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Regeneration of Hair Cell Epithelia in the Chick and Salamander

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REGENERATION OF HAIR CELL EPITHELIA IN THE CHICK AND SALAMANDER

A Thesis
Presented to
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Western Kentucky University
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of the Requirements for the Degree
Master of Science

by
Kenneth B. Detwiler

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REGENERATION OF HAIR CELL EPITHELIA IN THE CHICK AND SALAMANDER

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Director of Thesis

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The acousticolateralis sensory system is characterized by a specific receptor cell type called the sensory hair cell and is found in all vertebrates. There are two types of hair cell sensory epithelia based on location: those of the inner ear, such as the organs for hearing and balance, and the lateral line system located within the epidermis.

In mammals, including humans, loss or damage of the hair cells of the auditory system results in permanent hearing loss. However, this is not the case with birds and amphibians. Amphibians, with a lateral line system, are capable of replacing lost or damaged hair cells or hair cell epithelia, called neuromasts. This researcher is concerned with identifying the molecular clues used by the axolotl salamander to regulate this regeneration.

Alcian blue staining was used to compare the extracellular matrix of normal and regenerating neuromasts. Alcian blue staining at two different pHs can distinguish between highly sulfated and non-sulfated forms of glycosaminoglycans and proteoglycans. No differences could be found between control and regenerating hair cell epithelia. These results indicate that the level of sulfation is not involved in hair cell regeneration in the axolotl; however, it does not rule out a role for the extracellular matrix in this process, but rather suggests that more specific probes are needed for further investigation.
INTRODUCTION

Hair Cell Epithelia

The auditory systems of mammals and birds, as well as the lateral line systems of aquatic amphibians and fish, contain specialized sensory receptor cells called “hair cells” that respond to horizontal vibrations in the environment. In humans and other vertebrates, these epithelia are located in the inner ear. Within the inner ear, the sensory epithelia are found in two compartments: the cochlea, which is responsible for detecting sound, and the semicircular canals, which are responsible for detecting head position and motion. In addition to the inner ear hair cell epithelia, aquatic vertebrates, such as fish and amphibia, have an epidermally located sensory epithelium called the lateral line system.

The lateral line sensory epithelia are organized as discrete organs called neuromasts. In the axolotl salamander, *Ambystoma mexicanum*, these organs are located within the epidermis and function to detect vibrations in the water. The neuromasts are grouped in stitches (Harris and Milne, 1966) with three to seven organs in each stitch. The stitches are arranged in a system consisting of several rows on the head and three rows along the body. On the tail, only one row is present, which is a continuation of the middle body row (Jorgensen and Flock, 1973).

The chicken’s cochlear duct is a slightly curved finger-like epithelial tube, which is composed of three major components: the basilar papilla, the tegmentum vasculosum and the macula (Tanaka and Smith, 1978). The basilar papilla contains two major types of hair cells, tall hair cells and short hair cells (Tanaka and Smith, 1978). These hair cells contain stereocilia which make contact with an acellular mass known as the tectorial membrane. As sound waves make their way into the inner ear, their energy causes the basilar membrane to vibrate. This vibration of the basilar papilla causes a shearing motion.
relative to the overlying tectorial membrane, triggering the hair cells to produce electrical impulses that are conveyed to the brain and interpreted as sound (Travis, 1992).

**Commonalities of Hair Cell Epithelia**

The lateral line system of the axolotl salamander, *Ambystoma mexicanum*, and the sensory epithelia of the inner ear of the chicken, have very similar sensory organs in regard to cell type and structure. Both systems contain two types of cells: sensory hair cells and supporting cells.

In the salamander, the flask-shaped sensory hair cells are about 40 μm long and 10 μm wide and interposed between surrounding supporting cells, to which they are joined by desmosomes (Flock, 1965). According to Flock (1965), a bundle of sensory hairs protrudes into the overlying cupula from the apical surface of each hair cell. Several nerve endings make contact with the basal region of the hair cell. Each hair cell contains an oval-shaped nucleus that is located in the middle of the cell. Bundles of modified microvilli, containing an almost crystalline arrangement of actin, protrude from the apical surface of each hair cell. These modified microvilli are referred to as stereocilia. Each hair cell is composed of 40-50 stereocilia and one true cilium, called the kinocilium, which is located in the periphery of the bundle (Flock, 1965). The stereocilia are arranged in rows with the length increasing stepwise towards the kinocilium. The kinocilium is a typical nine plus two arrangement of microtubules (Flock, 1965). Each hair cell is innervated by several nerve endings that make contact with the cell at its basal surface.

In the lateral line system, hair cells are surrounded by supporting cells, which form desmosome cell junctions with the hair cells. The structure of the supporting cells suggests they are secretory in nature, due to the size and appearance of the endoplasmic reticulum (Hama, 1962; Pomes-Delaveuve 1964). It is also believed that the supporting cells secrete the cupular substance from their apical surface (Flock, 1965). In 1937, Denny described the cupula as a honeycomb framework derived from the borders of the supporting cells. Flock (1965) observed that a dense cupular matrix extends above each sensory hair cell with the sensory hair bundle interwoven through the stereocilia. Deflection of the cupula causes a proportional deflection of the contained stereocilia and depolarization of the hair cell.
The structure of the chicken hair cell epithelium is very similar to the lateral line system. The hair cells have a bundle of stereocilia arranged in a close pack that projects into the tectorial membrane from the cells' apical surface. The basilar papilla of the chick contains two types of hair cells: Tall hair cells and short hair cells. Tall hair cells are columnar in shape and approximately 20 μm in height (Tanaka and Smith, 1978) with a round nucleus located in the center of the cell. The short hair cells are pitcher-shaped and hemispherical, also containing a round nucleus located in the center of the cell (Tanaka and Smith, 1978). As with the lateral line hair cells, stereocilia project from the apical surface of the sensory hair cells. Also the stereocilia are arranged in step-like rows with the tallest stereocilia on the side of and adjacent to the kinocilium. Tanaka and Smith (1978) reported the number of stereocilia per hair bundle to be about 50-90, which is similar to the number seen in the salamander. However they did report that the chicken kinocilium did not have the typical nine plus two arrangement, as seen in the axolotl. The chicken’s kinocilium contains a variable number of outer microtubules and one central microtubule. As in the axolotl lateral line, the hair cells of the chicken are innervated with dendritic processes of the spinoid ganglion (Tanaka and Smith, 1978). Supporting cells are also present in sensory epithelium of the chicken. They are found surrounding the hair cells. These cells are quite similar to those found in the lateral line system.

Developmental Origin

In 1903, Harrison determined that the lateral line organs originate from a cephalic ectodermal thickening that he termed a placode. That placode produces a migratory primordium of cells that move posteriorly along the embryo’s flank, laying down the precursors to the cells of the lateral line neuromasts. In 1933, Stone found that the lateral line primordium migrates beneath the two strata in the epidermis of the embryo, which causes the epidermis to bulge outward where the primordium is passing. The primordium deposits bead-like groups of cells along its path of migration, and those cells give rise to the sensory and supporting cells of the neuromast organs (Jones and Corwin, 1993). By removing a patch of skin or amputating a segment of the tail that contained neuromasts, Stone (1937) investigated regenerative events that led to their replacement. He concluded
that regenerated neuromasts originated through a budding process.

**Regeneration**

In mammals and birds, the auditory hair cells of the ear are normally produced only during embryonic life. This process had been suspected from counts of standing populations of hair cells in the cochlea at different ages (Tinley et al., 1986) and was confirmed through radioactive tracing of DNA replication preceding the terminal mitoses that produce hair cells (Katayama and Corwin, 1989). The medical implication is that damage to the mammalian receptor cells results in hearing loss that is irreversible. The "nerve" deafness can be caused by acoustic over-stimulation, by antibiotic toxicity, or by other agents. More than 18 million Americans suffer from some form of deafness or hearing loss for which there is currently no cure (Travis, 1992). About 80% of these cases are due to sensorineural deafness, more often caused by the destruction of the sensory hair cells of the cochlea (Travis, 1992). Sensorineural deafness is caused by the destruction of the sensory receptor cells of hearing, the sensory hair cells. Hair cells are produced throughout life in the ears and the lateral line organs of fish and amphibia. In many of those animals, inner ear epithelia grow mainly through the addition of cells at the outer edges so populations can continually increase. Such epithelia are known to replace lost or damaged hair cells, because they are capable of producing new hair cells and making new neural connections (Corwin, 1985, 1986). Regeneration of that sort occurs in the lateral line systems of salamanders, where new hair cell epithelia are produced on demand when preexisting epithelia have been extirpated (Stone, 1933). On the other hand, it is known that regeneration of hair cells is not limited to fish and amphibia. In the ears of birds that have lost hair cells after acoustic over-stimulation (Cotanche, 1987) or as a result of ototoxic antibiotic treatment (Cruz et al., 1987), the epithelium is capable of replacing lost hair cells by regeneration.

In the lateral line system of the axolotl salamander, Balak et al. (1990) investigated the mechanisms that lead to the production of sensory hair cells during regeneration. They used two different procedures to deplete preexisting hair cells in individual neuromast sensory epithelial. The two methods of hair cell depletion were phototoxicity, using a
solution of DASPEI, which labels only the hair cells of the lateral line sensory epithelia, and laser ablation, in which only the sensory hair cells were subjected to a laser pulse to destroy existing hair cells.

After a one-hour treatment of the hair cells with DASPEI and high intensity light, the hair cells were killed and ejected from the epithelium. Loss of the hair cells resulted in a stimulation of supporting cell division. Some of the progeny of these divisions differentiated into new hair cells. The first regenerated hair cells appeared by day 6, and new hair cells were added approximately one per day over the 15 days (Balak et al., 1990).

With the use of the laser ablation method, hair cells from the lateral line were eradicated within 15 minutes and the first hair cell to be regenerated extended a kinocilium and a small bundle of stereocilia in just 3 days after laser treatment (Balak et al., 1990). In most cases, the first stereocilia of regenerated hair cells appeared 5 days after laser ablation.

It has been suggested that supporting cells are progenitors of regenerated hair cells. Following the elimination of hair cells from a neuromast, the supporting cells of that neuromast begin to divide with increased frequency, eventually giving rise to cells that differentiate as replacement hair cells. According to Balak et al. (1990), supporting cells in normal, undamaged lateral line sensory epithelia normally divide much less frequently than neuromasts in which the hair cells have been eliminated.

In the avian inner ear, hair cell regeneration after ototoxic treatment has also been clearly documented. Ototoxic agents, such as gentamicin, have resulted in loss and subsequent recovery of hair cells in the sensory epithelium. DNA labeling studies (Corwin and Cotanche, 1988) have provided convincing evidence that most recovery is due to cell division and differentiation of new hair cells. Also, the newly regenerated hair cells approach normal number (Ryals and Rubel, 1988) and show ultrastructural characteristics of normal hair cells (Ducert and Rubel, 1990), and even restore function (Tucci and Rubel, 1990). In an experiment by Janas et al. (1995), 16 chicks received a single subcutaneous injection of gentamicin on post-hatch days 0-1, and then divided into 3 survival groups: short term (3-5 days; n=6), intermediate term (2 weeks; n=6) and long term (5 weeks;
Several chicks from each group were treated with tritiated thymidine to observe actively dividing cells. In the short-term survival group, the cochlea showed damage from the single injection of gentamicin. Remnants of hair cells with disrupted stereocilia bundles could not be identified. New hair cells could be identified throughout the damaged segment where hair cells were once seen. In the intermediate term survival group (2 weeks), the cochlea of gentamicin treated chicks showed a return to normal appearance with some evidence of previous damage. Immature appearing hair cells were still present after 2 weeks. The long-term survival group at five weeks showed the appearance of maturing hair cells, although some evidence of previous damage still remained. Counts of the hair cells showed a return to numbers comparable to controls. The chicks that received the radioactive label of tritiated thymidine showed an abundance of labeled supporting cells and occasional hair cells throughout the damaged region, suggesting the supporting cells are possible progenitors of hair cell regeneration.

The extracellular matrix (ECM) has been implicated in a variety of cellular processes, such as cell shape, cell proliferation, and cell migration during both development and regeneration (Gilbert, 1991). The ECM is composed of glycoproteins, glycosaminoglycans (GAGs) and proteoglycans (PGs). Glycoproteins are high molecular weight proteins to which are attached small polysaccharide chains. The most ubiquitous and abundant ECM glycoproteins are fibronectin, laminin and the collagens. GAGs are large polysaccharides composed of repeating disaccharide units (Hook, et al. 1986). The most common GAGs are the chondroitin sulfates, heparin and dermatin sulfates. PGs are composed of a GAG attached to a core polypeptide. The PG families are continuing to grow and show both ubiquitous and tissue restricted distributions (Hay, 1991). For example, brevican and neurocan are primarily found in the central nervous system, whereas the syndeans (types 1-4), versican and the aggrecan family are more widely distributed (Margolis and Margolis, 1994; Lebaron, 1996; Bernfield et al., 1992; Yamada et al., 1994).

Recently, a number of studies have identified a role for GAGs and PGs in regeneration and response to injury. Chondroitin sulfate has been identified during regeneration of the sciatic nerve in the mouse and of the retinal axons of the goldfish
(Challocombe and Elam, 1990; Braunewell et al., 1995). Chondroitin sulfate and other GAGs and the glycoprotein tenascin have been found to be up-regulated during repair of the central nervous system (Lips et al., 1995; Stichel et al., 1995). Finally, chondroitin and heparin sulfates have been found to correlate with spinal cord repair in the embryonic chick (Dow et al., 1994). These studies indicate a growing appreciation of the importance of GAGs and PGs, especially those containing chondroitin sulfate in regenerative and injury responses.

In contrast to the above cited studies on the role of various GAGs and PGs on development and regeneration, only a single investigation has been done to examine the role of sulfation of GAGs in their functional role (Davies et al., 1995). This investigation found that two GAGs, heparin sulfate and chondroitin sulfate, were expressed during the development of the embryonic kidney, *in vitro*. To investigate the role of sulfation in this process they used the GAG sulfation inhibitor sodium chlorate. From these studies they found that both enteric bud epithelium growth and branching were inhibited by desulfation of GAGs and PGs.

The experiments described below were designed to investigate the role of sulfated GAGs and PGs in regeneration of hair cell epithelia. The studies initially were to investigate hair cell regeneration of the chick basilar papilla, however our attempts to use aminoglycoside hair cell depletion consistently resulted in the death of the animals. The hair cell epithelial of h4e axolotl salamander lateral line system was used instead for most of the experiments. The large human population that is affected by hearing loss due to hair cell damage, which is considered irreversible, provides a very compelling reason to seek those mechanisms.
MATERIALS AND METHODS

**Chickens.** One to two-day old chicks were obtained from Lowe’s Hatchery and maintained in an incubator until ready for use. They were fed and watered daily until ready for histological preparations. On the day of sacrifice, the chicks were asphyxiated in a CO₂ chamber and then their heads were removed and cochlea dissected out.

**Axolotls.** Axolotl salamanders (*Ambystoma mexicanum*) were obtained from the Axolotl Colony, University of Indiana. Axolotls between 2 and 4 cm in length were used for these experiments. They were maintained individually in plastic cups in Holtfreter’s solution and fed twice a week on freshly hatched brineshrimp (Duhon, 1996).

To induce hair cell epithelium regeneration tail tip amputations were performed as described previously (Stone, 1933). Briefly, axolotls were anesthetized in benzocaine/Holtfreter’s solution. The tip of the tail, containing 3 neuromasts, was amputated with a sharp, sterile razor blade. The axolotl was returned to Holtfreter’s solution to recover and examined the next day using bright-field microscopy. One day after tail tip amputation, wound healing is complete and tail regeneration begins over the next 2 days with the appearance of a regenerating blastema. Hair cell epithelium regeneration was allowed to proceed for 6 days, about the time when the first regenerated hair cells begin to appear (Balak et al. 1990). The axolotls were then anesthetized and the tail tip containing the regenerating neuromast was amputated and processed for histological examination as describe below.

**Histological Preparations.** After dissection, tissues were immediately fixed in Bouin’s
primary fixative for 12-24 hours at 4° C. The tissues were dehydrated in graded ethanol solutions of 30%, 50%, 70%, 80%, 95% and 100%. The tissues were washed in xylene, twice for 15 minutes each, to remove the ethanol and all traces of water. The tissues were embedded in liquid paraffin at 56-58° C, twice for 30 minutes each, and then transferred into tear-away paraffin molds containing fresh paraffin and allowed to solidify overnight at room temperature. Sectioning of the tissues was carried out on a Spencer “820” microtome. Cross-sectional sections of the tissue were cut at a thickness of 20 micrometers. Sections were collected on Myer’s protein albumin subbed slides with degassed water and allowed to dry overnight on a slide warmer at 40-45° C.

**Histological Staining.** A light microscopy study of the chick sensory epithelium and the axolotl lateral line system was carried out using three histological stains. Toluidine blue, Alcian blue, pH 1.0, and Alcian blue, pH 2.5. Toluidine blue stains nuclei dark blue and the cytoplasm light blue, while Alcian blue at pH 1.0 will preferentially stain sulfated proteoglycans light blue, and at pH 2.5, it will stain acidic proteoglycans a light blue color. The sections were deparaffinized on slides by two changes in xylene for 15 minutes each. The tissue sections were then rehydrated in graded ethanol series of 100%, 95%, 70%, 50% and then deionized water (dH₂O), twice for 5 minutes in each solution. The sections were then stained in 0.1% Toluidine blue in dH₂O for 5 minutes. The slides were then dehydrated in graded ethanol series for 5 minutes each and then cleared in xylene. The Toluidine blue stained slides were coverslipped using Eukitt mounting medium. The Eukitt was allowed to solidify overnight at room temperature prior to viewing with a light microscope.

Alcian blue staining at pH 1.0 and pH 2.5 was also performed on sections in a similar manner. The sections were deparaffinized and rehydrated to dH₂O in the same manner stated above. Once rehydrated, separate sections were stained in either 1% Alcian blue 8GCX solution in 0.1 N hydrochloric acid (pH 1.0 staining) or a 1% Alcian blue 8GCX solution in 3% acetic acid (pH 2.5 staining). The sections were stained in their respective dye for 30 minutes. After staining, the Alcian blue, pH 1.0 sections were washed in 0.1 N HCl and the Alcian blue, pH 2.5 sections were washed in 3% acetic acid.
Each was then drained, dehydrated through a graded series of ethanol and cleared in xylene. Coverslipping was done overnight with Eukitt mounting medium at room temperature and allowed to solidify overnight prior to viewing.

**Preparation of Probes for in situ hybridization.** The positive control probe for *in situ* hybridization was chick β-actin, which is represented in a moderately high level in the chicken. Chick β-actin was isolated from a pBluescript expression vector using the restriction enzyme EcoRI (Sigma). The 300 base pair β-actin insert was isolated after digestion using agarose gel electrophoresis and electroelution techniques followed by phenol/chloroform purification. As a negative control, the bacterial transcript pBR328 was used and was purchased in purified form from Boehringer Mannheim.

**Labeling of Probes for in situ hybridization.** The chick β-actin and pBR328 probes were labeled by random primed incorporation of digoxigenin-labeled deoxyuridinetriphosphate (dUTP). The kit was purchased from Boehringer Mannheim Biochemica. The purified DNA probes were denatured by heating in a boiling water bath (100° C) for 10 minutes and chilled quickly on ice. Complete denaturation is essential for efficient labeling. To 1.0 μg of DNA, the following were added as prescribed by the kit: 2 μL hexanucleotide mixture, 2 μL dNTP labeling mixture and 1 μL Klenow enzyme. The contents were then quickly micro-centrifuged and incubated for 60 minutes at 37° C. The labeling reaction was stopped by the addition of 2 μL of 0.2 M EDTA, pH 8.0. The labeled DNA was precipitated with 2.5 μL of 4 M LiCl and 75 μL prechilled ethanol. The precipitation took place at -70° C for 30 minutes. Once precipitated, the labeled DNAs were centrifuged at 12,000 x g and the pellets were washed with cold 70% ethanol, dried under a vacuum and dissolved in 50 mL TE (10 mM Tris HCl, pH 8.0; 1 mM EDTA) buffer. A dot blot was done to estimate the yield of DIG-labeled DNA to confirm that the labeling reaction had been successful in producing a DIG-labeled DNA probe. Serial dilutions of a supplied labeled control DNA were made at the following concentrations: 1.0 ng/μL, 100 pg/μL, 10 pg/μL, 1.0 pg/μL and 0.1 pg/μL. 1.0 μL of each dilution was spotted onto a positively charged nylon membrane. Serial dilutions of the newly labeled
experimental DNA probes were made in the same manner as the control labeled DNA. Each experimental dilution was spotted on the same nylon membrane underneath its corresponding control dilution. The DNAs were fixed to the membrane by baking for 30 minutes at +80°C. The membrane was then wet with Buffer 1 (100 mM Tris-HCl, 150 mM NaCl; pH 7.5). The membrane was incubated for 5 minutes at room temperature in Buffer 2 (2% (w/v) Blocking Reagent (Boehringer Mannheim) dissolved in Buffer 1). Antibody linking was carried out by incubating the membrane in diluted (1:5000) anti-DIG alkaline phosphatase in Buffer 2 for 5 minutes at room temperature. After incubation, the membrane was washed twice, 5 minutes per wash in Buffer 1 at room temperature. The membrane was then incubated in Buffer 3 (100 mM HCl, 100 mM NaCl, 50 mM MgCl$_2$; pH 9.5). Application of this buffer activates the alkaline phosphatase that is conjugated to the antibody. After 2 minutes, Buffer 3 was poured off and the Color Substrate Solution, 45 μL NBT (75 mg/mL nitroblue tetrazolium salt in 70% (v/v) dimethyformamide) and 35 μL X-phosphate solution (50 mg/mL 5-bromo-4-chloro-3 indolyl phosphate (x-phosphate), toluidinium salt in 100% dimethylformamide). The color was allowed to develop in the dark for 30-60 minutes. The spot intensities of the control and experimental samples were compared and the concentration of the experimental probes was calculated.

**In Situ Hybridization.**

**Preparation of paraffin-embedded tissue sections for hybridization.** Slides containing sections of chick cochlea were deparaffinized in two changes of xylene for 15 minutes each. The sections were then rehydrated in a graded ethanol series of 100%, 80%, 50%, dH$_2$O and PBS (100 mM Sodium Phosphate, pH 7.5; 150 mM NaCl) for 2 minutes in each. The sections were then covered with 10 μL/section of Proteinase K [10 μg/mL] and incubated at 37°C in a humidity chamber for 5 minutes. Sections were washed in 0.2% glycine/PBS for one minute and then PBS for 2 minutes. The tissues were post-fixed on the slide in 4% paraformaldehyde/PBS for 10 minutes at room temperature and then washed in PBS for 5 minutes. The tissues were washed again in 0.2% acetic anhydride/0.1 M triethanolamine, pH 8.0 for 10 minutes at room temperature and then
washed in PBS for 5 minutes and 2X SSC for 3 minutes.

**Hybridization.** Each section was covered with prehybridization solution (4X SSC, 1X Denhardt’s (1% (w/v) of Ficol, Polyvinylpyrrolidone and Bovine Serum Albumin Fraction V), 50% Formamide, 5% Dextran Sulfate, 250 µg/mL Salmon Sperm DNA, 150 µg/mL Yeast tRNA and dH₂O) and slides were placed in a humidity chamber and incubated at 42° C for one hour. After prehybridization incubation, the solution was removed and replaced with hybridization solution (prehybridization solution with [5 ng/µL labeled probe). Siliconized coverslips were placed over the hybridizing sections and placed in a humidity chamber in an oven at +90° C for 5 minutes and then quenched at 4° C by placing the chamber in an ice bath for 5 minutes. Incubation of the hybridization reaction was carried out at 42° C for 16-20 hours.

**Washing.** Washes were carried out by placing slides in vertical slide dishes with prewarmed 2X SSC at 42° C. Slides were washed three times for 15 minutes each in 1X SSC at 42° C, three times for 15 minutes each in 0.2X SSC at 42° C and then in TBS (0.1 M Tris HCl, pH 7.5; 0.15 M NaCl) for two minutes at room temperature. The slides were then incubated with blocking solution (2% Blocking Reagent in 0.1 M Tris-HCl, pH 7.5 and 0.15 M NaCl) for 30 minutes at room temperature.

**Detection of probe.** The slides were covered with a 1:400 dilution of anti-digoxigenin antibody-AP conjugate in blocking solution and incubated in the humidity chamber at room temperature for 2 hours. The sections were then washed in TBS 3 times for 10 minutes each and then in AP substrate buffer two times for 5 minutes each at room temperature. The slides were then incubated in the dark with the Color Substrate Solution for 8 hours. The colorimetric reaction was stopped by washing the slides in TE buffer for 5 minutes at room temperature. Sections were then counterstained in 0.1% Toluidine blue for 1 minute and dehydrated. After clearing in 2 changes of xylene, slides were coverslipped with Eukitt mounting medium.
RESULTS

To better understand the structure of the chick sensory epithelium and axolotl lateral line systems, light microscopy observations were made with Toluidine blue to identify and compare specific structures within each system. Toluidine blue is a basophilic dye that stains the nucleus dark blue, while staining the cytoplasm light blue. Figure 1 shows the cross-sections of the chick cochlea with a row of hair cells (HC) above a lower row of supporting cells (SC). The tectorial membrane (TM) is intact directly above the top row of hair cells. The approximate length of the epithelium is 1.8 millimeters, with approximately 40 hair cells. The basilar papilla is crescent shaped and is composed of both sensory and supporting cells situated on a fibrous basilar lamina (BL). The tegmentum vasculosum (TV) is also shown intact.

A study was also performed on normal chick cochlea tissue using Alcian blue. Alcian blue detects glycosaminoglycans (GAGs) and proteoglycans (PGs). At pH 1.0 it stains sulfated GAGs and PGs, while at pH 2.5 it stains many acidic GAGs and PGs. Figure 2A shows a cross section of normal chick cochlear tissue stained with Alcian blue, pH 1.0 and Figure 2B show the same tissue stained with Alcian blue, pH 2.5. Figure 2A shows light blue staining of the basilar lamina (BL) with very little, if any staining of hair cells and supporting cells, thus indicating the presence of sulfated proteoglycans in the basilar lamina. Figure 2B shows much more intense staining of the basilar lamina (BL), including deep staining of the tectorial membrane (TM), which is not seen in Figure 2A. Also, there appears to be no preferential staining of the sensory hair cells or supporting cells.

Figure 3A and B are Toluidine stained sections of the axolotl tail region showing
Figure 1. Light micrograph of cross-section through the cochlea of a chicken showing the normal sensory epithelium. The sections were stained with Toluidine blue. With this stain the nuclei are dark blue and the cytoplasm a light blue color. A. 50X magnification view showing the cochlear duct. B. 125X magnification view of normal chicken cochlear duct with hair cells (HC) supporting cells (SC) tectorial membrane (TM) basilar lamina (BL) and tegmentum vasculosum (TV).
Figure 2. Light micrograph of a cross-section through the cochlea of a chicken showing the normal sensory epithelium stained with Alcian blue. A. 125X magnification view stained with Alcian blue pH 1.0. B. 125X magnification view stained with Alcian blue pH 2.5. (HC) hair cells (SC) supporting cells (BL) basilar lamina.
Figure 3. Light micrograph of cross-section through the tail of an axolotl showing a normal neuromast. The sections were stained with Toluidine blue. With this stain the nuclei are dark blue and the cytoplasm a light blue color. A. 50X magnification view showing the neuromast organ (arrow heads) within the epidermis. B. 500X magnification view of a normal neuromast with hair cells (HC) and supporting cells (SC) and an underlying basilar lamina (BL). The arrow head indicates the cupula.
the structure of a normal neuromast organ. The neuromast organ consisting of hair cells (HC) and supporting cells (SC) is located within the epidermis. At larval stages the epidermis of the axolotl is only 1-2 cells thick with an underlying basal lamina. With Toluidine blue, the basal lamina (BL) is shown as a clear to opaque demarcation between the epidermis and the body cavity extending to the tail region.

Figure 4 shows sections through a normal developing larval stage axolotl tail region stained with Alcian blue at pH 2.5. The extracellular matrix of the body cavity of the tail is strongly stained. There appears to be little staining around any of the cells. Neither the hair cells (HC) nor supporting cells (SC) of the neuromast are stained. Also, there is strong staining of the surface layer of the epidermis at both pHs. There are differences in the staining of the basal lamina (BL) in the two preparations. The basal lamina at pH 2.5 is more darkly stained (Figure 4C) compared to the pH 1.0 (data not shown), indicating the presence of acidic GAGs and PGs.

Figures 5, 6 and 7 compare Alcian blue staining at pH 1.0 and pH 2.5 in regenerating tail tips in the region of a regenerating neuromast and placode. Figures 5A, 6A and 7A are photomicrographs of sections stained at pH 1.0, and Figures 5B, 6B and 7B are photomicrographs of sections stained at pH 2.5. Neuromast regeneration occurs concomitant with tail regeneration. These specimens were prepared at regeneration day 6 when a column of cells had moved out from the last neuromast of the tail stump and had formed a column of cells that subsequently migrated into the regenerating tail. This column of cells formed the regenerated neuromast called the regenerating placode (Corwin, 1986). Figures 5, 6 and 7 show Alcian blue stained sections at increasing magnifications and at different regions of the regenerating neuromast and placode.

The density of staining of Alcian blue in the extracellular matrix of the body cavity at pH 1.0 (Figure 5A) is much lighter than at pH 2.5 (Figure 5B) of the regenerating tail and compared to the normal non-regenerating tail (Figure 4). The reason could simply be an artifact of the staining or tissue preparation, though the same stain and procedure was used in all preparations. On the other hand, the difference could indicate a decrease in sulfated GAGs and PGs, though not likely. There is also less staining of the surface epidermis of the regenerating tail stains less at pH 1.0 (sulfated GAG and PG) than at pH
Figure 4. Light micrograph of a cross-section through the tail of an axolotl showing a normal neuromast stained with Alcian blue pH 2.5. A. 50X magnification view. B. 125X magnification view. C. 500X magnification view. Arrow heads indicate dark staining of the surface layer of the axolotl epidermis. The arrow indicates the very dark staining of the extracellular matrix of the peritoneal cavity. (HC) hair cell (SC) supporting cell (BL) basal lamina (NC) notochord (NM) neuromast.
Figure 5. A light micrograph of a cross-section through the tail of an axolotl showing a placode at regeneration day 6 on both sides of the tail (arrowheads). These figures are low magnification views (50X). A. Stained with Alcian blue pH 1.0. B. Stained with Alcian blue pH 2.5. (P) placode (NC) notochord (SC) spinal cord.
Figure 6. A light micrograph of a cross-section through the tail of an axolotl showing a neuromast at regeneration day 6 on one side of the tail. These figures are 125X magnification views. A. Stained with Alcian blue pH 1.0. B. Stained with Alcian blue pH 2.5. (HC) hair cell, (SC) supporting cell and basilar lamina (BL). Arrowhead indicates staining of the surface epidermis.
Figure 7. A light micrograph of a cross-section through the tail of an axolotl showing a placode at regeneration day 6 on one side of the tail. These figures are 500X magnification views). A. Stained with Alcian blue pH 1.0. B. Stained with Alcian blue pH 2.5. (P) regenerating placode (SC) spinal cord. The arrowheads indicate the position of the basal lamina (BL).
2.5. There is also less staining in the regenerating tail surface epidermis at pH 1.0 compared to the normal non-regenerating tail at the same pH. A similar difference is noted in the density of staining around the spinal cord (SC).

Figure 6A and B show cross sections through the regenerating neuromast stained with Alcian blue at pH 1.0 and pH 2.5, respectively. The hair cells (HC) and supporting cells (SC) are visible in both sections. But there is no significant difference in staining intensity of the basal lamina underlying this neuromast when compared with a control neuromast (Figure 4C).

Figure 7A and B show cross sections through the regenerating placode stained with Alcian blue at pH 1.0 (Figure 7A) and pH 2.5 (Figure 7B). As noted above, there is less staining throughout the section at pH 1.0 of the regenerating placode compared to the regenerating placode at pH 2.5 (Figure 8B) and the normal neuromast (Figures 4C). One observation that may be significant is the organization of the basal lamina (BL) in the region of the regenerating placode (P), a region where cells are migrating into the regenerating tail, compared to both the normal and regenerating neuromast (Figure 6A and 6B). The basal lamina (BL) underlying the regenerating placode is very disrupted compared to the neuromast basal lamina and the adjacent sub-epidermal basal lamina.

Figure 8 is a cross section of the normal chick cochlear duct analyzed by *in situ* hybridization. The tissue was probed with chick β-actin, which is found in virtually every cell. The hair cells (HC) and supporting cells (SC) are shown to be stained a purplish-brown. Identical observations were seen in the sections probed with a negative control (data not shown). It is quite possible that impure probe preparations and/or the type of tissue used for the development of an *in situ* protocol resulted in the lack of positive data.
Figure 8. Light micrograph of a cross-section through the cochlea of a chicken showing the normal sensory epithelium after in situ hybridization. A. 50X magnification view. Hair cell (HC) supporting cell (SC) endolymph (EN).
DISCUSSION

The aim of this research was to investigate the presence or absence of glycosaminoglycans (GAGs) and proteoglycans (PGs) in normal and regenerating hair cell epithelia and lateral line systems. Using dyes that stain for sulfated proteoglycans (Alcian blue, pH 1.0) and acidic proteoglycans (Alcian blue, pH 2.5), certain differences were observed, indicating a potential role for these molecules.

Upon comparison of the normal lateral line system with the regenerating system, it was noticed that Alcian blue, pH 1.0 (Figure 5A) stained the extracellular matrix of the body cavity in the regenerating tail much lighter than at pH 2.5 (Figure 5B) and compared to the normal non-regenerating tail (Figure 4). This difference could possibly represent the absence of or decreased amounts of sulfated GAGs and PGs during regeneration. Also, there is less staining at pH 1.0 (Figure 7A) of the regenerating placode compared to the regenerating placode at pH 2.5 (Figure 7B), again indicating the absence or decreased amounts of sulfated GAGs and PGs during regeneration.

Although the specificity of these dyes is limited to the type of molecule (sulfated vs. acidic GAGs and PGs) they can stain, there is good reason to investigate further specific GAGs and PGs to better understand the actual role they may play in regeneration.

Other research suggests alternative approaches that may be useful for examining the molecular mechanisms involved in regeneration. One study would be to examine specific components of the extracellular matrix during regeneration. The extracellular matrix (ECM) of all animals consists of a large variety of proteoglycans (PGs) and glycoproteins. All the proteoglycans consist of a core polypeptide attached to one of a
large number of different glycosaminoglycans (GAGs). The glycosaminoglycans are large polysaccharides consisting of repeated disaccharide units. All GAGs are attached to a polypeptide core and are consequently found only as PGs, except for the GAG hyaluronate. Previous studies of development and regeneration have suggested roles for particular components of the ECM in cellular processes such as cell migration, cell proliferation and differentiation (Hay, 1981).

The GAG hyaluronate has been implicated in cell migration and proliferation during development specifically in mesenchymal tissues (Mescher and Cox, 1988). It has been hypothesized that the hydration of this molecule creates spaces through which cells can migrate. This molecule also binds to a hyaluronate receptor on the cell surface of some cells facilitating cell adhesion and motility. This interaction has also been implicated in controlling cell differentiation in myogenesis and chondrogenesis. The synthesis and deposition of hyaluronate has also been observed to correlate with limb regeneration in the axolotl salamander and seems directly responsive to neural factors that are necessary for this regeneration. Mescher and Cox (1988) found that the synthesis and accumulation of hyaluronate was not present when neural innervation of the amputated limb stump was prevented and regeneration was prevented also. However, upon innervation, hyaluronate accumulated in the regenerating stump. From other studies, in vitro, hyaluronate has been shown to accumulate in response to a variety of growth factors. These studies suggest that the innervating nerve of the limb stump secretes a neural growth factor that causes an increase in hyaluronate, which directly affects cell proliferation, migration and possibly differentiation. However, further studies are needed to demonstrate this hypothesis.

Other GAGs and PGs have been implicated in both promoting and inhibiting regeneration. An investigation of axon regeneration within the mammalian central nervous system demonstrated that the accumulation of PGs had an inhibitory effect on axon outgrowth and migration in the adult (Davies, et al. 1997). However, the specific PGs involved were not identified.

The role of chondroitin sulfate in regeneration is unclear. In the amphibian and fish visual system, chondroitin sulfate promotes axon outgrowth and guidance during regeneration (Challacombe and Elam, 1997; Walz, et al. 1997). But in the regenerating
spinal cord of chicks, chondroitin sulfate was found to inhibit outgrowth, while heparin sulfate promoted this process (Dow et al. 1994). An investigation of axon regeneration in the rat brain found an up-regulation of various chondroitin sulfate proteoglycans at the site of the lesion, which correlated with an inhibition of regeneration (Stichel et al. 1995; Lips et al., 1995). On the other hand, a study of the mouse sciatic nerve regeneration show the up-regulation of chondroitin sulfate was correlated with this process (Braunewell et al., 1995). It is difficult to make any conclusions from these studies. They may indicate that there are different roles of chondroitin sulfate in different species and in the central nervous system and peripheral nervous system.

In addition to GAGs and PGs, the extracellular space also contains a variety of glycoproteins, two of which have recently been implicated in regeneration. Fibronectin is an adhesive extracellular glycoprotein that is used by migratory cells as a site of attachment. It has also been implicated in cell differentiation and proliferation (Hay, 1991). Two studies of regeneration, one in the salamander limb and the other in Hydra, have shown an up-regulation in the amount of fibronectin upon the initiation of regeneration (Nace and Tassava, 1995; Zhang et al., 1994). In limb regeneration, the fibronectin is associated with blastema cells, which are actively dividing cells that will produce the regenerated tissues. The other ECM glycoprotein is collagen type IV. This protein was found to be involved in regeneration of Hydra but has not been investigated in vertebrate regeneration. Such an investigation may be fruitful since this collagen binds to fibronectin and may play a supporting role with fibronectin in regeneration.

Another class of ECM molecules found in both developing and regenerating tissues is a group of proteases called the matrix metalloproteases (MMPs) (Werb et al., 1992). These molecules are thought to be involved in remodeling the ECM during development and have been implicated in such cellular activities as cell migration, cell differentiation and proliferation (Werb et al., 1992; Alexander and Werb, 1989). A recent study of salamander limb and tail regeneration found that one particular MMP, called MMP9, was expressed during regeneration and specifically during the early stages (Yang and Bryant, 1994). This finding suggests a role for this MMP in ECM remodeling during regeneration of these tissues.
The process of regeneration has been researched extensively in several animal systems; however the specific role of proteoglycans and glycosaminoglycans is still unclear. Some ECM molecules seem to be present during regeneration, as in the case of hyaluronate, which is present in the regenerating limb stump of the axolotl. In contrast, other GAGs and PGs have shown inhibitory properties. And in the case of chondroitin sulfate, it acts as a promoter and inhibitor, depending on the particular animal system. As mentioned above, the extracellular space also contains a variety of glycoproteins that have been shown to be involved in regeneration.

Obviously, there are many factors that may, or may not, contribute to regeneration. This research was designed to quickly and easily examine changes in the extracellular matrix during regeneration. The results do not show quantitative differences in GAGs and PGs; however, the interpretation should not be that there are no differences but rather that techniques need to be used to probe the specific composition of these broad classes of molecules.


