Epigenetics of Aging

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Aging represents the multifactorial decline in physiological function of every living organism. Over the past decades, several hallmarks of aging have been defined, including epigenetic deregulation. Indeed, multiple epigenetic events were found altered across different species during aging. Epigenetic changes directly contributing to aging and aging-related diseases include the accumulation of histone variants, changes in chromatin accessibility, loss of histones and heterochromatin, aberrant histone modifications, and deregulated expression/activity of miRNAs. As a consequence, cellular processes are affected, which results in the development or progression of several human pathologies, including cancer, diabetes, osteoporosis, and neurodegenerative disorders.

Keywords: Epigenetics ; Histones ; Histone Modifications ; Aging ; Aging-associated Diseases ; Diabetes ; CDKN2A ; Osteoporosis ; Gene Expression

1. Introduction

Aging is a multifactorial biological process of declining physiological functions increasing the susceptibility to aging-related chronic diseases, such as cancer, metabolic, cardiovascular, musculoskeletal, as well as neurodegenerative diseases [1]. Numerous studies have focused on the decipherment of the hallmarks of aging in order to identify potential therapeutic targets to mitigate the aging process. Hallmarks of aging include stem cell exhaustion, altered intercellular communication, senescence, genomic instability, and epigenetic deregulation [2].

Epigenetics refers to reversible heritable mechanisms, which can affect gene expression without underlying changes in DNA sequences, but rather via chromatin modifications. Eukaryotic chromatin is a highly condensed structure containing repeating structural subunits, the nucleosomes. Each nucleosome consists of a histone octamer assembled of two copies of each histone (H2A, H2B, H3, and H4, as well as histone variants, such as macroH2A, H3.3 and H2A.Z), wrapped around by 147 base pairs of DNA [3][4]. Each core histone possesses histone-fold domains serving for the interaction of the histones and N-terminal histone-tails. These tails can be subjected to post-translational modifications, which frequently affect gene expression. These modifications include, for instance, histone acetylation, methylation, phosphorylation and ubiquitination [5].

Epigenetics is a rapidly evolving research field and there is a profound interest in therapies targeting epigenetic as well as aging-related processes. In this study, we focus on aging-associated epigenetic regulatory mechanisms and highlight their implications in aging-related diseases.

2. Epigenetics of Aging and Aging-Related Diseases

2.1. Epigenetic Changes in Aging

2.1.1. Histone and Heterochromatin Loss

The DNA is organized into complex three-dimensional structures; however, for gene transcription, the DNA sequence has to be accessible to key regulators, such as transcription factors and RNA polymerases. Besides chromatin remodeling, which results in the rearrangement of chromatin structures, the global number of histones defines DNA accessibility [6]. In fact, the loss of histones during cellular aging is one of the key observations from simple eukaryotic models, including yeast, to mice and humans. In a micrococcal nuclease-DNA sequencing (MNase-seq) approach detecting protein-unbound DNA regions in young and old *Saccharomyces cerevisiae*, a nucleosome loss of approximately 50% was detected. As a consequence, global transcription levels were highly upregulated in aged cells [7]. Similarly, aging human fibroblasts grown in vitro showed a replication-associated reduction in histone biosynthesis and quiescent satellite cells displayed decreased histone expression [8][9].

Reduced synthesis of histones together with changes in chromatin structure leads to a global loss of constitutive heterochromatin, one of the earliest models associated with aging. Heterochromatin loss, the transition from highly condensed to tightly packed chromatin structures, during aging has been observed across many species. As a consequence, modified chromatin architecture, the de-repression of silenced genes and global gene expression changes can occur [10].

2.1.2. Histone Variants

Besides the loss of histones, the exchange of canonical histones (H2A, H2B, H3, and H4) with histone variants was observed in aging organisms. These histone variants display distinct primary sequence and properties compared to canonical histones, thereby regulating gene transcription programs. Various aging-related studies evaluating histone variants in murine, primate and human cells implicate a high enrichment of macroH2A (mH2A), H3.3 and H2A.Z. In general, the incorporation of histone variants into the chromatin can be replication-coupled or replication-independent. The replication-coupled process results in a genome-wide incorporation of new nucleosomes into gaps between pre-existing nucleosomes. In contrast, the replication-independent addition of nucleosomes or subunits occurs locally. Thus, during the replication-independent process, histone variants can replace canonical histones, thereby potentially altering gene expression programs [11][12]. The mH2A isoforms are characterized by the presence of a C-terminal 30 kDa non-histone macro domain [13][14], and were shown to facilitate the activation of transcription factors during differentiation processes [13] and the prevention of the reactivation of pluripotency-associated genes [15]. Notably, human fibroblasts undergoing replicative senescence in vitro as well as several tissues isolated from aging mice and primates displayed an enrichment in mH2A levels [16].

Another example is the H3 variant H3.3, which differs from the canonical form by only four amino acids. It was shown to be incorporated only in a replication-independent manner and to be enriched in transcriptionally active chromatin regions. Recent aging studies in mice revealed that H3.3 accumulates in various tissues during aging and that the canonical isoforms have been almost completely replaced by this histone variant by the age of 18 months [17]. Moreover, H3.3 was linked to aging processes in *Caenorhabditis elegans*. Here, the deletion of H3.3 resulted in profound transcription changes of longevity-associated genes and in decreased survival [18]. Similar results were found when analyzing postmortem human brains where H3.3 levels gradually increased over the first decade of life. In individuals who were 14 to 72 years old, H3.3 amounts remained stable [19].

2.1.3. DNA Methylation

Besides histone methylation, DNA can be directly methylated through the covalent linkage of a methyl group to the fifth position of the cytosine ring to generate 5-methylcytosine (5mC). This modification is mainly present in DNA regions rich in cytosine-phospho-guanine (CpG) dinucleotides. While there is extensive evidence that DNA methylation at promoter regions is associated with gene silencing, the decipherment of the function of gene body methylation is still ongoing [20][21][22]. The repression of transcription due to covalent addition of methyl groups onto the DNA can be mediated by interfering with the site-specific binding of transcription factors or by the recruitment of methyl-CpG-binding domain proteins [23][24]. The transfer of this heritable epigenetic mark is mediated by DNA methyltransferases (DNMTs) including DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L. While DNMT1 has a maintenance function, the de novo establishment of DNA methylation is exerted by DNMT3A and DNMT3B alone or in a complex with DNMT3L [25][26].

The conversion of 5mC to the unmodified state is thought to be mediated in an “active”, enzyme-dependent or in a “passive” demethylation process. The family of Ten Eleven Translocation (TET) proteins, TET1, TET2, and TET3, are able to erase DNA methylation in an “active” stepwise process [27][28]. These factors catalyze the oxidation of 5-methylcytosine (5mC) to the intermediates 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). After the recognition of 5fC and 5caC by the Thymine DNA Glycosylase (TDG), the oxidized cytosine base is excised. Finally, this abasic site will be recognized and replaced by an unmodified cytosine residue by Base Excision Repair (BER). During “passive” DNA demethylation, 5-methylcytosine is diluted in a replication-dependent process during cell division [29][30]. Interestingly, it has been demonstrated that a high abundance of 5hmC represses DNMT1 activity by 60-fold, suggesting a role of TET-mediated induction of “passive” demethylation [31].

While methylation-associated control of gene expression pattern is essential for mammalian development and further cellular processes, it was thought to be dispensable in several organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* [32]. Recent studies describe the methylation of exocyclic NH2 groups at the sixth position of the purine ring in adenines (6 mA) in *C. elegans*, which is thought to be regulated through the DNA demethylase NMAD-1 and the DNA methyltransferase DAMT1 [33]. In addition, there is emerging knowledge on species- and life cycle-dependent 5mC levels among the genomes of the members of genus *Drosophila* [34].

While it has been known for several decades that DNA methylation can regulate gene expression patterns, biological consequences are still not fully investigated. Generally, it has been described that CpGs at promoter regions display hypermethylation while other CpGs undergo hypomethylation during aging [35]. Remarkably, two large-scale studies significantly contributed to the understanding of the relevance of DNA methylation pattern during aging. The authors identified 353 and 71 CpG sites, respectively [36][37], which were differentially methylated during aging and, therefore, can serve as reliable age predictors in human tissues. In fact, this “epigenetic clock” displays a robust correlation to age (r = 0.96 and r = 0.91, respectively) with minor deviations from the calendar age of analyzed individuals (3.6 and 4.9 years, respectively). In a comparative study evaluating the robustness of biological age predictors (i.e., epigenetic clock, telomere length, composite biomarker predictors, as well as transcriptome-, proteome- and metabolome-based predictors), the epigenetic clock was suggested to be the most reliable readout. However, further confirmatory studies will be needed to additionally evaluate the predictive values of these biological hallmarks of aging [38].

2.2. Epigenetic Changes in Aging-Related Diseases

Epigenetic discoveries helped to lay the foundation for a deeper perception of multiple diseases. For instance, epigenetic events can contribute to the “hallmarks of cancer” (i.e., chromatin structure affecting cellular identity or methylation patterns leading to evasion from apoptosis), having led to a revised “hallmark” definition [39][40]. Here, we summarize the latest epigenetic discoveries in a selected range of medical conditions with a focus on certain cancer entities, inflammation, musculoskeletal disorders, neurodegenerative diseases, and nutritional diseases.

2.2.1. Cancer

DNA methylation patterns and miRNAs can influence chromatin state regulation [41][42]. In cancer development, the degenerated cell may unrestrictedly proliferate as a consequence of DNA hypermethylation or deregulation of epigenetic modifiers. Methylation of the promotor region of the tumor suppressor genes, for instance *VHL*, was associated with angiogenesis, leading to an enhanced supply for the tumor environment. Cell death was shown to be impaired by epigenetic modification of apoptotic or cell cycle key players including CDKN2A, hypermethylation of which leads to a loss-of-function gene in numerous cancers [41][42][43]. Here, we focus on two entities to exemplarily demonstrate age-related shifts and deregulations associated with leukemia and colorectal cancer.

2.2.2. Inflammation

It is broadly accepted that inflammation is a common event during aging, referred to as “inflamm-aging” [44]. This process is a underlying condition of several diseases such as sarcopenia, osteoarthritis, and cancer [45]. A hallmark of these processes is an increase in Tumor Necrosis Factor alpha (TNFα) levels. DNA methylation and histone acetylation modify the promoter region of TNFα [46]. The TNFα gene itself does not contain a classical CpG island, however, its promoter and first exon were described to be rich in CpG sequences [46]. Accordingly, methylation on these gene regions has been described to negatively regulate TNFα expression levels [47]. Wang et al. demonstrated in porcine spleens by bisulfite sequencing PCR and qPCR that the TNFα promoter region was increasingly methylated with age, correlating with decreased mRNA expression [48].

Similarly, NF-κB mediates acute, as well as chronic inflammation, and is proposed as one of the key regulators of aging. Via its transcriptional activity, the NF-κB family induces the expression of cytokines and genes associated with apoptosis and senescence as described elsewhere [49]. NF-κB levels can be regulated by various epigenetic mechanisms including the acetylation of histone H3 via the H3 lysine 4 methyltransferase SET7/9 [50] which represents a potential targeting strategy [47]. The link to aging has been validated in the skin where C57BL/6 mice exposed to UVB light for 16 days displayed accelerated aging of the skin via NF-κB activation through the mTORC2 pathway. The post-translational modification of the p65 member of NF-κB at Ser536 enhanced NF-κB activity via increased DNA binding activity. The same phenomenon was detected in physiological aged skin of these mice, demonstrating an accelerated inflammatory status in physiological as pathological (skin) aging [51][52].

Another epigenetic modification, which has been demonstrated to regulate NF-κB activity during inflammation, is the monoubiquitination at lysine 120 of histone H2B (H2Bub1). This monoubiquitination is performed by the RNF20/RNF40 E3 ligase complex and leads to increased chromatin accessibility. This results in eased passage of RNA Polymerase II and highly active transcriptional elongation [53]. Recently, it has been demonstrated that the monoubiquitination of histone H2B regulates NF-κB signaling in intestinal inflammation. However, the function of H2Bub1 in animal models for colitis remains inconclusive [54][55].

In general, the transformation from chronic inflammation to cancer can be promoted via DNA methylation, histone modifications, chromatin remodeling and noncoding RNA regulation, upon which the most important pathways are NF-κB- and STAT3-related [56][57]. The phosphorylation of Tyr705, Ser727, and Ser727 are known to positively activate transcriptional activity of STAT3 [57][58]. One downstream target of this signaling pathway is interleukin-6, which has been shown to be repressed via treatment with the DNMT1 inhibitor 5-azadeoxycytidine (5-AzaC) [56]. MicroRNAs are further key regulators of inflammatory responses and inflamed tissues are characterized by downregulation of TET gene expression due to the upregulation of TET-targeting miRNAs (e.g., MiR20a, MiR26B, MiR29C, Let-7 microRNA) [59][60].

Interestingly, the “epigenetic clock” concept of Horvath, has been demonstrated to be very accurate when methylation levels of CpG sites from white blood cells, the central regulators of immune response, were used [61][62][63]. These methylation levels were even able to predict mortality [64]. Despite a loss of T-cell diversity in old age, an exhausted/senescent $CD8^+$ T cell population increases with age, possibly giving rise to associated diseases [65][66][67][68][69].

Excessive inflammatory response, immunosenescence, and autoimmunity outline the other detrimental side of the inflammatory spectrum. The identification of hypomethylated apoptosis-related genes in naïve $CD4^+$ T cells led to the definition of an evolving autoimmune epigenotype [70][71][72]. Accordingly, in chronic nonbacterial osteomyelitis, a reduced expression of immunoregulatory cytokines (IL-10, IL-19) was centrally involved. The authors demonstrated in monocytes from chronic recurrent multifocal osteomyelitis patients that an altered SP1 activation negatively affected *IL10* and *IL19* expression. Mechanistically, the reduced phosphorylation of histone 3 serine 10 (H3S10P) and impaired SP1 phosphorylation at the *IL10* and *IL19* promoter regions impaired *IL10* expression. This causes an imbalance towards proinflammatory cytokines (compared to anti-inflammatory IL-10 and IL-19), leading to inflammatory bone loss [73]. Similar reductions of H3S1p levels have been identified in hippocampi of aged mice by Wu et al. [74], which were associated with the inflammation-related decline in spatial learning and memory.

2.2.3. Osteoporosis

Osteoporosis is tightly linked to aging via epigenetic changes in mesenchymal stem cells (MSCs) [75]. Physiologically, the Osterix promoter was shown to entail enriched levels of H3Ac/H3K4me3 and reduced levels of H3K9me3/H3K27me3, inducing the differentiation of MSCs into osteoblasts to mediate skeletal tissue homeostasis [75][76]. Among the key transcription factors for osteogenesis are HOX and RUNX2, both of which are hypermethylated in aged MSCs [77]. Bork and colleagues demonstrated in MSCs isolated from bone marrow aspirates from young and old human donors that long-term cell culture and regular aging result in similar epigenetic profiles. HOXA (2,5,6) and RUNX2, transcription factors involved in osteoblast differentiation, were the most prominent among genes hypermethylated during aging which leading to decreased gene expression and age-related bone loss [78].

To prevent the hypermethylation of *RUNX2*, the transcriptional activation of the methyltransferase DNMT1 can be inhibited via 5-AzaC treatment. As expected, 5-AzaC leads to a hypomethylation of genomic DNA resulting in increased expression of *RUNX2*, Osteocalcin (*OCN*) and Osterix (*OSX*). The beneficial effect of 5-AzaC was supported by Zhou et al. in cell culture experiments using MSCs. Besides a global reduction in methylation levels, an increase in osteogenic gene expression was detected as demonstrated via enhanced alkaline phosphatase (ALP) activity and, subsequently, aggrandized mineralization [77].

It was shown in human bone marrow stromal cells (BMSCs) that in osteoporosis the number of clonogenic BMSCs was reduced, corresponding to decreased levels of *Tet1* and *Tet2*, factors, which are able to erase DNA methylation. During normal osteogenesis, TET1 and TET2 levels were enhanced with an increased binding to the Osterix promoter [75][79]. Yang et al. detected an osteopenic phenotype and decreased *Runx2* expression in $Tet1^{-/-}Prx1^{cre}Tet2^{fl/fl}$ mice. After the authors demonstrated that the use of siRNAs against TET1 and TET2 led to reduced stem cell properties in bone marrow MSCs (BMMSCs), they found via RNA-seq and qPCR in their mouse model that miRNAs targeting *Runx2* gene expression were significantly higher in the knockout mice and a treatment with mimics of these miRNAs increased *Runx2* expression in BMMSCs. The authors used chromatin immunoprecipitation (ChIP)-qPCR to demonstrate that TET1 and TET2 directly bind to the CpG island of the *P2rX7* promoter, a gene which has been linked to exosome release in earlier studies. After a depletion of *Tet1* and *Tet2*, the subsequent methylation led to impaired self-renewal and differentiation potential on the stem cell level in the bone marrow, thus leading to an osteopenic phenotype [80]. Rising evidence shows a broad involvement of several miRNAs in osteoporosis, such as miR-297a-5p, miR-297b-5p, and miR-297c-5p. These are accumulating intracellularly, inhibiting *RUNX2* expression, and thereby promoting an osteoporotic phenotype. This TET/P2rX7/RUNX2 cascade may serve as a target for novel therapeutic approaches [80][81]. Again, the

link of Tet1/2 to aging has been made by Gontier et al. in the mouse brain, where a *Tet2* reduction was detected in the hippocampi of aged mice, and the application of high-titer lentivirus encoding for Tet2 shRNA in young adult mice caused deficits in short-term and long-term learning [82].

Mechanistically, an imbalance between histone modifications of osteogenic and adipogenic genes was proposed as an underlying mechanism of the development of musculoskeletal diseases. For instance, HDAC3 promotes osteogenesis and inhibits lipogenesis, while EZH2 and HDAC6 show opposite effects [77][83]. In bone marrow aspirates from human adults, it was demonstrated via retroviral-mediated enforced *Ezh2* expression in MSCs that the differentiation potential into adipocytes was higher compared to vector control cells, along with reduced *Runx2* transcription. Subsequent siRNA-mediated EZH2 depletion led to enhanced *RUNX2* expression. Using ChIP-qPCR, the authors demonstrated that enforced *EZH2* expression in MSCs resulted in increased H3K27me3 on transcriptional start sites of *RUNX2*, leading to a suppression of osteogenesis and marking EZH2 as positive regulator of adipogenesis and negative regulator of osteogenesis [83][84][85][86][87]. In another study, age-related bone loss was found linked to an increase in EZH2. In osteoporotic mice, Jing et al. detected via ChIP-qPCR that EZH2 was enriched at promoters of Wnt pathway members in BMSCs and a knockdown of EZH2 decreased H3K27me3 occupancy on these factors enhancing the *Runx2* and *Osterix* expression and subsequently osteogenic differentiation. Accordingly, the authors suggested the H3K27me3 inhibitor DZNep as a potential therapeutic substance for anti-osteoporotic treatment [84].

This translation of epigenetic discoveries into the clinic has already been established for several years. In fact, bisphosphonates (increasing MiR191c-5p and miR-497-5p) and monoclonal antibodies, such as denosumab, regulate—among others—DNA methyltransferases, histone acetylases, deacetalyses, and other key factors associated with detrimental epigenetic alterations [85][86][87].

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