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tooth-specific biology and development

Supplementary material

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Supplemental Information

Organoids from mouse molar and incisor as new tools to study tooth-specific biology and development

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Supplemental Figures and Legends

Figure S1

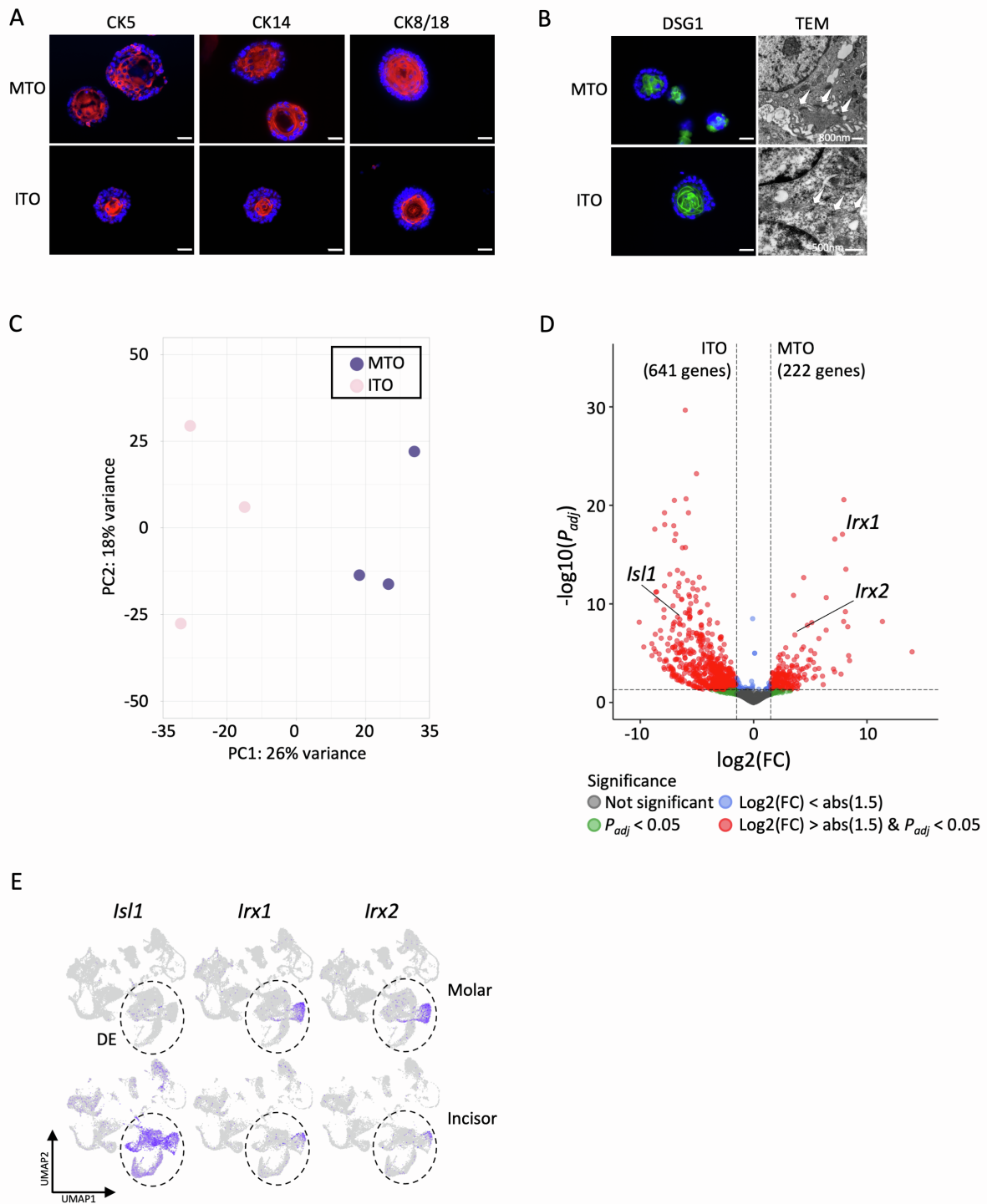


Figure S1. Additional characterization of organoids from mouse molar and incisor. Related to Figure 1. (A) IF analysis of indicated cytokeratins (CK; red) in TO. Nuclei of all IF images are counterstained with Hoechst33342. (B) IF (DSG1, green) and ultrastructural (TEM) characterization of desmosomes in TO. Arrows indicate desmosomes (TEM images). (C) PCA plot of bulk RNA-seq data from MTO and ITO (variance per component as indicated). (D) Volcano plot with $\log_2(\text{fold change (FC)})$ versus $-\log_{10}(P_{adj})$ value of RNA-seq data from MTO and ITO. Statistically upregulated genes (right for MTO and left for ITO) are indicated in red, as determined by a combination of $\log_2(\text{FC}) > \text{the absolute (abs) value of } \pm 1.5$, and $P_{adj} < 0.05$. (E) Projection of indicated genes on our previously published mouse tooth scRNA-seq atlas (Hermans et al., 2022). The dental epithelium (DE) is circled. Scale bars: 25 μm for IF images.

Figure S2

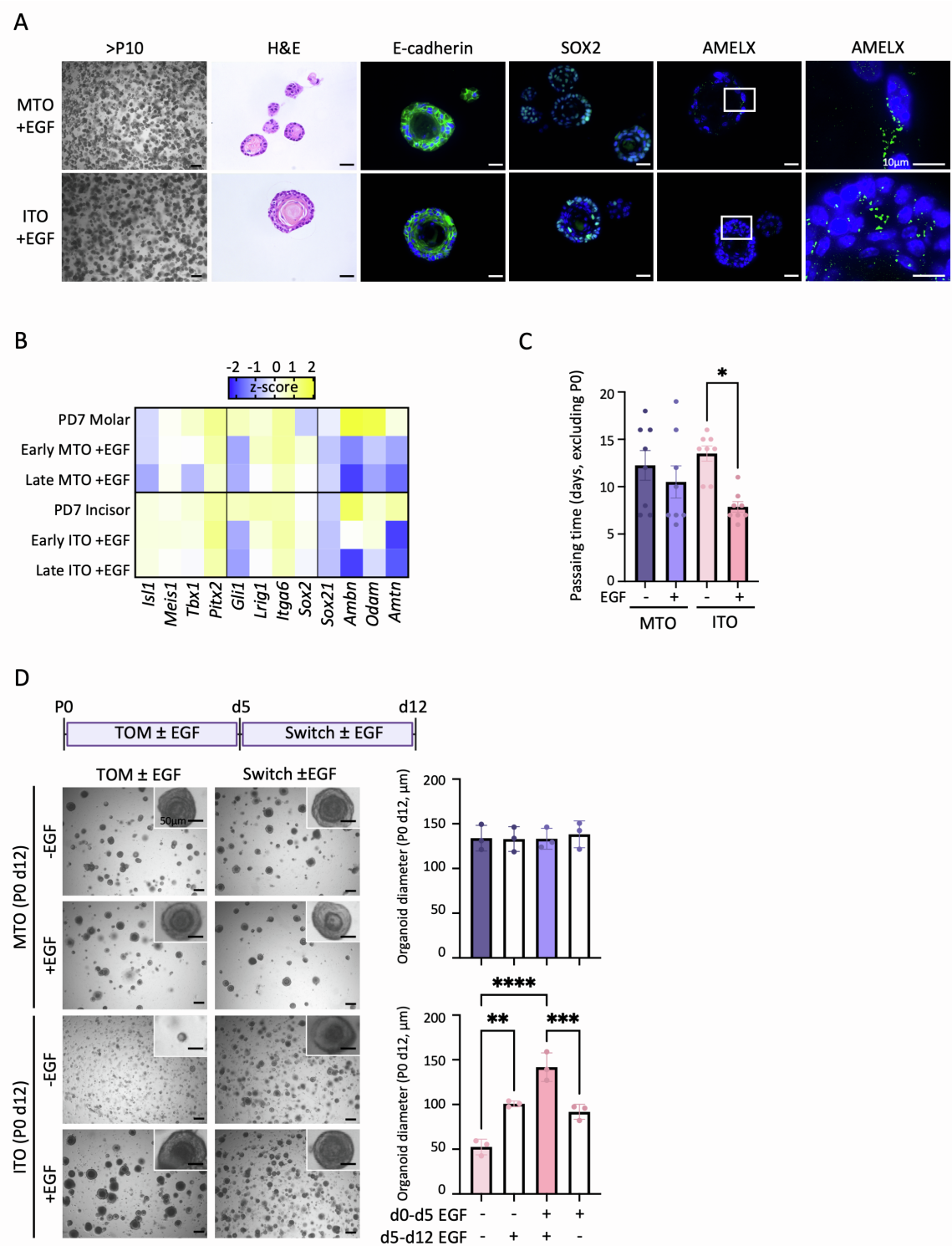


Figure S2. Characterization of TO grown in the presence of exogenous EGF. Related to Figure 2. (A) Brightfield images of MTO and ITO grown in the presence of EGF after long-term culture for more than 10 passages (>P10); H&E and IF evaluation (of indicated markers) of organoid morphology and characteristics. Nuclei are counterstained with Hoechst33342 (blue). Boxed areas are magnified. (B) Heatmap of gene expression of DE TF, proposed DESC markers and EMP in primary molar and incisor tissue, early- and late-passage TO grown in the presence of EGF, as quantified by RT-qPCR analysis. Data are presented as relative expression to *Gapdh* (ΔC_t) and z-score normalized. Colors range from blue (low expression) to yellow (high expression). (C) Bar graph showing time between organoid passaging (days, excluding P0; mean \pm SEM). Data points represent biological replicates from independently established organoid lines; one-way ANOVA with Šídák's multiple comparisons test. (D) *Top*: Timeline of experimental set-up. TO were seeded in P0 with or without EGF and grown for 12 days. After 5 days of initial growth in TOM with or without EGF, EGF was either removed or added from TOM ('switch'), respectively, for 7 more days. *Bottom-left*: Brightfield images of P0 d12 organoids grown in TOM without exogenous EGF (-EGF) or with EGF (+EGF), or alternatively switched from -EGF to +EGF and *vice versa*. *Bottom-right*: Bar graphs (mean \pm SEM) showing organoid diameter on d12. Data points represent biological replicates from independently established organoid lines; one-way ANOVA with Šídák's multiple comparisons test. Scale bars: 250 μ m for brightfield images and 25 μ m for H&E and IF images, unless indicated otherwise. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure S3

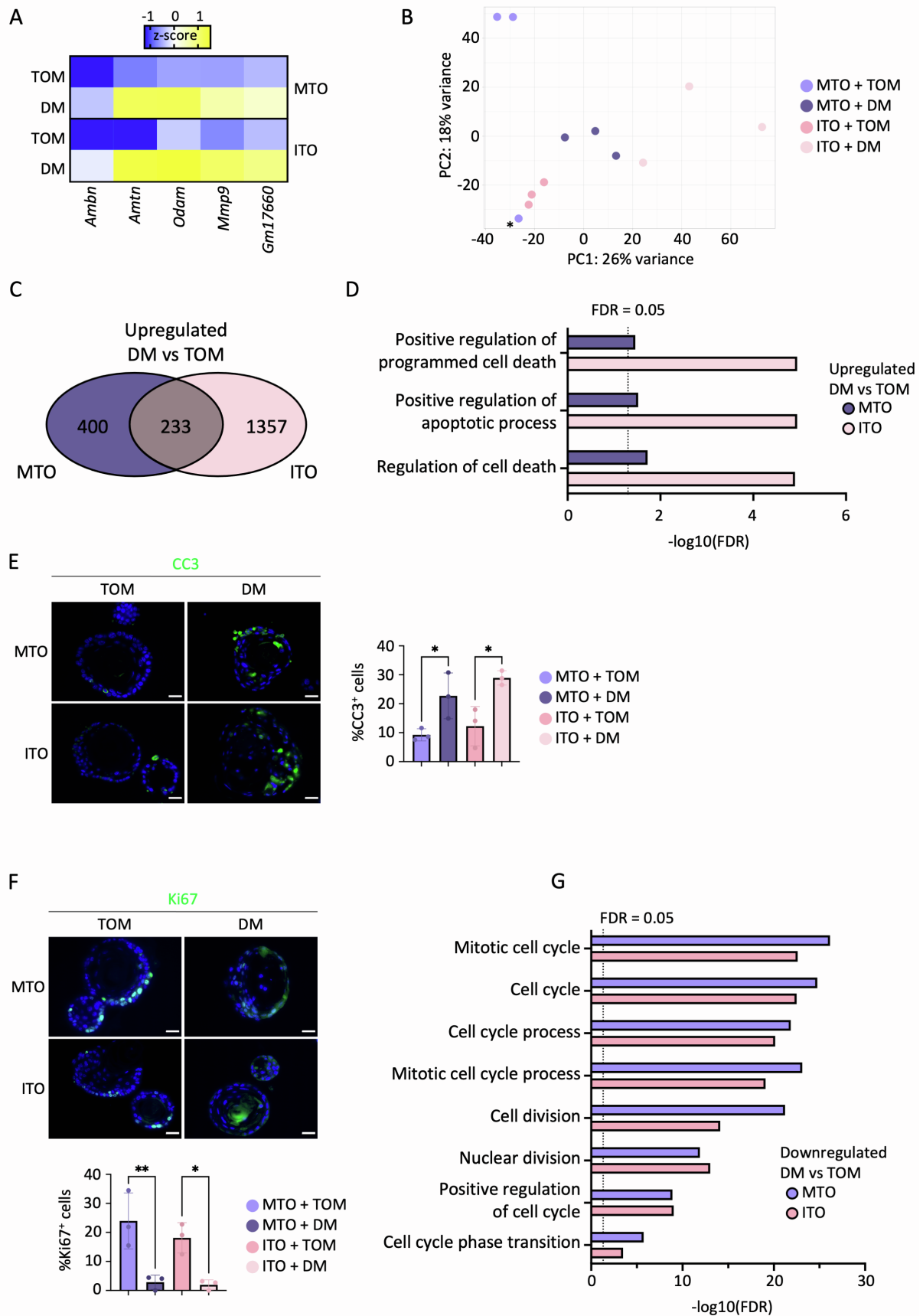


Figure S3. *In vitro* differentiation of TO toward AB-resembling cells. Related to Figure 3. (A) Heatmap of gene expression of AB markers in TO grown in TOM or switched to DM after 7d, as quantified by RT-qPCR analysis. Data are presented as relative expression to *Gapdh* (ΔC_t) and z-score normalized. Colors range from blue (low expression) to yellow (high expression). (B) PCA plot of bulk RNA-seq data from TOM- and DM-grown MTO and ITO (variance per component as indicated). * indicates an outlier which was removed from subsequent analyses. (C) Venn diagram indicating the number of DEG upregulated in DM-*versus* TOM-grown MTO and ITO, and tooth-type overlap. (D) Significant ($FDR \leq 0.05$, indicated by dotted line) DEG-based GO terms associated with apoptosis enriched in DM-grown TO compared to TOM-cultured controls. (E) *Left*: IF analysis of CC3 (green) in TO. Nuclei of all IF images are counterstained with Hoechst33342. *Right*: Bar graph (mean \pm SEM) showing proportion of CC3⁺ cells in organoids. Data points represent biological replicates from independently established organoid lines; one-way ANOVA with Šídák's multiple comparisons test. (F) *Top*: IF analysis of Ki67 (green) in TO. *Bottom*: Bar graph (mean \pm SEM) showing proportion of Ki67⁺ cells in organoids. Data points represent biological replicates from independently established organoid lines; one-way ANOVA with Šídák's multiple comparisons test. (G) Significant ($FDR \leq 0.05$, indicated by dotted line) DEG-based GO terms associated with proliferation and cell cycle downregulated in DM-grown TO compared to TOM-cultured controls. Scale bars: 25 μ m. * $P < 0.05$, ** $P < 0.01$.

Figure S4

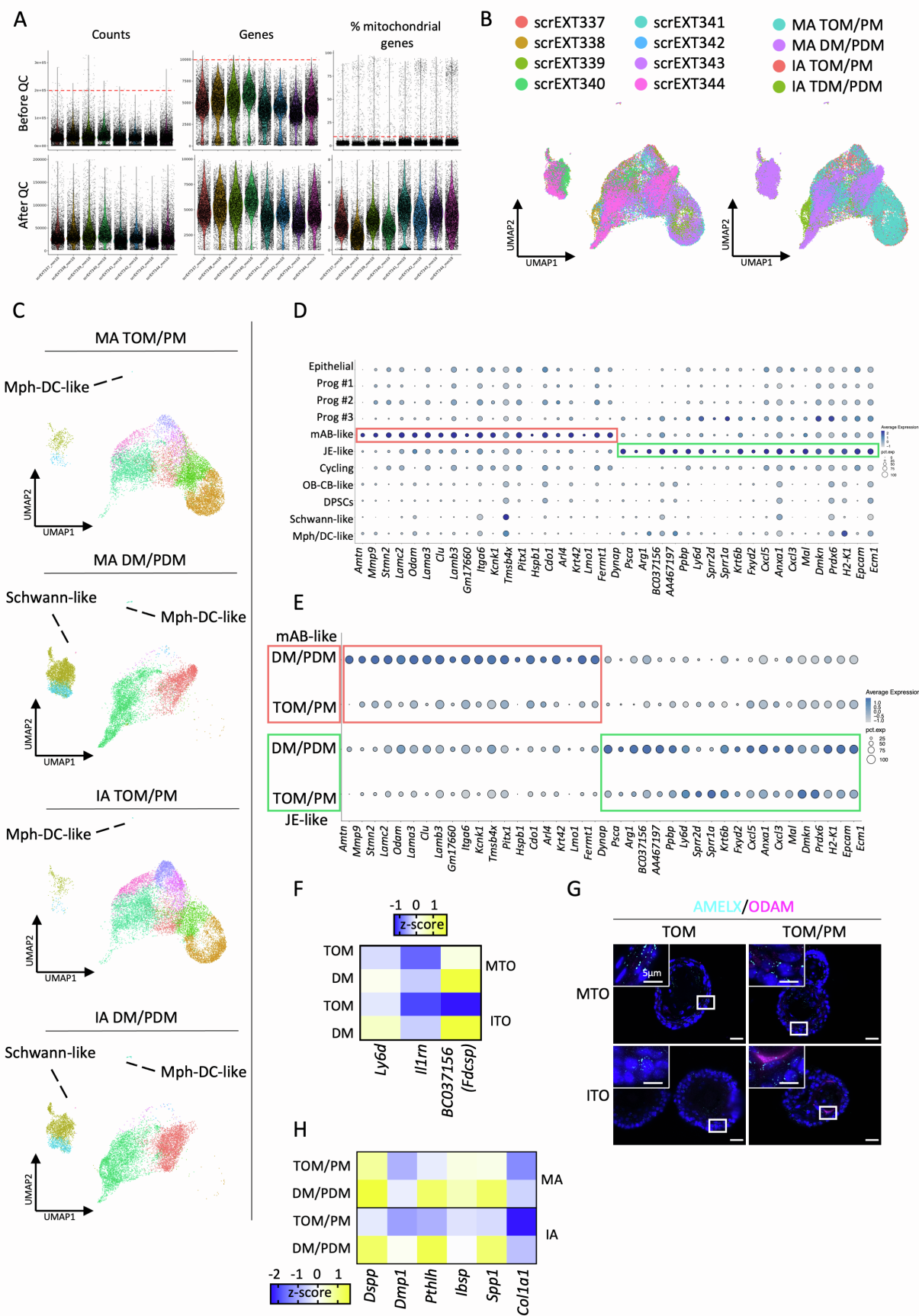


Figure S4. Quality control of tooth assembloid scRNA-seq analysis and additional characterization of developed assembloids. Related to Figure 4. (A) Violin plots showing the distribution of counts, genes and percent mitochondrial genes of the individual assembloid scRNA-seq samples before and after performing quality control (QC). Cells with values matching the following cut-offs were removed: counts >200,000, genes <1,000 and >10,000, percentage of mitochondrial genes >8%. (B) UMAP plots of integrated assembloid scRNA-seq data indicating individual samples (left, see Table S5) or experimental groups (right). (C) Annotated UMAP plots of integrated assembloid scRNA-seq datasets for MA TOM/PM- (*top*), MA DM/PDM- (*second*), IA TOM/PM- (*third*), and IA DM/PDM-grown (*bottom*) assembloids. See Figure 4C for cluster annotation. (D) Dotplot displaying the percentage of cells (dot size) expressing the top 20 DEG of mAB- (red box) and JE-like (green box) cells (average expression levels indicated by color intensity, see scale). (E) Dotplot displaying the percentage of mAB- (red) and JE-like (green) cells from TOM/PM- and DM/PDM-grown assembloids (dot size) expressing the top 20 DEG of mAB- (red box) and JE-like (green box) cells (average expression levels indicated by color intensity, see scale). (F) Heatmap of gene expression of JE markers in TO grown in TOM or switched to DM after 7d as quantified by RT-qPCR analysis (n=2 biological replicates from independently established organoid lines). Data are presented as relative expression to *Gapdh* (ΔC_t) and z-score normalized. Colors range from blue (low expression) to yellow (high expression). (G) IF analysis of AMELX (cyan) and ODAM (magenta) in TO controls grown in TOM or TOM/PM. Nuclei are counterstained with Hoechst33342 (blue). Boxed areas are magnified. (H) Heatmap of gene expression of markers of OB-/CB-like differentiation in tooth assembloids. Data are presented as relative expression to *Gapdh* (ΔC_t) and z-score normalized. Colors range from blue (low expression) to yellow (high expression). Scale bars: 25 μ m, unless otherwise indicated.

Supplemental Tables

Table S1. Serum-free defined medium (SFDM) composition. Related to Table 1.

Product	Concentration	Supplier	Catalogue number
Sterile H ₂ O			
DMEM 1:1 F12 without Fe	16.8 g/L	Invitrogen	074-90715A
Bovine Serum Albumin (BSA)	5 g/L	Serva	47330.03
Catalase from bovine liver	50 µL/L	Sigma-Aldrich	C100
Ethanol absolute, ≥99.8%	600 µL/L	Fisher Chemical	E/0650DF/15
Insulin from bovine pancreas	5 mg/L	Sigma-Aldrich	I6634
NaHCO ₃	1 g/L	Merck	106329
Penicillin	35 mg/L	Sigma-Aldrich	P3032
Streptomycin	50 mg/L	Sigma-Aldrich	S6501
Transferrin	5 mg/L	Serva	36760.01

Table S2. TO differentiation medium (DM) composition

Product	Concentration	Supplier	Catalogue number
SFDM			
B27 (without vitamin A)	2%	Gibco	12587-010
BMP2	100 ng/mL	Peprotech	120-02C
BMP4	50 ng/mL	Peprotech	120-05ET
EGF	20 ng/mL	R&D Systems	236-EG
FGF2 (=basic FGF)	20 ng/mL	R&D Systems	234-FSE
TGFβ1	4 ng/mL	R&D Systems	240-B

Table S3. Pulp medium (PM) composition

Product	Concentration	Supplier	Catalogue number
α-MEM		Sigma-Aldrich	M4526
ESGRO Recombinant LIF protein	10 ³ units/mL	Sigma-Aldrich	ESG1107
Fetal Bovine Serum (FBS)	10 or 20%*	Sigma-Aldrich	F7524
FGF2 (=basic FGF)	2.5 ng/mL	R&D Systems	234-FSE
Penicillin-streptomycin	1%	Gibco	P4333

*20% FBS used for initial passage (i.e entire P0) of DPSC culture only. 10% FBS used for subsequent passages and all experiments.

Table S4. Pulp differentiation medium (PDM) composition

Product	Concentration	Supplier	Catalogue number
PM (with 10% FBS)			For composition see Table S3
Ascorbic acid	100 μ M	Sigma-Aldrich	A4544
β -glycerophosphate	5 mM	Merck	35675-GM
Dexamethasone	10 μ M	Sigma-Aldrich	D4902

Table S5. Metadata of assembloid scRNA-seq

Sample	Tooth derived	Condition	Medium
scrEXT337_mm10	Incisor	Control	TOM/PM
scrEXT338_mm10	Incisor	Differentiation	DM/PDM
scrEXT339_mm10	Molar	Control	TOM/PM
scrEXT340_mm10	Molar	Differentiation	DM/PDM
scrEXT341_mm10	Incisor	Control	TOM/PM
scrEXT342_mm10	Incisor	Differentiation	DM/PDM
scrEXT343_mm10	Molar	Control	TOM/PM
scrEXT344_mm10	Molar	Differentiation	DM/PDM

Table S6. Antibodies used for immunofluorescence staining**Primary antibodies**

Antigen	Host	Company	Catalogue number	Dilution
AMELX	Mouse	Santa Cruz Biotechnology	365284	1:100
CC3	Rabbit	Sigma-Aldrich	AB3623	1:100
CK5	Rabbit	Biolegend	905501	1:1000
CK8/18	Guinea pig	Progen	GP11	1:200
CK14	Mouse	Thermo Fisher Scientific	MA5-11599	1:1000
DSG1	Rabbit	Proteintech	24587-1-ap	1:500
E-cadherin	Rabbit	Cell Signaling Technologies	24E10	1:400
ISL1	Rabbit	Abcam	ab20670	1:1000
Ki67	Mouse	BD Bioscience	556003	1:100
LAMC1	Rabbit	NovusBio (Biotechnne)	NBP1-877118	1:200
ODAM	Rabbit	Proteintech	16509-I-AP	1:200
SOX2	Rabbit	Abcam	AB97959	1:2000
VIM	Rabbit	Cell Signaling Technologies	D21H3	1:400

Secondary antibodies

Antigen	Host	Company	Catalogue number	Dilution
Mouse IgG (A488)	Donkey	Thermo Fisher Scientific	A-21202	1:1000
Mouse IgG (A555)	Donkey	Thermo Fisher Scientific	A-31570	1:1000
Rabbit IgG (A488)	Donkey	Thermo Fisher Scientific	A-21206	1:1000
Rabbit IgG (A555)	Donkey	Thermo Fisher Scientific	A-31572	1:1000
Guinea pig IgG (FITC)	Donkey	Jackson ImmunoResearch	706-096-148	1:500

Table S7. Forward and reverse primers used for qPCR gene expression analysis

Gene	Forward	Reverse
<i>Ambn</i>	CTGTCAACCAGGGAACCACT	TGTGATGCGGTTTAGCTGAG
<i>Amtn</i>	CTCAGACCGTCACATCCTCA	TGTGGATAAAGCAGGCTTCC
<i>Areg</i>	CCATCATCCTCGCAGCTATT	CTTGTCGAAGCCTCCTTCTT
<i>BC037156 (Fdcsp)</i>	AAAAC TCTTCTCCTGCTCGCT	CACTGTCACTGGCACTTCGT
<i>Btc</i>	TTCGTGGTGGACGAGCAAACCTC	CCATGACCACTATCAAGCAGACC
<i>Col1a1</i>	GCATGGCCAAGAAGACATCCC	GCATACCTCGGGTTTCCACG
<i>Dmp1</i>	CTCCTTGTGTTCTTTGGGGG	TCTGATGACTCACTGTTCTGTGG
<i>Dspp</i>	CAGGAACTGCAGCACAGAATGA	TATCTCACTGCCATCTGGGGA
<i>Egf</i>	ACTGGTGTGACACCAAGAGGTC	CCACAGGTGATCCTCAAACACG
<i>Epgn</i>	GAGCGAAGAAGCAGAGGTGATC	GGTCTTCCAGACAAGGATGAGAG
<i>Ereg</i>	CAGGCAGTTATCAGCACAACCG	CATGCAAGCAGTAGCCGTCCAT
<i>Gapdh</i>	ACCAGAGCATGATAAGGCAGCC	TGATGAGGCTGAAGGGTGTGAC
<i>Gm17660</i>	TTCCCGAATCTGTGCGCTCC	TGGAAC TCTCCTCCGGATTGTC
<i>Hbegf</i>	GTCCGTCTGTCTTCTTGTCTATC	CGCCCAACTTCACTTTCTCT
<i>Ibsp</i>	TACGGAGCAGAGACCACACC	TCTGCATCTCCAGCCTTCTTGG
<i>Il1rn</i>	GCTCATTGCTGGGTACTTACAA	CCAGACTTGGCACAAGACAGG
<i>Isl1</i>	GCTGCCTCTTTGATGGCTTCGA	CACATTCGGCACTGTTACAGCC
<i>Itga6</i>	CTCCTAATGCTATCTTCAAGGCG	ACCCTGAGATTGCCCAGAG
<i>Lrig1</i>	TTCAGCCAACGCTACCCTCACA	TAAGCCAGGTGATGCGTGGTGT
<i>Ly6d</i>	CAAAACCGTCACCTCAGTGGA	AGTCTGGCAGCATTGTGTGA
<i>Meis1</i>	GCAGTTGGCACAAGATACAGGAC	ACTGCTCGGTTGGACTGGTCTA
<i>Mmp9</i>	CTGGACAGCCAGACACTAAAG	CTCGCGGCAAGTCTTCAGAG
<i>Nrg1</i>	GCTCATCACTCCACGACTGTCA	TGCCTGCTGTTCTCTACCGATG
<i>Nrg2</i>	GGATGGCAAGGAACTCAACC	TCGGCCTCACAGACGTACT
<i>Nrg3</i>	CGAGACAAGGACCTGGCGTATT	TCACAACGGACTCCTTGGTAGC
<i>Nrg4</i>	TCCTCCTCACTCTTACCATCGC	GTCTCTACCAGGCTGATCTCAC
<i>Odam</i>	CCCTAAGATGCACAAC TCGGAG	GTAGTCGGGATGCTCCTTCATG
<i>Pitx2</i>	CGGCAGAGGACTCATTTTAC	TTCTTGAACCAAACCCGGAC
<i>Pthlh</i>	GGCGTTTCGGTGGAGGGGCTT	CAGATGGTGGAGGAAGAAACGG
<i>Sox2</i>	GAAGTGGCTGAACGAGGCATTG	TTGTCCGTGGAGGACCTTGCAT
<i>Sox21</i>	CCCTAAGATGCACAAC TCGGAG	GTAGTCGGGATGCTCCTTCATG
<i>Spp1</i>	GGCAGCTCAGAGGAGAAGAAGC	AGCATTCTGTGGCGCAAGG
<i>Tbx1</i>	CGAGATGATCGTCAACCAAGGCA	GTCATCTACGGGCACAAAGTCC

Supplemental experimental procedures

Isolation and dissociation of early-postnatal mouse molar and incisor tissue

C57BL/6 PD7 mice (pool of male and female) were used for tooth isolation, obtained by breeding in the Animal Housing Facility of the KU Leuven under conditions of constant temperature, humidity, 12-hour light-dark cycle and *ad libitum* access to food and water. Experiments were approved by the KU Leuven Ethical Committee for Animal Experimentation (P056/2022).

Pups were euthanized by decapitation, after which the mandibles were collected in Dulbecco's Modified Eagle Medium with 10 mM HEPES (DMEM; Gibco), supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1% penicillin-streptomycin (Gibco). Whole (unerupted) molars, including surrounding dental follicle and attached epithelium, as well as apical ends of (unerupted) incisors were carefully isolated, rinsed with phosphate-buffered saline (PBS; Gibco) and incubated with 2.5% Trypsin (Gibco) for 30 minutes at 37°C. Following trypsinization, the dental follicle and attached epithelium were manually recovered from the molars, and the dental pulp was separately collected from the molar pulp chamber. Subsequently, the molar (either dental follicle and attached epithelium or collected dental pulp tissue) and incisor tissues were further enzymatically digested with TrypLE Express (Gibco) supplemented with 5 μ M ROCK inhibitor (ROCKi, Y-27632; Thermo Fisher) for 15 minutes at 37°C, and mechanically triturated (using syringes with decreasing diameters: 18G (Terumo), 20G (BD) and 26G (BD)) to a suspension containing single cells and small cell clusters. The number of collected cells was determined with a Z2 Coulter Particle Count and Size analyzer using COULTER Z2 AccuComp software (Beckman Coulter).

Establishment and passaging of mouse TO

The dissociated molar and incisor DE cell material was resuspended in serum-free defined medium (SFDM; Thermo Fisher Scientific; Table S1) and growth factor-reduced Matrigel (Corning) at a 30:70 ratio, supplemented with 10 μ M ROCKi. Cells were seeded at 20,000 and 12,500 cells per 20 μ L drop from molars and incisors, respectively. After solidification, pre-warmed TOM (Table 1) was added. Organoid cultures were kept at 37 °C in a 1.9% CO₂ incubator (as required by the SFDM buffering system), and medium was refreshed every 2 days. To passage the TO as occurring every 7-10 days, Matrigel droplets were collected using ice-cold SFDM, followed by incubation with TrypLE containing 5 μ M ROCKi and mechanical trituration to dissociate the organoids. Remaining (large) organoid fragments were allowed to sediment, and the cell number in the supernatant, containing single cells and small fragments, was determined as described above. Upon reaching stable growth, dissociated MTO and ITO were seeded at 15,000-20,000 cells and 7,500 cells per 20 μ L droplet, respectively. Organoids could be cryopreserved to be stored in liquid nitrogen, and be brought again in culture after thawing, all as previously described (Boretto et al., 2017; Cox et al., 2019).

To inhibit EGFR signaling, TO were treated with AG-1478 (1 μ g/mL; Sigma-Aldrich) or EKI-785 (1 μ M; Selleckchem) for 12 days from initial DE cell seeding. To explore TO differentiation capacity, organoids from passage 5 (P5) were grown in TOM for 7 days, and subsequently switched to culture in DM (Table S2) for 7 days. The DM was optimized by removing multiple growth factors from TOM one by one, and subsequently adding BMP2, BMP4 and TGF β 1. As initial readout for DM optimization, gene expression of EMP was assessed (*Ambn*, *Amtn*, *Odsm*). Brightfield pictures of organoids were recorded using an Axiovert 40 CFL microscope (Zeiss).

DPSC culture

DPSC were obtained and cultured as previously described (Collignon et al., 2019). Briefly, molar pulp cells were seeded onto 0.1% gelatin-coated plates (gelatin from porcine skin; Sigma-Aldrich) and cultured in PM with 20% FBS (Table S3). Upon reaching 70-80% confluency, cells were dissociated using TrypLE and replated in PM with 10% FBS. The obtained DPSC were passaged every 7-10 days.

Establishment of mouse tooth assembloids

Tooth assembloids were established using a similar protocol as we previously described for human tooth assembloids (Hemeryck et al., 2022). Briefly, after dissociating TO (from P2-P3) and DPSC (from P2-P3), both matched and derived in the same tooth isolation experiment, into single cells as described above, both cell types were combined in round-bottom low-attachment plates (96-well; Greiner). Using a layered approach, 5,000 DPSC were first seeded by sedimentation (300 g for 1 min at 4 °C), followed by 5,000 MTO- or ITO-derived cells sedimented on top (300 g for 1 min at 4 °C) (Nakao et al., 2007). The cell composite was provided with 10% growth-factor reduced Matrigel and 90% of either a 1:1 mixture of TOM/PM or DM/PDM (Tables 1, S2-4). After 24 h incubation at 37 °C and 5% CO₂, the formed structures were collected and plated in 20 μ L droplets of 70% Matrigel to generate tooth

assembloids which were maintained in the respective 1:1 media mixtures at 37 °C and 5% CO₂. Brightfield pictures of assembloids were recorded using an Axiovert 40 CFL microscope (Zeiss).

Histochemical and immunostaining analysis

Organoids were fixed in paraformaldehyde (PFA, 4% in PBS; Merck) for 30 min at room temperature (RT) and subsequently paraffin-embedded with an Excelsior ES Tissue Processor (Thermo Fisher Scientific). Paraffin sections of 5 µm thickness were obtained with a Microm HM 30 microtome (Thermo Fisher Scientific) and subjected to hematoxylin and eosin (H&E) or immunofluorescence (IF) staining. For IF analysis, antigen retrieval with citrate buffer (pH6; at 95°C) and permeabilization (0.1% Triton X-100; Sigma-Aldrich) were performed. For detection of ISL1, antigen retrieval was done with Tris-EDTA (pH9; at 95°C). Next, blocking buffer (0.15% glycine (VWR), 2 mg/mL BSA (Serva), 0.1% Triton-X in PBS) with 10% donkey serum (Sigma-Aldrich) was added for 1 h at RT. After incubation with primary antibodies and subsequently secondary antibodies (Table S6), and nuclei counterstaining with Hoechst33342 (1:1000; Merck), sections were mounted with ProLong Gold Antifade Mountant (Thermo Fisher Scientific). Images were recorded using a Leica DM5500 upright epifluorescence microscope (Leica Microsystems) accessible through the Imaging Core (VIB, KU Leuven), and analyzed and converted to pictures with Fiji imaging software. For visualization of AMELX⁺ signals, the Fiji 'subtract background' function was used with a rolling ball radius of 3 pixels. Representative images are shown.

EdU labelling of TO

EdU labelling in TO was performed using the Click-iT EdU Alexa Fluor 488 kit (Thermo Fisher Scientific) according to the manufacturer's instructions, involving the incubation of organoids with EdU (10 µM) for 2 h. Images were recorded on a Zeiss LSM 780 – SP Mai Tai HP DS (Cell and Tissue Imaging Cluster (CIC), KU Leuven), and analyzed and converted to pictures with Fiji imaging software. Representative images are shown.

TEM analysis

Organoid samples were prepared for TEM as previously described in detail (Lambrichts et al., 1993; Cox et al., 2019). Samples were fixed in 2.5% glutaraldehyde (Sigma-Aldrich), dehydrated, embedded in epoxy resin, and cut into 40-70 nm sections. TEM images were acquired with the JEM1400 transmission electron microscope (JEOL) equipped with an Olympus SIS Quesmesa 11 Mpxl camera, or the Philips EM208 S electron microscope (Philips) equipped with the Morada Soft Imaging System camera with corresponding ITEM-FEI software (Olympus SIS), or a JEOL-1400 FLASH (JEOL) with Xarosa camera (EMSIS).

Gene expression analysis by qPCR

Total RNA was extracted from dissociated molar and incisor tissue, TO and tooth assembloids using the GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich) following manufacturer's instructions. RNA was reverse-transcribed (RT) using the Superscript III First-Strand Synthesis Supermix kit (Thermo Fisher Scientific). The resultant cDNA samples were analyzed with SYBR Green-based quantitative (q)PCR with the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen) and using specific forward and reverse primers (Table S7), as described before (Cox et al., 2019). Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was included as housekeeping gene. Gene expression levels were calculated as ΔC_t values relative to *Gapdh* ($C_{t\text{target}} - C_{t\text{housekeeping}}$). Z-score normalization was performed by subtracting the mean ΔC_t of the experiment and dividing by the standard deviation of the obtained ΔC_t values for each measurement.

Bulk RNA-seq analysis

RNA was isolated from P0 MTO and ITO grown in TOM (n=3 biological replicates per condition), or from P5 MTO and ITO grown in TOM+EGF and exposed to DM or not (n=3 biological replicates per condition). Total RNA was isolated using the GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich) following manufacturer's instructions, and RNA Integrity Number (RIN) determined with Agilent Picochips on an Agilent BioAnalyzer 2100 (Agilent Technologies). Samples with RIN > 7.5 were sequenced (Nucleomics Core, VIB/KU Leuven). TruSeq total stranded RNA library preparation was performed, followed by sequencing on a NovaSeq 6000 instrument (Illumina). Data are accessible from ArrayExpress database (accession number E-MTAB-12557). Following quality control, reads were aligned to the mouse reference genome (*Mus musculus*, GRCm38/mm10 release M21) and transcript abundancies were quantified using Salmon (1.4.0) (Patro et al., 2017). Gene-level count matrices were created using the tximport package (v1.18.0) and used as input for DEG analysis with DESeq2 (v1.30.1) as previously reported (Love et al., 2014; Sonesson et al., 2016). PCA analysis was performed using the

plotPCA function from the DESeq2 package after rlog transformation. Based on PCA analysis, one MTO+TOM sample was considered an outlier and omitted from further downstream analyses. For visualization and gene ranking, log fold change (LFC) estimates were shrunk using the aplegm (v1.12.0) method for effect size shrinkage as described (Love et al., 2014; Zhu et al., 2019). Volcano plots were generated using the EnhancedVolcano package (v1.6.0) (<https://github.com/kevinblighe/EnhancedVolcano>). GO analysis (FDR < 0.05) of DEG ($\log_2FC > 1.5$ and $P_{adj} < 0.05$) was executed with the GO webplatform (www.geneontology.org) (Ashburner et al., 2000; Carbon et al., 2021). Enriched pathway values are presented as $-\log_{10}(FDR)$.

scRNA-seq analysis

MA and IA, exposed to DM or not (2 replicates per condition), were dissociated into single cells using 0.25% Trypsin (Gibco; supplemented with 5 μ M ROCKi) treatment and mechanical trituration, collected in 0.04% BSA (in PBS) and filtered through a Flowmi 40 μ m cell strainer (Sigma-Aldrich). Single cells were loaded onto 10X Genomics cartridge according to the manufacturer's instructions. Generation of barcoded libraries was performed with the Chromium Single-cell 3' v2 Chemistry Library Kit, Gel Bead & Multiplex Kit and Chip Kit (10 \times Genomics). The libraries were sequenced on an Illumina NextSeq and NovaSeq6000. Data are accessible from ArrayExpress database (accession number E-MTAB-12544). Raw sequencing reads were demultiplexed, mapped to the mouse reference genome (mm10) and gene expression matrices were generated using CellRanger (v5.0.0; 10 \times Genomics).

Downstream analysis was performed in Seurat (v4.0.0) as previously described (Stuart et al., 2019; Hao et al., 2021; Hermans et al., 2022). Briefly, low quality cells and potential doublets were removed based on the number of counts (>200,000) and genes (<1,000 and >10,000) per cell, as well as the percentage of mitochondrial genes (>8%) expressed per cell. After quality control, data were normalized and variable features identified (using the NormalizeData and FindVariableFeatures functions), and the data were integrated by the combination of tooth type and medium condition (i.e. MA+TOM/PM, MA+DM/PDM, IA+TOM/PM and IA+DM/PDM) using the FindIntegrationAnchors (with default parameters and dims = 1:30) and the IntegrateData functions. After scaling of expression levels, integrated data were subjected to PCA. Uniform Manifold Approximation and Projection (UMAP) dimensionality reduction was performed (with umap.method=uwot, using the top 50 PC and with min.dist=0.5). Initial clustering and annotation were performed, prior to correction of count matrices for ambient/background RNA using the SoupX (v1.5.0) package (Young and Behjati, 2020). The global contamination fractions were estimated to be 1.7%, well within normal range (0–10%).

Using the SoupX corrected counts, data were integrated using the reciprocal PCA (rPCA) method (Hao et al., 2021). Therefore, after data normalization and identification of variable features, each individual dataset was scaled and subjected to PCA analysis, which was used as input to the FindIntegrationAnchors function (with dims=1:30 and reduction='rpca') after which the datasets were integrated across all features using the IntegrateData function. During the scaling of the data, cell cycle regression was performed. Lists of human S and G2M genes were obtained from Seurat and converted to their mouse orthologues as input for the CellCycleScoring function. Following integration, the dataset was subjected to PCA, after which the top 50 PC were used for UMAP dimensionality reduction (with umap.method=uwot and min.dist=0.5) and clustering. Clustering and annotation were finalized using resolution 1.2 and 50 (to allow separation of the Schwann-like cluster) and evaluation of marker expression. DEG analysis between AB-like and JE-like clusters was performed in Seurat with the SoupX-corrected counts using the FindMarkers function with logfc.threshold=0.5 and min.pct=0.5.

Subcutaneous transplantation of ITO

Matrigel with d7 ITO (P5) was pipetted into custom-made 3D-printed hydroxyapatite scaffolds (Sirris) which were s.c. transplanted in immunodeficient nu/nu mice (Janvier Labs), as in detail described elsewhere (Bronckaers et al., 2021; Hemeryck et al., 2022). Scaffolds with only Matrigel were used as negative controls. One week later, implants were resected, and grafts carefully extracted and fixed in 4% PFA before processing for H&E or IF staining as described above. This study was approved by the Ethical Committee on Animal Experiments (ECAE) of UHasselt (protocol 202138).

Chicken CAM assay

Fertilized chicken eggs (*gallus gallus*) were incubated for 3 days at 37 °C and constant humidity. Next, 3 ml of albumen was removed and eggs were again incubated until embryonic day (E) 9 as previously in detail described (Bronckaers et al., 2013). Subsequently, pre-solidified 30 μ l Matrigel droplets with d7 ITO (P5) were applied onto the CAM following the creation of a 1 cm² window into the shell to expose the CAM. Droplets of Matrigel alone served as negative controls. The window was covered with

cellophane tape and eggs were returned to the incubator. One week later, the CAM was removed and fixed in 4% PFA for H&E and IF analysis (described above).

Statistical analysis

Statistical analyses were performed using GraphPad Prism (v9.3.1) for macOS and are specified in the figure legends. All experiments were performed with ≥ 3 independent biological replicates (i.e. organoid lines established from independent mouse litters) unless otherwise indicated.

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