Supplemental Material and Methods

Quantitative real-time PCR

A commercial kit (RNeasy Kit, Qiagen) was used to isolate total RNA according to the manufacturer's instructions, including DNase treatment. 500 ng RNA was then reverse transcribed by reverse transcriptase (Takara) with random primers. Real-time PCR was performed in 20 μL SYBR Premix Ex Taq^{TM} (Takara) containing 0.4μLprimers and 2 μL cDNA using a 7500 Real-Time PCR System (Applied Biosystems). The thermal cycling program and PCR primers for DNA methyltransferases (DNMTs) are listed in Table 2 in the online-only Data Supplement. Each PCR reaction was performed in triplicates, and the mean Ct value was used for statistic analysis. Messenger RNA expression was standardized to β-actin expression levels, followed by normalization to the control group.

Western blotting analysis

Total protein was extracted using a commercial kit according to the manufacturer's instructions (Protein Extraction Kit, Millipore). 30 mg protein from each sample was mixed with a sample-loading buffer and loaded onto separate lanes on 10% sodium dodecyl sulfate-polyacrylamide gel. Proteins were electrotransferred onto polyvinylidene fluoride membranes (0.2 mm: Immun-Blot, Bio-Rad) and then immunoblotted with antibodies. The antibodies and dilution for α-smooth muscle actin (α-SMA), smoothelin, alkaline phosphatase(ALP), osteopontin (OPN), core binding factor α 1 (Cbfa1), Klotho, DNMT 1 and β-actin are listed in Table III in the online-only Data Supplement. The intensity of each band was quantified using the NIH Image software (Bethesda, MD) and the densitometric intensity corresponding to each band was normalized against β-actin expression.

Calcification staining

To visualize calcium deposition, paraffin-embedded aortic rings were serially sectioned into 4-mm-thick sections, deparaffinized, and stained with 1 mg/mLAlizarin Red S solution (Sigma, St. Louis, MO, USA) (pH 4.0–4.2). HASMCs were washed with 0.9 % NaCl isotonic solution at 37°C and fixed with 4% paraformaldehyde for 15 min, rinsed, and incubated with Alizarin Red S solution for 30 min. The results

were observed under light-microscopy and photographed by digital camera. Positive results were shown in an orange-red color in light microscope.

Hematoxylin-eosin and immunohistochemistry staining

Vascular morphologic alteration was examined by hematoxylin-eosin (HE) staining. Paraffin tissue sections were cut, mounted, deparaffinized, rehydrated, and stained with HE using standard histological techniques. Immunohistochemistry staining was performed using a biotin-streptavidin-peroxidase method. Rabbit monoclonal to Klotho (1:100 dilution; Abcam, Cambridge, MA, USA), rabbit monoclonal to DNMT1 (1:250 dilution; Cell signaling technology, Danvers, MA, USA) were used as primary antibodies. Biotinylated goat anti-rabbit immunoglobulin G was used as secondary antibody. Sections that were incubated with non-immune rabbit or mouse serum instead of the primary antibodies served as negative controls. All sections were stained under identical conditions together with control incubation. Nuclei were counterstained lightly with hematoxylin.

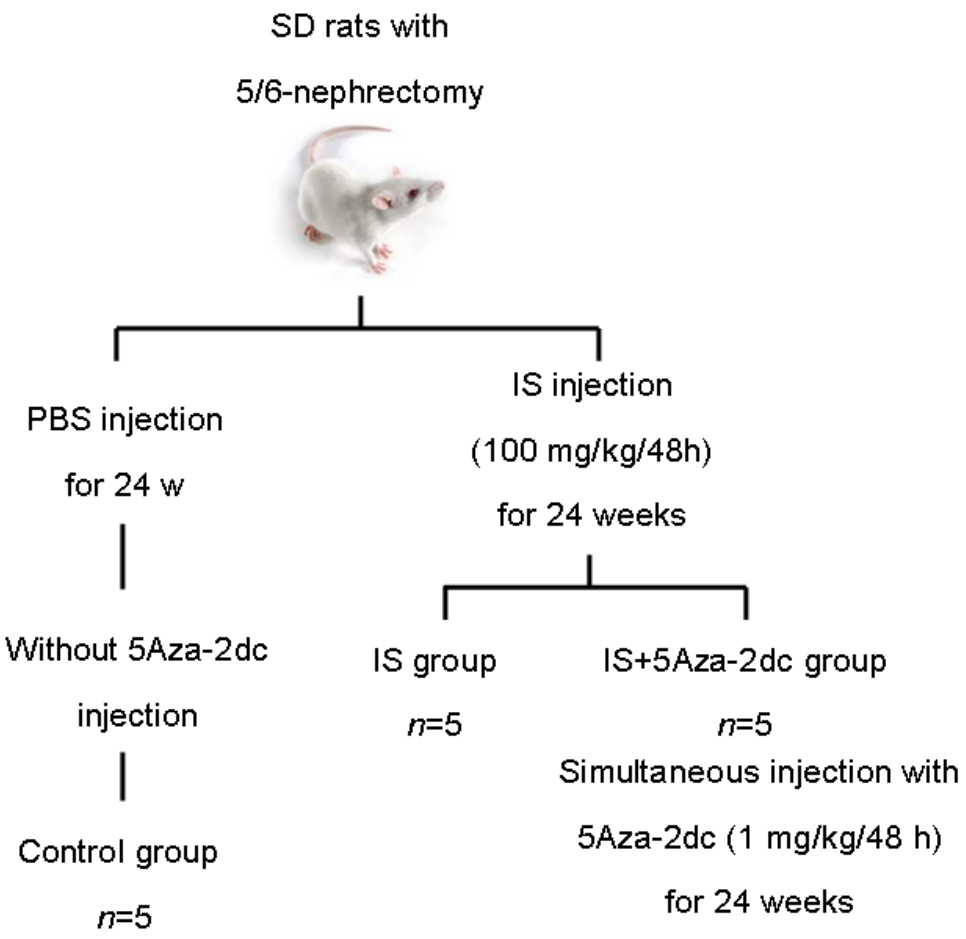

Figure S1. The flow diagram for animal study.

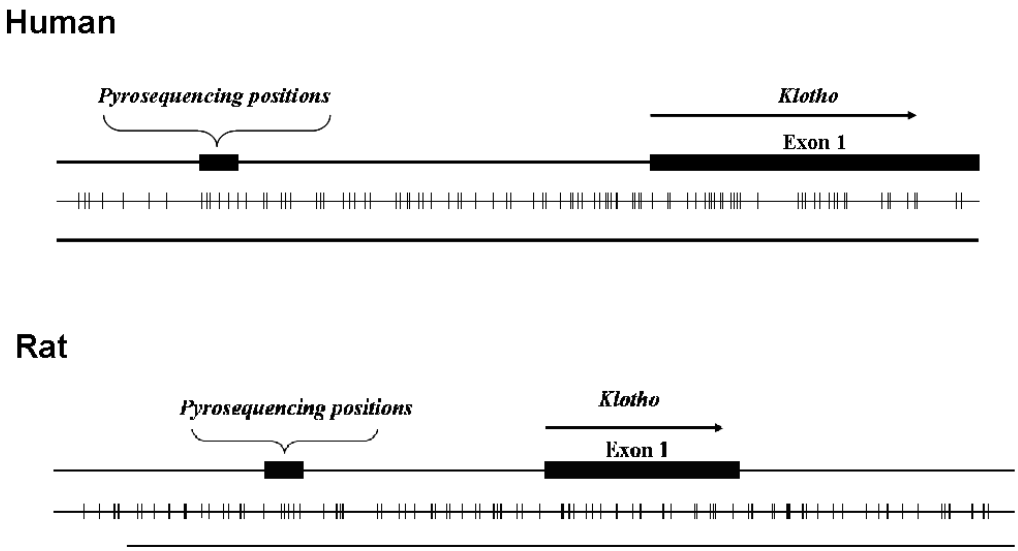

Figure S2. Map of sequencing sites of the Klotho gene in human and rat. Exon and pyrosequencing positions are shown in top line. CpG dinucleotides are shown in bottom line. Each short vertical bar represents a CpG site. The methylation levels of 6 CpG sites in human Klotho gene and 5 CpG sites in rat Klotho gene are determined. Map of sequencing sites of the Klotho gene in humans is cited from *Chen J et al. Elevated Klotho promoter methylation is associated with severity of chronic kidney Disease. PLoS One 2013, 8(11):e79856* with the permission of the authors.

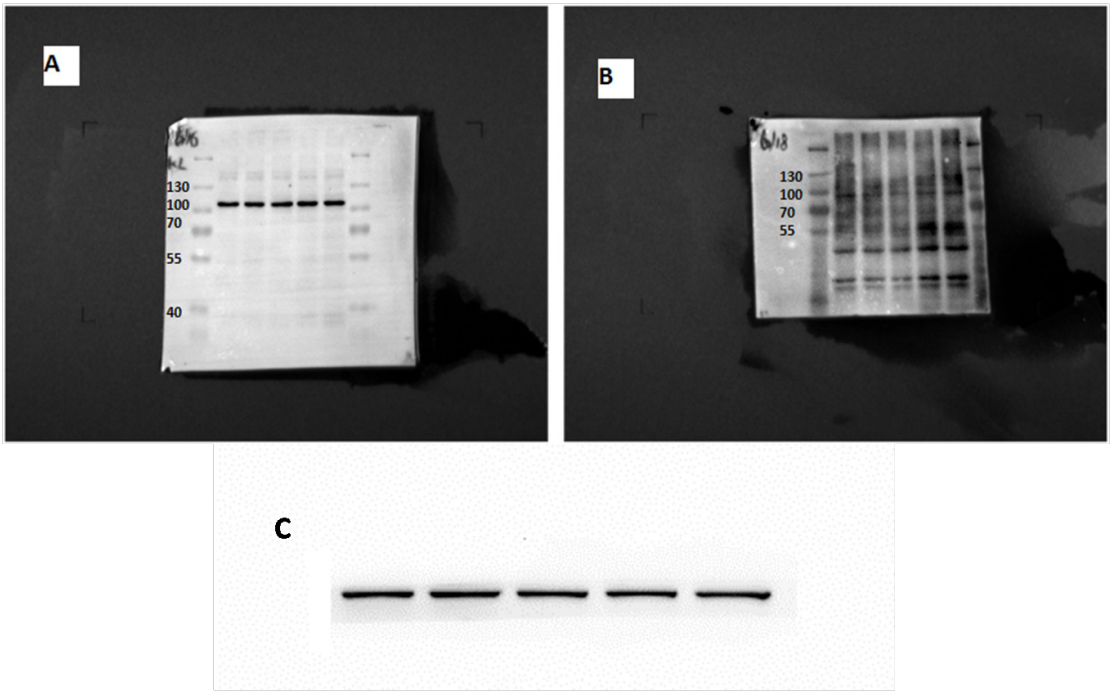

Figure S3. Full Western blot for Klotho protein. Figure A shows a representative result Klotho protein from an in vivo rat aortic experiment with Klotho antibody at a dilution of 1:500 (Catalogue No Ab69208; Abcam, USA). Figure B shows negative control without Klotho antibody. Figure C shows the experiment for the antibody using recombinant Klotho.

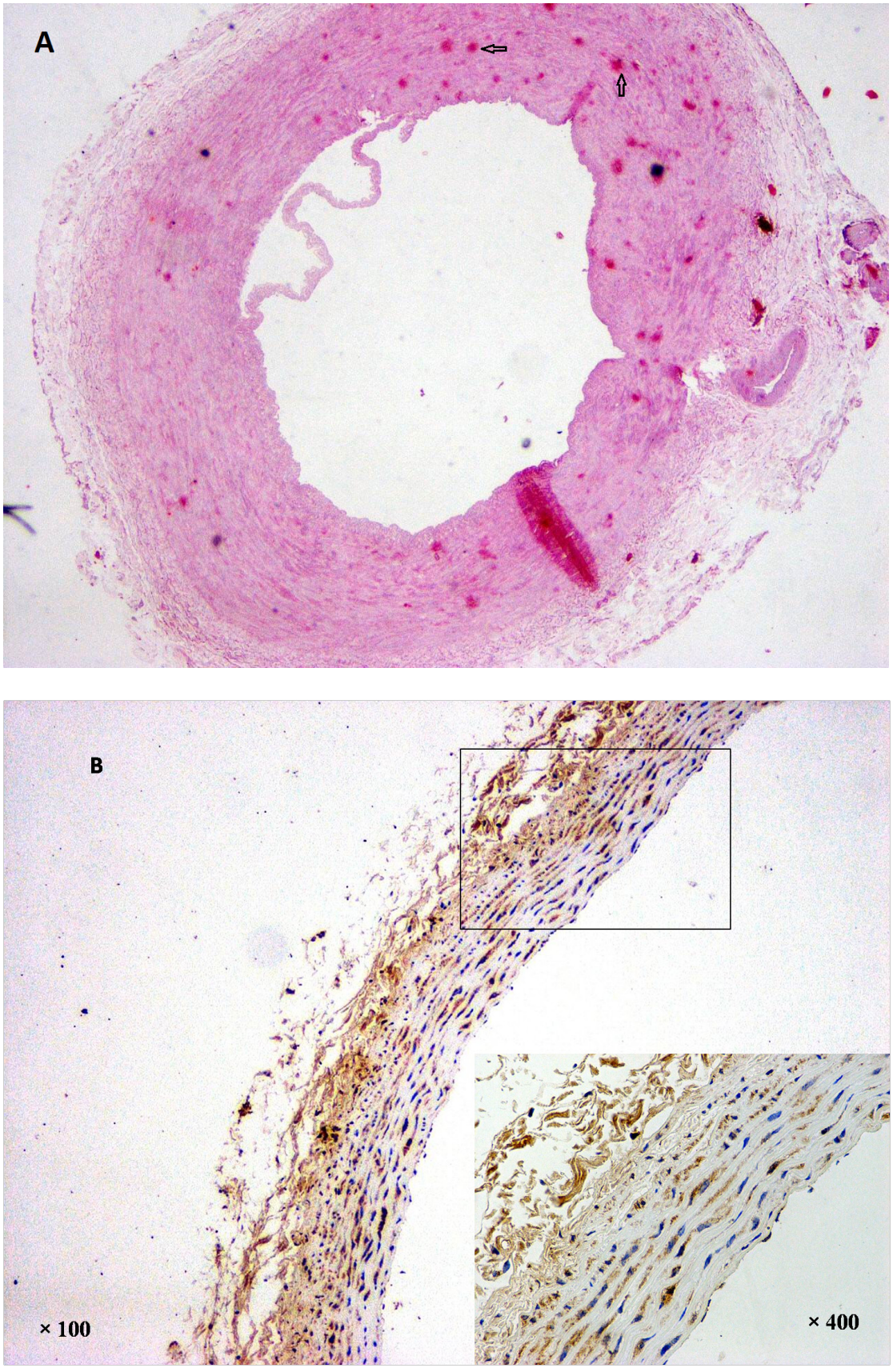

Figure S4. The calcification and Klotho expression in arteries from ESRD patients. Alizarin Red staining showed calcium nodule formation in radial medial layer of ESRD group (A). Immunohistochemical staining showed that Klotho mainly expressed in the media (B).

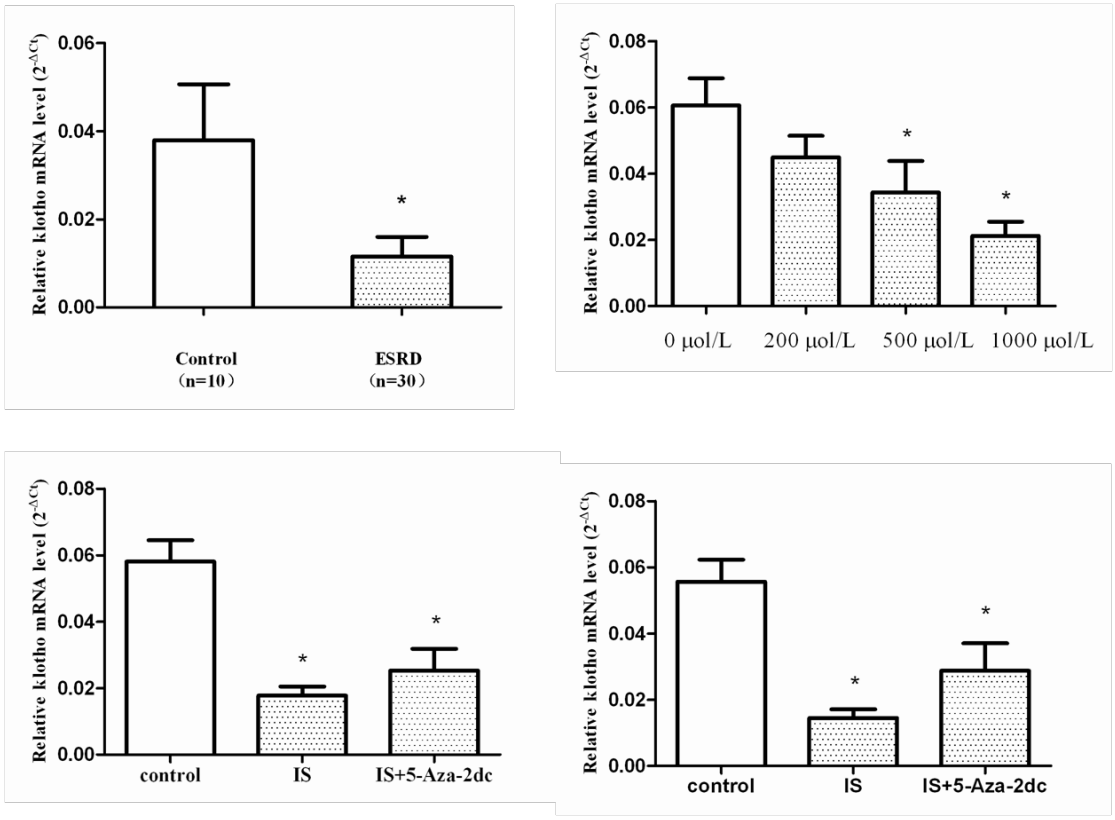

Figure S5. Real-time PCR analysis of Klotho mRNA expression.

(A) Vascular Klotho mRNA expression in ESRD patients (n=30) was significantly lower than in healthy individuals (n=10). (B) HASMCs were treated with IS at concentrations of 0, 200, 500, and 1000 μmol/L for 6 d. (C) IS reduces Klotho mRNA expression and 5Aza-2dc significantly increased the Klotho expression in HASMCs. (D) Real-time PCR analysis showed that 5Aza-2dc significantly increased the Klotho expression in IS-injected rats.

Compared with control group, *P<0.01.

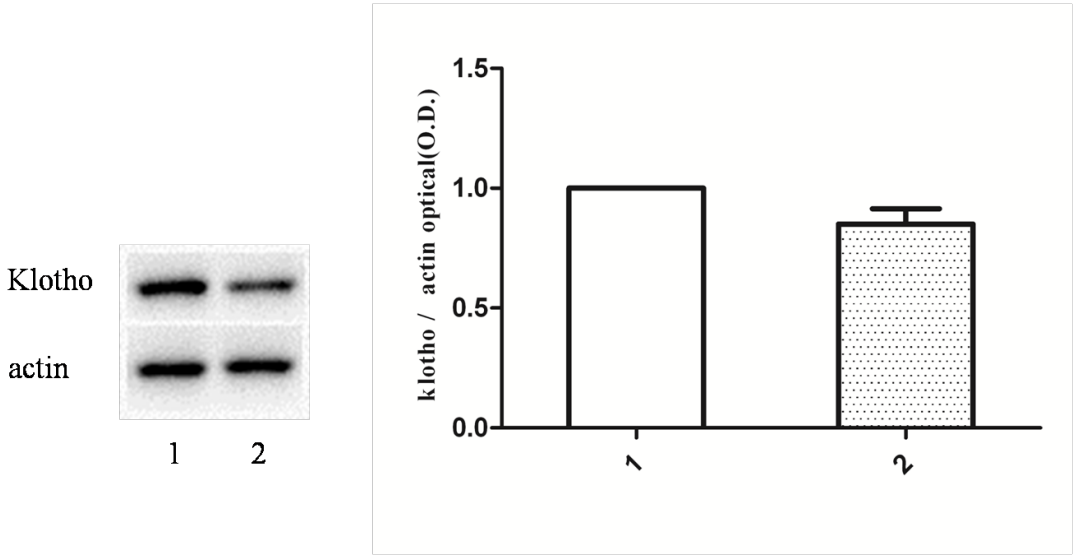

Figure S6. Klotho protein expression in non-nephrectomized control group.

The control group(1) has high Klotho expression, but no significantly different was found compared with 5/6 nephrectomized group(2).

Table S1. Summary of demographic and clinical data of patients included for vascular Klotho promoter methylation level analysis.

	Control group (n=10)	ESRD group (n=30)
Male (%)	80.00	73.33
Age (year)	55.20±7.45	54.03±15.61
BMI (kg/m^2)	22.68±6.06	22.49±3.25
Hypertension (%)	90.00	90.00
Diabetes (%)	50.00	33.33
MAP (mmHg)	89.67±7.81	94.83±9.40
eGFR (mL/min/ 1.73m^2)	77.50 (IQR 58.50-90.00)	7.00 (IQR 4.65-9.00)*
BG (mmol/L)	5.73±1.44	5.87±0.78
TC (mmol/L)	3.68±0.68	4.51±1.36
TG (mmol/L)	1.37±0.40	1.94±1.05
Ca (mmol/L)	1.96±0.08	1.98±0.10
P (mmol/L)	0.93±0.20	2.07±0.74*

ESRD, end-stage renal disease; BMI, body masss index; MAP, mean artery pressure; eGFR, estimated glomerular filtration rate; BG, blood glucose; TC, total cholesterol; TG, triglyceride; Ca, calcium; P, phosphate.

Compared with control group, *P<0.001.

Table S2: The primers and program for quantitative bisulfite pyrosequencing of Klotho.

	Forward, 5'-3'	Backward, 5'-3'	Pyrosequencing primers	Program
Human	GTGGGAGAAAAGTGAGAGTAG	AAACCCTCAAATTCATTCTCTTTACCTACC-biotinylated	AAGTGAGAGTAGGTG	94°C x 15 min; (95°C x 30 sec, 61°C x 30 sec, 72°C x 5min) x 35 cycles; 72°C 5 min.
Rat	GTGGGAGAAAATAGGTGAGAGG	CCCAAATCAAATTCATTCTCTTTACCTCCAA-biotinylated	GTGAAAGAGGTGTAGG	95°C×15min; (95°C×30sec, 58°C×40sec,72°C×1min)×35cycles; 72°C 5 min.

Table S3. The primers for thermocycler program of real-time PCR.

Genes	Species	Forward, 5'-3'	Backward, 5'-3'
Kotho	Human	CACGGCAAGGGTGCGTCCAT	TCGCGCCCACGAGATGGAGA
DNMT1	Human	AGGCGGCTCAAAGATTTGGAA	GCAGAAATTCGTGCAAGAGATTC
DNMT3a	Human	AGTACGACGACGACGGCTA	CACACTCCACGCAAAAGCAC
DNMT3b	Human	ACCTCGTGTGGGGAAAGATCA	CCATCGCCAAACCACTGGA
β-actin	Human	TCACCCACACTGTGCCCATCTACGA	CAGCGGAACCGCTCATTGCCAATGG

95º C for denaturation and 40 cycles consisting of 5 sec at 95ºC, 20 sec at 60 ºC, and 30 sec at 72 ºC.

Table S4. The antibodies for Western blot.

Protein	Species	Antibody	Source	Dilution	Product numbers
α-SMA	Rat	Rabbit polyclonal to α-SMA	Abcam, Cambridge, MA, USA	1:1000	Ab5694
Smoothelin	Rat	Mouse monoclonal to Smoothelin	Santa Cruz Biotechnology, Dallas, Texas, USA	1:1000	Sc-73042
ALP	Rat	Rabbit polyclonal to ALP	Abcam, Cambridge, MA, USA	1:3000	Ab95462
OPN	Rat	Mouse monoclonal to OPN	Abcam, Cambridge, MA, USA	1:300	Ab69498
Cbfα1	Rat	Mouse monoclonal to Cbfa1	Abcam, Cambridge, MA, USA	1:1000	Ab54868
Klotho	Rat	Rabbit polyclonal to Klotho	Abcam, Cambridge, MA, USA	1:3000	Ab69208
DNMT1	Rat	Rabbit monoclonal to DNMT1	Cell signaling technology, Danvers, MA, USA	1:5000	5032
β-actin	Rat	Mouse monoclonal to actin	Abcam, Cambridge, MA, USA	1:5000	