Comparison of MAP kinases in the proliferation of chick utricular epithelial cells

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COMPARISON OF MAP KINASES IN THE PROLIFERATION OF
CHICK UTRICULAR EPITHELIAL CELLS

by

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Abstract: Levels of activation of MAP kinase pathways and effects of inhibiting
these pathways were examined in chick utricular epithelial cultures in order to
determine the role of these pathways in proliferation.
Acknowledgements

“No one can whistle a symphony. It takes an orchestra to play it.” –H.E. Luccock

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<th>Description</th>
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<tr>
<td>ABR</td>
<td>auditory brainstem response</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal kinase</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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Introduction

The sensory epithelia of the avian inner ear have the capacity to regenerate after acoustic trauma or ototoxic injury (Cotanche, 1987; Cruz et al., 1987). Such regeneration is primarily the result of renewed supporting cell proliferation (Tsue et al., 1994; Warchol, 2007). The human inner ear lacks the ability to regenerate hair cells, and thus is permanently damaged by ototoxicity, aging, acoustic trauma, and other epithelial insults. Understanding the cellular signaling pathways involved in supporting cell proliferation is fundamental to developing treatments. This study examines the contribution of mitogen-activated protein kinases (MAPKs) toward the initiation of regenerative proliferation in chick utricular epithelia.

Mechanisms of hair cell regeneration

Proliferation of supporting cells gives rise to new hair cells in both the auditory and vestibular organs (reviewed by Matsui et al., 2005; Stone & Cotanche, 2007). Following hair cell injury, supporting cell proliferation rates increase and the daughter cells produced through mitosis differentiate into replacement hair cells or supporting cells. Auditory nerve fibers then connect to the new hair cells, leading to nearly complete functional recovery.

Hair cell regeneration may also occur through direct transdifferentiation of supporting cells. Roberson et al. (2004) reported that the earliest replacement hair cells (which are evident 4-6 days after gentamicin injection) are created via direct transdifferentiation. However, proliferation soon predominates as the primary means of regeneration. By ten days following gentamicin treatment, most regenerated hair cells are derived from supporting cell proliferation.

Proliferation has an advantage over direct transdifferentiation in that the ratio of hair cells to supporting cells is maintained. While transdifferentiation produces more immediate recovery,
one supporting cell is lost with the creation of every new hair cell. With proliferation, daughter
cells may become hair cells or supporting cells, allowing the sensory epithelium to maintain its
balance of hair cells and supporting cells.

Limited regeneration through supporting cell proliferation has also been reported in
mammalian utricles following aminoglycoside ototoxicity (Warchol et al., 1993). While this
finding provides hope for regeneration as a therapeutic target, more research is needed to
determine the mechanisms by which regenerative proliferation occurs. Currently, little is known
about the cell cycle control factors related to supporting cell proliferation.

Overview of mitogen-activated protein kinases

Mitogen-activated protein kinases (MAPKs) are a family of enzymes that phosphorylate
proteins, initiating cell-signaling cascades involved in cell-cycle entry. MAPKs are activated by
upstream kinase cascades located in the cytoplasm. These typically involve three protein kinases
organized by a scaffold protein. Each kinase in the cascade phosphorylates the next, culminating
in activation of the MAPK. Once activated, the MAPK may phosphorylate various proteins and
transcription factors, including those involved with cell-cycle control (Darnell, 2003).

There are three avian and mammalian MAPK pathways: extracellular signal-regulated
kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38. Each pathway contributes to specific
cellular responses. The particular response elicited by pathway activation is dependent on
several variables, including the intensity and duration of stimulation, the specific MAPK isoform
activated, the scaffold protein utilized, the pathway location within the cell, possible interaction
between MAPK pathways, and activity of other molecules within the cell (reviewed by Krishna
& Narang, 2008).
The extracellular signal-regulated kinase (ERK) subfamily is activated by a variety of stimuli, including growth factors, hormones, and neurotransmitters. The ERK cascade is involved in proliferation, cell cycle control (particularly G1-S phase transition), and differentiation (reviewed in Rubinfeld & Seger, 2005). Transient ERK activation promotes proliferation, while sustained activation induces differentiation (reviewed in Chambard et al., 2007).

The c-Jun NH2-terminal kinase (JNK) subfamily is activated in response to cellular stress, reactive oxygen species, cytokines, and growth factors. Its primary role is in stress-induced apoptosis, though it has also been implicated in proliferation, differentiation, and cell survival (reviewed in Vlahopoulos & Zoumpourlis, 2004). Ten JNK isoforms have been identified.

Six isoforms of p38 have been identified. Like JNK, the p38 subfamily is involved with inflammatory responses and cell death. Activation occurs in response to cytokines and extracellular stress. p38 plays a role in apoptosis, inflammation, cytokine production and differentiation (reviewed in Ono & Han, 2000). This pathway has also been associated with cell cycle arrest at the G1-S phase transition (reviewed in Zhang & Liu, 2002).

Purpose of the present study

While it is widely understood that the MAPK pathways play a role in cellular proliferation, there has not been a comprehensive comparison of the respective contribution of each kinase towards the regulation of proliferation in the avian inner ear. The aim of the study is to identify the relative activation of the three MAP kinase pathways and quantify changes in proliferation after blocking each pathway. The resulting data will contribute to growing
knowledge about the proliferative pathways in chick sensory epithelia. As the mechanisms of this process are discovered, researchers will be closer to developing treatment strategies for pathologies in the human inner ear.

MATERIALS AND METHODS

Preparation of epithelial cultures

Utricles were removed from chicks at 14-21 days post-hatch and incubated in thermolysin. The sensory epithelia were removed using a 30-gauge needle, then transferred to fibronectin- or laminin-coated culture wells containing Medium-199 with Earle’s salts and 10% fetal bovine serum. The epithelia were cut into 10-12 small pieces. The cultures were incubated at 37°C for three days, rinsed with Medium 199, and incubated an additional three days.

ERK, JNK, and p38 Pathway Quantification

The number of cells with activated forms of each kinase was assessed using immunocytochemistry. Data for each MAP kinase was obtained from eight separate cultures. Cultures were briefly rinsed with Medium-199, fixed for fifteen minutes in 4% paraformadehyde, and rinsed with phosphate buffered saline (PBS) five times over fifteen minutes. Prior to immunolabeling, cultures were treated for two hours with blocking solution that consisted of PBS with 0.2% Triton X-100 and 5% normal horse serum. Cultured supporting cells were labeled with rabbit anti-ERK (1:250, Cell Signaling Technologies, Beverly, MA, USA), rabbit anti-phospho-c-Jun (1:100, Cell Signaling Technologies), or rabbit anti-p38 (1:500,
Cell Signaling Technologies) antibodies in 2% normal horse serum and 1 mL 0.2% Triton-X (in PBS) were applied, and cultures were incubated overnight at 4°C.

The next day, cultures were rinsed five times over fifteen minutes with PBS. Secondary antibodies, consisting of cy3 anti-rabbit (1:500, GE Healthcare, Buckinghamshire, UK), Phalloidin (1:40, Invitrogen, Eugene, OR, USA), and DAPI (1:500, Sigma, St. Louis, MO, USA) in 0.2% Triton-X (in PBS) were applied over two hours at room temperature. Cultures were rinsed five times over fifteen minutes with PBS, and then coverslipped with 9:1 glycerol/PBS solution.

BrdU Immunocytochemistry

BrdU immunocytochemistry was used to examine the effects of inhibiting each MAP kinase pathway on supporting cell proliferation. In each experiment, four wells of dissociated chick utricular epithelia were treated with a small molecule inhibitor, and four wells acted as a control group. U0126 (10 μm, Calbiochem, La Jolla, CA, USA) was used to block ERK, SB203580 (20 μm, Calbiochem) to block p38, and SP600125 (10 μm, Calbiochem) to block JNK.

Cultures were incubated at 37°C for twenty hours. Each well was then treated with 3 μl/ml bromodeoxyuridine (BrdU, Sigma) to label cells in the S-phase of the cell cycle. Cultures were then incubated at 37°C for four hours. This procedure was repeated with three sets of cultures for each inhibitor, so that a total of twelve treated wells and twelve controls were obtained for each condition.

Cultures were rinsed with Medium-199 and fixed in 4% paraformadehyde for 15 minutes. Cultures were rinsed with PBS five times over fifteen minutes and treated with 100 μL
of 1N HCl for fifteen minutes to denature DNA. Cultures were then rinsed with PBS five times over fifteen minutes and treated for two hours with blocking solution containing 5% normal horse serum in 0.2% Triton-X (in PBS). Primary antibodies, consisting of 1:50 Anti-BrdU (BD Biosciences, San Jose, CA, USA) and 2% normal horse serum in 0.2% Triton-X (in PBS) were applied, and cultures were incubated overnight at 4°C.

The next day, cultures were rinsed five times over fifteen minutes with PBS and treated with secondary antibody (1:500 Alexa 488 anti-mouse (Invitrogen) and 1:500 DAPI in 0.2% Triton-X (in PBS)) over two hours at room temperature. Cultures were rinsed five times over fifteen minutes with PBS, then coverslipped with 9:1 glycerol/PBS solution.

**Imaging**

Images were obtained using a Nikon Eclipse TE2000-S immunofluorescence microscope equipped with a Q Imaging Retiga 1300 camera. Four to six images were obtained for each well using QCapture v2.8.1 software. Labeled cells were quantified using Image-Pro Plus v2.5.1.29 software. After quantification of the labeled cells in each image, the proliferation index for each treatment was calculated by dividing the number of BrdU-labeled cells by the number of DAPI-labeled cells. Statistical significance was assessed using a 2-tailed T-test, and was compared across treatment conditions.
RESULTS

Immunofluorescent microscopy revealed varying levels of cell density throughout each well. Images were obtained from regions bordering high-density zones, with attempt to minimize the amount of overlapping cells and maintain uniform cell density for each image.

Quantification of cells that were immunolabeled for activated members of the MAPK pathway revealed high levels of expression of ERK activation (98.83%, Figure 1A). Phospho-c-Jun was expressed in 5.66% of cells (Figure 1B), and p38 expression was rare (0.34%, Figure 1C).

Inhibiting these pathways had varying effects upon proliferation. Cultures treated with U0126 to block ERK contained 8.78 ± 5.25 BrdU+ cells/100,000 μm² (Figure 2A), while control cultures contained 21.66 ± 10.78 BrdU+ cells/100,000 μm² (Figure 2B). This treatment resulted in a statistically significant reduction in proliferation indices from 6.15% to 2.73% (p<0.001) (Graph 2).
Cultures treated with SP600125 to block JNK contained 19.2 ± 8.98 BrdU+ cells/100,000 μm² (Figure 3B), compared to 26.97 ± 11.31 BrdU+ cells/100,000 μm² in control wells (Figure 3A). Blocking JNK had a statistically significant effect on proliferation, reducing the percentage of BrdU-labeled cells from 11% to 7.66% (p<0.001) (Graph 3).

There was no statistically significant difference in proliferation between cultures treated with SB203580 to block p38 and control cultures (Graph 4). Control cultures contained 17.87 ± 8.52 BrdU+ cells/100,000 μm² (Figure 4A), while treated cultures contained 20.72 ± 10.26 BrdU+ cells/100,000 μm² (Figure 4B).

Proliferation of chick utricular epithelial cultures in control and MAPK-inhibited conditions. DAPI labeling (blue); BrdU labeling (red). Fig 2A: control culture, Fig 2B: ERK-inhibited. Fig 3A: control culture, Fig 3B: JNK-inhibited. Fig 4A: control culture, Fig 4B: p38-inhibited. Graphs 2, 3, and 4: Proliferation indices for control and treated cultures in ERK-, JNK-, and p38-inhibited conditions, respectively.
DISCUSSION

Hair cell regeneration in the inner ear occurs primarily through proliferation of supporting cells. MAP kinases are known to be involved in cellular proliferation, but their role in the inner ear is not yet understood. The purpose of this study was to examine the contribution of each MAP kinase pathway toward proliferation in chick utricular epithelia. The results obtained suggest ERK and JNK are key mediators in regulating this proliferation. Future research should further clarify the role of these kinases, as they have potential to become therapeutic targets for mammalian hair cell regeneration.

ERK

High levels of ERK expression were found in cultures of dissociated chick utricular epithelia. Blocking ERK with U0126 significantly reduced proliferation. This result supports findings by Witte et al. (2002), which reported that treating cultures with U0126 significantly reduced proliferation of chick supporting cells. Other ERK inhibitors, PD98059 and apigenin, also reduced S-phase entry in these cultures (Witte et al., 2002).

ERK inhibitors may also decrease proliferation of supporting cells in the avian basilar papilla (Bell and Oberholtzer, in press). In this study, researchers used forskolin to induce proliferation of auditory supporting cells in cultures treated with ERK inhibitors and control cultures. ERK-inhibited cultures had significantly fewer BrdU-labeled cells. Thus ERK may be important for proliferation in both the auditory and vestibular organs.
Phospho-c-Jun was expressed less frequently than ERK, and blocking JNK also reduced proliferation. JNK has been found to play a role in proliferation of other cell types, including human breast cancer cells (Mingo-Sion et al., 2004), rat hepatocytes (Schwabe et al., 2003), KB-3 human carcinoma cells (Du et al., 2004), and murine and human B-lymphoma cells (Gururajan et al., 2005). In each of these studies, inhibition of JNK with SP600125 reduced proliferation.

Studies of JNK in the inner ear have primarily focused on its role in apoptosis. Inhibition of JNK has been demonstrated to reduce hair cell death and hearing loss resulting from ototoxicity (Bodmer et al., 2002; Ylikoski et al., 2002; Sugahara et al., 2006), acoustic trauma (Wang et al., 2003; Coleman et al. 2007), and electrode insertion trauma (Eshraghi et al., 2007). JNK may be involved in both proliferation and apoptosis in the inner ear. Its role may depend on a variety of factors, including the time course of activation and cross-talk with other pathways.

The duration of MAPK pathway stimulation influences the cellular response (reviewed in Krishna & Narang, 2008). Within the JNK pathway, the length of activation may influence whether proliferation or apoptosis results. In a study of T-cells, Chen et al. (1996) found that transient JNK activation promoted proliferation, while prolonged activation resulted in increased apoptosis. It is possible that similar effects occur in supporting cells.

Cross-talk between JNK and ERK has been demonstrated to be responsible for proliferation of VEGF-stimulated bovine aortic endothelial cells (Pedram et al., 1998). This study demonstrated that, following VEGF stimulation, ERK activates upstream kinases in the JNK pathway, leading to activation of JNK. While ERK is necessary for JNK activation, it is JNK that is ultimately responsible for the majority of proliferation of these cells (Pedram et al., 1998). Further study is needed of MAPK pathway interaction in the inner ear.
Expression of p38 was comparatively low, and blocking p38 did not have a significant effect on proliferation. Although this suggests p38 does not have a primary role in avian supporting cell proliferation, this MAPK may still be a target for therapies aiming to ameliorate hair cell damage. A recent study by Tabuchi et al. (in press) implicates p38 in mammalian cochlear outer hair cell fate following acoustic overexposure. Acoustic overexposure activated p38 in murine cochlear hair cells, and inhibiting p38 with SB203580 diminished ABR threshold shifts following this insult. Similarly, Wei et al. (2005) found that gentamicin induced an upregulation of p38 in murine outer hair cells, and p38-inhibition with SB203580 protected hair cells from ototoxicity. Thus, while p38 may not be involved in supporting cell proliferation, further study of this kinase is warranted.

**Conclusion**

Together, these results suggest that ERK and JNK play key roles in the proliferation of chick utricular epithelia, while p38 alone does not regulate proliferation. Additional research involving multi-kinase inhibition is needed to establish whether these kinases together have additive or synergistic effects. Cross-talk between pathways should be examined, as well as the effects of duration of pathway activation. Future research should examine similarities and differences in the roles of the MAPK pathways in the chick basilar papilla. Finally, MAPK activation should be studied in the mammalian inner ear to determine whether differences in these pathways may be responsible for reduced proliferation. A better understanding of these kinases may lead to therapies for hair cell damage in the human ear.
References


Tabuchi, K., Oikawa, K., Hoshino, T., Nishimura, B., Hayashi, K., Yanagawa, T., Warabi, E.,


