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DNA protection epigenetic marks: "Youth-associated genomic stabilization DNA gaps" (youth-DNA-gaps)

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ABSTRACT: Global hypomethylation promotes genomic instability by increasing DNA damage. The accumulation of DNA damage contributes to cellular senescence, which is implicated in the aging process and various age-associated diseases, including age-associated non-communicable diseases (NCDs). Methylated youth-DNA-gap epigenetic mark produced by the molecular scissoring activity of Box A of HMGB1 molecule or rejuvenating DNA by genomic stability molecule to strengthen DNA (REDGEM-S-DNA) protects DNA from damage by relieving the double helix torsion stress during replication or transcription. The activation through active or passive release of intranuclear HMGB1 causes youth-DNA-gaps depletion. The reduction of youth-DNA-gaps results in DNA damage accumulation and global hypomethylation. Restoring the function of the youth-DNA-gap epigenetic mark through treatment of Box A of HMGB1 leads to reduced cellular senescence, rejuvenation of aging cells, and improved organ function. Therefore, HMGB1-Box A or REDGEM-S-DNA gene therapy to produce DNA gaps could be a promising strategy for treating aging conditions and age-related diseases.

KEYWORDS: Box A of HMGB1, youth-DNA-gap, REDGEM-S-DNA, global hypomethylation, senescence, DNA damage, DNA protection

INTRODUCTION

Global hypomethylation or loss of interspersed repetitive sequence (IRS) methylation level is associated with aging and carcinogenesis [1, 2]. A hypomethylated genome is prone to DNA damage and tends to have an elevated mutation rate, ultimately contributing to genomic instability [3, 4]. Genomic instability significantly affects cellular functions and the development of various age-related diseases, including cancers [1, 5]. Therefore, maintaining DNA methylation, especially at IRSs, is crucial for preserving genome stability. However, the mechanisms by which DNA methylation prevents DNA damage remain unclear [6].

Within the methylated genomic region, we discovered a new epigenetic mark, conserved in eukaryotic cells, known as naturally occurring replication-independent endogenous DNA double-strand breaks (RIND-EDSBs) [7]. This type of physical endogenous DNA double-strand break (EDSB) serves a biological role in DNA protection through DNA gaps production. RIND-EDSBs or DNA gaps stabilize the genome by reducing DNA damage from torsional stress which are similar to the small gaps between the joints of railway tracks that accommodate slight movements and prevent buckling from the contraction [7–9]. Because DNA gaps possess a stabilization role and their levels decrease with aging in eukaryotic cells, we have renamed them "youth-associated genomic stabilization DNA gaps" (youth-DNA-gaps) [9, 10].

Youth-DNA-gaps are generated by Box A domain of high mobility group box 1 (HMGB1) via its molecular scissoring activity, and the DNA gaps are retained in methylated IRS and heterochromatin by the function of Argonaute 4 (AGO4) and Sirtuin 1 (SIRT1), respectively [10, 11]. HMGB1 is a nuclear non-histone chromatin-binding protein and implicated in several DNA processes such as chromatin remodeling, replication, transcription, and DNA repair [12, 13]. Furthermore, HMGB1 can translocate from the nucleus to the cytoplasm through deacetylation modification by SIRT1 [14]. Loss of nuclear HMGB1 reduces youth-DNA-gaps, contributes global hypomethylation, accelerates DNA damage, and increases genomic instability [7, 8, 11, 15]. Moreover, HMGB1 release has been shown to promote cellular senescence [16]. Cellular senescence is driven by the accumulation of DNA damage, which influences the aging process and age-related diseases [17, 18]. Accumulation of damaged DNA induces the activation of DNA damage response (DDR), triggers cell cycle arrest and, finally contributes to cellular senescence [19]. Therefore, senescent cells promote aging and age-related diseases, possibly due to HMGB1-produced DNA gaps reduction.

Here, we review the role of DNA protection for youth-DNA-gaps. We will describe how they occur and form complexes, and discuss their role in preventing DNA damage, their connection to genomic instabil-

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ity, and their potential applications in rejuvenation through HMGB1 Box A gene therapy or rejuvenating DNA by genomic stability molecule to strengthen DNA (REDGEM-S-DNA).

GLOBAL HYPOMETHYLATION

DNA methylation is an epigenetic modification that involves adding a methyl group to the cytosine base of DNA, commonly at CpG dinucleotides [20, 21]. This modification plays many pivotal roles in cellular processes, cancer, aging and age-related diseases by influencing gene expression and genome stability [22–27]. Since the DNA methylation in the human genome is predominantly methylated at the IRS, global hypomethylation mainly reflects a reduction in the DNA methylation level of IRS such as long interspersed element-1s (LINE-1s), Alu elements (Alu), and several types of human endogenous retroviruses (HERVs) [23]. It has been indicated that methylation of LINE-1s and HERVs primarily functions in gene regulation [28, 29]. However, the Alu element was addressed to serve a role in maintaining genome stability [30–33]. Several pieces of evidence suggest that Alu repetitive elements are involved in aging and age-related diseases. Lower Alu methylation levels have been associated with pathological conditions such as inflammation and oxidative stress and have also been found in age-related diseases such as osteoporosis, diabetes mellitus, and cancers [30, 32, 34, 35].

Global hypomethylation is a widespread reduction in DNA methylation across the genome. Several mechanisms can cause global hypomethylation. The first is the downregulation of DNA methyltransferases (DNMTs) or DNMT inhibitors. These enzymes add methyl groups to DNA, and inhibiting their activity contributes to loss of genome-wide methylation [3]. Second, the demethylation process, which causes global demethylation, involves removing or modifying methyl groups from DNA. This process is driven by the ten-eleven translocation (TET) enzymes, which oxidize 5-methylcytosine [36, 37]. Moreover, exposure to oxidative stress or toxic chemical agents such as radiation, smoke, and benzene, promotes global hypomethylation [38–42]. When DNA damage occurs, DNA repair activates the repair process, which removes the lesions, triggers the demethylation process, and facilitates global hypomethylation [43, 44]. Additionally, a lack of methyl donors and co-factors such as vitamin B12, folate, betaine, and choline, contributes to the loss of genome-wide methylation [45, 46]. Previously, we revealed the distinctive functions of human Argonaute (AGO) proteins (AGO1-AGO4) in regulating IRS methylation [47]. We discovered later that AGO4 protein is a key player in human RNA-directed DNA methylation (RdDM) machinery which is primarily methylated IRS and maintained IRS methylation levels. The reduced expression of AGO4 renders IRS hypomethylation [48]. Furthermore, we recently showed that loss of nuclear HMGB1 displayed global hypomethylation [11].

DNA METHYLATION PREVENTS GENOMIC INSTABILITY

The hypomethylated genome is prone to various types of DNA damage [4, 49]. Previously, we demonstrated that DNA methylation prevents genomic instability by diminishing DNA damage [6, 11]. First, we found an inverse correlation between Alu methylation levels and endogenous DNA damage, including 8-hydroxy-2′-deoxyguanosine (8-OHdG) and AP sites [6]. Second, increased Alu methylation, mediated by AGO4 and RdDM, enhanced cell proliferation, reduced endogenous DNA damage, and improved resistance to DNA-damaging agents [6]. Furthermore, transfection with the AGO4 plasmid increased Alu methylation levels and reduced γ-H2AX expression, a marker of DNA breaks [11]. As a result, DNA methylation can prevent all types of DNA damage, including base changes, base loss, and DNA breaks. Regarding the protective effect of Alu methylation, it has been shown that DNA methylation protects damaged DNA over long distances. With a 10% increase in Alu methylation levels, endogenous DNA damage was reduced to 70% of the total genome [6].

The link between DNA methylation and youth-DNA-gaps has been established for over a decade. In the human genome, youth-DNA-gaps are located in regions of methylation, and the hypomethylated genome exhibits a lower level of youth-DNA-gaps [7]. Likewise, when chromatin become hyperacetylated, the number of youth-DNA-gaps is limited [8]. Interestingly, methylated youth-DNA-gaps serves the same function as DNA methylation, and their complex also forms with AGO4 protein [10, 11]. Consequently, global DNA hypomethylation inducing DNA damage, may result from the reduction of methylated youth-DNA-gaps [11].

FORMATION OF YOUTH-DNA-GAP COMPLEX

The molecular mechanism of youth-DNA-gaps was first studied in yeast cells. The number of youth-DNA-gaps declined in cells lacking high-mobility group box (HMGB) or histone deacetylase, SIR2 (the human SIRT1 homolog) [15]. Thus, these two proteins may be involved in the production of youth-DNA-gaps. Furthermore, our study indicated that the A box domain of HMGB1 is responsible for producing youth-DNA-gaps [10]. HMGB1, consisting of the A box, B box, and an acidic C-terminal domain, is the most abundant nuclear non-histone protein and plays essential roles in DNA binding, bending, and stabilization [50]. In addition, SIRT1, a NAD+-dependent deacetylase, regulates chromatin to maintain youth-DNA-gaps through histone deacetylation [10]. SIRT1 participates in vari-

Fig. 1 Youth-DNA-gap complex. The youth-DNA-gap complex comprises HMGB1-produced DNA gap, SIRT1, and AGO4 protein. Box A of HMGB1 generates a DNA gap by its nuclease activity. AGO4 uses RdDM to methylate the DNA sequences around the DNA gap. SIRT1 serves as histone deacetylase to mask the gap into heterochromatin. (Created with Biorender.com, https://BioRender.com/m98r673).

ous cellular functions related to aging and longevity. It modulates gene expression by deacetylating histones and non-histone proteins, affecting the activity of genes involved in inflammation, stress responses, and cell metabolism [51, 52]. Human youth-DNA-gaps are found in hypermethylated DNA regions and deacetylated heterochromatin. Moreover, youth-DNA-gap complexes limit γ-H2AX expression and do not activate the DDR [8]. Because the structure of youth-DNA-gaps is similar to DNA breaks, cells must have mechanisms to maintain these DNA gaps in compact chromatin through histone deacetylation to prevent DDR and the DNA repair process [8]. In addition, we recently reported the co-localization of youth-DNA-gaps, HMGB1, SIRT1, and AGO4 [11].

The youth-DNA-gap complex consists of HMGB1-produced DNA gap, SIRT1-deacetylated histone, and AGO4-methylated DNA [10, 11] (Fig. 1). To form the complex, Box A of HMGB1, possess nuclease activity and functions as molecular scissors to generate DNA gaps. AGO4, an essential protein involved in RdDM-methylated IRS, methylates the DNA sequences near youth-DNA-gaps [10]. Then, SIRT1 deacetylates histones, which retains youth-DNA-gaps within heterochromatin to avoid DNA break response [10]. In addition, AGO4 usually binds to IRSs [48]. The interaction between AGO4 and HMGB1 may be due to the role of AGO4 to guide the location of HMGB1-produced DNA gaps in the genome. Additionally, AGO4 interacts with SIRT1 to locate youth-DNA-gap complexes within methylated IRSs and heterochromatin. Notably, we observed that the interaction of AGO4-HMGB1 was much lower than that of the AGO4-SIRT1 interaction [11]. As a result, specific regulations regarding the number of youth-DNA-gaps may be established to prevent the effects of too many gaps, which could lead to unwanted DNA regulation.

Multiple DNA-based processes, including replication and transcription, modify the topology of DNA resulting in torsional forces from the twisting of the helical DNA strands. Torsional stress significantly affects the structure and stability of the nucleosome, and the excessive stress leads to DNA damage [53, 54]. Previously, we have demonstrated the role of youth-DNA-gaps in DNA protection. Youth-DNA-gaps prevent every types of DNA damage (base changes, base loss, single strand breaks (SSBs), and double-strand breaks (DSBs)) and their potential effects across a wide range of the genome [10]. Yeast cells with limited youth-DNA gaps had shearing of DNA, increased the production of pathologic DNA breaks, and decreased cell viability [15]. In mammal, the number of youth-DNA-gaps was inversely correlated with human age. Youth-DNA-gaps reduction was observed in elderly, aged rats, including D-gal-induced rats and naturally aged rats, and human cells induced senescence [10]. Moreover, the decrease of youth-DNA-gaps was detected in the white blood cells of patients with type 2 diabetes mellitus and its levels were inversely correlated with levels of hemoglobin A1c (HbA1c) [32]. To stabilize the genome, youth-DNA-gaps relieve torsional stress, thereby preventing damage and enhancing DNA durability. In aging, youth-DNA-gaps are found less frequently, and torsional force is higher in aged DNA.

Global hypomethylated genome promotes genomic instability. We have proposed that youth-DNA-gaps are DNA modifications within genomic methylation [55]. We used DNA-GAP PCR or IRS-EDSB PCR techniques to detect DNA gaps and the ligation-mediated PCR (LMPCR) from IRS to the EDSB. The results of the experiment showed that all EDSBs were hypermethylated and detectable in all cell types. Moreover, these hypermethylated EDSBs were detected in all cell cycle phases and were presented in non-dividing

cells that did not undergo replication [7].

For DNA repair process, the repair system for pathological EDSBs is Ku-mediated nonhomologous end-joining repair (NHEJ). However, methylated youth-DNA-gaps are repaired by a more precise Ataxia-telangiectasia mutated (ATM)-dependent NHEJ pathway [8]. Because the structure of youth-DNA-gaps is similar to that of pathological DSBs structure, youth-DNA-gaps are localized by histone deacetylation in heterochromatin to avoid DDR, including γ-H2AX, which promotes cellular senescence [8]. Due to the distinct characteristics of DNA lesions, youth-DNA-gaps are not considered DNA damage but rather epigenetic markers [55, 56].

HMGB1 RELEASE INDUCES GLOBAL HYPOMETHYLATION

The connection between HMGB1-produced DNA gap depletion and global hypomethylation has been observed in our previous study. Knockdown of HMGB1 using shRNA resulted in Alu hypomethylation, whereas increased HMGB1 expression was associated with Alu hypermethylation [11]. The primary IRS methylation in humans is AGO4-mediated RdDM and loss of AGO4-mediated RdDM causes IRS hypomethylation [48]. AGO4 is a critical protein in RdDM and integrates with small interfering RNA (siRNA) to direct the addition of methyl groups at IRSs [48, 57]. Since the youth-DNA-gaps are located in the hypermethylated regions, the primary targets of human RdDM may be near these DNA gaps [6, 11, 48]. So, we investigated whether AGO4-mediated RdDM is HMGB1-produced DNA-gap dependent by transfection of Alu siRNA in HMGB1-knockdown cells, and we found that Alu methylation could not be enhanced. Thus, human HMGB1-produced DNA gaps are methylated by AGO4-mediated RdDM machinery [11].

HMGB1 is released via active or passive processes. The active HMGB1 release occurs by the stimulation of immune cells or inflammatory cells. Furthermore, HMGB1 can be passively released from damaged cells or necrotic cells [14]. Two mechanisms can explain HMGB1 release driving genomic hypomethylation. One mechanism involves the reduction of HMGB1-AGO4 interaction. Intranuclear HMGB1 release diminishes AGO4-bound methylated youth-DNA-gaps. AGO4 mainly binds to IRS, and the DNA sequence around DNA gaps is hypermethylated [48]. Therefore, the loss of youth-DNA-gaps restricts AGO4 binding sites, leading to IRS demethylation and, consequently, global hypomethylation. Another mechanism is that a decrease in DNA gaps reduces DNA durability and enhance spontaneous DNA damage [9]. Elevated DNA damage can activate DNA repair pathways, which renders the DNA demethylation process and global hypomethylation (Fig. 2).

REJUVENATING DNA BY GENOMIC STABILITY MOLECULE TO STRENGTHEN DNA OR REDGEM-S-DNA

Youth-DNA-gaps protect DNA by preventing damage and promoting its durability [9, 10]. Reducing youth-DNA-gaps accelerates DNA damage accumulation and drives cellular senescence [9, 10]. Damage to DNA causes tissue dysfunction and contributes to aging phenotypes and senescence-associated diseases [19]. The accumulation of endogenous DNA damage contributes to cellular aging by activating DDR. While DNA damage promotes aging, limiting the DDR helps rejuvenation. Thus, inhibition of the DDR pathway could rejuvenate aging cells [58].

We previously developed a novel gene therapy that could rejuvenate aging cells called REDGEM-S-DNA, which consists of the molecules of HMGB1 Box A [10]. Introducing REDGEM-S-DNA *in vitro* or *in vivo* resulted in increased youth-DNA-gaps and revitalized aging phenotypes in senescent cells and aging rats. Overexpression of the HMGB1 Box A plasmid reduced senescent markers and increased cell viability in senescent cells [10].

Additionally, introducing REDGEM-S-DNA in two aging rat models, naturally aged rats and D-galactose-induced aging rats, diminished senescent markers, decreased DDR, and restored liver function, learning behavior, and memory [10]. Moreover, elevated HMGB1 Box A expression also minimized aging features such as visceral fat, liver fibrosis, lung fibrosis, and senescence-associated proteins [10, 59]. We also showed that transfection of Box A of HMGB1 expression plasmid improved stem cell properties in human mesenchymal cells [60]. In addition, introducing Box A of HMGB1 expression plasmid prevented lacrimal gland cellular senescence, an aging disorder that induces ocular pathogenesis [61]. These findings indicate that reducing youth-DNA-gaps retains the cellular senescence stage. As a result, the reduction of youth-DNA-gap is a part of the aging process [10]. Furthermore, a recent work from our group demonstrated the different outcome of HMGB1 Box A gene therapy between cancer and normal cells. Overexpression of HMGB1 Box A induced DSB production, promoting lung cancer cell apoptosis, and reducing cell survival. However, this overexpression did not cause DSB or harm to normal cells, suggesting the potential of REDGEM-S-DNA for cancer treatment [62].

THE THERAPEUTIC IMPLICATIONS OF YOUTH-DNA-GAP RESTORATION AND FUTURE DIRECTIONS

Youth-DNA-gap restoration abolishes the upstream driver of senescence-associated molecular pathogenesis by preventing DNA damage. DNA damage consequence of HMGB1-produced DNA gap reduction pushes DDR driving senescence cascade. Senescence

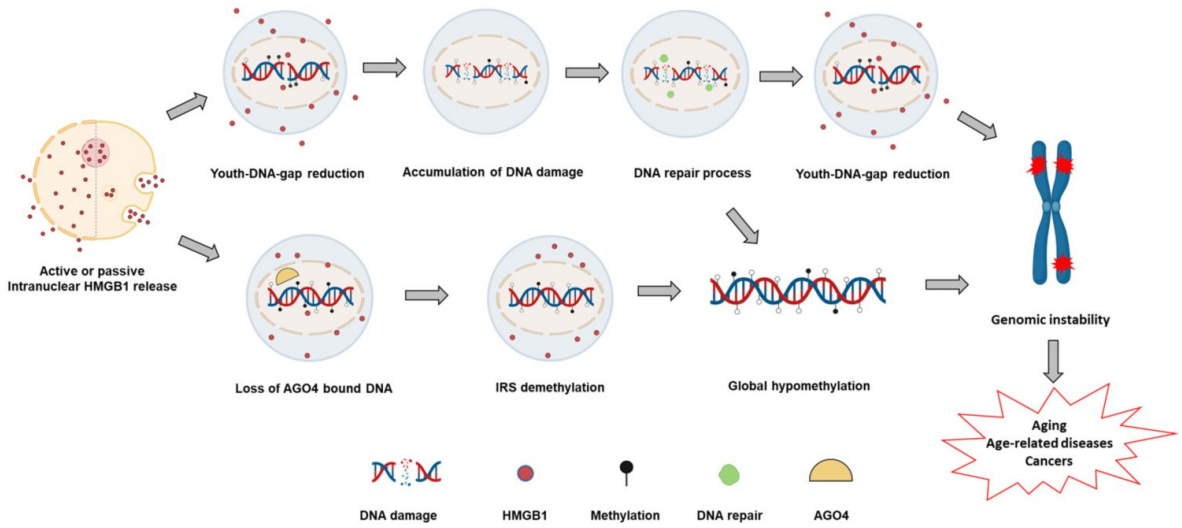

Fig. 2 HMGB1 release causes global hypomethylation and promotes genomic instability. HMGB1 release leads to genomic instability by two processes. First, it reduces youth-DNA-gaps and results in DNA damage accumulation, which facilitates global repair of these DNA gaps. Second, it limits AGO4-bound youth-DNA-gaps, leading to global hypomethylation. (Created with Biorender.com, https://BioRender.com/x35p584).

then causes poor cellular function and alters cell structure. Senescence cells also secrete inflammatory substances, including HMGB1. These processes lead to degenerative diseases and pathological findings in cellular and extracellular spaces, such as lung fibrosis, amyloid accumulation, and (neural) stem cell loss stemness. Before introducing Box A, aging cells had active damage, overcoming active healing processes. After Box A protects DNA, the detrimental process stops, but the healing continues. Thus, the outcome of HMGB1 Box A gene therapy is that it limits upstream pathogenesis process of all defects, including damaged DNA, cells, and tissues [55] (Fig. 3).

It can be concluded that restoring the DNA gaps by HMGB1 Box A gene therapy produces DNA protection epigenetic marks that stop the molecular flow of the senescence process from upstream. The remedy allows the cellular healing process to complete faster than damage. As a result, the organ defected by the cell stress-DNA gap reduction-DNA damage-senescence cascade is effectively healed.

Our ongoing research suggests that HMGB1 Box A gene therapy stops DNA damage and results in several unprecedented healing processes, including abolishing senescence-associated fibrosis and promoting neurogenesis in brain-damaged experimental animals. We proposed how Box A indirectly removes fibrosis by preventing its synthesis [10, 59]. For neurogenesis, the mechanism may be that youth-DNA-gap reduction usually prevents neural stem cells from proliferation. Therefore, Box A revitalizes the neural stem cells' dividing capacity, similar to improving stemness in other stem cell types [60] (Fig. 3).

HMGB1 Box A gene therapy outcome lasts longer than the presence of the Box A plasmid DNA. The plasmid DNA remained in cells for only one week, but the rejuvenation outcome lasted longer. By stopping DNA damage, the HMGB1 release is also prevented. As a result, the DNA protection process by endogenous nuclear HMGB1 is being restored.

HMGB1 Box A gene therapy is safe. Box A is derived from endogenous protein. Furthermore, targeting genome locations of HMGB1 Box A is controlled by SIRT1 and AGO4 [11]. So, the targets to producing DNA gaps is regulated naturally by endogenous proteins. Our coating system used Ca-P nanoparticles [10, 59]. This particle type can be found naturally in mammals, including humans [63]. Up to now, we have found no toxicity in our animals receiving therapeutic doses. We also found rejuvenation in all vital organ systems, including the brain.

CONCLUSION

In conclusion, youth-DNA-gaps play a crucial role in DNA protection and genome stabilization by reducing DNA damage and enhancing DNA durability. A decrease in HMGB1-produced DNA gap leads to global hypomethylation. The reduction of youth-DNA-gaps is commonly observed during aging and implicated in the molecular pathogenesis of cellular senescence and age-associated diseases. Restoring the function of the youth-DNA-gaps epigenetic mark by REDGEM-S-DNA enables cells to enhance their protective role for DNA, which could reduce cellular senescence, rejuvenate aging cells, and improve organ functions. Therefore, REDGEM-S-DNA could be a promising approach for

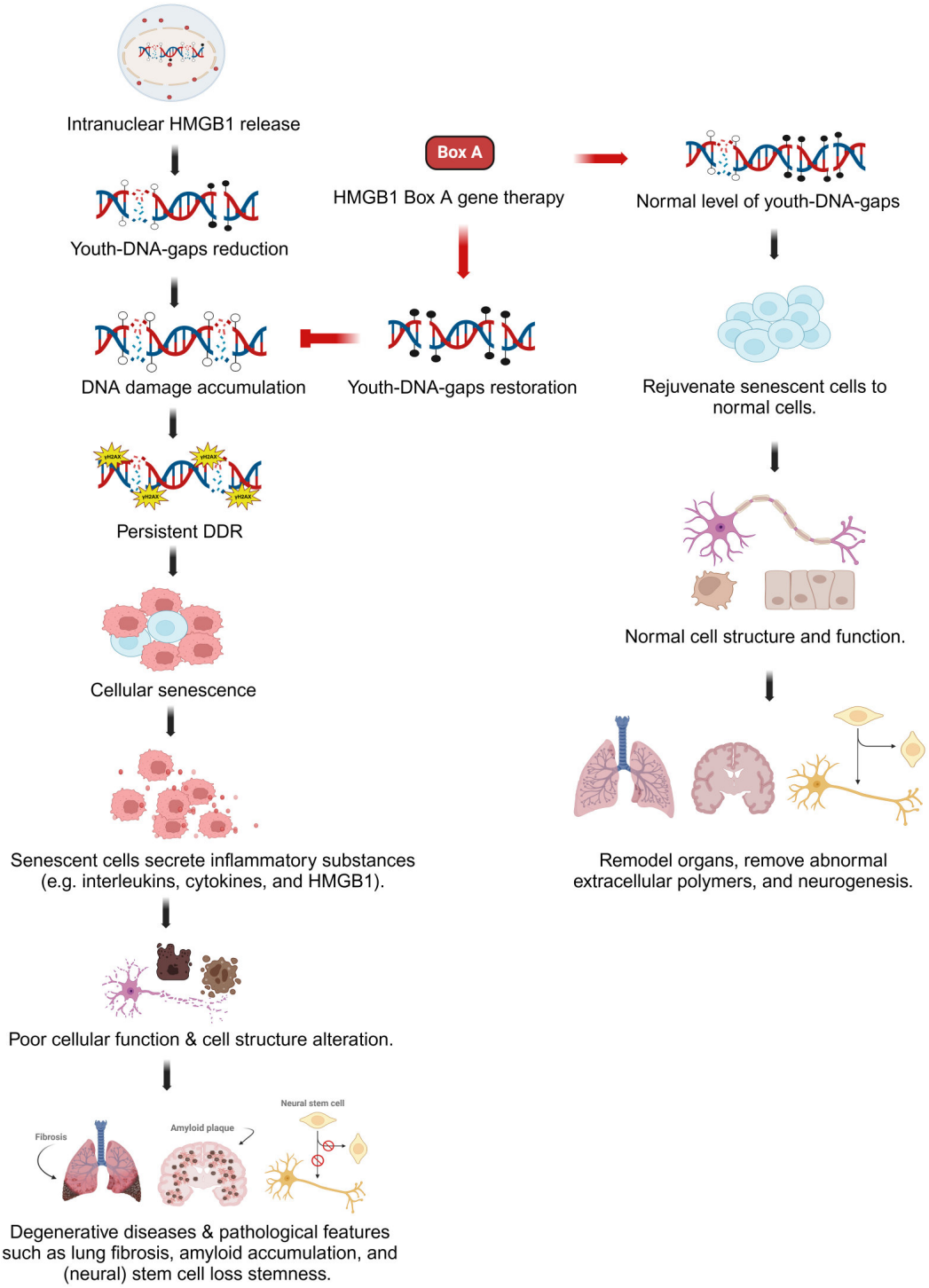

Fig. 3 Youth-DNA-gap restoration by HMGB1 Box A gene therapy abolishes the upstream driver of senescence-associated molecular pathogenesis by preventing DNA damage. Intranuclear HMGB1 release results in the reduction of youth-DNA-gaps which induces increased DNA damage. The accumulation of DNA damage stimulates DNA damage response (DDR) and the persistent DDR leads to cellular senescence that causes poor cellular function and cell structure alteration. Moreover, senescent cells can secrete inflammation substances, for example, interleukin, cytokines, and HMGB1, which results in degenerative diseases and pathological features such as fibrosis of lung cells, amyloid accumulation in the brain, and stemness loss of neural stem cells. Restoring youth-DNA-gaps by HMGB1 Box A gene therapy will protect DNA from damage and halt the pathogenesis processes. (Created with Biorender.com, https://BioRender.com/z36v807).

treating aging conditions and age-related diseases, including age-associated NCDs.

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