Eur Arch Otorhinolaryngol (2013) 270:1823–1830
DOI 10.1007/s00405-012-2222-1

OTOLOGY

Histone methylation and acetylation indicates epigenetic change in the aged cochlea of mice

Ken-ichi Watanabe · Wilhelm Bloch

Received: 7 August 2012 / Accepted: 4 October 2012 / Published online: 19 October 2012

Abstract It is currently accepted that epigenetics plays an important role in normal genetics and differentiation, and its failure triggers various diseases such as cancer, aging, metabolic diseases, and abnormal differentiations. The typical mechanism involves the modification of histones and the methylation of DNA. In this study, we investigated the modification of histones in the aged cochlea of mice using immunohistochemistry. Eight mice [C57BL/6(B6)] at the age of 8 weeks (young group) and 132 weeks (aged group) were used. Cochleas were fixed with paraformaldehyde and then decalcified. Hematoxylin-eosin staining was performed for the morphological study using a light microscope. After removing paraffin, the sections were incubated with the primary antibody to acetyl-histone H3 Lys9 or dimethyl-histone H3 Lys9. Confocal scanning microscopy was performed for observation. The degeneration was severest in the spiral ganglion cells and the organ of Corti of the basal turn as determined by light microscopy. Acetylated histone H3 was detected in the spiral ganglion cells and the organ of Corti of the young group, but not in those of the aged group. Dimethylated histone H3 was detected in the spiral ganglion cells and the organ of Corti of the aged group, but not in those of the young group. Acetylation was switched to methylation during ageing. Histone modification is known to have a critical role in neuro-degeneration. Our findings suggest that epigenetic change participates in the process of presbycusis.

Keywords Epigenetics · Cochlea · Presbycusis · Modification of histone · Acetylation · Dimethylation

K. Watanabe (✉)
Department of Otorhinolaryngology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan
e-mail: watanabe@nms.ac.jp

K. Watanabe · W. Bloch
Institute für Kreislaufforschung und Sportmedizin, Deutsche Sporthochshule Köln, Am Sportpark Müngersdorf 6, 50933 Köln, Germany

Introduction

Waddington [1] originally defined that the adaptive response to an environmental stimulus induced the selection of a suitable genetically controlled reactivity in 1942. It is currently accepted that epigenetics plays an important role in normal genetics and differentiation, and its failure triggers various diseases such as cancer, aging, metabolic diseases, and abnormal differentiations [2–5]. The typical mechanism is recognized as heritable changes in gene expression without changes in DNA sequences, and involves the modification of histones and the methylation of DNA. Issa et al. [4] first reported that the methylation of estrogen receptor gene was detected in aged colorectal mucosa. It has also been observed that elderly monozygotic twin pairs exhibited differences in the distribution of DNA methylation and modification of histone compared with young monozygotic twin pairs [6]. Lee et al. [7] reported that histone modification controlled the expression of miRNAs in cellular senescence. Slattery et al. [8] showed that the histone deacetylase positively regulated the proliferation of avian inner ear cells. These reports suggest that epigenetics is associated with pathological conditions of aged inner ear.

Presbycusis is one of the common diseases encountered at ENT clinics. Elderly adults exhibit reduced hearing ability and speech discrimination. With the increase of elderly adults in the population, presbycusis has become a

major social problem. Hearing loss starts from high frequencies in bilateral ears, spreading to the middle and low frequencies, and is irreversible. It is presumed to be caused by genetic factors and other risk factors such as environmental factors, otological diseases, and free radicals [6, 9, 10]. Many researchers have described cochlear disturbances; however, few have described age-related epigenetic changes in the inner ear [8, 11]. In this study, we investigated the epigenetics in the aged cochlea of mice using immunohistochemistry.

Materials and methods

Eight mice (C57BL/6(B6)) weighing between 20 and 35 g were used. They were kept in a specific pathogen-free area. The animals were anesthetized adequately with 5 % (w/v) ketamine hydrochloride (50 mg/kg body weight) before all procedures. The animals were divided into two groups: (1) a young group at the age of 8 weeks ($n = 4$) and (2) an aged group at the age of 132 weeks ($n = 4$). The protocol used was in accordance with the guidelines for research involving animals.

Immunohistochemical examination

Animals in each group were sacrificed under anesthesia. The tissues were fixed with 4 % (w/v) paraformaldehyde. The temporal bones were immersed in the same fixative overnight. The specimens were embedded in paraffin after decalcification by incubation in a solution of 10 % EDTA for 2 days. Each specimen was sectioned into slices of 6 μm thick with a microtome (Leica, Bartles and Stout, Washington). Hematoxylin-eosin (HE) staining was performed for morphological study using a light microscope (Zeiss, EL-Einsatz, Germany). After removing the paraffin, the sections were immersed in 3 % H_2O_2 for 20 min, then

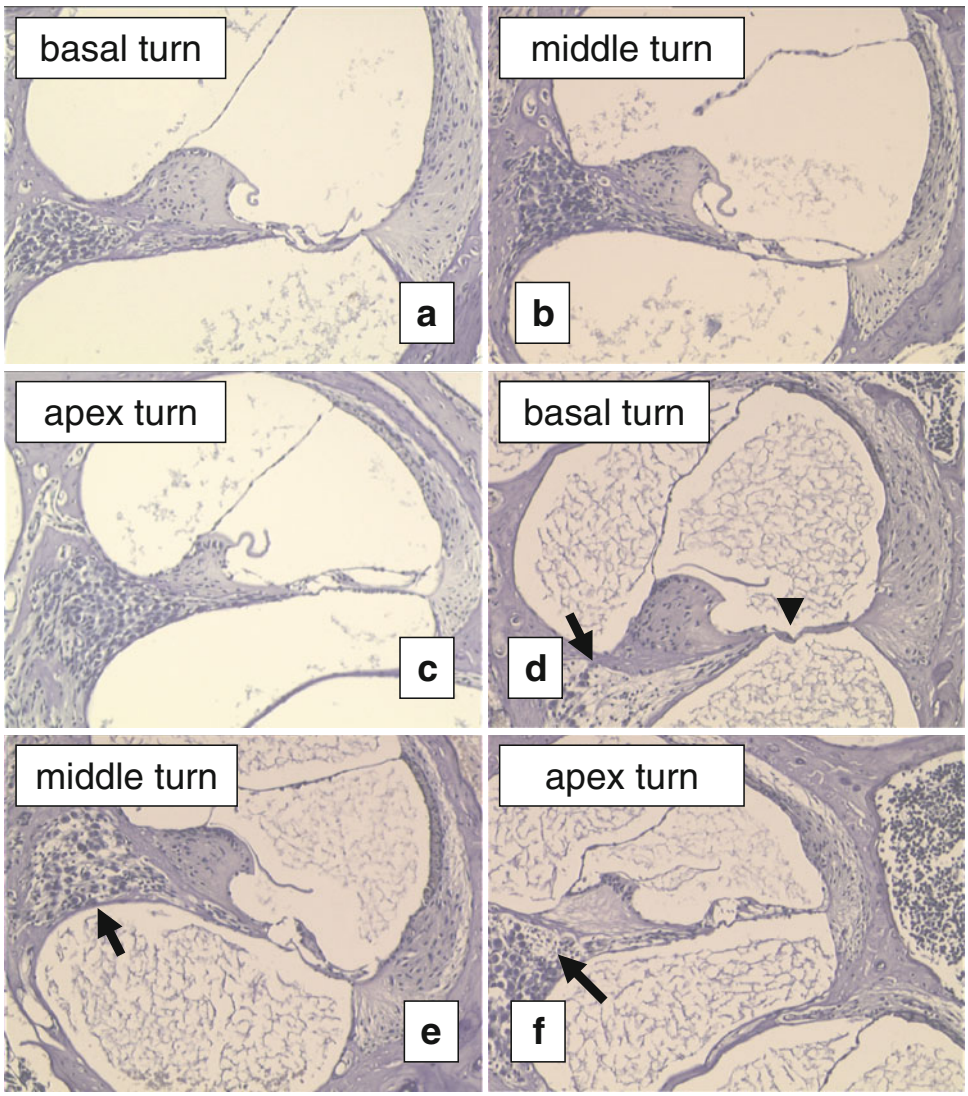

Fig. 1 Paraffin sections of the cochlea, 6 μm in thickness. HE staining. **a** Basal turn of the cochlea at the age of 8 weeks There was no morphological change. The structures of the spiral ganglion cells, organ of Corti, and the stria vascularis were maintained ×10. **b** Middle turn of the cochlea at the age of 8 weeks There was no morphological change ×10. **c** Apical turn of the cochlea at the age of 8 weeks There was no morphological change ×10. **d** Basal turn of the cochlea at the age of 132 weeks The organ of Corti (*arrowhead*) and the spiral ganglion cells (*arrow*) showed apparent degeneration. Most of the cells in the spiral ganglion cells had disappeared. The stria vascularis and the spiral ligament did not display changes ×10. **e** Middle turn of the cochlea at the age of 132 weeks. The spiral ganglion cells showed a slightly vacant area (*arrow*); however, the structure of the organ of Corti was maintained ×10. **f** Basal turn of the cochlea at the age of 132 weeks The spiral ganglion cells showed a vacant area (*arrow*). However, the degenerated area was narrower than that in the basal turn ×10

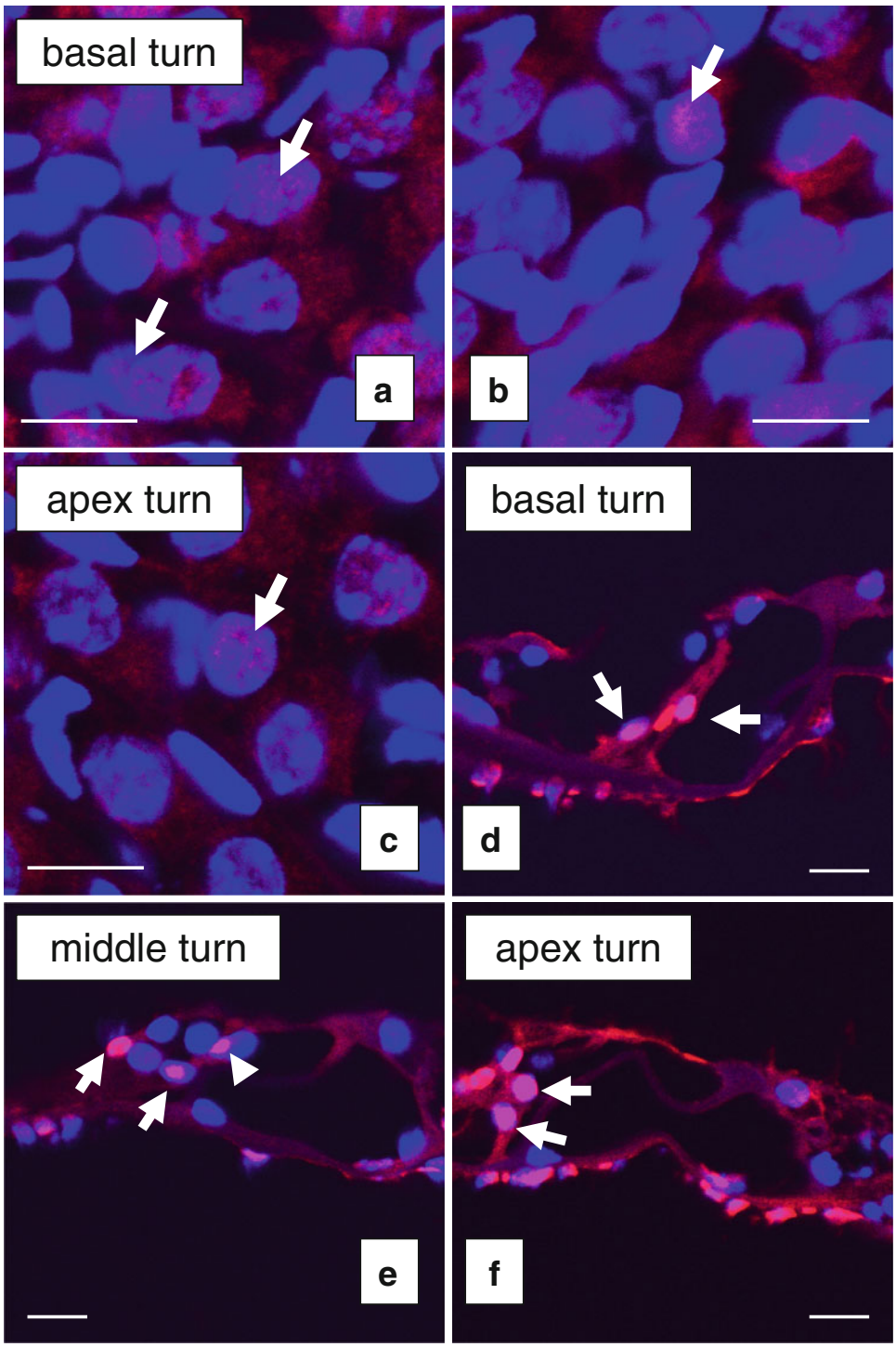

Fig. 2 Paraffin sections of the cochlea, 6 μm in thickness. Distribution of acetylated-H3 Lys9, at the age of 8 weeks; DNA stained by DAPI and DRAQ5 (*blue*), acetylated-H3 Lys9 stained by immuno-fluorescence Cy3-conjugated antibody (*red*). *Error bars* = 10 μm. **a** Spiral ganglion cells in the basal turn There was weak immunoreactivity for acetylated histone H3 within the nucleus (*arrow*). **b** Spiral ganglion cells in the middle turn There was weak immunoreactivity for acetylated histone H3 within the nucleus (*arrow*). **c** Spiral ganglion cells in the apical turn. There was weak immunoreactivity for acetylated histone H3 within the nucleus (*arrow*). **d** Organ of Corti in the basal turn The supporting cells showed positive staining for acetylated histone H3 (*arrow*). **e** Organ of Corti in the middle turn The supporting cells (*arrow*) and the outer hair cells (*arrowhead*) showed positive staining for acetylated histone H3. **f** Organ of Corti in the apical turn The supporting cells showed positive staining for acetylated histone H3 (*arrow*)

in Triton X for 10 min. Subsequently, they were incubated with the primary antibody to acetyl-histone H3 Lys9 at 1:1,000 dilution (rabbit polyclonal antibody, 9671, Cell Signaling, Germany) or dimethyl-histone H3 Lys9 at 1:1,000 dilution (rabbit polyclonal antibody, 6847, Cell Signaling, Germany) overnight at 4°. After rinsing with Tris buffer solution and normal goat serum, the tissues were incubated with the second antibody at 1:500 dilution (anti-rabbit, Cy3, Dako, Glostrup, Denmark). Then, they were immersed in DAPI at 1:50,000 dilution and DRAQ5 at 1:2,000 dilution (BOS-889-001-R200, Biostatus, UK). Confocal scanning microscopy was performed using a Zeiss microscope (Zeiss, Laser model LSM510, Germany). A laser was adjusted at wavelengths of 405 and 561 nm. The fluorescent images were captured by a program (Zen 2011, Ver.700285, Zeiss, Germany).

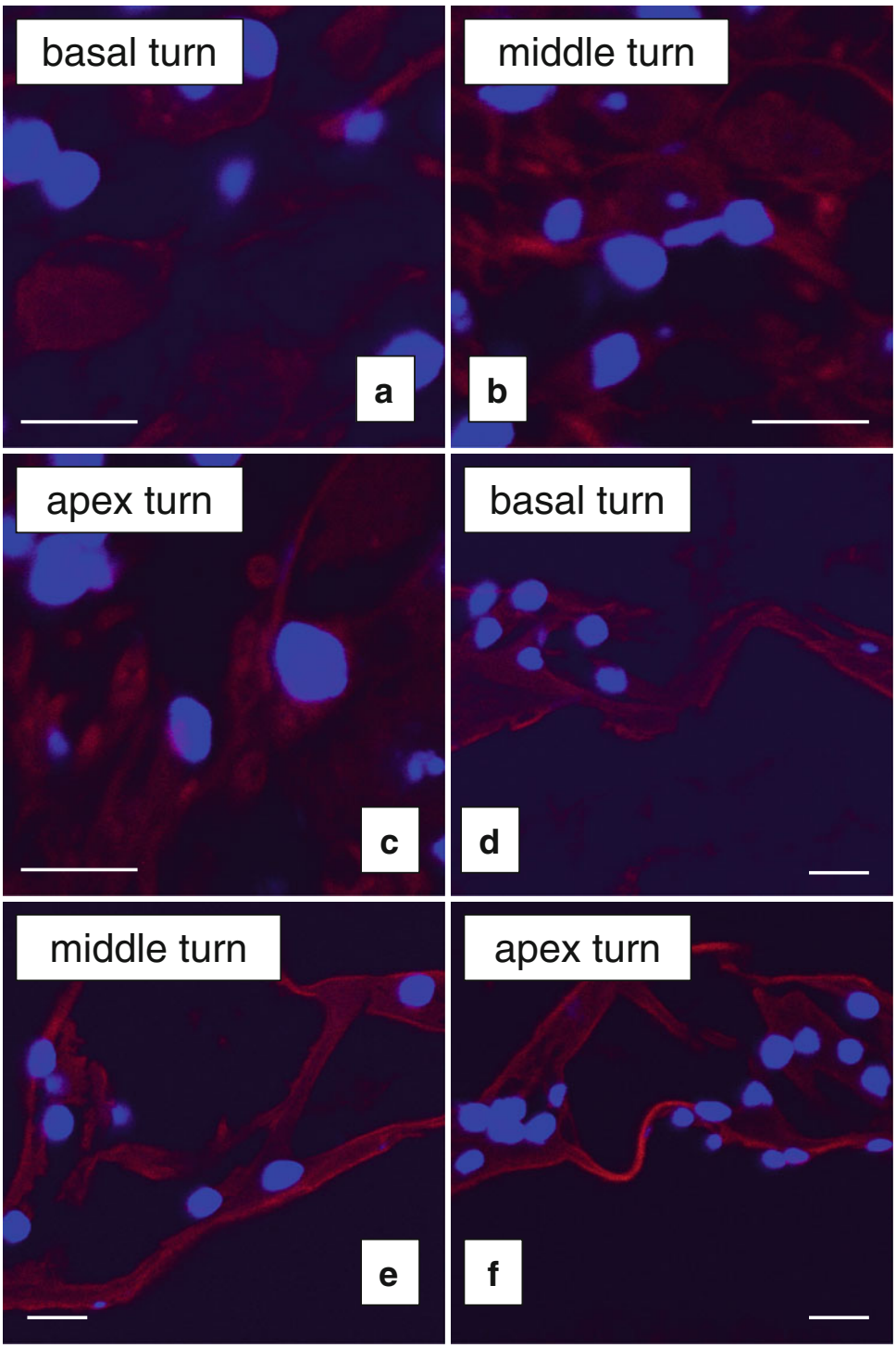

Fig. 3 Paraffin sections of the cochlea, 6 μm in thickness. Distribution of acetylated-H3 Lys9, at the age of 132 weeks; DNA stained by DAPI and DRAQ5 (*blue*), acetylated-H3 Lys9 stained by immuno-fluorescence Cy3-conjugated antibody (*red*). *Error bars* = 10 μm. **a** Spiral ganglion cells in the basal turn There was no immunoreactivity for acetylated histone H3. **b** Spiral ganglion cells in the middle turn There was no immunoreactivity for acetylated histone H3. **c** Spiral ganglion cells in the apical turn There was no immunoreactivity for acetylated histone H3. **d** Organ of Corti in the basal turn There was no immunoreactivity for acetylated histone H3. **e** Organ of Corti in the middle turn There was no immunoreactivity for acetylated histone H3. **f** Organ of Corti in the apical turn There was no immunoreactivity for acetylated histone H3

Results

Morphological changes in HE staining

In the young group, there were regular structures of cochlea in all turns (Fig. 1a–c). B6 mice showed degenerations of cochlea in the aged group, but not in the young group (Fig. 1). In the aged group, the organ of Corti in the basal turn exhibited an apparent structural change (Fig. 1d), whereas that in the middle and apical turn did not display any changes (Fig. 1e,f). There were no significant changes in the stria vascularis and the spiral ligaments (Fig. 1d–f). Some spiral ganglion cells disappeared in all turns (Fig. 1d–f). This degeneration was apparent in the basal turn (Fig. 1d).

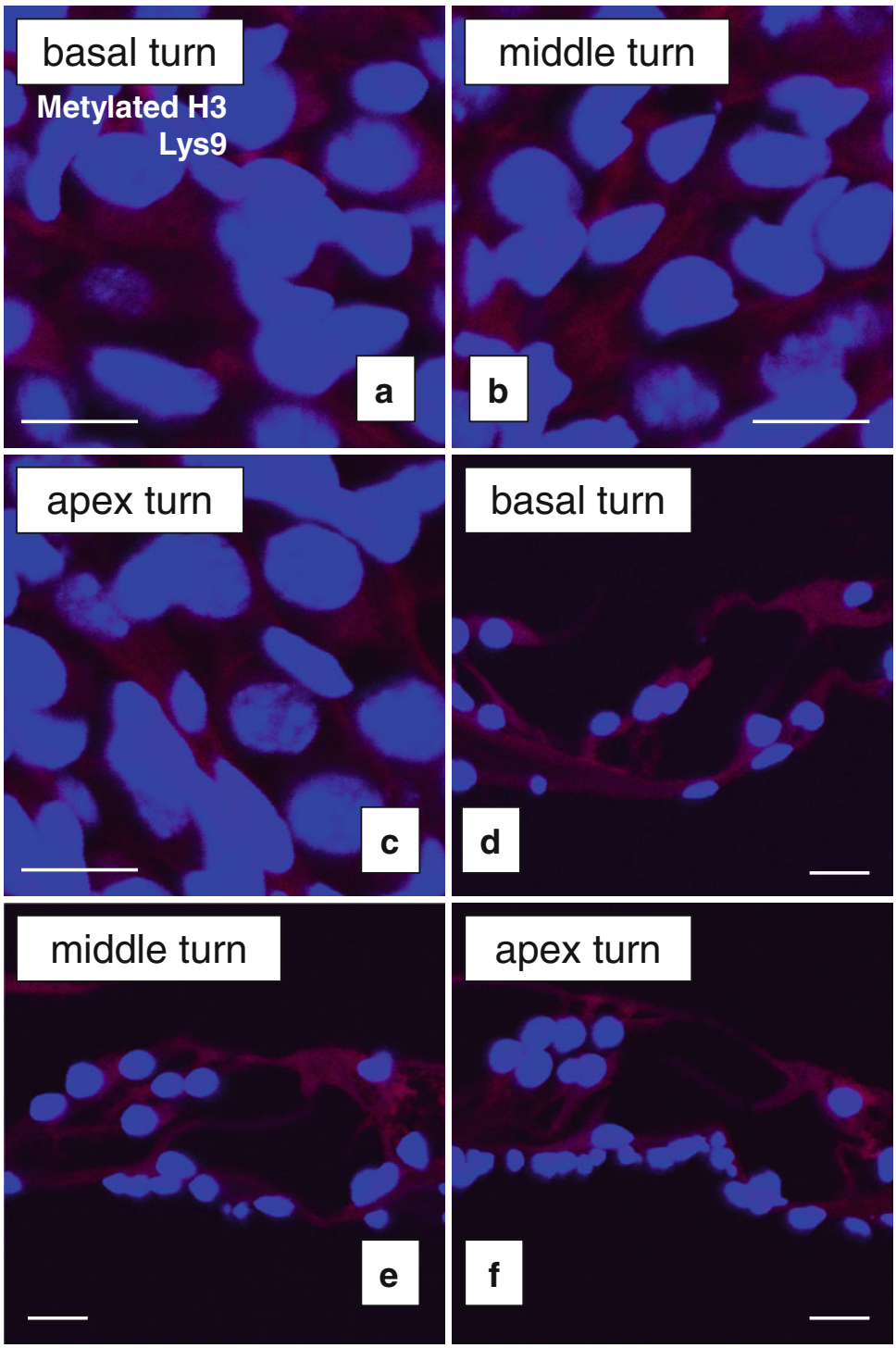

Fig. 4 Paraffin sections of the cochlea, 6 μm in thickness. Distribution of dimethylated-H3 Lys9, at the age of 8 weeks; DNA stained by DAPI and DRAQ5 (*blue*), acetylated-H3 Lys9 stained by immuno-fluorescence Cy3-conjugated antibody (*red*). *Error bars* = 10 μm. **a** Spiral ganglion cells in the basal turn There was no immunoreactivity for dimethylated histone H3. **b** Spiral ganglion cells in the middle turn There was no immunoreactivity for dimethylated histone H3. **c** Spiral ganglion cells in the apical turn There was no immunoreactivity for dimethylated histone H3. **d** Organ of Corti in the basal turn There was no immunoreactivity for dimethylated histone H3. **e** Organ of Corti in the middle turn There was no immunoreactivity for dimethylated histone H3. **f** Organ of Corti in the apical turn There was no immunoreactivity for dimethylated histone H3

Immunohistochemical expression of acetylated and dimethylated histone

The spiral ganglion cells in the young group showed immunoreactivity for acetylated histones. Some areas of the nucleus in all turns exhibited weak reactivity (Fig. 2a–c). The organ of Corti in all turns displayed immunoreactivity for acetylated histones (Fig. 2d–f). Supporting cells of the organ of Corti in all turns and outer hair cells in the middle turn showed positive staining (Fig. 2d–f).

The spiral ganglion cells in the aged group showed no immunoreactivity for acetylated histones (Fig. 3a–c). The organ of Corti in all turns displayed immunoreactivity for acetylated histones (Fig. 3d–f).

The spiral ganglion cells in the young group showed no immunoreactivity for dimethylated histones (Fig. 4a–c).

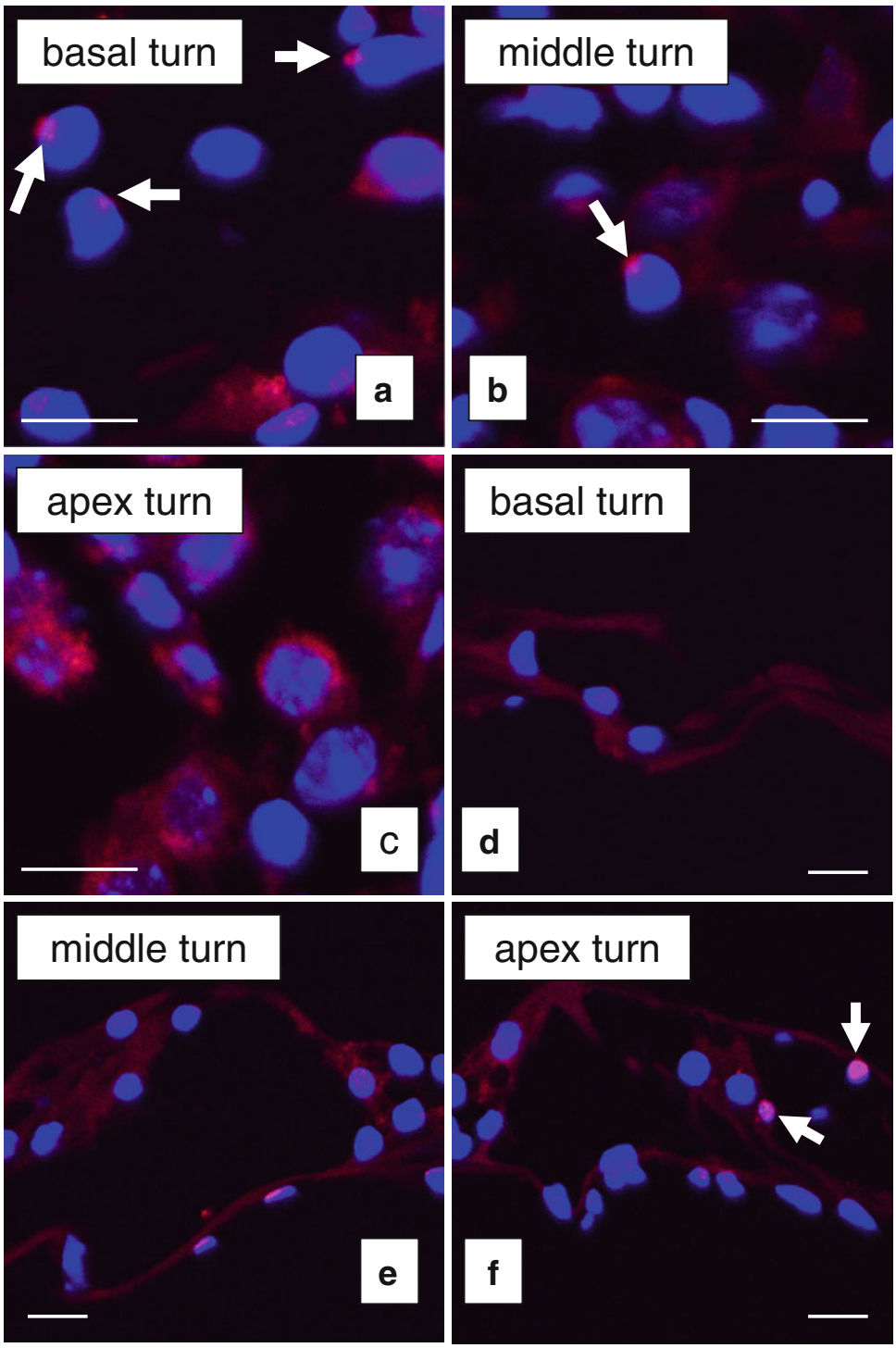

Fig. 5 Paraffin sections of the cochlea, 6 μm in thickness. Distribution of methylated-H3 Lys9, at the age of 8 weeks; DNA stained by DAPI and DRAQ5 (*blue*), dimethylated-H3 Lys9 stained by immunofluorescence Cy3-conjugated antibody (*red*). *Error bars* = 10 μm. **a** Spiral ganglion cells in the basal turn There was immunoreactivity for dimethylated histone H3 (*arrow*). **b** Spiral ganglion cells in the middle turn There was immunoreactivity for dimethylated histone H3 (*arrow*). **c** Spiral ganglion cells in the apical turn There was no immunoreactivity for dimethylated histone H3. **d** Organ of Corti in the basal turn There was no immunoreactivity for dimethylated histone H3. **e** Organ of Corti in the middle turn There was no immunoreactivity for dimethylated histone H3 **f** Organ of Corti in the apical turn The supporting cells showed positive staining for dimethylated histone H3 (*arrow*)

The organ of Corti in all turns displayed no immunoreactivity for dimethylated histones (Fig. 4d–f). The spiral ganglion cells in the aged group showed weak immunoreactivity for dimethylated histones (Fig. 5a,b). The organ of Corti in apex turn displayed immunoreactivity for acetylated histones (Fig. 5f).

Table 1 summarizes the results.

Discussion

In this study, we detected that acetylated histone H3 was expressed in the cochlea of the young group and its expression disappeared in the aged group. Dimethylated histone H3 was observed not in the young group, but in the aged group. These immunoreactivities were particularly

Table 1 Time-course changes of expression of acetylated H3 Lys9

Acetylated Histone 3 Lys 9				
	Young mice		Aged mice	
	SGC	Corti	SGC	Corti
Basal turn	±	+	−	−
Middle turn	+	+	−	−
Apex turn	±	+	−	−
Methylated histone 3 Lys 9				
	Young mice		Aged mice	
	SGC	Corti	SGC	Corti
Basal turn	−	−	+	−
Middle turn	−	−	+	−
Apex turn	−	−	±	+

Acetylated histone H3 was detected in the spiral ganglion cells and the organ of Corti of the young group but not in those of the aged group. Dimethylated histone H3 was detected in the spiral ganglion cells and the organ of Corti of the aged group but not in those of the young group. Acetylation was switched to methylation during ageing

SGC Spiral Ganglion Cells, *Corti* Organ of Corti, *SV* Stria Vascularis

detected in the spiral ganglion cells and the organ of Corti. Light microscopy also revealed that degeneration progressed in the spiral ganglion cells and the organ of Corti of the aged group. The auditory system transduces the sound pressure energy into electro-physiological signals [9]. These signals are conducted to the cochlear nerve and cochlear nuclei via spiral ganglion cells. The degeneration of the spiral ganglion cells and the organ of Corti cause the hearing disturbance. Epigenetic modifications are known to involve DNA methylation and the modification of histones in various age-related diseases [12]. DNA envelops the core, which is an octamer and is composed of histone H2A, H2B, H3, and H4. Histone proteins have amino-terminal tails that make them accessible to modification by acetylation and methylation [6, 13]. Histone modification may alter chromatin structure. The main acetylation sites of H3 include Lys 9, 14, 18 and 23. H3 Lys 4, 9 and 27 are sites of methylation [14]. Thus, we selected the histone H3 Lys 9 in this study.

We detected that acetylation was switched to methylation during aging. Gaikwad et al. [15] reported that increased acetylation of Histone H3 Lys 9 and decreased dimethylation were found in diabetic mice. They speculated that altered chromatin increased mRNA expression of cardiopathy related genes. Exposure to certain environmental factors, such as drugs, food, and toxins, during adulthood can affect epigenetic status [16, 17]. Neurotoxic peptide induces the acetylation of core histones, because hyperacetylation causes dopaminergic neuronal degeneration [18]. Hypo-acetylation of histone associates with the change of gene expression in Huntington's disease [19]. Although the degeneration of the spiral ganglion cells and organ of Corti was severe in the aged group, we could not detect acetylated histone in the cochlea. We could detect immunoreactivity for acetylated histone in the spiral ganglion cells and the organ of Corti in the young group. However, there was no immunoreactivity in the aged group. Additionally, morphological changes were observed in the spiral ganglion cells and the organ of Corti in the aged group. It is supposed that most of the cells that were positively stained were degenerated by aging, and there was no reactivity for acetylated histone. Expression of acetylated histone H3 may contribute to the degeneration of the ganglion cells and the organ of Corti. Renal failure is known to increase the dimethylation of histone H3 [15]. Dimethylated histone H3, which was observed in the aged cochlea, is considered to be a late phase of aging. The modified histone acetylation and dimethylation associated with DNA methylation leads to different gene expression [20].

Schuknecht [21] first classified the pathology of presbycusis into four categories. The first type, sensory presbycusis, is characterized by atrophy of the organ of Corti in the basal turn. The second type, neural presbycusis, is associated with loss of spiral ganglion cells. The third type, metabolic presbycusis, is due to the atrophic change of stria vascularis. The fourth type, mechanical presbycusis, is caused by the stiffness of basilar membrane. Nelson and Hinojosa [22] also reported that pathological changes were observed in the stria vascularis, spiral ganglion cells, and inner and outer hair cells of human temporal bones. B6 mouse is known to undergo degeneration in the cochlea from the basal turn to the apical turn [23]. This pattern is similar to human presbycusis. The morphological change shows hair cell loss and degeneration of spiral ganglion cells [24, 25]. We detected the degeneration of organ of Corti cells and spiral ganglion cells in the cochlear basal turn of the aged group. These changes are not contradictory to previous reports.

In conclusion, acetylated histone H3 was detected in the spiral ganglion cells and the organ of Corti of young cochlea, but not in those of aged cochlea. Dimethylated histone H3 was detected in aged group, but not in the young group. The degeneration was severest in the spiral ganglion cells and the organ of Corti of the basal turn as determined by light microscopy. Histone modification is known to have a critical role in neuro-degeneration.

Acknowledgments The authors thank Prof. Olaf Michel for his assistance.

References

1. Waddington CH (1942) Canaliyation of development and the inheritance of acquired characters. Nature 3811:563–565
2. Ushijima T, Okochi-Takeda E (2005) Aberrant methylations in cancer cells: where do they come from? Cancer Sci 96:206–211
3. Christensen BC, Marsit CJ (2011) Epigenomics in environmental health. Front Genet 84:1–10
4. Issa JPJ, Ottaviano YL, Celano P, Hamilton SR, Davidson NE, Baylin SB (1994) Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. Nat Genet 7:536–540
5. Jones PA, Baylin SB (2007) The epigenomics of cancer. Cell 23:683–692
6. Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML et al (2005) Epigenetic differences arise during the lifetime of monozygotic twins. PNAS 26:10604–10609
7. Lee S, Jung JW, Park SB, Roh K, Lee SY, Kim JH, Kang SK, Kang KS (2011) Histone deacerylase regulates high mobility group A2-targeting microRNAs in human cord blood-derived multipotent stem cell aging. Cell Mol Life Sci 68:325–336
8. Slattery EL, Speck JD, Warchol ME (2009) Epigenetic influences on sensory regeneration: histone deacerylases regulate supporting cell proliferation in the avian utricle. JARO 10:342–353
9. Huang Q, Tang J (2010) Age-related haring loss or presbycusis. Eur Arch Otorhinolaryngol 267:1179–1191
10. Staecker H, Zheng GY, Van De Water TR (2001) Oxidative stress in aging in the C57B16/J mouse cochlea. Acta Otolaryngol 121:666–672
11. Friedman LM, Avraham KB (2009) MicroRNAs and epigenetic regulation in the mammalian inner ear: implications for deafness. Mamm Genome 20:581–603
12. Rodriguez-Rodero S, Fernandez-Morera JL, Fernandez AF, Menendez-Torre E, Fraga MF (2010) Epigenetic regulation of aging. Discov Med 10:225–233
13. Kouzarides T (2007) Chromatin modifications and their function. Cell 128:693–705
14. Strahl BD, Allis CD (2000) The language of covalent histone modifications. Nature 403:41–45
15. Gaikwad AB, Sayyed SG, Lichtenkert J, Tikoo K, Anders HJ (2010) Renal failure increased cardiac histone H3 acetylation, dimethylation, and phosphorylation and the induction of cadiomyopathy-related genes in type 2 diabetes. Am J Pathol 176:1079–1083
16. Song C, Kanthasamy A, Anantharam V, Sun F, Kanthasamy AG (2010) Environmental neurotoxic pesticide increases histone acetylation tos promote apoptosis in dopaminergic neuronal cells: relevance to epigenetic mechanisms of neurodegeneration. Mol Pharmacol 77:621–632
17. Aguilera O, Fernandez AF, Munoz A, Fraga MF (2010) Epigenetics and environment: a complex relationship. J Appl Physiol 109:243–251
18. Garcia SN, Pereira-Smith O (2008) MRGing chromatin dynamics and cellular senescence. Cell Biochem Biophys 50:133–141
19. Sandri-Vakili G, Bouzou B, Benn CL, Kim WO, Chavia P, Overland RP, Glajch KE, Xia E, Qiu Z, Hersch SM, Clark TW, Yohrling GJ, Cha JHL (2007) Histone associated with down-regulated genes are hypo-acetylated in Huntingron's disease models. Hum Mol Genet 16:1293–1306
20. Clayton AL, Rose S, Barratt MJ, Mahadevan LC (2000) Phosphoacetylation of histone H3 on *c-fos*- and *c-jun*-associated nucleosomes upon gene activation. EMBO J 19:3714–3726
21. Schuknecht HF (1964) Further observations on the pathology of presbycusis. Arch Otolayngol 80:369–382
22. Nelson EG, Hinojosa R (2006) Presbycusis: a human temporal bone study of individuals with downward sloping audiometric patterns of hearing loss and review of the literature. Laryngoscope 116:1–12
23. Henry KR, Chole RA (1980) Genotypic differences in behavioral, physiological and anatomical expressions of age-related hearing loss in the laboratory mouse. Audiology 19:369–383
24. Kazee AM, Han LY, Spongr VP, Walton JP, Salvi RJ, Flood DG (1995) Synaptic loss in the central nucleus of the inferior colliculus correlates with sensorineural hearing loss in the C57BL/6 mouse model of presbycusis. Hear Res 89:109–120
25. Keithley EM, Canto C, Zheng QY, Fischel-Ghodsian N, Johnson KR (2004) Age-related hearing loss and *ahl* locus in mice. Hear Res 188:21–28