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A role for epigenetics in hearing: Establishment and maintenance of auditory specific gene expression patterns

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Abstract

Epigenetics is a large and diverse field encompassing a number of different mechanisms essential to development, DNA stability and gene expression. DNA methylation and histone modifications work individually and in conjunction with each other leading to phenotypic changes. An overwhelming amount of evidence exists demonstrating the essential nature of epigenetics to human biology and pathology. This field has spawned a vast array of knowledge, techniques and pharmaceuticals designed to investigate and manipulate epigenetic phenomena. Despite its centrality to molecular biology, little work has been conducted examining how epigenetics affects hearing. In this review, we discuss both the basic tenants of epigenetics and highlight the most recent advances in this field. We discuss its importance to human development, genomic stability, gene expression, epigenetic modifying agents as well as briefly introduce the expansive field of cancer epigenetics. We then examine the evidence of a role for epigenetics in hearing related processes and hearing loss. The article concludes with a discussion of areas of epigenetic research that could be applied to hearing research.

Keywords

Presbycusis; Hair Cells; Organ of Corti; DNA Methylation; Histone Modifications; Deafness

1. Introduction

The term epigenetics encompasses a variety of biological process and represents a vast and well developed field. It is perhaps best described as a change in phenotype that is not caused by a change in DNA sequence. The two most well understood mechanisms of epigenetic alterations that lead to these phenotypic changes are DNA methylation and histone modifications. While the two will initially be discussed separately, it must be remembered that epigenetic regulation is often a dynamic and coordinated process involving both as DNA resides in close association with histone proteins and the two together with the nuclear scaffold represent chromatin.

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Although a number of well written and informative review articles on epigenetics already exist in the literature, there is a noticeable absence of epigenetics work in the field of hearing research. Indeed, epigenetics is an essential player in the developmental process, and a well established biological process in the pathogenesis of cancer. Despite its importance to a number of research fields, epigenetics has yet to play a major role in explaining development, function and pathology of hearing. There are myriad benefits to discovering epigenetic mechanisms to diseases of hearing. Epigenetic mediated gene silencing is a naturally occurring process, which, in many instances, is non pathologic. As such, organisms retain a host of mechanisms to reverse these epigenetic modifications. A number of pharmaceuticals have also been developed to target these antagonistic processes. These compounds stand as an untapped resource for understanding hearing related illness. An epigenetically targeted pharmacologic intervention also avoids some of the known difficulties and complications associated with other experimental treatments for hearing loss such as gene therapy and stem cell transplantation. This review article aims to make a rapid tour of epigenetics, taking time to explore its role in development and hearing related malignancies, touch upon some of the epigenetic modifying agents that could be utilized in hearing research, explore the epigenetic basis for known causes of hearing loss and finish with possible areas of future intersections between epigenetics and hearing research. Epigenetics offers a relatively unexplored area of hearing research and presents vast opportunities to those endeavoring to better understand the normal and pathologic processes effecting hearing.

2. DNA Methylation

DNA methylation offers less complexity than the histone modifications that affect gene expression. Through DNA methylation, a single methyl group is added to cytosine on position 5 at CpG dinucleotides (Bird, 2002). This phenomenon is evident in a wide variety of plant and animal species and serves a number of useful roles in non pathologic genetic processes (Morel et al., 2000; Vaucheret et al., 2001). Upon methylation of the DNA, transcription is repressed either through the recruitment of methyl-CpG-binding proteins (MeCP) (Cross et al., 1997) or by preventing the transcriptional machinery, namely the transcription factors, from binding to the DNA (Tate et al., 1993). The methyl-CpG-binding proteins will be discussed in further detail in a later section.

The methylation of cytosine also increases the risk of mutational events occurring. When cytosine is unmethylated, deamination results in a cytosine to uracil change; uracil is recognized and removed from DNA by uracil DNA glycosylase. However, deamination of a methylated cytosine causes a cytosine to thymidine mutation. While mutations of cytosine to uracil are normally recognized and repaired by the body, the cytosine to thymidine mutations are not as easily recognized and repaired (Hermann et al., 2004). This allows them to go undetected and could explain why CpGs are mutational hotspots (Dean et al., 2005).

CpG location provides evidence of the role of DNA methylation in regulating gene expression. Genome wide analysis shows that the observed frequency of CpG sites occurs with a lower frequency than would be expected (Wilson et al., 2007). Moreover, where they do exist they tend to be clustered into groups called CpG islands. While most CpG contained in islands are unmethylated (Bird, 2002), those located outside of islands are usually methylated (Baylin et al., 2000). This association corresponds with the finding that most CpG islands are located at the 5' ends of genes. Indeed, about 50% of all human genes have a CpG island at their 5' end. Placement of the CpG islands within the 5' regulatory regions of genes provides a powerful tool for gene regulation. Some gene promoters are highly methylated during development while others undergo increasing methylation with age (Issa et al., 2001). While CpG location within promoters is well known and widely studied, their

position within introns and exons has also been documented to control gene expression (Januchowski et al., 2004; Uchida et al., 2004; Wutz et al., 1997).

2.1 DNA methyltransferases

DNA methylation occurs through the action of a family of proteins called the DNA methyltransferases (DNMT). These proteins are responsible for the two forms of DNA methylation: de novo methylation and maintenance of an established methylation pattern. DNMT1, is primarily responsible for maintenance methylation. The protein's importance is demonstrated by the lethality of the DNMT1 knock out mouse (Li et al., 1992). Its maintenance function is evidenced by its preference for hemimethylated DNA in contrast to unmethylated DNA. This allows DNMT1 to read methylated DNA strands during DNA replication and place a methyl group on the corresponding daughter strand (Bacolla et al., 1999; Bacolla et al., 2001; Pradhan et al., 1999). It has additional roles in interacting with other members of the chromatin remodeling machinery such as histone methyltransferases and MeCP proteins. This involvement in the interplay between histone modifications and DNA methylation enables cooperative repressive effects of DNA methylation and some histone modifications (Bird, 2002; Hermann et al., 2004). DNMT2's function remains to be fully described. Its weak DNA methylation capability appears to be non essential given the viability of knock out animals (Okano et al., 1998), but recently it has been shown to have an RNA methylating capacity (Goll et al., 2006). The third member of the DNMT family, DNMT3, actually consists of a number of different proteins involved in de novo DNA methylation (Okano et al., 1999). These proteins, essential for life, establish methylation patterns in CpG rich areas as well as isolated CpG sites. They play an essential role in development and are responsible for the DNA methylation patterns necessary for genomic imprinting and gene inactivation. DNMT3a is distributive in that it is targeted to a specific CpG location for DNA methylation, thereby resulting in de novo methylation (Hata et al., 2002). The DNMT3a protein is found as two different isoforms with DNMT3a expression found in adults while DNMT3a2 is expressed mostly in embryos (Chen et al., 2002). When expressed in adults, the enzyme is usually associated with condensed, transcriptionally inactive heterochromatin while its expression in developing embryos is usually isolated to open, transcriptionally active euchromatin (Dean et al., 2005).

While the enzymatic mechanisms of DNA methylation have been well described, to date there is no identified human demethylating enzyme. A mammalian DNA demethylase was reported to exist (Bhattacharya et al., 1999), but this finding has since been brought into question by subsequent work that has failed to reproduce the initial report (Boeke et al., 2000; Wade et al., 1999). Such an enzyme is known to exist in other, non human organisms through its activity as a methylcytosine glycosylase (Penterman et al., 2007). Despite this, it is well known that active demethylation occurs during human development, thus it is likely that there is a human enzyme with 5-methylcytosine DNA glycosylase activity that could be construed as a DNA demethylase (Vairapandi, 2004). It is also evident that global DNA methylation patterns change during carcinogenesis but it is unknown whether this is an active or passive process of DNA demethylation.

2.2. Biological Functions of DNA Methylation

As mentioned previously, DNA methylation is a normal biological process involved in regulating gene expression. It also plays an important role in genomic stability and can affect gene expression through interaction with the RNA interference machinery. Aberrant DNA methylation can prevent normal gene expression, leading to changes in phenotype (Xing, 2007) or malignancies (Oshiro et al., 2003). Methylation also has the secondary effect of recruiting a number of proteins that recognize methylated sections of DNA and remodel chromatin into a repressive, heterochromatin, conformation.

Instances of hypomethylation can also lead to pathological changes. Current evidence suggests that hypomethylation does not occur because of loss of expression or activity of the DNA methyltransferases. Instead, this phenomenon appears to result from a complex interplay of transcription factors, chromatin remodeling proteins and the DNA replication and repair machinery (Wilson et al., 2007). The consequences of hypomethylation could result not only from the inappropriate expression of previously silenced genes (Papageli et al., 2003; Roman-Gomez et al., 2007) but also from the perturbation of another function of DNA methylation; providing genome stability. Under normal cellular conditions, DNA methylation normally occurs in areas of long or short interspersed transposable elements (LINEs and SINES respectively), repetitive DNA elements and DNA satellites (Yoder et al., 1997). The normal methylation of these transposable elements within the genomes prevents transcription of the transposases and also places the chromatin into a conformation unfavorable to chromosomal rearrangements. When DNA is hypomethylated, especially in areas such as the Sat2 and Sat α sequences, chromosomal rearrangements are more likely to occur (Qu et al., 1999; Vilain et al., 2000).

DNA methylation and methyltransferases also interact with RNAi. Work in a number of different species has demonstrated that double stranded RNA, both miRNA and siRNA, can direct de novo DNA methylation (Bao et al., 2004; Wassenegger et al., 1994). In this way, the double stranded RNA can target genomic areas for a more permanent method of gene silencing. The fact that RNAi can also direct methylation of histones adds further credence to the relationship between DNA methylation and histone modifications (Ronemus et al., 2005). Just like mRNA destined for translation may be affected by DNA hypo or hypermethylation, so also might miRNA expression be altered. Perturbation in the expression of these regulatory RNAs has been shown to occur as a result of DNA methylation in cancer cells (Saito et al., 2006).

3. Histone Modifications

DNA is packaged within the nucleus as chromatin. The basic unit of chromatin is the nucleosome, consisting of 147 base pairs wound around a protein octamer composed of two each of histones H2A, H2B, H3 and H4. However, the histone composition of nucleosomes is not static. Removal and replacement of histones occurs depending upon the biological process involving chromatin. These replacement histones vary in amino acid sequence and post-translational modifications compared to the standard four histone components of the nucleosome. The histone changes can have wide ranging effects such as inhibiting binding of specific transcription factors like NF κ B, involvement in DNA replication, interfering with transcription or activating transcription (Groth et al., 2007; Li et al., 2007).

Histone modifications likely play a role in a wide variety of biological processes such as cancer, muscular dystrophy (Avila et al., 2007) and memory (Fischer et al., 2007). Post translational modification of the histones produces conformational changes and alters their relationship with DNA. Although a wide variety of post-translational modifications can occur on these proteins, we will focus upon the most well understood alterations that affect gene expression: acetylation and methylation. Brief attention will also be paid to histone phosphorylation and ubiquitination.

3.1. Histone Acetylation

Histone acetylation is perhaps the best known and well described method of histone modification. Acetylation occurs primarily on lysine residues of histones H3 and H4, thereby neutralizing their basic charge. When histones are acetylated, the associated DNA becomes more accessible for transcription. Acetylation levels are controlled through the action of two sets of antagonistic proteins. The histone acetyltransferases (HAT) place the

acetyl group while histone deacetylases (HDAC) remove the groups. Unlike some of the other histone modifications such as methylation and phosphorylation, the HATs are presumably less specific in their acetylation targets. Some studies suggest that acetylation occurs in specific areas to promote the coordinated transcription of certain genes (Kurdistani et al., 2004). However, the transcriptional effects may also be dependent upon the degree to which the histones are acetylated and not specific targets of acetylation (Dion et al., 2005). Acetylation can also affect the relationship of histones to one another, further altering the chromatin structure, thereby increasing accessibility for additional proteins to bind to the DNA. Some of these recruited proteins participate in transcription while others may further modify the chromatin. Histone acetylation is by no means an isolated phenomenon. Instead, it works in conjunction with additional histone modifications to enact chromatin remodeling and support biological processes.

3.2. Histone Methylation

Histone methylation is another widely described histone modification that works in concert with histone acetylation. In contrast to acetylation, methylation occurs on either lysine or arginine residues. While lysine can receive only one acetyl group, it can receive up to three methyl groups. Multiple methylation of lysine greatly increases the number of possible histone modifications. To further complicate matters, methylation can either promote or repress transcription depending upon the location of methylation. It also plays an important role in X chromosome inactivation and also DNA repair (Lachner et al., 2002). The histone methyltransferases (HMTases) are a diverse group of enzymes that exhibit specificity to amino acid type and location (Shilatifard, 2006). A second set of enzymes, the protein arginine methyltransferases (PRMTs) are responsible for methylating arginine residues on the histones.

Both HMTases and PRMTs utilize S-adenosyl-L-methionine (SAM) as a methyl source (Shilatifard, 2006). Methylation of histone H3 on lysine 9 is associated with heterochromatin formation and transcriptional repression (Richards et al., 2002). Further evidence suggests that transcriptional repression is a complex but coordinated process involving HDACs and RNAi, allowing for complete gene silencing through methylation (Shilatifard, 2006; Volpe et al., 2002). Other HMTases place methyl groups on lysines located on H3 and H4 that promote transcription. Mono, di or trimethylation at these lysine residues play important roles in transcriptional activation (Santos-Rosa et al., 2002), with trimethylation often found at active promoter sites (Kim et al., 2005). Although no enzyme has been found that removes methyl groups from DNA, a group of proteins does exist to demethylate histones. There are two known enzymes responsible for removing methyl groups from histones; lysine specific demethylase (LSD1) and Jumonji C (JmjC) domain containing proteins. LSD1 is unique in that it can remove mono- or di-methyl groups but has no activity against tri methylated lysines (Shi et al., 2004). The JmjC domain enzymes (JHDM1, JHDM2, JMJD2) are comprised of proteins with activity against mono-, di- or trimethylated histones. Currently, no enzyme has been discovered with an arginine demethylating activity, leaving a noticeable gap in the histone code hypothesis for gene regulation. Peptidylarginine deiminase 4 (PAD4) can convert monomethyl arginine residues to citrulline (Cuthbert et al., 2004). Because the final product is citrulline and not the original arginine, PAD4 is not considered to have true demethylase activity such as LSD1 or the JmjC proteins. Like its lysine counterpart, arginine methylation is also involved in chromatin remodeling, transcriptional regulation and the recruitment of various other proteins involved in these two processes. However, unlike lysine, arginine can only receive either one or two methyl groups, which can be symmetrical or asymmetrical, and arginine methylation is most often associated with activation of transcription (Bannister et al., 2002).

The polycomb (PcG) and trithorax (trxG) proteins are two additional and important members of the chromatin remodeling process. These proteins act to form large complexes involved with regulating gene expression and participate in a wide variety of cellular functions such as gene imprinting, cell proliferation and cancer (Schuettengruber et al., 2007). Formation of these complexes involves requirement of histone modifying enzymes.

3.3. Other forms of histone modification

Histones can undergo a number of additional modifications such as proline isomerization, deimination, sumoylation, phosphorylation, ubiquitination and ADP-ribosylation (Kouzarides, 2007). Although a detailed discussion of all of these histone modifications falls outside the scope of this review, brief mention must be made of the roles phosphorylation and ubiquitination play. Histone phosphorylation appears to play an important role in cell cycle regulation (Oki et al., 2007). Evidence suggests that phosphorylation is not only important in regulating mitosis, meiosis and apoptosis but also may be involved in directing other histone modifications such as acetylation (Oki et al., 2007). Ubiquitination of lysine residues in histones has also been implicated in X chromosome inactivation and also polycomb silencing (Wang et al., 2004). Ubiquitination is also important in the regulation of, and sometimes is essential for, histone methylation (Wang et al., 2004; Wood et al., 2003). These findings provide further evidence of the dynamic process of histone modification involving a variety of different changes over a large genomic landscape.

4. Interactions between DNA methylation and Histone Modifications

While DNA methylation and histone modifications have been discussed separately, epigenetic regulation relies upon the interaction of the two. There is now emerging evidence that DNA methylation and histone modifications work in concert. Some recent work has shown that DNA methylation can promote histone phosphorylation (Monier et al., 2007). DNA methylation also leads to the recruitment of methyl-CpG-binding proteins (MeCP) (Robertson, 2005). These proteins in turn allow for the formation of repressive complexes of histone methyltransferases, histone deacetylases and other proteins such as sin3, causing further transcriptional repression (Hellebrekers et al., 2006; Nicolas et al., 2007). In this way, the transcriptionally repressive nature of DNA methylation is reinforced through further chromatin condensation that comes from histone modifications such as deacetylation. This reinforcing repressive relationship can also be initiated by histone modifications that then lead to DNA methylation. Other work has shown that DNA methylation may occur after histone deacetylation (Mutskov et al., 2004).

However, epigenetic processes need not only work together to silence gene expression, sometimes they may cooperate to induce gene expression. Recent evidence suggests that histone hyperacetylation induces DNA demethylation of promoter regions (Dong et al., 2007). This substantiates previous findings that have shown that treatment with inhibitors to both DNA methylation and histone deacetylation results in a synergistic increase in the expression of some genes (Buschdorf et al., 2004; Ghoshal et al., 2002). Further evidence suggests that histone methylation may also be part to the coordinated interaction between histone modifications and DNA methylation (Zhang et al., 2005).

5. Epigenetics in Development

Epigenetic mechanisms also play an indispensable role in gene expression during embryogenesis and development. A number of well written, comprehensive review articles focusing specifically on this topic are available (Dean et al., 2005; Meehan et al., 2005). A brief review of epigenetics in development is warranted to understand the potential for hearing related research. The lethality of the DNMT1 knock out speaks to the importance of

epigenetic regulation to embryogenesis. DNA methylation can lead to genomic imprinting, thereby silencing a specific parental allele. Perturbation of this process can lead to inappropriate gene expression and, as will be discussed in a further section, is well described in a number of diseases such as Angelman syndrome (Robertson, 2005).

Although there is no evidence of a DNA demethylating enzyme in fully developed humans, there is abundant evidence that active demethylation is part of embryogenesis and development. Primordial germ cells undergo a series of DNA demethylation events as they migrate through the hindgut into the genital ridge. The rapid and selective targeting of specific genomic areas for demethylation suggests that this process is enzymatically controlled, and may be mediated by the aforementioned 5-methylcytosine glycosylase activity. The first stage of DNA demethylation during development leaves previously imprinted genes intact, while the remaining DNA methylation is erased within 24 hours (Dean et al., 2005; Hajkova et al., 2002). The final product is a completely erased genome, void of all parental epigenetic information, allowing the new embryo to establish appropriate, gender specific DNA methylation patterns. Once complete DNA demethylation has occurred, the gamete genomes then undergo remethylation (Lucifero et al., 2004). The final product is a gamete that has erased its previous epigenetic information in favor of a new methylation code. The process is then recapitulated after fertilization and during embryogenesis. The previously reestablished gamete methylation patterns are removed and the developing embryo begins the process of genomic imprinting (Dean et al., 2005).

Inactivation of the X chromosome to achieve gene dosage compensation during female development highlights the roles of DNA methylation and histone modification role in embryogenesis. Initially, RNA specific to X inactivation coats the chromosome and histone modifications occur followed by DNA methylation (Chow et al., 2005; Kohlmaier et al., 2004). These changes place the chromosome into a transcriptionally unfavorable confirmation. Histone modifications are not limited to the X chromosome, but also play important roles in chromatin remodeling throughout development.

6. Epigenetics of Cancer

The role of epigenetics in cancer is an expansive topic far exceeding the scope of this article. However, a number of recent review articles have addressed this topic (Jones et al., 2007; Shames et al., 2007). Cancer pathogenesis can result from a wide range of epigenetic changes including histone changes and DNA methylation affecting a number of genes. Much of this work has focused upon malignancies unrelated to hearing. However, recent findings on the role of epigenetics in the pathogenesis of vestibular schwannomas have direct application to hearing related work.

Vestibular schwannomas (VS), a form of benign tumor of the Schwann cells in the auditory vestibule, can lead to sensory neural hearing loss. There is emerging evidence that epigenetics may play a role in VS (Gonzalez-Gomez et al., 2003). DNA methylation studies have revealed that methylation patterns of two tumor suppressor genes, CASP8 and RASSF1A, were associated with VS tumor size and hearing loss (Lassaletta et al., 2006). Additional work has demonstrated that the gene NF2 displays aberrant DNA methylation in VS compared to normal Schwann cells (Kino et al., 2001). Histone perturbations may also contribute to NF2 related VS. The histone deacetylase inhibitor FK228 has been shown to block the growth of NF2 deficient cells (Hirokawa et al., 2005).

7. Epigenetic modifying Pharmacologic Agents

A number of pharmacologic agents have been developed to affect epigenetic processes and lead to gene reactivation. These agents have been primarily used in cancer treatment with

the goal of manipulating gene expression. They have also found use is promoting expression of epigenetically regulated developmental genes (Tsuji-Takayama et al., 2004) or those involved with normal physiology such as the sodium iodide symporter (Furuya et al., 2004). However, there appears to be an absence of literature describing their use in hearing related research.

7.1. DNA methyltransferase inhibitors

Like the chromatin modifications themselves, these agents fall within the broad categories of DNA methylation inhibiting agents and histone modifying drugs. Those drugs which affect DNA methylation do so by inhibiting the DNA methyltransferases enzymes. This prevents DNA methylation of the daughter strand from occurring during DNA replication (maintenance methylation). However, it necessitates that cells be actively dividing. Decitabine (5-aza-2'-deoxycytidine, often abbreviated 5-aza-dC) and its sister drug Vidaza (5-azacytidine, abbreviated as 5-azaC) have been used to treat a number of different malignancies (Issa et al., 2005) and to influence biological processes such as angiogenesis (Hellebrekers et al., 2006). However, these drugs are limited by their toxicities and by their instabilities under physiologic conditions (Yoo et al., 2004). Another agent, Zebularine, has also been used in the treatment of cancer and has a lower side effect profile than other DNA methyltransferases inhibitors (Yoo et al., 2004). Zebularine, like all DNA methyltransferases inhibitors, is non-specific in its targeting of gene activation. After stopping treatment with these agents, DNA methylation can reoccur, with gene expression reverting back to its pre-treatment state.

7.2 Histone modifying agents

Histone modifying agents encompass a large and diverse group of compounds (Hellebrekers et al., 2007), consisting mainly of drugs that inhibit histone deacetylase. This group includes both novel histone deacetylase inhibitors (HDAC inhibitors) such as Trichostatin A as well as medications used in non epigenetic applications such as valproic acid. These pharmaceuticals, unlike the DNA methyltransferases inhibitors, are not dependent upon DNA replication for their activity. They therefore could be more useful tools in targeting those cells that do not undergo regular cell division. These compounds, like the DNA methyltransferases inhibitors, effect global histone acetylation levels and suffer from the same lack of genetic specificity as other epigenetic modifying agents.

Combination use of these two classes of agents addresses both the individual processes and their mutually reinforcing relationship (Gilbert et al., 2004). Affecting DNA methylation interferes with the mechanism by which histone deacetylases are recruited and further repress gene expression through histone hypoacetylation. Such pharmacologic strategies have been employed in the treatment of cancer (Cameron et al., 1999); however, their application to hearing research remains to be tested.

8. Epigenetics in Hearing Related Diseases

A review of hearing related epigenetic research reveals only a handful of studies. There appears to be scant data to demonstrate a role for DNA methylation and histone modifications in ear development or hearing loss. However, it seems highly unlikely that such epigenetic phenomena would be important in every system of the human body except for hearing. The review of hearing related epigenetic work will focus upon hearing related disease processes that exhibit or suggest an epigenetic influence. We will provide examples of specific genes that have a role in hearing and are candidates for epigenetic control as well as an examination of how epigenetic tools and modifying agents could be employed in hearing related research.

Detection of specific DNA mutations has been invaluable in understanding causes of human deafness. Despite this important work, many hearing related diseases have no known mutation; it is possible that many will never have an identifiable mutation. It therefore seems reasonable to postulate that epigenetics could play an important role in those hearing related diseases and syndromes that have no identifiable perturbation to the DNA sequence. Even in those diseases with known mutations the possibility exists that epigenetic modifications could be important to phenotypic differences. Changes in expression of the mutated gene could have second order consequences leading to chromatin remodeling and changes to a host of genes that would not have been directly affected by the originally mutated gene. This idea has been born out in studies examining phenotypic differences of monozygotic twins. In one study involving twins with oculo-oto-radial syndrome, one sibling presented with a much more severe phenotype than the other sibling. The authors posited a possible epigenetic mechanism for the differences in gene expression (Elcioglu et al., 1997).

The lack of studies indicating an epigenetic cause of hearing loss speaks to the potential difficulty of establishing this mechanism as the culpable biological process. DNA methylation studies often employ sodium bisulfite conversion to identify patterns of genomic DNA methylation. In this process, methylated cytosine is conserved as cytosine during the conversion process while unmethylated cytosine is chemically modified to become uracil. A number of PCR techniques can then be employed to determine the DNA methylation pattern. The wide number of possible DNA methylation target regions, in conjunction with the difficulty of PCR on converted DNA, makes screening for aberrant DNA methylation a fairly laborious process.

Determining changes in histone modifications is an even more difficult task. Given the number of possible amino acid targets on the histones, the variety of histone modifications, and the possibility that chromatin conformation is actually a combination of a number of different modifications, determining the exact histone perturbation may be challenging. Western blotting, immunoprecipitation, chromatin accessibility studies and a number of different techniques have been used to study histone changes. There is also an abundance of research in yeast and plant models that have been used to study histone modifications. However, their applicability to hearing research would appear limited. Despite these difficulties, there is evidence to support a role for epigenetic mechanisms in hearing loss. A summary of these findings is presented in Table 1.

8.1. Hearing Related Syndromes

Analysis of DNA mutations has been important in demonstrating the importance of epigenetic mechanisms to hearing. The biological origins of Rett syndrome involve a series of mutations in the methyl-CpG-binding protein 2 (MeCP2) located on the X chromosome (Buschdorf et al., 2004). The phenotype of this disease includes typical hand motion such as wringing, and a host of motor and neurologic deficits and hearing loss. Hearing screening is an important component in the evaluation for Rett syndrome. Analysis of the mutations responsible for this disease, many occurring at CpG sites, also highlights the previously discussed finding that CpG dinucleotides are mutational hot spots (Buschdorf et al., 2004). Given the known function of the MeCP proteins, it is not surprising that additional work has found changes in gene expression in the brains of animal models of Rett syndrome. Animal models have demonstrated that symptoms begin only upon the completion of brain development (Chen et al., 2001). Given the time of onset of this disease, in conjunction with the known function of MeCP binding proteins, some have postulated that symptoms begin as the neurons shift from developmental gene expression patterns to maintenance patterns. Loss of MeCP2, and the subsequent changes to chromatin structure, may affect the required shift in gene expression from the developmental process to the maintenance process. If MeCP2 is absent, proper gene silencing may not be able to take place as the methylated

DNA cannot recruit the additional biological machinery needed for histone deacetylation and other associated transcriptionally repressive histone chromatin modifications.

Mutational hot spots, previously described as being associated with DNA methylation, also play an important role in Stickler syndrome. Described in the mid 20th century, Stickler syndrome consists not only of well described ophthalmologic problems but has also been described to include hearing loss in a large number of patients (Stickler et al., 2001). Analysis of these patients has shown that those with hearing loss are more likely to have changes in the COL11A1 gene (Annunen et al., 1999). Additional work on this syndrome has demonstrated that a methylcytosine deamination induced mechanism is responsible for mutations in Stickler syndrome associated genes (Wilkin et al., 2000). In one such case, the mutations result in the introduction of a stop codon.

While perhaps best known as a disease affecting the musculature, facioscapulohumeral muscular dystrophy (FSHD1) also has hearing related manifestations. Sensorineural hearing loss has been reported in patients suffering from this disease and it is now understood that deafness is a central finding in this form of muscular dystrophy (Meyerson et al., 1984). Additional studies have demonstrated this hearing loss to be between 4,000 and 6,000 Hz (Brouwer et al., 1991). Although the exact mechanism for this hearing loss remains incompletely understood, the importance of epigenetics in the etiology of this dystrophy has been reported. While it has been shown that substantial deletions in the D4Z4 repeat array are associated with FSHD1 (van Geel et al., 2002), it appears that hypomethylation in the region of D4Z4 is a key event leading to the disease phenotype (van Overveld et al., 2003). It has been hypothesized that this hypomethylation could be affecting chromatin structure and expression of additional genes.

DNA hypomethylation is also most likely also responsible for the conductive hearing loss seen in immunodeficiency-centromeric instability-facial anomalies syndrome (ICF syndrome) (Braegger et al., 1991). As has been described previously in this review, methylation plays an important role in the DNA areas known as SINES and LINES by stabilizing these regions and preventing recombination events. In this disease, the pericentromeric DNA in chromosomes 1, 9, and 16 is almost completely unmethylated (Jeanpierre et al., 1993), leading to dramatic chromosomal rearrangements. This change in DNA methylation also causes a change in chromatin confirmation, thereby resulting in uncondensed chromatin (Stacey et al., 1995). These recombination events and aberrant chromatin confirmation have an obvious effect on gene expression.

Epigenetic changes, in the form of imprinting errors also account for the abnormalities in Beckwith-Wiedemann syndrome. This syndrome, often associated with a number of anatomical anomalies at birth, also can present with posterior helical pits and stapes fixation leading to hearing loss (Best, 1991; Daugbjerg et al., 1984). Studies have demonstrated that defects in the imprinting of the potassium channel KCNQ1OT1 gene are associated with this syndrome (Weksberg et al., 2002). This is consistent with previous work which has implicated other members of this gene family with hearing loss (Beisel et al., 2005; Rivas et al., 2005). Additional work has demonstrated a number of additional genes involved in this syndrome. Interestingly, changes in DNA methylation and imprinting have been implicated in these other genes (Murrell et al., 2004), leading to the speculation that all patients may share a disorder with DNA methylation imprinting.

Another hearing related illness, CHARGE syndrome, may have an epigenetic connection. The syndrome is characterized by ocular coloboma, choanal atresia, hearing loss, cranial nerve deficits, and involvement of the genitals. Recent work has focused upon mutations in CHD7, a chromodomain helicase DNA protein, as a cause of this syndrome (Lalani et al.,

2006). The exact interaction between CHD7 and histone modifications or DNA methylation remains to be completely described; however, work on the CHD related proteins CHD3 and CHD4 has demonstrated their importance in histone acetylation (Tong et al., 1998). It is therefore possible that CHD7 may also play a role in histone modifications. This raises the interesting possibility that CHARGE syndrome, and its associated hearing loss, may be caused in part by changes in chromatin remodeling, and histone acetylation specifically, due to the CHD7 mutation.

All of the examples presented here involve hearing loss in the context of greater, syndromic abnormalities that affected a host of organ systems. This finding is a testament to the importance of epigenetics in the developmental process as well as the normal physiologic and biological processes. Aberrations in normal DNA methylation or histone modification processes would likely cause expression changes to a large number of genes and hence would lead to a number of abnormalities. Unlike epigenetic perturbations in cancer, which might give rise to a clonal population of pathologic cells, developmental problems would be more wide spread, changing the molecular mechanisms of a wide variety of tissues and organs. This is not to say that isolated hearing loss could not result from abnormalities of epigenetic mechanisms. However, this would most likely necessitate either an isolated gene being affected or an event specific to the ear.

8.2. Hearing related cancers

Epigenetic regulation also plays a role in a malignancy that has ramifications for hearing research. While often associated with retinoblastoma, perturbation to the Rb gene may also have consequences for hearing in humans (Schocket et al., 2003). Studies involving knock out of the Rb gene in the inner ear have shown profound hearing loss resulting from degeneration of the organ of Corti (Sage et al., 2006). Although a comprehensive review of this gene falls outside the scope of this article, there is ample evidence to demonstrate that epigenetic mechanisms play an important role in Rb expression (Lohmann et al., 1997; Sakai et al., 1991). It is possible that DNA methylation changes to this gene could also be playing a role inside the organ of Corti, leading to hearing loss.

As has been previously discussed, a number of studies have pointed to DNA methylation as important to vestibular schwannoma pathology. These findings, in conjunction with other work examining histone changes in a schwannoma cell line (Marushige et al., 1995), suggest that epigenetic changes may play an important role in Schwann cell dysfunction and the pathogenesis of VS. Although not a true malignancy, it raises the possibility of employing epigenetic modifying compounds in its treatment. Originally designed as cancer treatments, these drugs could find use in slowing or even reversing VS growth. This also raises the interesting possibility that these compounds could be used to manipulate normal SC function. Functional hearing requires not only hair cells and the auditory nerve, but also upon the supporting cells such as SC. Controlling normal SC growth through epigenetic modifying agents could provide a powerful mechanism by which to manipulate a vital component of the hearing system.

8.3. Use of epigenetic modifying agents in hearing research

Despite the toxicity profile and instability of some of these epigenetic modifying agents, they remain a powerful arsenal that could be employed in the re-expression of epigenetically silenced genes involved with hearing. A simple screening of gene expression before and after treatment with these agents could help identify a role for epigenetics in inner ear development and normal physiology. To date, there appears to be an almost complete lack of hearing research utilizing these powerful epigenetic modifier compounds. Even though they are non-specific, the administration of these epigenetic modifying agents could be a

more facile method of manipulating gene expression in the inner ear compared to other techniques such as gene transfer or RNA inhibition. Their use in other fields offers exciting possibilities for application to hearing research, such as in reestablishing a functional auditory nerve after deafness. Recent work has demonstrated that neuronal differentiation can be manipulated through the administration of histone deacetylases (Balasubramanian et al., 2006; Siebzehnubel et al., 2007). This finding carries important implications in the struggle to regenerate a functioning auditory nerve after deafening. It is possible that histone deacetylases could be employed to promote the growth of a new auditory nerve in a deafened inner ear.

Better understanding of epigenetic mechanisms affecting hair cell physiology could also lead to the use of the drugs in the effort to regenerate hair cells. Analysis of cDNA libraries from the cochlea and yeast two-hybrid experiments has suggested a role for chromatin remodeling proteins in regulating gene expression (Hibino et al., 2003). Additional work has demonstrated changes in gene expression via changes in histone acetylation after aminoglycoside mediated hair cell damage (Jiang et al., 2006). The histone change corresponds to a decrease in the cell survival pathway. This raises the possibility that some of these histone modifications could perhaps be manipulated through histone modifying drug such as a HDAC inhibitor. Early use of these drugs could perhaps rescue some hair cells and allow for the preservation of some hearing.

Additional work with these compounds has shown the re-expression of epigenetically regulated embryonic cell markers. This opens the possibility that epigenetically regulated developmental processes could be recapitulated in a deafened adult in order to reconstitute the components of a functioning inner ear. The *sox2* gene is essential for inner ear development and has been shown to be re-expressed after treatment with a DNA methyltransferase inhibitor (Kiernan et al., 2005; Tsuji-Takayama et al., 2004). A number of additional genes expressed in embryonic stem cells such as *nanog* and *oct4* have also been showed to have increased expression in differentiated cells after treatment with 5-AzaC. Recapitulation of the developmental process through administration of these drugs could perhaps be an alternative to stem cell transplantation.

8.4. Epigenetic mechanisms involving hearing development

A number of other genes important in inner ear development and hearing have additional epigenetic connections that could be useful in fostering the connection between hearing research and epigenetics. Two of these genes are β -catenin and members of the wingless (WNT) family of genes. Both of these genes are important to hearing research. β -catenin, found in cell adherens junctions in combination with a number of different proteins, has been studied for its role in the development of auditory epithelium and the inner ear. Immunostaining has demonstrated β -catenin expression in the inner ear of developing rat embryos (Matsuda et al., 2000). Further work has shown β -catenin localization into the nucleus corresponding to a reduction in cellular proliferation of the auditory epithelium (Takebayashi et al., 2005). Work with the vestibular epithelium demonstrated changes in β -catenin expression after aminoglycoside damage. Supporting cells showed an increase in β -catenin while the epithelium had a loss of expression as the cells entered apoptosis (Kim et al., 2002). In addition to its role in epithelial development, there is also evidence suggesting a role for β -catenin in cholesteatomas (Naim et al., 2005). Aside from its importance in hearing, β -catenin also plays a critical role in chromatin remodeling and epigenetic changes. It interacts with a number of different chromatin remodeling proteins such as CRB/p300 (Bienz et al., 2003) to change chromatin structure. To date, there appears to be an absence of information linking β -catenin's chromatin remodeling function to its role in auditory epithelium.

β -catenin's actions are often in association with WNT proteins. The WNT family of proteins is found across a number of species and has been studied extensively for its role in development. Studies with WNT and pax2 have demonstrated WNT's importance in development of the otic placode (Ohyama et al., 2006; Riccomagno et al., 2005) while others have shown its importance in the chicken inner ear (Stevens et al., 2003). Like β -catenin, WNT plays an important role in chromatin remodeling. WNT appears to play a role in histone modifications, specifically histone methylation, associated with genes under its control (Willert et al., 2006). Aside from its role in chromatin changes, WNT is an important member in a number of different signaling pathways. Although work has taken place to explain some of these pathways in relationship to hearing, there again appears to be little focus upon WNT's chromatin remodeling properties. WNT related epigenetic phenomenon stands as a prime opportunity for explaining the role that chromatin remodeling has upon hearing.

8.5. Hearing, reactive oxygen species and epigenetics

Although hearing research contains little information on epigenetic mechanisms, much work has already taken place examining the role of reactive oxygen species (ROS) and free radicals on hair cells and the hearing process. Causes of ROS mediated hearing damage include administration of ototoxic drugs such as cisplatin and sound induced trauma. There are now emerging connections between epigenetics and ROS. The connection between epigenetic mechanisms of transcriptional silencing of genes important to ROS such as *MnSOD* has been firmly established (Hitchler et al., 2006). Increases in ROS can also effect glutathione levels which in turn can change SAM synthesis and hence DNA methylation patterns. There are additional ROS related mechanisms involving hydrogen peroxide that can lead to further changes of the chromatin structure (Hitchler et al., 2007). The connection between ROS and epigenetic mechanisms offers new insight into this well established mechanism of hearing loss. A number of different compounds and vitamins have been studied for their hearing protective properties, so it is also reasonable to expect that the evaluation of epigenetic modifying agents might offer new insight and possible protection against this form of hearing loss.

9. Conclusion

Epigenetics offers vast opportunities for those engaged in understanding hearing, the development of the ear and hearing loss. Studies of DNA methylation and histone modifications can easily be conducted using a variety of commercially available kits and well established protocols. Many epigenetic related experiments utilize common laboratory techniques familiar to most molecular biologists. Treatment with the epigenetic modifying agents offers new opportunities for manipulating gene expression. Epigenetics mechanisms, central to many other biological systems, deserve renewed attention in the field of hearing research.

List of Abbreviations Used in the Text

CpG	The DNA sequence "CG" where DNA methylation occurs
SINES	Short interspersed transposable elements
LINES	Long interspersed transposable elements
DNMT	DNA Methyltransferase
siRNA	Small interfering RNA
miRNA	Micro RNA

H	Histone
HAT	Histone acetyltransferases
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
PRMTs	Protein arginine methyltransferases
SAM	S-adenosyl-L-methionine
LSD1	Lysine specific demethylase
JmjC	Jumonji C – a demethylase enzyme
PAD4	Peptidylarginine deiminase 4
PcG	Polycomb group protein
trxG	Trithorax group protein
MeCP	Methyl-CpG-binding proteins
VS	Vestibular Schwannoma
SC	Schwann cell
NF2	Neurofibromatosis type 2
5-aza-dC	Decitabine – a methyltransferases inhibitor
5-azaC	Vidaza – a methyltransferases inhibitor
FSHD1	Facioscapulohumeral muscular dystrophy
ICF Syndrome	immunodeficiency-centromeric instability-facial anomalies syndrome

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Table 1

Previous studies have implicated epigenetic mechanisms in hearing loss.

Disease	Epigenetic Mechanism	Gene Involved	Reference
Vestibular Schwannoma	DNA Methylation	CASP8	Lassaletta et al., 2006
	Histone Acetylation	RASSF1A	Kino et al., 2001
		NF2	Hirokawa et al., 2005
Retinoblastoma	DNA methylation	Rb	Sage et al., 2006
			Lohmann et al., 1997
Rett Syndrome	DNA Methylation	MeCP2	Buschdorf et al., 2004
Oculo-oto-radial syndrome	Unknown	Unknown	Elcioglu et al 1997
Stickler Syndrome	Methylcytosine deamination	COL2A1 COL11A1	Wilkin et al., 2000
Facioscapulohumeral	DNA	D4Z4	van Overveld et al., 2003
Muscular dystrophy	Hypomethylation	repeat array	
Immunodeficiency-centromeric	DNA	DNMT3b	Braegger et al., 1991
instability-facial anomalies syndrome	Hypomethylation		Stacey et al., 1995
Beckwith-Wiedemann syndrome	Imprinting Error	KCNQ1OT1	Weksberg et al., 2002
			Murrell et al., 2004
CHARGE syndrome	Histone Acetylation?	CHD7?	Tong et al., 1998