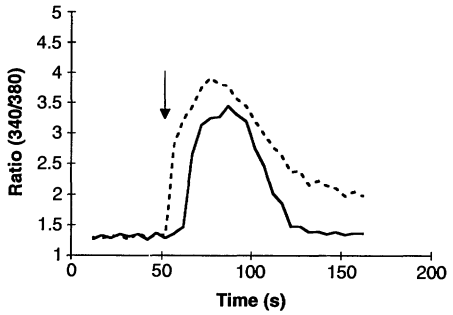

Fig. 4. Effect of glucagon on $[Ca^{2+}]_i$ in BHK cells expressing pLJ4. The ratio of emissions from excitation at two wavelengths (340 and 380 nm) is plotted versus time. The arrow indicates the time of addition of glucagon (100 nM final concentration). Representative traces for two different cells are shown. This experiment was repeated three times; at each time 6 to 40% of the cells in a field responded similarly. Similar responses were obtained with COS-7 cells expressing pLJ4 (*23*).

liver membrane preparations (*16*, *19*).

Glucagon causes an increase in the concentration of intracellular calcium ($[Ca^{2+}]_i$) (*4*, *21*) and small increases in the amount of inositol phosphates in hepatocytes (*2*, *3*). We monitored $[Ca^{2+}]_i$ in BHK cells transfected with pLJ4 with the fluorescent probe fura-2 (*22*). The addition of glucagon (100 nM) to BHK cells expressing the glucagon receptor caused a rapid increase in $[Ca^{2+}]_i$ (Fig. 4). Control cells expressing the β-adrenergic receptor showed no change in $[Ca^{2+}]_i$. Glucagon concentrations as low as 2 nM stimulated a similar increase in $[Ca^{2+}]_i$ (*23*). Glucagon (200 nM) also caused a rapid increase in $[Ca^{2+}]_i$ in COS-7 cells transfected with pLJ4, whereas COS-7 cells transfected with our vector, pZCEP, did not respond (*23*). These results indicate that activation of the cloned glucagon receptor leads to a rapid increase in $[Ca^{2+}]_i$, which is characteristic of receptors signaling via inositol phospholipid metabolites (*24*). Concentrations of intracellular Ca^{2+} and cAMP are both increased by signals from the related PTH (*5*) and CT receptors (*6*). It is possible that the alternate signaling pathways used by the glucagon receptor derive from its interaction with different G proteins, as previously suggested (*3*).

We have not detected a second glucagon receptor by expression cloning after screening 5×10^5 clones from our rat liver library, nor have we detected a different glucagon receptor by hybridization with pLJ4 as a probe. Thus, it is likely that only one receptor is responsible for both types of signaling observed in hepatocytes. We have also identified a human homolog (83% amino acid identity) of the rat hepatic receptor from human pancreatic islet cell cDNA (*25*). This suggests a role for the liver-derived receptor or a very similar receptor in the pancreatic islets as well.

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28. We thank T. W. Whitmore (ZymoGenetics) for advice and assistance.

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Ultrastructural Evidence for Hair Cell Regeneration in the Mammalian Inner Ear

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It has long been thought that hair cell loss from the inner ears of mammals is irreversible. This report presents scanning electron micrographs and thin sections of the utricles from the inner ears of guinea pigs that show that, after hair cell loss caused by treatment with the aminoglycoside gentamicin, hair cells reappeared. Four weeks after the end of treatment, a large number of cells with immature hair bundles in multiple stages of development could be identified in the utricle. Thin sections showed that lost type 1 hair cells were replaced by cells with a morphology similar to that of type 2 hair cells. These results indicate an unexpected capacity for hair cell regeneration in vivo in the mature mammalian inner ear.

The inner ears of vertebrates contain the organs for hearing and balance. Their mechanoreceptive auditory and vestibular epithelia are formed of mosaics of sensory "hair" and supporting cells. Hair cells can be damaged and lost after exposure to noxious agents including noise and ototoxic drugs such as aminoglycoside antibiotics. In cold-blooded animals, hair cells are continuously produced throughout life and damaged cells can be replaced (*1*). In birds,

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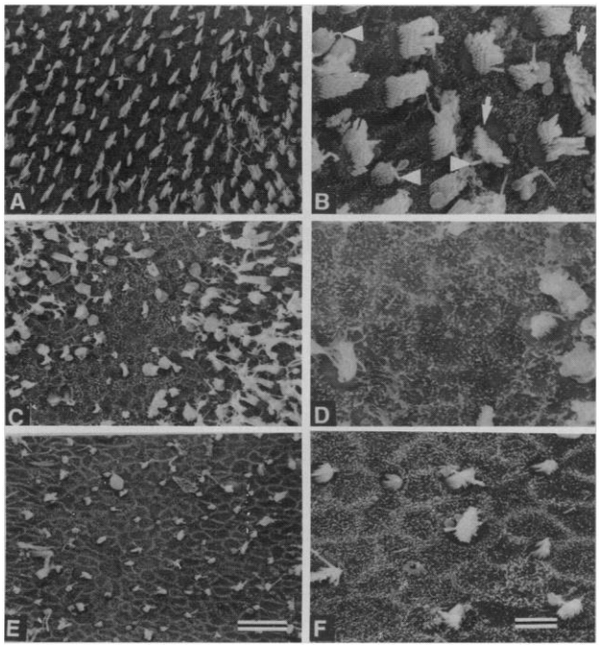

Fig. 1. Scanning electron micrographs of the striola of the utricular macula from a control animal (A and B) and at 4 days (C and D) and 4 weeks (E and F) after gentamicin treatment. (**A**) Low-power view of the normal macula. The hair cells, their bundles appearing white, are evenly distributed. The striola, down the center, can be distinguished because the hair bundles in this region are shorter than those on either side. (**B**) Normal morphology of the hair bundles at higher power. The stereocilia are organized in rows of increasing height toward the longer kinocilium (arrowheads). The field is to one side of the central line of the striola with most hair bundles oriented the same way, but the arrows indicate two bundles that are oriented in the opposite direction. (**C**) At 4 days after gentamicin treatment, hair cells have been lost from the striola and (**D**) most hair cells have been replaced by supporting cells. (**E**) At 4 weeks after treatment, the area that was affected by the drug is wider than that at 4 days, but small hair bundles reappearing across the striola are evident. (**F**) At higher power, cells with organized bundles of microvilli are clearly distinguishable from supporting cells. They show various stages of hair bundle development and emerge in locations where at 4 days supporting cells had replaced hair cells, and they reestablish a pattern of cell distribution similar to that seen in the control tissues. Scale bars: (A, C, and E), 20 μm; (B, D, and F), 5 μm.

after trauma-induced loss, hair cells reemerge and their number recovers through reinitiation of mitosis in the area of the lesion (*2*). However, it has generally been believed that mammalian hair cells are produced only during embryonic development and that they are not regenerated if lost from the mature mammalian inner ear (*3*). Permanent hearing impairment or balance dysfunction in humans who have been exposed to conditions that produce hair cell loss has contributed to this assumption.

The auditory epithelium in mammals has evolved separately from that in birds (*4*), and its structural organization differs from those of lower vertebrates. In contrast, the vestibular epithelia, present in the saccule, utricle, and the cristae of the three semicircular canals, are morphologically similar in all vertebrate classes. These similarities have led to the speculation that hair cell regeneration might take place in the mammalian vestibular system (*5*). Here we report morphological evidence showing that hair cells are replaced in the utricle of the mature guinea pig in vivo after gentamicin-induced hair cell loss. Warchol *et al.* (*6*) provide evidence that this probably occurs after reinitiation of mitosis.

Daily subcutaneous injections of gentamicin were given to guinea pigs (3 to 6 weeks old) for ten consecutive days (*7*). On the last day of treatment and at 2, 3, 4, and 7 days and then at 4 weeks after treatment, the inner ear tissues from gentamicin-treated animals and from untreated, age-matched guinea pigs were prepared for scanning electron microscopy (SEM) and transmission electron microscopy of thin sec-

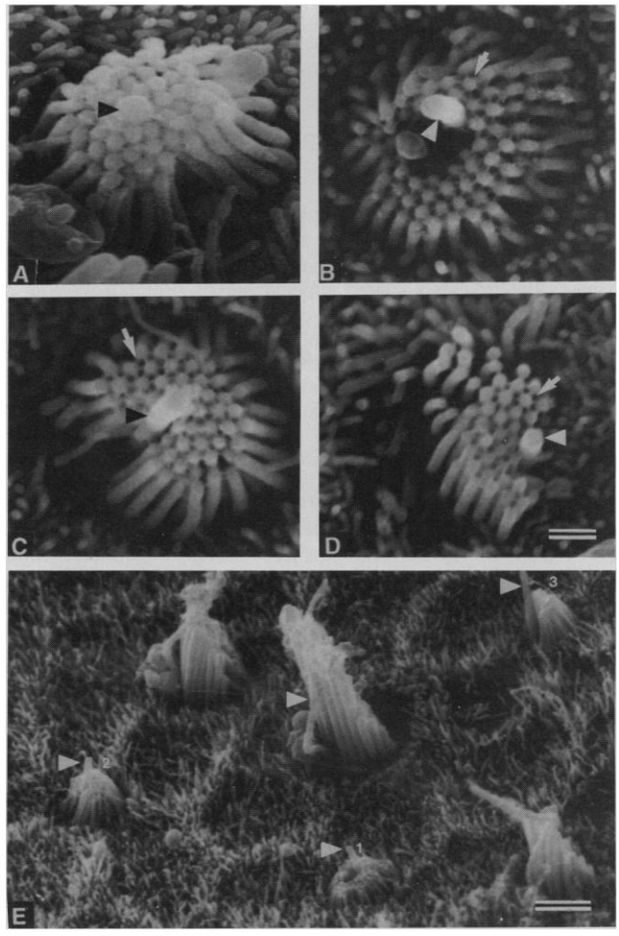

Fig. 2. Scanning electron micrographs of hair bundle morphologies in the region of reappearing hair cells at 4 weeks after gentamicin treatment. The bundles consist of immature stereocilia and a kinocilium. The kinocilium is distinguishable because it is thicker and longer than the stereocilia (arrowheads). (**A**) The kinocilium emerges from the center of the cell and is completely surrounded by the bundle of short stereocilia that are angled toward the cell center, that are closely apposed at their tips, and that cover the entire cell surface. (**B**) The cell surface has expanded relative to (A). The stereocilia are more regularly arranged and form a halo around a central space in which the kinocilium is located. Arrows indicate regularly arranged lateral cross-links between the tips of adjacent stereocilia that connect one stereocilium with six neighbors. (**C**) The kinocilium is close to the center of the cell surface but is positioned eccentrically relative to the stereociliary bundle. (**D**) The kinocilium is at one edge of the cell and behind the stereocilia. (**E**) Side view of hair bundles showing relative heights and orientations. A mature bundle in the center of the field is surrounded by three immature bundles (1, 2, and 3). Their stereocilia are thinner and shorter than those of the mature bundle. In the shortest (cell 1) the kinocilium is in the middle of the stereocilia. On cells 2 and 3 the stereocilia are longer and the kinocilium is on one side of the bundle, the same side as that of the kinocilium in the mature bundle. Scale bars: (A to D), 500 nm; (E), 2 μm.

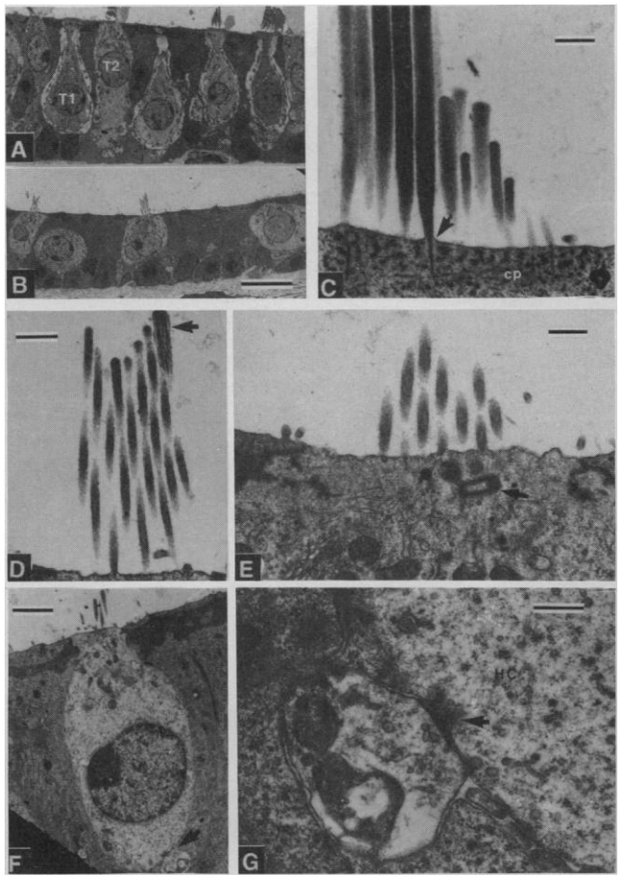

Fig. 3. Transmission electron micrographs of thin sections of the striola of the utricle in controls (A and C) and at 4 weeks after gentamicin treatment (B and D through G). (**A**) In the striolar region of the controls, type 1 hair cells (T1) predominate over type 2 hair cells (T2). This micrograph is from a region near the edge of the striola in order to illustrate type 2 cells; in the center of the striola there were no type 2 cells. (**B**) At 4 weeks after treatment, the epithelium is thinner than normal. There are no type 1 cells within the striola, but hair cells similar to type 2 cells are easily identified. (**C**) Apical end of a normal type 2 hair cell. The stereocilia are supported on the cuticular plate (cp), a meshwork of microfilaments in the apical cytoplasm that supports all the stereocilia. Each stereocilium is densely packed with microfilaments, tapers where it joins the cell body (arrow), and has an electron-dense rootlet running into the cuticular plate. (**D**) The hair bundle on a cell in the striolar region at 4 weeks after treatment; this bundle consists of a kinocilium (arrow) containing parallel microtubules and stereocilia with parallel microfilaments. The stereocilia are much thinner and less densely packed with filaments than normal. (**E**) In the apical cytoplasm of a striolar hair cell at 4 weeks after treatment, microfilaments are present but there is no well-defined cuticular plate. Stereocilia show no constriction or rootlet where they join the cell body. The arrow indicates a centriole close to the center line of the cell. (**F**) Hair cell with relatively mature stereocilia forming a synapse with a bouton nerve ending (arrow). (**G**) At higher power, the synapse arrowed in (F) is seen to be fully developed with thickened synaptic membranes and presynaptic vesicles (arrow) on the hair cell side (HC), demonstrating that it is an afferent nerve ending. Scale bars: (A and B), 10 μm; (C, D, and E), 500 nm; (F), 2 μm; (G), 400 nm.

tions (8). In this work, attention was focused on the utricular macula.

In SEM of the luminal surfaces of the utricular macula, hair cells are recognized by the organized bundles of stereocilia [modified microvilli (9)] that project from their apical ends. In the maculae of the control animals, hair cells were regularly and evenly distributed (Fig. 1A). Each hair cell was separated from its neighbors by intervening supporting cells (Fig. 1, A and B).

Gentamicin treatment caused a loss of hair cells from the utricle that began within the striolar region (Fig. 1C). The striola is a band through the middle of the macula, on opposite sides of which hair bundles are oriented in opposite directions. With time after gentamicin treatment, the loss became progressively more extensive and spread toward the periphery of the epithelia (Fig. 1, C and E). By 4 to 7 days, most of the hair cells in the striolar region had been replaced completely by the expanded surfaces of supporting cells; only a few degenerating hair cells remained (Fig. 1D). At 4 weeks after treatment, however, in the regions with extensive cell loss at earlier times, there were signs of recovery (Fig. 1E). At this time, within the striolar region, cells had emerged whose apical surfaces were smaller than those of mature hair cells but which bore distinct, organized bundles of small stereocilia (Fig. 1F). These cells were present at locations that reestablished the normal alternating pattern of hair cells and supporting cells (compare Fig. 1, E and F, with Fig. 1, A and B). Their hair bundles resembled the developing stereocilia seen during normal hair cell development and during early stages of regeneration in other species (*10*). Each bundle consisted of closely packed immature stereocilia that were all almost equal in height and a single thicker, longer kinocilium (Fig. 2). A progression in the development of the hair bundles could be identified (Fig. 2, A to D). The kinocilium appeared to emerge initially from the center of the cell and was surrounded by short stereocilia that covered almost the entire cell surface (Fig. 2, A and B). When the stereocilia were longer, they were organized in a regular fashion with lateral cross-links radiating from their tips, and the kinocilium was positioned eccentrically relative to the stereociliary bundle, sometimes located close to the center of the cell (Fig. 2C) but on other cells at one edge (Fig. 2D). The orientation of these asymmetric, immature bundles was often the same as that of mature-appearing bundles in their immediate vicinity (Fig. 2E).

Quantitative data were obtained from the analysis of 2000-μm^2 fields within the striolar regions of controls and at 4 weeks after gentamicin treatment (*11*), and the number of hair bundles with the easily recognizable, distinctive morphology of the most immature forms, that is, with stereocilia covering the entire cell surface and angled toward the center (similar to Fig. 2, A and B) was assessed. In control utricles, such bundles were present in 8 of 33 fields examined (from 11 different animals), but in none of these fields were there more than two immature bundles. In total, 13 immature hair bundles were counted, a frequency of approximately one per 5000 μm^2. At 4 weeks after treatment, of 35 similar fields (from nine different animals), 34 contained between two and eight immature bundles, and the total number was 153, equivalent to a frequency of approximately 11 per 5000 μm^2. This substantially larger number confirms that immature hair bundles appeared after the gentamicin treatment.

Thin sections of the control tissues (Fig. 3A) showed the presence both of type 1 hair cells (each with a pear-shaped cell body enclosed within a single, large nerve ending) and of cylindrical type 2 hair cells (each surrounded by supporting cells and synapsing at its base with several small, bouton nerve endings). Across the striolae of the control utricles, type 1 cells predominated to the almost complete exclusion of type 2 cells (Fig. 3A). In contrast, in these areas in the animals that had survived for 4 weeks after the end of the gentamicin treatment, there were almost no intact type 1 cells (Fig. 3B). The hair cells that were present resembled type 2 hair cells; their lateral membranes were surrounded by closely apposed supporting cells (Fig. 3, B and F). It has been suggested that during hair cell regeneration in the avian vestibular system, type 2 hair cells replace lost type 1 hair cells (*12*).

In comparison with mature hair cells in the control tissue (Fig. 3C), most of these reemerging hair cells appeared immature.

Their hair bundles did contain a single true cilium (kinocilium) (Fig. 3D), but their stereocilia were thin, with a relatively low density of microfilaments (Fig. 3, D and E). Many joined the cell body without any constriction at their proximal end or an obvious rootlet, and some arose in the absence of a well-differentiated underlying cuticular plate (Fig. 3E). Whereas most of the apparently maturing hair cells had no obvious synaptic contacts, some of those that possessed more mature stereocilia had a few fully developed synapses with afferent nerve boutons at their bases (Fig. 3, F and G).

These morphological observations demonstrate that replacement hair cells can develop after ototoxic damage to the utricle in guinea pigs. The replacements appeared in the regions from which the original hair cells were lost. Similar observations have been made in cristae and saccules, which suggests that these phenomena occur throughout the mammalian vestibular system. At least some of the new hair cells become innervated, making it likely that they could contribute to a recovery of sensory function. DNA labeling studies described by Warchol *et al.* (6) indicate that the new hair cells are produced after mitoses occurring in response to trauma and thus that regeneration of hair cells does occur in the mammalian inner ear.

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11. Quantitative analyses were made from scanning electron micrographs of the striolar regions of the utricles at a standard magnification that provided a field size of 2000 μm^2. In control utricles, the total density of hair cells estimated from these fields was 27.5 ± 1.92 per 1000 μm^2 (33 fields from 11 different animals). The vast majority of hair bundles showed the normal mature morphology; stereocilia occupied about half of the cell surface and were organized in typical staircase fashion. There was, however, in addition to the very small number of bundles that showed the distinctive morphology of the most immature forms (equivalent to those shown in Fig. 2, A and B), a subpopulation of bundles that were noticeably shorter than normal and with relatively small surface areas. In many of these, the kinocilium was as long as that of normal hair bundles and the stereocilia were organized in staircase fashion, but these (or at least some of them) may represent immature hair bundles. If these are included with those showing the most immature bundle form, then the density of immature hair cells in controls was 3 ± 1 per 1000 μm^2. At 4 weeks after treatment the density of hair cells was reduced to 16 ± 2 per 1000 μm^2 (35 fields from nine different animals). However, in addition to the bundles with the most immature morphology, there were numerous bundles in which stereocilia were erect but of equal height (equivalent to those shown in Fig. 2D) and which in terms of bundle height and stereocilia organization were clearly immature. If we include all these bundle forms and exclude bundles that showed the typical staircase form (regardless of relative height and cell surface size), then the total density of immature hair bundles at 4 weeks after treatment was 9 ± 1.5 per 1000 μm^2 (that is, 56% of the total number of hair cells present). These analyses not only confirm that mature hair cells are lost and replaced by immature cells after gentamicin treatment but also they suggest there may be ongoing development of hair cells in undamaged utricles of normal mature guinea pigs.
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Regenerative Proliferation in Inner Ear Sensory Epithelia from Adult Guinea Pigs and Humans

Mark E. Warchol, Paul R. Lambert, Bradley J. Goldstein, Andrew Forge, Jeffrey T. Corwin

Supporting cells in the vestibular sensory epithelia from the ears of mature guinea pigs and adult humans proliferate in vitro after treatments with aminoglycoside antibiotics that cause sensory hair cells to die. After 4 weeks in culture, the epithelia contained new cells with some characteristics of immature hair cells. These findings are in contrast to expectations based on previous studies, which had suggested that hair cell loss is irreversible in mammals. The loss of hair cells is responsible for hearing and balance deficits that affect millions of people.

Sensory hair cells are essential for the transduction of mechanical stimuli into hearing and balance signals in the internal ear. Sound is transduced by hair cells in the cochlea, and movements of the head are transduced by hair cells in the five vestibular sensory epithelia. Tritiated thymidine labeling in mice in vivo has provided support for the belief that the cells of the sensory epithelia in the mammalian ear cease proliferation in the embryo or shortly after birth and do not proliferate in the adult (*1*).

Here we report that cells in the utricular sensory epithelia from adult guinea pigs and adult humans proliferate after treatment with ototoxic antibiotics when maintained in vitro and that some of the progeny of the proliferating cells begin to differentiate as

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