Hyaluronan as a propellant for epithelial movement: the development of semicircular canals in the inner ear of *Xenopus*

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Summary

The membranous labyrinth of the inner ear, with its three semicircular canals, originates from a simple spheroidal otic vesicle. The process is easily observed in *Xenopus*. The vesicle develops three dorsal outpocketings; from the two opposite faces of each outpocketing pillars of tissue are protruded into the lumen; and these paired 'axial protrusions' eventually meet and fuse, to form a column of tissue spanning the lumen of the outpocketing like the hub of a wheel, with a tube of epithelium forming the semicircular canal around the periphery. Each axial protrusion consists of epithelium encasing a core of largely cell-free extracellular matrix that stains strongly with alcian blue. In sections, at least 60% of the stainable material is removed by treatment with *Streptomyces* hyaluronidase. When *Streptomyces* hyaluronidase is microinjected into the core of a protrusion in vivo, the protrusion collapses and the corresponding semicircular canal fails to form. Hyaluronan (hyaluronic acid) in the core of the protrusion therefore seems to be essential in driving the extension of the protrusion. Autoradiography with tritiated glucosamine indicates that the hyaluronan-rich matrix is synthesised by the epithelium covering the tip of the protrusion; the basal lamina here appears to be discontinuous. These findings indicate that the epithelium of the axial protrusion propels itself into the lumen of the otocyst by localised synthesis of hyaluronan. Hyaluronan may be used in a similar way in the development of other organs, such as the heart and the secondary palate.

Key words: hyaluronan, inner ear, semicircular canals, *Xenopus*.

Introduction

All vertebrates are equipped in a similar way with an elaborately shaped inner ear. This has its beginnings in a patch of thickened ectoderm, the otic placode, on the side of the head of the embryo adjacent to the hindbrain. The placode invaginates and pinches off from the surface to become the otic vesicle, which then develops into the membranous labyrinth of the inner ear through an extraordinary process of epithelial growth and remodelling, as shown in Fig. 1. The simple spheroidal vesicle is transformed into a shape with precisely positioned bulges and projections and, most strikingly, with three toroidal 'handles', the semicircular canals. In this paper, we examine how the semicircular canals are created. They provide a very clear illustration of how a localised biochemical activity brings about a transformation of shape.

The geometry of semicircular canal formation has often been described before (von Noorden, 1883; Krause, 1903; Streeter, 1907; Paterson, 1948; Knowlton, 1967; Waterman and Bell, 1984; Hertwig, 1987). The otic vesicle develops a set of three flattened outpocketings, one for each future duct. The centres of the two opposite faces of each outpocketing become indented, forming axial protrusions into the lumen of the vesicle from opposite sides. These protrusions eventually contact one another and, at the site of contact, the two apposed epithelial sheets fuse and reorganise, creating a squat pillar of tissue spanning the lumen of the outpocketing (see Fig. 2). Around this pillar, like the tube of a car tyre around the wheel hub, lies the epithelium of the newly formed semicircular canal, derived from the peripheral part of the epithelium of the outpocketing, and communicating at each end with the lumen of the body of the otic vesicle. The topological transformation is the same in all vertebrates; there are, however, variations in the quantitative details of the geometry. In higher vertebrates, the initial outpocketings are pronounced and strongly flattened, and the axial protrusions are merely low, broad mounds, whereas in fish (Waterman and Bell, 1984) and amphibians, including *Xenopus* (on which we focus here), the outpocketings are much less apparent, and the axial protrusions are finger-shaped and much more clearly defined (Fig. 3; see also Paterson (1948) and Hertwig (1987)).

The protrusions are the crucial structures in the
standard methods (Godsabe et al. 1988) and allowed to develop in dechlorinated tap water. They were staged according to the developmental tables of Nieuwkoop and Faber (1967).

Histology
Embryos to be used for histology were either fixed at 4°C in half-strength Karnovsky fixative (2% paraformaldehyde, 1.25% glutaraldehyde, 0.25% calcium chloride in 0.1 M cacodylate buffer; Karnovsky, 1965) and subsequently embedded in Araldite or Fibrowax (BDH), or were fixed in Bodian's fixative (75% absolute alcohol, 18% distilled water, 2% formaldehyde, 5% glacial acetic acid) or Sainte Marie fixative (1% glacial acetic acid in 95% alcohol) and then embedded in Fibrowax (Tuckett and Morriss-Kay, 1988). Araldite sections were cut at 3 μm and stained with toluidine blue. Fibrowax sections of embryos fixed in half-strength Karnovsky fixative were cut at 6 μm and stained for glycosaminoglycans (GAGs) with alcian blue at pH 2.5 (1% alcian blue 8GX, 3% glacial acetic acid, in distilled water) for 30 min (Humason, 1972). Some sections were further counterstained with neutral red. To assess how much of the alcian-blue stainable material was hyaluronan, sections were incubated, before alcian-blue staining, with Streptomyces hyaluronidase (Sigma, type IX) at concentrations ranging from 0.015 to 1.0 mg ml⁻¹ in sodium acetate buffer (pH 5.2, 0.1 M), for 4 h at 37°C. These sections were compared with adjacent control sections incubated in buffer alone. The stain intensity was quantified by spot-metering of the light transmitted through the regions of interest, using the accurate photomultiplier-based digital light-meter incorporated in a Leitz Vario-Orthomat camera system on a Leitz Diaplan microscope with a ×100 objective. The diameter of the metered spot was 11 μm. The intensity of light, I₀, transmitted through the stained core of a protrusion was compared with the intensity, Iᵦ, transmitted through the empty lumen of the otic vesicle, and the absorption coefficient, A = (Iᵦ - I₀)/I₀, was calculated. For our specimens, values of A were relatively small — in the range 0.1 to 0.3 depending on the enzyme treatment used — and should therefore be very nearly linearly proportional to the amount of stained material present. The fraction of stained material remaining after enzyme treatment is therefore given by the ratio Aᵦ / A₀, i.e. the ratio of the A value for the enzyme-treated specimen to the A value for an adjacent control section treated with buffer only. This ratio is the 'relative stain intensity' referred to in the Results section below.

Materials and methods

Xenopus laevis embryos were obtained and dejellied by standard methods (Godsabe et al. 1988) and allowed to develop in dechlorinated tap water. They were staged according to the developmental tables of Nieuwkoop and Faber (1967).

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Immunohistochemistry
For the localisation of chondroitin sulphate, wax sections of Ste. Marie-fixed embryos were stained with the CS56 monoclonal antibody (Sigma, C8035) followed by a goat anti-mouse IgM, μ-chain specific FITC-conjugate (Sigma, F9259), according to the method of Morriss-Kay and Tuckett (1989). Sections were counter-stained with DAPI (5 μg ml⁻¹ in PBS) and mounted in gelvatol (Canning and Stern, 1988). To monitor the laminin distribution, we used a rabbit anti-laminin antiserum (ICN, 68–125) on Fibrowax sections of larvae fixed in Bodian's fixative, detected with an affinity-purified, goat anti-rabbit IgG-specific, FITC-conjugated secondary antibody (ICN, 61–640–1). The sections were counterstained with DAPI and mounted in gelvatol as above. For precise localization, some of the immunofluorescent sections were viewed using a BioRad MRC 500 confocal scanning laser microscope.
The inner ear develops rapidly in Xenopus; some of these were anaesthetised with MS222, killed and fixed at 4°C in half-strength Karnovsky fixative; they were then critical-point dried and sputter-coated in the usual way.

**Electron microscopy**

For scanning E.M., embryos were dissected to expose the lumen of the otic vesicle and the tip of an epithelial protrusion and fixed at 4°C in half-strength Karnovsky fixative; the specimens were then critical-point dried and sputter-coated in the usual way.

**In vivo injections**

Living tadpoles were anaesthetised in MS222 (Sandoz) and immobilized in gelatin wells at stage 46, when axial protrusions were well defined but not yet fused. The enzymes chondroitinase ABC (Sigma, C2905) and Streptomyces hyaluronidase (Sigma, type IX, H1136) were dissolved in full-strength NAM saline (Slack et al. 1973) at varying concentrations, with the addition of a small amount (approximately 0.01 %) of trypan blue to make the injected fluid visible. According to the manufacturer’s data, the Streptomyces hyaluronidase had a specific activity of 1050 hyaluronidase units mg⁻¹, while the chondroitinases ABC had a specific activity of 0.5 chondroitinase units mg⁻¹. To avoid confusion between the two types of activity unit, based on quite different assays, we specify enzyme concentrations in mg ml⁻¹ in this paper. Enzyme solution was microinjected by mouth-applied pressure into the acellular core of a protrusion using a glass microelectrode, bevelled to a tip diameter of roughly 3 µm. Approximately 0.01 nl fluid was injected although the volume was hard to regulate exactly. Control injections were made using NAM medium with trypan blue but without any enzymes. The effect of the injections on the morphology of the axial protrusions and the subsequent semicircular canal development was monitored by light microscopy of living embryos and by analysis of transverse sections.

**Autoradiography**

To localise sites of GAG synthesis by incorporation of a radioactive GAG precursor, we used stage 46 larvae in which semicircular canal protrusions are just beginning to elongate. To facilitate the uptake of the radioactive precursor by the inner ear, some of these were anaesthetised with MS222, killed surgically and used to prepare explants taken in the form of thick transverse sections that included both ears, together with the neural tube and ventral tissues; other larvae were kept alive and intact. Specimens were placed in either half-strength NAM solution or in a solution containing 60% L15 medium (Sigma), 10% fetal calf serum and 30% distilled water. To both solutions we added tritiated glucosamine (glucosamine hydrochloride, D-[1,6-3H(N)], specific activity 52.7 Ci mmol⁻¹, from NEN Research Products) diluted to give a final activity of 0.09 mCi ml⁻¹. The larvae and tissue explants were incubated at 22°C in the labelled media for 1 h and either fixed straight away or incubated for a further 20 h in unlabelled media (whole tadpoles in NAM/2, explants in L15/fetal calf serum medium) before fixing. We used half-strength Karnovsky fixative at 4°C and embedded in Araldite; to localise sites of GAG synthesis by incorporation of a radioactive GAG precursor, we used stage 46 larvae in which some of these were anaesthetised with MS222, killed and fixed at 4°C in half-strength Karnovsky fixative, post-fixed in osmium tetroxide and stained with uranyl acetate and lead citrate.

**Results**

**Sequence of anatomical events**

The inner ear develops rapidly in Xenopus and the head of the tadpole soon becomes transparent so that the later stages of ear morphogenesis can be observed easily in vivo, as seen in Fig. 3. The sequence and timing of the key events, based on the standard descriptions of Nieuwkoop and Faber (1967) and of Paterson (1948) as well as on our own observations, is as follows for animals reared at 22–24°C. The otic vesicle closes and pinches off from the surface ectoderm at stages 27–28 (31–32.5 h). The first signs of sensory differentiation are seen at stage 33/34 (44 h), when sensory hair cells begin to be visible by scanning E.M. At stage 41 (76 h), a slight outpocketing of the epithelium on the lateral wall of the dorsal part of the vesicle marks the first step in the formation of the horizontal (lateral) semicircular canal. A little later, at stage 42 (80 h), a similar but more extended outpocketing develops along the topmost edge of the vesicle; this soon becomes divided by a slight depression into an anterior and a posterior outpocketing, corresponding to the future anterior (superior) and posterior semicircular canals.

The paired axial protrusions belonging to the lateral outpocketing appear first, at stage 43 (87 h), grow towards one another, and finally fuse at their tips at about stage 46 (106 h) to form the hub of the lateral duct system. The other two semicircular canals develop in the same way, the anterior usually a little before the posterior; both their pairs of protrusions originate at approximately stage 45 (98 h) and have fused by about stage 46.5 (120 h) although there is some random variability in the timing. For each of the three ducts, the paired protrusions, having fused at their tips, then become moulded over the course of a few hours into smooth pillars linking opposite faces of the original outpocketings. Transverse sections illustrating the steps in this process are shown in Fig. 4.

**Each pillar has a largely acellular core filled with fibrous matrix**

One can see by viewing the living tadpole with interference contrast optics that each axial protrusion is covered by a thin shell of epithelium and that its core is usually empty of cells, or contains at most one or two.

This is confirmed by serial sections, which show the protrusions to have a core of extracellular matrix, visible also by scanning E.M., as shown in Fig. 5. Alcian-blue staining at pH 2.5 (Fig. 6A) indicates that the matrix is rich in glycosaminoglycans (GAGs) (Pearse, 1968). In at least one other system – the developing heart – where an epithelium becomes locally separated from mesenchyme by an accumulation of acellular matrix, the matrix has been shown to consist largely of one particular GAG, hyaluronan (Markwald et al. 1978). To test whether hyaluronan was a major ingredient of the matrix in the cores of our protrusions, we treated sections with the highly specific hyaluronidase from Streptomyces (Pintar, 1978; Derby and Pintar, 1978) before staining with alcian blue (Fig. 6B), and compared the resultant intensity of staining with that seen in adjacent control sections that had not been treated with the enzyme. The amount of stain bound in
the core of the protrusion in treated and control sections was measured photometrically for a series of different concentrations of hyaluronidase, each applied for 4h, as described in Materials and methods. The results are shown in Fig. 7. This indicates that for concentrations of *Streptomyces* hyaluronidase above

![Fig. 4. Araldite sections showing the formation of a semicircular canal. (A) A pair of axial protrusions (arrows) filled with extracellular matrix grow into the ear lumen. Note the thickened appearance of the epithelium covering the rounded tips of the protrusions. (B) The protrusions extend and meet, fusing at their tips. Cell death occurs at the fusion plate (arrow). (C) Epithelial remodelling occurs creating a smooth cylinder of epithelium covering a column whose core, initially acellular, later becomes seeded with mesenchymal cells. The completed canal lies perpendicular to the plane of the page. Scale bar=80 μm.](image)

![Fig. 5. S.E.M. of an axial protrusion with its apical epithelium removed, viewed end-on from the luminal side. Note the finely fibrous matrix in the core. Scale bar=10 μm.](image)

![Fig. 7. Graph showing the amount of alcian-blue stainable material remaining in the core of an axial protrusion following a 4-h digestion with *Streptomyces* hyaluronidase at various concentrations. The intensity of stain was measured photometrically as described in ‘Materials and methods’; the ‘relative stain intensity’ plotted along the abscissa is the stain intensity for the given enzyme concentration divided by the intensity for an adjacent control section exposed to zero enzyme concentration. (Thus the relative stain intensity for zero enzyme concentration is exactly 1.0 by definition). The solid circles represent a series of measurements made on multiple sections of a single tadpole. The hollow square at 1.0 mg ml⁻¹ represents a set of measurements on sections of 10 different tadpoles. The error bars represent the standard error of the mean. The curve is drawn by eye. 0.5 mg ml⁻¹, at least 60% of the alcian-blue-stainable material is lost. We can conclude that this proportion of the GAGs in the core of the protrusion either consists of hyaluronan or depends on binding to hyaluronan for its retention in situ.](image)

*When hyaluronidase is injected into the core of an axial protrusion in vivo, it collapses, aborting the development of the semicircular canal.*

A further experiment served to test whether the hyaluronan is functionally important. We immobilised tadpoles in anaesthetic at stage 46, when the anterior and posterior pairs of axial protrusions are usually well-formed (about twice as long as they are wide) but have not yet both fused. Using a fine glass micropipette, we then injected roughly 0.01 nl of enzyme solution into the core of the lateral protrusion of either the posterior or the anterior pair. Two different GAG-degrading enzymes were tested: chondroitinase ABC and *Streptomyces* hyaluronidase, both at a variety of concentrations. Chondroitinase ABC acts on several substrates, degrading chondroitin sulphates A and C, dermatan sulphate and hyaluronan (Derby and Pintar,
Fig. 3. Dorsal view of an intact tadpole at stage 46, showing the location of the ears adjacent to the hindbrain. A blue pigment (monastral blue) has been injected into the lumen of the ear to make its outline clear. Scale bar=100 μm.

Fig. 6. Fibrowax sections stained with alcian blue at pH 2.5 and counterstained with neutral red. (A) Control section. (B) Adjacent section treated with *Streptomyces* hyaluronidase (1 mg/ml−1) for 4 h at 37°C before alcian blue staining. The enzyme has removed most of the alcian-blue-stainable material from the core of the axial protrusion (arrow). Scale bar=75 μm.
Fig. 9. Collapse of an axial protrusion after *Streptomyces* hyaluronidase (0.01 nl, 1 mg ml⁻¹) is injected into its core, as seen in dorsal views of the ear of a live, anaesthetised stage 46.5 tadpole. (A) Just before injection of enzyme into the lateral posterior axial protrusion (arrow). (B) The same specimen 1 min after injection: the protrusion is already beginning to deflate. (C) After 10 min the protrusion has collapsed to less than half its former size. Scale bar=70 μm.
Hyaluronan in Xenopus ear morphogenesis

1978; Morriss-Kay and Tuckett, 1989) while Streptomyces hyaluronidase degrades hyaluronan only (Ohya and Kaneko, 1970). As a control, buffer devoid of enzymes was injected into the same site in sibling tadpoles at the same developmental stage.

Injection of buffer in all 16 control specimens had no apparent effect: the puncture wound made by the micropipette healed rapidly and the protrusion continued to elongate normally, resulting in the formation of a morphologically normal canal. By contrast, in 13 out of 17 specimens injected with Streptomyces hyaluronidase at a concentration of either 0.5 or 1 mg ml\(^{-1}\), the injected protrusions had collapsed to less than half their original length within 4 h and usually much sooner. No recovery was seen over the following 48 h and the semicircular canal failed to form, as shown in Fig. 8. The other protrusions in the same ear usually also retracted, although not so rapidly, presumably through the effects of enzyme that had diffused from the site of injection. A typical example of the collapse is shown in Fig. 9, for a specimen injected with the hyaluronidase at a concentration of 1 mg ml\(^{-1}\). The injected protrusion has begun to retract within 1 min, and by 10 min has collapsed to a low pimple. When Streptomyces hyaluronidase was injected at a concentration of only 0.1 mg ml\(^{-1}\), effects were still apparent but much milder; 3 out of 5 injected protrusions showed a partial collapse or slight delay of outgrowth. 12 out of 16 specimens injected with chondroitinase ABC at concentrations of 20 or 40 mg ml\(^{-1}\) caused protrusions to collapse as rapidly as Streptomyces hyaluronidase at 0.5 or 1.0 mg ml\(^{-1}\); similarly, injections of chondroitinase ABC at 2 mg ml\(^{-1}\) caused a very mild effect in 3 out of 5 protrusions, analogous with 0.1 mg ml\(^{-1}\) Streptomyces hyaluronidase injections. The chondroitinase ABC thus had a collapsing activity about 20 times lower (per mg of protein) than the Streptomyces hyaluronidase. (Note that according to Morriss-Kay and Tuckett (1989), the degradative activity of chondroitinase ABC on hyaluronan is approximately 1/50th of its activity on chondroitin sulphates A or C.)

**Chondroitin sulphate is absent from the cores of the protrusions but present on their epithelium**

It is still possible that other extracellular matrix molecules, and in particular other charged GAGs such as chondroitin sulphate, may also contribute to the inflation of the protrusions. We examined the distribution of chondroitin sulphate by immunofluorescence with the CS56 monoclonal antibody, which binds specifically to the GAG moiety of chondroitin sulphate proteoglycans (Morriss-Kay and Tuckett, 1989). A typical result is shown in Fig. 10. The cartilage of the

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**Fig. 8.** Transverse section of a tadpole 2 days after an injection of *Streptomyces* hyaluronidase (~0.01 nl, 1 mg ml\(^{-1}\)) into the lateral axial protrusion in the right ear. On the control (left) side, the axial protrusions have met and fused to form the hub (arrow) of a semicircular canal (asterisk). By looking at serial sections, we confirmed that nothing remained of the injected protrusion and its partner except the two low pimples visible in this section. On the injected side, the protrusions have regressed and no canal has been formed. Scale bar=160 μm.

**Fig. 10.** (A) The chondroitin-sulphate distribution in the inner ear at stage 46.5 as revealed by immunofluorescence with the CS56 antibody. (B) The pattern of cell nuclei revealed by DAPI staining. Note that there is CS56 immunoreactivity in the epithelium of an axial protrusion (arrow) and as expected in the cartilage around the ear but not in the core of the protrusion. Scale bar=50 μm.
developing otic capsule is strongly labelled as expected, since chondroitin sulphate is known to be the predominant GAG in cartilage matrix. By contrast, no immunostaining is seen in the core of the adjacent protrusion. The epithelium covering the protrusion, however, is strongly labelled; the rest of the otic epithelium is also labelled but not quite so strongly. Evidently chondroitin sulphate is absent, or occurs only at very low levels in the cores of the protrusions but is present in their epithelial covering.

**Epithelial cells at the site of fusion are removed by cell death**

Sections of tadpoles fixed within a couple of hours after the moment of fusion show pycnotic cells and debris at the site of fusion (see Fig. 4B), clearly indicating that the majority of epithelial cells here are eliminated by cell death, although it is possible that a few may be retracted back into the epithelium as occurs in the mouse (P. Martin, personal communication). Through removal of cells at the site of fusion, the two opposing axial protrusions become joined into a single squat pillar, with a continuous core of extracellular matrix and a cladding of epithelium. The matrix, initially mostly acellular, soon becomes invaded by fibroblasts.

**The basal lamina appears discontinuous beneath the epithelium covering the tips of the axial protrusions**

Transmission E.M. (Fig. 11) reveals a continuous basal lamina beneath most of the otic epithelium and in particular beneath the epithelium at the bases of the axial protrusions. But towards the tips of the protrusions the basal lamina seems to be absent or discontinuous. The findings were similar (Fig. 12) when we stained sections by immunofluorescence with an anti-laminin antiserum. The basal lamina appears to be initially intact, but as the protrusion extends the lamina seems to break down under the apex of the protrusion while remaining intact elsewhere.

**Autoradiographs**

Which cells make the matrix in the cores of the axial protrusions — the cells of the epithelium or those of the mesenchyme? To find out, we examined, by autoradiography, the pattern of incorporation of tritiated glucosamine, a precursor that is incorporated into GAGs including hyaluronan and into glycoproteins. The labelled compound was supplied for 1 h at stage 46, when the anterior and posterior pairs of axial protrusions are about half-way in their outgrowth. Uptake by intact tadpoles was very weak irrespective of the medium used, but dissected tissue explants (comprising...
ears, hindbrain and associated ventral tissues) showed strong incorporation. In autoradiographs of specimens fixed immediately after pulse-labelling, silver grains were found concentrated over the epithelium of the protrusions, and present at a much lower density over the epithelium forming the rest of the otic vesicle (Fig. 13A). Immediately beneath the heavily labelled epithelium of the protrusion there seemed to be a thin zone of labelling of the extracellular matrix. Elsewhere in the cores of the protrusions and in the mesenchyme beneath them the labelling was scarcely above background levels. In autoradiographs of specimens in which the radioactively labelled medium was removed after one hour and replaced with nonradioactive medium for a further 20 h before fixation, fewer silver grains were seen over the epithelium of the protrusions but now there were silver grains over the whole of the extracellular-matrix cores of the protrusions (Fig. 13B). These observations strongly suggest that GAGs (presumably including hyaluronan) in the cores of the axial protrusions are made by the epithelium, and that the epithelium of the protrusion is distinguished from the rest of the otic epithelium by a high rate of incorporation of glucosamine into GAGs. This fits in with the histological picture: if the mesenchyme cells were making the matrix, one would expect them to be embedded in it, whereas in fact the material in the cores of the protrusions is largely acellular.

**Discussion**

We have demonstrated that formation of the semicircular canals depends on movements of the otic epithelium that are driven by localised accumulation of extracellular matrix at specific sites in each ear. The matrix at these locations occupies a cell-free space between the epithelium and its underlying mesenchyme, causing the two to separate and thereby forcing the epithelium to protrude into the lumen of the vesicle. Hyaluronan is an essential component of this matrix: when the hyaluronan is destroyed enzymatically, the cell-free space disappears, the protrusion collapses, and the corresponding semicircular canal fails to form. From the pattern of incorporation of glucosamine, it appears that the matrix in the protrusions is made by the epithelium: localised GAG synthesis by the epithelium seems to provide the driving force for the localised epithelial movement.

**Comparison with previous observations of ear development**

While many authors have described the morphology of semicircular canal formation, only a few (von Noorden, 1883; Krause, 1903; Hertwig 1987 and Waterman and Bell, 1984) have commented on the accumulation of extracellular matrix beneath the developing axial protrusions. Waterman and Bell (1984) have reported in the zebrafish a pattern of events very similar to that in *Xenopus*; but while they note the presence of an acellular matrix in the cores of the protrusions they do not investigate its composition. They do suggest, however, that the matrix is made by the epithelium at the tips of the protrusions, which they find to be rich in rough endoplasmic reticulum, and they note the absence of basal lamina here. McPhee *et al.* (1987) have shown that hyaluronan is synthesized by the inner ear of...
the mouse, with a peak at E12.5, approximately the time when semicircular canals are forming, although they have not reported the distribution of the hyaluronan or related it to semicircular canal formation. We ourselves see, in analogy with Xenopus, cell-free, matrix-filled spaces at sites of semicircular canal formation in the chick embryo (unpublished data); such spaces are also visible in Knowlton’s published photographs (1967), though she makes no comment on them.

**Hyaluronan as an appropellant for epithelial morphogenesis**

Hyaluronan is a linear glycosaminoglycan polymer that differs fundamentally from other GAGs in respect of its giant size, the disproportionately large volume that it occupies, and its mode of synthesis. A single molecule at the upper end of the hyaluronan size range has a relative molecular mass \( M_r \) of about \( 4 \times 10^6 \), corresponding to \( 10^4 \) repetitions of the disaccharide \( N\)-acetyl-D-glucosamine +D-glucuronate, and forms a random-coil configuration with a diameter of about 0.5 \( \mu \) m (Comper and Laurent, 1978). Thus hyaluronan acts as a space-filler, and by synthesizing relatively small quantities of it a cell can quite efficiently push itself away from other structures. In particular, an epithelium sitting on a stationary substratum can propel itself away from that substratum by emitting hyaluronan basally.

For this function, it is necessary that the hyaluronan should not diffuse freely away from the site of its production. Hyaluronan is exceptional among the extracellular polymers found in animal tissues in that it is not accumulated inside the cell and then secreted, but rather is spun out from the cell surface by a synthetase located in the plasma membrane (Prehm, 1984). Throughout the synthetic process, the molecule is therefore tethered to the exterior of the cell. Once it has been released from the synthetase, the hyaluronan may be retained by binding to another cell-surface hyaluronan-binding protein such as the hyaluronan receptor, CD44 (Aruffo et al., 1990), or through sparse protein-mediated linkages to other components of the extracellular matrix (Underhill, 1989; Toole, 1990).

**Hyaluronan as an agent of morphogenesis in other systems**

Hyaluronan has been implicated in several other morphogenetic movements in embryos (Toole, 1981). In the developing chick cornea, for example, it accumulates between the external corneal epithelium and the corneal endothelium, creating a space between these layers that is then invaded by neural crest cells (Toole and Trelstad, 1971; Bard and Abbott, 1979). Similarly, it accumulates beside the neural tube and beneath the ektoderm of the very early rodent embryo, and creates a space through which neural crest cells will migrate (Pintar, 1978; Derby and Pintar, 1978; Morriss-Kay and Tuckett, 1986). It has also been suggested that hyaluronan plays some part in bringing about the closure of the neural tube (Morriss and Solursh, 1978; Schoenwolf and Smith, 1990; Copp and Bernfield, 1988; but see also Morriss-Kay and Tuckett, 1986) and in the moulding of the nasal folds (Burk, 1985). Its accumulation in the palatal shelves is thought to be necessary to drive their elevation to form the secondary palate (Pratt et al., 1973; Brinkley and Morris-Wiman, 1987a,b; Ferguson, 1988). In addition, it has a multitude of effects on cell differentiation and proliferation (Toole, 1990).

There is an especially close parallel between our system and the developing heart. The heart originates as a simple bilayered tube, which becomes subdivided into separate chambers through the formation of septa and valves. These are derived from the endocardial cushions (Markwald et al., 1981; Van Mierop et al., 1963), protrusions of the endocardial inner lining of the heart tube that extend into the lumen, analogous to the axial protrusions in the inner ear. The formation of the cushions is driven by localised accumulation of an acellular, avascular extracellular matrix, the cardiac jelly, which consists largely of hyaluronan (Markwald et al., 1978). When hyaluronidase is injected into the jelly (Nakamura and Manasek, 1981), the endocardial cushion collapses, just like the protrusions in the inner ear.

**Hyaluronan-driven morphogenesis is coupled with basal lamina breakdown and changes of epithelial cohesion**

There is a further parallel between our system and several of the others where epithelial morphogenesis involves hyaluronan accumulation: the movement of the epithelium as a coherent sheet is followed by a rearrangement of the contacts between the epithelial cells. Thus in the inner ear, when the tips of a pair of protrusions meet, they adhere and the epithelium at the site of contact breaks down and disappears to allow continuity between the core of one protrusion and that of the other. This means that the epithelial cells must form new junctions at their luminal surfaces with their opposite numbers on the other protrusion and must lose their old lateral attachments to their original neighbours on the same protrusion. A similar rearrangement of epithelial cell contacts occurs in the heart when two endocardial cushions meet and fuse to form a septum (Hay, 1978; Kinsella and Fitzharris, 1980), and during neurulation, when the neural folds come together and fuse (Schoenwolf and Alvarez, 1989), and in the development of the palate, when the two palatal shelves meet and fuse (Fitchett and Hay, 1989). We have shown here for the axial protrusions in the ear, and others have shown for the other systems listed, that the epithelial rearrangements are accompanied by a local breakdown of the basal lamina. Moreover, in each of the latter three systems, some cells are released from the epithelium, become mesenchymal and migrate into the hyaluronan-rich matrix (Markwald et al., 1978; Bernanke and Orkin, 1984; Krug et al., 1985; Fitchett and Hay, 1989; Pintar, 1978). Thus there seems to be some general correlation between hyaluronan production, dissolution of the basal lamina, and changes in epithelial cohesion.

In our system, one can see a plausible mechanism...
that might explain this coupling: if the epithelial cells are secreting large volumes of hyaluronan at their basal surface, they can hardly fail to push away or at least disrupt the basal lamina on which they sit. Since contact with the basal lamina is thought to be important in maintaining the polarity of epithelial cells and coordinating their organisation, one would expect a breakdown of epithelial structure to be a common result. Of course, other factors such as TGF-β (Potts and Runyan, 1989; Fitzpatrick et al. 1990) and chondroitin sulphate (Morriss-Kay and Tuckett, 1989) may also play a part in such transformations.

The ear, the heart, the neural tube and crest, and the secondary palate are at first sight radically different structures. Yet in all of them we find a similar pattern of events, in which localised hyaluronan accumulation goes hand in hand with, and in the former two systems appears to cause, a localised movement of epithelium as a coherent sheet, and this movement is followed by a change of cohesiveness and a rearrangement of the epithelial cell–cell contacts. This hints at the existence of a common developmental subroutine that involves hyaluronan production and is evoked at many different sites of epithelial morphogenesis in the embryo.

The example of semicircular canal development shows especially clearly how, for such systems, the problem of explaining the pattern of morphogenetic movement reduces to a problem of chemical pattern formation. To understand how the labyrinth acquires its strange shape, we have now to discover what causes cells at a restricted set of sites in the epithelium of the otic vesicle to manufacture large quantities of hyaluronan.

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References


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