

# Signals mediating Klotho-induced neuroprotection in hippocampal neuronal cells

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The erythropoietin (Epo) receptor (EpoR) is expressed in the brain and was shown to have neuroprotective effects against brain damage in animal models. A recent study indicated that EpoR and its activity are the downstream effectors of Klotho for cytoprotection in the kidney. Thus, we propose that Klotho can stimulate the expression of EpoR in neuronal cells to enhance Epo-mediated protection. H19-7 hippocampal neuronal cells were treated with recombinant Klotho. In H19-7 cells, Klotho increased the expression of both the EpoR protein and mRNA. Klotho also enhanced the transcription activity of the EpoR promoter in H19-7 cells. Moreover, Klotho augmented the Epo-triggered phosphorylation of Jak2 and Stat5 and protected H19-7 cells from hydrogen peroxide cytotoxicity. The silencing of EpoR abolished the protective effect of Klotho against peroxide-induced cytotoxicity. Finally, the silencing of GATA1 diminished the Klotho-induced increase in EpoR protein and mRNA expression as well as its promoter activity. In conclusion, Klotho increased EpoR expression in neuronal cells through GATA1, thereby enabling EpoR to function as a cytoprotective protein against oxidative injury.

**Key words:** Klotho, EpoR, GATA1, STAT5, Jak2, H19-7 cells, neuroprotection

## ABBREVIATIONS

EPO – erythropoietin;  
EpoR – erythropoietin receptor;  
STAT5 –transducer and activator of transcription 5;  
PBS – phosphate-buffered saline;  
BSA – bovine serum albumin;  
RIPA – radioimmunoprecipitation assay;  
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis;  
ECL – enzymatic chemiluminescence;  
SD – standard deviation;  
SEM – standard error of mean;  
GATA-1– GATA binding protein 1;  
CSF – cerebrospinal fluid.

## INTRODUCTION

Erythropoietin (Epo) is a glycoprotein hormone originally considered to act as the primary regulator of erythropoiesis by stimulating proliferation and inhibiting apoptosis and differentiation in erythroid precursor cells (Moritz et al. 1997, David et al. 2002). Nonetheless, Epo signaling has been recognized in many nonerythroid tissues, including neural, endothelial, and muscle cells (Ogilvie et al. 2000, Shingo et al. 2001, Chen et al. 2006, Cokic et al. 2014). Recently, Epo has been shown to contribute to the development and protection of the nervous system (Yang et al. 2007). The Epo receptor (EpoR) is expressed on cultured cortical neurons and hippocampal cells and mediates Epo-induced protection against apoptosis (Digicaylioglu and Lipton 2001, Dzietko et al. 2004). The neuroprotective effect of Epo is associated with its anti-apoptotic activities (Yu et al. 2002). *In vitro* and *in*

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*vivo* experiments suggest that Epo/EpoR signaling may have a positive effect on ischemia- or hypoxia-induced brain injury (Brines et al. 2000, Bartsaghi et al. 2005).

There is convincing evidence that EpoR is expressed in non-hematopoietic tissues (Arcasoy 2008) such as pulmonary tissue, cardiac tissue (Zhang et al. 2008), the nervous system (Liu et al. 1997), skeletal muscle cells (Rundqvist et al. 2009, Christensen et al. 2012), vascular endothelial cells (Anagnostou et al. 1994), smooth muscle cells (Ammarguella et al. 1996), and kidney (Westenfelder et al. 1999). Paracrine and autocrine Epo/EpoR signaling has been suggested to be involved in many biological processes, including angiogenesis, organogenesis, cell proliferation, apoptosis, cytoprotection, and tissue repair (Jelkmann et al. 2008). The genetic knockout of EpoR leads to reduced angiogenesis, slows tissue regeneration, and results in severe tissue damage after ischemia in mice (Tsai et al. 2006, Nakano et al. 2007). Neuronal EpoR is up-regulated under harmful stress conditions, such as ischemia and inflammation, and thus provides protection against harmful stress conditions by preconditioning (Chen et al. 2010, Kim et al. 2010, Jantzie et al. 2013).

Klotho is a recently discovered anti-aging gene (Wang and Sun 2009), as demonstrated by the use of the *Klotho* mouse, which has a mutated Klotho gene and exhibits multiple disorders similar to a human premature-aging syndrome (Kuro-o et al. 1997). Genetic mutations in Klotho cause general premature-aging phenotypes and drastically shorten the lifespan, whereas the overexpression of Klotho extends lifespan (Kuro-o et al. 1997, Kurosuo et al. 2005). Klotho is predominantly expressed in the kidney, parathyroid gland, and choroid plexus, but is not detectable in other organs. However, non-expressing organs can still be severely affected by mutations in this protein (Kuro-o et al. 1997). It has been demonstrated that the choroid plexus produces cerebrospinal fluid (CSF), which serves as the extracellular fluid for neurons, and secreted Klotho protein has recently been detected in the CSF (Imura et al. 2004). We found that the baroreflex could be restored in rats with higher cerebral Klotho (Chen et al. 2014b). Thus, Klotho appears to be involved in neuronal regulation. Additionally, *in vivo* and *in vitro* studies have previously shown that Klotho up-regulates EpoR expression in the kidney and a kidney cell line, thus amplifying Epo-triggered

signaling pathways (Hu et al. 2013). However, the subcellular mechanism(s) for these results remains obscure.

Because both Klotho and EpoR are also expressed in the brain, it is possible that EpoR is a downstream neuroprotective effector of Klotho. Thus, in the present study, we investigated the subcellular effects of Klotho on EpoR expression and Epo-mediated protection against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity in cultured neuronal cells.

## METHODS

### Materials

Antibodies to the erythropoietin receptor (EpoR), phosphorylated signal transducer and activator of transcription 5 (p-STAT), GATA binding protein 1 (GATA1), phosphorylated janus kinase 2 (p-JAK2), activate caspase 3,  $\beta$ -actin (actin) and recombinant human Klotho were the products of Abcam (Cambridge, MA, USA). The siRNA (siGENOME SMARTpool) for GATA1 and TurboFect plasmid transfection reagent were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). siRNA Transfection Reagent (TransIT-TKO) was purchased from Mirus Bio LLC. (Pittsburgh, PA, USA). TRIzol reagent was purchased from Invitrogen Corp. (Carlsbad, CA, USA).

### Cell culture

Rat hippocampal neuroblasts (H19-7 cells) (ATCC No. CRL-2526) were obtained from the Culture Collection and Research Center of the Food Industry Institute (Hsin-Chiu City, Taiwan) and were maintained in Dulbecco's modified Eagle's medium (Gibco, BRL, USA) supplemented with 10% fetal bovine serum and 0.2 mg/ml G418. The cells were grown on a poly-l-lysine (0.015 mg/ml)-coated surface and maintained in the exponential growth phase as described previously (Pan et al. 2012). To induce differentiation, the cells were placed in serum-free defined medium (N2) and shifted to 39°C prior to treatment with differentiating agents. As previously described (Choi et al. 2012), differentiated cells were defined as cells with round and retractile cell bodies containing at least one neurite. The differentiated cells were used for the following experiments.

Table I

Real-time PCR primers and universal library probes (UPL) for target genes			
Gene name	Forward	Reverse	UPL number
Hydroxymethylbilane synthase (HBMS)	tccctgaaggatgtgcctac	aagggtttcccgtttgc	79
Erythropoietin receptor (EpoR)	ttccagctatggctgttgc	tcagggaaggggctacttg	22

H19-7 cells were treated with 0.4 nmol/L recombinant Klotho (rKl) and/or 100 IU/ml erythropoietin (Epo) for 24 hours, the most effective condition as previously described (Hu et al. 2013). Following the previous studies, the rat IgG was used as control of exogenous peptide (Chen et al. 2014a,b). At the end of this treatment, the cells were harvested for further analysis.

### Western blotting analysis

Protein was extracted from cell lysates using an ice-cold radio-immuno-precipitation assay (RIPA) buffer supplemented with phosphatase and protease inhibitors (50 mmol/L sodium vanadate, 0.5 mmol/L phenyl-methylsulphonyl fluoride, 2 mg/mL aprotinin, and 0.5 mg/mL leupeptin). The protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The total proteins (30 µg) were separated by SDS/polyacrylamide gel electrophoresis (on a 10% acrylamide gel) using a Bio-Rad Mini-Protean II system. The protein was transferred to expanded polyvinylidene difluoride membranes (Pierce, Rockford, IL, USA) with a Bio-Rad Trans-Blot system. The membrane was blocked with 5% non-fat milk in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T) and incubated in the room temperature (25°C) for two hours. The membrane was then washed in PBS-T and incubated for 16 hours with primary antibodies in 4°C, which were diluted to a suitable concentration in PBS-T. Specific antibodies for p-JAK2, p-STAT5, activate caspase 3, GATA1, EpoR (1:1000), and β-actin (1:5000) were used. The membranes were then incubated with the corresponding secondary antibody, and the detection of the antigen–antibody complexes was performed using an ECL kit (Millipore, Billerica, MA, USA). The immunoblot densities were quantified using a laser densitometer. The expression of β-actin was used as an internal standard.

### RNA interference

Duplexed RNA oligonucleotides for rat EpoR and GATA1 (siGENOME SMARTpool™) were synthesized by Thermo Fisher Scientific Inc. H19-7 cells were transfected with 40 pmol of GATA1-specific siRNAs (siGATA1), 40 pmol of EpoR-specific siRNAs (siEpoR) or scrambled siRNA (Sc) using transfection reagents (TransIT-TKO, Mirus) according to the manufacturer's protocols. After transfection, the cells were then incubated for 48 hours prior to their use in further investigations. The efficiency of the EpoR and GATA1 protein silencing was evaluated by immunoblotting to optimize the experimental conditions (siRNA dose and time after transfection) following the previous studies (Rujitanaroj et al. 2013).

### Real-time reverse transcription-polymerase chain reaction

The total RNA was extracted from cell lysates with TRIzol reagent (Carlsbad, CA, USA) and treated with RNase-free DNaseI (Promega, Madison, WI, USA). The concentration of RNA was measured using a spectrophotometer (Pharmacia Biotech, a division of GE Healthcare, Uppsala, Sweden) 260/280 ratio ranged in 1.9–2.5. Two microgram of total RNA was used for the reverse transcription reaction, along with Superscriptase II (Invitrogen), oligo-dT, and random primers. Web-based assay-design software from the Universal Probe Library Assay Design Center (<http://www.roche-applied-science.com/sis/rtpcr/upl/adsc.jsp>) was used to design TaqMan primer pairs and to select appropriate hybridization probes. All PCR experiments were performed using a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany). The reactions were performed in 20 µL of a mixture consisting of 13.4 µL of PCR buffer, 0.2 µL of each of the Universal Probe Library probes (10 mmol/L), 0.2 µL of each primer (20 µmol/L) (Table I), 4 µL of the LightCycler TaqMan Master (Roche Diagnostics GmbH),

and 2  $\mu$ L of the template cDNA. Following the previous studies (Chen et al. 2013, Niu et al. 2014), the thermal cycling conditions consisted of an initial denaturation step at 95°C for 10 min, followed by 45 cycles of 94°C for 10 s, 60°C for 20 s, and 72°C for 1 s following the instruction of manufactory. The crossing point for each amplification curve was determined by the second derivative maximum method. The concentration of each PCR product was calculated by reference to the respective standard curve with the aid of the LightCycler software. Relative gene expression was expressed as the ratio of the concentration of the target gene to that of a house-keeping gene, hydroxymethylbilane synthase (HMBS).

### EpoR gene transfection and activity assay

An EpoR gene construct containing the 5'-untranslated region (UTR) flanking the EpoR coding region and extending to -1,778 bp 5' of the transcrip-

tion start site (Chin et al. 1995) was inserted into the pGL3-based (Promega, Madison, WI, USA) luciferase reporter construct. This construct was co-transfected with a Renilla luciferase construct (pRL-TK) as a control into H19-7 cells using the TurboFect transfection reagent (Thermo Fisher Scientific, USA). The transfected H19-7 cells were treated with 0.4 nmol/L recombinant Klotho (rKl) and/or 100 IU/ml erythropoietin (Epo) for 24 hours according to a previous study (Hu et al. 2013). After treatment, the cells were harvested, and the activity of the EpoR reporter gene was determined using the Dual Luciferase Assay System (Promega, Madison, WI, USA). A non-promoter construct containing only the 5' UTR beginning at +3 of EpoR connected to the luciferase gene ( $\Delta$ EpoR) was used as a negative control. Each test was repeated in triplicate. The signals from the Renilla luciferase were used as a transfection control to normalize the signals of the firefly luciferase.

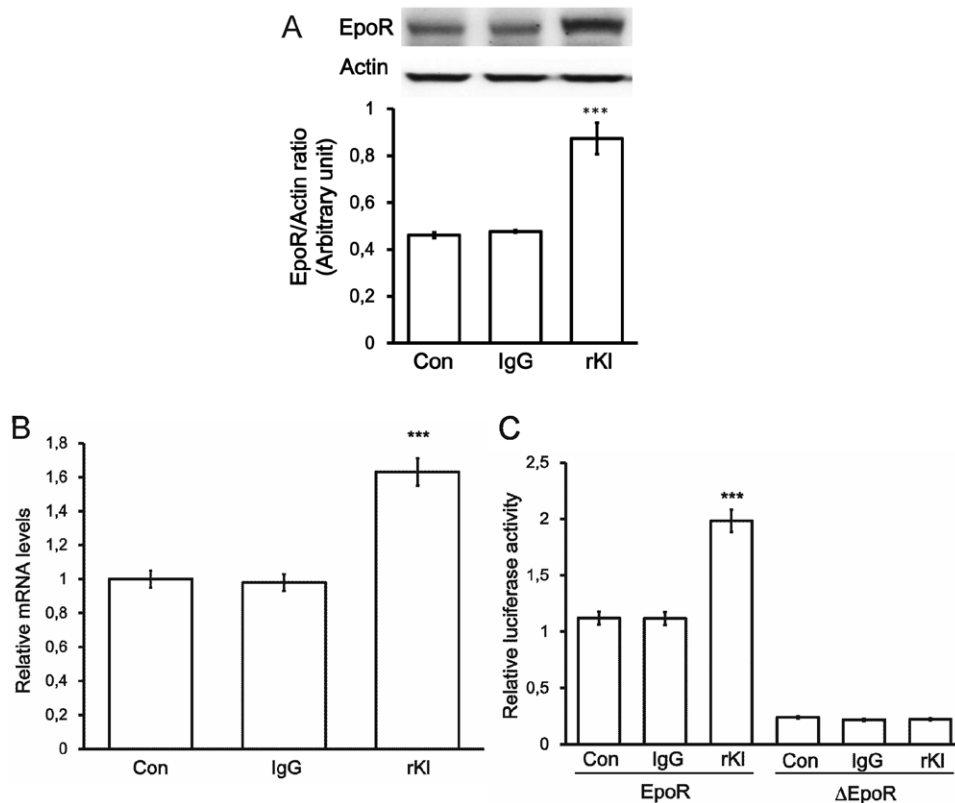


Fig. 1. EpoR expression and regulation in H19-7 cells. The changes in EpoR expression in cultured H19-7 cells by incubating them with recombinant Klotho (rKl) or IgG, each at a concentration of 0.4 nmol/L. Quantification of the protein levels, reported as the means  $\pm$  SE ( $n=6$  per group) of the values for EpoR normalized to  $\beta$ -actin in each column, is shown in the lower panel (A). The related mRNA expression of EpoR normalized to HMBS, is shown as the means  $\pm$  SE ( $n=6$  per group) (B). The luciferase activity of EpoR reporter gene or the  $\Delta$ EpoR promoterless -reporter gene in H19-7 cells (C). \*\*\* $P<0.001$  compared to the control (Con).

### Assessment of H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity in the H19-7 cells

EpoR-silenced H19-7 cells and normal H19-7 cells were used in this experiment. The viability of the cells was determined using CellTiter-Glo Assay (Promega, Madison, WI, USA) (Liu et al. 2013). H<sub>2</sub>O<sub>2</sub> is a common factor for induction of neural disease (Marlatt et

al. 2004). Cells were plated in a 96-well plate in triplicate (20 000 cells/well) and cultured overnight before being treated with 50 nmol/L H<sub>2</sub>O<sub>2</sub> and co-treated with 0.4 nmol/L recombinant Klotho (rKl) and/or 100 IU/ml erythropoietin (Epo) for 24 hours according to a previous study (Hu et al. 2013), their viability was then assessed. Each group was mixed with the CellTiter-Glo reagent using orbital shaking (500 rpm for 30 s) and incubated for 1 h at 37°C. The fluorescence was measured at two wavelengths: 380Ex/510Em (viability) using the Synergy HT Multi-Mode Microplate Reader (BioTek, USA). The cells were also harvested for subsequent western blotting analysis.

### Staining of life and dead cells

To imaging the live and dead cells, we using a LIVE/DEAD viability assay kit (Molecular Probes; Eugene, Oregon, U.S.) according to the manufacturer's instructions. H19-7 cells were incubated with two probes, calcein-AM (green color) and ethidium homodimer-1 (EtdD-1, red color), for intracellular esterase activity and plasma membrane integrity, respectively. Then, specimens were observed under a fluorescence microscope (Olympus IX71; Olympus, Japan). All experiments were performed in triplicate.

### Statistical analysis

Data are expressed as the mean  $\pm$  SE for the number (*n*) of animals in one group as indicated. Statistical analysis was carried out using one-way ANOVA analysis and Newman-Keuls *post-hoc* analysis. A *P*-value of 0.05 or less was considered significant.

## RESULTS

### Klotho increases EpoR protein and mRNA expression in H19-7 neuronal cells

To investigate whether Klotho regulates the expression of EpoR, recombinant Klotho (rKl) or IgG was added to the culture medium for 24 hours. The expression of the EpoR protein was up-regulated in rKl-treated cells (Fig. 1A) but was not changed by treatment with IgG only. Then, RNA was isolated from the rKl-treated cells, and the level of EpoR expression was determined with real-time quantitative RT-PCR. We

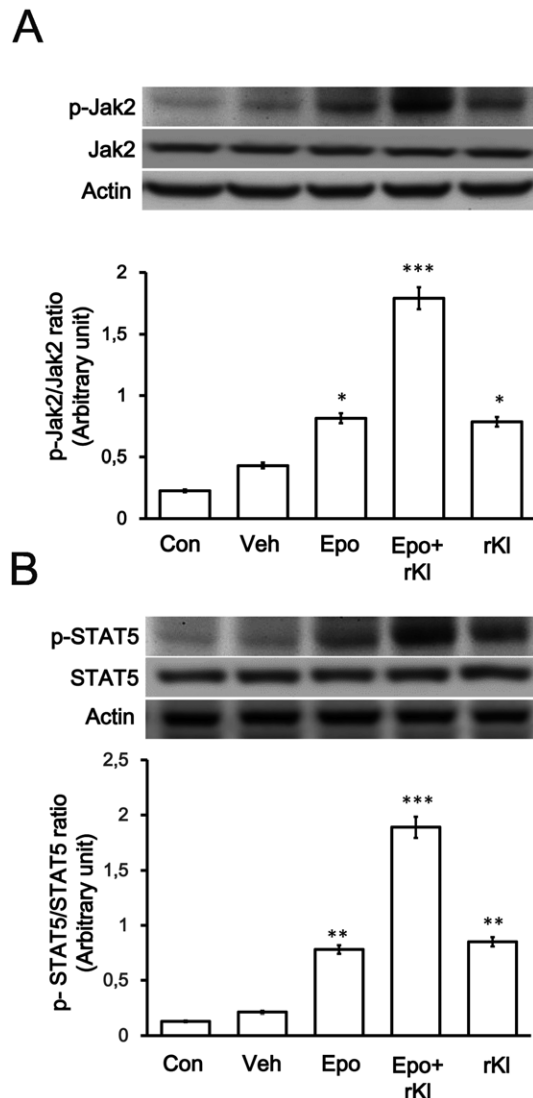


Fig. 2. Klotho enhances the activation of downstream EpoR effectors. The changes in EpoR expression in cultured H19-7 cells by incubation with recombinant Klotho (rKl) (0.4 nmol/L) and/or erythropoietin (Epo) (50 nmol/L). Quantification of the protein levels, for p-Jak2 (A) or p-STAT5 (B), normalized to total protein, and reported as the mean  $\pm$  SE (*n*=6 per group) for each column, is shown in the lower panel. \**P*<0.05, \*\**P*<0.01 and \*\*\**P*<0.001 compared to the control (Con).

observed that rKl, but not IgG, increased EpoR expression (Fig. 1B). Furthermore, we used EpoR gene promoter assays to determine whether Klotho regulated EpoR expression at the transcriptional level. The luciferase activity was assumed to indicate the promoter activity (Fig. 1C). Treatment of the transfected H19-7 cells with rKl increased the EpoR promoter activity by approximately 80%, while IgG failed to affect the EpoR promoter activity (Fig. 1C). These results are consistent with the data from the Western

blotting experiments and suggest that Klotho regulates EpoR expression at the transcriptional level.

### Klotho enhances the activation of EpoR downstream effectors

To define whether the up-regulation of EpoR by Klotho is functional, we examined the effect of rKl on the downstream effectors of EpoR. Figure 2 shows that in the presence of exogenous Epo, both the janus kinase 2 (JAK2)

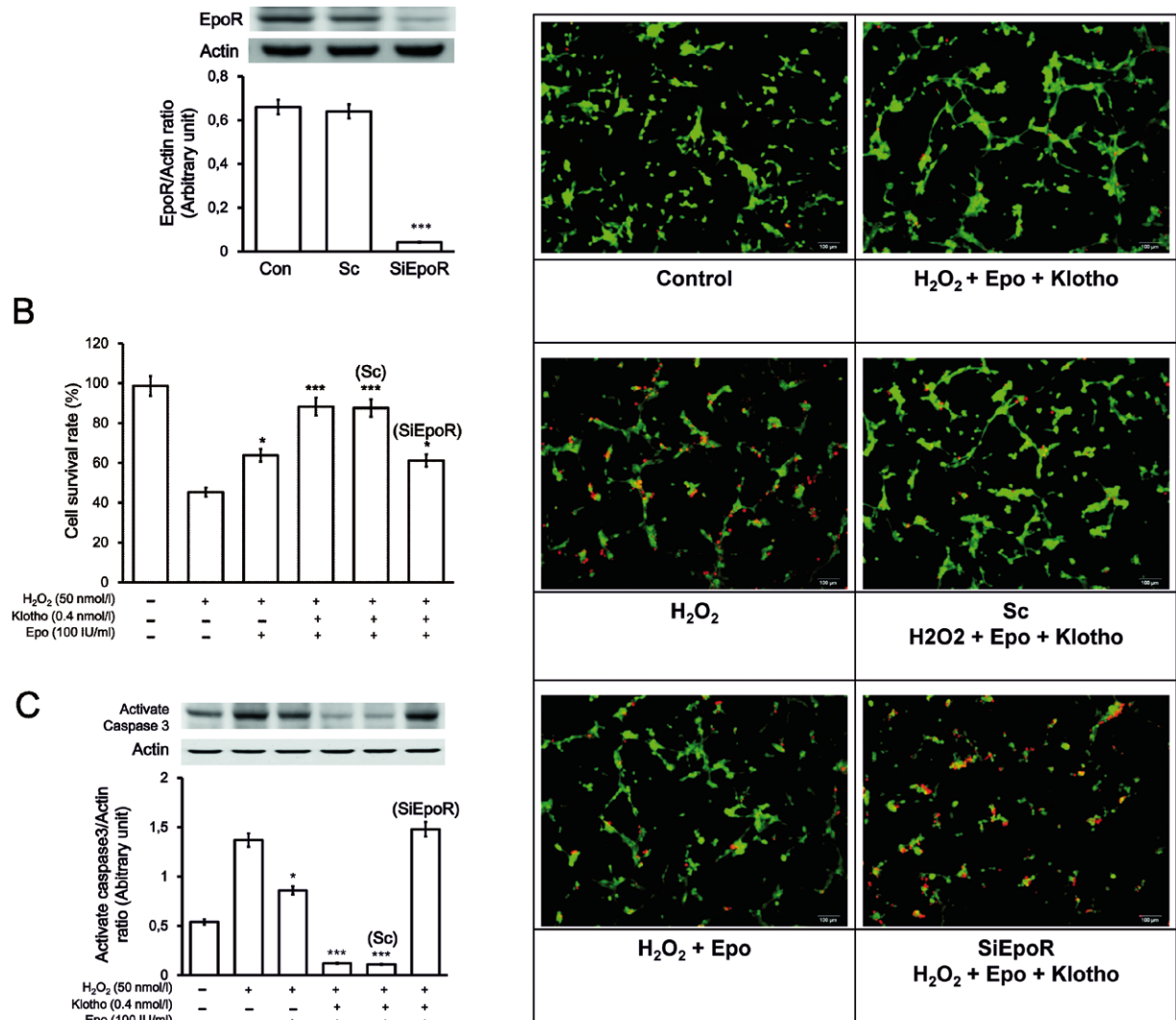


Fig. 3. Klotho protects H19-7 cells from H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity *via* EpoR. H19-7 cells were incubated with siRNA specific to EpoR (SiEpoR) or a siRNA-scramble (Sc) control for 48 h. Normal H19-7 cells, siEpoR- H19-7 cells and Sc H19-7 cells were incubated with H<sub>2</sub>O<sub>2</sub> and co-treated with erythropoietin (Epo) and/or recombinant Klotho (rKl). (A) The silencing efficiency of EpoR in H19-7 cells. (B) The cell viability. (C) The activate caspase 3 protein expression in H19-7 cells. More, the images of H19-7 cells were showed to reflect live and dead cells. Live cells stained green, whereas dead cells stained red (D). All values are expressed as the mean  $\pm$  SE ( $n=6$  per group). \* $P<0.05$  and \*\*\* $P<0.001$  compared to the control (normal cells without Epo and rKl).

and Signal Transducer and Activator of Transcription 5 (STAT5) were activated, consistent with the findings of previous reports (Breggia et al. 2008, Vera et al. 2008, Lai et al. 2009). Moreover, Klotho augmented Jak2 and STAT5 activation in the presence of exogenous Epo, which was paralleled by a Klotho-induced increase in the expression of EpoR. However, the effect of rKl on Jak2 and STAT5 activation was not completely abolished in the absence of Epo. This observation suggests that there were factors in the medium other than Epo, that may also contribute to EpoR-mediated signaling (Stewart et al. 2004).

### Klotho protects H19-7 cells from H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity *via* EpoR

To study the role of Klotho in cytoprotection, we incubated H19-7 cells with H<sub>2</sub>O<sub>2</sub>. The cell survival rate was increased when the cells were treated with Epo, and were

enhanced by Klotho (Fig. 3B). Additionally, the immunoreactivity of activated caspase 3 was decreased after these treatments, consistent with the results of the cell survival experiments (Fig. 3C). To examine whether EpoR is required for Klotho to exert its cytoprotective effect, we tested the effect of Klotho in H19-7 cells in which EpoR had been silenced. EpoR expression was markedly reduced in the H19-7 cells that had received siEpoR, thus confirming the transfection efficiency (Fig. 3). The parallel cellular images for with calcein-AM (green stain, live cells) and ethidium homodimer-1 (EtdD-1, red stain, dead cells) staining are shown in Figure 3D.

### The effects of GATA1 siRNA on the Klotho-induced expression of EpoR in H19-7 cells

H19-7 cells were transfected with siRNA specific to GATA1 (siGATA1) or the siRNA-scramble (Sc) as a

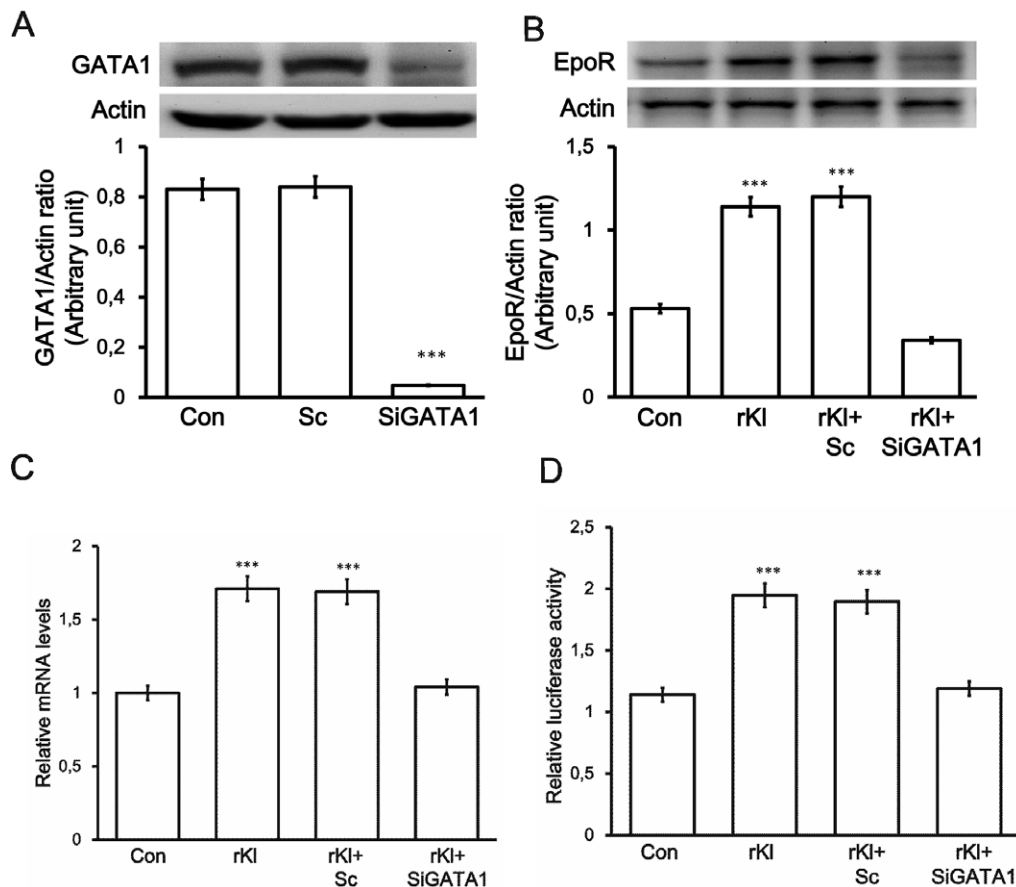


Fig. 4. The effects of GATA1 siRNA on recombinant Klotho-induced EpoR expression. H19-7 cells were incubated with siRNA specific to GATA1 (SiGATA1) or the siRNA-scrambled (Sc) control for 48 h before incubation with recombinant Klotho (rKl). (A) The knockdown efficiency of the GATA1 in cells. (B) and (C) EpoR protein and mRNA expression after rKl treatment of the H19-7 cells. The luciferase activity of H19-7 cells was determined (D). All values are expressed as the mean  $\pm$  SE ( $n=6$  per group). \*\*\* $P<0.001$  compared to the control (Con).

control for 48 hours, as previously reported (Yu et al. 2008). GATA1 expression was markedly reduced in the H19-7 cells that received siGATA1, thus confirming the transfection efficiency (Fig. 4A). Additionally, the silencing of GATA1 attenuated the Klotho-induced changes at both the EpoR protein and mRNA levels (Fig. 4B,C). Moreover, the EpoR promoter activity was reduced after GATA1 was silenced (Fig. 4D). Thus, GATA1 may mediate Klotho-induced EpoR expression.

## DISCUSSION

In the present study, we describe for the first time that the Klotho protein can induce the marked expression of EpoR protein and mRNA in H19-7 neuronal cells. Additionally, increased EpoR promoter activity further confirmed these findings (Fig. 1). To demonstrate that this increase in EpoR expression has a cellular effect, we measured the downstream signals (Jak2 and STAT5 phosphorylation) by Western blotting. Our results showed that Klotho augmented Epo-mediated Jak2 and STAT5 activation (Fig. 2). Thus, functional EpoR expression can be increased in H19-7 cells by Klotho, and Klotho can be considered to induce EpoR expression. Treatment with Klotho increased Epo-induced cell survival under conditions of H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity in H19-7 cells. This finding was further confirmed by a western blotting analysis of activate caspase 3. Furthermore, the Klotho-induced cytoprotection in H19-7 cells was abolished by EpoR silencing, suggesting role of EpoR in the Klotho-induced cytoprotection (Fig. 3). Finally, the Klotho-induced expression of the EpoR protein and mRNA was diminished by GATA1 silencing in H19-7 neuronal cells (Fig. 4). These results indicate that Klotho-induced EpoR expression is mediated by the transcription factor GATA1. Thus, Klotho increased functional EpoR expression through GATA1 and enabled EpoR to function as a cytoprotective protein against oxidative stress. This process has not previously been described.

Epo, a member of the type 1 cytokine superfamily, was first recognized as an endocrine erythropoietic factor produced mainly by the kidney. It is required for the maintenance of erythrocyte production by acting as a ligand for the Epo receptor (Goldwasser 1975). EpoR belongs to the cytokine

receptor superfamily (Longmore et al. 1993). Epo engagement at the cell surface induces the activation of Jak2 kinase activity, which phosphorylates STAT5 to trigger the transcription of its downstream target genes (Breggia et al. 2008). Thus, phospho-Jak2 and phospho-STAT5 are consistent indicators for the activation of Epo/EpoR signal transduction (Kondyli et al. 2010, Hu et al. 2013). In this study, we demonstrated that Klotho increased EpoR gene promoter activity and the expressions of the EpoR protein and transcript in H19-7 neuronal cells (Fig. 1). Additionally, Epo-mediated downstream signals, including the phosphorylation of Jak2 and STAT5, were significantly enhanced (Fig. 2). These results suggest that Klotho induces functional EpoR expression in H19-7 cells.

The cytoprotection mediated by Epo is associated with its anti-apoptotic activity (Pallet et al. 2010). Epo has numerous actions in the nervous system, including reducing the production of molecules such as reactive oxygen species and glutamate that cause tissue injury, modulating neurotransmission, reversing vasospasm, stimulating angiogenesis, attenuating apoptosis, modulating inflammation, and recruiting stem cells (Chen et al. 2010, Zhang et al. 2012, Traudt and Juul 2013, Tugyan et al. 2013, Wenker et al. 2013). In animal models, Epo has been shown to be neuroprotective during blunt force trauma and ischemia (Grimm et al. 2002). Our study shows that Klotho enhanced Epo-mediated cell survival and reduced the immunoreactivity of activated caspase 3 in H19-7 cells. The silencing of endogenous EpoR diminished the observed Klotho-induced cytoprotection (Fig. 3). Thus, Klotho enhanced Epo-mediated cytoprotection by increasing the expression of EpoR in a manner similar to that previously reported in the kidney (Hu et al. 2013).

GATA transcription factors are a family of six zinc-finger proteins, that bind to the (T/A) GATA (G/A) consensus sequence and play important roles in cellular differentiation and proliferation (Takahashi et al. 1998, Rylski et al. 2003). Analysis of the *EpoR* 5'-flanking region revealed several potential GATA binding sites. The transcription factor GATA1 activates EpoR expression in hematopoietic cells and in neuronal cells (Zon et al. 1991, Wallach et al. 2009). In this study, the Klotho-induced increase in both EpoR protein and mRNA



levels were prevented by the silencing of GATA1. Moreover, the silencing of GATA1 also attenuated the EpoR promoter activity (Fig. 4). Thus, GATA1 may mediate the Klotho-induced EpoR expression. The association between GATA1 and EpoR expression is consistent with the results of previous studies (Zon et al. 1991, Wallach et al. 2009). However, the detail mechanisms underlying the Klotho-induced GATA1 activation need to be investigated in the future.

In H19-7 cells, Klotho increases EpoR expression and activates well-established downstream components of the Epo/EpoR signaling pathways. The administration of Epo in cell culture medium is prerequisite for Klotho to augment Epo/EpoR signaling activity. Additionally, Klotho-induced EpoR expression is primarily mediated by the GATA1 transcription factor. Thus, we are the first to disclose that GATA1 is an intermediate for Klotho to modulate EpoR transcript in neuronal cells. Klotho serves as a co-receptor for fibroblast growth factor (FGF), but it also functions as a humoral factor that regulates cells activities. Several Klotho biological roles have been determined, but the underlying molecular mechanisms are not fully understood and have yet to be clarified. However, many studies have revealed lots of Klotho novel effects to be involved in different intracellular signaling pathways, including: insulin/IGF-1, cAMP, PKC, p53/p21, and Wnt signaling pathway (Dermaku-Sopjani et al. 2013). However, the detail mechanism involved in Klotho-induced GATA1 activation is still unclear. More experiment in molecular level should be done in the future.

The downstream effectors of EpoR are partially activated in Klotho-induced H19-7 cells in the absence of Epo treatment. It is likely that other factors in the serum (10% FBS) may increase the effect of Klotho. This result is consistent with previous report in kidney cells (Hu et al. 2013).

## CONCLUSIONS

Klotho exerts beneficial cytoprotective effects on H19-7 neuronal cells. Our studies indicate that Klotho up-regulates EpoR expression in this neuronal cell line, magnifies the Epo-triggered signaling cascade, and protects these neuronal cells from oxidative injury in an EpoR-dependent manner. These results provide novel insights into the subcellular mechanisms of Klotho action in neurons.

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