Smad7 Regulates Dental Epithelial Proliferation during Tooth Development

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Appendix

Antibody information

Primary antibodies used in the study were:

1. Anti-Smad7 1:300 (IHC-P), Abcam, ab216428;
2. Anti-Ki67 1:500 (IHC-P), Abcam, ab15580;
3. Anti-p-Smad2/3 1:100 (IHC-P), Abcam, ab52903;
   Anti-p-Smad2/3 1:1000 (WB), CST, D27F4;
4. Anti-Smad2/3 1:500 (WB), Santa Cruz, sc-133098;
5. Anti-Cyclin D1 1:100 (IHC-P), 1:1000(WB), Abcam, ab16663;
6. Anti-p21 1:250 (IHC-P), 1:1000(WB), Abcam, ab188224;
7. Anti-GAPDH 1:5000 (WB), GeneTex, GTX100118;
8. Anti-Cleaved caspase-3 1:300 (IHC-P), CST, 5A1E.

Secondary antibodies, all from Thermo Fisher Scientific and used at 1:500 dilution, were: goat-anti rabbit (A11037), donkey-anti rabbit (A21207, A21206, and A32795).

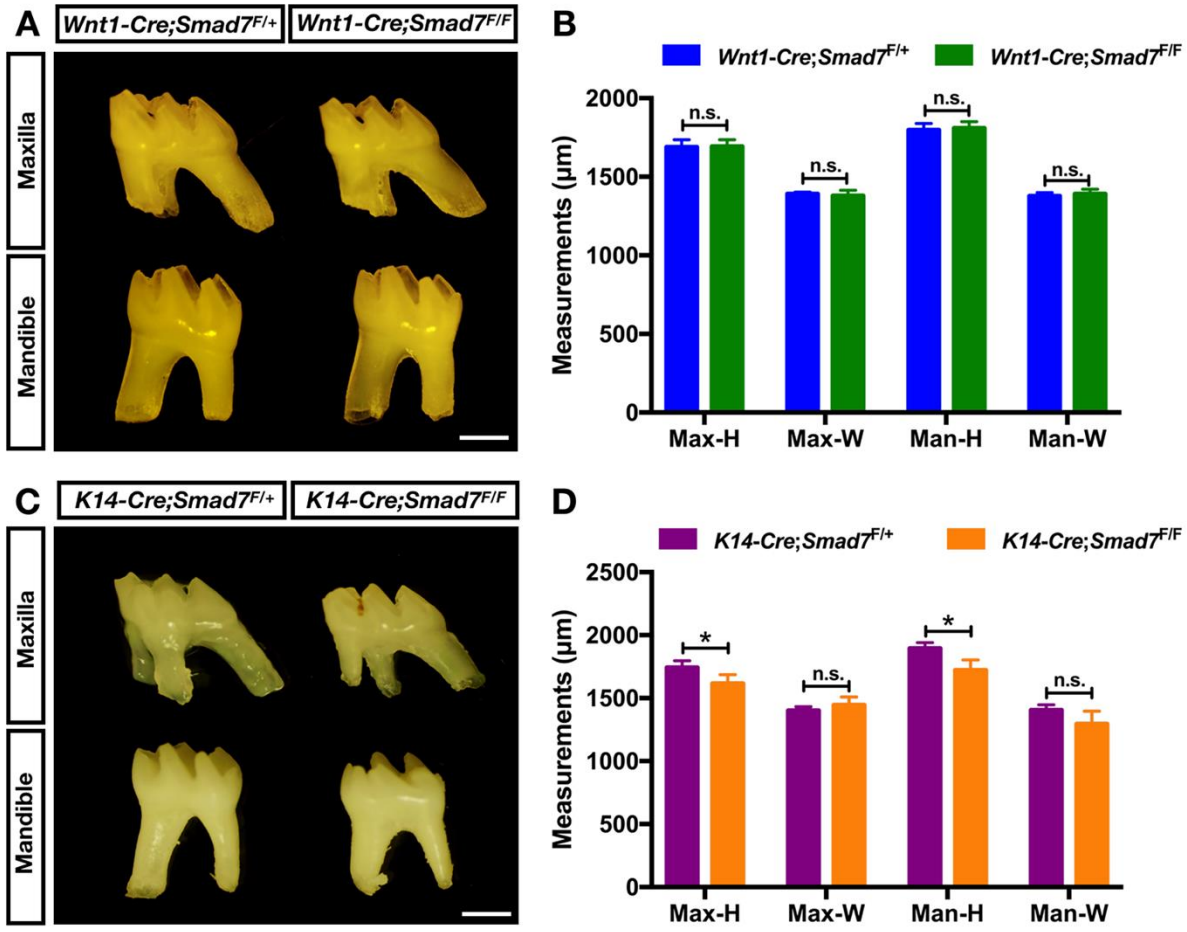
Western blotting

Protein was extracted and normalized according to manufacturer's instructions. 20-80 μg of protein were separated by SDS-PAGE and transferred to 0.45 μm PVDF membrane. Membranes were blocked in TBS with 0.1% Tween 20 and 5% BSA for 1 hr, followed by overnight

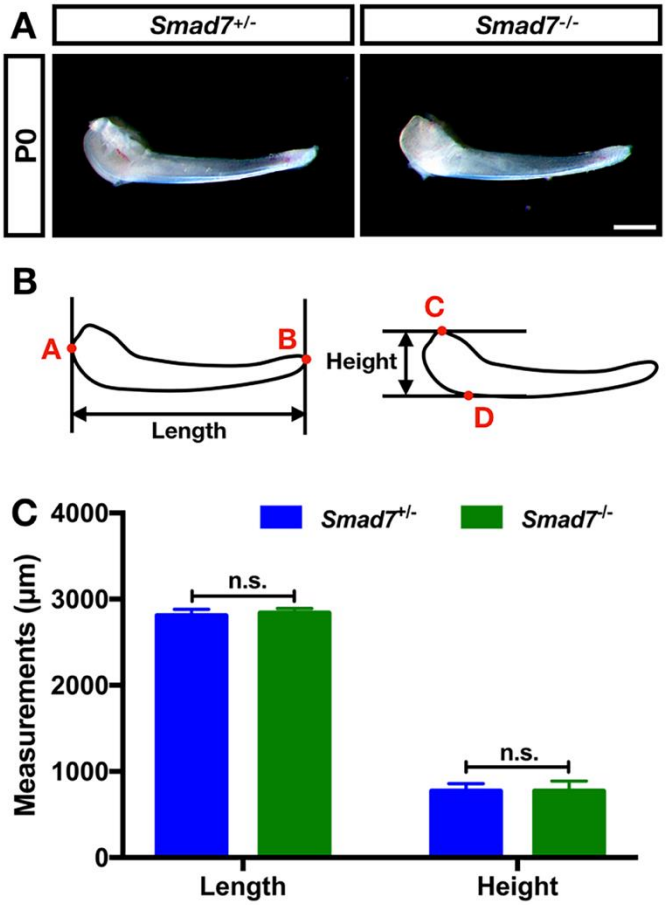
incubation with primary antibodies (see Appendix for antibody information) in blocking solution, and 1 hr incubation with HRP-conjugated secondary antibody diluted at 1:5000. Immunoreactive protein was detected by Odyssey Imaging System (LI-COR). Densitometry analysis on the bands was performed by the NIH image J software with normalization to total protein levels.

CCK8 assay

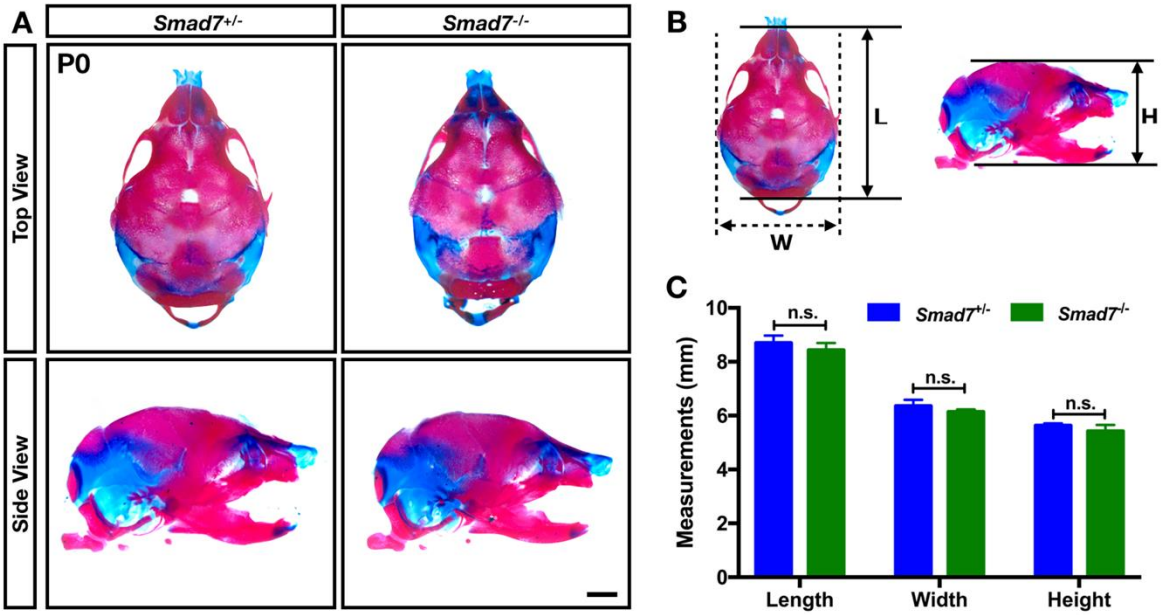
CCK8 assay was performed to determine cell proliferation rate. Briefly, tooth germ cells from either E15.5 or P0 were plated in flat-bottomed 96-well microplates at 2×10^3cells/well and divided into four groups: *Smad7*$^{+/-}$, *Smad7*$^{+/-}$ + DMSO, *Smad7*$^{-/-}$, and *Smad7*$^{-/-}$ + SB431542. After 1, 2, 3, 4, 5, 6, 7 and 8 days in culture, 10 μl fresh CCK8 solution (Dojindo, Japan) was added into each well at the end of the experiment. After incubation at 37°C for 2 hr, the absorbance of each well was determined using a microplate reader at 450 nm. The rate of cell proliferation was determined as the percentage of absorbance of treated cells to that of control cells. Five independent experiments were performed for each group, and the graph was plotted according to the average value.



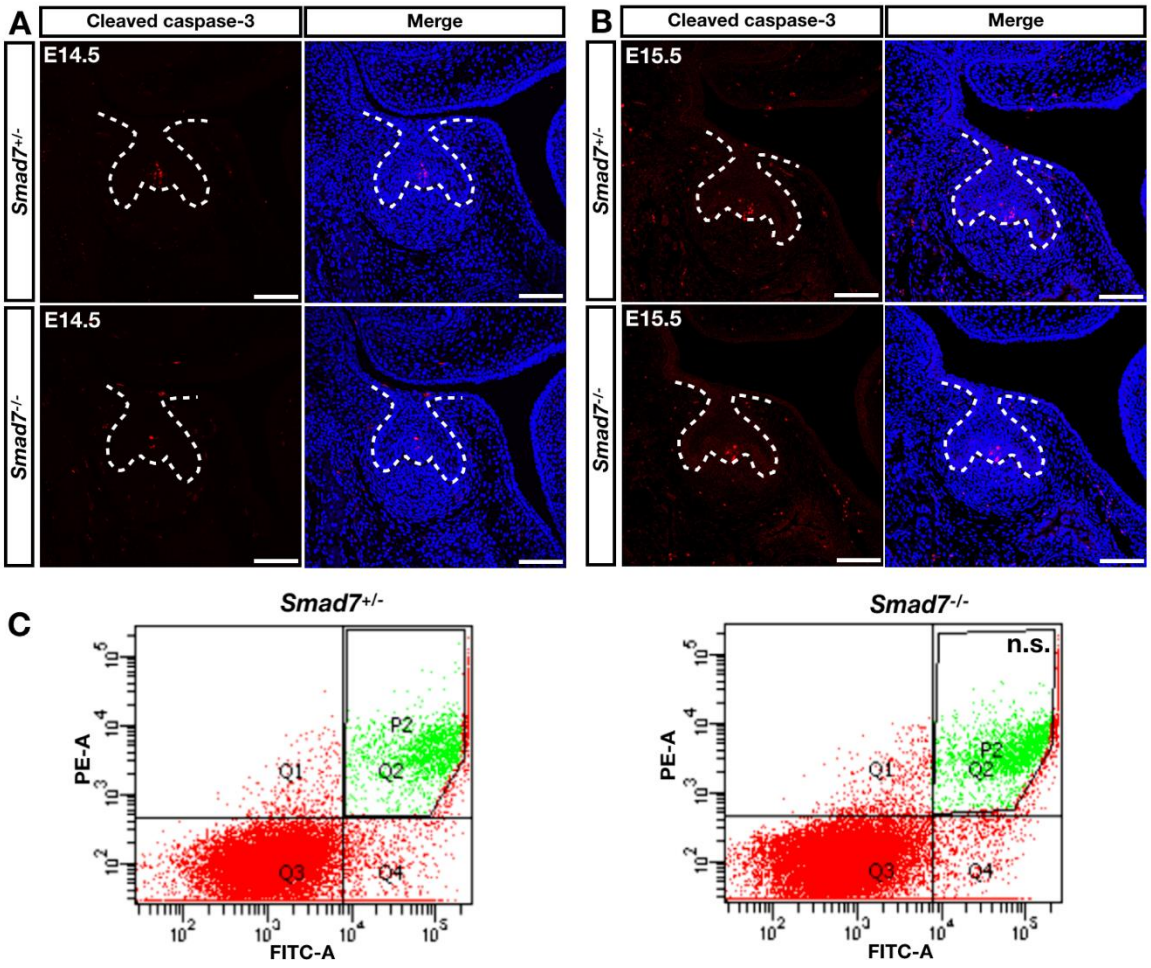
Appendix Figure. 1 Ablation of *Smad7* either in the dental epithelium or mesenchyme does not cause obvious tooth phenotype. (A, B) Representative images and statistical analyses (n = 4 for each genotype) of control and *Wnt1-Cre;Smad7*$^{F/F}$ lower first molar at weaned age show unaltered dental patterning and size in both groups. (C, D) Representative images and statistical analyses (n = 4 for each genotype) of control and *K14-Cre;Smad7*$^{F/F}$ lower first molar at weaned age show unaltered dental patterning but a recognizable reduction only in tooth height in mutants compared to controls. *: $P < 0.05$. n.s.: no statistical significance. Scale bars = 500-μm.



Appendix Figure. 2 Loss of *Smad7* does not affect incisor development. (A) Representative images of control and *Smad7*$^{-/-}$ lower incisors at P0 show unaltered morphology and size in both groups. (B) Schematic representation of the method used for measuring incisor size including length and height. Point A: the most proximal point of the incisor; point B: the distal tip of the incisor; point C: the top point of the lingual cervical loop; point D: the buccal bottom of the incisor. (C) Statistical analyses of the measurements of incisors from control and *Smad7*$^{-/-}$ mice at P0 (n = 3 for each genotype). n.s.: no statistical significance. Scale bars = 500-µm.



Appendix Figure. 3 *Smad7* deficiency does not affect head size at P0. (A) Whole-mount skeletal preparations of control and *Smad7*$^{-/-}$ heads at P0 show comparable size in both groups. (B) Schematic representation of the method used for measuring the head size including length, width, and height. (C) Statistical analyses of the measurements of the heads from control and *Smad7*$^{-/-}$ mice at P0 (n = 3 for each genotype). n.s.: no statistical significance. Scale bars = 1-mm.



Appendix Figure. 4 Inactivation of *Smad7* does not increase cell apoptosis in the developing tooth. (A and B) Immunostaining shows the comparable levels of cell apoptosis in the dental epithelium of the *Smad7*$^{-/-}$ molar at E14.5 (A) and E15.5 (B). (C) Flow cytometry histograms of cell apoptosis distribution by Annexin V-FITC/PI double staining in control and *Smad7*$^{-/-}$ tooth germ cells freshly isolated at E15.5. n.s.: no statistical significance. Scale bars = 100-μm

Appendix Table. Primer sequences for Quantitative RT-PCR.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
Cdkn1a	CCTGGTGATGTCCGACCTG	CCATGAGCGCATCGCAATC
Ccnd1	GCGTACCCTGACACCAATCTC	CTCCTCTTCGCACTTCTGCTC
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA