Spironolactone ameliorates PIT1-dependent vascular osteoinduction in klotho-hypomorphic mice

Supplementary Material

Supplementary Methods

Materials

Spironolactone, aldosterone and β-glycerophosphate were obtained from Sigma-Aldrich. Human FGF23 was purchased from R&D Systems. The ELISA kit for determination of calcitriol was obtained from Immunodiagnostic Systems, the ELISA kit for FGF23 was purchased from Immutopics, the ELISA kit for cystatin C from Biovendor and the alkaline phosphatase colorimetric assay kit from Abcam. The following primary antibodies were used: rabbit anti-NF-kB p65, rabbit anti-β-catenin, rabbit anti-phospho-p38 MAPK (Thr^{180}/Tyr^{182}), rabbit anti-p38 MAPK or rabbit anti-GAPDH antibody (all purchased from Cell Signaling), rabbit polyclonal anti-Sp7/osterix (Abcam), rabbit polyclonal anti-Cbfa1 (Santa Cruz Biotechnology) and goat polyclonal anti-Msx2 (Santa Cruz Biotechnology). The secondary antibodies used are: anti-rabbit HRP-conjugated antibody (Cell Signaling), goat anti-rabbit Alexa488-conjugated antibody and donkey anti-goat Alexa488-conjugated antibody (both purchased from Invitrogen). RT-PCR primers were obtained from Invitrogen. Primary human aortic smooth muscle cells were kindly provided by Dr. Dorothea Siegel-Axel (Department of Endocrinology, Diabetology, Angiology, Nephrology and Clinical Chemistry, University of Tübingen, Tübingen, Germany). Silencing was performed using validated siRNA for PIT1 (ID no. s13087, Ambion), siRNA for klotho (ID no. s225119, Ambion) or negative control siRNA (ID no. 4390843, Ambion).

Quantitative RT-PCR

The following mouse primers were used (5'→3' orientation) for quantitative RT-PCR measurements:

Alpl fw: TTGTGCCAGAGAAAGAGAGAGA;

Alpl rev: GTTTCAGGGCATTTTTCAAGGT;

Cbfa1 fw: AGAGTCAGATTACAGATCCCAGG;

Cbfa1 rev: AGGAGGGGTAAGACTGGTCATA;

eNos fw: TCAGCCATCACAGTGTTCCC;

eNos rev: ATAGCCCGCATAGCGTATCAG;

Gapdh fw: AGGTCGGTGTGAACGGATTTG;

Gapdh rev: TGTAGACCATGTAGTTGAGGTCA;

Msx2 fw: TTCACCACATCCCAGCTTCTA;

Msx2 rev: TTGCAGTCTTTTCGCCTTAGC;

Osx fw: TCCCTGGATATGACTCATCCCT;

Osx rev: CCAAGGAGTAGGTGTGTTGCC;

Pai1 fw: TTCAGCCCTTGCTTGCCTC;

Pai1 rev: ACACTTTTACTCCGAAGTCGGT;

Pit1 fw: TTTGACAAACTTCCTCTGTGGG;

Pit1 rev: GGACTTTCAGACGGACTAGACTT;

Tnfa fw: CTGAACTTCGGGGTGATCGG;

Tnfa rev: GGCTTGTCACTCGAATTTTGAGA;

Wnt3a fw: AATTTGGAGGAATGGTCTCTCGG;

Wnt3a rev: CAGCAGGTCTTCACTTCACAG;

Wnt7a fw: GGCTTCTCTTCGGTGGTAGC;

Wnt7a rev: TGAAACTGACACTCGTCCAGG.

The following human primers were used (5'→3' orientation) for quantitative RT-PCR measurements:

ALPL fw: GGGACTGGTACTCAGACAACG;

ALPL rev: GTAGGCGATGTCCTTACAGCC;

CBFA1 fw: GGAAGGGCTTGATTGACGTG;

CBFA1 rev: CAGAACCAAACATAGCACTGACT;

GAPDH fw: GAGTCAACGGATTTGGTCGT;

GAPDH rev: GACAAGCTTCCCGTTCTCAG;

KL fw: GGTGTCCATTGCCCTAAGCTC;

KL rev: TCGGTCATTCTTCGAGGATTGA;

MSX2 fw: TGCAGAGCGTGCAGAGTTC;

MSX2 rev: GGCAGCATAGGTTTTGCAGC;

PIT1 fw: GGAAGGGCTTGATTGACGTG;

PIT1 rev: CAGAACCAAACATAGCACTGACT;

TNFA fw: GAGGCCAAGCCCTGGTATG;

TNFA rev: CGGGCCGATTGATCTCAGC.

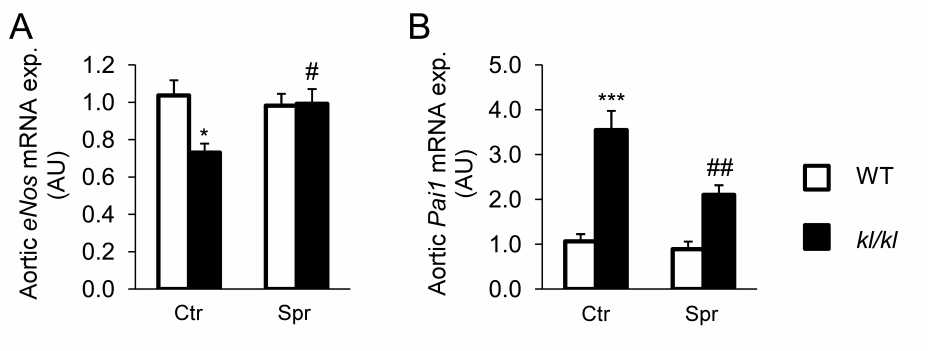
Western blot analysis

After sacrificing the animals, kidney tissues from WT and *kl/kl* mice with or without spironolactone treatment were immediately snap frozen in liquid nitrogen. Samples were lysed with ice-cold lysis buffer (Thermo Fisher Scientific) supplemented with complete protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). After centrifugation at 10000 rpm for 5 min, 30 µg of proteins were boiled in Roti-Load1 Buffer (Carl Roth GmbH) at 100ºC for 10 min. Proteins were separated on SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were incubated overnight at 4°C with the following primary antibodies: rabbit anti-NF-kB p65, rabbit anti-β-catenin, rabbit anti-phospho-p38

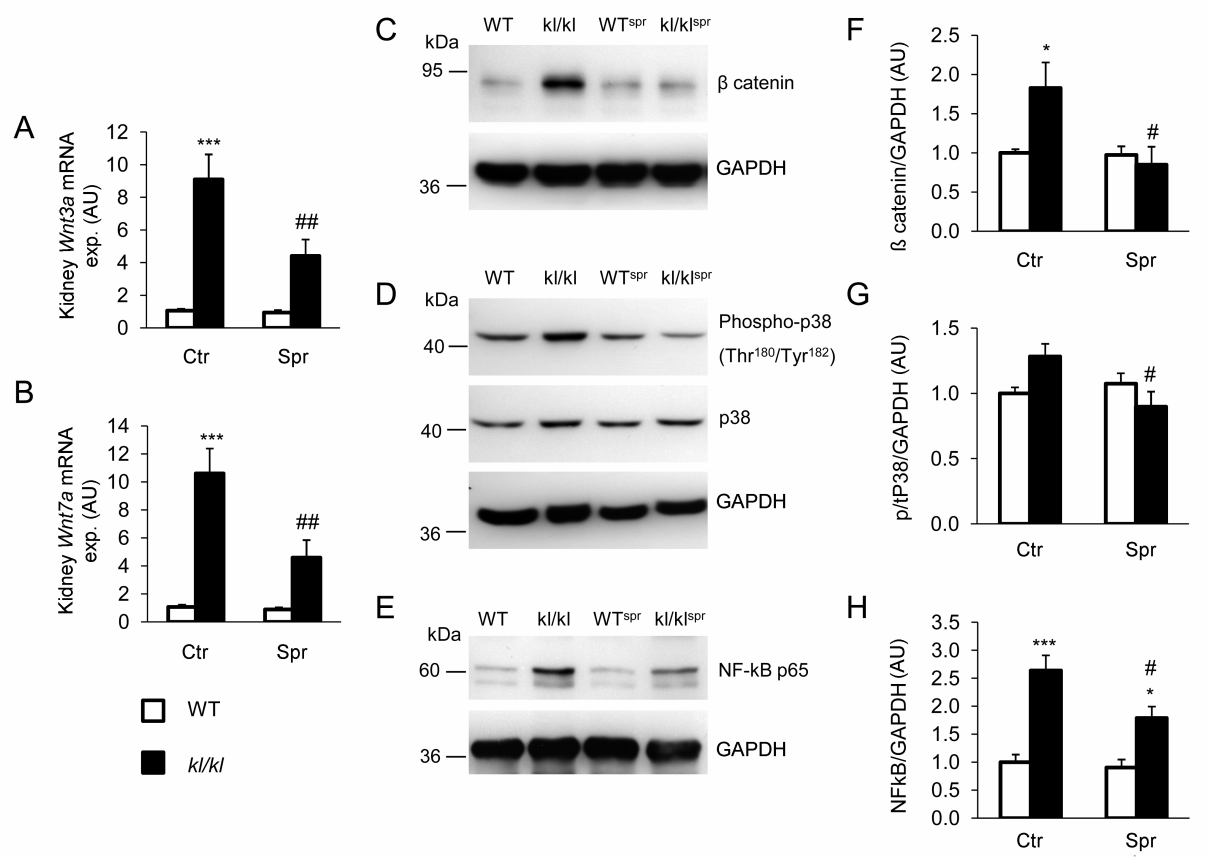
MAPK (Thr^{180}/Tyr^{182}), rabbit anti-p38 MAPK or rabbit anti-GAPDH antibody (used at a 1:1000 dilution, Cell Signaling) and then with secondary anti-rabbit HRP-conjugated antibody (diluted 1:1000, Cell Signaling) for 1 hour at room temperature. For loading controls, the membranes were stripped with stripping buffer (Carl Roth GmbH) at 56°C for 5 min. Antibody binding was detected with the ECL detection reagent (Amersham). Bands were quantified with Quantity One Software (Bio-Rad Laboratories).

Supplementary figures:

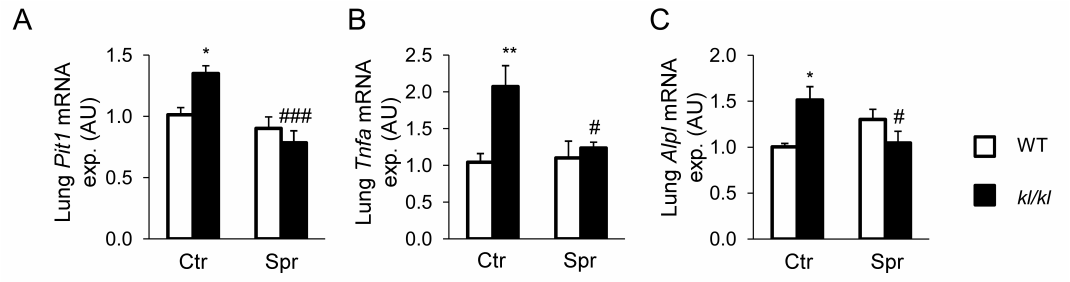
Supplementary figure 1: Effect of spironolactone treatment on aortic *eNos* and *Pai1* expression. Arithmetic means ± SEM (n= 7-15; arbitrary units) of aortic (**A**) *eNos* and (**B**) *Pai1* mRNA levels of wild-type mice (WT, white bars) and klotho-hypomorphic mice (*kl/kl*, black bars), treated with control solution (Ctr, left columns) or spironolactone (Spr, right columns). #(p<0.05), ##(p<0.01) compared with *kl/kl* mice; *(p<0.05), ***(p<0.001) compared with WT control-treated mice.



Supplementary figure. 2:
Effect of spironolactone treatment on renal calcification signaling in *kl/kl* mice. Arithmetic means ± SEM (n=7-8; arbitrary units) of mRNA levels encoding *Wnt3a* (**A**) and *Wnt7a* (**B**) in renal tissue of wild-type mice (WT, white bars) and klotho-hypomorphic mice (*kl/kl*, black bars), treated with control solution (Ctr, left columns) or spironolactone (Spr, right columns). Representative original western blots showing β-catenin/Gapdh protein abundance (**C**), phospho-p38/total-p38/Gapdh protein abundance (**D**) and Nf-kB p65/Gapdh protein abundance (**E**) in renal tissue of untreated wild-type mice (WT) and klotho-hypomorphic mice (*kl/kl*) as well as spironolactone treated wild-type (WT^{spr}) and klotho-hypomorphic mice (kl/kl^{spr}). Arithmetic means ± SEM (n=6-8; arbitrary units) of β-catenin/Gapdh protein ratio (**F**), phospho-p38/total-p38/Gapdh protein ratio (**G**), and Nf-kB p65/Gapdh protein ratio (**H**) in renal tissue of wild-type mice (WT, white bars) and klotho-hypomorphic mice (*kl/kl*, black bars), treated with control solution (Ctr, left columns) or spironolactone (Spr, right columns). #(p<0.05), ##(p<0.01) compared with *kl/kl* mice; *(p<0.05), ***(p<0.001) compared with WT control-treated mice.



Supplementary figure 3:
Spironolactone-sensitive *Pit1*, *Tnfa* and *Alpl* gene expression in lung tissue of *kl/kl* mice**.** Arithmetic means ± SEM (n= 7-10; arbitrary units) of mRNA levels encoding: (**A**) *Pit1*, (**B**) *Tnfa* and (**C**) *Alpl* in lung tissue of wild-type mice (WT, white bars) and klotho-hypomorphic mice (*kl/kl*, black bars), treated with control solution (Ctr, left columns) or spironolactone (Spr, right columns). #(p<0.05), ###(p<0.001) compared with *kl/kl* mice; *(p<0.05), **(p<0.01) compared with WT control-treated mice.



Supplementary figure 4:
Effect of spironolactone treatment on osteoinductive signaling in lung tissue of *kl/kl* mice. Arithmetic means ± SEM (n= 7-9; arbitrary units) of mRNA levels encoding: (**A**) *Msx2*, (**B**) *Cbfa1* and (**C**) *Osx* in lung tissue of wild-type mice (WT, white bars) and klotho-hypomorphic mice (*kl/kl*, black bars), treated with control solution (Ctr, left columns) or spironolactone (Spr, right columns). # ($p<0.05$) compared with *kl/kl* mice; *($p<0.05$), ** ($p<0.01$) compared with WT control-treated mice. (**D**) Immunohistochemical analysis and confocal microscopy (original magnification, x400) of Msx2, Cbfa1, and osterix expression in lung tissue of wild-type mice (WT), klotho-hypomorphic mice (*kl/kl*) and klotho-hypomorphic mice treated with spironolactone (*kl/kl*^{spr}). Osteoblastic markers expression is represented by green labelling, nuclei are labelled in blue, and actin staining is labelled in red. Scale bar represents 20µm.