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Activation of the anti-aging and cognition-enhancing gene Klotho by CRISPR-dCas9 transcriptional effector complex

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Abstract

Multiple lines of evidence show that the anti-aging and cognition-enhancing protein Klotho fosters neuronal survival, increases the anti-oxidative stress defense and promotes remyelination of demyelinated axons. Thus, upregulation of the Klotho gene can potentially alleviate the symptoms and/or prevent the progression of age-associated neurodegenerative diseases such as Alzheimer's disease and demyelinating diseases such as multiple sclerosis. Here we used a CRISPR-dCas9 complex to investigate single-guide RNA (sgRNA) targeting the Klotho promoter region for efficient transcriptional activation of the Klotho gene. We tested the sgRNAs within the –1 to –300 bp of the Klotho promoter region, and identified two sgRNAs that can effectively enhance Klotho gene transcription. We examined the transcriptional activation of the Klotho gene using three different systems: a Firefly luciferase (FLuc) and NanoLuc luciferase (NLuc) coincidence reporter system, a NLuc knock-in in Klotho 3'-UTR using CRISPR genomic editing, and two human cell lines: neuronal SY5Y cells and kidney HK-2 cells that express Klotho endogenously. The two sgRNAs enhanced Klotho expression at both the gene and protein levels. Our results show the feasibility of gene therapy for targeting Klotho using CRISPR technology. Enhancing Klotho levels has a therapeutic potential for increasing cognition and treating age-associated neurodegenerative, demyelinating and other diseases, such as chronic kidney disease and cancer.

Keywords

Alzheimer's disease; multiple sclerosis; neuroprotection; myelin; chronic kidney disease; cancer

Introduction

The anti-aging protein Klotho was named after the goddess who spins the thread of life (Kuro-o et al., 1997). Klotho knockout mice have an accelerated aging phenotype recapitulating many of the features observed in aged humans (Kuro-o et al., 1997). Conversely, lifespan was extended by ~30% and an increased resistance to oxidative stress

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was observed in Klotho overexpressing mice (Kurosu et al., 2005). The single copy gene Klotho is a Type I transmembrane protein which is mainly expressed in the brain, kidney and reproductive organs (Masuda et al., 2005). Klotho is also shed by proteolytic cleaving resulting in a soluble form that is detectable in serum and CSF (Bloch et al., 2009; Chen et al., 2007; Matsumura et al., 1998). A third form of Klotho, found mainly in the brain, results from differential mRNA splicing and is secreted from the cell into the blood and CSF (Masso et al., 2015). Both the transmembrane and soluble forms of Klotho have pleiotropic actions throughout the body and are essential for many homeostatic functions (for extensive reviews see (Abraham et al., 2016; Kuro-o, 2012). Our lab demonstrated that Klotho protects neurons from oxidative stress by increasing expression of antioxidant factors, and promotes oligodendrocyte maturation *in vitro*. Furthermore, we showed that Klotho induces re-myelination *in vivo* in the cuprizone-induced demyelination model of MS (Zeldich et al., 2015). We further reported that Klotho overexpression reduces cognitive deficits in a mouse model of Alzheimer's disease, and that Klotho enhances cognition in humans and mice (Dubal et al., 2014; Dubal et al., 2015). Together, these findings suggest that increasing Klotho levels in the brain would have a beneficial effect to prevent cognitive impairment associated with normal aging and neurodegenerative diseases.

Recent advances in genome editing technology may provide a means to increase Klotho expression using CRISPR (clustered regularly interspaced short palindromic repeat) technology. CRISPR technology utilizes RNA guide sequences to target a specific gene or genomic locus where CRISPR-associated (Cas) proteins are utilized for genomic editing (Cho et al., 2013; Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013). Single or multiple loci can be targeted simultaneously by the simple base-pair complementarities between an engineered single-guide RNA (sgRNA) and a target genomic DNA sequence. A nuclease-deficient Cas9 (dCas9) and the VP64 fusion transcription activator in complex with guide RNA (gRNA) is used to specifically activate single or multiple genes simultaneously (Gersbach and Perez-Pinera, 2014; Kearns et al., 2014; Konermann et al., 2015). A recent study demonstrated that structure-guided engineering of a CRISPR-Cas9 complex mediated efficient transcriptional activation at endogenous genomic loci (Konermann et al., 2015). This result is based on the incorporation of bacteriophage coat protein MS2 RNA aptamers into the stem loop and loop two of the single-guided RNA (sgRNA2.0) that are exposed in the ternary Cas9 complex. The complex consists of three components: a dCas9-VP64 fusion, a MS2-P65-HSF1 activation helper protein, and a sgRNA. The incorporation of three distinct activation domains - VP64, P65 and HSF1 – into the complex aids robust transcriptional activation through synergy (Konermann et al., 2015).

In this study, we report the application of the CRISPR-Cas9 complex to activate human Klotho gene expression. We found that two sgRNAs enhanced Klotho expression in two different cell reporter systems, the neuronal cell line SY5Y, and the kidney cell line HK-2. These two cell lines express Klotho endogenously, but to different extents. The findings provide support for Klotho gene activation and demonstrate a potential therapeutic strategy for Klotho upregulation which may mitigate the symptoms of some neurodegenerative diseases and demyelinating disorders.

Materials and Methods

Design and cloning of sgRNA plasmid—Guides were designed to target the first 300 bp upstream of the Klotho translation initiation site (“A” in ATG as number +1). Target sequences were selected according to predicted off-target scores using online CRISPR design tool (developed by Zhang lab, <http://crispr.mit.edu> (Ran et al., 2013) and subsequently filtered for a maximum GC content of 25%, and minimal overlap of the target sequence. After filtering, four guides on the sense strand with the best off-target scores were selected, and then cloned into sgRNA (MS2) backbone (gift from Feng Zhang, Addgene #61424) at the BbsI site. Final plasmid constructs were confirmed by DNA sequencing. The primers used for cloning are:

sgRNA1 sense: 5'-CACCGGGCATAAAGGGGCGCGGCGC-3'

sgRNA1 anti-sense: 5'-AAACGCGCCGCGCCCTTTATGCCC-3'

sgRNA2 sense: 5'-CACCGGGCGGGGCGCGGGCATAAAG-3'

sgRNA2 anti-sense: 5'-AACTTTATGCCC GCGCCCCGCGC-3'

sgRNA3 sense: 5'-CACCGTGCCTTTCTCCGACGTCCGG-3'

sgRNA3 anti-sense: 5'-AAACCGGACGTCCGAGAAAGGCACC-3'

sgRNA4 sense: 5'-CACCGAAACGTCCTGCACGGCTCCG-3'

sgRNA4 anti-sense: 5'-AAACGGAGCCGTGCAGGACGTTTCC-3'

Cell culture—Cell lines were maintained under standard growth conditions and propagated in DMEM (Dulbecco's modified Eagle's medium) (4.5 g/ml glucose) containing 10% FBS (fetal bovine serum) (Atlanta Biologicals) and 1% penicillin/streptomycin (100 units/ml). For HK-2 kidney cells, DMEM:F12 (1:1) medium was used. All cell culture solutions were obtained from Cellgro unless otherwise noted in the text.

Cloning of Klotho (KL) 4 kb promoter into FLuc and NLuc luciferase coincidence reporter and stable cells generation—To clone the SV40 enhancer into pNLCoI1[luc2-P2A-NlucP/Hygro] Vector (Promega), the SV40 enhancer was amplified from pGL3 using the following primers using Clontech HiFi according to the manufacturer's protocol:

- 5'-AAATCGATAAGGATCCGATGGAGCGGAGAATGGGCGG-3' (forward primer)
- 5'-ATACGCAAACGGATCCGCTGTGGAATGTGTGTCAG-3' (reverse primer)

The PCR product was isolated and ligated with pNL vector by digestion with BamHI to generate the pNLCoI1-SV40 vector. The KL 1.8 kb promoter was digested from pGL3-KL1800 (King et al., 2012) with HindIII and XhoI, and subcloned into pNLCoI1-SV40 vector using the same restriction sites to generate pNLCoI1-SV40-KL1800.

To clone the KL 4 kb promoter, the additional 2.2Kb (–1800 to –4000) and the vector were amplified from HEK293 genomic DNA and pNLCoI1-SV40-KL1800, respectively, with the following primers and ClonAmp HiFi polymerase according to the manufacturer's protocol:

- 5'-CTCGCTAGCCTCGAGATCTATAGTGCCACATGGTGAC (forward primer for 2.2kb KL promoter insert)
- 5'-AGTATCACATTTCCTTCTAGAAGTGAAGATTGGAGTG (reverse primer for 2.2kb KL promoter insert)
- 5'-GGGAAATGTGATACTCCATGTAG-3' (forward primer for vector)
- 5'-CTCGAGGCTAGCGAGCTCAGGTACC-3' (reverse primer for vector)

The insert and vector bands (100 ng each) were ligated together using In Fusion kit (Clontech) to generate pNLCoI1-SV40-KL4000. Stable HEK-293 cells expressing 4kb of the KL promoter or the PGK promoter with the coincidence reporter were generated from single clones and selected using Hygromycin (Invivogen, USA) at a concentration of 75µg/ml for two weeks following Hygromycin 25µg/ml for maintenance.

Generation of a NLuc knock-in HEK293 cell line by CRISPR genomic editing

A double nicking strategy with Cas9n was used to introduce double-stranded breaks (DSB) by a pair of appropriately spaced and oriented sgRNAs at Klotho 3'-UTR to minimize off-target activity. Guides were designed to target Klotho 3'-UTR using the online CRISPR design tool as stated above and the best off-target scores (with a score of 100, and 0 off-target) gRNA pair was selected, and then cloned into pSpCase9n(BB)-2A-Puro (PX462) V2.0 plasmid (gift from Feng Zhang, Addgene #62987) digested with BbsI, and confirmed by DNA sequencing. The primers used for cloning were: gRNA pair 1 sense: 5'-CACCGGTCTCACTGGCATCTTGTTG-3'; gRNA pair 1 anti-sense: 5'-AAACCAACAAGATGCCAGTGAGACC-3'; gRNA pair 2 sense: 5'-CACCGCAGGGACACAGGGTTTAGAC-3'; gRNA pair 2 anti-sense: 5'-AAACGTCTAAACCCTGTGTCCCTGC-3'. The P2A-NLuc sequence was inserted at the DSB site of the Klotho 3'-UTR by co-transfection of a template DNA with 1kb homology arms to each side of the DSB site of the Klotho genomic DNA sequence. The template DNA containing the homology arms flanking the P2A-NLuc sequence was amplified from HEK cells genomic DNA or pNLCoI1[luc2-P2A-NLucP/Hygro] and constructed into pcDNA3.1 plasmid without CMV promoter region using ClonAmp HiFi and In Fusion cloning kit (Clontech) with the following primers: pcDNA3.1 forward: 5'-CTCGAGTCTAGAGGGCCCGCGTTTC-3'; pcDNA3.1 reverse: 5'-AAGCTTCGTATATCTGCCCCGTACATCGCG-3'; KL 2710 forward: 5'-AGATATACGAAGCTTCCCACATACTGGATGGTATCAATC-3'; KL 3700 reverse: 5'-AGTAGCTCCGCTTCCGACAGGACCTCAAAAATCATATAA-3'; P2A NLuc forward: 5'-GGAAGCGGAGCTACTAAGTTCAGCC-3'; P2A NLuc reverse: 5'-TTAGACGTTGATGCGAGCTGAAGC-3'; KL 3743 forward: 5'-CGCATCAACGTCTAATTGAGGGCCTTGACATAGGAAAC-3'; KL 4978 reverse: 5'-CCCTCTAGACTCGAGATTATGAAAGAAGGCAAAAAGTTGC-3'.

Isolation of clonal cell lines with P2A-NLuc insertion was achieved after co-transfection of the two pairs of gRNA and template plasmids into HEK293 cells by isolating single cells through serial dilutions in 96 well plates, followed by an expansion period to establish a new clonal cell line. NLuc positive lines were confirmed by PCR using Terra PCR direct kit (Clontech) with the following primers: Klotho intron 4 forward: 5'-GTGTTGTGTGCAAAATACGTAATAA-3'; NLuc reverse: 5'-TGACATGGATGTCGATCTTCAG-3'. The forward primer is located in intron 4 of Klotho gene upstream to the left homology arm, and can therefore avoid false positive stable colonies with random insertion into genomic DNA. As a control for a specific activation of the Klotho promoter in a coincidence reporter vector we have used pNLCoI4[luc2-P2A-NLucP/PGK/Hygro] Vector (cat. 1492, Promega, USA). For validation of the dual luciferase system we used as positive controls Ataluren (PTC124) (cat. S6003, SelleckChem, USA) for FLuc and Cilnidipine (cat. S1293, SelleckChem, USA) for NLuc luciferase.

Transfection—Cells were grown on poly-D-lysine-coated plates in 96, 12 or 6-well formats. Twenty-four hours after plating, cells reached 70–80% confluency and were transfected with a 1:1:1 ratio of Klotho specific targeting sgRNA plasmid or control sgRNA cloning backbone plasmid, MS2-P65-HSF1effector plasmid (Addgene, #61423), and dCas9-VP64 effector plasmid (Addgene, #61422). For positive controls, cells were transfected with a 1:2 ratio of Egr1 (a transcription factor known to activate Klotho transcription) or pcDNA3.1 empty vector. Transfections were carried out using Mirus TransIT-X2 with 100 ng, 1µg, or 2µg of total plasmid DNA per well in 96, 12, or 6-well plates, respectively. Transfection medium was removed and replaced with fresh medium after 5 hours.

Luciferase assays

For measurement of FLuc and NLuc expression the coincidence reporter vector under Klotho promoter, Nano-Glo® Dual-Luciferase® Reporter Assay System (cat. N1620, Promega) was used according to manufacturer's instructions. Briefly, 24 hours after transfection in white 96 well culture plates, the medium was replaced with 70µL of the fresh medium and assay was performed after an additional 24 hours. The 96 wells plates and the reagents were equilibrated to room temperature and 70µL ONE-Glo™ EX was added to the culture medium. The samples were incubated for 10 minutes and Firefly luminescence was measured with a plate reader (GloMax® Discover System, Promega). For measuring NLuc luciferase activity, 70µL of NanoDLR™ Stop& GloR Reagent was added to each well and the luminescence was measured after 20 minutes.

Klotho promoter-induced NLuc expression was measured in a NLuc knock-in HEK293 cell line using the Nano-Glo Luciferase Assay System (cat. N1110, Promega) according to the manufacturer's instructions. Briefly, 24 hours after transfection in white 96-well culture plates, the medium was replaced with 70µL of the fresh medium and assay was performed after an additional 24 hours. The 96-wells plates and reagents were equilibrated to room temperature and 70µL of Nano-Glo® Luciferase Assay Reagent was added to the culture medium. The samples were incubated for 10 minutes and the luminescence was measured using a plate reader (GloMax® Discover System, Promega).

qPCR Experiments and Analysis

48 hours after transfection, total RNA was isolated using the RNeasy mini plus Kit (QIAGEN) and 1 µg of total RNA was reversed transcribed using the SuperScript™ VILO™ cDNA Synthesis Kit according to the manufacturer's instructions (cat. 11754050, ThermoFisher scientific). Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was carried out for all genes of interest in each sample using human TaqMan Gene Expression Assays (Life Technologies): Klotho (Assay ID Hs00934627_m1 FAM); Peptidylprolyl isomerase A (PPIA) (Assay ID Hs04194521_s1; FAM) and beta actin (ActinB) (Assay ID Hs01060665_g1; VIC), on a BioRad 7900HT Real-Time PCR system using Fast Advanced Master Mix (Life Technologies), according to the manufacturer's protocol. The Klotho transcript was normalized to PPIA, and ActinB that were used as endogenous controls. Samples were run in triplicates at 1 µg of cDNA per reaction. The presence or absence of transcripts was assessed by whether a critical threshold (CT) value was determined or undetermined, respectively, at the threshold chosen by BioRad software v2.4. To normalize sample input, CT values were calculated for each gene. Data were analyzed further by the Ct method and fold changes in Klotho gene expression were determined by Gene Expression Module of CSX Manager software (BioRad) and a p-value 0.05 was considered significant.

Western Blotting—Protein Western Blotting was performed as described. Protein expression in Western blots was assessed and normalized by densitometry using ImageJ.

The antibodies used were for anti-Klotho (KO603, clone number KM2076, 1:500, Transgenic) and anti-Actin (1:1000, Cell Signaling, Danvers, MA).

Statistical Analysis—For Western Blotting and Luciferase assay, the significance was calculated using the traditional Student's t-test. Quantitative data are expressed as the means ± SD. Statistical comparisons between experimental groups were made using the two-tailed, unpaired Student's t-test. Probability values of $p < 0.05$ were considered significant.

Results

sgRNA target site selection

To activate endogenous Klotho gene transcription using CRISPR/Cas9 Synergistic Activation Mediator (SAM) system, we designed sgRNA in human Klotho promoter region using the online CRISPR design tool <http://crispr.mit.edu>. As recently reported targeting the proximal promoter region closer to the transcription start site (Konermann et al., 2015) results in the maximal level of gene activation using the SAM system. We therefore selected sgRNA within -300 to +1 of the Klotho promoter region (Figure 1). Four guides on the sense strand with the best off-target scores were selected for gene activation experiments.

Evaluation of Klotho gene activation using a dual luciferase coincidence reporter system

Cloning 4,000 bp of the Klotho promoter resulted in the stoichiometric expression of two orthologous reporters, firefly luciferase (FLuc) and PEST-destabilized NLuc luciferase (NLuc). The reporters are expressed from the same Klotho promoter using ribosome

skipping mediated by the P2A peptide (Figure 2A). To examine the efficiency of the sgRNA on Klotho gene activation, we introduced the four target site sequences into sgRNA expression plasmids and co-transfected them with the dCas9-VP64 and MS2-P65-HSF1 expression plasmids into stable FLuc NLuc coincidence reporter HEK293 cells. The results showed that all sgRNAs activated Klotho gene transcription, with sgRNA3 and 4 performing better (2–3 fold increase) (Figure 2B). In the 4K Klotho promoter reporter system, the positive control transcription factor Egr-1 for Klotho gene activation (Choi et al., 2010) resulted in 3–4 fold increase of Klotho gene expression, comparable to sgRNA activation (Figure 2B). We tested the specificity of Klotho gene activation in HEK293 cells stably transfected with a control promoter of the yeast gene encoding phosphoglycerate kinase (PGK) in the FLuc NLuc coincidence reporter vector. The results in the PGK promoter system showed that none of the sgRNAs increased PGK promoter activity. sgRNA3 lightly reduced PGK-1 promoter activity (Figure 2C). We validated the PGK promoter system using two known luciferase inhibitors: Cilnidipine, a NLuc luciferase inhibitor, and Ataluren (PTC124), a FLuc inhibitor. Both agents produced the expected luciferase responses in the PGK promoter system. Cilnidipine elicited NLuc-specific increase that fits the 7-parameters bell-shape, while the treatment with Ataluren led to stabilization of the FLuc reporter manifested in an enhanced signal at intermediate concentrations followed by a signal decrease at high concentrations (Figure 2D). Our validation results using Cilnidipine and Ataluren are in line with the results obtained by us with the Klotho 4kb promoter in the coincidence reporter vector (data not shown) and with the previously published protocol (Schuck et al., 2017). These results suggested that sgRNAs specifically activated Klotho gene expression but not the PGK promoter.

Evaluation of Klotho gene activation using a CRISPR NLuc knock-in HEK293 cell line

To monitor endogenous Klotho gene expression, we used CRISPR genomic editing technology to generate a HEK knock-in line with NLuc inserted into 3'-UTR of the Klotho gene (Figure 3A). In this system, Klotho transcript and NLuc are expressed off the same Klotho promoter by the P2A sequence. Thus, we can monitor Klotho gene transcription using NLuc activity. The positive lines were selected by NLuc assay. Further confirmation of successful CRISPR genomic editing vs random incorporation into genome was performed by direct PCR amplification from NLuc positive cells using two primer pairs, the forward primer located in the intron 4 of Klotho gene upstream of the left homology arm, and the NL reverse primer located on the NLuc sequence (Figure 3B). To examine the efficiency of the sgRNA on Klotho gene activation, we introduced sgRNA /dCas9-VP64 /MS2-P65-HSF1 plasmids into NLuc knock-in HEK293 cell line. The results showed that all sgRNAs activated Klotho gene transcription, with sgRNA3 and 4 performing better (2–3 fold increase) (Figure 3C). Egr-1 was used as positive control (Figure 3C).

Evaluation of Klotho gene activation in HK-2 and SY5Y cells

Klotho is mainly expressed in brain and kidney (Masuda et al., 2005). Therefore, we sought to investigate whether the sgRNA targeting Klotho promoter region can activate Klotho expression in cell lines from these organs that express Klotho endogenously. We transfected the SAM complex into HK-2 cells and examined Klotho mRNA and protein levels, and found that sgRNA3 and 4 enhanced Klotho mRNA 2–4 fold (Figure 4A) by qPCR. Klotho

protein level was evaluated by Western Blot. The results showed that sgRNA increased Klotho protein expression about 4–6 fold (Figure 4B and C). The Egr-1 overexpression resulted in higher Klotho gene expression than either sgRNA targeting by SAM complex (Figure 4C). We then tested the effects of sgRNAs on Klotho activation in neuronal SY5Y cells. In this system, Klotho mRNA level is detectable by qPCR, however the protein level is undetectable by Western blot. We transfected the SAM complex into SY5Y cells and examined Klotho mRNA levels by qPCR, and confirmed that sgRNA3 and the positive control Egr-1 enhanced Klotho mRNA 15–20 fold (Figure 4B). sgRNA4 enhanced Klotho gene expression about 4 fold but did not reach significance (Figure 4B). In both HK-2 and SY5Y cells, sgRNA3 increased mRNA expression more than sgRNA4, however, in terms of protein level enhancement, sgRNA4 worked better than sgRNA3 in HK-2 cells (Figure 4C and D), suggesting there was potential post-transcriptional regulation of Klotho protein expression.

Discussion

In this study, we upregulate Klotho gene expression using a CRISPR-dCas9 SAM complex to investigate whether we could employ sgRNA targeting of the Klotho promoter region for efficient transcriptional activation of the Klotho gene. We identified two sgRNAs that can effectively enhance Klotho gene expression on the gene and protein level using three different assessments: a FLuc and NLuc coincidence reporter system, a NLuc luciferase knock-in in Klotho 3'-UTR using CRISPR genomic editing, and two cell lines that endogenously express Klotho: the human neuronal cell line SY5Y, in which the levels of Klotho are detectable by qPCR, but not detectable at the protein level, and a human kidney cell line HK-2 that endogenously expresses Klotho in an amount sufficient to be detected on the mRNA and protein levels. It is not surprising that we were unable to detect Klotho in SY5Y on the protein level, but on the mRNA level only: the Klotho transcript in SY5Y cells reaches a critical threshold (CT) value at around the 32nd cycle, providing evidence for the low abundance in these cells, which we have observed with other cell lines where Klotho is not detectable with the existent antibodies by Western Blot. In contrast, in HK-2 cells the Klotho transcript crosses a critical threshold (CT) value at the 26th cycle, and that explains the protein detection on Western Blot.

Potential applications of CRISPR/RNA-guided genomic editing are diverse across many areas of science and biotechnology. CRISPR/Cas9 technology enables inexpensive and high-throughput interrogation of gene function, likely due to the simplicity, high efficiency and versatility of the system. In this study, we used CRISPR technology to activate Klotho gene expression, and monitor its activation with a P2A-NLuc CRISPR knock-in cell line. The precise homology-directed repair pathway allows insertion of NLuc reporter in the Klotho 3'-UTR, and monitor of Klotho gene transcript from the endogenous Klotho promoter using ribosome re-entry via the P2A sequence. The knock-in line has potential to be used for high-throughput drug screening to study Klotho gene regulation in a more physiologic system.

Taking advantage of the therapeutic potential of secreted proteins, such as Klotho, poses particular challenges for translational neurology, because of the profound difficulty in delivering these proteins across the blood-brain barrier (BBB). Another way to address this

issue is by identifying small molecule compounds that are able to penetrate the BBB and stimulate the production of Klotho within the brain as a possible pathway in drug discovery. The luciferase reporter is commonly used in high throughput screens to identify compounds that increase gene expression. However, many compound libraries contain molecules that may activate the reporter, either luciferase or other reporters used in screens (Hasson et al., 2015). These compounds present a potential challenge in the gain-of-signal assays since by binding reporters they increase the half-life of the reporter, producing a higher signal, which is translated to an activation-like signal in the cell based assays (Cheng and Inglese, 2012, Hasson et al., 2015).

In our previously published work we conducted a high-throughput screen (HTS) to identify compounds that activate Klotho transcription using the Klotho 1.8kb promoter to drive expression of firefly luciferase, however the rate of false positive hits increasing the luciferase signal independently of Klotho promoter was high and the discrimination between the real and false positive hits was challenging (King et al., 2012). This limitation can be mitigated with a ‘coincidence reporter:’ a system that allows expression of both firefly luciferase and NanoLuc® Luciferase from the same mRNA transcript. The stoichiometric expression of both luciferases is achieved by use of the P2A sequence, which promotes a ribosomal skip and expression of the two unfused enzymes with distinct compound interaction profiles. We are currently utilizing this system for a new HTS of a library of 50,000 compounds. Transfections using Egr-1 transcription factor, known to activate Klotho transcription, provide a reliable positive control for the assay leading to consistent and significant increase in the expression of both luciferases. In this current assay we are able to identify false hits caused by direct interaction with one or the other luciferases and to distinguish them from true hits that show a similar response for both luciferases, adding reproducibility to the assay.

Compared to the CRISPR NLuc knock-in HEK293 cell line, the 4kb Klotho promoter coincidence reporter system appears more suitable for HTS purpose. The signal observed in the 4kb Klotho promoter coincidence reporter system is higher than in the NLuc knock-in line likely because endogenous the Klotho promoter is not very active due likely to the presence of repressors or methylation since the Klotho gene is mostly expressed in specific tissues such as kidney and brain and is downregulated with age and in disease.

Modulation of the levels of one protein, Klotho, in mice results in dramatic changes in lifespan and cognition (Dubal et al., 2015; Kuro-o et al., 1997; Masso et al., 2017). Knocking-out the gene, while not affecting development or adolescence, results in premature death in adulthood (Kuro-o et al., 1997). In contrast, overexpression of Klotho increases lifespan by ~30% over normal mice (Kurosaki et al., 2005). In the CNS Klotho has pleiotropic functions, it is neuroprotective and anti-oxidative (Zeldich et al., 2014) and is involved in oligodendrocytes maturation and myelination *in vivo* and *in vitro* (Chen et al., 2015; Chen et al., 2013; Zeldich et al., 2015). Klotho overexpression reduces cognitive deficits in a mouse model of Alzheimer’s disease, and enhances cognition in humans and mice (Dubal et al., 2014; Dubal et al., 2015; Masso et al., 2017). Increasing Klotho levels in the brain would have a beneficial effect to prevent cognitive impairment in the aged population and protect against neurodegeneration. In a high throughput screen of small molecules that would

enhance Klotho expression, we have identified a number of promising compounds that elevate Klotho expression at the RNA, protein and functional levels (Abraham et al., 2012; King et al., 2012). However, most compounds have inevitable off-target effects and cytotoxicity. Here we provide information of activation of the anti-aging gene Klotho via CRISPR/RNA-guided transcription activation. This system presents an alternative assay for identifying specific Klotho gene activation. The results described here are valuable for the study of Klotho gene regulation and have great potential in gene therapeutics. The technology could be tested *in vivo* in animal models of various diseases, including the currently untreatable multiple sclerosis and neurodegenerative diseases such as Alzheimer's disease.

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Abbreviations

CRISPR	clustered regularly interspaced short palindromic repeat
Cas	CRISPR-associated
dCas9	nuclease-deficient Cas9; sgRNA
FLuc	firefly luciferase
NLuc	NLuc luciferase
3'-UTR	3'-untranslated region
PCR	polymerase chain reaction
DMEM	Dulbecco's modified Eagle's Medium
PBS	phosphate buffered saline
FBS	fetal bovine serum
BSA	bovine serum albumin
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
PGK	phosphoglycerate kinase

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Figure 1. sgRNA target site selection for Klotho gene activation. Schematic view of the Klotho genomic area surrounding the promoter region, showing the sgRNA targets. The 20-nt sgRNA recognition sequence (blue), and protospacer-adjacent motif (PAM) (pink) are indicated. The number indicates the first nucleotide of the gRNA relative to Klotho translation start site ("A" in ATG as number +1).

Figure 2.

Klotho gene activation using a dual luciferase coincidence reporter system. (A) Schematic view of the Firefly, NLuc coincidence reporter system under control of Klotho 4kb promoter. The P2A ribosome skipping sequence is indicated. (B) Fold activation of Klotho gene expression by SAM evaluated with the dual luciferase coincidence reporter system in stable HEK293 cells. Cells were analyzed by dual luciferase assay 2 days after transfection with dCas9-VP64, MS2-P65-HSF1 and the indicated sgRNA. Negative control: sgRNA cloning backbone empty vector. Positive control: Egr-1 transfected cells. Data are expressed as fold over negative control. Error bars show standard deviation among 6 replicates. (C) Fold change of the PGK promoter activities by SAM evaluated with dual luciferase coincidence reporter system in stable HEK293 cells as in (B). (D) Validation of the PGK promoter system in stable HEK293 cells using Cilnidipine and Ataluren that were added to the cells 24 hours after plating and results assayed 24 hours later. Asterisks (*) indicate statistical significance of $p < 0.05$ by t -test.

Figure 3.

Klotho gene activation using a CRISPR NLuc knock-in HEK293 cell line. (A) Schematic view of the Klotho genomic area surrounding the 3'-UTR region, showing the stop codon, the sgRNA targets, and polyA tail. The P2A-NLuc sequence was inserted at the DSB site of the Klotho 3'-UTR. (B) PCR confirmation of positive clones using forward primer located at Klotho intron 4 (Int4) and NLuc reverse primer (NLrv) indicated in A. Cell line numbers are indicated. (C) Fold activation of Klotho gene expression by SAM evaluated with a CRISPR NLuc knock-in HEK293 line. Cells were analyzed by NLuc luciferase assay 2 days after transfection with dCas9-VP64, MS2-P65-HSF1 and the indicated sgRNA. Negative control: sgRNA cloning backbone empty vector. Positive control: Egr-1 transfected cells. Data are expressed as fold over negative control. Error bars show standard deviation among 6 replicates. Asterisks (*) indicate statistical significance of $p < 0.05$ by t -test.

Figure 4.

Klotho gene activation in kidney HK-2 (A, C, D) and neuronal SY5Y cells (B). (A) Fold activation of Klotho mRNA by SAM evaluated in the HK-2 line by qPCR 2 days after transfection with SAM complex. Data are expressed as fold over negative control. Error bars show standard deviation among 6 replicates. (B) Fold activation of Klotho mRNA by SAM evaluated in neuronal SY5Y line by qPCR 2 days after transfection with SAM complex. Data are expressed as fold over negative control. Error bars show standard deviation among 6 replicates. (C) Western blot analysis of Klotho gene expression by SAM evaluated in HK-2 cells. Two days after transfection with SAM complex, cell lysates were analyzed by WB against anti-Klotho antibody using Actin as internal control. (D) Statistical analysis of the results from B. The intensities of the 130 kDa Klotho bands were analyzed and normalized to the Actin bands using the average intensity of the controls as 1 from 3 independent experiments. Error bar indicates standard deviation. Significance of results using student's *t*-test: *, $p < 0.05$.