Selection criteria are not clear as to which microtubules remain in the centrosomal MTOCs, and which migrate to the other MTOCs because the centres have lost their control function. During the stage of centrosomal MTOC activity, a marked surplus of microtubules is present, a cause for which could be:

Firstly, it is known that microtubules are dynamic and unstable structures, and therefore larger numbers of these are needed to form microtubular bundles, even after depolarization of a certain amount of microtubules.

Secondly, larger numbers of microtubules are involved in conformational changes in the cell under the influence of forces, generated by the spindles with a larger number of tubular elements. After maturation, a part of them become inactive and are eliminated.

Thirdly, larger numbers of microtubules are distributed among the spindles without depolarization of any of their parts.

During the third stage (days 9-12 after birth) inner pillar cells maturation marks the completion of microtubular assembly. After development surface-associated cytoskeletal bodies connect the ends of the microtubular bundles to one or more cell junctions. These structures are called surfoskelosomes. They are located in the areas of the phalangeal processes, heads and cell bases. Even though there are marked differences in the shape and size, the ultrastructure of all surfoskelosomes is similar. They are composed of fibrous material tufts, attached to the plasma membrane and its intercellular junctions. Pillar cells surfoskelosomes contain α- and β-isoforms of the nonmuscle actin and α-actinin. Their texture very much resembles the cuticular plates of hair cells, connected by intercellular junctions. Cuticular plates, however, have a different structure, composed of actin, α-actinin, fodrin, profilin, myosin, tropomyosin. Surfoskelosomes at the apical ends of the supporting cells and the cuticular plates of the hair cells together contribute to formation of a dense sturdy layer, called lamina reticularis [10-12, 21, 27-29]. The surfoskelosomes development gives the impression that fibrous material and adhesion junctions are interdependent throughout the maturation period. This relationship is important for the correct cell positioning and formation during the organ of Corti development. It is believed that the completion of this process is facilitated by the negative feedback received when the cytoskeletal junction with neighboring cells is successful [30, 31].

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