Supporting Information

A novel transgenic model to study thyroid axis activity in early life stage medaka

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Materials and Methods:

Appendix A: Characterization and cloning of *thyroglobulin* promoter.

The promoter region of the medaka *tg* gene has not been previously characterized. I*n silico* analysis was performed using genomic medaka sequences potentially containing the *tg* promoter and presumably involved in the regulation of *tg* transcription (upstream *thyroglobulin* cDNA, Gene ID: 101172845, RefSeq: NC_019874.2). Briefly, we analyzed a 10 kb region directly upstream from the *tg* coding sequence in order to identify the position of binding elements for transcriptional factors known to be involved in *tg* regulation (Unipro UGENE v36.0, Novosibirsk, Russia). We compared both the medaka sequence and the putative regulatory region of zebrafish *thyroglobulin* used to develop the Tg(*tg:mCherry*) transgenic line[12]. ATACseq results of this medaka genomic region were analyzed in order to assess potential chromatin accessibility [13] and to match identified putative binding elements. We focused on putative binding sites of three transcription factors that have been identified as regulators for *tg* transcription: thyroid transcription factors 1 (TTF1, also referred to as NKX2.1) and 2 (TTF2 also referred to as FOXE1) and paired box 8 (PAX8).[14–17] The binding site of PAX8 overlaps the binding site of TTF1 and TTF2 is bound to the complex NF1/CTF. [17,18] We identified two putative Pax8/TTF1 binding sites as well as one putative TTF2 site close to a NF1 site in the 3 kb upstream *tg* sequence from zebrafish. The genome of medaka and zebrafish are highly divergent in this region and they contain different repeated sequences upstream of the putative *tg* start codon (Figure S1A). This made this localization of common regulatory sequences for both species more complex, but we identified four putative Pax8/TTF1 binding sites and two putative TTF2 sites close to NF1 sites in a 5 Kb 5'-upstream region of the *tg* gene. From this analysis we decided to focus on the 5 kb-long sequence upstream of the *thyroglobulin* ATG. This 5kb-long sequence was synthetized with the addition of 5' Xho1 and 3' Sac2 sites and was subcloned into the pEGFP1 plasmid (GenBank Accession #U55761) by GenScript (GenScript Biotech, Leiden, Netherlands). Specifically, it was subcloned into the multi-cloning site between Xho1 and Sac2 sites and upstream of the GFP coding sequence, in order to generate the pTG-eGFP plasmid (Figure S1B) and to establish the Tg(*tg:eGFP*) medaka transgenic line.

Appendix B: Immunofluorescence *in toto* (or whole-mount immunofluorescence)

Whole-mount antibody staining was performed as previously described[1,2]. Newly hatched embryos were euthanized by overdose of tricaine (1 g/L) and fixed overnight at 4°C in PBS containing 4% paraformaldehyde. Embryos were then rinsed three times in PBS and stored in 100% methanol at -20°C after serial incubations with increasing concentrations of methanol. Prior to immunofluorescence staining, embryos were incubated in 10% H_2O_2 in PBS for 5 h at room temperature, to block endogenous peroxidases and rinsed three times for 10 min with 0.3% triton in PBS (PBS-T). To block unspecific binding sites, eleuthero-embryos were incubated in goat and mouse serum (4% each) in PBS-T for 2 h. Overnight incubation with primary antibodies was performed with an anti-thyroxine antibody (anti-T_4, 1:200 rabbit anti-thyroxine BSA serum #658501; MB Biomedicals, Solon, OH, USA) and a polyclonal anti-human thyroglobulin (TG) antibody (anti-TG, 1:200 rabbit anti-human thyroglobulin #A0251; Dako, Agilent Technologies, Santa Clara, CA, USA) either alone, or with Chicken anti-GFP antibody (1:1000, Abcam #13970, Cambridge, UK). Embryos were rinsed in PBST-T six times for 1 hour. The anti-TG antibody was developed for human thyroglobulin, but was reported to stain zebrafish[3] and medaka[4] thyroid follicles at hatching stage. Embryos were then incubated in the dark with secondary antibodies for 4 h at room temperature using a mouse anti-rabbit IgG-R (1:200, Santa Cruz Biotechnology #sc-2492, Santa Cruz, CA, USA) and an Alexa Fluor 488-conjugated goat anti-chicken IgG antibody (1:1000, Invitrogen #A11039, Carlsbad, CA, USA). Finally, embryos were rinsed

six times for 1 hour with PBS-T and twice in PBS. Shortly after, images of the eleuthero-embryos were acquired at 10x, 20x or 40x magnification using an inverted confocal microscope (LSM-880 Airyscan, Zeiss, Oberkochen, Germany), equipped with an Argon laser (488nm, for Alexa Fluor 488 antibody) and DSSP laser (561nm, for IgG-R). Zen Black Software from Zeiss and FIJI software [5] were used for image acquisition and analysis.

Appendix C: Histological analyses of thyroid follicles

At 72 h post-hatching, embryos were euthanized by overdose of tricaine (1 g/L), fixed for 12 h in Modified Davidson's fixative at 4°C, then stored in 70% ethanol at 4°C. Afterwards, embryos were embedded in an agarose mold[6] in order to place them in the same orientation in blocks of 12 embryos. Agarose blocks were processed in a tissue processor machine (TP1020, Leica, Nussloch, Germany) to replace ethanol with paraffin (Paraplast, Carl Roth, Karlsruhe, Germany). The blocks were then embedded in bigger paraffin blocks using an embedding machine (EG1140H, Leica, Nussloch, Germany). Paraffin 4 µm sections were cut with a rotating microtome (Microm HM 355S, Thermo Fischer Scientific, Waltham, MA, USA) and transferred to microscope glass slides. Slides were stained with Haematoxylin & Eosin (Cellstain-15, Tharmac, Limburg an der Lahn, Germany), and then imaged using a slide scanner AxioScan Z1 (Zeiss, Oberkochen, Germany).

Appendix D: RNA extraction and qPCR.

To perform RNA extractions a stainless-steel ball (INOX AISI 304 grade 100 AFBMA) and 500 µL TRIzol (Invitrogen #15596-026, Carlsbad, CA, USA) were added in reaction tubes containing 20 whole embryos. Samples were lysed with Tissue Lyser II apparatus (Qiagen, Courtaboeuf, France), as described before.[7] The mix was deposited on RNA purification columns (RNeasy Mini Kit, Qiagen #74104, Courtaboeuf, France) according to the manufacturer's instructions and RNA was eluted with RNAse free water. The RNA concentration was measured with a Nano-Drop ND-1000UV-Vis spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA) and RNA integrity was assayed using an Agilent Bioanalyser, (Agilent RNA 6000 Nano, Agilent: 5067-1511, Santa Clara, CA, USA). DNAse treatment, reverse transcription of mRNA and quantitative PCR were carried out to quantify RNA abundance as previously described[7]. Primers were designed using Primer-BLAST[8] (SI, Table-S1). Raw results were processed using the ΔCt method as described previously, [9,10] in order to compare the expression levels of genes from each other, and at any time point. Briefly, ΔCt is equal to the difference in threshold cycles for the target gene and the reference gene $\Delta C_T = (C_{T,X} - C_{T,R})$. No calibrator sample is purposely defined here, in order to provide information about levels of mRNA from 0 dph to 3 dph, as fold-change would evaluate expression changes relatively to the calibrator sample (usually control sample or 0 dph sample). Data were normalized using the endogenous reference gene *tbp*, which codes for TATA-binding protein. The endogenous control was selected based on geNorm[11] analysis of a panel of candidate genes (data not shown). The results are the means of four different runs, presented as heatmap with -ΔCt for each time point, in order to give the same direction of variation as the mRNA levels.

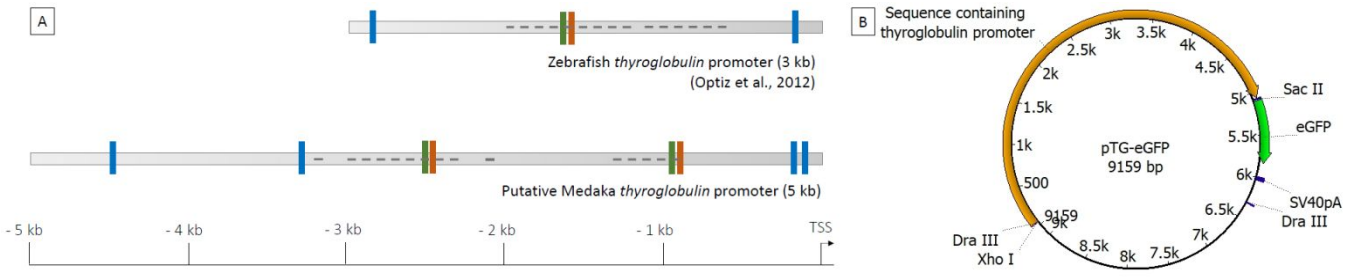

Figure S1: Characterization and cloning of the thyroglobulin promoter. (A) Schematic representation of the zebrafish thyroglobulin (*tg*) genomic locus including the promoter, previously used in the Tg(tg:mCherry) transgenic line[12], and the putative medaka tg genomic locus selected for the transgene (predicted NF1/CTF binding site (BS) (green), TTF-2 BS (orange), Pax8/TTF-1 BS (blue), after *in silico* analysis. Dotted lines represent repeated sequences. (B) Plasmid map of the DNA construct microinjected in one-cell medaka embryos to perform the transgenesis.

Gene ID	Full Gene name	Name	Sequence Forward	Sequence Reverse
101171658	tshba	*tsh*	5'-CAGCTGCCACTGTAGTGCC-3'	5'-GTACACCTCCCTCCGCCTG-3'
101170888	*tshr*	*tshr*	5'-AGGAGCAAAGCGATGGAGAT-3'	5'-CGAAGTATGTGTGGTAGAGG-3'
101161389	solute carrier family 5 member 5	*slc5a5*	5'-CCAGGCTCTGAAAAAGAAATCAG-3'	5'-GACAGGCAAAGCAGGCATACT-3'
105356959	thyroid peroxidase	*tpo*	5'-GGCCTGCCCTTGATTTCTCT-3'	5'-GCTTCTGCAATTCTGTTGTGTTCT-3'
101172845	thyroglobulin	*tg*	5'-AACTGGGCCGAAAGTAGAGC-3'	5'-TGAACCGCTGCGAGTAAAGT-3'
101174678	monocarboxylate transporter 8	*slc16a2*	5'-TTCGCAAAGGAGCAGTTCAA-3'	5'-CAGATGAGGCTCCAATGCAA-3'
101156322	solute carrier organic anion transporter family member 1C1	*slco1c1*	5'-AGCTGCGACAAAGTCTTTCCA-3'	5'-CTCCGAGAGAAATGACGAAGGA-3'
100192348	iodothyronine deiodinase 2	*dio2*	5'-TTCTGGATGCCTACAAGCAG-3'	5'-GCCACGAGGAACACTAGTCA-3'
101165608	iodothyronine deiodinase 3a	*dio3*	5'-ACGGAGCCTTTTTCGACAGA-3'	5'-GGACCCCTACCACCTTGATAAAC3'
101167294	thyroid hormone receptor alpha a	*thraa*	5'GATGACGTCAGGATGTCCTGTTT3'	5'CCTGAATAAAGCACCTGCACAC3'
100049275	thyroid hormone receptor alpha b	*thrab*	5'-GGGTTTCTTTCGTAGGACCA-3'	5'-CTGGTTGCGAGTGATCTTGT-3'
100049254	thyroid hormone receptor beta	*thrb*	5'-GCTTTATGCGTGTGCAAGTT-3'	5'-CGCGTACGAAGTCAAGGTTA-3'
100049222	TATA box binding protein	*tbp*	5'GGTAACTGCAGACCTCGTGAC3'	5'ACTAGAACAGAAATAAGAGCGCGA3'

Table 1: RT-qPCR primer sequences.

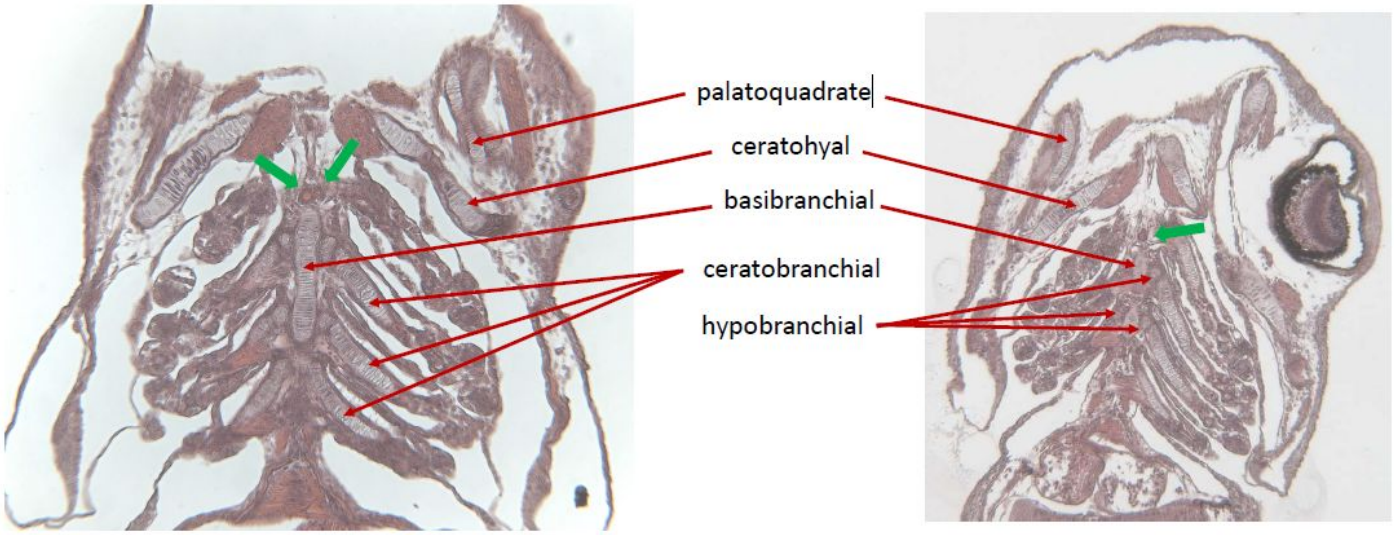

Figure S2: Histology section of a 3 dph wild-type medaka, showing thyroid follicles (green arrow) and their position regarding cartilage structures.

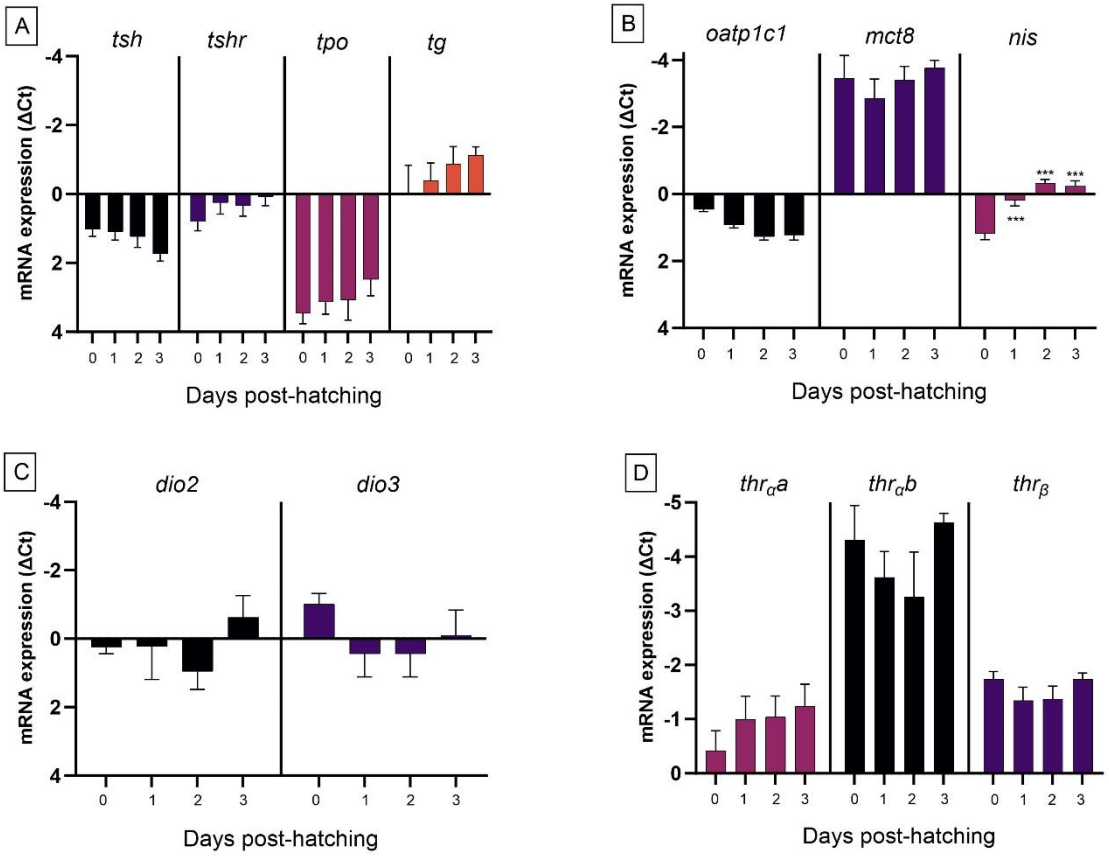

Figure S3. RT-qPCR analysis of genes involved in thyroid hormone signaling. Expression of the genes during the first three days post-hatching represented as mean of ΔCt (for n=7). Y axis is reversed to be more intuitive: the more negative ΔCt is, the lower the Ct of the gene of interest is compared to the Ct of gene of reference (TATA-binding protein), therefore the higher the expression of the gene is. Thrαb is the more expressed gene here of the 12 genes compared to the reference gene. ***: $P < 0.001$. Mean ± SEM are given.

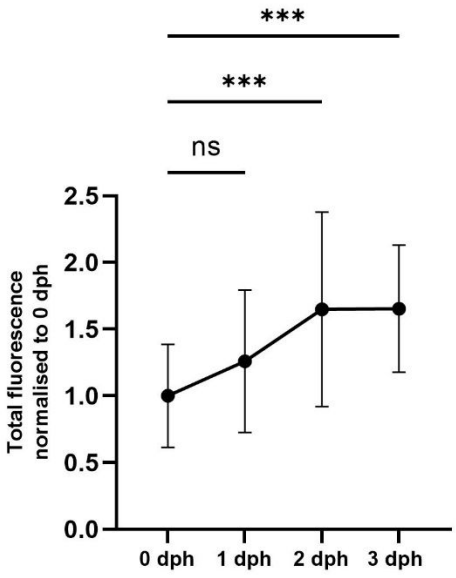

Figure S4: Mean of total fluorescence of thyroid follicles normalized to the group 0 dph, from medaka eleuthero-embryos Tg(*tg:eGFP*) from the hatching day, and for 72 hours, n= 30-40 embryos) (2 runs of n=15/20). ***: P < 0.001. Mean ± SD are given.

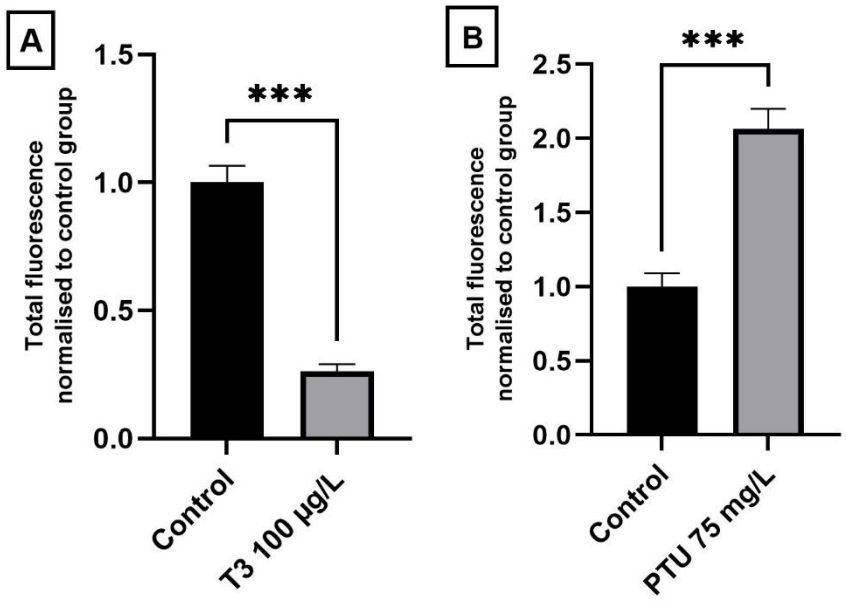

Figure S5: Mean of total fluorescence of thyroid follicles normalized to the control group from medaka eleuthero-embryos Tg(*tg:eGFP*) exposed to T3 100 µg/L (A) or PTU 75 mg/L (B) for 72 hours, n= 15. One run was performed, as it was part of range-finding tests, no sublethal effects was observed at those concentrations.). ***: $P < 0.001$. Mean ± SEM are given

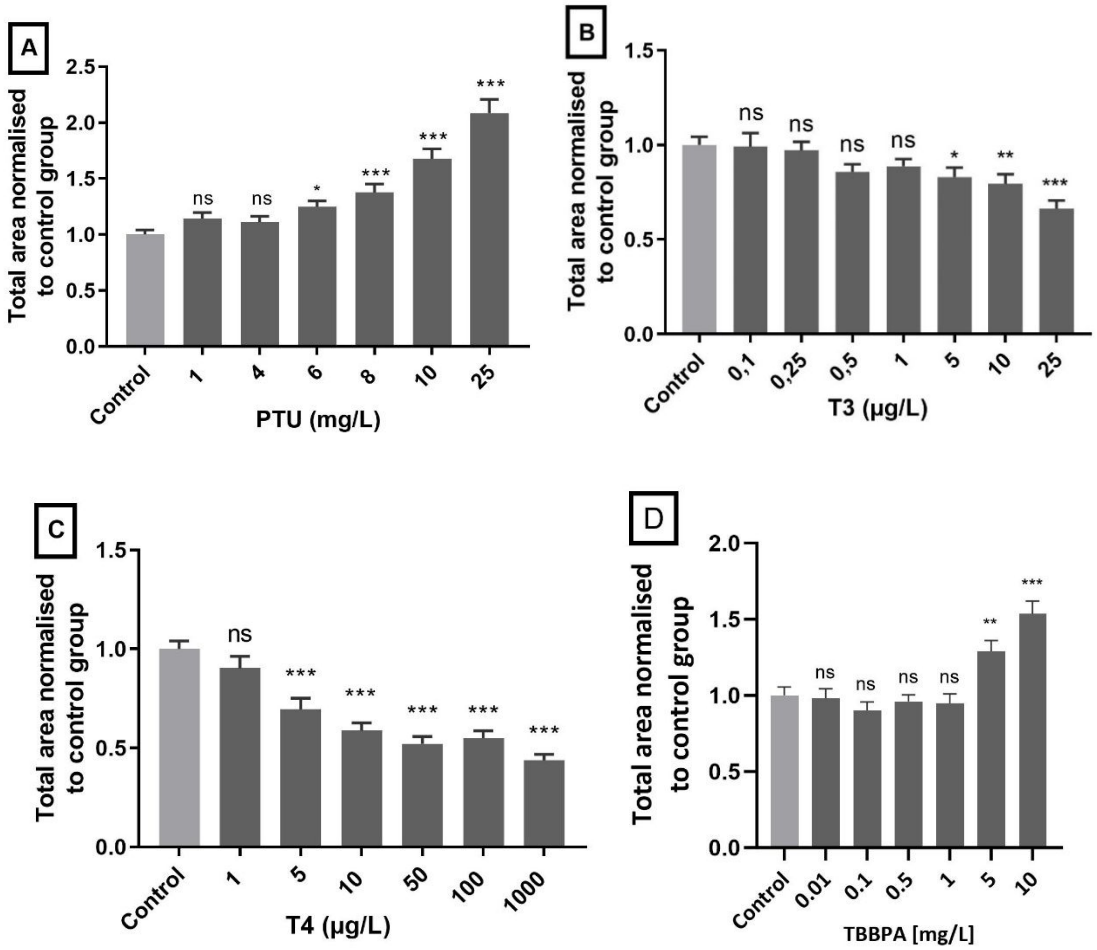

Figure S6: Mean of total area expressing GFP normalized to the control group from medaka eleuthero-embryos Tg(*tg:eGFP*) exposed to increasing concentrations of PTU (A), T3 (B), T4 (C), TBBPA (D) for 72 hours, n= 45 (n=15 per run, with three independent runs performed and pooled, from the same experiments shown in Figure 5-D). ***: P < 0.001 **: P < 0.01 and *: P < 0.05. Mean ± SEM are given.

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