Establishment of a cell-based assay to screen regulators for Klotho gene promoter

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KEY WORDS Klotho protein; aging; biological factors; drug screening

ABSTRACT

AIM: To discover compounds which can regulate Klotho promoter activity. Klotho is an aging suppressor gene. A defect in Klotho gene expression in the mouse results in the phenotype similar to human aging. Recombinant Klotho protein improves age-associated diseases in animal models. It has been proposed that up-regulation of Klotho gene expression may have anti-aging effects. **METHODS:** Klotho promoter was cloned into a vector containing luciferase gene, and the reporter gene vector was transfected into HEK293 cells to make a stable cell line (HEK293/KL). A model for cellular aging was established by treating HEK293/KL cells with H_2O_2. These cells were treated with extracts from Traditional Chinese Medicines (TCMs). The luciferase activity was detected to identify compounds that can regulate Klotho promoter. **RESULTS:**The expression of luciferase in these cells was under control of Klotho promoter and down-regulated after H_2O_2 treatment. The down-regulation of luciferase expression was H_2O_2 concentration-dependent with an IC_{50} at approximately 0.006 %. This result demonstrated that the Klotho gene promoter was regulated by oxidative stress. Using the cell-based reporter gene assay, we screened natural product extracts for regulation of Klotho gene promoter. Several extracts were identified that could rescue the H_2O_2 effects and up-regulated Klotho promoter activity. **CONCLUSION:** A cell -based assay for high-throughput drug screening was established to identify compounds that regulate Klotho promoter activity, and several hits were discovered from natural products. Further characterization of these active extracts could help to investigate Klotho function and aging mechanisms.

INTRODUCTION

Klotho gene encodes a membrane protein that shares sequence similarity with beta-glucosidase enzymes. A defect in Klotho gene expression in the mouse results in a syndrome that resembles human aging, including a short lifespan, infertility, arteriosclerosis, skin atrophy, osteoporosis, and emphysema[1-3]. The Klotho protein protects the cardiovascular system through endothelium-derived NO production by humoral pathways[1]. In addition, Klotho protein may regulate B lymphopoiesis via its influence on the hematopoietic microenvironment[2]. It has also been shown that Klotho plays important roles in the maintenance of normal energy homeostasis[3]. Local expression of the Klotho gene retards or partially improves pathological abnormalities in several organs of Klotho mutant mice[4].

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Klotho protein functions through a signaling pathway involving a circulating hormonal factor. Administering recombinant Klotho protein into limited organs rescued aging phenotypes of Klotho mutant. Recently, we found that the Klotho gene expression in the hippocampus was down-regulated in aged and memory impaired rats, suggesting that the protein may also play important roles in regulating high brain functions, such as learning and memory. Furthermore, recent studies suggested that Klotho is a candidate gene involved in several common age-related diseases in humans[5].

Since Klotho gene deficit leads to aging phenotype and recombinant Klotho protein improves age-related diseases, we propose that Klotho promoter activator should increase Klotho gene expression and have beneficial effects on age-related disorders. To test this hypothesis, we established a cell-based reporter gene assay to screen natural product extracts that can activate Klotho gene promoter. The natural product library was derived from Traditional Chinese Medicine (TCM). SBG530 (serial number in our lab) extract was identified which can activate human Klotho promoter. The extract has no effects on other promoters such as VIP promoter, cAMP response element, multiple response element and serum response element. The active extracts should provide useful tools to investigate the functions of Klotho gene during aging process.

MATERIALS AND METHODS

Cloning of Klotho promoter Human genomic DNA was used to clone Klotho promoter. Primers 5'-GGG AAG CTT GCT GCG CGG GAG CCA GGC TCC GGG GC-3' and 5'-GGC CTC GAG TGC TAA TAT ATG CTG GCT GGA GTT GG-3' were used for PCR cloning. An expected 1.6 kb PCR product was produced and cloned into pMD18-T vector (TaKaRa). The identity of the PCR product was confirmed by DNA sequencing. The vector was digested with *Hind*III and *Xho*I, and the insert was subcloned into *Hind*III and *Xho*I digested pGL_3 vector (Promega) to generate the luciferase based reporter gene construct pGKL.

Cell culture, transfection and stable cell line generation HEK293 cells were maintained in DMEM with 10 % FBS (Gibco) and 1 % non-essential amino acid at 37 ºC, 5 % CO_2. HEK293 cells were transfected with 2 μg of pGKL and pcDNA3.1 mixture in a ratio of 5:1 using lipofectin (Invitrogen) according to the manufacturer's protocol. Twenty-four hours after transfection, cell culture medium was replaced with fresh medium containing G418 at final concentration of 800 mg/L. Single cell was selected using limited dilution in the presence of G418 to generate stable cell line HEK293/KL.

Luciferase assay The luciferase activity was measured according to the protocol from Promega. Briefly, 100 μL of HEK293/KL cells at 1×10^5 cells/mL were added into each well of white 96-well plate (flat, clear bottom, Costar). The cells were maintained in DMEM containing 10 % FBS (Gibco) and 1 % non-essential amino acid, and incubated at 37 ºC, 5 % CO_2 overnight. Then, 10 μL of natural product extract was added into each well and incubated for 6-8 h. Finally, 110 μL of luciferase substrate Bright Glo™ (Promega) was added into each well. Luciferase activity was measured using Analysit HT (Molecular Device).

Natural product extracts All natural product samples were derived from Traditional Chinese Medicine (TCM). Samples were purchased from local TCM pharmacy. TCM samples were extracted with water at 80 ºC for 2 h. Each sample was extracted two times, each time with about 250 mL. Two extracts were combined to 500 mL and concentrated to 100 mL under decompression. The crude extracts were used for the reporter gene assay as described above.

RESULTS

Establishment of a cellular model of aging It is well known that high levels of oxidative stress drive aging process. We treated the HEK293/KL cells with H_2O_2 to generate a cellular model of aging. Our result showed that H_2O_2 treatment reduced the expression of luciferase (Fig 1). The reduction of luciferase activity by H_2O_2 was in a dose-dependent manner with an IC_{50} at approximately 0.006 %. It is possible that oxidative stress may down-regulate the Klotho promoter activity and lead to the reduction of luciferase gene expression.

There are several possible ways that H_2O_2 treatment reduces the luciferase activity in HEK293/KL cells. One possibility is that H_2O_2 could directly affect the interaction between luciferase with its substrate, therefore decreased its enzymatic activity. To test this possibility, we added H_2O_2 and luciferase substrate Bright Glo™ to HEK293/KL cells at same time and measured the luciferase activity. The result showed that addition of H_2O_2 did not affect the activity of luciferase (Fig 2). Another possibility is that long-term treatment of cells with H_2O_2 may oxidize luciferase and decrease its activity. To test this, we first lysed HEK293/KL cells to

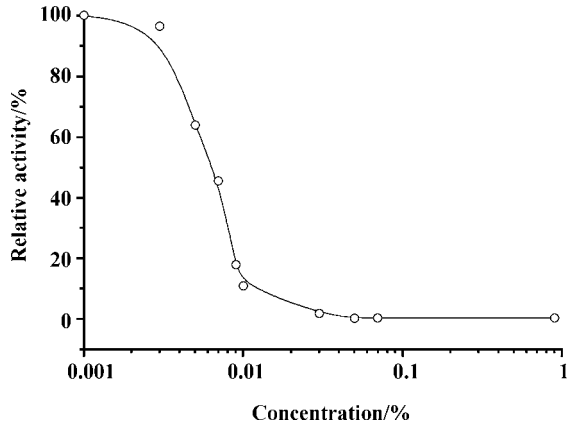

Fig 1. Luciferase activity of HEK293/KL cells treated with different concentrations of H_2O_2. HEK293/KL cells were treated with different concentrations of H_2O_2 for 4 h and luciferase activity was measured thereafter (IC_{50}=0.006 %).

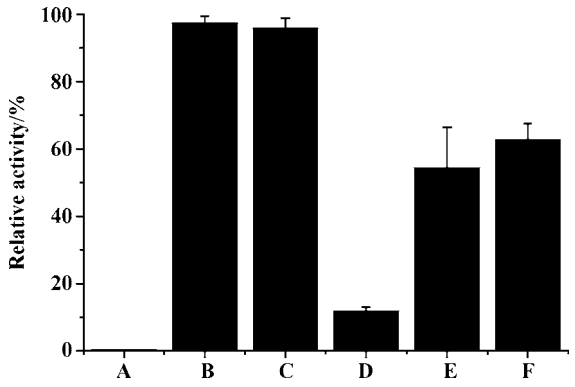

Fig 2. Effects of H_2O_2 on luciferase activity. A) HEK293 cells without treatment; B) HEK293/KL cells without treatment; C) HEK293/KL cells without treatment, luciferase activity was measured using Bright Glo™ in the presence of 0.006 % H_2O_2; D) HEK293/KL cells treated with 0.006 % H_2O_2 for 6 h; E) HEK293/KL were lysed with lysis buffer, incubated with PBS for 1 h; F) HEK293/KL cells were lysed with lysis buffer, incubated with PBS and 0.006 % H_2O_2 for 1 h.

release luciferase into the cell culture medium. We then treated the medium with 0.006 % H_2O_2 for one hour and measured the luciferase activity. The result demonstrated that H_2O_2 treatment did not affect luciferase activity (Fig 2). Taken together, our results suggest that H_2O_2 treatment generated oxidative stress to HEK293/KL cells and lead to the down-regulation of Klotho gene promoter. This cellular model of aging has been further modified and used for compounds screening to identify Klotho promoter activator.

Identification of extracts for activation of Klotho promoter We have used the cell-based reporter gene assay to screen natural product extracts and to identify activators for human Klotho promoter. HEK293/KL cells were treated with 0.006 % H_2O_2 for one to nine hours and luciferase activity was determined. The result showed that six-hour incubation gave the best result (Fig 3). Therefore, we have used six-hour incubation time for all of our experiments unless otherwise indicated. We prepared extracts from over three hundred different TCM samples to screen Klotho promoter activators. Our previous experiments from other screens showed that although most of the active extracts showed activities at high concentration, some of the extracts had no or even inhibitory effects at high concentrations, but high activity at low concentrations. To increase the chances for finding Klotho promoter activators, we used original concentration as well as 5-fold diluted extract samples for the screening. Indeed, we found that some of the samples had high activities after dilution (Fig 4). We have screened extract samples using HEK293/KL cells in the presence of 0.006 % H_2O_2. After 6-h treatment, luciferase activity was measured. We defined active samples as the luciferase activity higher than (mean+3SD) compared with H_2O_2 treatment alone. Five active extracts were identified from more than three hundred samples. The active extracts increased luciferase activity either through activation of Klotho promoter or by inhibition of H_2O_2 activity. To test these possibilities, HEK293 cells were treated with 0.006 % H_2O_2 for 3 h, then H_2O_2 was removed by washing cells with PBS, and finally the cells were treated with the positive extracts for another 5 h. Luciferase

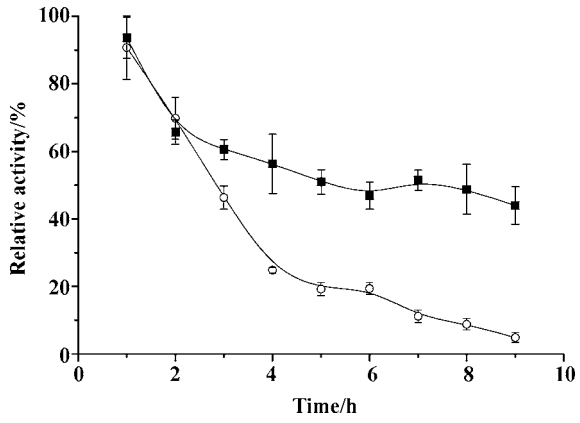

Fig 3. Luciferase activity of HEK293/KL cells treated with H_2O_2 or SBG530 extract for different period of time. ○: HEK293/KL cells were treated with 0.006 % H_2O_2. ■: HEK293/KL cells were treated with 100 μg/L SBG530 extract and 0.006 % H_2O_2 (*t*-test, P<0.01 after 4 h-treatment).

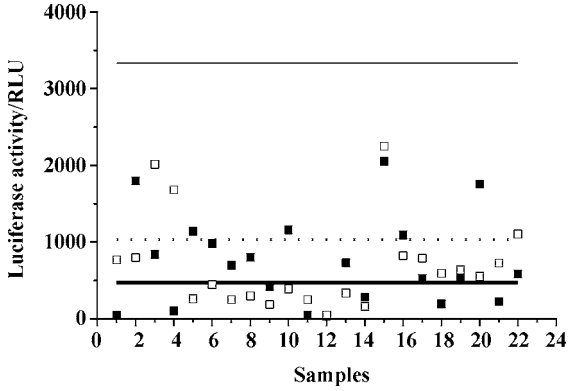

Fig 4. Extracts screening for Klotho promoter activator. HEK293 cells were treated with 0.006 % H_2O_2 and 10 μL plant extracts for 6 h in a total volume of 110 μL. Two different concentrations of extracts were used for the screening, each concentration tested in duplicate. □ represents original extracts and ■ represents 5-fold diluted samples. The mean (n=4) of untreated cells is represented by the thin line. The (mean+3SD) of untreated cells is represented by the dot line. The mean (n=4) of H_2O_2-treated cells is designated by the solid line.

activity was determined after these treatments. It was shown that extract of SBG530 could still increase luciferase activity, suggesting that the plant extract directly activated Klotho promoter (Fig 5).

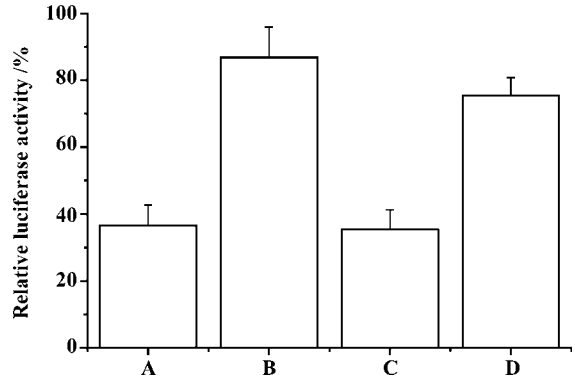

Fig 5. Effect of the SBG530 extract on Klotho promoter activity. A) HEK293/KL cells were pre-incubated with 0.006 % H_2O_2 for 3 h, washed with PBS, incubated in fresh medium for another 5 h. B) HEK293/KL cells were pre-incubated with 0.006 % H_2O_2 for 3 h, washed with PBS, incubated with 100 μg/L SBG530 for another 5 h. C) HEK293/KL cells were treated with 0.006 % H_2O_2 for 8 h without SBG530 extract. D) HEK293/KL cells were treated with 0.006 % H_2O_2 for 8 h with 100 μg/L SBG530 extract.

Characterization of Klotho promoter activator We firstly determined the dose-response effects of SBG530 extract on luciferase activity in H_2O_2-treated HEK293/KL cells. Cells were treated with different concentrations of SBG530 extract for 6 h in the presence of 0.006 % H_2O_2, and then luciferase activity was measured. Fig 6 shows the dose-response curve with

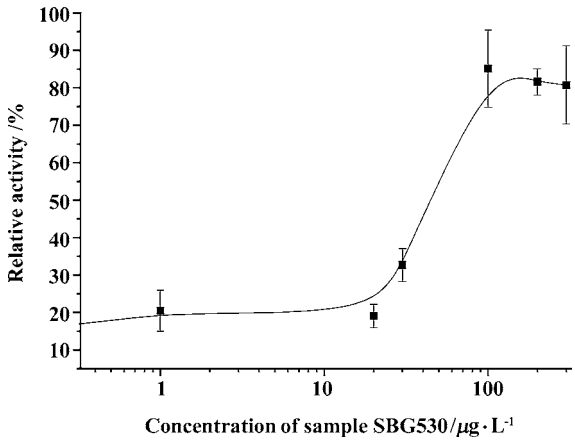

Fig 6. Concentration-dependent effect of gentian root extract on luciferase activity. HEK293/KL cells were treated with different concentrations of SBG530 extract and 0.006 % H_2O_2 at the same time, incubated at 37 ºC, 5 % CO_2 for 6 h, and luciferase activity was measured.

an EC_{50} at about 50 μg/L. To further characterize its activity, the extract sample from SBG530 was tested for the effects on other transcriptional responsive elements and promoters. We have generated a stable HEK293 cell line containing a reporter gene construct. This construct includes serum response element (SRE), cAMP response element (CRE), multiple response element (MRE), and VIP promoter, which linked to luciferase gene (HEK293/SMC/Luci). Forskolin stimulated the luciferase expression (Fig 7), indicating that the reporter gene construct can be activated in the stable cell line. Next, we tested the effects of H_2O_2 on the cell line. Our result indicated that H_2O_2 had no effects on luciferase expression in HEK293/SMC/Luci cell line (Fig 7), suggesting that the down-regulation of luciferase expression in HEK293/KL cells was not caused by cell damage, but most likely through inhibition of Klotho promoter. Finally, we demonstrated that the SBG530 extract could not activate the luciferase expression in HEK293/SMC/Luci cells (Fig 7). These results suggested that the SBG530 extract could not enhance the luciferase expression through SRE, CRE, MRE, and VIP promoters.

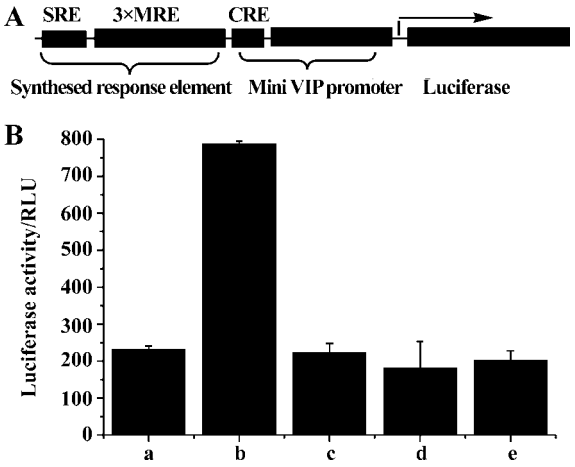

Fig 7. Effect of SBG530 extract on luciferase activity in HEK293/SMC/VIP/Luci cells. A) Construct used to make stable cell line HEK293/SMC/VIP/Luci. The construct contains serum response element (SRE), multiple response element (MRE), cAMP response element (CRE) and VIP promoter linked to luciferase gene. B) Effect of SBG530 extract on MRE, SRE, CRE, and VIP promoter. a-e: represent luciferase activity of the HEK293/SMC/VIP/Luci cells with different treatment. a, without treatment; b, treated with forskalin; c, treated with 0.006 % H_2O_2; d, treated with 0.006 % H_2O_2 and 100 μg/L SBG530 extract; and e, treated with 100 μg/L SBG530 extract.

DISCUSSION

Klotho gene was mainly expressed in the kidnety[6]. We have chosen human kidney cell line HEK293 for the Klotho promoter studies. This cell line should contain necessary transcription factors and other modulators for the regulation of Klotho gene expression. Indeed, HEK293/KL cells that contain Klotho promoter linked to luciferase reporter gene displayed high luciferase activity. Aging and age-related diseases are associated with decrease of Klotho expression[7], but the mechanism of the gene down-regulation is not clear. It is well known that oxidative stress plays an essential role in aging process. To test whether oxidative stress can regulate Klotho promoter activity, we generated a cellular model of aging by treating HEK293/KL cells with H_2O_2. Our results demonstrate that H_2O_2 treatment could decrease reporter gene luciferase expression through inhibition of the Klotho promoter activity.

Using a computer program, we have identified several potential promoter sequences including 4 Sp-1 transcription factor binding sites in Klotho promoter. Sp-1 is a DNA binding protein that has three zinc finger motifs and binds to GC-rich DNA. Sp-1 is ubiquitously expressed in many mammalian tissues with different abundances. It plays a critical role in the transcriptional regulation of a class of genes lacking classical TATA or CAAT boxes in their proximal promoters. These genes include glycolytic enzymes, dihydrofolate reductase (DHFR), thymidylate synthase, and adenine deaminase[8]. The Klotho promoter does not contain TATA box, but contains 4 Sp-1 binding sites. Therefore, Sp-1 transcription factor may play essential roles in regulating Klotho gene expression. It has been shown that Sp-1 in rat liver becomes progressively oxidized during aging. An *in vitro* analysis showed that Sp-1 binding activity was reversibly inhibited by the oxidization[9]. In addition, *in vivo* studies demonstrated that oxidative transcriptional repression of some endogenous genes was related to oxidative inactivation of Sp-1 binding[10]. It is possible that Klotho expression decline during aging could be associated with Sp-1 inactivation by progressive oxidation.

We took chemical genetic approaches to further characterize the Klotho promoter. Using a cell-based reporter gene assay, we have identified a potential activator of Klotho promoter from the SBG530 extract. This method could be used to identify Klotho promoter selective activators if enough control experiments were performed. For example, SBG530 extract does not show any activity on some promoters such as VIP promoter, cAMP response element, multiple response element, and serum response element. However, it is not clear whether the SBG530 extract activate the Klotho promoter through the activation of Sp-1 or other factors. It is essential to test other promoters that contain Sp-1 binding sites. Another approach is to generate a series of deletions of the Klotho promoter and to identify the essential elements that can be regulated by the SBG530 extract. Furthermore, we are in the process to separate and identify the active compound responsible for the observed effect from the extract. These compounds may provide useful tools to investigate the functions of Klotho gene during the aging process.

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REFERENCES

1 Saito Y, Yamagishi T, Nakamura T, Ohyama Y, Aizawa H, Suga T, *et al*. Klotho protein protects against endothelial dysfunction. Biochem Biophys Res Commun 1998; 248: 324-9.

2 Okada S, Yoshida T, Hong Z, Ishii G, Hatano M, Kuro OM, *et al*. Impairment of B lymphopoiesis in precocious aging (klotho) mice. Int Immunol 2000; 12: 861-71.

3 Mori K, Yahata K, Mukoyama M, Suganami T, Makino H, Nagae T, *et al.* Disruption of klotho gene causes an abnormal energy homeostasis in mice. Biochem Biophys Res Commun 2000; 278: 665-70.

4 Shiraki-Iida T, Iida A, Nabeshima Y, Anazawa H, Nishikawa S, Noda M, *et al*. Improvement of multiple pathophysiological phenotypes of klotho (kl/kl) mice by adenovirus-mediated expression of the klotho gene. J Gene Med 2000; 2: 233-42.

5 Ogata N, Matsumura Y, Shiraki M, Kawano K, Koshizuka Y, Hosoi T, *et al.* Association of klotho gene polymorphism with bone density and spondylosis of the lumbar spine in postmenopausal women. Bone 2002; 31: 37-42.

6 Koh N, Fujimori T, Nishiguchi S, Tamori A, Shiomi S, Nakatani T, *et al*. Severely reduced production of klotho in human chronic renal failure kidney. Biochem Biophys Res Commun 2001; 280: 1015-20.

7 Manya H, Inomata M, Fujimori T, Dohmae N, Sato Y, Takio K, *et al*. Klotho protein deficiency leads to overactivation of mu-calpain. J Biol Chem 2002; 277: 35503-8.

8 Swick AG, Blake MC, Kahn JW, Azizkhan JC. Functional analysis of GC element binding and transcription in the hamster dihydrofolate reductase gene promoter. Nucleic Acids Res 1989; 17: 9291-304.

9 Ammendola R, Mesuraca M, Russo T, Cimino F. Sp1 DNA binding efficiency is highly reduced in nuclear extracts from aged rat tissues. J Biol Chem 1992; 267: 17944-8.

10 Wu X, Bishopric NH, Discher DJ, Murphy BJ, Webster KA. Physical and functional sensitivity of zinc finger transcription factors to redox change. Mol Cell Biol 1996; 16: 1035-46.