**Online Data Supplement**

# Anti-aging Gene Klotho Enhances Glucose-induced Insulin Secretion by Upregulating Plasma Membrane Retention of TRPV2

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Running title: Klotho Enhances Insulin Secretion

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## Methods

**Reagents and antibodies**. Dulbecco's modified Eagle's medium (DMEM) were obtained from Cellgro® Mediatech (Manassas, VA). Fetal bovine serum (BCS) was obtained from ATCC (Manassas, VA). Ethidium bromide, Tranilast, Histopaque 1077 and Histopaque 1119, streptomycin and penicillin, and protease inhibitors were purchased from Sigma (Saint Louis, MO). Insulin antibody (clone H-86) and GLUT2 antibody (clone H-67) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-TRPV2 antibody was ordered from Biomol (Plymouth Meeting, PA). Calcium Orange, Pluronic® F-127, Optifect™ reagent, OPTI-MEM® I reduced serum medium TRIzol® Reagent, RNaseOUT™ Recombinant Ribonuclease Inhibitor, SuperScript™ III Reverse Transcriptase, and OligodT20 were obtained from Invitrogen (Carlsbad, CA). Anti-Klotho antibody was obtained from R&D System (Minneapolis, MN). 57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Insulin ELISA kit from ALPCO (Salem, NH). SDS-PAGE (4-20% Tris-HCL precast gel) and ChemiDoc System BioRad Imager were obtained from Bio-Rad (Hercules, CA). The pAAV empty vector and pAAV-GFP were obtained from Stratagen (La Jolla, CA, USA). The siPort™ NeoFX™ transfection kit and control siRNA were obtained from Ambion (Austin, TX). Collagenase-P was obtained from Roche (Penzberg, Germany). The RNeasy® Mini Kit and Plasmid Maxi kit were purchased from Qiagen (Valencia, CA, USA). BCA assay was ordered from Pierce (Rockford, IL). The Taq 2X Mater Mix was obtained from New England Biolabs (Ipswich, MA).

**RNA Isolation, RT-PCR, and real time RT-PCR**. For detecting insulin gene expression, MIN6 cells transfected with plasmid DNA pAAV-GFP or pAAV-mKL for 72 hours were washed twice with PBS and then incubated with DMEM containing 0.5% FBS and 2.8 or 25 mM glucose for 15 hours. Total RNA was purified from MIN6 cells using TRIzol® Reagent, followed by Qiagen RNeasy® Mini Kit. RNA (500 ng) was reverse-transcribed using SuperScript™ III Reverse Transcriptase with OligodT20 in the presence of 10ul dNTP. The resulting cDNAs were used as templates for PCR with oligonucleotides primers to amplify *β-actin* mRNA, *insulin I* mRNA, *insulin II* mRNA, and *insulin II* pre-mRNA.

One specific primer pairs for mouse *insulin I* mRNA were used (F: 5′-CCTGTTGGTGCACTTCCTAC-3′ and R: 5′-TGCAGTAGTTCTCCAGCTGG-3′), which generated a PCR product with 317 bps (1). One specific primer pairs for mouse *insulin II* mRNA (F-5'- AGCCCTAAGTGATCCGCTACAA-3' and R-5'-CATGTTGAAACAATAACCTGGAAGA-3') were used (2), whihh generated a pCR product with 178 bps. One specific primer pair for mouse

*Insulin II* pre-mRNA was used (F: 5'-GGGGAGCGTGGCTTCTTCTA-3' and R: 5'-GGGGACAGAATTCAGTGGCA-3'), which generated a PCR product with 86 bps. This primer pair targets *insulin II* extron 2/intron 2 mRNA, which has been shown with a shorter half life compared to mature *insulin* mRNA (3). The primers for the *β-actin* mRNA were used as the internal control (F: 5'-AGGTATCCTGACCCTGAAGT-3' and R: 5'-ACTGTGTTGGCATAGAGGTC-3'), which generated a PCR product of 708 bps. PCR reactions (50-μL volume) contained 3 ul of above cDNA, 0.2 μM of appropriate oligonucleotide primer pair, and 1x of New England Biolab Taq 2X Mater Mix. PCR amplification conditions were as follows: 5 min at 95 °C followed by 30 cycles of 95 °C for 1min, optimized annealing temperature for each primer pair for 1 min, and 68 °C for 1 min. The PCR products were separated on 1.5% agarose gels and stained with ethidium bromide (Sigma). The bands were visualized using a ChemiDoc System BioRad Imager (Bio-Rad) and quantified using Image J software (NIH).

For real-time RT-PCR, one microgram of total RNA from above purification was reverse-transcribed using SuperScript™ III Reverse Transcriptase with random hexamer in the presence of 10ul dNTP. The resulting cDNAs were used as templates for real time PCR. Real-time RT-PCR was performed on a Bio-Rad CFX96™ Real-Time PCR Detection Systems, using the same forward and reverse primers to amplify *insulin II* pre-mRNA as described above, and the primer pairs (F: 5'-AGGTCATCACTATTGGCAACGA-3' and R: 5'-CACTTCATGATGGAATTGAATGTAGTT-3') for *β-actin* mRNA, which generated a PCR product of 118 bps. Bio-Rad SsoFast™ EvaGreen® Supermix was used. PCR were cycled 40 times using the following conditions: 95 °C for 5 s, 60 °C for 5 s. Homogeneity of products from each reaction was confirmed by melt curve analysis and 1.5% agarose gel analysis of PCR products.

**Western blotting analysis.** Briefly, at 70 to 80% confluence, MIN6 cells were washed twice with PBS. Cells were lysed with Ripa buffer containing the protease inhibitor cocktail. Protein concentration was measured with the Pierce BCA assay. Lysates (40 μg protein/well) under the reducing condition were directly subjected to SDS-PAGE (4-20% Tris-HCL precast gel) followed by Western blotting with rabbit antibody against insulin (H-89, Santa Cruz). The same blot was re-probed with antibody against β-actin after stripping the blot.

## Results

**Overexpression of Klotho did not alter insulin mRNA and protein expression**.

Mouse has two *insulin* genes, *insulin I* and *II*. Overexpression of mouse Klotho did not affect *insulin I* or *insulin II* mRNA levels. Given that half life of mature *insulin* mRNA levels is relative long, we also examine *insulin II* pre-mRNA, which has shorter half life and is considered a good indicator for *de nevo* synthesis of *insulin* gene in MIN6 cells (3). Glucose (25 Mm) did increase *insulin II* pre-mRNA levels compared to 2.8 mM glucose group, but overexpression of mouse Klotho did not affect *insulin II* pre-mRNA levels in MIN6 cells (Fig. S1). Similar results were also observed in real time RT-PCR (Fig. S1E.). These results indicated that Klotho did not affected *insulin* gene levels in MIN6 cells.

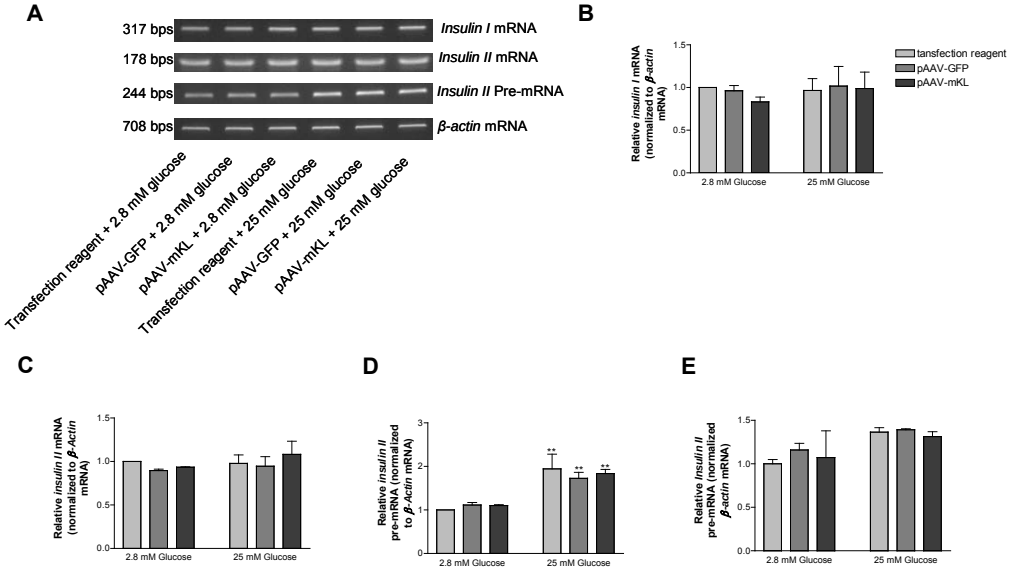



**Figure 1. Overexpression of mKL did not affect *insulin I* and insulin II mRNA levels and *insulin II* pre-mRNA levels in MIN6 cells.** MIN6 cells transfected with plasmid DNA pAAV-GFP or pAAV-mKL for 72 hours were washed with PBS and then incubated with DMEM containing 0.5% FBS and 2.8 or 25 mM glucose for 15 hours. ***A*:** RT-PCR analysis of *insulin I* and *II* mRNA, *insulin II* pre-mRNA, and *β-actin* mRNA levels in MIN6 cells. The quantification of three independent experiments was displayed in ***B, C, and D*** after normalized to the *β-actin* mRNA levels. **p<0.01 *vs* the control group (transfection agent). ***E:*** Real time RT-PCR analysis of *insulin II* pre-mRNA, after normalized to the *β-actin* mRNA levels in MIN6 cells.

In Western-blotting, overexpression of Klotho did not affect insulin protein levels in MIN6 cells (Fig. 2).

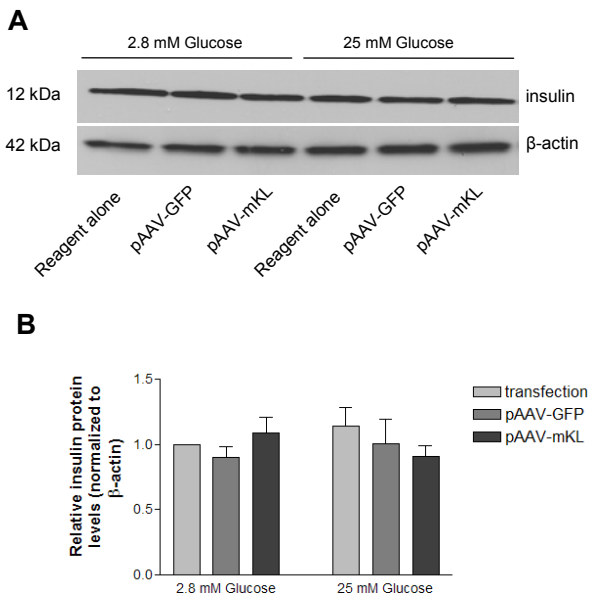
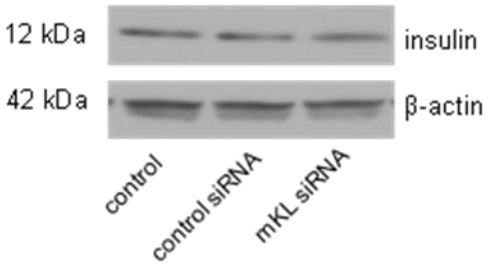



**Figure 2. Overexpression of mKL did not affect insulin protein levels in MIN6 cells.** Following transfection with plasmid DNA pAAV-GFP or pAAV-mKL for 72 hours, MIN6 cells were washed with PBS and then incubated with DMEM containing 0.5% FBS and 2.8 or 25 mM glucose for 15 hours. MIN6 cells were lysed with Ripa buffer. ***A*:** Insulin from the lysates was immunoblotted (upper panel). The same blot was re-probed with antibody against β-actin after stripping the blot (lower panel). ***B:*** Quantification of insulin. Data=means±SEM. Results were normalized to β-actin level and expressed as fold changes vs the control group (transfection reagent alone) (n = 3).

**Suppression of Klotho did not alter insulin protein levels**.

To explore the effect of suppression of Klotho expression on insulin protein levels, we silenced *Klotho* gene expression by transfection of MIN6 cells with siRNA against mouse *Klotho*. As shown in Figures S3 (next page), transfection with mKL siRNA for 72 hours did not alter the insulin protein levels significantly in MIN6 cells.

A




B

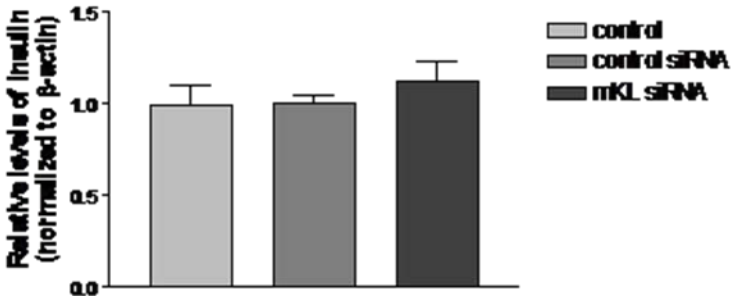



**Figure 3. Suppression of mKL did not alter insulin protein levels in MIN6 cells.** Following transfection with mKL siRNA for 72 hours, MIN6 cells were lysed with Ripa buffer. ***A*:** Insulin from the lysates was immunoblotted (upper panel). The same blot was re-probed with antibody against β-actin after stripping the blot (lower panel). ***B:*** Quantification of insulin expression. Data=means±SEM. Results were normalized to β-actin level and expressed as fold changes vs the control group (control) (n = 3).

# References

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