2011

Heat Shock Protein Induced Protection against Cisplatin-Induced Hair Cell Death

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Heat shock protein-induced protection against cisplatin-induced hair cell death

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A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirement for the degree of Ph.D. in the College of Graduate Studies.

Department of Pathology and Laboratory Medicine

2011

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TIFFANY GRAY BAKER. Heat shock protein-induced protection against cisplatin-induced hair cell death. (Under the instruction of Lisa L. Cunningham)

Cisplatin is a highly successful and widely used chemotherapy for the treatment of various solid malignancies in both adult and pediatric patients. Side effects of cisplatin include nephrotoxicity and ototoxicity. Cisplatin's ototoxic effect results in part from damage to and death of cochlear hair cells. Mechanisms underlying cisplatin-induced hair cell death are poorly understood and have been attributed to DNA damage, oxidative stress, and inflammation. This study was designed to determine the role of p53 in cisplatin-induced hair cell death and to investigate heat shock proteins (HSPs) as potential protectants against cisplatin-induced hair cell death using adult mouse utricle as an *in vitro* model of mature mammalian hair cells. p53 is a well-known transcription factor involved in the DNA damage response. Using $p53^{-/-}$ mice and wild-type litter mates, results indicate that p53 is not necessary for cisplatin-induced death of hair cells and hearing loss. Heat shock has been previously shown to inhibit cisplatin-induced hair cell death. Since HSP70 is upregulated following sublethal heat shock, the role of HSP70 in heat shock-conferred protection against cisplatin was investigated. HSP70 is necessary for the protective effect conferred by heat shock against cisplatin-induced hair cell death. Constitutive expression of inducible HSP70 offered modest protection against cisplatin-induced hair cell death, indicating that HSP70 is sufficient to protect against cisplatin. HSP32, a stress-inducible protein responsible for the catabolism of free heme, has been shown to protect against oxidative and inflammatory stress in multiple systems. Cobalt protoporphyrin IX (CoPPIX) –induced HSP32 was previously shown to inhibit cisplatin-induced death of hair cells from neonatal rat cochlear explants. Results indicate that

HSP32 offers significant protection against cisplatin-induced hair cell death in cultured adult mouse utricle at multiple cisplatin concentrations, that CoPPIX induces expression of HSP32 primarily in resident macrophages of mouse utricle, and that macrophages are necessary for the protection conferred by CoPPIX-induced HSP32 against cisplatin. Due to the robust protection conferred by HSP32, it may prove promising in the design of a co-therapy for the prevention of cisplatin-induced hearing loss.

CHAPTER 1: INTRODUCTION

How the ear senses sound and balance

The vertebrate organ responsible for detecting sound and balance is the ear. The ear can be subdivided into three parts: the outer ear, the middle ear, and the inner ear. These three components of the ear work together to transmit acoustic stimuli through air and fluid interfaces until they, ultimately, result in neuronal impulses sent to the brain.

The outer ear, consisting of the pinna and ear canal, is the only portion of the ear that is externally visible. The pinna, made primarily of cartilage and skin, is the funnel-like portion of the outer ear that collects and directs sound waves to the ear canal. Functions of the outer ear include a mild increase in the sound pressure level of acoustic stimuli and protection of the tympanic membrane and other more internal ear anatomy from the environment. The sound waves continue through the ear canal until they reach its most internal limit, the tympanic membrane. The tympanic membrane is a thin connective tissue sheet that serves as the interface between the outer ear and the middle ear.

The middle ear is an air-filled space which contains the three middle ear bones or ossicles: malleus, incus, and stapes. Sound stimuli cause vibration of the tympanic membrane. The sound vibration is transmitted through the middle ear as a result of direct physical contact between the tympanic membrane and the inner ear bones. Thus, when sound pressure from the ear canal pushes on the tympanic membrane, the malleus moves within the middle ear. As all of the middle ear ossicles are connected to one another via small ligaments, movement of one results in movement of the others. The stapes, the last

in the ossicular chain, has direct contact with the oval window, a thin membrane separating the air-filled middle ear cavity from the fluid-filled inner ear. Movement of the ossicular chain, results in a piston-like action of the stapes on the oval window, an energy which is transferred to an inner ear fluid, perilymph. Due to the impedance mismatch between air and perilymph, sound vibrations traveling through the middle ear must be amplified in order to be effectively transmitted to the fluid medium of the inner ear. Such amplification is achieved in two ways: (1) The surface area of the oval window is smaller than the surface area of the tympanic membrane, thus resulting in the transfer of increased energy per unit area at the oval window when compared to the tympanic membrane, and (2) the ossicles are arranged so that their combined movement results in a lever action which also boosts the energy contained in the already existing sound stimulus. Due to the delicate anatomy of the middle ear ossicular chain, mechanisms also exist which effectively attenuate vibrations that could potentially damage the middle ear. For instance, two muscles, the tensor tympani and the stapedius muscle, will contract in the presence of loud sound in order to attenuate the vibrations transmitted to the inner ear.

The inner ear, a collection of highly specialized organs designed to sense sound and balance, is housed in the bony labyrinth of the temporal bone. Each of the inner ear organs contains sensory hair cells which are responsible for the transduction of mechanical stimuli resulting from sound (organ of Corti) or head movement (ampullae, utricle, and saccule) into neuronal impulses. Hair cells are columnar, highly polarized cells with hair-like projections from their apical surfaces called stereocilia. Stereocilia are arranged in bundles. The stereocilia bundles of the cochlea and vestibular system are deflected in response to sound or head movement, respectively, which leads to the

opening of mechanically-gated ion channels located near the tips of the stereocilia (Hudspeth 1989). The mechanotransduction channel, which is yet to be identified, allows the influx of ions rather nonspecifically into the hair cells. This influx of K^+, Ca^{++}, and Na^+ ions into the hair cell is a consequence of the electrochemical gradient existing between the specialized fluid media of the inner ear, the endolymph (high K^+) and the perilymph (low K^+). Hair cells are arranged within inner ear sensory epithelia such that only their apical surfaces (containing stereocilia) are exposed to endolymph. Apical tight junctions between hair cells and neighboring supporting cells prevent the mixing of endolymph and perilymph. Specialized cells and cycling pathways in the inner ear work to maintain the ionic gradient between endolymph and perilymph (Hibino et al 2010; Wangemann 2006). Following the depolarization of hair cells, synaptic vesicles are released into synaptic clefts found between the hair cells and adjacent neurons. The stimulated auditory neurons then propagate action potentials which send the sound or balance stimulus information to the brain.

The snail shell-shaped region of the inner ear is the cochlea which is home to the auditory organ known as the organ of Corti. The organ of Corti, like the balance organs, features a hair cell-containing sensory epithelium. However, the organ of Corti exhibits an extremely complex arrangement of hair cells and other cell types. The cochlea is a coiled bony tube lined with membranous structures that subdivide it into three smaller tubes: scala vestibuli, scala media, and scala tympani. The scala media is found in the middle of these three canals, and it is lined by Reissner's membrane superiorly and the basilar membrane inferiorly. Scala media contains K^+ -rich endolymph. Superior to Reissner's membrane is the scala vestibuli. The scala vestibuli is filled with perilymph, a

relatively K^+ -poor fluid which also fills the scala tympani. As the stapes footplate pushes on the oval window, that energy is then transferred to the scala vestibuli at the base of the cochlea. Upon reaching the most apical point of the cochlea (the helicotrema), the energy begins to move basally again through the scala tympani. Thus the scala vestibuli and scala tympani meet at the apex of the cochlea. The energy is finally dispersed by way of the fluid of the scala tympani pushing outward on the round window membrane which then bulges out from the basal portion of the scala tympani to the air-filled middle ear. The specialized morphology of the basilar membrane, the membrane segregating scala media from scala tympani, results in the tonotopic arrangement of the cochlea (i.e. high frequencies sensed at the base of the cochlea, low frequencies sensed apically). The basilar membrane oscillates in response to the sound energy moving through perilymph. The point of maximal oscillation in response to an individual stimulus is directly related to the frequency of the sound. When the basilar membrane is exposed to a multi-frequency stimulus, it will exhibit several oscillatory maxima along its length. Thus, the basilar membrane is dissecting a single sound into its constituent frequencies. These oscillations in the basilar membrane are often referred to as traveling waves. The louder a sound, the greater the amplitude of the traveling wave at the locations of the frequencies involved. The lateral wall of the scala media contains the stria vascularis, a group of specialized cells which are integral to maintaining the high K^+ concentration in the endolymph, the fluid which fills the scala media. Reissner's membrane and the basilar membrane are fused at the apex of the cochlea, thus resulting in the sac-like character of the scala media. The hair cells of the organ of Corti have a specific arrangement of three to four rows of outer hair cells and a single row of inner

hair cells. The stereocilia of the outer hair cells are embedded in the ribbon-like tectorial membrane that runs the entire length of the organ of Corti. As mentioned before, deflection of the stereocilia bundles of hair cells results in their depolarization and subsequent signaling to adjacent neurons. The deflection of the stereocilia of hair cells in the organ or Corti results from the shearing forces created as a result of the movement of the tectorial membrane (superior to the hair cells) and the basilar membrane (inferior to the hair cells). The inner hair cells are those directly responsible for turning a sound stimulus into a neuronal signal to be sent to the brain. The outer hair cells act to amplify sound stimuli directed to the inner hair cells. Although still a debatable topic, this is likely the mechanism behind what has long been dubbed the cochlear amplifier. Outer hair cells contain a motor protein called prestin in their lateral cell membrane. Upon depolarization, outer hair cells contract, thus pulling down on the tectorial membrane, and changing its position relative to the hair bundles of the inner hair cells.

The balance organs are found at the bases of the three semicircular canals (cristae ampulari) and in the vestibule (utricle and saccule). Each of these organs contains a small monolayer patch of hair cells and supporting cells. The three semicircular canals are oriented in different planes: horizontal, anterior, and posterior, and this unique arrangement allows their sensory epithelia (cristae ampulari) to detect angular acceleration of the head. Each semicircular canal terminates in a dilated chamber called an ampulla, which contains the crista. A narrow band of tissue, the cupula, spans the diameter of each ampulla, contacting the crista at one end. Angular head movement causes fluid within the narrow canals to move, thus applying pressure to the cupulae and triggering mechanotransduction in hair cells of the cristae. The utricle and saccule detect

linear acceleration of the head, as well as gravitational forces. The hair bundles of these otolith organs project into a gel-like medium. The gel layer is topped by a layer of small crystals or otoconia. Collectively, these otoconia act as a weight which, when moved, results in deflection of the hair cell bundles. The orientation of the hair cells in the otolith organs and the position of each organ in the skull determine the direction of movement sensed by these organs. In the case of both the semicircular canals and the vestibular otolith organs, it is the combination of signals from each sensory patch that ultimately allows humans to sense the orientation of their head in space.

All non-mammalian vertebrates (i.e. fish, amphibians, reptiles, and birds) have the capacity to replace hair cells throughout their lives, a capability lost in postnatal mammals. Thus, a mammal that loses hair cells can never regain them, resulting in permanent hearing loss. Outer hair cells tend to be more sensitive to ototoxic insults than inner hair cells. Loss of either type of hair cell results in the worsening of an individual's hearing capacity. Outer hair cell loss, alone, results in decreased sensitivity to sound (due to loss of the cochlear amplifier), as well as a decrease in frequency resolution. Loss of inner hair cells results in loss of the capacity to sense sound at those frequency places along the cochlea that were affected. Currently, hair cell regeneration is a hot topic in the field of hearing research. Hearing loss in humans can occur as a result of: (1) genetics, (2) infection (bacterial/viral), (3) ototoxic drugs (aminoglycosides/cisplatin), (4) acoustic trauma, and (5) aging. Due to the morphological complexity of the mammalian cochlea, the creation of new hair cells is not sufficient to regain cochlear function in the face of the afore-mentioned insults. Such newly derived hair cells would have to be present in the proper organization within the already-existing cochlea and gain associations with the

appropriate neighboring cell types, such as neurons and supporting cells. Therefore, it will be a long time before mammalian hair cell regeneration will become a practical clinical reality. It is for this reason that the preservation of hair cells in the inner ear epithelia is critical to the preservation of human hearing for years to come.

Cisplatin, an ototoxic drug

Cisplatin (*cis*-$PtCl_2(NH_3)_2$) is a 300 d molecule that was first discovered in 1845 by Michel Peyrone. In the 1960's Rosenberg and colleagues discovered that cisplatin inhibits cell division (Rosenberg et al 1965). Cisplatin was accidentally formed during an experiment designed to test the effects of electricity on bacteria. The result was the formation of a platinum compound in the culture media which originated from the platinum coating on the submerged electrodes. Subsequent investigation revealed that the *cis* form of the platinum compound was the most effective at inhibiting bacterial cell division (Rosenberg et al 1967). A few years later, cisplatin was investigated as a potential chemotherapeutic agent. Cisplatin entered clinical trials for the treatment of cancer in 1971 and was approved by the FDA as an anti-neoplastic agent to treat ovarian cancer in 1978. Since its original approval, cisplatin remains a widely and successfully used chemotherapeutic drug for numerous adult and pediatric malignancies. Cancers commonly treated with cisplatin include bladder, ovarian, testicular, lung, head and neck, osteosarcoma, neuroblastoma, retinoblastoma, and medulloblastoma.

Cisplatin has numerous effects on cells, due to the indiscriminating manner in which it covalently binds different types of molecules. This feature of cisplatin is what allows it to so readily bind DNA, thus performing the tell-tale antitumor role for which

cisplatin is known. In addition, cisplatin inhibits protein synthesis by interfering with polypeptide elongation (Heminger KA et al 1997; Rosenberg JM 1993). *In vitro* analysis of this phenomenon has revealed that cisplatin disrupts the formation of the initiation complex required for protein elongation to occur (Rosenberg JM 1993). Furthermore, cisplatin can inhibit protein translation by inhibiting synthesis of the translation machinery, itself. Due to the affinity for high mobility group (HMG) domains to interact with cisplatin-DNA adducts, proteins containing these domains are sequestered by the cisplatin-DNA adducts, thus preventing them from undergoing their normal activities in cells. Upstream binding factor (UBF) is a transcription factor responsible for initiating the transcription of ribosomal RNA (rRNA). UBF and other rRNA transcription machinery is hijacked by cisplatin-DNA adducts, thus preventing rRNA transcription in cisplatin-treated cells (Jordan P 1998).

Cisplatin and cancer cells

The primary mechanism by which cisplatin kills cancer cells stems from the direct interaction of cisplatin molecules with DNA. Cisplatin hydrolysis seems to be a rate-limiting step in the formation of cisplatin-DNA adducts (Butour et al 1985; Knox et al 1986). The very reactive aquated cisplatin forms inter- and intrastrand DNA adducts which cause deformation of the DNA double helix. The majority of these adducts, and those believed responsible for the cytotoxic effects of cisplatin, are 1, 2-intrastrand cross-links (Eastman 1986).

Cells have intrinsic DNA repair mechanisms which may or may not improve the quality of damaged DNA. Certain DNA damage recognition and/or repair molecules are known to interact with cisplatin-DNA adducts. The major DNA repair mechanism

responsible for the removal of cisplatin adducts is nucleotide excision repair (NER) (Huang et al 1994; Zamble et al 1996). This discovery has been further substantiated by evidence that some NER-deficient cells are more sensitive to cisplatin-induced death than those with intact NER (Furuta et al 2002; Koberle et al 2010; Selvakumaran et al 2003; Stubbert et al 2010; Wu et al 2003). Moreover, cisplatin resistance has been attributed to enhanced NER capacity (Ferry et al 2000; Mukai et al 2002). Another class of DNA-binding proteins that interact with cisplatin adducts are a family of the high mobility group proteins dubbed high mobility group box proteins (HMGBs), due to the presence of a B-box DNA-binding motif. HMGBs bind with very high affinity to the 1, 2-intrastrand cross-links formed by cisplatin (Billings et al 1992; Hughes et al 1992; Pil & Lippard 1992). HMGB proteins, once bound to platinated DNA, are thought to act as a shielding mechanism which prevents NER-mediated repair of cisplatin-DNA adducts (Arioka et al 1999). The existence of the shielding mechanism is supported by the fact that the presence of HMGB1 on cisplatin adducts inhibits the ability of NER proteins to bind the DNA, thus directly inhibiting the DNA repair process (Huang et al 1994; Zamble et al 1996). Interestingly, evidence exists linking HMGB proteins to the function of p53, a transcription factor and major mediator of the DNA damage response. p53 seems to enhance the ability of HMGB1 to bind cisplatin adducts, and HMGB1 can stimulate binding of p53 to its target sequences on DNA (Imamura et al 2001; Jayaraman et al 1998). As with NER, the relative amount of HMGB protein expressed by cells has been correlated with cisplatin sensitivity (Arioka et al 1999). Although a lot is known about the removal or lack of removal of cisplatin-DNA adducts, it should be noted that many proteins recognize and interact with platinated DNA. In fact, novel proteins that

bind to these sites, such as TOX4, are still being discovered (du Puch et al 2011). The exact mechanisms linking these proteins to cell death are still not known.

The DNA damage response involves recognition of the damage, cell cycle arrest, and, upon insufficient repair of the damage, activation of apoptosis. Cell cycle arrest and apoptosis are regulated by p53. p53 is a transcription factor that is normally degraded as quickly as it is transcribed, keeping total p53 protein levels fairly low in normal cells (Oren et al 1981). This rapid and perpetual degradation of p53 is mediated by ubiquitin ligases (e.g. murine double minute 2 (MDM2)) and the proteosome (Honda et al 1997; Maki et al 1996). However, once a cell sustains DNA damage, p53 is stabilized via phosphorylation by phosphatidylinositol-3-kinase related kinases (PIKK), such as ataxia telangiectasia mutated protein (ATM) and ataxia telangiectasia and Rad3 related protein (ATR). ATM, ATR, and downstream serine/threonine-protein kinases Chk1 and Chk2, can phosphorylate p53 at Ser15 (ATM, ATR), Ser 37 (ATR), and Ser 20 (Chk1/Chk2) (Banin et al 1998; Canman et al 1998; Hirao et al 2000; Khanna et al 1998; Sakaguchi et al 1998; Shieh et al 2000; Tibbetts et al 1999). p53 phosphorylation inhibits the binding of MDM2 to p53, thus preventing its ubiquitination and subsequent degradation and allowing it to accumulate in the cell (Shieh et al 1997). MDM2 not only inhibits p53 activity by inducing its degradation, it also inhibits its transcriptional activity (Momand et al 1992). Thus, once free of MDM2, p53 protein is allowed to undergo nuclear translocation to induce transcription of a variety of genes. Gene targets of p53 include regulators of the cell cycle, such as p21, pro-apoptotic proteins, such as the p53 upregulated modulator of apoptosis (Puma) and the Bcl-2-associated X protein (Bax), as well as its own negative regulator MDM2 (Barak et al 1993; el-Deiry et al 1993;

Miyashita & Reed 1995; Nakano & Vousden 2001). At this first sign of DNA damage, p53 transcriptionally activates p21, resulting in cell cycle arrest (Ozaki & Nakagawara 2011). Irreparable DNA damage ultimately leads to the transcription of pro-apoptotic targets of p53, thus killing the cell. The molecular steps that trigger p53 to make the switch from cell cycle arrest to activation of pro-apoptotic transcriptional targets have only recently been elucidated. In 2010, a group discovered that for a period just after the onset of DNA damage, p53 is bound by a nuclear factor with BRCT domain 1 (NFBD1), thus preventing the phosphorylation of p53 by ATM (Nakanishi et al 2007). At a later time point following the onset of DNA damage a sharp decline in NFBD1 protein allowed ATM to activate p53. The tumor suppressive function of p53 arises from its ability to prevent the inheritance of damaged DNA by daughter cells via the disruption of cell cycle progression and/or triggering pro-apoptotic events (Ozaki & Nakagawara 2011). It is, therefore, no surprise that nearly half of all tumors harbor a p53 mutation (Olivier et al 2010; Soussi et al 2006).

Due to the wide variety of tumors commonly treated with cisplatin, the exact role of p53 in cisplatin-induced tumor cell death is somewhat variable and ultimately depends on the tumor cell. However, p53 sensitizes certain tumors to the cytotoxic effects of cisplatin. Cisplatin-resistant tumor cell lines, including esophageal, bladder, and osteosarcoma cells, with non-functional p53 can be sensitized to cisplatin treatment upon exogenous expression of wild-type p53 or p53 targets (Ganjavi et al 2006; Pagliaro et al 2003; Wang et al 2006). Further evidence for p53's role in cisplatin-induced cancer cell death stems from investigations into testicular germ cell tumors (TGCTs) which are hypersensitive to cisplatin treatment. Kerley-Hamilton et al. (2005) examined changes in

gene expression following cisplatin treatment of testicular germ cell-derived human embryonal carcinoma cells. This group identified 46 upregulated genes following cisplatin treatment, and it was postulated that over half of them were downstream of p53. p53 knockdown experiments revealed that inhibition of p53 not only decreased the sensitivity of these cells to cisplatin but also eliminated or lessened the upregulation of the cisplatin-responsive genes. A more recent study demonstrated direct links between p53 and the cisplatin-sensitive nature of TGCTs (Gutekunst et al 2011). In this study siRNA knockdown of p53 rescued TGCT cell lines from cisplatin-induced apoptosis in a dose-dependent manner. Furthermore, cisplatin-induced apoptosis of the cells was attributed to two transcriptional targets of p53, Puma and Noxa, and concomitant knockdown of Puma and Noxa completely rescued the cells from cisplatin-induced death.

Reactive oxygen species (ROS) have also been implicated in the cisplatin-induced death of cancer cells (Pak et al 2011; Santandreu et al 2010). ROS are intermediates or byproducts of cellular respiration that, if not transformed into more stable molecules or eliminated by antioxidant systems, can cause damage to lipids, proteins, and nucleic acids. The types of damage incurred by ROS on cells includes lipid peroxidation, protein oxidation, and breaks in DNA. Thus, ROS could be a secondary mechanism by which cisplatin incites the DNA damage response. Examples of ROS include the superoxide anion and the hydroxyl radical.

Cisplatin-induced nephrotoxicity

Nephrotoxicity is a major dose-limiting side effect of cisplatin treatment that was first documented while cisplatin was in clinical trials (Pabla N and Dong 2008; Lippman, AJ

1973; Talley RW 1972). Approximately 30% of patients who receive cisplatin experience renal toxicity as a result (Arany I Et al 2003; Beyer J et al 1997). Symptoms of cisplatin-induced nephrotoxicity, which can occur as early as 10 days into treatment, include decreased glomerular filtration rate, increased serum creatinine, increased blood urea nitrogen, and unbalanced electrolytes (Arany I et al 2003; Meyer and Madias, 1994; Hanigan and Devarajan 2003). Numerous aspects of cisplatin dynamics have been implicated in its ability to harm renal cells, including its route of entry by way of copper transporters and organic cation channels, its ability to incite the DNA damage response, its ability to tip the redox balance of a cell toward oxidative stress, as well as its ability to induce inflammation.

The primary cell type affected by cisplatin in kidney is the renal proximal tubule cell (Wei Qet al 2007). These cells can die apoptotically and/or necrotically as a result of cisplatin treatment (Lieberthal W et al 1996). At least half of cisplatin molecules are thought to enter renal proximal tubule cells via the copper transporter Ctr1 and the organic cation transporter OCT2 (Pabla and Dong 2008; Pabla N et al 2009; Yonezawa A et al 2005; Ciarimboli G et al 2010). Once inside the cell, the nature of cisplatin-induced renal cell death is multifactorial. Molecular pathways responsible for the injury and death of proximal tubule cells include the DNA damage response, oxidative stress, and inflammation.

p53, a major mediator of the DNA damage response in cells, is a major mediator in cisplatin-induced nephrotoxicity. Although the exact mechanism of p53 upregulation following the exposure of renal tubule cells to cisplatin is unclear, it is most likely the result of the formation of cisplatin-DNA adducts and activation of DNA damage

recognition machinery, such as ATR (Pabla N 2007; Pabla and Dong 2008 review). The potential role of p53 in cisplatin-induced nephrotoxicity was first proposed in a study by Schnellmann and Cummings (2002). The results of this study demonstrated the early nature of p53 upregulation (4 h after the onset of cisplatin treatment) in rabbit proximal tubule cells, an event which occurred in advance of key apoptotic events. In addition, a pharmacological inhibitor of p53, pifithrin-α, protected the cultured cells from cisplatin-induced apoptosis and death. Later studies confirmed these findings and demonstrated that dominant negative p53 with a point mutation in the DNA-binding domain also alleviated cisplatin-induced tubular cell apoptosis, thus demonstrating the role of DNA-induced transcription in cisplatin-induced nephrotoxicity (Jiang et al 2004). Transcriptional targets of p53 that have been implicated as mediators of cisplatin-induced nephrotoxicity, include Puma-α and p53-induced protein with death domain (PIDD) (Seth et al 2005; Jiang et al 2006).

Oxidative stress has been a known contributor to cisplatin-induced nephrotoxicity for nearly two decades. The sources of cisplatin-induced oxidative stress in the kidney are multiple and include depletion of glutathione and disruption of the electron transport chain in mitochondria (Pabla and Dong 2008). Cisplatin, once inside the cell, becomes aquated and is highly reactive. Glutathione (GSH) is an endogenous non-enzymatic antioxidant responsible for aiding in cellular redox homeostasis. Glutathione reductase (GR) is an enzyme responsible for generating GSH in cells. Cisplatin reduces GR activity in porcine proximal tubular cells, resulting in a marked reduction in GSH levels (Kruidering M et al 1997). Cisplatin-induced increases in reactive oxygen species (ROS) have been attributed to such a drop in GSH levels in renal cells. Cisplatin also inhibits

activity of Complex I in the electron transport chain, redirecting the flow of electrons through complex II, and, ultimately, increasing superoxide formation (Kruidering M et al 1997). Along with those already discussed, other sources of increased ROS exist following the exposure of renal proximal tubule cells to cisplatin. Due to the apparent role of ROS in cisplatin-induced nephrotoxicity, many studies have been performed to investigate the potential protection conferred by antioxidants in this context.

Inflammation is a major player in cisplatin-induced nephrotoxicity. Tumor necrosis factor α (TNFα) is secreted by resident kidney cells, such as renal tubular cells, as a result of cisplatin treatment (Ramesh G and Reeves 2004; Zhang B et al 2007). TNFα can induce extrinsic apoptotic cell death by way of interaction with TNF receptors (TNFR) which are classified as death receptors. TNFR1 and TNFR2, each of which are expressed in kidney, have each been implicated as necessary for cisplatin-induced tubular cell death (Ramesh G et al 2003; Tsuruya K et al 2003). Pharmacological inhibitors and neutralizing antibodies against this well-known pro-inflammatory cytokine suppresses the secretion of other inflammatory mediators following cisplatin treatment in addition to ameliorating cisplatin-induced renal damage (Ramesh G and Reeves 2004). This group had similar results using TNFα-deficient mice.

Cisplatin-induced ototoxicity

Cisplatin is a widely-used and successful chemotherapeutic drug for treating a variety of solid tumors in both adult and pediatric patients. Side effects of cisplatin include ototoxicity, nephrotoxicity, and peripheral neuropathy (Helson et al 1978; Kedar et al 1978; Lippman et al 1973; Talley et al 1973). The ototoxic effect of cisplatin is most

detrimental to patients in the years following treatment, since it has a direct impact on their quality of life. Such patients experience bilateral, progressive, and irreversible hearing loss. These effects are particularly devastating to the social and educational development of individuals younger than 5 years old who are still learning language and social skills (Li et al 2004; Neuwelt & Brock 2010).

Cisplatin causes significant permanent hearing loss in anywhere from 20-80% of patients, with an average of about 30% (Coradini et al 2007; Fausti et al 1994; Knight et al 2005; Lewis et al 2009; reviewed in Neuwelt & Brock 2010). This statistic is highly variable, in part, due to the risk factors that influence the likelihood of a patient to exhibit cisplatin-induced hearing loss. Such risk factors include genetics, patient age, cumulative cisplatin dose, and prior radiation to the base of the skull (Caronia et al 2009; Huang et al 2007; Li et al 2004; Ross et al 2009; Schaefer et al 1985; Schell et al 1989). The progressive nature of cisplatin-induced ototoxicity has only come to light fairly recently, as long-term follow up studies have been completed. A patient's hearing can continue to worsen up to 11 years following the cessation of cisplatin treatment (Bertolini et al 2004).

Another reason for the wide range of incidence of cisplatin-induced hearing loss is the lack of standardized and/or effective monitoring protocols. These statistics may not accurately represent the proportion of patients experiencing hearing loss as some patients perceive a greater hearing deficit following cisplatin treatment than is indicated by their audiology exams (Einarsson et al 2010). Therefore, more patients are likely experiencing hearing loss than is indicated by the literature. Several grading scales have been established over the years in an attempt to effectively and accurately monitor cisplatin-induced hearing loss in patients in a clinical setting, including: The National Cancer

Institute Common Terminology Criteria for Adverse Events (CTCAE), the American Speech-Language-Hearing Association Criteria (ASHA), Brock (Brock et al 1991), and Chang (Chang & Chinosornvatana 2010). Recently, the need for a standardized audiologic monitoring protocol of cisplatin-treated individuals on an international level has been recognized (Neuwelt & Brock 2010).

Cisplatin and the inner ear

Systemically-administered cisplatin damages multiple sites in the cochlea, including the organ of Corti, lateral wall, and spiral ganglion. Though the hair cells of the organ of Corti seem to die apoptotically (Garcia-Berrocal et al 2007; Wang et al 2004), the events triggering apoptosis are not completely understood. Evidence from the literature points to the involvement of the three major effectors of cisplatin-mediated cochlear damage: the DNA damage response, an increase in reactive oxygen species (ROS), and the propagation of inflammatory mediators.

Apoptosis

Apoptosis, or programmed cell death, is a highly regulated process which allows cells to be removed from a tissue without interfering with the health of neighboring cells (Devitt & Marshall 2011). This ritualistic depletion of cells occurs naturally to insure proper development of multicellular organisms, for the purpose of cell turnover throughout an organism's life, as well as for the removal of damaged or stressed cells (Conradt 2009; Fulda et al 2010; Jacobson et al 1997; Steller 1995). Apoptosis was originally identified based on morphological changes within cells, including nuclear condensation, DNA fragmentation, cell shrinkage, and the formation of apoptotic bodies (Fulda et al 2010;

Kerr et al 1972). Currently, apoptosis is known as a complex array of molecular pathways all diverging on the tidy deconstruction of a cell.

Two apoptotic pathways are commonly discussed: extrinsic and intrinsic. Both of these pathways require the activity of caspases. Caspases are aspartate-specific cysteine proteases that cleave a wide variety of proteins, ultimately for the purpose of efficiently dismantling cells (Kumar 2007). Apoptosis requires the activation of initiator caspases (2,8, 9, and 10) that subsequently activate executioner caspases (3, 6, and 7). The executioner caspases are responsible for the disassembly of cell infrastructure as well as the activation of other proteolytic enzymes in the cell that also work to break it down piece by piece. The major difference between the extrinsic and intrinsic apoptotic pathways is that the signal for the extrinsic pathway comes from outside the cell and is mediated by a death receptor at the cell surface, whereas the signals triggering the intrinsic pathway originate intracellularly (ex. DNA damage). The two pathways are not entirely distinct from one another in that they may converge on the mitochondria.

The extrinsic pathway is activated when extracellular ligands bind to and activate their associated death receptors (ex. Fas receptor (FasR) & TNFR) (Ashkenazi & Dixit 1998; Grell et al 1994; Itoh et al 1991). The death receptor then recruits its associated death domain protein to the intracellular side of the cell membrane (ex. Fas recruits Fas-associated via death domain protein (FADD); TNFR recruits TNFR type 1-associated death domain protein (TRADD)) (Chinnaiyan et al 1995; Hsu et al 1995). This series of events ultimately leads to the formation of a death-inducing signaling complex (DISC) at the cell membrane that consists of the death receptor, the death domain, and some recruited initiator caspase (Kischkel et al 1995). Once recruited, the initiator caspases

autoactivate by cleaving one another (Muzio et al 1998). At this point, activated initiator caspases, such as caspase-8, are sufficient to activate caspase-3 (Stennicke et al 1998). Thus, the extrinsic pathway can stop at the direct activation of caspase-3 by initiator caspases, or it can converge on the intrinsic pathway and induce mitochondrial permeabilization. To perform the latter, the activated initiator caspases cleave BH3-interacting domain death agonist (Bid) molecules to form truncated Bid (tBid) in the cytosol (Li et al 1998; Luo et al 1998). tBid interacts with proapoptotic members of the Bcl-2 family, such as Bax and Bcl-2 homologous antagonist/killer (Bak), causing them to oligomerize and form pores in outer mitochondrial membrane (Kluck et al 1999; Lovell et al 2008; Mikhailov et al 2003; Wei et al 2000). This results in mitochondrial permeability transition which allows the release of proteins from the mitochondrial intermembrane space into the cytosol. Proteins released into the cytosol as a result of the mitochondrial permeabilization include cytochrome c, Smac/Diablo, HtrA2/Omi, endoG, and AIF (Du et al 2000; Li et al 2001; Susin et al 1996; Suzuki et al 2001; Yang et al 1997). Cytochrome c, normally a member of the electron transport chain of mitochondria, plays a very different role in apoptotic cell death. Once in the cytosol, cytochrome c associates with apoptotic protease activating factor 1 (Apaf-1) to form the apoptosome. The apoptosome is a complex of molecules (cytochrome c, heptameric Apaf-1, and adenosine triphosphate (ATP)) which, once formed, causes a conformational change in Apaf-1 that allows for activation of caspase-9 (Zou et al 1999). Caspase-9 then activates the executioner caspase, caspase-3 (Li et al 1997). As mentioned previously, the intrinsic apoptotic pathway is initiated inside of the cell. Signals that can trigger intrinsic apoptosis include DNA damage and increased ROS. Exposure to such stimuli

results in activation of transcription factors with pro-apoptotic gene targets. For instance, DNA damage results in upregulation of p53 which induces the transcription of Bax. Thus, the intrinsic mechanism of apoptosis completely bypasses the death receptors and begins at the mitochondrial level.

Apoptosis is a highly regulated pathway, and a cell's fate is determined by the balance between pro- and anti-apoptotic signals. Pro-apoptotic proteins include the BH3-only B-cell lymphoma-2 (Bcl-2) family members, such as Bid, Bcl-2-like protein 11 (Bim), Bcl-2 antagonist of cell death (Bad), Puma, and Noxa (Westphal et al 2011). Anti-apoptotic signals include Bcl-2 family pro-survival proteins, such as Bcl-2 and B-cell lymphoma-extra large (Bcl-xL), as well as non-Bcl-2 family proteins, such as heat shock proteins (Garrido et al 2006; Westphal et al 2011).

Heat shock proteins

Heat shock proteins (HSPs) are a highly conserved group of molecules responsible for the maintenance of cellular homeostasis in response to multiple stressors, including thermal and oxidative stress (Martindale & Holbrook 2002; Richter et al 2010). Six families of HSPs have been documented in mammalian cells, and they are classified according to molecular weight. There are 5 families of high molecular weight HSPs: HSP100, HSP90, HSP70, HSP60, HSP40, and one family referred to as the small HSPs (15-30kD) (Khalil et al 2011; Young 2010). HSPs come in both constitutive and inducible forms. The constitutive forms are involved in normal cell functions, whereas the inducible forms are transcriptionally upregulated in response to stress. Most of these proteins act as molecular chaperones, and, therefore, act to regulate the folding, transport,

and activity of proteins. Induction of HSPs, using an initial stress such as sublethal heat shock, has been shown to inhibit cell death caused by subsequent stress, an effect termed cross-tolerance. Due to the ability of HSPs to counteract cytotoxic mechanisms, HSPs are being investigated as potential therapies and therapeutic targets for a number of diseases.

The HSP70 family represents one of the largest and most evolutionarily conserved of the HSPs (Karlin & Brocchieri 1998). HSP70 proteins are remarkably ubiquitous, in that they are present across phyla in both pro- and eukaryotic cells, with few exceptions (Macario et al 1999). Both constitutive (heat shock conjugate protein (Hsc) 70) and inducible forms of HSP70 proteins exist (Ingolia & Craig 1982; Lengyel et al 1980). HSP70 works to guide folding of newly synthesized polypeptides (Sousa & Lafer 2006), transport proteins across membranes (Pilon & Schekman 1999), and to unravel tangles of misfolded protein aggregates (Ben-Zvi et al 2004). The HSP70 family has a well-known structure and chaperoning mechanism which is dependent on ATP hydrolysis and co-chaperones. The HSP70 proteins have a specialized N-terminal ATP-binding domain (NBD), as wells as a C-terminal substrate binding domain (SBD), which are connected by a flexible 10-residue linker region (Jiang et al 2006). The SBD is made up of a helical lid and a beta-sheet base, whose relative conformations allow the SBD to assume an open or closed state (Zhu et al 1996). The opening and closing of the SBD is an ATP-dependent process (McCarty et al 1995). When ATP is bound to HSP70, the chaperone is considered to be in a low-affinity state. However, upon ATP hydrolysis or in the presence of ADP, HSP70 binds with high affinity to hydrophobic regions of client polypeptides. Two types of co-chaperones mediate ATP-cycling and client binding to

HSP70 proteins, J proteins and nucleotide exchange factors (NEFs). J proteins encourage hydrolysis of ATP and keep substrates readily available to HSP70, thus keeping HSP70 in a high-affinity state and allowing for high turnover rates of protein folding (Karzai & McMacken 1996; Liberek et al 1991). NEFs, such as Bcl-2-associated athanogene 1 (BAG-1) and HSP70-binding protein (HspBP1) in eukaryotes, are responsible for the exchange of ADP for ATP which allows for release of the client protein (Brehmer et al 2001; Harrison et al 1997; Shomura et al 2005; Sondermann et al 2001).

Evidence exists implicating apoptosis as the primary mechanism of inner ear hair cell death as a result of cisplatin treatment. The first evidence that hair cells were dying apoptotically from cisplatin treatment came in 1998 (Liu et al 1998). In this study the investigators observed that cisplatin treatment of P3 rat cochlear explants exhibited increased TUNEL-positive staining and decreased hair cell survival. When caspase inhibitors were implemented alongside cisplatin treatment, the tissue exhibited virtually no TUNEL staining. Shortly after these investigations, several other groups reported similar findings. Alam et al. (2000) reported the presence of TUNEL-positive staining and condensed, pyknotic nuclei in cochleae of gerbils that had received systemic cisplatin injections. This group took the mechanistic story a little further by attempting to investigate the roles of pro-apoptotic Bax and anti-apoptotic Bcl-2 by immunochemistry. Their data indicated that cisplatin treatment increased levels of Bax and decreased levels of Bcl-2 in the cochlea, thus tipping the balance in these cells toward apoptotic death. Watanabe et al. (2001) reported executioner caspase 3 activation in the vestibular organs of cisplatin-treated guinea pigs. That same year, a group showed that vitamin E reduced cisplatin-induced TUNEL staining in hair cells of the organ of Corti, an observation

which indicated a potential link between reactive oxygen species production and cisplatin-induced apoptosis in the inner ear (Teranishi et al 2001). Due to difficulties associated with culturing inner ear sensory organs, such as the large number of animals required to achieve an appropriate sample size, Devarajan et al. published the first paper in which an auditory cell line was used to elucidate mechanisms behind cisplatin-induced apoptosis in cells of the inner ear (2002). As seen in other cell types undergoing apoptosis, Devarajan et al. revealed that cisplatin treatment of HEI-OC1 cells caused p53 stabilization, initiator caspase 8 activation, truncation of Bid, mitochondrial translocation of Bax, cytochrome c release to the cytosol, and caspase 9 activation, in that chronological order. p53 activation following cisplatin treatment of neonatal rat cochlear and utricular explants was demonstrated in 2003, although the localization of the stabilized p53 (i.e. in which cell types p53 was expressed) were not clear (Zhang et al). Wang et al. performed what is probably the most convincing study for the role of apoptosis in cisplatin-induced hair cell death and hearing loss (2004). TUNEL-positive staining was observed in ears of cisplatin-treated guinea pigs, and hair cell nuclear morphology in these samples was described as apoptotic in the basal and middle cochlear turns. Organ of Corti immunostaining revealed that cisplatin caused Bax to move from the cytosol to the mitochondria, cytochrome c to be released from mitochondria, and caspase 3 to become activated. These data represent the first *in vivo* evidence to corroborate the mechanistic findings from HEI-OC1 cells. Furthermore, Wang et al. provided evidence for the cleavage of fodrin, the activation of c-jun N-terminal kinase (JNK), and the phosphorylation of c-jun by western blot. Finally, these investigators implemented the use of minipumps for perfusion of caspase and JNK inhibitors into the

fluids of the inner ear. Cisplatin-induced hearing loss was prevented by inhibitors of caspase 3 and caspase 9. Interestingly, inhibitors of caspase 8 and JNK did not improve the hearing of cisplatin-treated animals. On the contrary, the JNK inhibitor actually worsened the cisplatin-induced hearing loss. In summary, inner ear studies have shown that cisplatin induces morphological changes indicative of apoptosis, translocation of Bax, release of cytochrome c, and caspase activation in hair cells. The next step to understanding cisplatin-induced ototoxicity, is knowing how cisplatin causes apoptosis. Three modes of action have been touched on in the literature: DNA damage, ROS production/antioxidant depletion, and inflammation (Fig. 1-1).

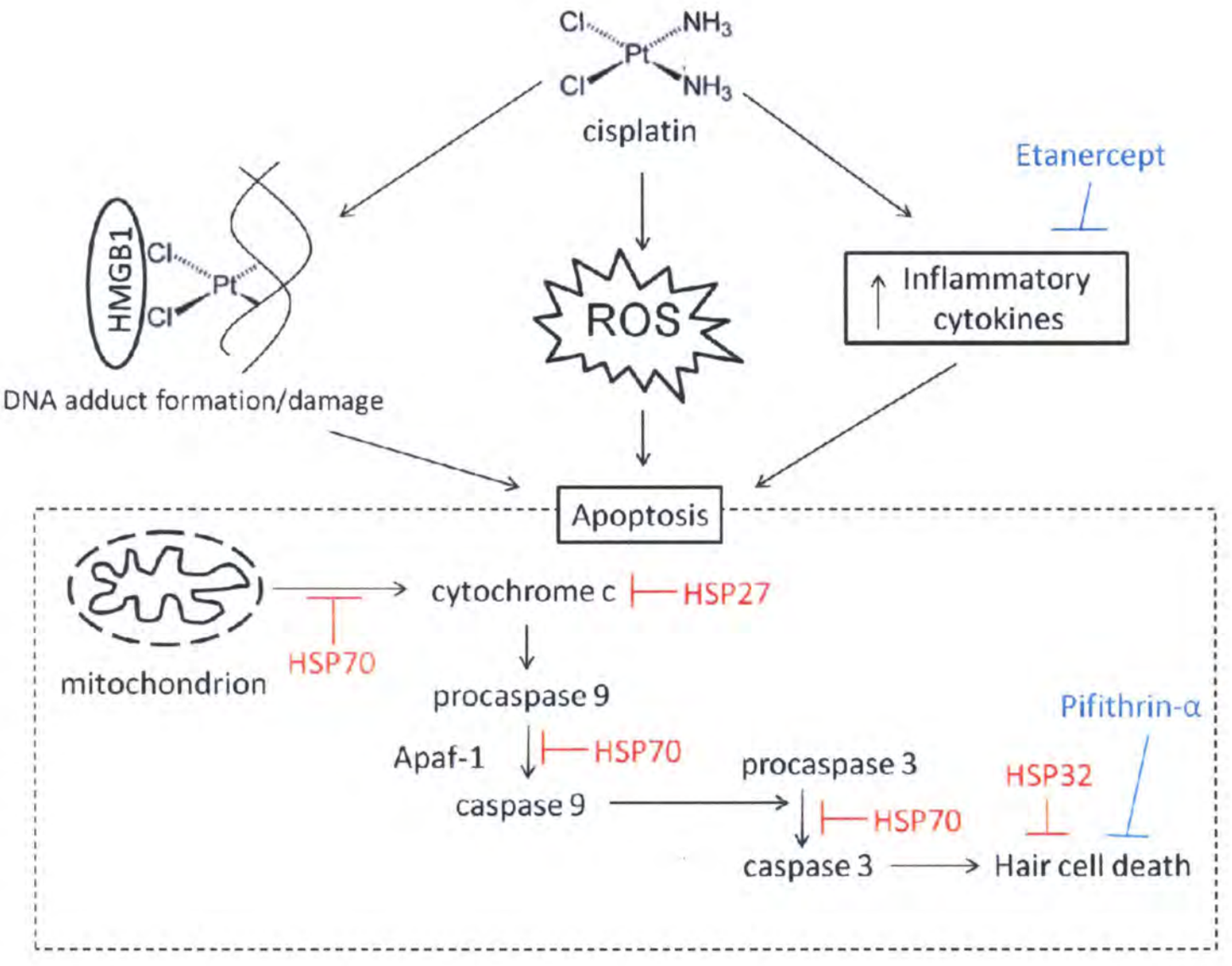

Figure 1-1. Cisplatin-induced hair cell death. Cisplatin has been shown to induce the apoptotic death of hair cells by inducing DNA damage, producing reactive oxygen species (ROS), and inducing production of inflammatory cytokines. HSPs have been shown to prevent apoptosis in various model systems at different points in the apoptotic pathway. The points at which HSPs inhibit apoptosis are included in this figure. Furthermore, two pharmacological agents that protect against cisplatin-induced hair cell death, pifithrin-α and etanercept, are depicted here.

Apoptosis of hair cells: evidence for DNA damage

DNA damage has been implicated as a major player in cisplatin-induced hair cell death. However, little knowledge exists concerning how the DNA damage response affects hair cell survival, and the identities of DNA damage-associated proteins involved in pathways leading to hair cell death are unknown. As with cancer cells, cisplatin-DNA adduct formation has been documented in guinea pig cochleae (van Ruijven et al 2005a). Also, HMGB1 levels increase in cochleae of cisplatin-treated rats (Li et al 2006). The increase in HMGB1 could be indicative of a high concentration of cisplatin-DNA adducts and/or disruption of NER mechanisms. The p53 inhibitor pifithrin-α inhibits cisplatin-induced death of hair cells of cochlear and utricular explants from neonatal rats (Zhang et al 2003). This is of particular interest due to the notoriety of p53 as a tumor suppressor and as a major mediator of the DNA damage response in many cell types. The current study includes experiments designed to define the role of p53 in cisplatin-induced hair cell death.

Apoptosis of hair cells: evidence for ROS production

In the early 1990's, indirect evidence of a role for oxidative stress in cisplatin-treated cochleae was reported (Ravi et al 1995; Rybak et al 1995). Ravi et al. is the first example of a study designed to investigate cisplatin-induced ototoxicity in a rat model. They eventually determined an ototoxic dose of cisplatin (16mg/kg) and investigated the antioxidant protein levels and activity in the cochleae of these cisplatin-treated animals. The results of this study indicated that glutathione (GSH) levels decreased in correlation with increased threshold shift (i.e. increased hearing loss). Furthermore, they revealed that cisplatin caused a decrease in activity of GSH peroxidase and GSH reductase, while

increasing activity other antioxidant enzymes (superoxide dismutase (SOD), catalase, and malondialdehyde). In a follow-up paper, this group demonstrated that a potential chemoprotectant protected rats from cisplatin-induced hearing degeneration, in part due to the prevention of GSH depletion in the cochlea (Rybak et al 1995). To summarize, these studies were the first to link antioxidants and, indirectly, reactive oxygen species (ROS) to cisplatin-induced ototoxicity.

The first direct evidence of cisplatin-induced reactive oxygen species production by inner ear tissue was documented in 1996 (Clerici et al). In this study, electroparamagnetic resonance spectrometry (EPR) was used to directly measure the amount of hydroxyl radicals produced by guinea pig cochlear explants following ten minutes of cisplatin exposure. The results of this study indicated that cisplatin-treated cochlear explants had $OH^{\cdot}$ spin adduct peak magnitudes that were greater than those of explants in cisplatin-free culture conditions. Since then, the majority of investigation into cisplatin-induced hearing loss has been focused on inhibiting the formation of and/or neutralizing reactive oxygen species production.

Apoptosis of hair cells: propagation of inflammatory mediators

Several cell lines have been established as model systems for hair cells. One cell line, House Ear Institute-organ of Corti 1 (HEI-OC1), was designed to represent hair cells from the cochlea, whereas UB/UE-1 cells are a model for vestibular hair cells. Each of these cell lines was established from inner ear sensory organs of the transgenic Immortomouse™ and is conditionally immortal in that they are capable of differentiating under specific culture conditions. The UB/UE-1 cell line, originally described in 1999,

was established from P2 immortomouse utricles (Lawlor et al). UB/UE-1 cells were originally established for the purpose of developing a model useful to the study of hair cell differentiation. These cells proliferate readily at 33°C, and differentiate into three different utricular cell types at 39°C: one resembling supporting cells, and two resembling precursors to sensory hair cells. HEI-OC1 cells were established in 2003 from P7 Immortomouse cochleae (Kalinec et al). HEI-OC1 cells express proteins in common with supporting cells, sensory cells, and ganglion cells of the organ of Corti. Due to the apparent sensitivity of this cell line to ototoxic agents, the authors suggested that it be used as a model system for investigating potential mechanisms associated with ototoxic drug-induced cell death, as well as screening potential otoprotectants. As with any model system, there are pro's and con's to using cell lines as models for inner ear sensory epithelia. Due to limitations regarding the use of organotypic cultures in research, these cell lines are very useful for working out potential molecular pathways exhibited by hair cells. For instance, cochleae and utricles are comprised of heterogeneous populations of cells, and it is difficult to separate the specific cell types from one another for the purpose of culturing them. In addition, many animals must be sacrificed in order to complete an experiment using inner ear organ culture. However, these cell lines are not hair cells as they express markers of multiple cell types and are therefore more primitive than the terminally differentiated hair cells they are commonly used to represent. Also, while HEI-OC1 cells have proven to be susceptible to cisplatin-induced death, they are not sensitive to the aminoglycoside antibiotics, the other major class of ototoxic drugs. Finally, these cell lines are homogeneous, monolayer cell populations, and, therefore, cannot truly represent the interactions between the multiple

cell populations present in the highly organized inner ear. For these reasons, studies performed using these cell lines should always be confirmed with animal work in differentiated sensory epithelia.

Cisplatin treatment results in the production of pro-inflammatory cytokines in inner ear-derived cell lines, as well as in sensory organs of the inner ear. HEI-OC1 and UB/UE-1 cells exhibit increased secretion and transcription of pro-inflammatory cytokines, most notably tumor necrosis factor alpha (TNFα), following cisplatin treatment (Kim et al 2008; So et al 2007). Increased expression of these cytokines was also detected *in vivo*. Kim et al. (2008) demonstrated that rats treated with cisplatin plus Etanercept, a commercially available TNFα inhibitor, exhibited decreased serum levels of TNFα compared to rats treated with cisplatin alone. Furthermore, cochleae from Etanercept-treated rats contained lower levels of cytokine mRNA. Exogenous TNFα reduces viability of HEI-OC1 cells, as well as hair cells of neonatal cochlear explants (Dinh et al 2008; So et al 2007). As a whole, these data suggest that the propagation of inflammatory cytokines, such as TNFα, may play an important role in cisplatin-induced hair cell death.

The current study was designed to further elucidate the mechanism(s) by which cisplatin kills hair cells, as well as to examine the potential protective effects of specific heat shock proteins against cisplatin-induced hair cell death. The project is divided into three aims: (1) to determine the role of p53 in cisplatin induced hair cell death, (2) to identify specific heat shock proteins capable of protecting against cisplatin-induced hair cell death, and (3) to determine the mechanism(s) by which HSP32 inhibits cisplatin-induced hair cell death.

CHAPTER 2: GENERAL METHODS (The following methods were used in each of the three aims of this project, any methods specific to one aim is discussed in the chapter dedicated to that aim.)

Animals

All mice were maintained in the central animal care facility at the Medical University of South Carolina (Charleston, SC, USA). Mice were euthanized via carbon dioxide asphyxiation and then decapitated. All animal protocols were approved by the MUSC Institutional Animal Care and Use Committee.

CBA/J mice

Adult CBA/J mice (4 to 6 weeks old) were obtained from Harlan Laboratories, Inc. (Indianapolis, IN, USA).

C57Bl/6J mice

Adult C57Bl/6J mice (4 to 6 weeks old) were obtained from The Jackson Laboratory (Bar Harbor, ME).

Genotyping

Tail clips, acquired from mice at three weeks of age, were lysed using DirectPCR Lysis Reagent (Tail; Viagen Biotech 101-T, Los Angeles, CA, USA). DNA contained in the resultant lysates was amplified by one-step polymerase chain reaction (PCR) using the appropriate primers (TaqMan Core Reagent Kit, Applied Biosystems no. N808-0228, Foster City, CA, USA). PCR products were then subjected to electrophoresis on a 2% low-melt agarose gel. Following electrophoresis, gels were incubated in ethidium

bromide and photographed under UV light to visualize the PCR products. The migration pattern of the PCR products was used to determine the genotype.

Culture of adult mouse utricle

Culture of utricles from adult mice has been previously described (Cunningham 2006). To summarize, dissections were performed in a sterile tissue culture hood. Once collected, the utricles were cultured free-floating in culture medium in a 24-well tissue culture plate. The culture medium was a 2:1 v/v mixture of basal medium Eagle (Sigma, St. Louis, MO, USA) and Earle's balanced salt solution (Invitrogen, Carlsbad, CA, USA) which was supplemented with 5% fetal bovine serum (Invitrogen) and 50 U/mL penicillin G (Sigma). Later in the project, these culture media were replaced with a dissecting medium composed of M199 (Invitrogen) and a culture medium of DMEM/F12 (Invitrogen), each supplemented with 5% fetal bovine serum (Invitrogen) and 50 U/mL penicillin G. The dissecting medium was changed from the bicarbonate-buffered BME/EBSS medium to a HEPES-buffered M199-based medium. This change was made in order to keep the pH of the utricle-containing medium as constant as possible during dissections, which, depending on the number of utricles collected, could last from 30 min to over 2 h. Utricles were maintained in an incubator at 37°C in a 5% CO_2/95% air environment.

Cisplatin was supplied as a 1 mg/mL stock solution (Teva Parenteral Medicine, Inc., Irvine, CA, USA) and diluted in culture medium. Final cisplatin concentrations ranged from 10-60 μg/mL (33.3-200 μM). Cisplatin was not added to control cultures. Co (III) protoporphyrin IX chloride (CoPPIX) was provided in powder form (Frontier

Scientific, Inc., Logan, UT, USA). A 1mM CoPPIX solution was prepared by dissolving the powder in 0.2 M NaOH, lowering the pH below 8 with 1 M HCl, and bringing the solution to volume with 1X phosphate buffered saline (PBS). The 1 mM solution of CoPPIX was then filter-sterilized with a 0.22 μm filter. The 1 mM CoPPIX was further diluted in culture medium resulting in final concentrations ranging from 10-40 μM. CoPPIX-treated utricles were cultured in CoPPIX for 12 h (Kim et al., 2006). Utricles treated with CoPPIX alone were returned to culture media following CoPPIX incubation. For utricles undergoing both CoPPIX and cisplatin treatments, the CoPPIX media was replaced with cisplatin-containing culture media and incubated in cisplatin for 24 h. Zn (II) protoporphyrin IX (ZnPPIX) was provided in powder form (Frontier Scientific, Inc.) and made as previously described (Francis et al, in press). Briefly, 3.1 mg ZnPPIX was dissolved using DMSO and 0.2 M NaOH to make a 50 mM stock solution. The stock solution was brought to a 100 μM working concentration with culture media and filter sterilized.

Immunohistochemistry

Immunohistochemistry was performed similarly to the protocol described in Taleb et al. (2008). Utricles were fixed in 4% paraformaldehyde for a short (1 h at room temperature (RT)) or long (overnight at 4°C) fix depending on the antibody being used. They were then washed in 0.1 M Sorensen's phosphate buffer (SPB). Otoconia were dissolved by incubating utricles in Cal-ex decalcifying solution for 2 min (Fisher Scientific, Fair Lawn, NJ, USA). Otoconia removal was followed by washes in 0.1 M SPB. Utricles were subjected to 0.1 M sodium borohydride solution followed by washes in 0.1 M SPB. Utricles were incubated in blocking solution (2% bovine serum albumin, 0.8% normal

goat serum, 0.4% Triton X in 1X PBS) at room temperature for 3 h. Utricles were exposed to the primary antibodies simultaneously overnight at 4°C. (Primary antibodies: calmodulin (Sigma C 3545; 1:150), calbindin (Chemicon no. AB1778, Temecula, CA, USA; 1:200), myosin 7a (Proteus 25-6790; 1:100), p53 (Cell Signaling 2524; 1:1000), HSP32/HO-1 (Abcam ab13248; 1:300), SOX2 (Santa Cruz sc17320; 1:500))). Following primary antibody incubation, utricles were washed with blocking buffer before incubation with secondary antibodies. Secondary antibodies were diluted in blocking solution (Alexa 488-conjugated goat anti-mouse IgG (Invitrogen no. A11001, 1:500), Alexa 488-conjugated goat anti-rabbit IgG (Invitrogen no. A11008;1:500), Alexa 488-conjugated goat anti-rat 488 (Invitrogen no. A11006; 1:500), Alexa 594-conjugated goat anti-rabbit IgG (Invitrogen no. A11012; 1:500), Alexa 594-conjugated goat anti-mouse IgG (Invitrogen no. A11005; 1:500) Alexa 594-conjugated donkey anti-goat IgG (Invitrogen no. A11058), Alexa 647-conjugated goat anti-rabbit IgG (Invitrogen no. A21244)). Utricles were incubated in secondary antibody solution for 4 h in the dark at RT on a rocker and then mounted on glass slides using Fluoromount G (Southern Biotech, Birmingham, AL, USA). Utricles were visualized using a Zeiss Axioplan 2 fluorescent microscope and a high resolution monochrome digital camera (Zeiss Axiocam MR). Imaging software was used for the purpose of performing hair cell counts (AxioVision 40 V 4.6.3.0). Hair cells were counted in each of ten 900 μm^2 areas (five striolar (calmodulin and calbindin-positive), five extrastriolar (calmodulin-positive, calbindin-negative). (Alternatively, hair cell counts were performed on myosin 7a-labeled utricles, in which case only hair cells of the extrastriolar region were counted.) Cell counts from the 5 striolar and 5 extrastriolar regions were averaged separately and

reported as striolar or extrastriolar hair cell densities. Hair cell density is reported as the mean number of hair cells per unit area for each utricle ± SEM.

Western blotting

Cultured utricles were homogenized in a 0.1mL dounce homogenizer containing RIPA buffer (150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate, 0.1% SDS), 1mM sodium orthovanadate, and 1mM sodium fluoride. The resultant supernatants were resuspended in 5X SDS Laemmli sample loading buffer. The samples were subjected to SDS PAGE using 4-20% Tris-HCl minigels (Bio-Rad, Hercules, CA, USA). The proteins were then transferred to a 0.45 or 0.2 μm-pore Immobilon-P PVDF membrane (Millipore, Billerica, MA, USA). Membranes were blocked in 5% milk in 1X phosphate buffered saline with 0.1% Tween 20 (PBST). Protein bands were visualized by chemiluminescence using either SuperSignal® West Dura Extended Duration Substrate, or SuperSignal® West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL, USA) and developed using CL-XPosure™ Film (Pierce Biotechnology). All antibodies were diluted in 5% milk PBST. An antibody against actin was used as a loading control (#A2066, Sigma). Membranes were incubated in the appropriate HRP-conjugated secondary antibodies. Membranes were stripped between antibodies using Re-Blot Plus Strong Solution (10X) (#2504, Millipore). Results were quantified by densitometry using ImageJ software (Image Processing and Analysis in Java, http://rsb.info.nih.gov/ij/index.html).

Real-time quantitative RT-PCR (qRT-PCR)

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) experiments were performed similarly to Cunningham and Brandon (2006). Treated utricles were preserved and stored in RNAlater RNA Stabilization Reagent (Qiagen, Valencia, CA, USA). Following utricle homogenization in a 1mL dounce homogenizer, total RNA was collected (RNAEasy, Qiagen). RNA was reverse-transcribed (TaqMan, Applied Biosystems, Foster City, CA, USA), and the resulting cDNA was used for SYBR Green (Applied Biosystems) real-time PCR amplification mRNA transcript (Table 2-1). The ΔΔCt method was used to calculate the fold change in the target transcript for each experimental group relative to the control group. Each ΔCt value was calculated by subtracting the mean Ct value of the normaliser (18S or GAPDH) from the individual Ct values for a target. Because each experiment was performed in triplicate, each treatment group had three Ct values. The three ΔCt values for each target were then averaged. At that point the ΔΔCt values for a particular target were calculated by subtracting the mean ΔCt value of each treatment group from the mean ΔCt value of the control group. Finally, assuming 100% efficacy of the primer set, the calculation $2^{\Delta\Delta Ct}$ was performed to give the fold change in the target relative to control. When at least three biological replicates were performed, the mean $2^{\Delta\Delta Ct}$ value for those replicates are graphed and error bars represent the SEM for those values. When less than three biological replicates were performed, the $2^{\Delta\Delta Ct}$ values from a representative experiment are depicted graphically with no error bars.

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). Data were analyzed using statistical software SYSTAT 8.0 (San Jose, CA, USA) and Graphpad 3.0 (La Jolla, CA, USA). Tests included ANOVA, 2-way analysis of variance (ANOVA), 3-way ANOVA, and 2-way repeated measures analysis of variance (RM-ANOVA). Post-hoc testing included Tukey's and Dunnett's multiple comparisons. Results were considered significant when the p-value was less than 0.5.

Gene	Primer set	Product size	Reference
18S	F: 5'-TTCGGAACTGAGGCCATGATT-3' R: 5'-TTTCGCTCTGGTCCGTCTTG-3'	100	(Yoshida et al 1999)
GAPDH	F: 5'-TGTGTCCGTCGTGGATCTGA-3' R: 5'-TTGCTGTTGAAGTCGCAGGAG-3'	150	(Han et al 2010)
HSP90	F: 5'-GTGCGTGTTCATTCAGCCAC-3' R: 5'-GCAATTTCTGCCTGAAAGGC-3'	100	(Cunningham & Brandon 2006)
HSP70	F: 5'-AGGCCAGGGCTGGTATTACT-3' R: 5'-AATGACCCGAGTTCAGGATG-3'	170	(Yoshida et al 1999)
HSP32	F: 5'-CTCACAGATGGCGTCACTTCGTCA-3' R: 5'-TTGCCAACAGGAAGCTGAGA-3'	152	(Kim et al 2006)
HSP27	F: 5'-GAGAACCGAACGACCGTCC-3' R: 5'- CCCAATCCTTTGACCTAACGC-3'	100	(Cunningham & Brandon 2006)
IL-10	F: 5'-GACAACATACTGCTAACCGACCTC-3' R: 5'-ATCACTCTTCACCTGCTCCACT-3'	252	(Yin et al 2010)

Table 2-1. Primer sets used in qRT-PCR experiments.

CHAPTER 3: Cisplatin and p53

Cisplatin is a widely and successfully used chemotherapeutic drug, a major side effect of which is ototoxicity. Cisplatin causes damage to multiple cell types in the cochlea, including the sensory hair cells. Though the ototoxic effects of cisplatin have been recognized for over three decades, the molecular mechanisms behind cisplatin-induced hair cell death are largely unknown.

The primary mechanism by which cisplatin kills cancer cells is through DNA damage, and some evidence exists supporting the involvement of DNA damage in cisplatin-induced hair cell death. Cancer cells and hair cells differ greatly in their mitotic activity. Cancer cells are rapidly dividing, whereas mammalian hair cells are terminally differentiated and undergo no division postnatally. It is for this reason that the mechanism(s) of cisplatin-induced death of these two cell types might differ. As in cisplatin-treated cancer cells, cisplatin-DNA adducts have been observed in cochlear hair cells of cisplatin-treated animals (van Ruijven et al 2005a). Furthermore, HMGB1, a protein involved in DNA damage recognition, was shown to increase in cochleae of cisplatin-treated rats (Li et al 2006). In 2003 p53 was implicated in cisplatin-induced hair cell death (Zhang et al 2003). This group demonstrated that a pharmacological inhibitor of p53, pifithrin-α, protected against cisplatin-induced death of hair cells in explants of neonatal organ of Corti. Recently, pifithrin-α was shown to exhibit some off-target effects. In other words, this compound has been shown to act on molecules other than p53 (Davidson et al 2008). Thus, the current study was designed to examine the role of p53 in cisplatin-induced hair cell death using $p53^{-/-}$ mice.

Methods

$p53^{-/-}$ *mice*

p53 knockout breeders ($p53^{+/-}$) were ordered from Jackson Laboratories. This strain was constructed via insertion of a neomycin cassette into the Trp53 gene locus (Jacks et al 1994). The neomycin resistance cassette disrupts 40% of the p53 coding sequence, resulting in a complete lack of p53 protein synthesis (Jacks et al 1994). As $p53^{-/-}$ and $p53^{+/-}$ mice are predisposed to tumor development, all mice were monitored for tumor formation, and sacrificed in the case that tumors developed and/or the mice reached 6 months of age for $p53^{-/-}$ and 10 months for $p53^{+/-}$. Genotyping was performed as suggested on the JAX website, using the same primers as originally described:

5' ACA GCG TGG TGG TAC CTT AT 3' oIMR7777

5' TAT ACT CAG AGC CGG CCT 3' oIMR7778

5' CTA TCA GGA CAT AGC GTT GG 3' oIMR8306

Cisplatin administration and auditory brainstem response (ABR)

Numerous labs have tried various protocols in an attempt to achieve a cisplatin-induced hearing loss in mice with little success. After amassing a list of those protocols that had been tried and failed (both published and not), several new protocols were attempted to no avail. Finally, through correspondence with Nichole Schmitt, the following protocol was implemented which results in minimal mortality of the mice and a threshold shift in the 32 kHz frequency of about 25 dB. Prior to the publication of this protocol by Dr.

Schmitt, More et al. (2010) published the same protocol, with the exception of saline supplementation.

ABR thresholds of anesthetized $p53^{-/-}$ and $p53^{+/+}$ mice were measured prior to (pre-test) and 72 h after (post-test) cisplatin treatment. Anesthesia was given as an IP injection of a ketamine chloride (100 mg/kg; Fort Dodge Animal Health, Dodge, IO, USA) and xylazine chloride (20 mg/kg; Ben Venue Laboratories, Bedford, OH, USA) cocktail. ABR measurements were performed following the protocol described in Francis et al. (in press) using software and equipment from Intelligent Hearing Systems (Miami, FL, USA). Following anesthesia, the mouse subject was kept warm using a heating pad and placed in a sound-proof chamber. Three platinum subdermal needle electrodes were then placed at appropriate positions for performing the ABR: a non-inverting electrode at the vertex, an inverting electrode near the mastoid process behind the left ear, and a reference electrode at the hip (FH-E2-12 Grass Technologies, West Warwick, RI, USA). A high-frequency transducer was used to deliver sound stimuli (IHS). Pure tones at 8, 16, and 32 kHz were presented at a rate of 19.3/s. Each wave form represented an average of 1024 stimulus presentations. Responses were filtered using a 300 Hz high pass filter and a 3000 Hz low pass filter. Responses were recorded beginning with the highest intensity and decreasing at 10 dB intervals until nearing threshold. 5 dB intervals were used to pinpoint the hearing threshold. The hearing threshold for a particular frequency was determined as the lowest intensity level at which a response was detectable in the ABR waveform. The cisplatin-induced hearing loss protocol was modified from More et al. (2010) by adding a pre-cisplatin hydration as suggested by Nichole Schmitt, as well as post-cisplatin saline supplementation. Mice

were given 36 h to recover from anesthesia and then received 400 μL saline intraperitoneally followed 30 min later by a single intraperitoneal injection of cisplatin (20 mg/kg) or saline. Subcutaneous saline (3 mL) was given every 24 h following the cisplatin injection. ABR thresholds were measured 72 h after the cisplatin was administered (ABR post-test). Threshold shifts were calculated by subtracting the ABR threshold at a given frequency as determined at the pre-test from the ABR threshold of that same frequency as determined from the post-test. Following the ABR post-test mice were sacrificed without recovering from anesthesia. The cochleae were removed from each animal and processed for whole mount immunochemistry using anti-myosin 7a as previously described in Taleb et al. (2009).

p53 is not necessary for cisplatin-induced hair cell death in vitro

In order to determine if p53 is necessary for cisplatin-induced hair cell death *in vitro*, utricles from $p53^{-/-}$ mice and their wild-type littermates were exposed to cisplatin for 24 h. The utricles were then fixed and cell counts were performed. Utricles from both $p53^{-/-}$ and $p53^{+/+}$ mice exhibited similar amounts of hair cell death following cisplatin treatment (Fig. 3-1) *(2-way ANOVA:* $F_{3,47}=2.72$, $p=0.055$*)*. Therefore, p53 is not necessary for cisplatin-induced hair cell death *in vitro*.

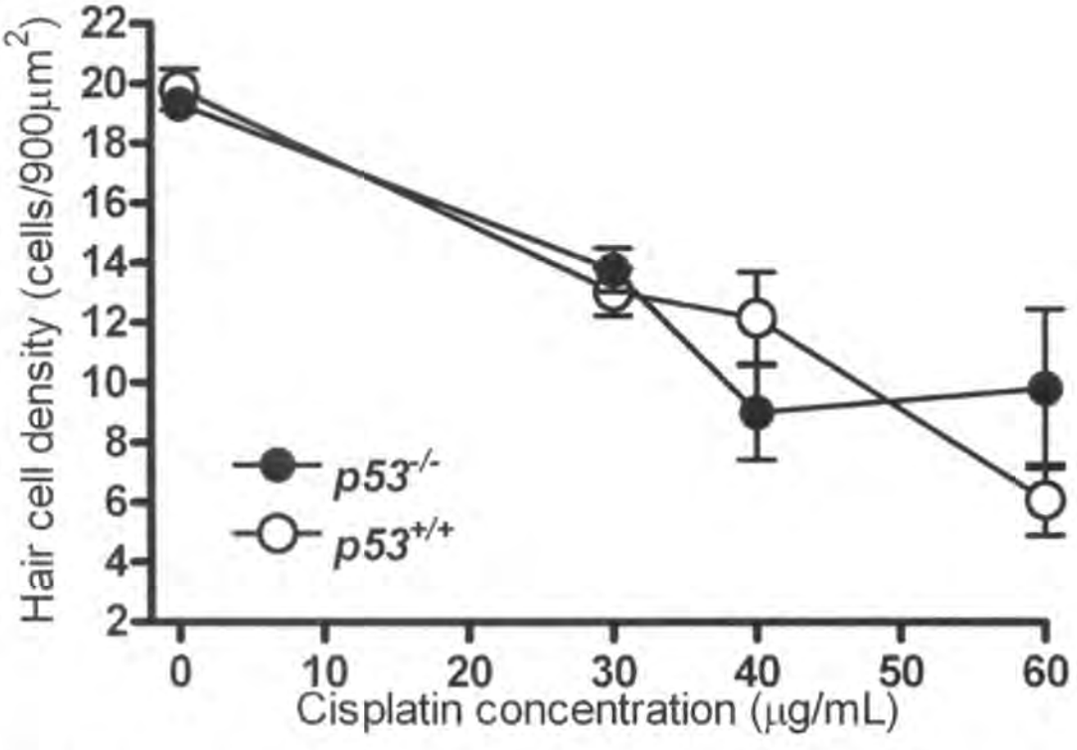

Figure 3-1. p53 is not required for cisplatin-induced hair cell death *in vitro*. Utricles from *p53*$^{-/-}$ mice and their wild-type littermates were treated with 0, 30, 40, or 60 μg/mL (0, 100, 133.3, or 200 μM) cisplatin for 24 h. Cell counts were performed on stained utricles. *p53*$^{-/-}$ and *p53*$^{+/+}$ utricles exhibited similar hair cell densities following cisplatin treatment *(2-way ANOVA: $F_{3,47}=2.72$, $p=0.055$). Data points represent the mean ± SEM for n = 4-13 utricles per condition.*

p53 is not necessary for cisplatin-induced hearing loss or cochlear hair cell death in vivo

In order to examine the role of p53 in cisplatin-induced hearing loss and cochlear hair cell death *in vivo*, $p53^{-/-}$ mice and their wild-type littermates were treated with systemic cisplatin. Mice underwent hearing testing via pretest ABRs prior to receiving a single cisplatin injection (20 mg/kg, IP). Post-test ABRs were performed 72 h after the cisplatin injection. Control mice received IP saline as a vehicle control for cisplatin. Cochleae from these mice were preserved for immunochemistry. ABR results indicate significant threshold shifts in both $p53^{+/+}$ and $p53^{-/-}$ mice following cisplatin treatment ($F_{1,18} = 17.39$, $p = 0.0006$) (Fig. 3-2). A significant effect of frequency was observed, with greatest threshold shift occurring at 32 kHz ($F_{2,36} = 6.35$, $p = 0.0038$). There was no significant difference in threshold shift as a result of cisplatin treatment between the two genotypes (*RM-ANOVA:* $F_{1,18}=1.72$, $p=0.21$). Although it seems as though a difference may exist between ABR threshold shifts of saline-injected control $p53^{+/+}$ and $p53^{-/-}$ mice, this is not likely the case. Statistically, there was no main effect of genotype across frequencies (*RM-ANOVA:* $F_{1,18}=0.25$, $p=0.62$). In the first group of saline-injected animals tested, two $p53^{+/+}$ individuals exhibited a threshold shift greater than would be expected by natural variation in the testing (~10db). However, it is possible that this threshold shift was a result of otitis media and not an effect of genotype on the hearing capacity of these animals. Such a threshold shift was not seen in saline-injected $p53^{+/+}$ mice in later experiments. These data indicate that p53 is not required for cisplatin-induced hearing loss.

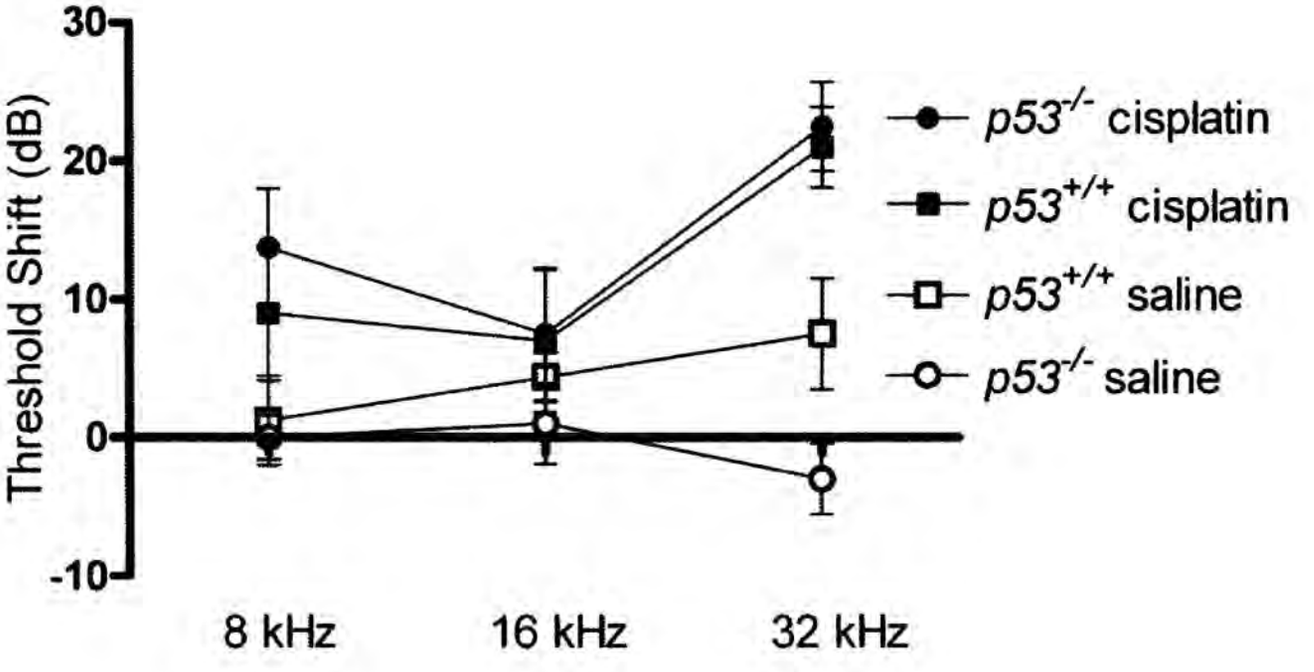

Figure 3-2. *p53*$^{-/-}$ mice are not protected from cisplatin-induced hearing loss. *p53*$^{-/-}$ mice and their wild-type littermates were injected with 20 mg/kg cisplatin or saline. Mice underwent ABR measurements prior to, as well as 72 h following injection. Cisplatin induced significant threshold shifts in both genotypes *($F_{1,18}$ = 17.39, p = 0.0006)*. No significant difference in threshold shifts existed between *p53*$^{-/-}$ and *p53*$^{+/+}$ mice *(RM-ANOVA: $F_{1,18}$=1.72, p=0.21)*. Statistical analysis revealed no main effect of genotype *(RM-ANOVA: $F_{1,18}$=0.25, p=0.62)*. *Data points represent the mean ± SEM for n = 4-8 mice per condition.*

Cochleae were harvested from $p53^{+/+}$ and $p53^{-/-}$ mice following post-test ABR measurements. A control (or saline) cochlea reveals a typical pattern of three rows of outer hair cells (OHCs) and a single row of inner hair cells (IHCs) present from base to apex. Previous studies have shown that cisplatin-exposed cochleae typically exhibit loss of OHCs in the base with subsequent OHC loss progressing toward the apex and loss of IHCs occurring only at very high cisplatin doses (Cardinaal et al 2000; van Ruijven et al 2005b). Cochleae from cisplatin-treated $p53^{+/+}$ and $p53^{-/-}$ mice exhibited loss of OHCs in the base, but no OHC loss in the middle or apical turns (Fig. 3-3). No IHC loss was observed. No outer hair cells were missing in the apex and middle turns of both genotypes treated with either saline or cisplatin. The finding that cochleae from $p53^{-/-}$ and $p53^{+/+}$ mice exhibited similar loss of hair cells in the basal turns indicated that p53 is not necessary for cisplatin-induced cochlear hair cell death. The ABR results were consistent with the pattern of hair cell loss as seen by the cochlear whole mounts. The most significant cisplatin-induced threshold shift occurred at the highest frequency tested, 32 kHz. The 32 kHz place, according to mathematical derivation, is located in the basal third of the mouse cochlea (Muller et al 2005).

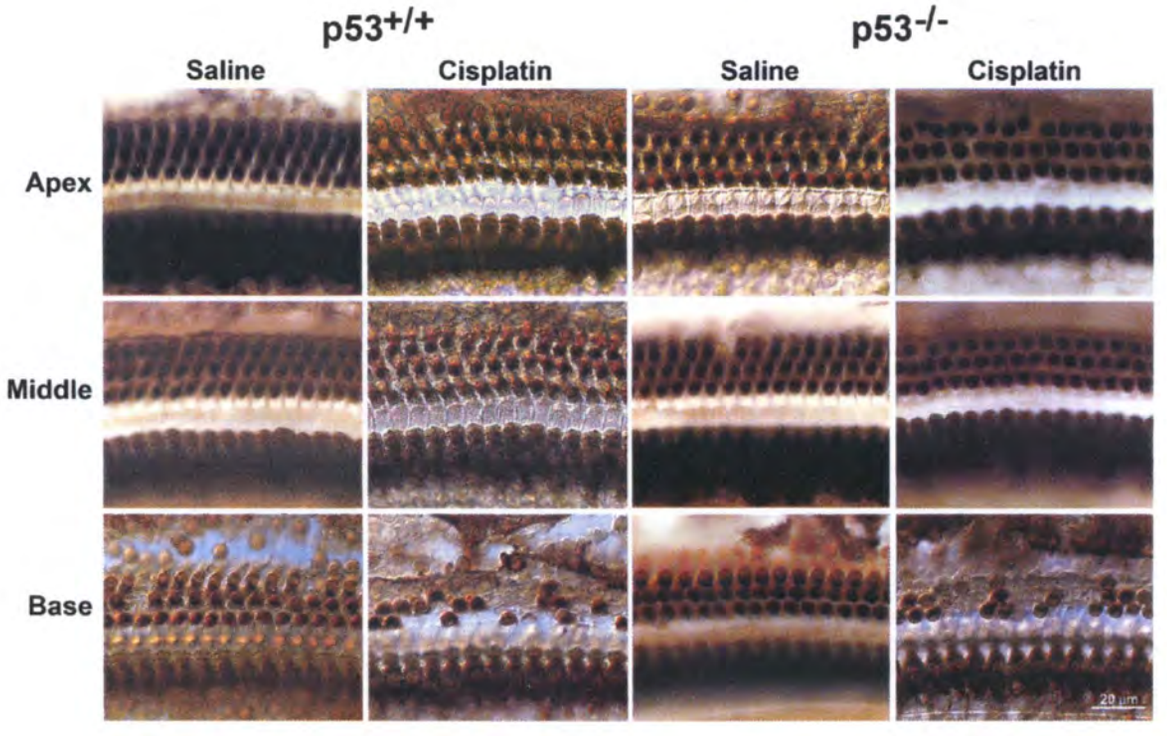

Figure 3-3. *p53*$^{-/-}$ mice are not protected from cisplatin-induced cochlear hair cell death. Cochleae were removed from cisplatin- and saline-injected *p53*$^{-/-}$ and $^{+/+}$ mice following ABR post-test. Organ of Corti whole mount preparations were stained with primary antibody against myosin 7a, and visualized with DAB. Representative images from each treatment group are presented in the micrograph. Dramatic hair cell loss is evident in the basal turns of cochleae from mice of both genotypes following cisplatin treatment.

p53 is upregulated in stroma, but not in sensory cells of cultured utricle

Because there are multiple cell types in the utricle, it was necessary to identify the specific cell types in which p53 is upregulated following cisplatin treatment. Utricles were treated with cisplatin for 6 h, fixed, and stained with antibodies against p53 and myosin 7a (Fig. 3-4). Hoechst was used for visualization of nuclei. These utricles were imaged by confocal microscopy at three specific planes in the z dimension: (1) hair cell nuclei, (2) supporting cell nuclei, and (3) stroma. Results indicate that p53 is not stabilized in hair cells or supporting cells, whereas p53 is markedly upregulated in the nuclei of cells in the stroma. According to these data, cisplatin does not induce p53 activation in adult sensory epithelium, which supports the idea that cisplatin-induced hair cell death occurs independently of p53.

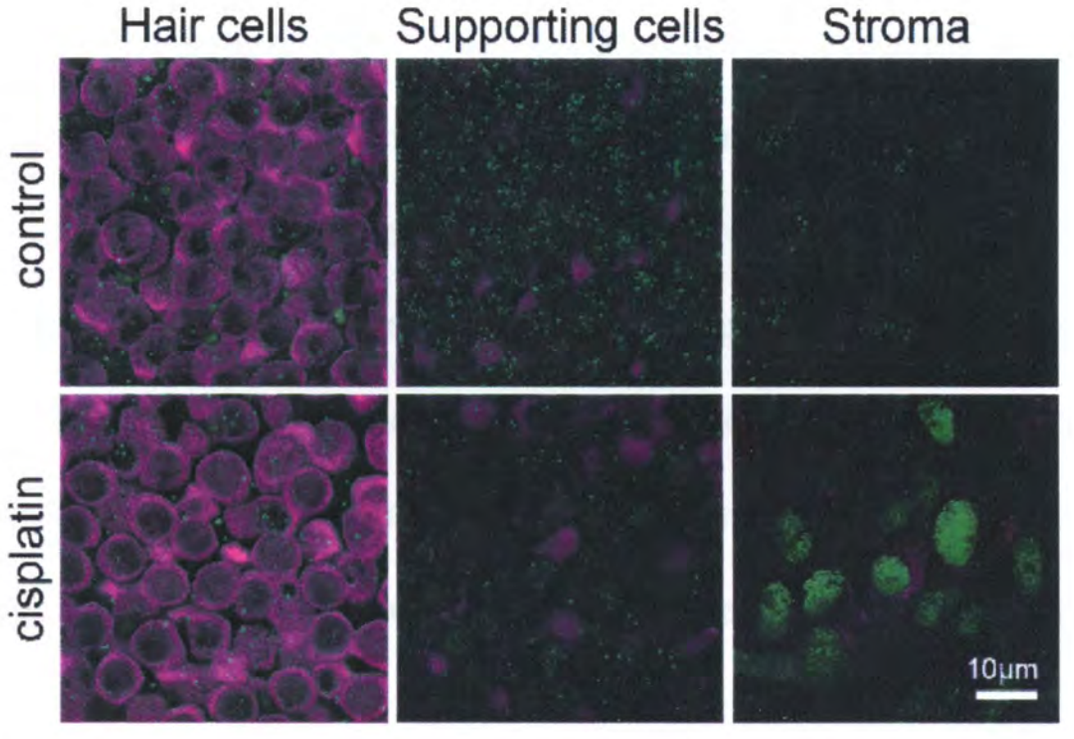

Figure 3-4. Cisplatin-treated mouse utricles exhibit p53 stabilization in stroma. Utricles were treated with 0 or 25 μg/mL (83.3 μM) cisplatin for 6 h and processed for immunochemistry (myosin7a (purple), total p53 (green)). As indicated in these confocal micrographs, cisplatin-induced p53 stabilization is restricted to stroma. No upregulation of p53 was observed in either the hair cell or supporting cell layers.

Discussion: Cisplatin and p53

The transcription factor p53 was investigated as a potential player in the death of cisplatin-treated hair cells. Both *in vitro* and *in vivo* data acquired by using $p53^{-/-}$ and $p53^{+/+}$ utricles and mice indicate that p53 is not necessary for cisplatin-induced hair cell death or hearing loss (Figs. 3-1, 3-2, & 3-3). The lack of p53 upregulation in hair cells and supporting cells of cisplatin-treated utricles lends further support to these findings, especially in light of the marked upregulation of p53 in the stroma (Fig. 3-4). Historically, the DNA damage response has been implicated in cisplatin-induced death of rapidly dividing tumor cells. In addition, evidence for involvement of the DNA damage response in cisplatin-induced hair cell death exists. A previous report indicated that a p53 inhibitor, pifithrin-α, protected against cisplatin-induced hair cell death *in vitro* (Zhang et al 2003). Based on the fact that pharmacological inhibitors can exhibit off-target effects, one must consider that the protective effect of pifithrin-α against cisplatin-induced hair cell death may not be p53-mediated. A recent report demonstrated that pifithrin-α not only inhibits p53 function but also the function of another p53 family member, p73 (Davidson et al 2008). p73 is upregulated in response to specific modes of DNA damage, including cisplatin treatment (Gong et al 1999; Strano et al 2005). Like p53, p73 is stabilized via a phosphorylation event that results in reduced degradation of the protein, followed by its accumulation in cells and subsequent ability to transcriptionally activate its targets. Many of the transcriptional targets of p73 and p53 overlap and include the proapoptotic proteins Bak (Graupner et al 2011), Noxa (Martin et al 2009), and Puma (Melino et al 2004). In light of the lack of any apparent role for p53 in cisplatin-induced hair cell death, it is possible that the protection conferred by

pifithrin-α against cisplatin-induced hair cell death is mediated by p73 inhibition. Thus, p73 may be a major mediator of cisplatin-induced hair cell death.

The use of *p53*$^{-/-}$ mice is a potential limitation to this study, in that such long-term knockouts are known to develop compensatory mechanisms to replace the function(s) of the missing protein. The expression levels of other p53 family members, such as p73, were not examined in this study; however, their expression could be different than that observed in wild-type mice due to the absence of p53.

The results of the present study indicate that p53 is not a major instigator of hair cell death caused by the chemotherapeutic drug cisplatin. This conclusion was reached using *in vitro* and *in vivo* studies in a *p53*$^{-/-}$ mouse model to investigate the role of this notorious transcription factor and mediator of the DNA damage response. *p53*$^{-/-}$ mice were equally as susceptible to cisplatin-induced hair cell death and hearing loss as their wild-type littermates. Furthermore, immunochemical studies revealed a complete lack of p53 upregulation in hair cells and supporting cells of cultured utricles in response to cisplatin treatment.

CHAPTER 4: Cisplatin and HSPs

Heat shock proteins are a class of molecules intrinsic to cells which, once upregulated, can play a protective role. Non-lethal heat shock preconditioning results in the upregulation of heat shock proteins (HSPs), a group of molecules known to be protective against a variety of cellular stresses (Martindale & Holbrook 2002; Richter et al 2010). Previously, we have shown that heat shock protects against both cisplatin- and aminoglycoside-induced hair cell death (Cunningham & Brandon 2006).

The most highly-inducible HSP in response to heat shock is HSP70. HSP70 is the most ubiquitous and evolutionarily-conserved HSP (Karlin & Brocchieri 1998). In a number of systems, HSP70 inhibits apoptotic cell death by preventing oligomerization of Bax, release of cytochrome-c and second mitochondria-derived activator of caspases (Smac) from mitochondria, formation of a functional apoptosome, and even cell death subsequent to caspase 3 activation (Beere et al 2000; Evans et al 2010; Jaattela et al 1998; Jiang et al 2009; Stankiewicz et al 2005; Tsuchiya et al 2003). Moreover, HSP70 inhibits aminoglycoside-induced hair cell death *in vitro* and hearing loss *in vivo* (Taleb et al 2009; Taleb et al 2008).

Another heat shock protein, HSP32, has potential as a protectant against cisplatin-induced hair cell death and, potentially, hearing loss. Like other heat shock proteins, HSP32 (also called heme oxygenase-1, HO-1), is upregulated in response to a variety of stressors, including thermal stress. However, unlike many heat shock proteins, HSP32 is not a molecular chaperone. HSP32 is an enzyme responsible for heme catabolism, the products of which include bilirubin, carbon monoxide (CO), and free iron (Tenhunen et al 1968; 1969). Bilirubin and CO have known antioxidant and anti-inflammatory

properties (Hayashi et al 1999; Kirkby & Adin 2006; Otterbein et al 2000; Stocker et al 1987). Pharmacological induction of HSP32 is protective against multiple insults in many tissue types, including ischemia-reperfusion injury in liver and retina (Sun et al 2010; Tsuchihashi et al 2007). HSP32 has been shown to protect human renal proximal tubule cells from cisplatin-induced toxicity *in vitro*, and HSP32 knockout mice are more susceptible to cisplatin-induced nephrotoxicity than their wild-type counterparts (Shiraishi et al 2000). HSP32 has recently been shown to mediate the protective effect of celastrol against aminoglycoside-induced hair cell death (Francis et al, in press). In addition pharmacological induction of HSP32 using cobalt protoporphyrin IX chloride (CoPPIX) protects neonatal cochlear explants from cisplatin-induced hair cell death (Kim et al 2006).

The current study was designed to identify whether specific heat shock proteins, such as HSP70 and/or HSP32, can protect against cisplatin-induced hair cell death.

Methods

HSP70.1/3$^{-/-}$ *mice*

HSP70.1/3$^{-/-}$ mice have inactivated *HSP70.1* and *HSP70.3* genes. Both *HSP70.1* and *HSP70.3* were inactivated by insertion of a single *neo* gene on chromosome 17 (Hunt et al 2004). *HSP70.1/3*$^{-/-}$ mice were obtained from the Mutant Mouse Regional Resource Center at the University of California at Davis. Since *HSP70.1/3*$^{-/-}$ mice are viable and fertile, mating pairs were established between knockout mice, and all offspring were *HSP70.1/3*$^{-/-}$ genotype. Wild-type B6129SF2/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and used as strain controls.

rHSP70i-expressing transgenic mice

rHSP70i-expressing transgenic mice were provided by Dr. Wolfgang Dillmann (University of California San Diego). rHSP70i-expressing transgenic mice constitutively express rat inducible HSP70 (rHSP70i) (Marber et al 1995). rHSP70i-expressing transgenic mice are on a BALB/c × C57BL/6 background. Wild-type female CB6F1 mice (Jackson Laboratory) were mated with male rHSP70i-expressing transgenic mice. Wild-type littermates served as controls. Genotyping was performed as described in the general methods section. The following primers resulted in a 280-bp product of the CMV-IE sequence of the transgene: forward primer 5'-ATTACGGGGTCATTAGTTCATAGCC-3', reverse primer 5'-GTAGGAAAGTCCCAGTAGGAAAGTCCCATAAGGTCATGT-3.'

Heat shock preconditioning

The heat shock preconditioning protocol was performed as described by Cunningham and Brandon (2006). Utricles and surrounding media (~1 mL total volume) were transferred from 24-well culture plates into sterile 1.5-mL microcentrifuge tubes. Tubes containing utricles to be heat shocked were placed in a 43°C water bath for 30 min. Immediately following heat shock, utricles and media were transferred back into their original wells in the 24-well plate and allowed to recover at 37°C/5% CO_2 for 6 hours before any additional treatment. Control utricles were transferred to sterile 1.5-mL microcentrifuge tubes, and they were placed in the 37°C incubator for 30 min before they were transferred back to their original wells in the 24-well plate.

Results

Heat shock inhibits cisplatin-induced hair cell death

We previously showed that heat shock preconditioning inhibits cisplatin-induced hair cell death at a single cisplatin concentration (Cunningham & Brandon 2006). HSP induction in response to heat shock was examined by western blotting (Fig. 4-1). The heat-shocked utricles were exposed to 43°C for 30 min, followed by a 6 h recovery period at 37°C. Heat shock resulted in upregulation of HSPs 27, 32, 40, and 70 with little change in HSPs 90 and 60.

In order to examine the protective effect of heat shock across the cisplatin dose-response curve, heat-shocked and control utricles were treated with cisplatin at a range of concentrations for 24 h. Following cisplatin treatment, the utricles were fixed, stained with calmodulin and calbindin, and hair cells were counted. In control (non heat-shocked) utricles, cisplatin treatment resulted in a dose-dependent loss of hair cells. Utricles that were heat-shocked prior to cisplatin treatment had significantly greater hair cell survival across the dose-response curve *(2-way ANOVA:* $F_{6,160} = 5.778$, $p<0.0001$*)*(Fig. 4-2).

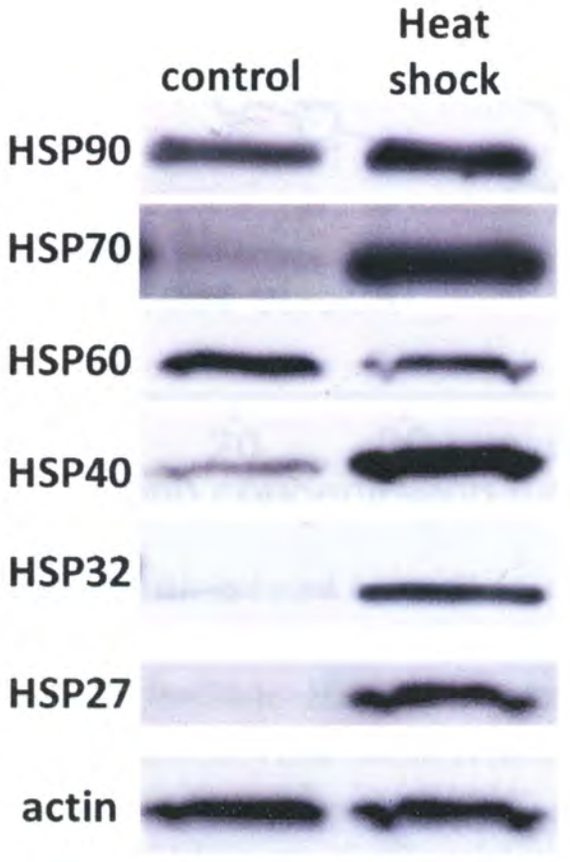

Figure 4-1. HSP expression following heat shock. Control and heat-shocked utricles were homogenized, and the resultant lysates were analyzed by western blot using antibodies against HSP27, HSP32, HSP40, HSP60, HSP70, HSP90, and actin. Results indicate that sublethal heat shock of adult mouse utricle upregulates HSP70, HSP40, HSP32, and HSP27.

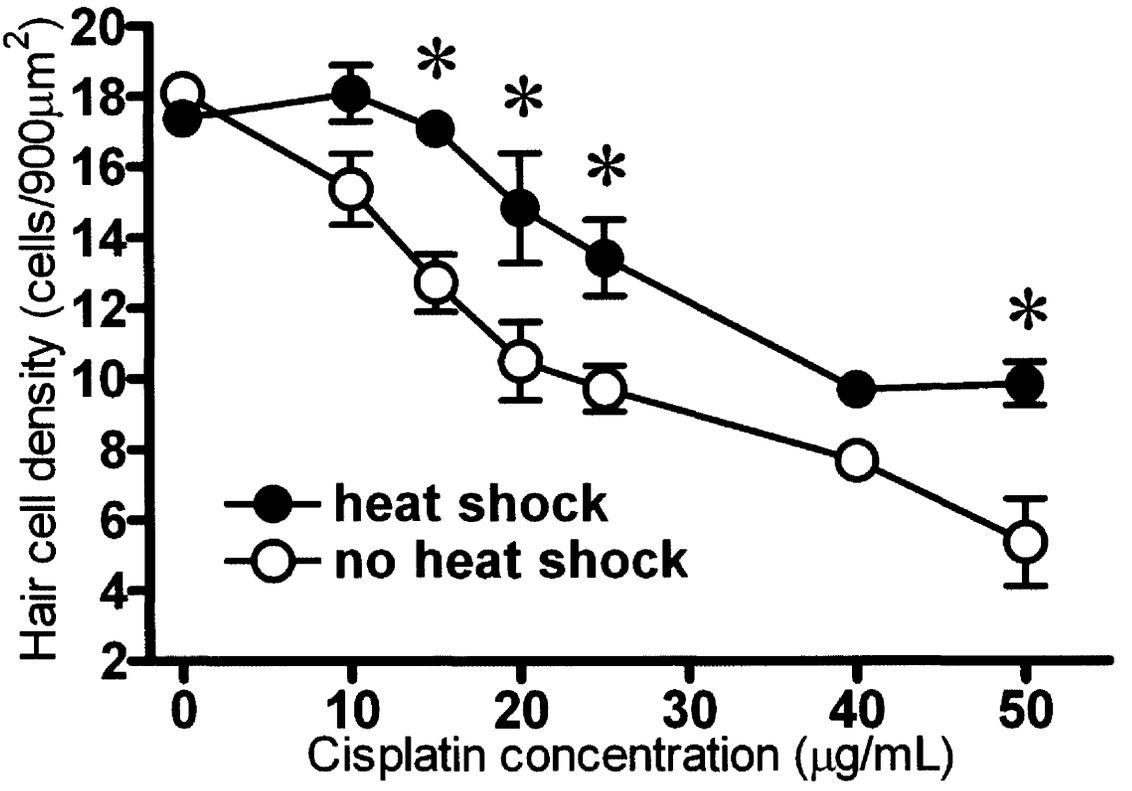

Figure 4-2. Heat shock inhibits cisplatin-induced hair cell death. Control and heat-shocked utricles were exposed to 0, 10, 15, 20, 25, 40, or 50 μg/mL (0, 33.3, 50, 66.7, 83.3, 133.3, or 166.6 μM) cisplatin for 24 h. Hair cell counts were performed on calmodulin and calbindin-stained utricles. Heat shock resulted in significant protection against cisplatin-induced hair cell death *(2-way ANOVA:* $F_{6,160} = 5.778$, $p<0.0001$*). Data points represent the mean ± SEM for n = 5-40 utricles per condition. Asterisks (*) denote significant differences in hair cell density between utricles that were heat-shocked and those that were not.*

HSP70 is required for the protective effect of heat shock

HSP70 was highly-induced in response to heat shock (Fig. 4-1). In order to determine whether HSP70 is necessary for the protective effect conferred by heat shock against cisplatin-induced hair cell death, we used utricles from *HSP70.1/3*$^{-/-}$ and wild-type mice. Utricles of both genotypes were either maintained at 37°C or heat-shocked. Utricles were treated with either 0 or 20 μg/mL (66.7 μM) cisplatin for 24 h. Heat shock had a significant protective effect against cisplatin-induced hair cell death in wild-type utricles *(2-way ANOVA:* $F_{1,54} = 8.975$, $p<0.01$*)*(Fig. 4-3). However, there was no significant protective effect of heat shock in the *HSP70*$^{-/-}$ utricles *(2-way ANOVA:* $F_{1,39} = 0.029$, $p>0.05$*).* These data indicate that HSP70 may be necessary for the protective effect of heat shock against cisplatin-induced hair cell death.

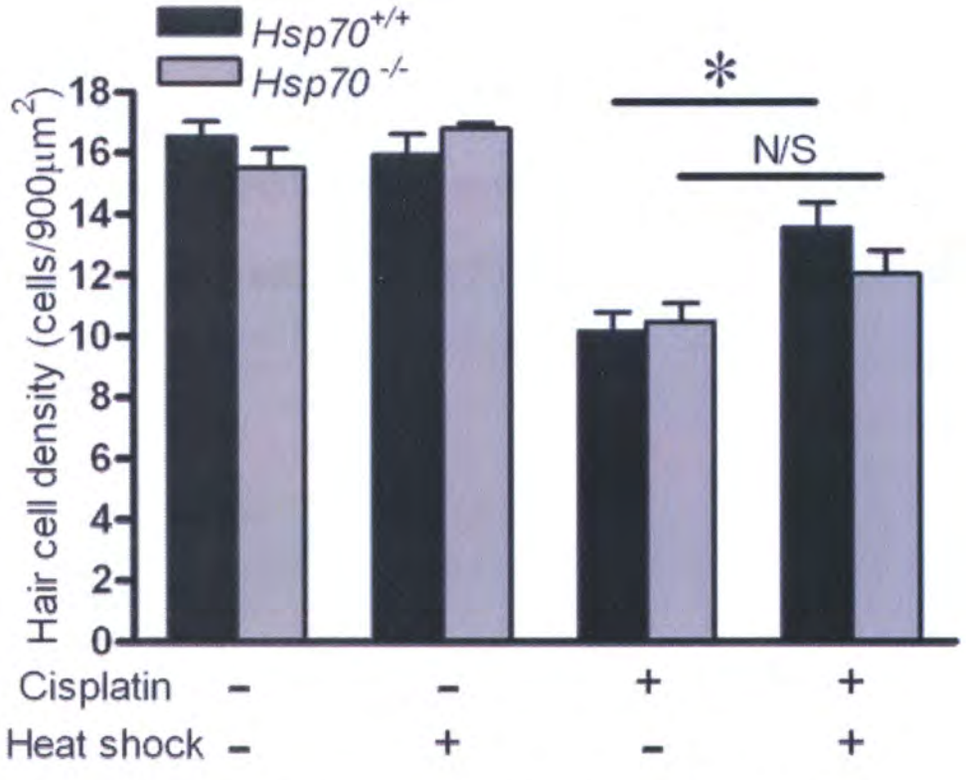

Figure 4-3. Hsp70 is required for the protective effect of heat shock. Control and heat-shocked utricles from adult wild-type and *Hsp70*$^{-/-}$ mice were exposed to 20 μg/mL (66.7 μM) cisplatin for 24 h. Cell counts were performed on stained utricles. Heat shock inhibited cisplatin-induced hair cell death in *HSP70*$^{+/+}$ utricles *(2-way ANOVA:* $F_{1,54} = 8.975$, $p<0.01$*)*, but not in utricles from *HSP70*$^{-/-}$ mice *(2-way ANOVA:* $F_{1,39} = 0.029$, $p>0.05$*). Data points represent the mean ± SEM for n = 5-18 utricles per condition. Asterisks (*) denote significant differences in hair cell density in the extrastriolar region. "N/S" Not significant .*

Constitutive expression of rHSP70i inhibits cisplatin-induced hair cell death

In order to investigate whether HSP70 is sufficient to confer protection against cisplatin-induced hair cell death, utricles from transgenic mice that constitutively express rHSP70i (and their wild-type littermates) were treated with cisplatin for 24 h. Constitutive rHSP70i expression had a significant protective effect against cisplatin-induced hair cell death *(2-way ANOVA:* $F_{4,116} = 59.063$, $p<0.0001$*)* (Fig. 4-4). These results demonstrate that HSP70 is sufficient to provide partial protection against cisplatin-induced hair cell death, although the protective effect of HSP70 expression doesn't appear to be as robust as that of heat shock.

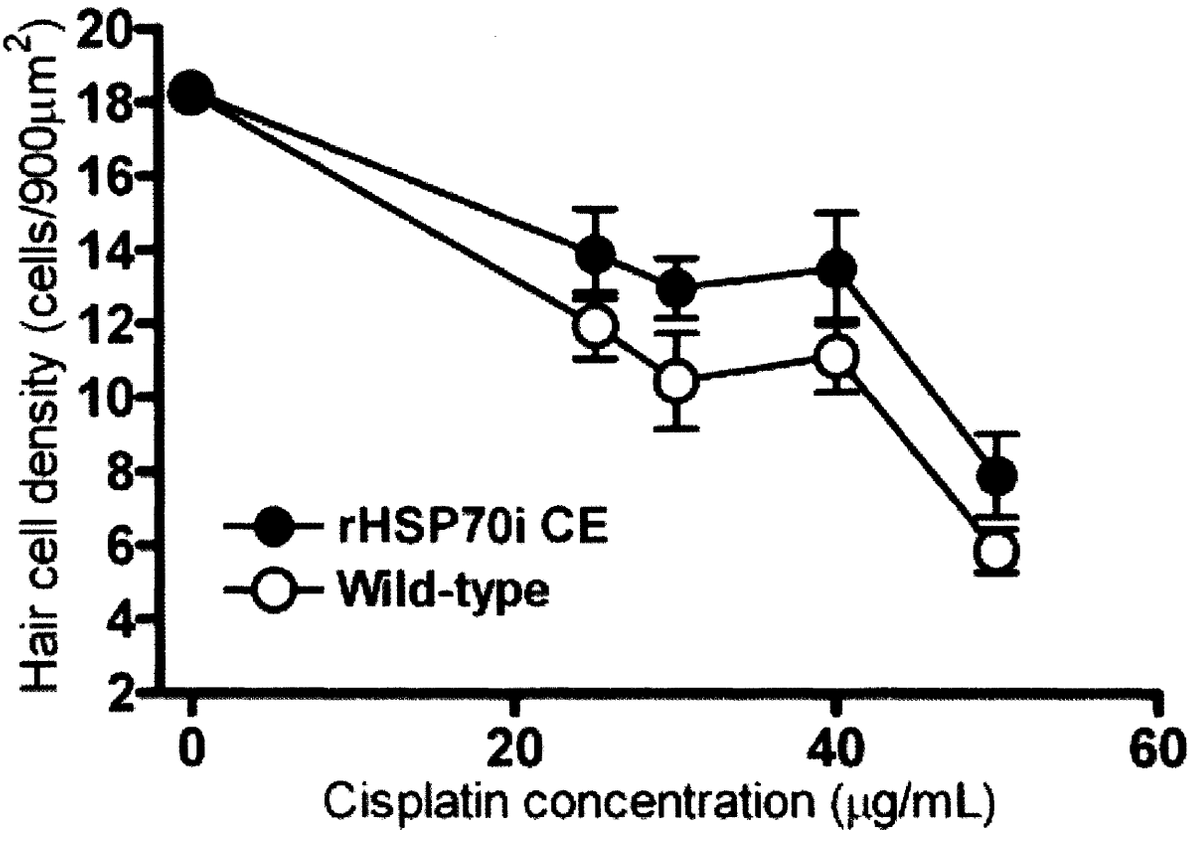

Figure 4-4. rHsp70i constitutive-expression (CE) inhibits cisplatin-induced hair cell death. Utricles from rHsp70i transgenic mice and their wild-type littermates were treated with 0, 25, 30, 40, or 50 μg/mL (0, 83.3, 100, 133.3, or 166.6 μM) cisplatin for 24 h. Cell counts were performed on calmodulin and calbindin-stained utricles. Results reveal a main effect of genotype, indicating that constitutive expression of rHSP70i protects against cisplatin-induced hair cell death *(2-way ANOVA: $F_{4,116}$ = 59.063, $p<0.0001$). Data points represent the mean ± SEM for n = 7-24 utricles per condition.*

HSP32 induction inhibits cisplatin-induced hair cell death

In addition to HSP70, heat shock also results in induction of HSP32 (Fig. 4-1 & 4-5). In order to examine the protective effect of HSP32 against cisplatin-induced hair cell death, we utilized the chemical HSP32 inducer CoPPIX (Drummond & Kappas 1982; Ferrandiz & Devesa 2008). We first tested the specificity of HSP32 induction with CoPPIX by qRT-PCR for which utricles were exposed to 10 μM CoPPIX for 12 h (Fig. 4-6 A). Next, we tested for CoPPIX specificity at the protein level by western blot for which utricles were treated with 20 μM CoPPIX for 12 h (Fig. 4-6 B). After treatment, utricles were processed for western blotting using antibodies against HSP27 (Upstate Cell Signaling Solutions #06-517, 1:1000), HSP32 (R & D Systems #MAB3776, 1:1000), HSP40 (Cell Signaling #4868, 1:1000), HSP60 (Cell Signaling #4870, 1:1000), HSP70 (Cell Signaling #4872, 1:1000), HSP90 (Cell Signaling #4874, 1:1000), and actin (Sigma #A2066, 1:1000). HSP32 was robustly upregulated following CoPPIX treatment of utricles, while the other HSPs were not induced, thus confirming that CoPPIX is a specific inducer of HSP32 in utricles.

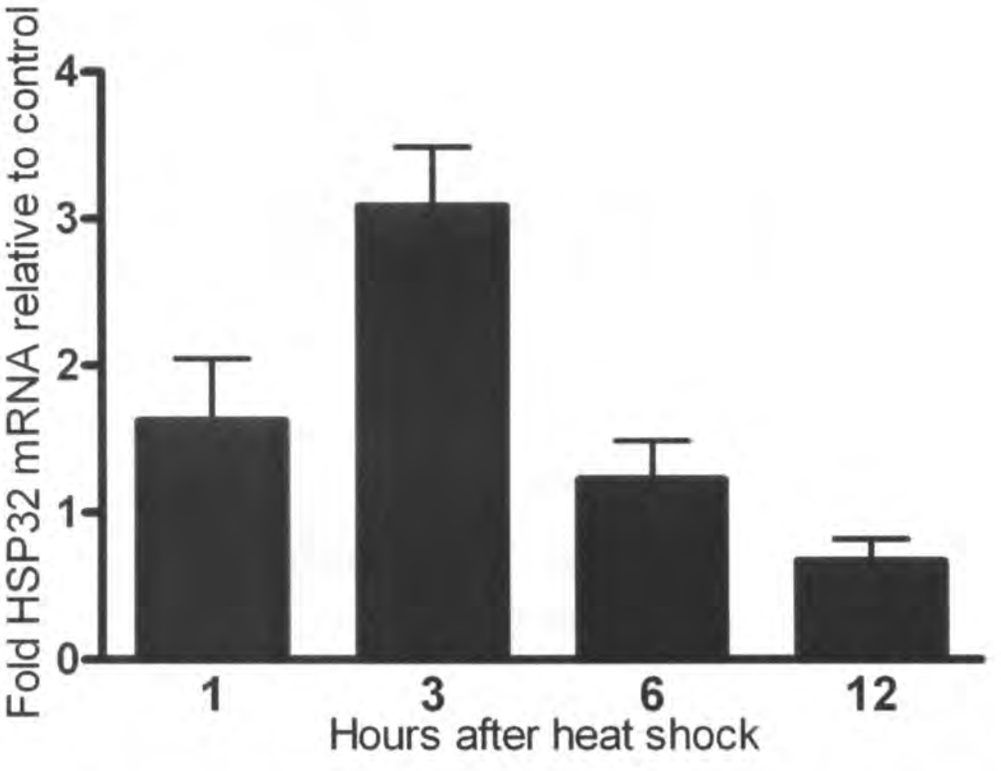

Figure 4-5. HSP32 is transcriptionally upregulated in adult mouse utricle following heat shock. Utricles were preserved in RNA later at 1, 3, 6, and 12 h following heat shock and processed for qRT-PCR. *Data points represent the mean ± SEM for three biological replicates.*

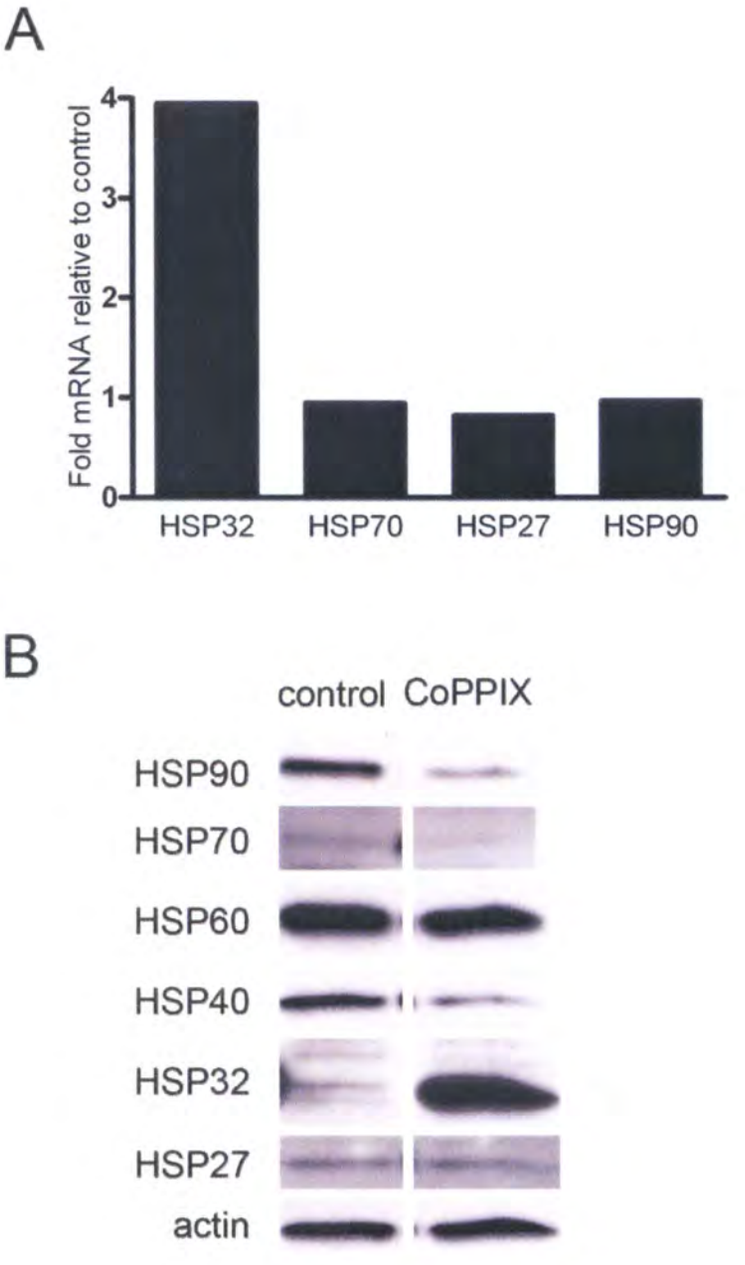

Figure 4-6. CoPPIX induces HSP32 expression in adult mouse utricle. (A) Utricles were treated with 10 μM CoPPIX for 12 h and processed for qRT-PCR. (B) Utricles were treated with 20 μM CoPPIX for 12 h and processed for western blotting.

We next used CoPPIX treatment to determine if HSP32 induction is protective against cisplatin-induced hair cell death. Utricles were treated with CoPPIX for 12 h and then treated with cisplatin for 24 h. Utricles were processed for immunohistochemistry and hair cells were counted. Results indicate a significant protective effect of CoPPIX treatment against cisplatin-induced hair cell death *(2-way ANOVA:* $F_{4,69} = 4.24$, $p=0.004$*)* (Fig. 4-7). These data indicate that HSP32 induction by CoPPIX inhibits hair cell death caused by cisplatin.

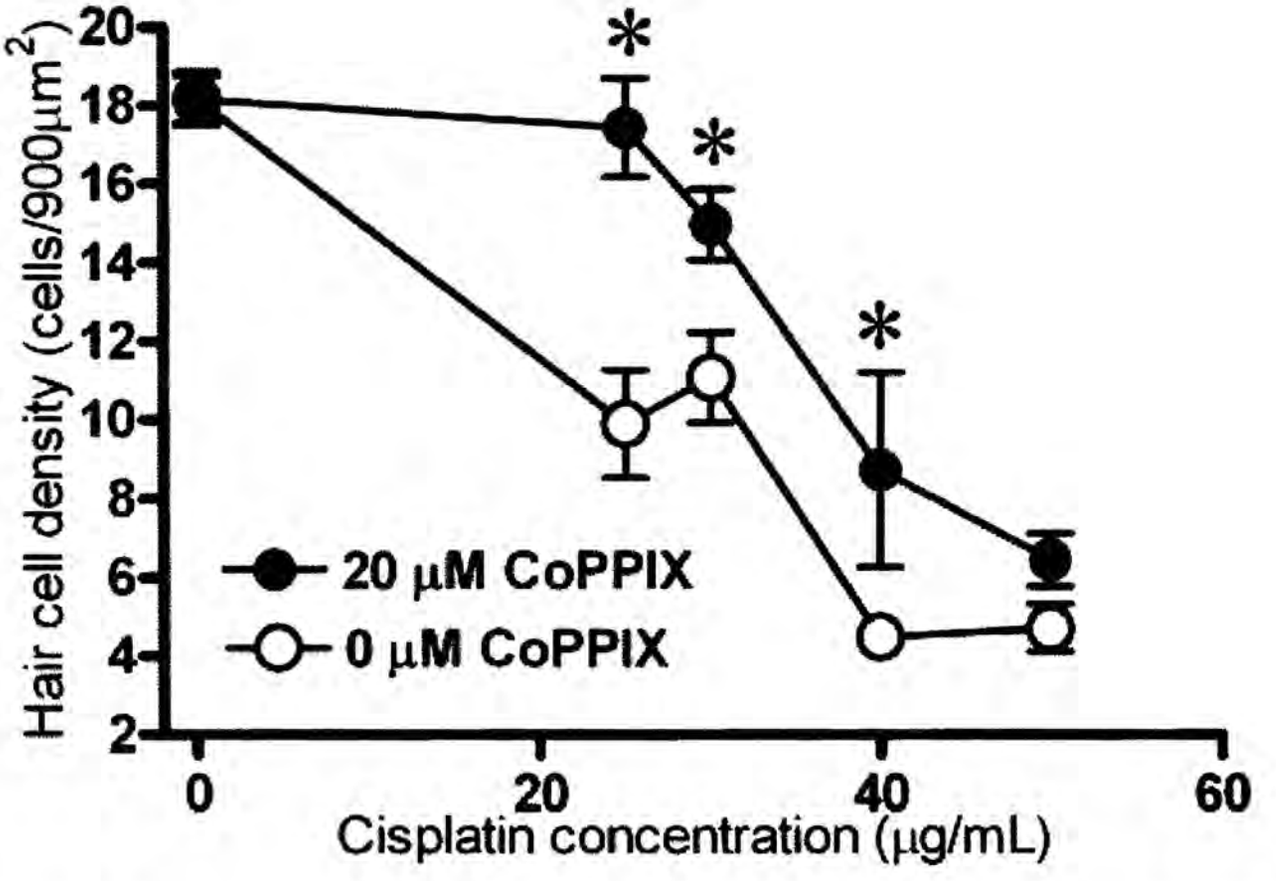

Figure 4-7. CoPPIX inhibits cisplatin-induced hair cell death. Control and CoPPIX-treated (20 μM for 12 h) utricles were exposed to 0, 25, 30, 40, or 50 μg/mL (0, 83.3, 100, 133.3, or 166.6 μM) cisplatin for 24 h. Hair cell counts were performed on calmodulin and calbindin-stained utricles. These data indicate that CoPPIX offers robust protection against cisplatin-induced hair cell death *(2-way ANOVA: $F_{4,69}$ = 4.24, p=0.004). Bars represent the mean ± SEM for n = 5-7 utricles per condition. Asterisks (*) denote significant differences in hair cell density between utricles that received CoPPIX and those that did not.*

In order to further confirm that the protective effect of CoPPIX was due to the induction of HSP32, we utilized an inhibitor of heme-oxygenase activity, zinc protoporphyrin IX (ZnPPIX) (Maines 1981; Wong et al 2011). Utricles were treated with 20 μM CoPPIX for 12 h followed by 25 μg/mL (83.3 μM) cisplatin for 24 h. 0 or 10 μM ZnPPIX was present for the entire 36 h. Utricles were processed for immunohistochemistry and hair cells were counted. ZnPPIX abrogated the protective effect of CoPPIX (Fig. 4-8). ZnPPIX and cisplatin treatment combined exhibited more toxicity than cisplatin treatment, alone *(Tukey's multiple comparison test: $p<0.001$)*. These results indicate that HSP32 mediates the protection conferred by CoPPIX against cisplatin-induced hair cell death.

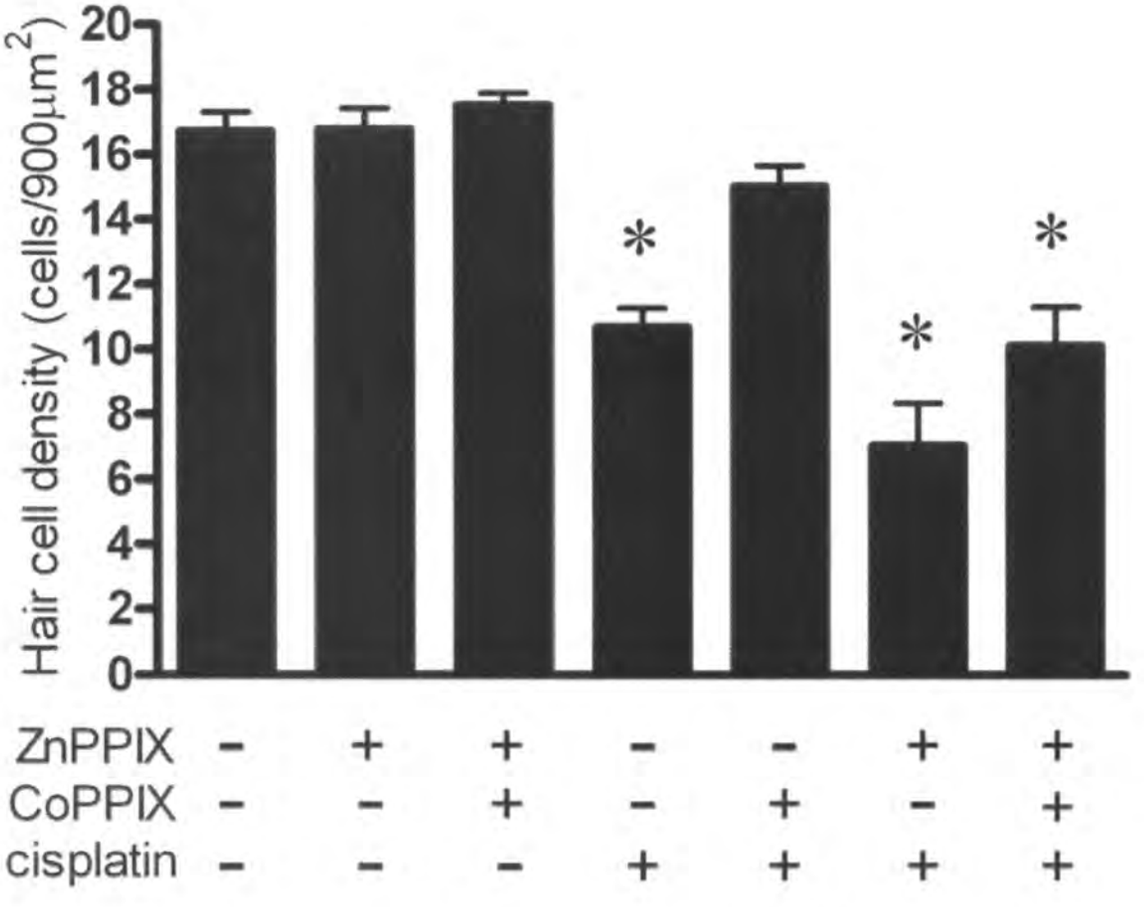

Figure 4-8. ZnPPIX inhibits protection conferred by CoPPIX against cisplatin-induced hair cell death. Utricles were treated with 10 μM ZnPPIX for 36 h in the presence of 20 μM CoPPIX and/or 25 μg/mL (83.3 μM) cisplatin. Hair cell counts were performed on myosin7a-stained utricles. The HSP32 inhibitor ZnPPIX abolished the protective effect of CoPPIX against cisplatin-induced hair cell death *(Tukey's multiple comparisons: $p<0.001$). Bars represent the mean ± SEM for n = 7-12 utricles per condition. Asterisks (*) denote significant differences in hair cell density from control.*

HSP70 and HSP32 do not demonstrate synergistic protection against cisplatin-induced hair cell death

In order to investigate whether HSP70 and HSP32 might work synergistically to protect against cisplatin-induced hair cell death, utricles from rHSP70i transgenic mice and their wild-type littermates were exposed to 0 or 20 μM CoPPIX for 12 h, followed by treatment with cisplatin for 24 h. Hair cell counts revealed that, as seen in previous experiments, CoPPIX was protective against cisplatin-induced hair cell death at 25 μg/mL (83.3 μM) cisplatin *(3-way ANOVA: $F_{1,106}$ =14.47 , $p<0.001$)* (Fig. 4-9). In addition, rHSP70i CE utricles had significantly more hair cells remaining following treatment with 25 μg/mL (83.3 μM) cisplatin when compared to wild-type utricles *(t-test: $p<0.05$)*. However, CoPPIX pretreatment of rHSP70i transgenic utricles did not offer greater protection than either rHSP70i or HSP32 alone *(3-way ANOVA: $F_{1,106} = 0.74$, $p = 0.39$)*. These data indicate that rHSP70i and HSP32 together do not demonstrate a synergistic protection.

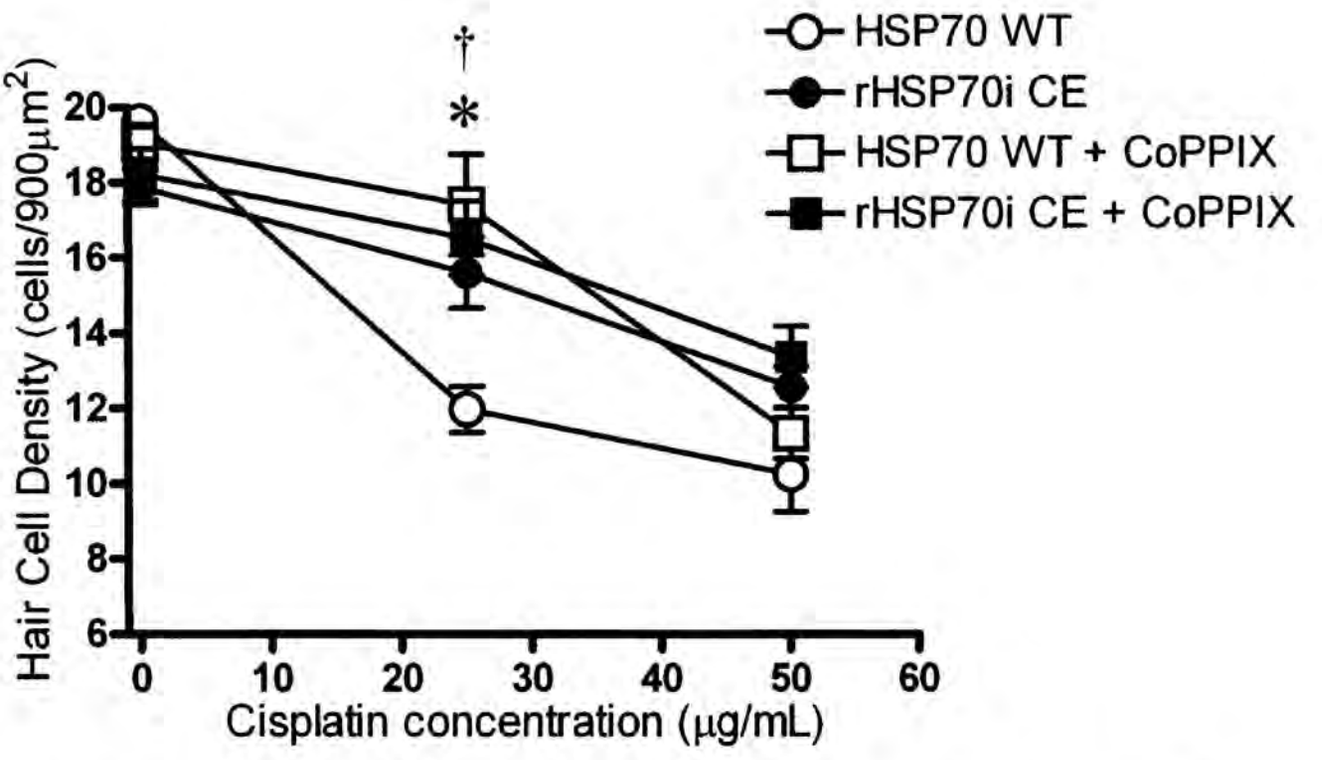

Figure 4-9. Combined expression of HSP70 and HSP32 does not protect against cisplatin-induced hair cell death more than either condition alone. rHSP70i CE mice and their wild-type littermates were treated with 0 or 20 μM CoPPIX for 12 h, followed by treatment with 0, 25, or 50 μg/mL (0, 83.3, or 166.6 μM) cisplatin for 24 h. CoPPIX was protective against cisplatin-induced hair cell death at 25 μg/mL (83.3 μM) cisplatin *(3-way ANOVA: $F_{1,106}$ =14.47 , $p<0.001$).* rHSP70i CE utricles had significantly more hair cells remaining following treatment with 25 μg/mL (83.3 μM) cisplatin when compared to wild-type utricles *(t-test: $p<0.05$).* CoPPIX pretreatment of rHSP70i transgenic utricles did not offer greater protection than either rHSP70i or HSP32 alone *(3-way ANOVA: $F_{1,106} = 0.74$, $p = 0.39$). Bars represent the mean ± SEM for n = 4-14 utricles per condition. Asterisks (*) denote significant differences in hair cell density between* HSP70 WT *utricles that received CoPPIX or not. † denotes significant differences between hair cell density of* HSP70 WT *and* rHSP70i CE *utricles.*

Discussion

Heat shock provides robust protection against cisplatin-induced hair cell death at multiple cisplatin doses in adult mouse utricle (Fig. 4-2). Protection of heat shock against cisplatin-induced hair cell death was reported previously at a single cisplatin dose (Cunningham & Brandon 2006). In addition, heat shock has been shown to protect against other ototoxic agents, such as aminoglycoside antibiotics (Cunningham & Brandon 2006). Previous studies of cisplatin-induced ototoxicity have implicated DNA damage, ROS, and inflammation in the apoptotic death of hair cells. Thus, the mechanism of protection by HSPs against cisplatin-induced hair cell death is likely multi-faceted. HSPs can protect cells from oxidative stress by preventing the accumulation and aggregation of ROS-damaged proteins, as well as by helping denatured and/or damaged proteins return to their native conformations (Jolly & Morimoto 2000; Martindale & Holbrook 2002; Mayer & Bukau 2005; Yamamoto et al 2000). The protection conferred by HSPs is not limited to protein interactions; HSPs have been shown to inhibit lipid peroxidation and oxidative damage to DNA, as well (Martindale & Holbrook 2002; Park et al 1998; Su et al 1999). In addition, individual HSPs, such as HSP90, HSP70, and HSP27, have been shown to inhibit apoptosis at multiple points along the apoptotic pathway (Beere et al 2000; Concannon et al 2003; Concannon et al 2001; Evans et al 2010; Jaattela et al 1998; Jiang et al 2009; Pandey et al 2000a; Pandey et al 2000b; Pasupuleti et al 2010; Rodina et al 2007; Stankiewicz et al 2005; Tsuchiya et al 2003). Furthermore, a heat shock protein with a chaperone-unrelated function, HSP32, is a known inhibitor of oxidative stress and inflammation in multiple tissue types (Blancou et al 2011; Gozzelino et al 2010; Kirkby & Adin 2006; Paine et al 2010; Ryter et al 2006).

Unfortunately, heat shock is not the most practical of inner ear therapies, in that perfusion of the inner ear with warm liquid would be invasive and uncomfortable for patients prior to receiving chemotherapy. Therefore, it is necessary to identify specific HSPs capable of protecting against cisplatin-induced hair death that might be upregulated pharmacologically.

HSP70, a highly upregulated HSP following heat shock of adult mouse utricle, was shown to be necessary for the protection conferred by heat shock and sufficient to protect against cisplatin-induced hair cell death (Figs. 4-3 & 4-4). Though statistically significant, the results of these experiments are not striking. One reason for this could lie in the model systems tested. The inducible HSP70 that is constitutively expressed by the transgenic HSP70 mice is only 2.5 fold higher than what is normally expressed in the mouse utricle (Fig. 4-10). Therefore, HSP70 may be more protective against cisplatin-induced hair cell death at higher concentrations. On the other hand, HSP70 constitutive expression was shown to inhibit aminoglycoside-induced hair cell death and hearing loss (Taleb et al 2009; Taleb et al 2008). The robust protective effect of rHSP70i constitutive expression against aminoglycosides but not cisplatin, may be indicative of a different mechanism of cell death from these two well-known ototoxic agents. The results of the $HSP70^{-/-}$ experiment may have been more convincing had the sample size been larger. Mice of this genotype are smaller at birth than wild-type counterparts, and mutant males exhibit improper spermatocyte morphology (Hunt et al 2004). These are potential reasons for the small brood sizes we experienced when mating exclusively homozygous knockout mice. Future experimentation with alternative model systems, such as adenoviral transfections for overexpression of HSP70 and conditional

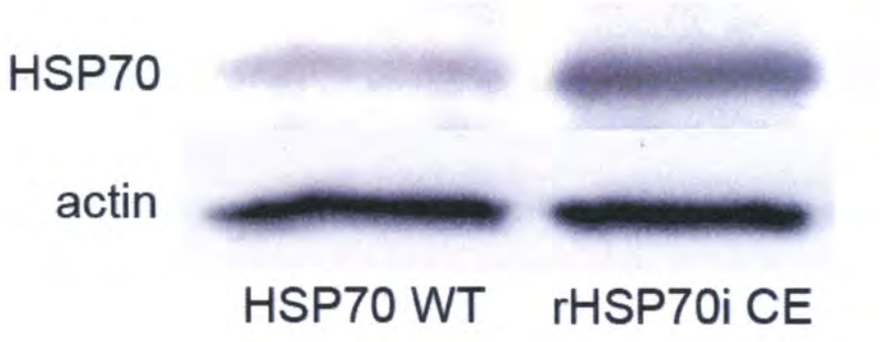

Figure 4-10. Expression of HSP70 by HSP70 WT and rHSP70i CE utricles. Utricles from HSP70 WT and rHSP70i CE mice were processed for western blotting using antibodies against HSP70 and actin.

HSP70 knockout or HSP70 siRNA to inhibit HSP70 upregulation following heat shock, may offer more definitive results regarding the potential protection of HSP70 against cisplatin-induced hair cell death.

The use of *HSP70*$^{-/-}$ mice was a limitation of this study, in that germline knockouts typically develop compensatory mechanisms to replace the function(s) of the missing protein. Due to the ubiquitous and multifunctional nature of HSP70, it is possible that other heat shock proteins are upregulated in the *HSP70*$^{-/-}$, in order to compensate for the loss of HSP70. The current study did not incorporate an examination of such compensatory mechanisms.

HSP32 (a.k.a. heme oxygenase-1) offered robust protection against cisplatin-induced hair cell death (Figs. 4-7 & 4-8). The mechanism of protection conferred by HSP32 could be two-fold, due to the well-documented antioxidant and anti-inflammatory effects of the byproducts of heme catabolism bilirubin and CO. Cisplatin is known to cause oxidative stress in the inner ear (Clerici et al 1996; Ravi et al 1995; Rybak et al 1995). Thus, bilirubin may effectively counteract the production of reactive oxygen species induced by cisplatin. Cisplatin treatment of auditory cells is known to cause secretion of pro-inflammatory cytokines, most notably TNFα (Kim et al 2008; So et al 2008; So et al 2007). TNFα is toxic to hair cells, and inhibition of TNFα has been shown to protect hair cells from cisplatin-induced injury (Dinh et al 2008; Haake et al 2009; So et al 2007). HSP32 expression in tissues is known to suppress TNFα release (Devey et al 2009; Ferenbach et al 2010; Inoue et al 2001). CO, alone, recapitulates this feature of HSP32 upregulation (Chen et al 2010b; Otterbein et al 2000). Furthermore, CO has been shown to protect against ototoxic drugs in inner ear model systems. The CO scavenger,

hemoglobin, was shown to abrogate the protection of HSP32 against cisplatin-induced hair cell death in neonatal rat cochlear explants (Kim et al 2006). In addition, CO has been shown to inhibit aminoglycoside-induced hair cell death (Francis et al, in press).

Since the pharmacological agent CoPPIX was used to upregulate HSP32 in the utricles, it was necessary to confirm that the protective effect of CoPPIX could be attributed to heme oxygenase activity. Therefore, a known inhibitor of heme-oxygenase activity, ZnPPIX, was used. Results indicate that ZnPPIX abrogated the protective effect of CoPPIX against cisplatin-induced hair cell death (Fig. 4-8). Therefore, CoPPIX protects hair cells from cisplatin by inducing HSP32 expression in the utricle.

One may use the findings of this study when comparing the mechanisms by which hair cells die from cisplatin and aminoglycosides. HSP70 strongly protects against aminoglycoside-induced hair cell death and hearing loss (Taleb et al 2009; Taleb et al 2008). However, our data indicate that HSP70 does not offer strong protection against cisplatin-induced hair cell death. Thus, heat shock proteins are providing evidence in support of different mechanisms of cell death by these two highly-studied ototoxins. One notable difference between the mechanisms of cell injury caused by cisplatin and aminoglycosides is the initial DNA adduct formation by cisplatin. As this is not an event that occurs in aminoglycoside-induced ototoxicity, this is a good place to begin comparing and contrasting otoprotectants that differentially protect against these two agents.

In conclusion, heat shock preconditioning, as well as the induction of individual heat shock proteins, is protective against cisplatin-induced hair cell death. The results of

this study imply that HSPs, particularly HSP32, are good candidates for the design of a future co-therapy to prevent cisplatin-induced hearing loss in human patients.

CHAPTER 5: Cisplatin and macrophages

Heme oxygenases are responsible for the catabolism of free heme in cells. Three heme oxygenases exist: HSP32 (HO-1), HO-2, and HO-3. HSP32 is a stress-inducible heme oxygenase that is expressed by myeloid and endothelial cells of many organ systems, including eye, liver, spleen, vasculature, lung, gastrointestinal tract, and central nervous system (Aztatzi-Santillan et al 2010; Durante 2010; Fan et al 2011; Ferenbach et al 2010; Immenschuh et al 2010; Maines et al 1986; Raval & Lee 2010; Takagi et al 2010). HO-2 is constitutively expressed, with particularly high expression levels in testis and brain (Trakshel et al 1986; 1988). The third member of the heme oxygenase family, HO-3, is likely an HO-2 pseudogene that has yet to be definitively detected at the protein level (Hayashi et al 2004; McCoubrey et al 1997).

The inducible heme oxygenase, HSP32, is known to have antioxidant and anti-inflammatory effects on tissues through the production of bilirubin and CO, thus acting as a protectant against various insults (Kirkby & Adin 2006). HSP32-deficient mice and humans exhibit pro-inflammatory phenotypes (Agarwal et al 1996; Kapturczak et al 2004; Koizumi 2007; Poss & Tonegawa 1997; Radhakrishnan et al 2011; Yachie et al 1999; Yet et al 1999). Many studies in the literature suggest that HSP32-inducing agents are protective against pro-oxidative and -inflammatory insults, such as ischemia/reperfusion (I/R) injury, bacterial infection, and atherosclerosis (Devey et al 2009; Durante 2010; Fan et al 2011; Ferenbach et al 2010; Roach et al 2009; Sun et al 2010). The ability of HSP32 to protect against liver I/R injury has been attributed to the resident macrophages of the liver, Kupffer cells (Devey et al 2009). In this study, liposomal clodronate was administered intravenously to deplete kupffer cells from the

liver, and results indicated that Kupffer cells were required for hepatocyte survival following I/R. As HSP32 was expressed exclusively in kupffer cells, HSP32-null mice were used to determine the role of HSP32 in liver I/R injury. HSP32-null mice exhibited increased hepatic injury following I/R than did their wild-type counterparts. The ability of HSP32 to induce macrophage-mediated protection against these injuries has been attributed to a change in macrophage phenotype that results in a switch from pro- to anti-inflammatory cytokine secretion following HSP32 upregulation. Devey et al. (2009) went on to further investigate the macrophages of HSP32-null and wild-type animals to determine the role of HSP32 in macrophage differentiation. They found that there was a difference in several macrophage markers, thus segregating HSP32-null macrophages from HSP32-expressing macrophages into "resident" or non-inflammatory and "inflammatory" groups, respectively. Several reports have indicated that inhibition or deficiency of HSP32 results in increased TNFα secretion, whereas induction of HSP32 results in decreased TNFα and increased IL-10 (Drechsler et al 2006; Inoue et al 2001; Sheikh et al 2011). CO, alone, has similar effects on cytokine expression to HSP32 (Chen et al 2010b; Otterbein et al 2000).

The role for heme oxygenase molecules in inner ear pathology has only begun to be investigated. It wasn't until the early 2000's that HSP32 and HO-2 were known to be expressed in cochlea (Fairfield et al 2004; Watanabe et al 2003). Soon after in 2006, a group of investigators set out to determine the efficacy of an HSP32-inducer, cobalt protoporphyrin IX (CoPPIX), as a protectant against cisplatin-induced hair cell death in neonatal rat cochlear explants (Kim et al 2006).

Efforts have been made, mostly in immortalized auditory cell lines, to work out the mechanism(s) behind HSP32-mediated protection of hair cells against drug-induced toxicity. The protective effect of HSP32 in inner ear has been attributed to the antioxidant and anti-inflammatory effects of this enzyme. Kim et al. demonstrated that the CO scavenger hemoglobin was able to inhibit the protective effect of CoPPIX-induced HSP32 against cisplatin in both HEI-OC1 cells and neonatal cochlear explants (2006). These results led them to the conclusion that the protection conferred by HSP32 against cisplatin was primarily through the antioxidant properties of CO. Later, the same group of investigators began looking at the role of HSP32 on cytokine production by HEI-OC1 cells. They used ELISA and PCR in order to demonstrate the effect of HSP32 expression on the secretion of pro-inflammatory cytokines by cisplatin-treated HEI-OC1 cells. Their results indicated that HSP32 inhibits cisplatin-induced pro-inflammatory cytokine (TNFα, IL-1β, and IL-6) transcription and secretion in cisplatin-treated HEI-OC1 cells and mouse cochleae (So et al 2008).

Previous studies of HSP32-conferred protection against inner ear insults have yet to link protective mechanisms in auditory cell lines to intact sensory epithelia. In addition, it is necessary to link such mechanisms of HSP32-mediated protection to the protein's site of expression within inner ear epithelia. The current study was designed to identify cell types responsible for CoPPIX-induced HSP32 expression and to further elucidate the mechanism behind this protection against cisplatin-induced hair cell death. The hypothesis is that CoPPIX induces HSP32 expression in macrophages of adult mouse utricle, resulting in a change in cytokine expression that is protective against cisplatin-induced hair cell death.

Methods

$CX_3CR1^{GFP/+}$ *mice*

$CX_3CR1^{GFP/GFP}$ mice have an EGFP gene in place of the CX_3CR1 gene, therefore they express GFP instead of CX_3CR1 (Jung et al 2000). Mice with GFP in place of each CX_3CR1 allele express no CX_3CR1 protein. CX_3CR1 is a seven-transmembrane G-protein-coupled receptor for the chemokine fractalkine. CX_3CR1 is expressed by monocytic, natural killer, dendritic and microglial cells in mice. The homozygous CX_3CR1 knockout mice (*$CX_3CR1^{GFP/GFP}$*) exhibit normal development and fertility. *$CX_3CR1^{GFP/GFP}$* male mice were acquired from Jackson Laboratories and bred with c57Bl/6 females to produce CX_3CR1 heterozygous mice (*$CX_3CR1^{GFP/+}$*). This model system has been used in previous studies of inner ear macrophages (Sato et al 2008; 2010; Sautter et al 2006). *$CX_3CR1^{GFP/+}$*utricles were used for studies of utricular macrophages, since previous studies of inner ear macrophages indicated that the *$CX_3CR1^{GFP/GFP}$* macrophages have different behavior in response to ototoxic drugs compared to heterozygote and wild-type macrophages (Sato et al 2010). *(Note : $CX_3CR1^{GFP/GFP}$ = homozygous null for CX_3CR1, but express GFP; $CX_3CR1^{GFP/+}$=heterozygous expression of CX_3CR1 and GFP; $CX_3CR1^{+/+}$= wild-type expression of CX_3CR1, with no GFP expression.)*

Results

CoPPIX upregulates HSP32 in resident macrophages of utricle

Due to the protective nature of HSP32 against cisplatin-induced hair cell death, it was important to identify which cell type(s) in the utricle upregulate HSP32 in response to CoPPIX. We treated utricles with 20 μM CoPPIX for 12 h. The utricles were subsequently fixed and stained for HSP32 (Fig. 5-1). HSP32 immunoreactivity was not detected supporting cells, and minimal upregulation was observed in hair cells after CoPPIX treatment. However, HSP32 immunoreactivity was most prominent in another cell type in the stromal layer beneath the sensory epithelium that was morphologically dissimilar to hair cells or supporting cells. These HSP32-expressing cells were morphologically similar to macrophages (Bhave et al 1998; Hirose et al 2005; Warchol 1997). In order to confirm the identity of the primary HSP32-expressing cells as macrophages, *CX3CR1*$^{GFP/GFP}$ utricles were treated with 0 or 20 μM CoPPIX for 12 h. Some macrophages expressed HSP32 in the absence of CoPPIX, whereas many more expressed HSP32 following CoPPIX treatment. Some unknown cell type(s) around the periphery of the utricle also upregulated HSP32 following CoPPIX treatment (data not shown). These data suggest that the main cell type responsible for HSP32 upregulation following CoPPIX treatment of the adult mouse utricle is the macrophage.

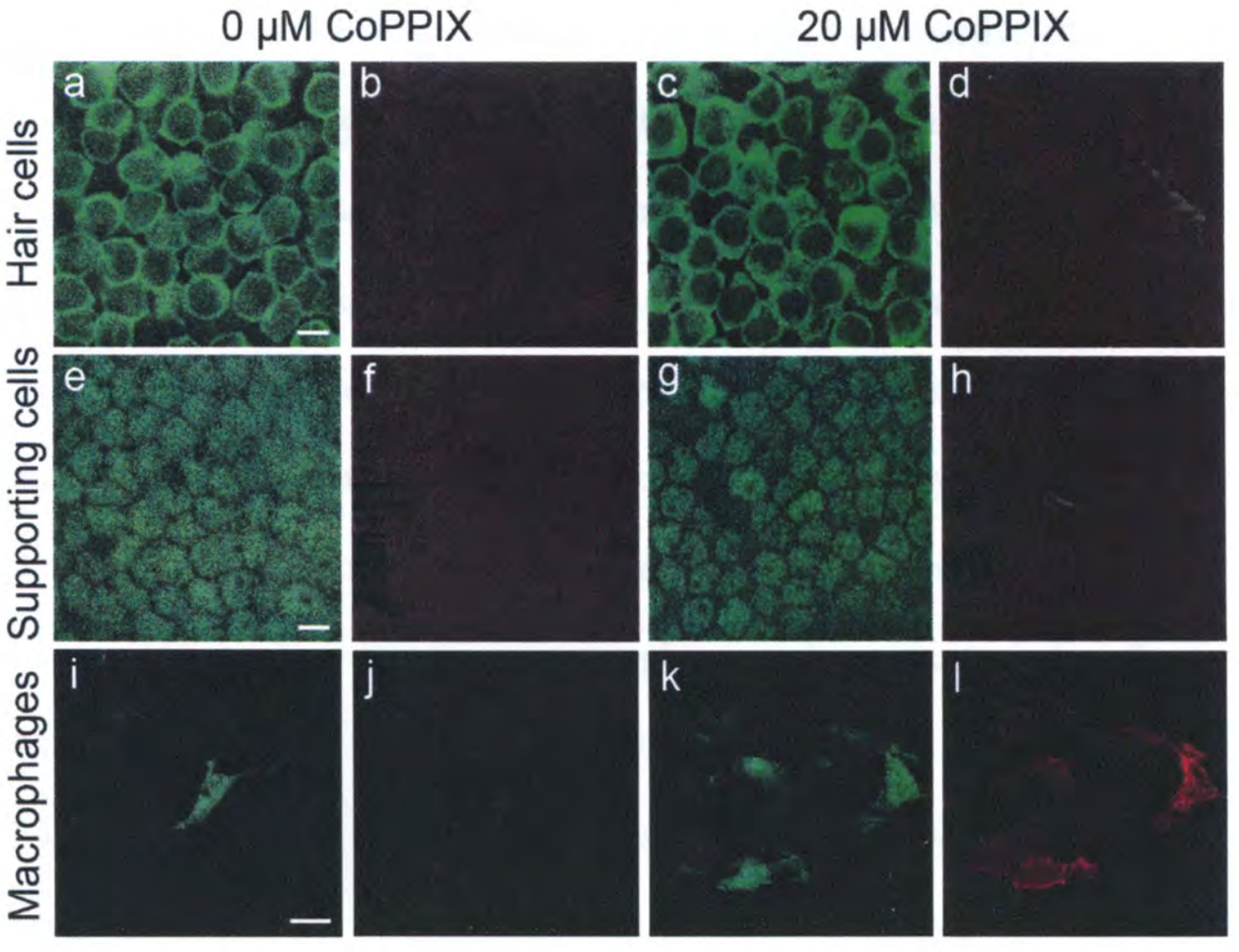

Figure 5-1. HSP32 is upregulated primarily in resident macrophages of adult mouse utricle. Utricles were treated with 0 (a, b, e, f, i, j) or 20 µM (c, d, g, h, k, l) CoPPIX. Utricles were stained for the hair cell marker myosin 7a (a, c), the supporting cell marker SOX2 (e, g), or were *CX3CR1*$^{GFP/GFP}$ utricles which have GFP-expressing macrophages (i, k). All utricles were stained with an anti-HSP32 antibody (b, d, f, h, j, l). These data indicate that HSP32 results in upregulation of HSP32 in macrophages of adult mouse utricle. No HSP32 upregulation was detectable in the hair cells or supporting cells. *Confocal micrographs are presented. Scale bars represent 5 µm in the hair cell and supporting cell rows (a-h) and 10 µm in the macrophage row (i-l).*

Liposomal Clodronate depletes macrophages without killing hair cells

In order to examine the role of macrophages in mediating the protective effect of HSP32 induction, we utilized liposomal clodronate (LC) to deplete resident macrophages. LC (a.k.a. liposomal dichloromethylene-bisphosphonate) selectively kills macrophages because only cells with phagocytic activity take up liposomes (van Rooijen & Hendrikx 2010; Van Rooijen & Sanders 1994). Once inside macrophages, the lipid membranes of these multilamellar liposomes are broken down by lysosomal enzymes, thus allowing accumulation of the very polar clodronate molecule in the macrophages. Upon accumulation of enough clodronate, the macrophages undergo apoptosis (van Rooijen et al 1996). Utricles from *CX3CR1*$^{GFP/+}$ mice were treated with 0, 0.1, 0.5, 1, 2, 4, or 8 mM LC for 48 h. Utricles were fixed and stained for myosin 7a and GFP-positive macrophages and hair cells were counted from maximum intensity projections of confocal z-stacks taken of entire utricles. Liposomal clodronate resulted in depletion of macrophages at 2, 4, and 8 mM concentrations *(Dunnett's multiple comarisons: $p<0.01$)* (Fig. 5-2A) without loss of hair cells *(Dunnett's multiple comarisons: $p>0.05$)* (Fig. 5-2B).

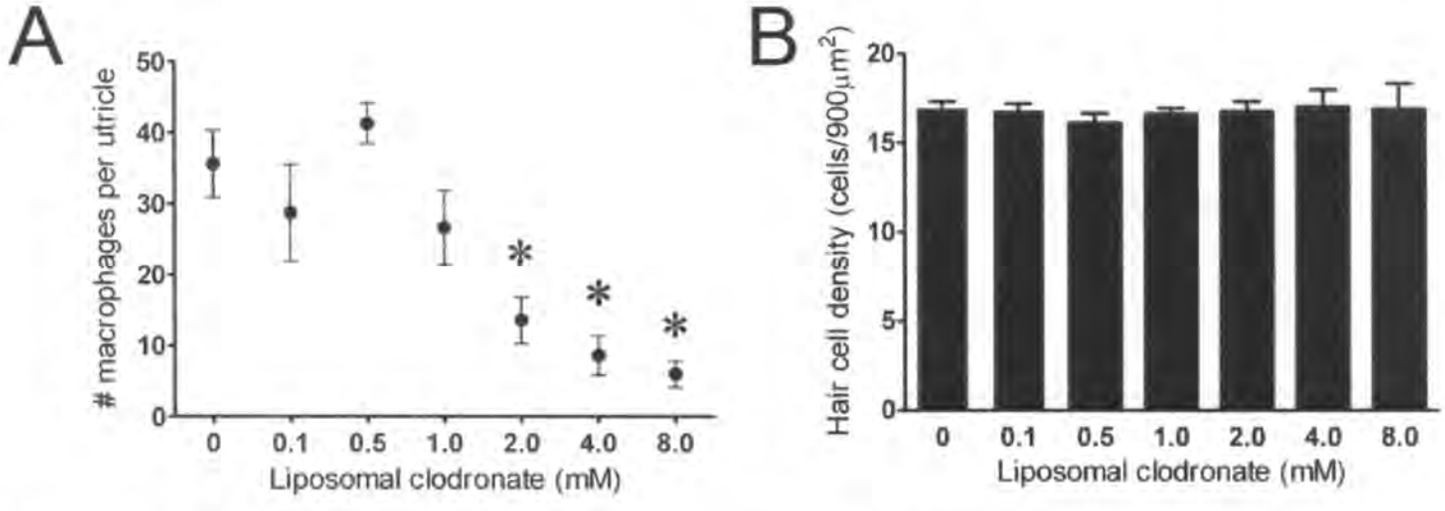

Figure 5-2. Liposomal clodronate (LC) depletes macrophages from utricle without killing hair cells. *CX3CR1$^{GFP/+}$* utricles were treated with 0, 0.1, 0.5, 1, 2, 4, and 8 mM LC for 48 h. Utricles were fixed and stained for myosin7a. (A) Confocal z-stack images were acquired for each utricle. Macrophage counts were performed on maximum intensity projection images from each z-stack. Liposomal clodronate depleted macrophages from the utricular epithelium at 2, 4, and 8 mM concentrations *(Dunnett's multiple comparisons: $p<0.01$)*. (B) Hair cell counts were performed for each utricle. Liposomal clodronate had not significant effect on hair cell density at the concentrations investigated *(Dunnett's multiple comparisons: $p>0.05$). Bars represent the mean ± SEM for n = 4-10 utricles per condition. Asterisks (*) denote significant differences in cell numbers compared to control.*

Depletion of resident macrophages inhibits the protective effect of HSP32 against cisplatin-induced hair cell death

In order to determine whether macrophages are necessary for the protective effect of HSP32 against cisplatin-induced hair cell death, 4 mM LC was used to deplete macrophages from *CX3CR1*$^{GFP/+}$ utricles prior to CoPPIX treatment. Utricles were treated with 4 mM LC for 48 h. Following this period in LC, the utricles were allowed to sit in drug-free culture media for 24 h. Then the utricles were exposed to 20 μM CoPPIX for 12 h with subsequent incubation in 30 μg/mL (100 μM) cisplatin for 24 h. These utricles were then fixed and underwent immunochemical staining for the hair cell marker myosin 7a. LC was not toxic to hair cells at 4 mM concentration *(Tukey's Multiple Comparisons: $p>0.05$)*. CoPPIX + cisplatin treatment resulted in increased hair cell density compared to cisplatin treatment, alone *(Tukey's Multiple Comparisons: $p<0.01$)*. Significantly fewer hair cells remained in the LC + CoPPIX + cisplatin group when compared to the CoPPIX + cisplatin group *(Tukey's Multiple Comparisons: $p<0.01$)*, as well as the control group *(Tukey's Multiple Comparisons: $p<0.001$)*. These results indicate that LC abrogates the protective effect of CoPPIX, thus indicating a role for macrophages in hair cell survival (Fig. 5-3).

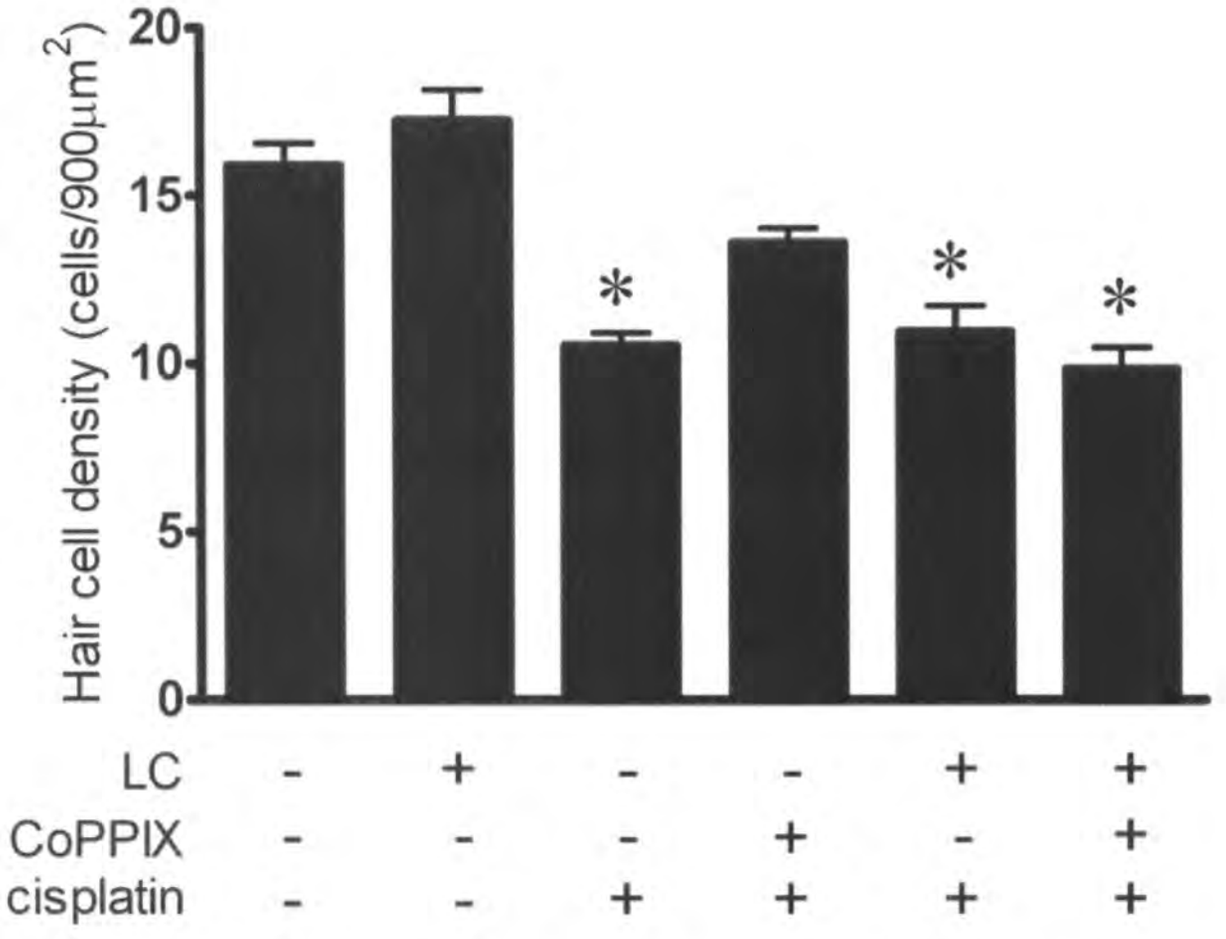

Figure 5-3. Liposomal clodronate-mediated macrophage depletion inhibits protection conferred by CoPPIX against cisplatin-induced hair cell death. Utricles were treated with 4 mM LC for 48 h, allowed to incubate in drug-free culture media for 24 h, then treated with 0 or 20 μM CoPPIX for 12 h, followed by 0 or 30 μg/mL (100 μM) cisplatin for 24 h. Utricles were fixed and processed for immunochemistry. Hair cell counts were performed on myosin7a-stained utricles. LC abolished the protective effect of CoPPIX against cisplatin induced hair cell death *(Tukey's multiple comparisons: $p<0.01$). Bars represent the mean ± SEM for n = 4-6 utricles per condition. Asterisks (*) denote significant difference in hair cell density relative to control utricles.*

HSP32 causes a change in cytokine expression of cisplatin-treated utricles

In order to determine the mechanism by which increased HSP32 expression protects against cisplatin-induced hair cell death, utricles were treated with 0 or 20 μM CoPPIX for 12 h followed by 0 or 30 μg/mL (100.0 μM) cisplatin for 18 h and processed for qRT-PCR to detect changes in transcript of the anti-inflammatory cytokine IL-10. Results indicate that IL-10 mRNA is twice as abundant in cisplatin-treated utricles that also underwent CoPPIX pretreatment than in utricles treated with cisplatin, alone (Fig. 5-4).

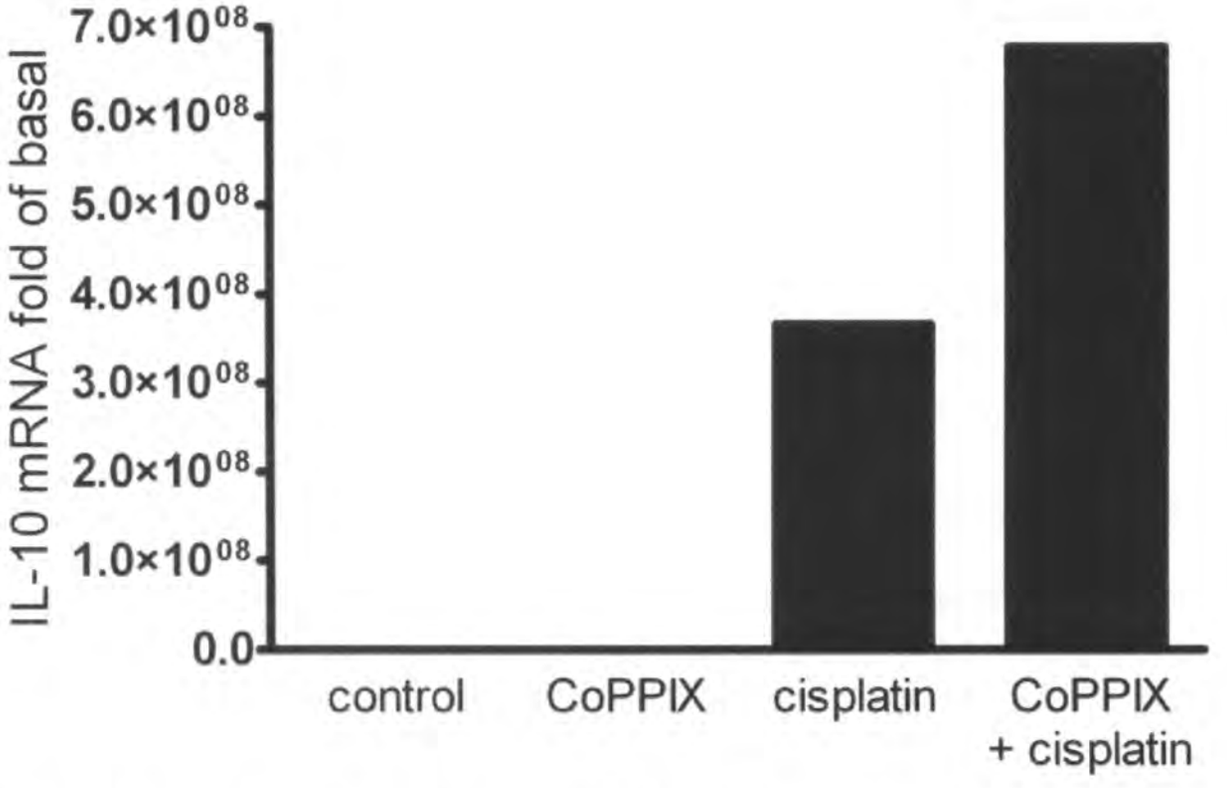

Figure 5-4. HSP32 increases transcription of IL-10 in presence of cisplatin. Utricles were treated with 0 or 20 μM CoPPIX for 12 h followed by treatment with 0 or 30 μg/mL (100 μM) cisplatin for 18 hours. Utricles were then processed for qRT-PCR. Each treatment group was plated in triplicate.

Discussion

Immunochemistry revealed and increase in expression of HSP32 in macrophages of adult mouse utricle following CoPPIX treatment (Fig. 5-1). While HSP32 expression is known to occur primarily in macrophages of other organs, such as liver, lung, and spleen, this is the first report of such a phenomenon in the utricle.

qRT-PCR experiments revealed a role for cytokine production in the protective effect of HSP32 expression by macrophages against cisplatin-induced hair cell death (Fig. 5-4). The increase in IL-10 transcript in the cisplatin + CoPPIX treatment group when compared to the cisplatin group falls in line with the results of other similar studies (Drechsler et al 2006; Inoue et al 2001). It seems as though HSP32 or CO triggers a change in macrophage phenotype, causing increased IL-10 secretion (Chen et al 2010b; Drechsler et al 2006; Inoue et al 2001; Otterbein et al 2000; Sheikh et al 2011). TNFα has been implicated as a potential mediator of cisplatin-induced ototoxicity. HSP32 induces IL-10 expression, an event which seems to coincide with the ability of HSP32 to inhibit TNFα expression (Drechsler et al 2006; Inoue et al 2001). Recently, a publication emerged in which IL-10 was shown to inhibit transcriptional elongation of TNFα, thus supporting a direct role for IL-10 in this process (Smallie et al 2010). Furthermore, *IL-10*$^{-/-}$ mice exhibit worse hearing than their wild-type counterparts in a model of experimental autoimmune hearing loss (EAHL) (Zhou et al 2011). The same study demonstrated that exogenous IL-10 can improve hearing in mice with EAHL. Thus, IL-10 inducers may prove useful as potential co-therapies for the prevention of cisplatin-induced hearing loss.

Removal of macrophages from utricle using LC effectively demonstrated the protective nature of macrophages against cisplatin-induced hair cell death (Fig. 5-3). The finding that the protective effect of HSP32 is mediated by resident macrophages indicates that macrophages are important determinants of hair cell survival versus death in response to cisplatin. This is especially true in light of recent reports regarding the importance of other cell types, such as supporting cells, in governing hair cell life and death (Lahne & Gale 2008). These findings also illustrate the importance of studying whole tissues rather than monolayer cells, to test potential otoprotectants and ototoxins. Studies of intact tissues are necessary in order to fully understand hair cell death and survival in the context of these important signals from surrounding cell types.

In conclusion, cisplatin-induced hair cell death is a complex process that involves not only intracellular hair cell signals, but also interactions between hair cells and neighboring macrophages. The current study provides evidence that macrophages offer protection against cisplatin-induced hair cell death, a feature which could be manipulated for use in the future as a co-therapy to prevent cisplatin-induced hearing loss in humans.

Chapter 6: Future directions toward designing a co-therapy for the prevention of cisplatin-induced hearing loss

Cisplatin is an extremely successful and widely-used chemotherapy for the treatment of many solid tumors in both adults and children. Ototoxicity, though not necessarily dose-limiting, is a major side-effect of cisplatin that negatively affects quality of life following treatment in at least one-third of patients receiving the drug. Due to the efficacy of cisplatin in treating such a wide range of tumors and the lack of an effective replacement for the drug, it is unlikely that cisplatin will fall out of favor as a chemotherapy. Therefore, it is imperative to design a co-therapy for the prevention of cisplatin-induced ototoxicity. It is important to note that a co-therapy designed for use with cisplatin must not interfere with the anti-neoplastic properties of this chemotherapeutic drug. Thus, either the mechanism of action of the co-therapy must not aid in tumor growth or cisplatin tolerance, or the drug must be administered locally to the ear.

Uncovering mechanisms behind cisplatin-induced hair cell death is imperative for the design of the most effective co-therapy. Now that p53 has been ruled out as an effector of the DNA damage response in hair cells, other candidates can be investigated. p73 is a promising player as an initiator of apoptosis following cisplatin treatment. This p53 family member is known to be inhibited by the pharmacological agent pifithrin-α. Future experiments designed to test the relevance of p73 to cisplatin-induced hair cell death involve the use of pifithrin-α on *p53*$^{-/-}$ utricles. If pifithrin-α protects hair cells of cultured utricles in the absence of p53 protein, this would be a good indication of a potential involvement of p73 in cisplatin-induced hair cell death. Subsequent studies would use western blot to detect p73 activation in cisplatin-treated utricles, as well as

immunochemistry to determine cell types in which p73 is activated. Preliminary data indicate that, unlike p53, p73 is expressed in utricular hair cells (Fig. 6-1).

HSP70 was shown protect against cisplatin-induced hair cell death; however, this protection was not as good as HSP32. The model systems used to determine the potential of HSP70 as a potential protectant against cisplatin-induced hair cell death were not ideal. Therefore, future studies regarding HSP70 as a potential co-therapy for use against cisplatin-induced hearing loss should include studies designed to more definitively determine the efficacy of HSP70 against cisplatin-induced hair cell death. Such model systems could include viral transfection of HSP70 to overexpress it in the utricular epithelium, as well as silencing studies to demonstrate the necessity of HSP70 in the protection conferred by heat shock.

HSP32 offers robust protection against cisplatin-induced hair cell death, thus implying the potential for HSP32, its byproducts, or HSP32-related mechanisms of protection for use as a co-therapy against cisplatin-induced hair cell death. The first step toward designing a cisplatin co-therapy involving HSP32 would require *in vivo* studies designed to further test hypotheses presented herein. It is necessary to determine that the preservation of hair cells *in vitro* translates to preservation of hearing in the face of cisplatin treatment. LC is used more often in *in vivo* than *in vitro* settings, and would, therefore, be an extremely useful tool for the depletion of resident macrophages from the inner ear prior to HSP32 upregulation in order to confirm that the *in vitro* evidence for a macrophage-mediated protection in the utricle translates to the functional cochlea. However, a protocol for administration of LC to the inner ear has not, yet, been

established. Such a protocol would require a route of administration that would introduce LC into blood circulation and allow LC to efficiently reach inner ear macrophages.

Known experimental inducers of HSP32, such as CoPPIX and heme, are not promising for use as clinical therapies (Paine et al 2010). It is therefore necessary to begin experimenting with HSP32-inducing agents that are already approved for human use, such as statins and polyphenols. Statins are commonly used to lower LDL cholesterol levels in blood by inhibiting a rate-limiting enzyme, 3-hydroxy-3-methylglutaryl-CoA reductase, in cholesterol synthesis. Recently, statins have been shown to have multiple effects aside from that of lowering cholesterol, including regulating inflammation (Bu et al 2011). Furthermore, several statins have been shown to induce HSP32 expression (Chen et al 2010a; Kwok et al 2011). Current evidence strongly indicates that statins are excellent candidates for a co-therapy to prevent cisplatin-induced hearing loss. First, statins have been shown have anticancer effects, as well as to act synergistically with cisplatin against osteosarcoma and ovarian cancer, a feature that would allow systemic administration of this potential co-therapy (Fromigue et al 2008; Roudier et al 2006; Taylor-Harding et al 2010). Secondly, statins have been shown to protect against cisplatin-induced nephrotoxicity in rodents, thus inhibiting a dose-limiting side effect of cisplatin treatment (An et al 2011; Iseri et al 2007). Thirdly, statins have been approved for long-term use by humans. Due to the progressive nature of cisplatin-induced ototoxicity, it is likely that patients would benefit from taking statins for an extended period of time following cisplatin treatment. The benefits of such a long-term statin treatment may extend beyond prevention of hearing loss to prevention of cancer recurrence, as statins have been shown to prevent cancer metastasis and

development (Demierre et al 2005). Currently, no investigations into statins as protectants against cisplatin-induced ototoxicity have been published. Polyphenols are a group of plant-derived molecules with antioxidant capabilities. A polyphenol found in green tea, EGCG, is a known HSP32-inducer, and it has been shown to inhibit cisplatin-induced hair cell death *in vitro* (Romeo et al 2009; Schmitt et al 2009). Furthermore, like statins, EGCG has been shown to inhibit cisplatin-induced nephrotoxicity, as well as to sensitize tumors to cisplatin therapy (Chan et al 2006; El-Mowafy et al 2010; Sahin et al 2010; Singh et al 2011; Yunos et al 2011).

In addition, if the protection conferred by HSP32 is primarily due to an IL-10-mediated inhibition of TNFα, known therapeutic TNFα inhibitors, such as etanercept, infliximab, and cardiac glycosides, may be beneficial. The role of TNFα in cisplatin-induced hearing loss has yet to be definitively determined. Due to the existence of multiple pro-inflammatory cytokines, germ-line TNFα knockout mice have likely compensated for the absence of TNFα by the time they have matured. In fact, such compensation has been documented (Miyoshi et al 2005). For this reason, the use of a conditional TNFα knockout mouse strain would be much more effective in determining the role of this cytokine in cisplatin-induced hair cell death and hearing loss.

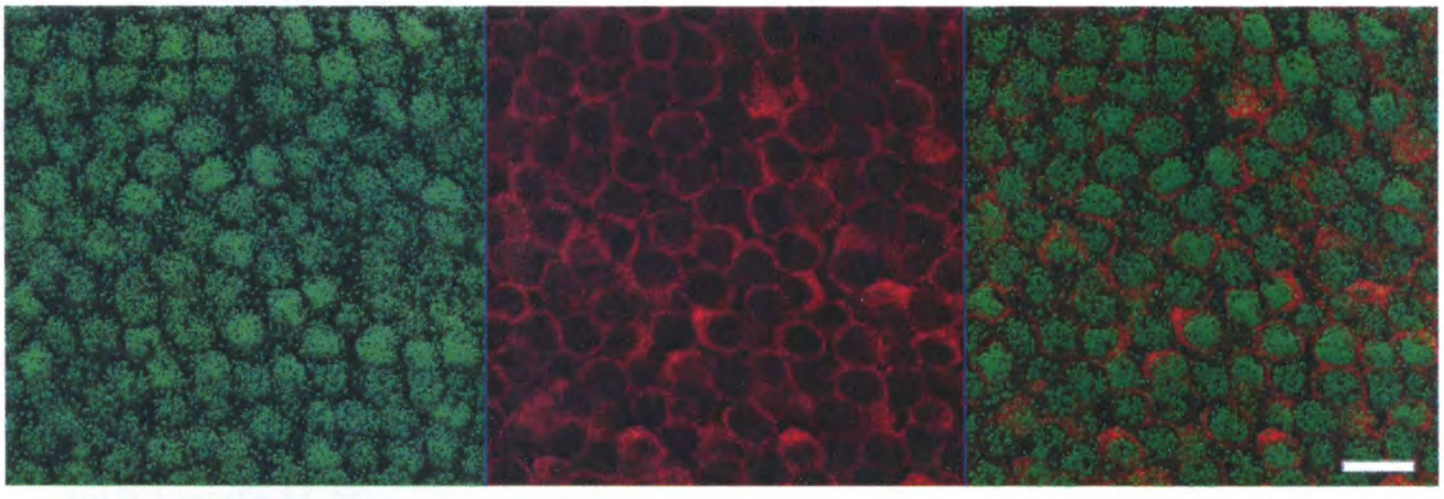

Figure 6-1. p73 expression by hair cells of adult mouse utricle. Untreated utricles were fixed and stained with antibodies against total p73 (green) and myo7a (red). Confocal images are presented. *Scale bar represents 10μm.*

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Acknowledgements

This work was supported by NIH/NIDCD R01 DC007613, F30DC010522, and NIH/NCRR extramural research facilities construction (C06) grants C06 RR015455 and C06 RR14516 from the National Center for Research Resources. Thanks to the MUSC GAANN fellowship in Cell and Neurobiology. This work was supported in part by the NIDCD Intramural Research Program. Imaging facilities for this research were supported, in part, by Cancer Center Support Grant P30 CA138313 to the Hollings Cancer Center, Medical University of South Carolina. Thanks to Dr. Lina Obeid and Dr. Linda Heffernan-Stroud for their help in starting the *p53* $^{-/-}$ mouse colony. Thanks to Dr. Suhua Sha for sharing her lab space during the completion of these studies. Thanks to the members of my lab, Carlene Brandon, Inga Kramarenko, Shimon Francis, and Mona Taleb for taking the time to help me in the lab with both experiments and intellectual exercises. Thank you to my mentor, Dr. Lisa Cunningham, for her unwavering dedication and support during the development of this work. Lisa is a remarkable mentor who is always eager to stop whatever she is doing to sit down and think thoroughly and critically about hypotheses and experiments. Her influence has made a significant impact on both my professional and scientific growth. Thank you to my committee: Dr. Craig Beeson, Dr. Debra Hazen-Martin, Dr. Hainan Lang, Dr. Rick Schnellmann, and Dr. Christina Voelkel-Johnson, each member of which was invaluable to the progression and design of this work. Each member of this committee gave generous and thoughtful insight into these experiments. Thank you to the College of Graduate Studies, namely Dr. Perry Halushka, for providing me the opportunity to continue to advance my graduate-level education. Thank you to my mom, Coleen Gray Baker, my dad, Jesse David Baker, III, M.D., and my brother, David Jackson Baker, for their constant love and support throughout my life and during the preparation of this work. Thank you to my love, Antony John Ferro, for loving me and helping me to remain focused on life's most important things.