Inhibition of DNA methyltransferase aberrations reinstates antioxidant aging suppressors and ameliorates renal aging

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Abstract
DNA methylation alterations play mechanistic roles in aging; however, the epigenetic regulators/mediators causally involved in renal aging remain elusive. Here, we report that natural and D-galactose (D-gal)-induced aging kidneys display marked suppression of antiaging factor NRF2 (nuclear factor erythroid-derived 2-like 2) and KLOTHO, accompanied by upregulations of DNA methyltransferase (DNMT) 1/3a/3b and NRF2/KLOTHO gene promoter hypermethylations. Administration of a DNMT inhibitor SGI-1072 effectively hypomethylated the promoters, derepressed NRF2/KLOTHO, and mitigated the structural and functional alterations of renal aging in D-gal mice. Moreover, oleuropein (OLP), an olive-derived polyphenol, also displayed similar epigenetic modulation and antiaging effects. OLP inhibited the epigenetic NRF2/KLOTHO suppressions in a gain of DNMT-sensitive manner in cultured renal cells, demonstrating a strong DNA-demethylating capacity. In NRF2 knockout and KLOTHO knockdown D-gal mice, OLP exhibited reduced antiaging effects with KLOTHO displaying a prominent gene effect and effect size; consistently in KLOTHO knockdown mice, the antiaging effects of SGI-1027 were largely abrogated. Therefore, the KLOTHO recovery is critical for the antiaging effects of DNA demethylation. Collectively, our data indicate that aberrant DNMT1/3a/3b elevations and the resultant suppression of antiaging factors contribute significantly to epigenetic renal aging, which might be targeted for epigenetic intervention by synthetic or natural DNA-demethylating agents.

KEYWORDS
DNA methylation, epigenetics, KLOTHO, NRF2, renal aging

1 INTRODUCTION

Development of renal aging is a slow process and manifests as declined renal functions and increased susceptibility to various acute or chronic kidney diseases (Nitta et al., 2013). The aging kidney is histomorphologically characterized by glomerulosclerosis, tubular atrophy, interstitial fibrosis, arteriosclerosis, and loss of cortical mass, and exhibits reduced anti-oxidative stress potentials and accumulation of inflammatory and fibrotic factors (Abdel-Rahman & Okusa, 2014). In addition, the aging kidney seems to lose part of its repair ability owing to deficient renal cell proliferation and autophagy, enhanced senescence, and apoptosis (Gekle, 2017), which

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are attributed to expression changes of many aging-related genes. Lately, several studies have demonstrated that epigenetic modifications, especially DNA methylation, substantially affect gene expression that are associated with aging (Pal & Tyler, 2016; Zampieri et al., 2015), suggesting an additional control of renal aging process.

DNA methylation, among other epigenetic modifications including protein acetylation/methylation and microRNA interference, is the most stable epigenetic modification that affects the expression of more than 60% of genes (Portela & Esteller, 2010). Three bioactive DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b) add a methyl group from S-adenosyl-methionine to the cytosine residue of CpG dinucleotides to form 5-methylcytosine (5mC) (Maunakea et al., 2010). In the context of gene promoters, hypomethylated CpGs are generally associated with active and constitutively expressed genes, while hypermethylated CpGs correlate with lowly expressed/silenced genes (Bird, 2002). Although genome-wide association studies and gene-targeted investigations detect significant changes in DNA methylation with aging and aging-related kidney diseases in humans and rodents (Unnikrishnan et al., 2019), the direct evidence that alteration of a particular gene expression with its genomic DNA methylation causally affecting renal aging is still lacking.

Oxidative stress is a major pathological factor for premature aging and aging-associated diseases (Liguri et al., 2018). Past studies have revealed that NRF2 (Nuclear factor erythroid-derived 2-like 2, gene Nfe2l2) and KLOTHO, two major antiaging factors with antioxidant capacities, are suppressed with aging which correlates with increased incidence of aging-related kidney disorders (Semb et al., 2011; Semb et al., 2016; Silva-Palacios et al., 2018). Moreover, NRF2 and KLOTHO deficiencies exacerbate mouse renal aging, while their restorations by a number of strategies reduce aging phenotype and increase mouse life span (Makoto Kuro-o et al., 1997; Kurosu et al., 2005; Tarantini et al., 2018), indicating their essential roles in controlling aging processes. NRF2 is a ubiquitously expressed transcriptional factor and a key regulator of redox response. NRF2 positively regulates a number of antioxidant molecules and enzymes by binding to the antioxidant-response element (ARE) on target gene promoters, thereby elevating the host anti-oxidative stress and antiaging potentials (Silva-Palacios et al., 2018). KLOTHO is enriched in distal convoluted tubules in kidney (Azuma et al., 2012) and exists as both a transmembrane and a secreted protein known to exert the antiaging functions mainly by inhibiting insulin/insulin-like growth factor-1 (IGF-1) signaling, excessive inflammation, and oxidative stress (Liu et al., 2011; Masuda et al., 2005). The promoters of both NRF2 gene (Nfe2l2) and KLOTHO gene (Kl) contain typical CpG islands and their suppressions due to DNA methylation aberrations are reported in various aging-related disorders (Reyes-Aguirre & Lamas, 2016; Zhang et al., 2017); however, whether the suppressions involve aberrant DNA methylation modification mechanistically relevant to renal aging remains to be determined.

In this study, we investigated the altered DNA methylation modifications of NRF2 and KLOTHO expression in kidneys of natural and D-galactose (D-gal)-treated accelerated aging mice (Azman & Zakaria, 2019). We observed that both NRF2 and KLOTHO were markedly suppressed, which correlated with their promoter hypermethylations and aberrant DNMT1/3a/3b elevations. We then assessed a synthetic DNA-demethylating agent SGI-1072 and a small compound oleuropein (OLP) found in natural olive for their epigenetic regulations of the KLOTHO/NRF2 suppression and anti-renal aging efficacies. Our data might reveal important epigenetic characters of renal aging and provide novel insights into potential prophylactic and therapeutic anti-renal aging strategies.

2 | RESULTS

2.1 | KLOTHO expression is suppressed in natural and accelerated mouse aging kidney

We first examined the protein expression of KLOTHO and NRF2 in mouse aging kidneys. The kidneys from both natural aging mice (25 months old, n = 8 per group) and an accelerated mouse aging model induced by D-galactose (D-gal) injection (n = 8 per group, 8 weeks) displayed increased collagen depositions in renal sections, especially in glomerulus, as demonstrated by Masson's trichrome staining (Figure 1a and b, the upper panels, indicated by arrows), as well as SA-β-galactosidase (SA-β-gal) staining (Figure 1a and b, the lower panels, indicated by arrows). Notably, the natural aging kidney (25 m) showed more fibrotic collagen depositions (Figure 1a and b, comparing panel 2 and 4). Moreover, the natural aging kidney displayed time-dependent increases (2, 7, 16, and 25 months) of myofibroblast marker α-SMA and aging-associated DNA double-strand break marker γH2AX (phosphorylated histone H2AX), as well as decreased KLOTHO and NRF2 (Figure 1c and d, the left panels). The similar protein expression alterations were also observed in D-gal-treated aging kidneys (Figure 1c and d, the right panels). We also examined kidney sections by immunohistochemical staining and confirmed that both KLOTHO and NRF2 were enriched in renal distal tubular epithelial cells in young control mice, but the levels were noticeably reduced in old (25 m) and D-gal-treated aging mice (8 weeks, Figure 1e). In addition, both natural and D-gal-induced aging kidneys displayed inductions of inflammatory cytokines TNF-α and IL-6 (Figure 1f). These results clearly demonstrate that suppressions of antiaging factor KLOTHO and NRF2 are features of renal aging.

2.2 | Aging kidneys exhibit KLOTHO gene promoter hypermethylation and aberrant DNMT1/3a/3b elevations

To explore the possibility that aberrant DNA methylation modification might cause the KLOTHO and NRF2 suppressions, we first analyzed the promoters of both mouse KLOTHO gene (Kl) and NRF2 genes (Nfe2l2) by online software MethPrimer (http://www.urogene.org/methprimer). Both Kl and Nfe2l2 promoters contain typical CpG islands located at −110/800 (Kl) and −550/−150 (Nfe2l2)
loci, respectively (Figure 2a). We then examined the promoter DNA methylation status of renal tissues from natural (2, 16, and 25 m) and D-gal-treated aging mice (8 weeks), and found that the promoter methylation levels of *Kl* (594/777) increased from 23.11% ± 1.76% of 2 m mice to 69.47% ± 4.88% of 25 m mice (*p* < 0.05) and from 24.92% ± 1.37% of control to 49.29% ± 5.16% of D-gal mice (*p* < 0.05), respectively. In addition, the promoter methylation of *Nfe2l2* (−389/−265 locus) increased from 23.55% ± 4.36% of 2 m mice of 2 m mice to 69.47% ± 4.88% of 25 m mice (*p* < 0.05) and from 24.92% ± 1.37% of control to 49.29% ± 5.16% of D-gal mice (*p* < 0.05), respectively. In addition, the promoter methylation of *Nfe2l2* (−389/−265 locus) increased from 23.55% ± 4.36% of 2 m mice
FIGURE 2 Aging kidneys exhibit KLOTHO gene promoter hypermethylation and aberrant DNMT1/3a/3b elevations. (a) Schematic diagrams of mouse Kl and Nfe2l2 promoters. The positions of CpG islands (gray area) and MSP/BSP primers were depicted relative to the transcription-starting site. (b and d) MSP analysis of renal tissues of mice (8 weeks, the left) or HK2 cells (48 h, the right) treated with vehicle, D-gal (100 mM), SGI-1027 (SGI, 10 μM, the upper panel)/OLP (50 μM, the lower panel), or SGI-1027/OLP plus D-gal. Representative agarose gel analysis of methylated (Methyl), unmethylated (Unmet) and input PCR products for (b) Mouse tissue Kl/human HK2 cell KL or (d) Nfe2l2. (c and e) Quantifications for (b; c) or (d; e). Values were presented as mean percentages ±SEM after adjusted with input PCR products, *p < 0.05, Two-way ANOVA. (f) BSP analysis of kidney tissues of mice from Figure 3a for Kl promoter. For each group, 3 randomly selected mice were analyzed (M1, M2, and M3 were shown). After PCR, 5 clones from each animal were sequenced, of which three clones were presented and additional two clones were presented as Figure S2. One box represented one mouse. Each row of dots in the boxes represented one single sequenced clone, and each dot represented one CpG site. Empty or dark dots indicated unmethylated or methylated CpGs, respectively. (g) Quantification of (f). Data are presented as ±SEM. Each circle represented the percentage of methylated CpGs over total CpGs of all 15 cloned fragments from 3 animals in each group. *p < 0.05, two-way ANOVA. (h) Western blots of renal tissues of mice (the left panel) or HK2 cells (the right panel) treated with vehicle, OLP, D-gal, or both as in Figure 3a for DNMT1(Dt1), DNMT3a (Dt3a), and DNMT3b (Dt3b). Two random samples from kidneys and one representative sample from cell assay were shown. (i) Quantitation of (h). The values were presented as means ±SEM. *p < 0.05, two-way ANOVA.
to 68.37% ± 5.84% of 25 m mice (p < 0.05) and from 26.46% ± 6.79% of control to 56.17% ± 5.11% of D-gal mice (p < 0.05), respectively (Figure S1A and B). Since DNA methylation is positively regulated by DNMTs, we further assessed the renal protein levels of three bioactive DNMTs and found that DNMT1 started to increase on 7th month, and DNMT3a and DNMT3b also increased later (16th and 25th months) in natural aging and D-gal-treated (8 weeks) mice (Figure S1C and D). These results indicate that the increased DNMT1/3a/3b expression might be responsible for the promoter hypermethylation and the suppression of KLOTHO in aging kidneys.

To confirm that the KLOTHO and NRF2 suppressions are due to the DNMT aberrations, we tested whether SGI-1027, a quinoline-based DNMT inhibitor with IC_{50} of 12.5 μM, 8 μM and 7.5 μM, for DNMT1, DNMT3a, and DNMT3b (Datta et al., 2009), inhibits the epigenetic alterations. The results showed that SGI-1072 administration effectively demethylated K/β promoters from 55.45% ± 3.45% of D-gal-treated kidney to 26.2% ± 1.82% (p < 0.05), and from 48.71% ± 2.25% of D-gal-treated HK2 cells to 25.18% ± 3.38% (p < 0.05, Figure 2b and c, the upper panel), and Nfe2l2 promoter from 44.95% ± 4.48% of D-gal-treated kidney to 22.81% ± 2.58% (p < 0.05, Figure 2d and e, the upper panel), supporting that the promoter hypermethylation of both K/β and Nfe2l2 in D-gal mouse kidneys are mainly caused by aberrant DNMT1/3a/3b elevations.

SGI-1027 is a synthetic DNMT inhibitor. Because synthetic epigenetic drugs, such as decitabine (5-Aza-2’-deoxycytidine), might be potentially cytotoxic, and also because many dietary or medicinal plant components, especially polyphenols, possess antiaging and epigenetic modulating activities with tolerable side effects, we also tested OLP, a polyphenol isolated from olive leaves with antiaging capacity (Leri et al., 2020), for its demethylating and anti-renal aging potencies. Similarly, we treated D-gal-treated mice and HK2 cells with OLP and found that OLP effectively demethylated the K/β promoters from 61.92% ± 7.77% of D-gal-treated kidney to 35.09% ± 4.55% (p < 0.05), and from 61.39% ± 4.38% of D-gal-treated HK2 cells to 34.82% ± 2.83% (p < 0.05, Figure 2b and c, the lower panels), and the Nfe2l2 promoter from 55.68% ± 7.19% of D-gal-treated kidney to 30.19% ± 3.17% (p < 0.05, Figure 2d and e, the lower panels). We also analyzed the K/β promoter methylation of the same locus by BSP, the gold standard for DNA methylation analysis. The K/β promoter region analyzed (466/700) contained 21 CpG sites and displayed increased methylation in D-gal-treated kidney from 1.58% ± 0.59% to 18.73% ± 3.06% (p < 0.05), whereas SGI-1027 and OLP treatments reduced the level to 4.76% ± 1.14% and 6.03% ± 1.09%, respectively (p < 0.05, Figure 2f,g and Figure S2). Further, the results from Western blotting showed that OLP treatment significantly lowered the elevated DNMT1/3a/3β in D-gal-treated kidneys (Figure 2h and i), suggesting that OLP possesses strong DNA-demethylating capacity.

### 2.3 SGI-1027 and OLP derepress KLOTHO and reduce renal aging in D-gal-treated mice

To test whether the demethylation of K/β and Nfe2l2 promoters by SGI-1027 and OLP affects their expression and the functional relevancies to renal aging, we treated D-gal mice with SGI-1027 or OLP separately (n = 8 per group, 8 weeks). As anticipated, the kidney sections from D-gal mice showed increased collagen deposits in interstitium and glomerulus areas (Figure 3a, the left panel) and β-gal-positive cells (Figure 3a, the right panel). SGI-1027 and OLP treatments significantly reduced the deposits from 15.73% ± 0.97% to 7.69% ± 0.51% (p < 0.05) and to 8.07% ± 0.59% (p < 0.05), respectively (Figure 3b), as well as the numbers of β-gal-positive cells. Since aging kidneys are featured by infiltrated macrophages, which also express β-gal, we also stained the sections for macrophage marker CD68 and found that CD68-positive macrophages appeared in D-gal-treated kidney, but largely disappeared after SGI-1027 or OLP treatment (Figure 3a). These macrophages only accounted for a small portion of β-gal-positive cells, suggesting that renal aging occurs mainly in kidney parenchymal cells. We further calculated the effect sizes of SGI-1027(β1) and OLP (β2) on the fibrosis severities, which were η^2_1 = 0.280 and η^2_2 = 0.326 (Figure 3b, the insert), respectively, indicating that both SGI-1027 and OLP effectively reduce the fibrosis intensities with OLP showing a stronger capacity. We also found that serum levels of creatinine (Cr) and blood urea nitrogen (BUN), two key parameters of renal functions, increased in D-gal-treated mice, which were significantly lowered by SGI-1027 or OLP treatment (Figure 3c). Moreover, SGI-1027 and OLP treatments inhibited the abnormal renal expression of KLOTHO, NRF2, α-SMA, and γH2AX proteins (Figure 3d and e) and TNF-α (Tnfa) and IL-6 (Il6) mRNAs in D-gal-treated mice (Figure 3f). Since KLOTHO and NRF2 inhibit aging, these results suggest that the preservations of KLOTHO and NRF2 by SGI-1027 or OLP might contribute to their anti-renal aging functions.

### 2.4 OLP preservation of KLOTHO is sensitive to gain of DNMT function in renal cells

OLP displays multiple pharmacological activities. We confirmed that OLP reversed the D-gal-induced KLOTHO and NRF2 suppressions in HK2 cells in a dose-dependent manner, similarly to that of SGI-1027 (Figure 4a and b). We further found that both SGI-1027 and OLP effectively reduced the induction of TNFA and Il6 mRNAs in D-gal-treated HK2 cells (Figure 4c), suggesting that the KLOTHO/NRF2 restoration by DNMT inhibition mitigated the inflammatory response, and renal epithelial cells contributed to the production of inflammatory cytokines. To assess whether OLP preservations of KLOTHO and NRF2 are due to its inhibition of the DNMT elevations, we tested whether gain of DNMT function affects the OLP restoration of KLOTHO and NRF2 by two strategies. We first pre-treated HK2 cells with or without DMOG (dimethyloxallyl glycine), a small molecule of TET enzyme inhibitor (Amouroux et al., 2016) that supposedly counteracts the activity of DNMT inhibition (Hideyuki Takeshima et al., 2020), and found that DMOG treatment blocked OLP alleviation of the KLOTHO and NRF2 suppressions induced by D-gal (Figure 4d and e, the left panel). Further, overexpression of flag-tagged DNMT1 or DNMT3a diminished OLP recovery of the KLOTHO and NRF2 losses,
respectively (Figure 4d and e, the middle and right panels). These data strongly support that a considerable portion of the OLP effects on KLOTHO and NRF2 suppressions in aging kidney is due to its inhibition of the aberrant DNMT elevations.

2.5 | KLOTHO is critical for the anti-renal aging effects of OLP

To further assess the role of KLOTHO and NRF2 preservation by OLP in renal aging, we compared the renal-protective effects of OLP between KLOTHO knockdown (siKl) and the control mice (siCtrl, n = 6 per group), as well as between Nfe2l2WT and Nfe2l2KO mice (n = 8 per group). As revealed by the renal fibrotic scoring, siKl mice displayed more collagen depositions than siCtrl mice under control condition (9.44% ± 1.69% vs. 3.3% ± 1.16% of siCtrl mice, p < 0.05, Figure 5a and b, the left and the upper panels), while the fibrotic extents of Nfe2l2KO and Nfe2l2WT control mice were similar (Figure 5a and b, the right and lower panels). After D-gal treatments, both siKl and Nfe2l2KO mice showed significant increases of fibrotic depositions than their control littermates (24.79% ± 2.0% vs. 13.09% ± 3.3% of siCtrl D-gal mice, p < 0.05; and 20.63% ± 2.82% vs. 12.96% ± 3.0% of Nfe2l2WT D-gal mice, p < 0.05). OLP treatments effectively reduced the fibrotic
areas in siCtrl and Nfe2l2WT D-gal mice (Figure 5b, comparing the 2nd and 3rd columns); however, the inhibitions were significantly reduced in siKl or Nfe2l2 KO D-gal mice (Figure 5a and b, the 3rd and 6th columns). Since siKl control mice showed spontaneous renal fibrotic alterations and siKl D-gal mice developed more severe fibrotic depositions, we calculated the group main effect of genotype (P1), effect of group interactions between genotype and D-gal treatment (P2) and between genotype and OLP intervention (P3, Figure 5b, the inserts), and the corresponding effect size ($\eta^2$). The results showed that the renal fibrotic alterations were significantly affected by Kl and Nfe2l2 genotypes ($p < 0.00001$), and by interactions between Kl and D-gal treatment ($P2 = 0.00115$, $\eta^2 = 0.225$) and between Kl and OLP intervention ($P3 = 0.01085$, $\eta^2 = 0.145$), which were greater than that of the interactions between Nfe2l2 and D-gal treatment ($P2 = 0.01116$, $\eta^2 = 0.144$) and between Nfe2l2 and OLP intervention ($P3 = 0.04708$, $\eta^2 = 0.091$). Consistently, OLP effectively normalized the adverse expression of KLOTHO, NRF2, $\alpha$-SMA, and $\gamma$H2AX (Figure 5c and d), as well as the induction of Tnfa and Il6 (Figure 5e) in siCtrl and WT D-gal mice, but the effects were largely obliged in siKl and Nfe2l2KO D-gal mice. Taken together, these results suggest that KLOTHO affects renal aging more than NRF2 and the derepression of KLOTHO by OLP provides stronger anti-renal aging effects than that of NRF2.

2.6 | KLOTHO deficiency abrogates the anti-renal aging effects of SGI-1027

To further prove that the KLOTHO suppression due to aberrant DNA methylation is a major causative factor of renal aging, we compared the antiaging effects of SGI-1027 between siKl and the control mice (siCtrl, n = 8 per group). The results confirmed that SGI-1027 effectively minimized the renal fibrotic areas in siCtrl D-gal mice (7.76% ± 0.29% vs. 12.58% ± 0.75% D-gal mice, $p < 0.05$); however, the effects were largely reduced in siKl mice (7.76% ± 0.29% siCtrl vs. 19.82% ± 0.82% siKl, $p < 0.05$. Figure 6a and b). We also calculated the effect of interaction between genotype and SGI-1027 intervention ($p = 0.03222$, Figure 6b, the insert), and the corresponding effect size ($\eta^2 = 0.105$), which were smaller than that of OLP ($p = 0.01085/\eta^2 = 0.145$). Moreover, SGI-1027 effectively corrected the abnormal expression of KLOTHO, NRF2, $\alpha$-SMA, and $\gamma$H2AX in
siCtrl D-gal mice, but the effects were significantly reduced in siKl D-gal mice (Figure 6c and d). Collectively, these results not only suggest that KLOTHO suppression due to DNA hypermethylation causally affects renal aging, but also support that OLP preservation of KLOTHO via DNA demethylation plays a significant role in its anti-renal aging activities.

FIGURE 5 KLOTHO preservation is critical for the anti-renal aging effects of OLP. Mice receiving siRNA-Control (siCtrl) or siRNA-KLOTHO (siKl, n = 8), or Nfe2l2WT and Nfe2l2KO mice (n = 8) were grouped into vehicle control, D-gal, and OLP/D-gal mice as before (8 weeks). (a) Representative photomicrographs of kidney sections (Masson's trichrome staining) for siCtrl/siKl mice (the left panel) and Nfe2l2WT/KO mice (the right panel). The arrows indicated fibrotic collagen deposition areas. (b) Quantitation of renal fibrotic areas of siCtrl/ siKl (the upper panel) and Nfe2l2WT/KO mice (the lower panel) in (a). The effects of Kl and Nfe2l2 genotypes (P1), the effects of interactions between Kl or Nfe2l2 genotype and D-gal treatment (P2), or the interactions between Kl or Nfe2l2 genotype and OLP intervention (P3), as well as their corresponding effect sizes were indicated. (c) Western blots. The renal tissues from experimental mice in (a) were assayed for KLOTHO, NRF2, α-SMA, and γH2AX. Two samples from each group were shown. (d) Quantification of (c). Data were presented as means ± SEM. *p < 0.05, three-way ANOVA followed by Tukey’s post hoc test. (e) qRT-PCR of the renal tissues from siCtrl and siKl mice (n = 6) for Tnfa and Il6 mRNAs. Data were presented as box-and-whisker plots. *p < 0.05, three-way ANOVA followed by Tukey’s post hoc test.
In this study, we made several meaningful findings toward a better understanding of the epigenetic mechanisms of renal aging. (1) KLOTHO and NRF2, two antiaging factors with antioxidant properties, were suppressed in aging kidneys due to DNMT1/3a/3b elevation-incurred transcriptional inhibition; (2) DNA-demethylating agent SGI-1027 and OLP effectively reduced the epigenetic losses of KLOTHO and NRF2, and mitigated the D-gal-induced renal aging alterations; (3) OLP possesses strong DNMT-inhibiting capabilities, as it effectively lowered the elevated DNMT1/3a/3b in aging kidneys; (4) In KLOTHO gene knockdown and NRF2 gene knockout mice, NRF2 and KLOTHO were mutually repressed and the antiaging effects of OLP were significantly abrogated, suggesting that KLOTHO and NRF2, and possibly their mutual regulations, are crucial in the development of renal aging; (5) KLOTHO had a greater genotype effect and effect size of interaction between KLOTHO genotype and OLP intervention comparing to that of NRF2, and KLOTHO knockdown in mice significantly reduced the anti-renal aging effects of DNMT inhibition by SGI-1027. Thus, our study demonstrated that aberrant DNMT1/3a/3b elevations and the resultant KLOTHO suppression and renal aging. Epigenetic cues upregulate DNMT1, DNMT3a, and DNMT3b, resulting in the promoter hypermethylation and expression suppressions of KLOTHO and NRF2, which accelerate D-gal-induced renal aging. DNA hypomethylations by DNA-demethylating agents correct the epigenetic alterations, recover the repressions of KLOTHO and NRF2 losses, and reduce the renal aging.

3 | DISCUSSION

In this study, we made several meaningful findings toward a better understanding of the epigenetic mechanisms of renal aging. (1) KLOTHO and NRF2, two antiaging factors with antioxidant properties, were suppressed in aging kidneys due to DNMT1/3a/3b elevation-incurred transcriptional inhibition; (2) DNA-demethylating agent SGI-1027 and OLP effectively reduced the epigenetic losses of KLOTHO and NRF2, and mitigated the D-gal-induced renal aging alterations; (3) OLP possesses strong DNMT-inhibiting capabilities, as it effectively lowered the elevated DNMT1/3a/3b in aging kidneys; (4) In KLOTHO gene knockdown and NRF2 gene knockout mice, NRF2 and KLOTHO were mutually repressed and the antiaging effects of OLP were significantly abrogated, suggesting that KLOTHO and NRF2, and possibly their mutual regulations, are crucial in the development of renal aging; (5) KLOTHO had a greater genotype effect and effect size of interaction between KLOTHO genotype and OLP intervention comparing to that of NRF2, and KLOTHO knockdown in mice significantly reduced the anti-renal aging effects of DNMT inhibition by SGI-1027. Thus, our study demonstrated that aberrant DNMT1/3a/3b elevations and the resultant suppressions of KLOTHO and NRF2 contribute significantly to renal aging, which can be effectively targeted by epigenetic intervention with synthetic or natural DNA-demethylating agents (Figure 6e).

Demonstrating the causative role of aberrant DNMT1/3a/3b elevations in aging kidney is an important discovery of our study. Past studies have established that aging is accompanied by a global decrease in DNA methylation in aging humans (Bjornsson, 2008) and mice (Singhal et al., 1987). However, DNA methylation modifications occur in a tissue and/or gene-specific manner and loss of DNA methylation of genome-wide is accompanied by a gain of methylation in CpG islands in or near gene promoters (van Otterdijk et al., 2013). DNMT inhibitor 5-Azacytidine reverses the aging phenotypes of mesenchymal stem cells (Kornicka et al., 2016), suggesting that gain of DNMT function promotes aging. However, DNA methylation status is affected by multiple regulatory proteins, including
DNA insulator proteins, DNMTs, methyl-CpG-binding protein, and TET (ten-eleven translocation) enzymes (Unnikrishnan et al., 2019). The information regarding the DNMT expression in aging kidney is limited, except that one study reported upregulated DNMT1 and DNMT3a (Pushpakumar et al., 2020). We found that DNMT1/3a/3b were all upregulated in natural and D-gal aging kidneys, which caused hypermethylation of KLOTHO and NRF2 gene promoters and renal aging, as DNA-demethylating agents corrected the epigenetic aberrations and improve the renal aging alterations in a KLOTHO and NRF2-dependent fashion. These observations provide a solid basis to further investigate whether the downregulation of other antiaging molecules is affected by the similar DNMT alterations in aging kidney or extrarenal organs.

One intriguing observation of our study is that both KLOTHO and NRF2 are repressed in aging kidneys and mutually inhibited during renal aging as demonstrated in KLOTHO gene knockdown or NRF2 gene knockout mice (Figure 5). To our knowledge, this is the first evidence that two major antiaging factors are functionally and mechanistically connected during renal aging process. Previous studies have shown that oxidative stress suppresses KLOTHO (Song et al., 1999) and KLOTHO deficiency or reactivation inversely affects oxidative stress by acting on NRF2 signaling (Maltese et al., 2017). Together with our data, these observations suggest that KLOTHO and NRF2 suppressions are affected by aberrant DNMT elevations, which form a regulatory loop in a positive fashion to additionally control renal aging and the antiaging effects of DNA demethylation. The antiaging effects of NRF2 are mainly attributed to its beneficial regulation of oxidative stress and inflammatory responses (Swamy et al., 2016), whereas KLOTHO seemingly affects renal aging through multiple signaling pathways and cellular processes, such as insulin/insulin-like growth factor-1 signaling, oxidative stress, inflammation, fibrosis, and apoptosis (Kurosu et al., 2005; Liu et al., 2011; Masuda et al., 2005; Seok-Jo Kim et al., 2017), likely through both NRF2-dependent (Maltese et al., 2017) and NRF2-independent (Yamamoto et al., 2005) signaling pathways. Therefore, although deficiency of either KLOTHO or NRF2 individually reduces the anti-renal aging effects by DNA demethylation, KLOTHO has a greater genotype effect and effect size of interactions between KLOTHO genotype and D-gal treatment and OLP intervention (Figure 5b, P2/η2 and P3/η2), and is the key target of the epigenetic anti-renal aging intervention.

The bioactive components from dietary food or medicinal plants are emerging as the rich sources of epigenetic drugs with tolerable side effects (Chistiakov et al., 2017). Several plant phenolic compounds, such as curcumin, epigallocatechin-3-gallate, and resveratrol, possess impressive antiaging and epigenetic modulating capacities (Casamenti & Stefani, 2017). An oleuropein analogue decarboxymethyl oleuropein aglycone is capable of inhibiting DNA methylation activity (Corominas-Faja et al., 2018). The olive phenolic compounds have shown effective protective potencies against aging-related diseases, including neurodegenerative disorders, atherosclerosis, and cancer (Casamenti & Stefani, 2017). OLP is the most prevalent phenolic component found in olive leave, seed, pulp, and peel with impressive anti-inflammatory and anti-oxidative stress activities (Ghanbari et al., 2012). We discovered that OLP inhibited the aberrant DNMT1/3a/3b elevations and DNMT sensitively recovered the KLOTHO and NRF2 losses in D-gal-treated renal cells. Experiments with kKLOTHO gene knockdown and NRF2 gene knockout mice further demonstrated that its regulations of KLOTHO and NRF2 suppression are critical for its antiaging functions. These data provide strong evidence that olive phenolic compounds contain potent antiaging activities and a significant part of which is attributed to its preservation of antiaging factors through a mode of DNA hypomethylation action.

In conclusion, we have demonstrated that aberrant DNMT1/3a/3b elevations and the resultant suppression of antioxidant aging suppressor KLOTHO and NRF2 promote renal aging, as DNA-demethylating agent SGI-1027 and OLP effectively reverse the epigenetic alterations and reduce the renal aging alterations in a KLOTHO and NRF2-dependent manner in D-gal mice, since synthetic demethylating compounds, such as decitabine (Ornstein et al., 2015), are not suitable for prophylactic use due to uncertain side effects and potential cytotoxicity. Future exploration of bioactive DNA-demethylating components from dietary or medicinal plants might yield effective epigenetic strategies to delay or alleviate renal aging and the aging-associated kidney disorders.

4 | MATERIALS AND METHODS

4.1 | Animal studies

The use of animals and the animal protocols complied with the ARRIVE guidelines, conformed to the European Directive 2010/63/EU, and were approved by the Institutional Animal Care Committee (IACUC) of Nanjing University. C57BL/6J and ICR mice were from Model Animal Research Center of Nanjing University. Mice were maintained under a standard environmental condition (25 ± 2°C; 12-h light-dark cycle) and allowed free access to water and regular sterile chow diets containing 20.6% protein, 12% Fat, and 67.4% carbohydrate (SWS9102; Xietong Pharmaceutical Bio-engineering Company).

Studies of natural renal aging were performed with young and old C57BL/6J mice of 2–25 months of age available in laboratory. The accelerated renal aging was induced by D-galactose (D-gal) treatment, which is a well-established aging model in vitro and in vivo (Azman & Zakaria, 2019). The experiments were performed with three sets of mice, namely 8- to 10-week-old ICR male mice, ICR male mice treated with siRNAcontrol (siCtrl) or siRNALKLOTHO (siKLO), and NRF2 knockout male mice and the control littersmates (Cai et al., 2015). The mice of each set were randomly divided into six groups (8 mice in each group): (1) vehicle control; (2) oleuropein (OLP, 50mg/kg daily by oral gavage) (Fiorella Casamenti & Stefani, ); (3) D-gal aging mice (D-gal subcutaneous injection, 500mg/kg daily) (Zhao et al., 2020); (4) OLP intervention (OLP-treated D-gal mice); (5) SGI-1027 (SGI, intraperitoneal injection at 2.5 mg/kg daily) (Reyes-Aguirre & Lamas, 2016); and (6) SGI intervention...
(SGI-treated D-gal mice). The effective dosages of OLP, D-gal, and SGI were based on previously published studies and optimized in our preliminary investigations (data not shown). The experiment went on for 8 weeks.

For in vivo siRNA-mediated mouse KLOTHO gene (klotho) knockdown, a small interfering RNA (siRNA) targeting klotho mRNA (5′ GCGACTACCCGAGGAGTA T-3′, 10 nm in 200 μl of phosphate-buffered saline; Genescript) was injected through tail vein one day before D-gal treatment and then twice a week during the experimental period. The control siRNA contained a scrambled RNA sequence.

4.2 | Renal histology and senescence-associated β-galactosidase (SA-β-Gal) staining

Paraffin-embedded kidney sections were stained with Masson's trichrome to determine the fibrosis-related collagen deposition as before (Zhang et al., 2017). For SA-β-Gal staining, the kidney sections (8 μm thickness) embedded in optimal cutting temperature compound were processed with a commercial kit (C0602; Beyotime Biotech) following the manufacturer’s instructions. Images were captured with a light microscope (Olympus, Japan). The extents of fibrosis and positive SA-β-Gal signals were blindly assessed and calculated as the ratio of collagen deposition or β-Gal-positive areas over the whole field based on ten randomly selected non-overlapping fields and averaged for each animal.

4.3 | Immunohistochemical and immunofluorescent staining

Immunohistochemical (IHC) and Immunofluorescent (IF) staining of kidney sections was performed essentially as before (Zhang et al., 2017) with primary antibodies to KLOTHO (A12028; Abclonal), NRF2 (sc-722; Santa Cruz), and CD68 (25747- I- AP; Proteintech) following routine procedures.

4.4 | Cell culture

Human renal tubule epithelial HK2 cells and human embryonic kidney HEK293 cells (ATCC) were maintained in DMEM/F12 or DMEM medium (Hyclone), respectively, with 10% FBS at 37°C in a humidified 5% CO₂ incubator. Cells were treated with D-galactose (G0750; Sigma), OLP (JBZ-0396; Jin Yibai BioTech), or SGI-1027 (HY13962; Genescript) following the manufacturer’s instructions. Images were captured with a light microscope (Olympus, Japan). The extents of fibrosis and positive SA-β-Gal signals were blindly assessed and calculated as the ratio of collagen deposition or β-Gal-positive areas over the whole field based on ten randomly selected non-overlapping fields and averaged for each animal.

4.5 | Western blot analysis

Protein expression of renal tissues or cells was analyzed by Western blotting following a regular procedure. The primary and secondary antibodies used were as following: KLOTHO (A12028), γH2AX (phosphorylated histone H2AX, AP0099; Abclonal); NRF2 (sc-722), α-SMA (sc-32251; Santa Cruz Biotech); DNMT1(ab188453), DNMT3b (ab79822; Abcam), DNMT3a (bs-0497R; Bioss); GAPDH (60004–1-lg; Proteintech); goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP (YFSA02 and YFSA01; Yifeixue Biotech).

4.6 | Plasmid constructions and cell transfection

The plasmids overexpressing flag-tagged DNMT1 (F-Dt1) and DNMT3a (F-Dt3a) were obtained from VectorBuilder. The plasmids were transfected into HEK293 cells with Lipofectamine 2000 (11668–019, Invitrogen, USA) according to manufacturer’s instruction.

4.7 | Quantitative real-time PCR (qRT-PCR)

Total RNAs from mouse kidneys or human renal tubular epithelial HK2 cells were isolated using a Total RNA Extraction kit (R401-01; Vazyme) according to the manufacturer’s instructions. Equal amounts of mRNA were reversely transcribed to cDNA using a HiScript RT SuperMix kit (R122-01; Vazyme) on a Viia 7 quantitative real-time PCR instrument (Thermo-Fisher Scientific). The primer sequences for TNF-α (Mouse gene Tnfa, mTnfaF and mTnfaR; human gene TNFA, hTNFAF, and hTNFAR), IL-6 (mouse gene Il6, mIl6F, and mIl6R; human IL6, hIL6F, and hIL6R), and the control GAPDH (mouse gene Gapdh, mGapdhF, and mGapdhR; human gene GAPDH, hGAPDHF, and hGAPDHHR) were listed in the Table 1. For each detection, a 20 μl of reaction volume included 10 μl of master mixture, 2 μl of diluted cDNA, 0.6 μl each primer, and sterile distilled water. The mRNA levels were calculated using the 2−ΔΔCt method and expressed as relative fold changes.

4.8 | Methylation-specific PCR and bisulfite-sequencing PCR

Prediction of CpG islands in Klotho (gene name for mouse KLOTHO) and Nfe2l2 (gene name for mouse NRF2) promoters and primer design for methylation-specific PCR (MSP) and bisulfite-sequencing PCR (BSP) were performed with the online software MethPrimer (http://www.urogene.org/methprimer). Genomic DNA was isolated from kidneys or cells using the Animal Tissues/Cells Genomic DNA Extraction Kit (D1700; Solarbio), and then modified by bisulfite treatment and purified by the SanPrep Column PCR Product Purification Kit (B518141; Sangon Biotech). The mouse Klotho promoter methylation on +594/+777 locus was assayed by MSP with methylated primer pair mKlotho-MF/mKlotho-MR (184 bp); unmethylated primers mKlotho-UNMF/mKlotho-UNMR (188 bp); and input DNA control primers Inp-mKlotho-MF/Inp-mKlotho-MR (180 bp). The Klotho promoter methylation (∼315/∼99 locus) of HK2 cells (human) was assayed with
| Primer Type | Sequences |
|-------------|-----------|
| PCR primer | mTnfoF: CATCTTCTAAATTCGAGTACAA |
|            | mTnfoR: TGGGAGTAGCAGGTTACAAAC |
|            | mL6F: GAGGATACATCTCCAAAGAGAC |
|            | mL6R: AAGTGCATACGTGTTCCATAAC |
|            | mGapdhF: TATGGTGGAGTCTAAGGTGT |
|            | mGapdhR: GCTATCATCTGAGCCAGGTTCT |
|            | hTNFAF: TGACATTGAGTGATCGCC |
|            | hTNFAR: GGCGCAGGACTGATTAGAA |
|            | hIL6F: TGAGGAGCTTGGTGTGTTG |
|            | hIL6R: ATTTTGTTTGGGTCAAGGG |
|            | hGAPDH: AAGTGCCCTTCTCTGATTTT |
|            | hGAPDHR: CTGTGCTGTAGCCAAATTCC |
| MSP primer | mKl-MF: GGTATCCGGGCTATTATTATAC |
|            | mKl-MR: CGACATAATCCCATAAAATCGAC |
|            | mKl-unMF: TTAATGGTATTGTGGGTATTTTTAATTG |
|            | mKl-unMR: CAACATAATCCCATAAAATCAAC |
|            | Inp-mKLF: TAGTTTTAGGAAGTAAAGGGTG |
|            | Inp-mKLR: AAATCCCCAAAAAACACACAAAA |
|            | hKl-MF: AAAGAGAAGTAAAGGAGGTTAC |
|            | hKl-MR: ACTCCGCTAACAATAATCATTAGC |
|            | hKl-unMF: AAGAGAAGTAAAGTGTTATGATAG |
|            | hKl-unMR: TCCACATAATCTACATAACAAAA |
|            | Inp-hKLF: CCAACTCACAATCCCTCCTAT |
|            | Inp-hKLR: TGGTTAATTAGGTTGGTTGGTTGAGG |
|            | mNfe2i2-MF: GTTTTAAAGGTTAGGTTTGGGAG |
|            | mNfe2i2-MR: AAATCAAAATAACTAAAATCGAC |
|            | mNfe2i2-unMF: TGGTTTTAACAGGTTAGGTTAGT |
|            | mNfe2i2-unMR: ATCCATTTAAAAACTACACAAAA |
|            | Inp-mNfe2i2F: ATTCTGTTAGGCTGTTG |
|            | Inp-mNfe2i2R: GGATGAGTCCAGCAGTCCAAAA |
| BSP primer | Bis-mKIF: TTTTGTGTTTTATTGGAGATTGG |
|            | Bis-mKIR: TCCCAATAATCAAATAACACACC |

Three randomly selected mice from control, D-gal, SGI, SGI/D-gal, OLP, or OLP/D-gal group were subjected for BSP assay. The PCR products were separated by electrophoresis, and the target DNA fragments were purified and cloned into pGEM T Easy Vector (A1360; Promega). Five colonies from each mouse/PCR reaction were randomly chosen for sequencing, and the percentages of methylated cytosines over total cytosines within the cloned fragment were calculated.

### 4.9 Serum biochemistry

Measurements of blood urea nitrogen (BUN, D799850-0100; Sangon Biotech) and serum creatinine (ab65340; Abcam) were performed with respective commercial assay kit, following manufacturer’s assay protocols. In particular for creatinine assay, creatinine is converted by creatinine to creatine that is further converted to sarcosine, whose oxidized product reacts with a probe to generate red color ($\lambda_{\text{max}} = 570$ nm) and fluorescence (Ex/Em = 538/587 nm), which were recorded with a microplate reader.

### 4.10 Statistical analysis

All data are expressed as means ±SEM or box-and-whisker plots as follows: Midline represents median, box is the 25th-75th percentiles, and whiskers are minimum and maximum. The data normal distributions and homogeneity test of variances were determined by Shapiro–Wilks test and Levene’s test, respectively. The calculation of main effect (P) and effect size (large effect size, $\eta^2 \geq 0.1379$; medium effect size, $0.0588 \leq \eta^2 < 0.1379$; small effect size, $0.0099 \leq \eta^2 < 0.0588$; Cohen, 1988) and statistical analysis, including Student’s $t$ test, two-way analysis of variance (ANOVA), or three-way ANOVA followed by Tukey’s post hoc test, were performed using SPSS V.22.0 software. Results were considered significant if the $p$ values were <0.05.

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### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### AUTHOR CONTRIBUTIONS

Qi Gao performed the investigation, data analysis, and draft writing; Fang Chen, Lijun Zhang, Ai Wei, Yongxiang Wang, and Zhwei Wu provided technique supports and research resources; Wangsen Cao designed the study, arranged the data, and wrote the manuscript. All authors have approved the final version of the manuscript.
Reyes-Aguirre, L. I., & Lamas, M. (2016). Oct4 methylation-mediated silencing as an epigenetic barrier preventing Müller Glia dedifferentiation in a murine model of retinal injury. *Frontiers in Neuroscience*, 10, 523. https://doi.org/10.3389/fnins.2016.00523

Semba, R. D., Cappola, A. R., Sun, K., Bandinelli, S., Dalal, M., Crasto, C., Guralnik, J. M., & Ferrucci, L. (2011). Plasma klotho and mortality risk in older community-dwelling adults. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 66A(7), 794–800. https://doi.org/10.1093/gerona/glq058

Semba, R. D., Ferrucci, L., Sun, K., Simonsick, E., Turner, R., Miljkovic, I., Harris, T., Schwartz, A. V., Asao, K., Kritchevsky, S., & Newman, A. B. (2016). Low plasma klotho concentrations and decline of knee strength in older adults. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 71(1), 103–108. https://doi.org/10.1093/gerona/glv077

Seok-Jo Kim, P. C., Eren, M., Jablonski, R. P., Yeldandi, A., Ridge, K. M., Scott Budinger, G. R., Kim, D.-H., Wolf, M., Vaughan, D. E., & Kamp, D. W. (2017). Klotho, an antiaging molecule, attenuates oxidant-induced alveolar epithelial cell mtDNA damage and apoptosis. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, 313(1), L16–L26. https://doi.org/10.1152/ajplung.00063.2017. Alveolar

Silva-Palacios, A., Ostolga-Chavarría, M., Zazueta, C., & Königsberg, M. (2018). Nrf2: Molecular and epigenetic regulation during aging. *Ageing Research Reviews*, 47, 31–40. https://doi.org/10.1016/j.arr.2018.06.003

Singhal, R. P., Mays-Hoopes, L. L., & Eichhorn, G. L. (1987). DNA methylation in aging of mice. *Comparative Study*, 41(3), 199–210. https://doi.org/10.1007/BF00573943

Song, X., Bao, M., Li, D., & Li, Y. (1999). Advanced glycation in D-galactose-induced mouse aging model. *Mechanisms of Ageing and Development*, 108(3), 239–251. https://doi.org/10.1016/S0047-6374(99)00222-6

Swamy, S. M., Rajasekaran, N. S., & Thannickal, V. J. (2016). Nuclear factor-erythroid-2-related factor 2 in aging and lung fibrosis. *The American Journal of Pathology*, 186(7), 1712–1723. https://doi.org/10.1016/j.ajpath.2016.02.022

Tarantini, S., Valcarcel-Ares, M. N., Yabluchanskiy, A., Tucsek, Z., Hertelendy, P., Kiss, T., Gautam, T., Zhang, X. A., Sonntag, W. E., de Cabo, R., Farkas, E., Elliott, M. H., Kinter, M. T., Deak, F., Ungvari, Z., & Csiszar, A. (2018). Nrf2 Deficiency exacerbates obesity-induced oxidative stress, neurovascular dysfunction, blood-brain barrier disruption, neuroinflammation, amyloidogenic gene expression, and cognitive decline in mice, mimicking the aging phenotype. *Journals of Gerontology. Series A, Biological Sciences and Medical Sciences*, 73(7), 853–863. https://doi.org/10.1093/gerona/glx177

Unnikrishnan, A., Freeman, W. M., Jackson, J., Wren, J. D., Porter, H., & Richardson, A. (2019). The role of DNA methylation in epigenetics of aging. *Pharmacology & Therapeutics*, 195, 172-185. https://doi.org/10.1016/j.pharmthera.2018.11.001

van Otterdijk, S. D., Mathers, J. C., & Strathdee, G. (2013). Do age-related changes in DNA methylation play a role in the development of age-related diseases? *Biochemical Society Transactions*, 41(3), 803-807. https://doi.org/10.1042/bst2012035

Yamamoto, M., Clark, J. D., Pastor, J. V., Gurnani, P., Nandi, A., Kurosu, H., Miyoshi, M., Ogawa, Y., Castrillon, D. H., Rosenblatt, K. P., & Kuro-o, M. (2005). Regulation of oxidative stress by the anti-aging hormone klotho. *Journal of Biological Chemistry*, 280(45), 38029–38034. https://doi.org/10.1074/jbc.M509039200

Zampieri, M., Ciccarone, F., Calabrese, R., Franceschi, C., Burkle, A., & Caiafa, P. (2015). Reconfiguration of DNA methylation in aging. *Mechanisms of Ageing and Development*, 151, 60–70. https://doi.org/10.1016/j.mad.2015.02.002

Zhang, Q., Liu, L., Lin, W., Yin, S., Duan, A., Liu, Z., & Cao, W. (2017). Rhein reverses Klotho repression via promoter demethylation and protects against kidney and bone injuries in mice with chronic kidney disease. *Kidney International*, 91(1), 144–156. https://doi.org/10.1016/j.kint.2016.07.040

Zhao, M., Tang, X., Gong, D., Xia, P., Wang, F., & Xu, S. (2020). Bungeanum improves cognitive dysfunction and neurological deficits in D-galactose-induced aging mice via activating PI3K/Akt/Nrf2 signaling pathway. *Frontiers in Pharmacology*, 11, 71. https://doi.org/10.3389/fphar.2020.00071

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