hair cell nuclei. The morphological features of those labeled cells were similar to immature hair cells. The most parsimonious explanation of these findings is that the immature hair cells that appear after treatment in vivo develop from cells that have undergone proliferation, as had been suggested in the studies of organotypic cultures. The number of labeled cells that could be "putatively" identified as hair cells in the organotypic cultures was rather less than the number of cells with characteristics of immature hair cells that were identified from the in vivo morphological study, but this could be a result of differing capacities for proliferation and subsequent differentiation in vitro and in vivo. Alternatively, the apparent discrepancy could result because, in addition to proliferation, there are, as Rubel *et al.* suggest, other processes that lead to development of hair cells after damage, including self-repair of nonlethally damaged cells, or the direct conversion of some nonsensory cell type in the epithelium into a hair cell. Self-repair through a process that involves redevelopment of their apical structures has never previously been recognized in the many, varied studies of hair cell injury in vivo. If such does occur, it seems unlikely that it is confined to the mammalian vestibular system, and this would have implications for studies of those sensory epithelia where recovery of hair cell numbers through proliferation has been reported. However, phenotypic conversion of supporting cells directly into hair cells has been suggested previously (*5*) as a supplementary mechanism for hair cell production, although at present the evidence is indirect and inconclusive.

Rubel *et al.* however, appear to contend that in vivo most of the immature hair cells arise from some process other than from the proliferating nonsensory cells. None of the labeled cells in their study could be identified as hair cells. That they did not find any labeled hair cells is an unexpected and potentially significant result.

However, the drug treatment protocol used by Rubel *et al.* appears to have induced extensive damage over prolonged periods, with marked interanimal variability in the amount of damage caused. The variability between animals in the pattern of damage could be reflected in a variability in the number and timing of appearance of immature hair cells. Some of the data do appear to show that the pattern of damage influences the pattern and extent of proliferation. In one of the two thymidine-exposed, drug-treated animals examined at 6 weeks after treatment, there are no labeled cells. The density of mature, remaining hair cells in the utricle in this animal is low, suggesting damage was extensive. The other animal examined at this time is the only sample showing pairs of labeled nuclei, and it is suggested that progression to cell division had occurred by this time. This animal is also the one that shows the highest density of hair cells after treatment, which suggests that it suffered the least damage. Furthermore, we have found significantly greater numbers of hair cells at 12 weeks than at 4 weeks after treatment, as assessed from SEM (*6*) and from thin sections (*7*). If this is correct, then it may be that the development of hair cells after injury to the epithelium continues over a period more prolonged than that examined by Rubel *et al.* It would be of interest to determine the developmental fate of those daughter cells that Rubel *et al.* found had been produced 6 weeks after drug exposure.

It is difficult from the results presented by Rubel *et al.* to draw firm conclusions as to the extent to which proliferative regeneration is involved in the appearance of immature hair cells in the drug-damaged mammalian urtricles. Nevertheless, the results of their study indicate that proliferation might not be the only process involved. This raises important questions that need to be considered in future work.

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Regeneration and Mammalian Auditory Hair Cells

Until recently, it was assumed that deafness in mammals resulting from the loss of auditory receptors (by administration of ototoxic drugs or by physical injury) was permanent, although such is not the case for cold-blooded vertebrates and birds (*1*). P. Lefebvre *et al.* report that retinoic acid (RA) stimulated the regeneration of hair cells on the cochleas of neonatal rats maintained in vitro after ototoxic poisoning (*2*). Finding a molecule or molecules that could initiate regeneration of hair cells would have a great impact on the treatment of deafness.

To test whether RA can stimulate hair cell regeneration, we performed tests with RA and neomycin separately and then compared the effects of RA and neomycin together (Fig. 1). Corti explants from 3-day-old rats were maintained for 10 to 13 days in vitro with fetal bovine serum (FBS) and N1 (*3*). Normal cochlear organization with one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs) was observed by phalloidin staining (*4*). The apical surface of hair cells with their cuticular plates and stereocilia bundles were normal. However, some qualitative differences were observed compared to in vivo control cochleas. For example, stereocilia of IHCs were more elongated and stereocilia of OHCs sometimes still had an immature shape, irrespective of in vitro conditions. The mean number density of hair cells in vitro compared to 3 days after birth (DAB) in vivo control cochleas is slightly lower, because an increase in the intercellular spaces between hair cells. No dying hair cells were observed. Postnatal cochlea explants may have the potential to produce supernumerary hair cells in vitro, (*5*), but previous observations (*6*) have shown that explants from postnatal cochleas, even from the apical part, do not reliably produce supernumerary hair cells. Moreover, our control explants from three DAB, maintained 10 to 13 days in vitro, have less hair cells whatever the origin of the explant in the cochlea (Fig. 2).

We investigated whether RA is able to stimulate production of supernumerary hair cells in postnatal explants (*7*). Explants exposed to RA alone for 7 days (*8*) did not show an increase in the number of hair cells and no extra rows of hair cells were observed (Fig. 1).

Is antibiotic treatment, as used by Lefebvre *et al.* (*2*), able to destroy virtually all hair cells? After treatment (*9*), the apical surface of the former sensory epithelium is replaced by polygonal cells. Few hair cells were visible in the basal turn (Figs. 1 and 2A). However, explants from the last half of the apical turn showed remaining hair cells. Over half

of the hair cells survived the drug treatment compared to control explants (Fig. 2B). The cochlear area containing surviving cells varied in length from 160 to 1800 μm (the average was 750 μm).

Explants from 34 cochleas were treated with neomycin and subsequently treated with 10^{-8} M RA for 7 days. The explants were examined by phalloidin staining and by scanning electron microscopy (SEM) (*10*). The surface of the former sensory epithelium in all cultures showed the same cells with polygonal borders as observed with neomycin treatment alone (Fig. 3, A and B). We did not observe at any time, with any explantation methods or culture solutions, any signs of hair cell regeneration caused by RA. There were no differences between explants treated with only neomycin and those treated with neomycin plus RA. Phalloidin-stained dots were often present in the center of cells that replaced lost hair cells in both conditions. Quantitative analyses (Fig. 1) indicated that few hair cells persisted in the basal turn after either treatment, and a high percentage of hair cells in the last half of the apical turn survived.

Differentiation and regeneration are likely to depend on a number of factors such as the culture methods, the presence of chemical mediators, and the microenvironment to which the explant is exposed. Some of these factors could explain why we were not able to replicate the observations of Lefebvre *et al.* (*2*). One answer may come from technical differences. However, our conditions did preserve the organotypy of the explant, as well as hair cell integrity and the afferent innervation (*11*).

One difference may be culture supports; we used dialyzed rat tail collagen or glass and plastic coated with poly-D-lysine, while many other coating substances exist such as other molecules involved in the extracellular matrix (*12*). We know of no information about the effect of such substances on the preservation of cochlear organotypy and hair cell integrity, although there are many observations concerning the good preservation, over many days, in vitro of hair cells in collagen matrices (*13*).

A further critical point is the culture medium. One medium corresponds as closely as possible to the one used by Lefebvre *et al.* (*2*), with a cocktail of hormones mainly used for neurons in vitro. Despite this, we did not find indication of hair cell regeneration due to RA, and there was negligible difference in these cultures compared to the use of other media. We were unable to find hair cell regeneration with different explantation techniques and different media; possible minor differences between our culture techniques and those of Lefebvre *et al.* (*2*) seem unlikely to account for the difference in the results.

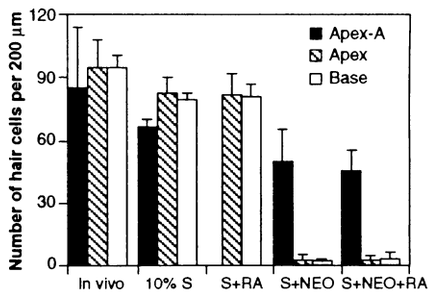

Fig. 1. Average number of hair cells computed from segments of a standard length of 200 μm of 3 DAB cochleas for several feeding solutions with N1. Explants cultured 10 days with 10% fetal bovine serum and N1 (10% S). Explants treated with 10^{-3} M neomycin (Neo) for 2 days after 2 days in culture and subsequently maintained 7 days in feeding solution (S + Neo). Explants treated for 7 days with 10^{-8} M retinoic acid (RA) after 4 days in culture in feeding solution (S + RA). Explants maintained 2 days in feeding solution followed by 2 days with 10^{-3} M neomycin and 7 days with 10^{-8} M RA (S + Neo + RA). Three parts of the cochlear partition were distinguished, that is, the basal (hook and first turn), the apical (second turn and a part of the apical one), and the A-apical (the last 2 mm of apex with hair cells resistant to neomycine). Hair cell counts were made from several segments along each part. Because of identical results, we did not separate data according to culture supports, that is, collagen or poly-D-lysine–coated plastic or glass supports. Hair cells were identified at the end of the experiment after fixation from TRITC-phalloidin–stained stereocilia bundles or cuticular plates (*4*) and were counted directly from the microscope. In vivo controls were used for comparison with in vitro in order to ascertain the viability of our cultures. Then, the mean number of hair cells were counted from in vivo 3-days-old Corti's organ set at a magnification of 1000 through a calibrated reticulum. There is no statistically significant difference between S + Neo and S + Neo + RA ($P > 0.05$, Student's t test) or between 10% S and S + RA. For each mean value we analyzed more than 20 segments, except for Base and Apex-A of 10% S. The error bars correspond to the standard deviation.

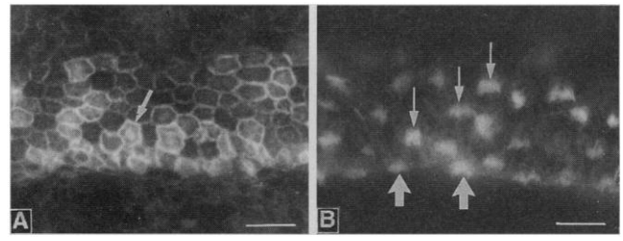

Fig. 2. Three DAB cochlear explants treated for 2 days with neomycin. (**A**) At the base of the cochlea no hair cells were visible, but a polygonal organization is present. A few cells present a stained dot in their center (arrow). Scale bar, 20 μm. (**B**) The last mm of the apical turn shows rows of both types of hair cells, OHCs (small arrows), IHCs (large arrows). Scale bar, 20 μm.

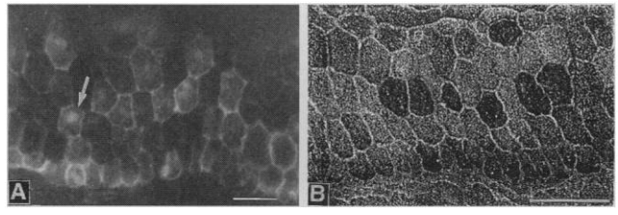

Fig. 3. Three DAB explants treated with neomycin and later RA. (**A**) Phalloidin-stained basal explant shows a cellular formation with a polygonal shape replacing the former sensory epithelium. A few cells present a stained dot in their center (arrow). Scale bar, 20 μm. (**B**) SEM micrograph from an apical turn. The same polygonal organization can be observed on the previous location of the sensory epithelium without any regenerated hair cells. Scale bar, 20 μm.

The evidence presented of hair cell regeneration in figure 3, C and D, of the report by Lefebvre *et al.* (*2*) is not convincing. An apical cell surface with microvilli and a kinocilium cannot be taken as proof of hair cell regeneration because cells of Kölliker's organ (progenitor cells of hair cells) and young hair cells as well as supporting cells present microvilli and a kinocilium. The kinocilium was seen from the embryonic stage to at least 1 week after birth in rat (*14*). Moreover, cells with polygonal shape after only neomycin treatment also presented a kinocilium (Fig. 4).

Finally, the survival of apical hair cells must be considered as a possible reason for the difference between our results and those reported earlier. We and others (*15*) have

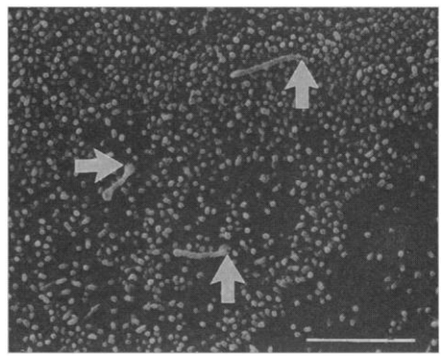

Fig. 4. SEM micrograph of the first half of apical turn from a neomycin-treated explant. Apical surface of cells replacing the former sensory epithelium show many microvilli and some kinocilia (arrows). Scale bar, 2 μm.

shown that hair cells from the apex of the cochlea are less sensitive to neomycin and other ototoxic drugs (Figs. 1 and 2B). It is possible that remaining apical hair cells after neomycin treatment could have been taken as regenerated cells in Lefebvre's study (*2*).

With regard to auditory receptors, regeneration may be possible. However, how hair cells differentiate from progenitor cells needs to be understood first, as well as the signals responsible for it. The finding of spontaneous supernumerary hair cells after explantation of fetal mammal cochleas (*6*) looks promising. The nature of the trigger signal of this enhanced proliferation remains to be investigated in order to design new approaches to regeneration strategies in adult mammals which finally may become applicable in humans.

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3. Sprague-Dawley rat cochleas were explanted 3 days after birth (DAB). Cultures were prepared according to several procedures because the protocol described by Lefebvre *et al.* (*2*) was not complete. First, we used the recently described organotypic method (D. Rastel, A. Abdouh, D. Dahl, R. Romand, *J. Neurosci. Methods* **47**, 123 (1993), which gives good results on the preservation of the organotypy of the explant including its innervation. The distinguishing feature of this method is that the explants are put on a drop of photo-reconstituted collagen floating over a feeding medium. In addition, we used other procedures; explants were placed in a 24-mm COSTAR transwell-col transparent collagen-coated membrane insert with 0.45-μm pores, or on glass lamellae and plastic lamellae (Thermanox) coated with poly-D-lysine. In all cases, the spiral lamina was dissected in minimum essential medium. Stria vascularis and Reissner's membrane were removed. The spiral lamina was cut in two pieces (base included the hook and the first turn; apex the second and apical turns) and each part was cultivated in a separate well. Explants were cultivated with two different batches of heat-inactivated fetal bovine serum (Sigma F 4135). For some experiments, we used Dulbecco's modified Eagle's medium (DMEM) (Sigma D1152) 50%/Hanks balanced salt solution (Sigma H6135) 40% with high glucose and L-glutamine. In experiments that served as a basis for Fig. 1, we used the same feeding solution as described by Rogister *et al.* [B. Rogister, J. M. Rigo, P. P. Lefebvre, P. Leprince, D. Marin, J. Schoenen, G. Moonen, in: *Neuromethods*, vol. 23, *Practical Cell Culture Techniques*, A. Boulton, G. Baker, W. Waltz, Eds. (Humana, Totowa, NJ, 1992), p. 173] and Lefebvre *et al.* (*2*) with DMEM/F-12 1:1 mixture (Sigma D 8900) plus a cocktail of hormones (N1) usually used for neuron cultures with final 6g/l of D-glucose and 4 mM L-glutamine. Culture media were changed every days.
4. For tetramethylrhodamine isothiocyanate (TRITC)–labeled phalloidin (Sigma) staining, explants were fixed 45 min in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.2), and after washing, stained with phalloidin (3 μg/ml in PBS) for 45 min at room temperature. Observations were made with a Nikon microscope with a blue excitation filter.
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8. Control explants with RA from 18 cochleas were cultured simultaneously using the same procedures and mediums as described (*3*). After 4 days in culture, 10^{-8} M RA (all-transs, ref. R. 2625, Sigma) was added for 6 to 9 days. Two different batches of RA were used, and contact with light was avoided as much as possible.
9. Explants 3 DAB from 25 cochleas, after 48 hours in vitro were exposed to neomycin 10^{-3} M for an additional 48 hours and seven more days in culture with different neomycin-free feeding solution. This should have been an effective dose for the destruction of 99% of auditory hair cells in cochlear explants in organotypic cultures per Lefebvre *et al.* (*2*).
10. Cultures were fixed for 1 to 4 hours in 2% (w/v) paraformaldehyde-2% (v/v) glutaraldehyde buffered in a 0.15 M Sorensen phosphate buffer, (pH 7.4). After four washes and postfixation with 2% (w/v) osmium tetroxide in 0.1 M phosphate buffer, the explants were dehydrated with ascending concentrations of ethanol and then with acetone before being critical point–dried with liquid CO_2 and plated with gold. The specimens were observed with a Cambridge stereoscan 360 SEM.
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16. We thank E. W Rubel and G. Ehret for helpful suggestions in the preparation of this manuscript and Dr. M.-Y. Mu for sharing in vivo data and C. Delattre and A.-M. Mafille for technical assistance. Supported by La Fondation pour la Recherche Médicale and by l'Association Recherche et Partage.

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Response: The fact that Chardin and Romand did not replicate the results of our report (*1*) may be explained by essential differences in technique. The organotypic explants in our study were maintained as free floating cultures of whole Corti's organs. In a series of 45 cultures (where the basal, mid, and apical segments were assayed for hair cell counts) these explants consistently showed regeneration-repair of the auditory sensory epithelium (*1*). The cultures of Chardin and Romand were divided into three portions, thereby introducing additional trauma to these explants.

In our initial attempts to stimulate regeneration-repair of ototoxin-damaged auditory hair cells, we used organotypic cochlea cultures fixed to either a polyornithine-coated plastic substratum or a collagen type I matrix to allow for the sequential observation of any regeneration-repair that occurred in response to treatment with retinoic acid (RA) and fetal calf serum (FCS). None of the 30 cultures grown while fixed to a substratum showed signs of regeneration or repair despite the use of the same medium and supplements that were described in our report (*1*). The explants in the study of Chardin and Romand were all adhered to a substratum. Adherence to a substratum, and most particularly interaction with matrix molecules such as collagen type I, can have profound effects on cellular behavior and even result in changes in cellular phenotype, for example, epithelial-mesenchymal transformation (*2*). There are also differences in the culture medium that could effect the outcome of the regeneration-repair process. We have found that handling of the insulin supplement and the selection of batches of FCS to be used as a growth supplement affected our results (*3*). Insulin has been shown to be an important factor for potentiating the stimulation of cell proliferation by two members of the epithelial growth factor (EGF) family. EGF and transforming growth factor–α (TGF-α), in organotypic cultures of utricles (*4*); therefore, proper handling of insulin would be a critical factor. To overcome the problem of variable quality of FCS batches, we have attempted to replace serum with a growth factor.

Western blot studies of noise- and ototoxin-lesioned juvenile chick cochlea suggest that a protein with TGF-α–like immunoreactivity may play a role in the regeneration of chick auditory hair cells (*5*). Initial histological studies localized EGF receptor to the inner and outer hair cells of the 3-day-old (PP) rat Corti's organ and free floating 3 PP rat organ of Corti cultures exposed to neomycin 10^{-3} M for 48 hours showed immunolocalization of EGF receptor to the area of ototoxin damage (*6*). Two separate studies have also shown that TGF-α may participate in the regeneration-repair of vestibular hair cells in mammals (*4*, *7*). These observations prompted us to test whether any members of the EGF family of trophic factors could be substituted for FCS.

A series of 3 PP rat Corti's organ cultures were exposed for 48 hours to neomycin 10^{-3} M followed by an additional 8 days in Dulbecco's modified Eagle's medium + N_1 + glucose (*8*) plus RA (10^{-8} M) and either EGF, TGF-α, platelet derived growth factor (PDGF), or basic fibroblast growth factor (bFGF) at varying concentrations, in 10 to 500 ng/ml amounts (*9*). Control cultures

not exposed to neomycin showed maintenance of hair cell integrity (Fig. 1A), whereas cultures treated with neomycin 10^{-3} M for 48 hours followed by either 8 days in defined medium (Fig. 1B) or 8 days in defined medium supplemented with RA 10^{-8} M showed almost complete destruction of all hair cells in the sampled midportion of Corti's organ. In agreement with the observations of Chardin and Romand, we observed that there were auditory hair cells that survived the 48 hours of exposure to neomycin (10^{-3} M) located only in the most apical portion of Corti's organ. Therefore, these apical areas were excluded from our counts of stereocilia bearing cells. Addition of EGF, PDGF, or bFGF (10 to 500 ng/ml) to the RA 10^{-8} M supplemented medium did not result in any detectable regeneration-repair of hair cells. Addition of TGF-α at concentrations of 10 to 50 ng/ml (9) resulted in the presence of cells bearing disorganized bundles of stereocilia in the mid-portion (Fig. 1C) of the explants (Table 1). Retinoic acid could be entirely eliminated in these cultures without impairment of the TGF-α induced hair cell regeneration-repair process, however higher doses of TGF-α were needed to achieve a similar degree of regeneration-repair (Table 1). This suggests that retinoic acid potentiates, but does not initiate, the regeneration-repair process observed in these cultures. Several recent studies on epithelial regeneration have observed the interaction of TGF-α–EGF receptor in regeneration-repair of gastric mucosa, liver, and kidney (*10*), thus setting a biological precedent for our observations.

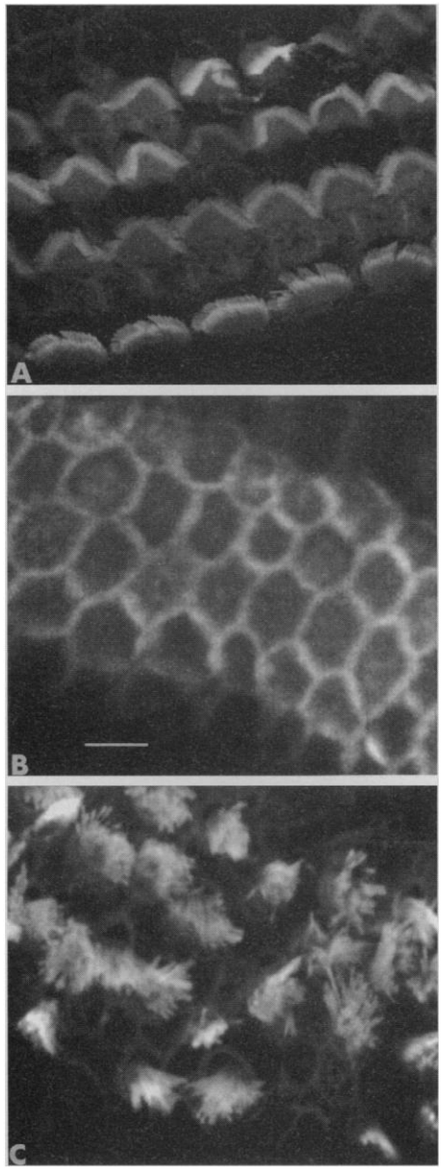

Fig. 1. Corti's organ explants from 3-day-old rats after 10 days in vitro. Hair cells are stained with phalloidin–fluorescein isothiocyanate (FITC). Confocal microscopic images represent samples taken from the mid-turn segments of explants. (**A**) Control organ of Corti cultured in defined medium. The four rows of normally oriented auditory hair cells show no evidence of degeneration or disorganization of stereocilia bundles. (**B**) Explant exposed to neomycin 10^{-3} M for 48 hours and then cultured for 8 days in defined medium shows only a reticulated pattern of staining in the area of Corti's organ. (**C**) Explant exposed to neomycin 10^{-3} M for 48 hours and subsequently treated for 8 days with defined medium supplemented with TGF-α (25 ng/ml) + RA 10^{-8} M shows staining of a group of cells with disorganized bundles of stereocilia in the area of Corti's organ. Scale bar, 5 μm.

Table 1. Effect of TGF-α concentration with and without retinoic acid 10^{-8} M on the number of hair cells in the mid-turn segments of ototoxin-damaged Corti's organ explants.

TGFα (ng/ml)	Hair cells per millimeter*	
	−RA, 0 M	+RA, 10^{-8} M
0	0	0
10	29 ± 6†	70 ± 23
25	145 ± 29	325 ± 29
50	330 ± 52	388 ± 52
Unlesioned	428 ± 47	–

*Hair cell counts were determined by averaging total number of stereocilia bundles in three 250-μm segments of the mid-turn portions of Corti's organ explants. Number of specimens per group is 15 explants. † ± Sample standard deviation.

Table 2. Cells labeled with BrdU in whole mount cultures of 3 PP rat Corti's organ Corti explants.

Culture condition	BrdU-labeled nuclei/×40 field*	*P* value
Untreated	2.7 ± 5.2†	–
Neo > DMEM	3.8 ± 7.4	$P > 0.05$
Neo > DMEM+ TGF-α	53.1 ± 28.4	$P < 0.001$

*Each group represents the average of counts from 12 specimens. Four ×40 fields were counted from each whole mount specimen representing the basal, mid, and apical segments. † ± Sample standard deviation.

To determine if the TGF-α initiated regeneration-repair process is the result of cell division as shown for avian hair cell regeneration (*11*), TGF-α treated organotypic cultures were labeled with bromodeoxyuridine (BrdU) (*12*). Uptake of BrdU was low in both control and neomycin exposed cultures (Table 2). A 19-fold increase in BrdU labeling was seen in the combined epithelial and mesenchymal layers of ototoxin exposed organ of Corti explants that were treated with TGF-α during the period of BrdU exposure (Table 2), however, no labeled hair cells have been observed in serial sections of explants. This supports our earlier observations that treatment with cytosine arabinoside could inhibit the regeneration-repair process (*1*). A mitotic event may be involved in the regeneration-repair process, but the regenerated-repaired auditory hair cells themselves do not appear to be a direct product of a mitotic event. There may be a fundamental difference between the process occurring in our cultures (*1*) and the process of avian hair cell regeneration (*11*).

It appears that the regeneration-repair observed in our cultures is mediated by TGFα and the EGF receptor and that retinoic acid modulates, but does not initiate this process. This data potentially provides further clarification as to why Chardin and Romand did not reproduce our results (*1*). Potentially, the serum used in their cultures may not have had adequate levels of TGFα. With the use of the free-floating culture system under the conditions described, we have consistently observed the regeneration-repair of stereocilia bearing cells in hundreds of explants using either FCS 10% + RA 10^{-8} M or TGF-α + RA 10^{-8} M as supplements. Whether or not this process represents regeneration from a stem cell population or a regenerative-repair process as reported by Sobkowicz (*13*) remains unclear, as does whether or not this process can occur in the adult animal. However, in agreement with Chardin and Romand, we believe that an increased understanding of the molecular basis of hair cell differentiation signals and the process of hair cell regeneration-repair are essential for progress in this field.

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3. Each of the N1 components was prepared separately as either 500 × (insulin, progesterone) or 1000 × (putrescine, transferrin, Na_2SeO_4) solutions. These N1 components with the exception of insulin were combined into 1.25-ml aliquots and stored at −20°C for a maximum storage period of 1 mon. Insulin stock solution was acidified with 1 N NCl until the suspension went into solution and was stored at 4°C. (Insulin solution cannot be frozen and must be prepared fresh every week. We sample six batches of FCS at a time, and of these only one on average is suitable as a culture supplement.) FCS batches were selected with the use of several neuronal survival and cell proliferation assays (G. Moonen, University of Liège, Belgium). All transretinoic acid (Sigma, St. Louis, MO) stock solution was prepared at a concentration 0.01 M in ethanol, stored in a light-proof container at −20°C, and thawed immediately before use. RA was freshly diluted each time the medium was exchanged and cultures were protected from light.
4. H. Yamamshita and E. Oestele, paper 522 presented at the 17th Midwinter Research Meeting of the Association for Research in Otolaryngology, St. Petersburg Beach, FL, 6 to 10 February 1994.
5. X. Xu and J. T. Corwin, paper 588.15 presented at the 22nd Annual Meeting of the Society for Neuroscience, Anaheim, CA, 25 to 30 October, 1992; X. Xu and J. T. Corwin, paper 649.11 presented at the 23rd Annual Meeting of the Society for Neuroscience, Washington, DC, 7 to 12 November, 1993.
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7. P. R. Lambert, *Laryngoscope* **104**, 701 (1994).
8. J. E. Bottenstein and G. H. Sato, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 514 (1979).
9. Corti's organ explants from 3-day-old rats were set up as previously described (*1*). The explants were exposed to neomycin (10^{-3} M) in defined medium (DMEM + N1 + glucose) for 48 hours and subsequently incubated for another 8 days in defined medium; defined medium supplemented with RA 10^{-8} M and growth factor; or defined medium supplemented with only TGF-α. Growth factors: PDGF, EGF, and bFGF (R&D Systems, Minneapolis, MN) were added in concentrations of 10, 25, 50, 100, and 500 ng/ml defined medium. After 10 days in vitro cultures were fixed, stained with FITC-phalloidin and analyzed with the aid of Bio-Rad MRC 600 confocal microscope.
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11. J. T. Corwin and D. A. Cotanche, *Science* **240**, 1772 (1988); B. M. Ryals and E. W Rubel, *ibid.*, p. 1774.
12. Corti's organ explants from 3-day-old rats were prepared as previously described (*1*). Explants were cultured for 48 hours in either DMEM + N1 + glucose or in this defined medium plus neomycin 10^{-3} M. During the second 48 hours of culture, explants were either exposed to defined medium plus BrdU (2 μg/ml) or BrdU plus a supplement of TGF-α (25 ng/ml). After 4 days of culture, all explants were methanol fixed, permeabilized with a detergent (Tween 80; Sigma, St. Louis, MO), stained with a monoclonal antibody against BrdU, mounted in glycerin and analyzed for immunolabeled nuclei (counts per ×40 field) in the area of Corti's organ with the use of a Zeiss Axiophot microscope.
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