

Review

Apoptosis in inner ear sensory hair cells

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Abstract

Apoptosis, or controlled cell death, is a normal part of cellular lifespan. Cell death of cochlear hair cells causes deafness; an apoptotic process that is not well understood. Worldwide, 1.3 billion humans suffer some form of hearing loss, while 360 million suffer debilitating hearing loss as a direct result of the absence of these cochlear hair cells (Worldwide Hearing, 2014). Much is known about apoptosis in other systems and in other cell types thanks to studies done since the mid-20th century. Here we review current literature on apoptosis in general, and causes of deafness and cochlear hair cells loss as a result of apoptosis. The family of B-cell lymphoma (Bcl) proteins are among the most studied and characterized. We will review current literature on the Bcl2 and Bcl6 protein interactions in relation to apoptosis and their possible roles in vulnerability and survival of cochlear hair cells.

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Keywords: Apoptosis; Hair cell; Bcl2; Bcl6

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1. Introduction

In normal tissues, there is a balance between the generation of new cells via cell division and the loss of cells via cell death. Old cells become damaged over time and are eliminated. Programmed cell death, or apoptosis, is an orderly process during which the genome of the cell is broken down, the cell is fragmented into smaller pieces and the debris is consumed by nearby cells (phagocytes) that clean up the cell fragments. Apoptosis is crucial for embryological development, renewal and aging, as well as for maintaining hemostasis. In the inner ear, loss of the sensorineural cells, known as inner hair cells (IHC) and outer hair cells (OHC), is a key factor contributing to hearing loss. Apoptosis appears to be the most common mechanism of hair cell loss. Cell death can also occur in a chaotic manner, necrosis. Although either mechanism can cause hair cell death, we will first review apoptosis, and its known pathways. We will then discuss possible mechanisms of hair cell apoptosis and how the B-Cell Lymphoma (Bcl) family of proteins is involved and possible mechanisms in the second part of this review.

2. Apoptosis

Apoptosis is a genetically controlled mechanism involving an enormous collection of signaling pathways, triggers, genes, proteins, and molecules. There are two distinct phases in apoptosis, the initiation phase and the execution phase. The initiation phase involves many different proteins and is quite complex. It is started by various “stresses” from either outside the cell (extracellular) or inside the cell (intracellular). Some examples of extracellular signals that trigger apoptosis include loss of growth factors, low oxygen levels (hypoxia), and radiation. Intracellular signals include DNA damage, the damage caused by chemotherapy drugs, telomere malfunction, and infection with viruses. The initiation phase triggers the execution phase. The execution phase involves the activation

of specialized enzymes (caspases and others) that directly result in cell death.

2.1. Apoptotic pathways

There are two main pathways that apoptosis can take: intrinsic and extrinsic pathways. These pathways, although initiating at different places and with different mechanisms, both meet up with caspase activation leading to protein cleavage. See Fig. 1.

2.1.1. Extrinsic pathway

The extrinsic, or death receptor, pathway is principally facilitated through signaling of the tumor necrosis factor (TNF) super family (or membrane bound death) receptors. Several examples of types of receptors found in the TNF superfamily are Fas receptor, TNF R1, TNF-related apoptosis

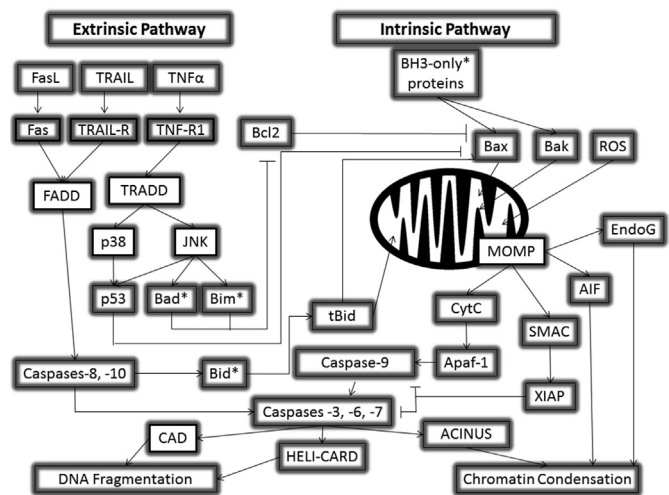


Fig. 1. Extrinsic and Intrinsic Apoptotic Pathways. The extrinsic and intrinsic pathways of apoptosis are graphically shown with their associated receptors, proteins, molecules, and cross talk.

inducing ligand receptor 1 (TRAIL R1), and receptor activator of NF κ B (RANK). Each of these receptors is associated with a ligand to activate them; Fas ligand (FasL), TNF, TRAIL, and RANK ligand (RANKL) (Locksley et al., 2001). Each of these receptors initiates a cascade of activity that can lead to apoptosis via the death inducing signaling complex (DISC) (Ashkenazi and Dixit, 1998; Bharwaj and Aggarwal, 2003; Kischkel et al., 1995). FasL binding to the Fas receptor generates the Fas-associated Death Domain (FADD), while TNFR binding of TNF induces the TNFR-associated death domain (TRADD) (Hsu et al., 1995). These both activate caspase-8 and -10. Curiously, caspase-10 is not activated in mice (Reed et al., 2003). Caspase-8 can activate tBid to recruit the help of the intrinsic pathway (more on this later). Caspase-8 and -10 can also activate caspases-3, -6, and -7. Paradoxically, TNF α can also activate the TNFR-1 receptor, which can activate a cascade leading to NF κ B stimulation and pro-survival gene activation (Marques Fernandez et al., 2013). The extrinsic pathways are depicted in Fig. 1.

2.1.2. Intrinsic pathway

The intrinsic pathway is initiated via intracellular mechanisms, including reactive oxygen species (ROS) and activation from the extrinsic pathway via Bid (Wu and Bratton, 2013). DNA damage is a major inducer of apoptosis as a mechanism to prevent the cell from undergoing mitosis with imperfect DNA (Pistritto et al., 2016). The intrinsic pathway of apoptosis is heavily regulated by the Bcl2 family of proteins. Apoptotic stimuli signal Bcl2 family proteins Bax and Bak to oligomerize and insert into the mitochondrial outer membrane (Antonsson et al., 1997; Eskes et al., 2000; Fesik, 2000; Green and Kroemer, 2004; Kuwana et al., 2002; Minn et al., 1997; Muchmore et al., 1996; Schendel et al., 1997; Wei et al., 2000). These proteins form a pore causing mitochondrial outer membrane permeabilization (MOMP) (Green and Kroemer, 2004) that will allow for release of cytochrome C (CytC) from the mitochondria. CytC released will bind to Apaf-1, creating the “apoptosome” complex in the cytosol. The apoptosome will activate caspase-9, which will activate caspases-3, -6, and -7, aligning with the extrinsic pathway. Caspase-3 moves apoptosis into the nucleus to initiate caspase-activated DNase (CAD), DNA condensation, chromatin condensation via apoptotic chromatin condensation inducer in the nucleus (ACINUS), and acceleration of DNA degradation by cutting cytosolic helicase with an N-terminal caspase-recruitment domain (HELI-CARD) (Enari et al., 1998; Kovacsovic et al., 2002; Liu et al., 1997; Sahara et al., 1999).

The SMAC protein, which is encoded from the *DIABLO* gene, is also released from the mitochondria following MOMP. SMAC will inhibit several apoptosis inhibitors, like XIAP (Galluzzi et al., 2008). Another protein that acts in conjunction with SMAC is mammalian homolog of bacterial high temperature requirement protein A2 (Omi/HtrA2) (Deveraux et al., 1999). XIAP, when not inhibited, will inhibit caspase-9 activity, preventing the initiator caspase from

activating the executioner caspases. XIAP can also inhibit the executioner caspases (Jost et al., 2009). The intrinsic pathways are summarized in Fig. 1.

2.2. Phases of apoptosis

2.2.1. Initiation phase

2.2.1.1. Extrinsic or receptor-mediated pathway. Members of the tumor necrosis factor (TNF) receptor superfamily of transmembrane proteins control the extrinsic pathway. All TNF receptors, also known as death receptors, share a region of 80 amino acids called the “death domain”. This region plays a critical role in transmitting death signals across the cell membrane. Inside the cell a cascade of proteins is turned on. At the end of these pathways, initiator caspase-8 is activated and the execution phase of apoptosis is triggered.

2.2.1.2. Intrinsic or mitochondrial pathway. The Bcl-2 family of proteins controls the intrinsic pathway. There are 25 known proteins in the Bcl-2 family. The different members function to either stimulate apoptosis (pro-apoptotic) or block apoptosis (anti-apoptotic). There is a delicate balance between pro-apoptotic and anti-apoptotic proteins within a cell. BH3-only proteins sense intrinsic signals to undergo apoptosis, such as DNA damage. They travel to the mitochondrial membrane and activate the pro-apoptotic proteins Bax or Bak, or inhibit anti-apoptotic proteins. When activated, Bax and Bak bind, and cause mitochondrial outer membrane permeabilization (MOMP). This perforates the mitochondrial membrane, and induces the release of a crucial pro-apoptotic factor, cytochrome *c*, into the cytosol. Cytochrome *c* joins another pro-apoptotic factor, APAF1, to form the “apoptosome” complex, which in turn activates a series of caspases, leading to cell destruction. The cell death proteins are closely regulated by the tumor suppressor protein p53.

2.2.1.3. Perforin/granzyme. In some cases, immune cells called cytotoxic T lymphocytes can start apoptosis. This happens when the lymphocytes secrete a protein called perforin and small particles containing specialized enzymes. Perforin creates holes in plasma membrane of the target cell. The additional particles use the holes to enter the cell. After entering the cell, they release their enzymes (granzymes A and B) that start the execution pathway and wreak havoc on cell structure and function.

2.2.2. Execution phase

The extrinsic and intrinsic pathways both stimulate the execution phase. During this phase a group of protein-cutting enzymes called caspases lead directly to cell death. The main execution caspases are caspases. Caspases are present in lethal doses within each cell, but they only become active via the initiation process. Caspase-3 is considered the most important of all the caspases. It can cause DNA and chromatin damage, re-arrange the cytoskeleton, and disrupt intracellular transport,

cell division, and signal transduction. Once activated the execution caspases cannot be stopped, cell death is certain. Cell fragments produced during the final stage of apoptosis are quickly recognized, engulfed, and digested by macrophages or surrounding epithelial cells.

2.3. Caspases

One thing relating all the pathways, triggers, and proteins is that the grunt work of cutting up the cellular proteins for apoptosis are a class of enzymes termed cysteine-aspartic protease (caspase) enzymes. Within the caspase class, there are two types: Initiator (or apical) caspases and effector (or executioner) caspases (Riedl and Salvesen, 2007; Timmer and Salvesen, 2007). Initiator caspases include caspases -8 and -9. Following an initial signal for apoptosis, these enzymes target scaffold proteins. Additionally, initiator caspases cleave executioner caspases into 10 and 20 kDa sections that can bind into a tetrameric protease. Executioner caspases include caspases -3, -6, and -7. The cleavage of executioner caspases begins a proteolytic cascade that will lead to the lysis of nearly all parts of the cell. As part of this cascade, executioner caspases will also activate caspase activated DNases.

2.4. p53 in apoptosis

The gene encoding p53 is encoded on the short arm of chromosome 17, and the corresponding p53 protein has a molecular mass of 43.7 kDa (Levine and Oren, 2009). It has 3 main domains; a DNA binding domain for binding to other genes, an N-terminal trans-activational domain that forms binding sites for positive or negative regulators, and a C-terminal oligomerization domain that undergoes post-translational modifications and alternative splicing (Mollereau and Ma, 2014; Pflaum et al., 2014). In normal, healthy cells, levels of p53 are low because turnover is high. However, when damage occurs, p53 stabilizes and the levels rise (Haupt et al., 2003). Stability is conferred to p53 by the mouse double minute 2 (MDM2) protein (Perry, 2010), a

protein that is also overexpressed in many cancers (Jansson et al., 2014; Riley et al., 2016).

3. Bcl2 and Bcl6

3.1. Bcl2 family of proteins

The Bcl2 family of proteins can be broadly categorized into 2 groups: pro-apoptotic and anti-apoptotic. All Bcl2 family members have certain sequence homology domains, termed Bcl2 homology (BH) domains. There are currently 4 known BH domains. The anti-apoptotic proteins generally contain 3-4 BH domains and include Bcl-2, Bcl-x_L, Bcl-W, Mcl-1, A1, and Bcl-B. The pro-apoptotic groups can be further subdivided based upon these BH domains; a BH3-only group, including Bid, Bim, Bad, Noxa, and Puma, and the multi-domain group, comprising Bax, Bak, and Bok.

3.1.1. BH domains

The functions of the various domains have been elucidated (Danial, 2007; Lomonosova and Chinnadurai, 2009). The BH1 and BH2 domains are critical for dimerization with other pro-apoptotic members, then subsequent pore formation and insertion into the mitochondrial membrane for MOMP. The BH3 domain is contained by all Bcl-2 family proteins and is crucial for homo- and hetero-dimerization among Bcl-2 family member interactions. The BH4 domain is essential for interaction and regulation of several proteins involved in apoptosis.

3.1.2. Interactions of Bcl2 family of proteins

Many members of the Bcl2 family of proteins interact with each other to either drive apoptosis, or to prevent apoptosis (see Fig. 2). The most notable of these interactions is homo- and hetero-dimerization between Bax and Bak proteins to form proteinaceous or lipidic pores. Upon oligomerization, these pores will insert into the mitochondrial membrane, allowing for the escape of CytC, and other proteins, from the mitochondria (Eskes et al., 2000; Reed, 2006; Wei et al., 2000, 2001). MOMP requires only 1 of the multi domain pro-apoptotic proteins in order for apoptosis to occur (Wei et al.,

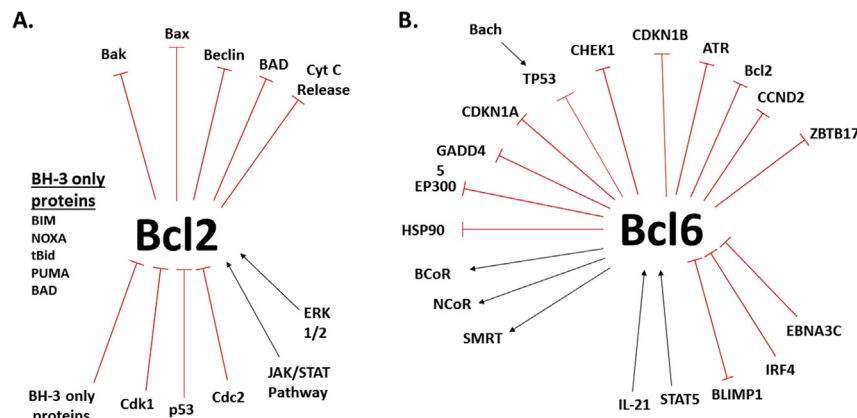


Fig. 2. Interactions of Bcl2 and Bcl6. Shown are some of the known interactions of Bcl2 (panel A) and Bcl6 (panel B). Shown are protein interactions relating to cell cycle and cell death.

2001). The Bcl2 family protein Bid serves as an agonist for Bax oligomerization and insertion into membranes (Kuwana et al., 2002; Wei et al., 2000). Bcl2 protein itself is an integral membrane protein (Chen-Levy et al., 1989; Tsujimoto et al., 1987), but it can also serve as an inhibitor of CytC release from the mitochondria (Yang et al., 1997) as it binds to and inhibits Bax oligomerization (Eskes et al., 2000; Nechushtan et al., 1999; Wei et al., 2000; Wolter et al., 1997). In addition to Bid serving as an agonist for Bax oligomerization, both Bid and Bim are direct activators of Bax and Bak. Additionally, all the BH3-only proteins can bind and deactivate the Anti-apoptotic Bcl2 family members (Du et al., 2011; Green and Kroemer, 2004). In recent years, the Bcl2 protein has been referred to as the apoptotic switch (Adams and Cory, 2007) in reference to the regulation of MOMP by Bcl2 as it can bind to MOMP effectors Bax and Bak.

3.1.2.1. p53 interactions with Bcl proteins. There are many interactions between p53 and the Bcl2 family of proteins. Among those being transcriptionally activated by p53 in the nucleus are *PUMA*, *Bid*, *Bax*, *TRAILR2*, *CD95* (Beckerman and Prives, 2010; Nakano and Vousden, 2001; Oda et al., 2000; Sax et al., 2002; Thornborrow et al., 2002; Yu et al., 2003). Because *Bax* is a direct target of p53, in p53 mutant cancers, the Bax levels can drop and MOMP can be abolished, stopping apoptosis from occurring. When p53 is constitutively active, Bax levels rise and MOMP will be up regulated and apoptosis will occur. *Bid* can be induced by p53 following irradiation by gamma radiation and aids in sensitizing cells to chemotherapeutic agents (Sax et al., 2002). Other agents are not able to induce apoptosis, but sensitize cells to chemotherapeutic agents, improving apoptotic results, include Apaf-1 and caspase-6 (MacLachlan and El-Deiry, 2002; Moroni et al., 2001; Sax and El-Deiry, 2003). p53 can also aid in apoptosis in a transcriptionally independent manner. It can act directly in the mitochondria by binding Bcl-x_L and Bcl2, freeing up Bax and Bak proteins to activate MOMP, eliciting CytC release (Tomita et al., 2006).

In addition to the Bcl2 family of proteins, p53 interacts with Bcl6 proteins (see Fig. 2). In chronic myeloid leukemia, Bcl6 represses p53 in the initiation process of colony formation (Hurtz et al., 2011). Additionally, Bcl6 and BACH2 are binding competitors, with BACH2 reversing Bcl6 repression of p53 in normal healthy cells (Swaminathan et al., 2013, 2014). The Bcl6 and BACH axis expression are implicated in controlling p53 expression in acute lymphoblastic leukemia (Ge et al., 2017).

4. Possible Bcl6 mechanisms

The exact mechanism of Bcl6 action is unknown. This may be due to the possibility of Bcl6 having various mechanisms in different cell types. For example, IL-21 mediates Bcl6 regulation in follicular helper T-cells as well as in germinal center B-cells to turn on Bcl6 (Linterman et al., 2010; Ozaki et al., 2004; Spolski and Leonard, 2010; Zotos et al., 2010). Alternatively, STAT5 is responsible for induction of Bcl6

expression in tonsillar B-cells (Scheeren et al., 2005). In development, it is known that Bcl6 is necessary for embryological development (Sakano et al., 2010). Mouse models deficient in Bcl6 have no germinal centers and are unable to produce high affinity antibodies (Toney et al., 2000). Subpopulations of CD4⁺ T-cells require Bcl6 for development (Mondal et al., 2010), as well as do Follicular T-helper cells (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009).

There are several factors that repress Bcl6 expression. Acetylation of Bcl6 can deactivate it (Bereshchenko et al., 2002). In breast epithelium, STAT5a represses Bcl6 expression (Tran et al., 2010). In bone marrow antibody production, STAT5 represses Bcl6 (Duy et al., 2010). This interaction also leads to a non-normal repertoire of primary antibodies in bone marrow. B-cell crosslinking reduces Bcl6 expression via ubiquitylation by extracellular signal-related kinases 1 and 2 (ERK 1/2; MAPK1) activation (Niu et al., 1998). Additionally, CD40 signaling triggers reduction of Bcl6 expression due to induction of transcription factor interferon regulatory factor 4 (IRF4) via NFκB pathway (Saito et al., 2007).

Bcl6 is transcriptional repressor that works by reducing mRNA expression of target genes (Deweindt et al., 1995; Seyfert et al., 1996). It binds to target genes as a homodimer and then recruits co-repressors molecules such as SMRT (silencing mediator of retinoid and thyroid receptor), nuclear receptor co-repressor (NCoR), and Bcl6 co-repressor (BCoR) (Dhordain et al., 1997, 1998; Huynh et al., 2000). The N-terminal POZ virus and zinc-finger protein Bric-a-brac, Tramtrack, broad complex protein (POZ/BTB) domain is where homo-dimerization occurs as well as co-repressor recruitment (Dhordain et al., 1995). Co-repressors recruit deacetylases 1 and 2 (HDAC1 & HDAC2). The deacetylation of histones leads to transcriptional repression of Bcl6 target genes (Miles et al., 2005).

Bcl6 can bind to and repress several oncogenes and tumor suppressors: CDKN1A (Phan et al., 2005), CDKN1B (Shaffer et al., 2000), CCND1 (Shvarts et al., 2002), CCND2 (Shaffer et al., 2000), TP53 (Phan and Dalla-Favera, 2004), and Bcl2 (Saito et al., 2009). When Bcl6 binds to the Bcl2 locus, it disrupts the transcriptional activator ZBTB17, and by this means, repressing Bcl2. It is possible that by disrupting ZBTB17, or mutation of the Bcl6 binding site in the Bcl2 locus, that constitutive expression of Bcl2 will occur, resulting in clinically aggressive diseases that are resistant to apoptosis. There is a mechanism by which Bcl6 aids in transcriptional repression, even though a Bcl6 binding site is not present. Two separate reports show that Bcl6 can bind to Miz-1 (Phan et al., 2005; Wu and Jelinek, 2005). Binding of Bcl6 to Miz-1 can promote cell proliferation by repressing cyclin-dependent kinase inhibitor 1A (CDKN1A). At the very least, this is a way for Bcl6 to influence the cell cycle, in addition to inhibiting other DNA repair pathways (Ranunolo et al., 2007, 2008).

5. Bcl2 and Bcl6 protein deregulation and disease

Deregulation of Bcl6 and Bcl2 family of proteins can lead to many types of cancers. These deregulations can give an

insight into possible therapeutics. I will briefly describe some of the diseases and refer the reader to several reviews written to treat the subject of possible therapeutics.

Malignant gliomas have been shown to use Bcl3 to positively regulate Bcl2 downstream. Specifically, astrocytic glioma cells proliferate and apoptotic inhibition through the STAT3 pathway occurs (Wu et al., 2016). Also notable is the fact that Bcl2-x_L has been linked to the STAT pathway (Grad et al., 2000; Zaanani et al., 2015). Cells of the hippocampus are vulnerable to ischemia-reperfusion (I-R) injury. Overexpression of Bcl2 in hippocampal tissues resulted in Granule cells resisting apoptosis, while CA1 cells only showed a delay in apoptosis (Wang et al., 1999). What could be regulating Bcl2 in I-R injury? A 7-mer peptide that acts as a glutathione peroxidase (GPX) mimetic that was given in I-R injury. The result was an increase in both mRNA and protein levels of Bcl2 while Bax was down regulated (Jiang et al., 2016). This suggests a possibility for GPX regulation of Bcl2 and Bax in liver tissue, as well as suppression of reactive oxygen species. Integrin-linked kinase (ILK) overexpression with treatment of temozolomide (TMZ) in glioma cells resulted in an increase of Bcl2 (anti-apoptotic) expression and a decrease of Bax (pro-apoptotic) expression (Liang et al., 2017). This suggests that the overexpression of ILK reduces sensitivity of the tissues to the anti-cancer drug, though no hypotheses as to a mechanism have been given.

5.1. Bcl2 in disease

The Bcl2 family of proteins, as discussed previously, is heavily involved in the intrinsic pathway of apoptosis. As such, they are not only involved in mechanisms related to lymphomas, but also to many other disease states, such as colorectal cancers, ischemia, diffuse large B-Cell lymphoma, malignant gliomas, and more. Many of these disease states have mechanisms that are still unknown. Such an example is in ovarian serous carcinoma where high expression of TRAIL-receptor 2 (TRAIL-R2) has been associated with the disease state (Braga et al., 2014). This study also showed that higher levels of Bcl2 were associated with TRAIL-R2 and the disease state. In other correlative studies, high expression of Bcl2, concurrently with Myc, has been correlated with pathogenesis of a family of diffuse large B-cell lymphoma (Bogusz et al., 2017).

In colorectal cancers (CRC), the effects of the Bcl2 and Bax were correlated to weight and size of xenograph tumors in a murine model (Ye et al., 2016). As a result of many studies like these, many studies looking into therapeutics that modulate Bcl2 and Bax expression in hopes of viable treatments. Both in vitro and in vivo models have shown promise for possible drug therapies (Kim et al., 2014; Li et al., 2017; Moghtaderi et al., 2017).

5.2. Bcl6 in disease

In attempting to elucidate the pathway of Bcl6 in Follicular Lymphoma, it was determined that Bcl6 represses the NOTCH2 signaling pathway (Valls et al., 2017). This

repression signals for cell survival and growth, suggesting that suppression of Bcl6 will allow for normal apoptosis to occur, preventing lymphoma growth. Additionally, in follicular lymphoma, a knock down of endogenous Bcl6 resulted in a 6.8-fold increase of RUVBL1 (aka pontin, Tip49, NMP238) protein expression (Baron et al., 2016). RUVBL1 is involved in cell activities like DNA repair, gene transcription regulation, and chromatin remodeling. Disruption of this relationship may be enough to cause lymphoma growth, though further studies into these effects and pathway are needed for support.

Another question is how much dysregulation is needed in the Bcl6 system to cause oncogenesis? In a murine model of transient expression of *Bcl6*, it was noted that the experimental mice lived much shorter lives than their wild type littermates. Accompanying these shorter lives was the presence of splenomegaly and lymphomas in the form of enlarged white pulp nodules with pleomorphic large B-cells (Green et al., 2014). In the realm of prognostics, it was revealed that Bcl2 positive expression and negative Bcl6 expression are associated with poor prognostics in DLBCL (Fang et al., 2017).

Bcl6 is not only involved in B-Cell lymphoma, but also in other cancers such as breast cancer. It was recently observed that expression of Bcl6 mRNA can serve as a marker for breast cancer (Badr et al., 2016). In breast cancer cell lines, miRNA-127 repression of Bcl6 showed significant reduction in cell proliferation (Chen et al., 2013). Although the exact mechanism of Bcl6 function in breast cancer is unknown, it is known that Bcl6 can activate ZEB1, a transcriptional repressor. And when ZEB1 binds to the E-cadherin promoter, E-cadherin expression is repressed. Activation of Bcl6 and ZEB1, along with the subsequent repression of E-cadherin transcription results in activation of epithelial to mesenchymal transition (EMT), and induction of EMT lead to growth, invasion, and migration of breast cancer cells (Yu et al., 2015). Another mechanism of Bcl6 action in breast cancer is through STAT3 and STAT5 proteins as they have opposing effects in regulating Bcl6 (Nelson et al., 2009; Walker and Frank, 2015). Bcl6 is also known to be involved heavily with the STAT family of proteins in primary mediastinal B-Cell lymphoma, bone marrow B-cells, and tonsillar B-cells (Ritz et al., 2013; Wagner et al., 2011).

In mammals, the ear is separated into three sections: the outer, middle, and inner ear. The outer ear is lined on the medial side by the tympanic membrane. The middle ear is limited by the tympanic membrane laterally, and the oval window medially. The inner ear consists of the membranous labyrinths; vestibule, semi-circular canals, and the cochlea. Within the cochlea, there are three compartments: scala tympani, scala vestibuli, and the cochlear duct (see Fig. 3). The scala tympani and the scala vestibuli both contain an extracellular fluid with a high sodium (Na⁺) and low potassium (K⁺) ion content called perilymph. The cochlear duct is limited by Reissner membrane medially and the stria vascularis and spiral ligament laterally. The cochlear duct contains endolymph, which holds a higher concentration of K⁺. Accordingly, the transduction current in the hair cells is carried by K⁺ ions. The K⁺ concentration is generated and

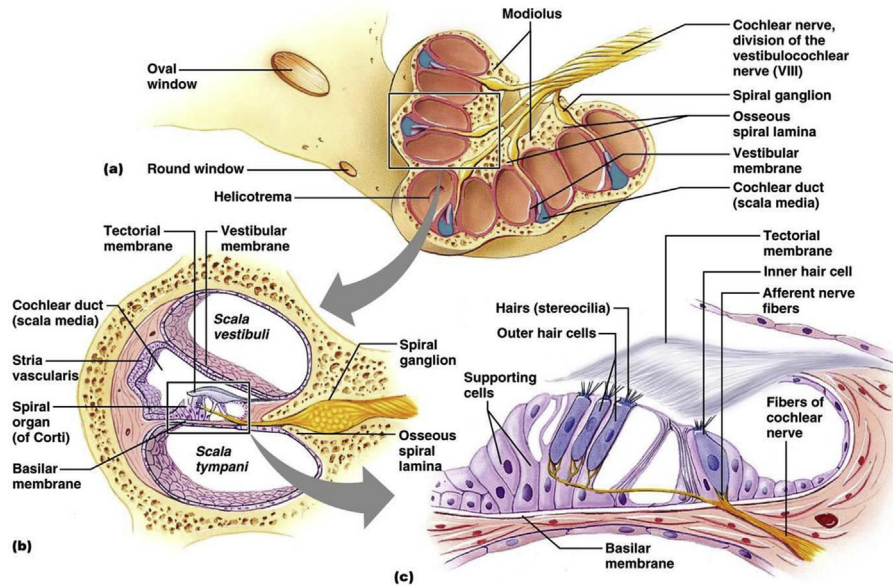


Fig. 3. Anatomy of the Cochlea. Cartoon illustration of the cochlea. *Panel a.* A split cochlea showing the various turns, base, and helicotrema. *Panel b.* Cartoon illustration of the 3 compartments of the cochlea. *Panel c.* Relation of the cochlear hair cells, basilar membrane, and tectorial membrane. (Anatomybody-charts, 2016).

maintained by the stria vascularis. Within the cochlear duct lies the basilar membrane on which the sensory epithelium, the organ of Corti is located. The organ of Corti contains sensory hair cells and supporting cells. Hair cells transduce mechanical stimuli into electrical activity (Hudspeth and Corey, 1977; Hudspeth, 1989). Mechano-electrical transduction is mediated by the hair bundle, an array of modified microvilli or stereocilia arranged in a staircase on the apical surface of the hair cell (Fettiplace and Hackney, 2006). In addition to the stereocilia specialization critical for mechano-electrical transduction in the apical membrane, all hair cells also have specializations in the basolateral and synaptic membranes that are responsible for electrical activities and synaptic transmission. The supporting cells, which include pillar cells, Deiters cells, and inner phalangeal cells, contribute to the stiffness of the cochlear partition and the homeostasis of the ionic and chemical environment (Raphael and Altschuler, 2003; Slepecky, 1996).

There are two hair cell types in the organ of Corti (see Figs. 3 and 4); inner hair cells (IHCs) and outer hair cells (OHCs). One row of IHCs and three rows of OHCs are tonotopically arranged along the entire basilar membrane in the cochlea. IHCs and OHCs are distinct morphologically (see Figs. 3 and 4) and functionally (Dallos, 1992). IHCs predominantly possess afferent innervation (Spoendlin, 1970), and are considered to be the major sensory receptor as they transmit information to the brain. OHCs predominantly possess efferent fibers (Spoendlin, 1970), serving as an effector cell that amplifies input to IHCs by a receptor potential-driven somatic motility (Brownell et al., 1985; Dallos et al., 2008; He et al., 2003; Liberman et al., 2002; Zheng et al., 2000). The human cochlea contains on the order of 3,500 IHCs and 12,000 OHCs at birth. The mechanosensitive HCs are lost with age and are highly susceptible to damage from noise and ototoxic drugs. Hair cell loss is the most common cause of hearing loss. Mammalian hair cells are no longer able to regenerate once

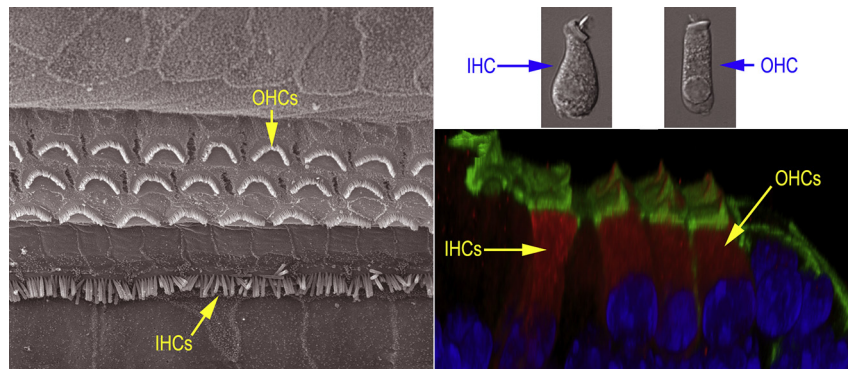


Fig. 4. Anatomy of the Cochlear Hair Cells. Left *panel:* organization of the inner and outer hair cells that line the tonotopic axis of the cochlea. Top right: morphological differences of the IHC and OHC. IHC are more pear-shaped with a central nucleus and apical stereocilia. OHC are more cylindrical with a basal-located nucleus and apical stereocilia. Bottom right: image showing grouping of outer hair cells versus inner hair cells.

lost. Lower vertebrates such as bird and fish can spontaneously regenerate lost hair cells through proliferation and trans differentiation of supporting cells.

6. Hair cell loss

Sensory hearing loss is the result of deficient, damaged, or inadequate cochlear hair cell function, and affects 1.3 billion humans worldwide ([Worldwide Hearing, 2014](#)). There are 4 main causes of hair cell loss: aging, ototoxicity, noise insult, and genetic defect.

6.1. Genetic disposition in hearing loss

One of the most common birth defects (or congenital defects) is hearing loss or deafness. This congenital abnormality can affect as many as three of every 1000 babies born. Inherited genetic defects contribute to about 60% of deafness/hearing loss occurring in infants. So these genes play an important role in congenital hearing loss. The two most common classifications of hearing impairment are syndromic and non-syndromic.

6.1.1. Non-syndromic hearing impairment

Non-syndromic hearing impairment is responsible for a considerable majority of inherited hearing loss, approximately 70%. Autosomal-recessive inheritance consists of about 80% of cases of non-syndromic hearing impairment, while autosomal-dominant genes make up 20%. Less than two percent of cases are the result of X-linked and mitochondrial genetic malfunctions.

One example of non-syndromic hearing impairment, DFNA3, is found in the autosomal dominant mutations of *GJB2* or *GJB6*. These genes encode for connexins, specifically for connexin 26 and connexin 30 respectively ([Smith and Ranum, 1998](#)). Within the mitochondria, two genes are specific to non-syndromic hearing loss, *MTTS1* and *MTRNR1*. *MTRNR1* translates to the mitochondrial ribosomes ([Ballana et al., 2006](#)) and *MTTS1* correlates to mitochondrial tRNA for serine ([Reid et al., 1994](#)).

6.1.2. Syndromic hearing impairment

Syndromic hearing impairment means that the hearing impairment is correlated with additional clinical abnormalities. Approximately 15–30% of hereditary hearing impairments are syndromic. Over 400 syndromes are known to include hearing impairment and can be classified as: syndromes due to cytogenetic or chromosomal anomalies, syndromes transmitted in classical monogenic or Mendelian inheritance, or syndromes due to multi-factorial influences, and finally, syndromes due to a combination of genetic and environmental factors.

SLC26A4 is the gene that is often mutated in Pendred Syndrome. It is characterized by congenital deafness and the development of a goiter during puberty. Waardenburg Syndrome is an autosomal dominant disease that includes hearing

loss. It can be accompanied by pigment abnormalities of the skin and/or irises. In the 4 identified types of Waardenburg Syndrome, some of the mutated genes include *Pax3*, *MITF*, *EDNRB*, *EDN3*, and *Sox10*. Mutation of the *NF2* gene can cause neurofibromatosis; a disease that, if caught early enough, is treatable. Mohr-Tranebjaerg syndrome is caused by a mutation in the *TIMM8A* gene and is characterized by hearing loss as well as movement of cytosolic proteins into the mitochondrial matrix across the inner mitochondrial membrane.

6.2. Age-related hearing loss

Many physiological changes occur in the aging process. Age-related hearing loss (ARHL), or presbycusis, occurs is one of the most common conditions affecting older and elderly adults. In the United States population between the ages of 65 and 74, roughly one in three people has hearing loss. Of those older than 75, nearly half have difficulty hearing.

Presbycusis is characterized by decreased hearing sensitivity, decreased processing of acoustic impulses, and reduced speech recognition (especially in noisy environments). The earliest signs of presbycusis are tinnitus and high frequency hearing loss. Over time, the hearing threshold can progress to lower frequencies. Presbycusis is also bilateral and symmetrical. There is no known single cause for age-related hearing loss. Most commonly, it is caused by loss of mechanosensitive hair cells in the inner ear as one grows older. However, genetic deficits and repeated exposure to loud noises may play a major role ([Fransen et al., 2003](#)). Smoking and certain medical conditions and medications can aggravate presbycusis ([Gates et al., 1993](#); [Agrawal et al., 2009](#); [Sha et al., 2008](#)). Recent studies have shown that microRNAs (miRNA) can also play a role in age-related hearing loss ([Zhang et al., 2013](#)). Several miRNAs (such as miR-34 and miR-29 families) that are pro-apoptotic are up regulated, while anti-apoptotic microRNAs (such as miR-181 and miR-183 families) are downregulated during aging.

As just noted, there are numerous factors that can contribute to presbycusis. As a result, a single mechanism is difficult to isolate. However, multiple studies using various model organisms have indicated that increases in caspases-3, -7, and -9 accompany this ailment. Additionally, an increase in Bax expression and a decrease in Bcl-2 expression have both been found to accompany ARHL ([Alam et al., 2001](#); [Nevado et al., 2006](#); [Someya et al., 2009](#)). For further treatment of the subject, the reader is referred to a recent review that weaves epidemiological data with mechanistic information to paint a broader story of the causes of ARHL ([Yamasoba et al., 2013](#)).

With aging, it is possible that loss of Bcl2 expression can lead to increased Bax expression. With less Bcl2 binding to Bax, Bax can then oligomerize and insert into the mitochondrial membrane leading to MOMP, shifting the hair cell to apoptosis. As p53 also binds to Bcl2 in the mitochondria, loss of Bcl2 expression can free p53 to act as a transcriptional activator of *Bax* and *Bid*, pushing the hair cell towards apoptosis and presbycusis.

6.3. Ototoxicity

Ototoxicity has long been known to be a cause of hair cell and hearing loss. Some examples of drugs that cause ototoxicity are cisplatin, carboplatin, bumetanide, and aminoglycosides (i.e. gentamicin, neomycin, kanamycin). Some studies show that kanamycin-induced hair cell loss is accompanied by features of both classical apoptosis and necrosis, but also lacked critical features of the apoptotic pathway (Jiang et al., 2006). A subsequent study revealed the presence of some apoptotic features, TUNEL labeling and caspase-3 activation, as well as some necrosis features, large amounts of cell debris, following injection of kanamycin and a loop diuretic, bumetanide (Taylor et al., 2008). A common class of antibiotics, aminoglycosides, which also induce hearing loss, have been recently described being transported into cochlear hair cells via mechanotransducer channels and endocytosis (Alharazneh et al., 2011; Hailey et al., 2017). The transient receptor potential vanilloid 1 and 4 (TRPV 1 and 4) participates in transport of the antibiotic, and ototoxic, drug gentamicin into the basal hair cells (Lee et al., 2013). Finally, basal hair cells show an increased vulnerability to free-radical damage, as described by lower expression of the anti-oxidant glutathione (Sha et al., 2001). Ototoxicity-induced hair cell loss only shows a general trend, that is OHCs are more vulnerable than IHCs and basal turn hair cells are more vulnerable than apical turn hair cells. The mechanism of this differential vulnerability is unknown. Currently, models are being created to study for therapeutics for cisplatin-induced hearing loss (Callejo et al., 2017; Wu et al., 2017). Many studies target possible therapeutic drugs for platinum-induced hearing loss (Theunissen et al., 2015; van As et al., 2016a,b).

The mechanism of ototoxicity, though not perfectly understood, is very similar to apoptosis in many other cells. Specifically, aminoglycosides induce Bax activation with subsequent MOMP and release of CytC. Caspase-3 activation will result in DNA degeneration (Mangiardi et al., 2004; Matsui et al., 2004; Coffin et al., 2013). In cisplatin induced hair cell and hearing loss, cisplatin triggers free radical production in the cochlea, Bax activation, and subsequent intrinsic pathway apoptosis (Rybak et al., 2007). Additionally, the increase of Bax expression could possibly bind all possible Bcl2 proteins, leaving p53 to activate further Bax and Bid. This could also affect Bcl6 binding of p53, leaving Bcl6 to further repress Bcl2 and push the hair cell towards cell death via apoptosis.

6.4. Acoustic trauma

Noise induced hair cell loss shares many of the same features that ototoxicity induced hair cell loss contains. It is unclear what proportion of hair cells die via apoptotic intrinsic pathway versus apoptotic extrinsic pathway versus the necrotic pathway, although it is clear that all are involved. Some studies show that the inhibiting caspases increases the necrosis proteins RIP1 and RIP3 and number of necrotic nuclei. The opposite holds true as well, inhibiting necrosis proteins RIP1 and RIP3 increases the number of apoptotic

nuclei with an up regulation of caspase-9 and no increase in caspase-8 activity (Zheng et al., 2014).

Loud noises initiate large displacements of the tympanic membrane, creating waves of mechanical energy into the inner ear. These waves cause shearing forces to cause possible damage to the basilar membrane, organ of Corti, hair cells, and alteration of the endocochlear potential. Intrinsic pathway activation can appear via ROS activity (Fetoni et al., 2013), caspase independent activation via EndoG and AIF action (Yamashita et al., 2004), and necrosis (Bohne et al., 2007). In extrinsic pathway, activation can transpire via activation of the TNF α and Fas receptors, TNFR1 and FasR (Li et al., 1998; Nicotera et al., 2003; Jamesdaniel et al., 2011). TNF α -mediated p38 and JNK signaling can be up regulated following loud noise exposure. This signaling occurs in the sensory epithelium of the inner ear and propagates Bax-induced mitochondrial release of CytC, and other apoptotic proteins, as well as pro-death gene transcription (Wang et al., 2007a,b; Dinh et al., 2008a,b; Jamesdaniel et al., 2011).

Most likely, ROS released from shearing forces of loud noise lead to an up regulation of p53 in cochlear cells, leading to an up regulation of Bid and Bax that will lead to MOMP and hair cell death. Additionally, anti-apoptotic molecules such as Bcl2 and Bcl-x_L are down regulated in response to oxidative stress, possibly allowing for more pro-apoptotic molecules to push a hair cell towards cell death via apoptosis.

7. Bcl6 and Bcl2 family of proteins in cochlear hair cells

Recently, transcriptome and transcription factor studies have shown differential expression of both transcription factors and genes in inner versus outer hair cells corresponding to both *bcl2* and *bcl6* in IHC (Liu et al., 2014; Li et al., 2016). The difference of these molecules in between inner and outer hair cells is a possible explanation to OHC susceptibility to early cell death. It is also possibly the key to unlocking the exact mechanisms of Bcl2 and Bcl6 involvement in hair cell loss. The current hypothesis is that IHC are more resilient against cell death due to their increased expression of *Bcl2* and *Bcl6*. We have generated a conditional knock out of Bcl6 and plan on examining the effects the knockout has on the cochlear hair cells. We hope to elucidate the role of Bcl6, and its up- and down-stream effects on other molecular signals.

8. Concluding comments

Recently, Bcl6 has become of interest as it is an oncoprotein that can be targeted as a cancer therapeutic (Cardenas et al., 2017). In this article, the common causes of sensorineural hair cell loss and their relation to the apoptotic pathway have been reviewed. Other cell types within the inner ear, such as the cells of the stria vascularis, spiral ganglion cells, Deiters' cells, and other supporting cells, may also contribute to cell death and hearing loss. Routes leading to cell death include necrosis and apoptosis, including the various pathways within apoptosis and necrosis. Many potential targets for clinical treatment, including the Bcl2 and Bcl6 families of

proteins, have been noted. Further studies will lead to more advanced therapeutic treatments to mitigate hearing loss.

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