Organoids from human tooth showing epithelial stemness phenotype and
differentiation potential

Cellular and Molecular Life Sciences

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SUPPLEMENTARY FIGURES

a) Various treatments and controls:
- Noggin
- SB202190
- RSPO1
- IGF1
- SHH
- Nicotinamide
- FGFs

b) Time points:
- d0
- d1
- d7

(c) DF:

(d) P6, d0, d14:
- Brightfield
- eGFP

(e) P0, P1:
- 200 µm

Legend:
- TOM
Supplementary Figure 1 Establishment of organoids from human dental follicle

**a** Brightfield images of the development of organoid structures (P0; day 14) after seeding dissociated dental follicle (DF) in the medium as indicated (see text).

**b** Organoids growing out from DF-derived cell clusters (top) or from single cells (bottom) in tooth organoid medium (TOM; passage 0, P0; d, day).

**c** Histological (H&E) analysis of DF. Boxed area is enlarged. Arrows indicate Epithelial Cell Rests of Malassez (ERM).

**d** Brightfield and epifluorescence pictures of eGFP⁺ and eGFP⁻ cells generated from...
dissociated organoids and cultured as mixture in TOM (P6; day 0 and day 14). Arrow indicates a fluorescent (eGFP⁺) organoid and arrowheads point to non-fluorescent (eGFP⁻) organoids. e Brightfield image of organoid culture after seeding of the dissociated DF tissue in TOM (P0; day 14), showing attachment of spindle-formed mesenchymal cells at the bottom of the culture plate (arrows), which are lost at passaging, being not present anymore in the first passage (P1; day 14) Consecutive magnifications are indicated by black and blue boxes, respectively. f Progressing organoids’ development in TOM during a single-passage (P4) 14-day culture period showing brightfield pictures, organoid diameters (mean ± SEM; n=3 biological replicates), and proportions of proliferative and apoptotic events as quantified through Ki67 and cleaved caspase-3 (CC3) immunostaining, respectively (dots indicate biological replicates; n=2). g Proportion of apoptotic (CC3⁺) cells and diameters of day-14 organoids over different passages (mean ± SEM; n=3 biological replicates). h Organoids derived from DF of erupted wisdom teeth from patients as indicated. I Immunofluorescence staining for markers as indicated in primary DF tissue and organoids (P0, day 14). DAPI (blue) was used to label nuclei. j Gene expression levels (relative to GAPDH) of indicated markers in DF and organoids (P1) (mean ± SEM; n=3 biological replicates). Scale bars: 50µm, unless indicated otherwise.
**Supplementary Figure 2** Single-cell transcriptomic analysis of primary DF and corresponding organoids

**a** Dot plot displaying the percentage of cells (dot size) expressing indicated marker genes with average expression levels (color intensity) (see scales) of the annotated cell clusters. UMAP representation of the distinct cell clusters, and UMAP plot of the different patients (Pat). **b** Violin plots showing the distribution of the number of genes detected per cell (nGene), the total unique molecular identifier counts per cell (nUMI) and the percentage of mitochondrial content (percent.mito) per sequenced sample as indicated. Dashed lines show cut-off values (see Methods). **c** Significant (FDR ≤ 0.05) DEG-
based GO term enriched in the lower-quality cell cluster based on the top 10 DEGs. Ultrastructural (TEM) analysis of full-grown organoids (P5; day 15). Boxed area is enlarged. Arrowhead indicates an apoptotic nucleus. d-e Projection of indicated genes on UMAP plot. ERM cluster is enlarged at the bottom. f Projection of ITGA6 expression on UMAP plot. ERM cluster is enlarged at the bottom. Brightfield pictures of organoid cultures from FACS-isolated ITGα6+ or ITGα6− cells in TOM (P0; day 17). Boxed area is enlarged. Arrows indicate attached spindle-formed mesenchymal cells in the ITGα6− cell culture at the bottom of the plate. g Projection of indicated genes on UMAP plot. ERM cluster is enlarged at the bottom. Immunofluorescence staining of DF and (day-14) organoids for indicated markers. DAPI (blue) was used to label nuclei. h Violin plots displaying activity of indicated regulons in the ERM and organoid clusters. Scale bars: 50µm, unless indicated otherwise.
Supplementary Figure 3 Effect of EGF on tooth organoid cultures

a Projection of indicated genes on UMAP plot. b Timeline of experimental set-up (d, day) and brightfield pictures of organoid cultures (day 14) as indicated. Right: diameter of organoids in specified cultures (violin plot; n=3 biological replicates). c Immunofluorescence staining for indicated markers in organoids cultured in TOM+EGF (P0). Boxed area is enlarged. DAPI (blue) was used to label nuclei. Arrows indicate double P63*VIM* cells. d Expression levels (relative to GAPDH) of indicated genes in organoids (day 14) cultured as denoted (mean ± SEM; n=4 biological replicates). Scale bars: 50µm.
Supplementary Figure 4 Ameloblast differentiation-mimicking process in tooth organoids

a Immunofluorescence staining for AMELX in organoids cultured as denoted. DAPI (blue) was used to label nuclei. b Gene expression levels (relative to GAPDH) of indicated markers in organoids cultured in TOM at indicated time points (mean ± SEM; n=3 biological replicates). c Timeline of in vivo experimental set-up (d, day). ARS and Masson’s trichrome (TCM) staining of depositions in recovered hydroxyapatite scaffolds which had been seeded with organoids before subcutaneous implantation, or only with Matrigel (empty). Boxed areas are enlarged. Negative control of ARS involves hematoxylin staining only. Arrow indicates a still discernible organoid. d Timeline of experimental set-up (d, day). Immunofluorescence staining for indicated markers in full-grown organoids as specified. DAPI (blue) was used to label nuclei. Quantification of SOX2+ cells in organoids cultured as indicated (mean ± SEM;
Gene expression levels (relative to GAPDH) of indicated markers in organoids cultured as specified (mean ± SEM; n=3 biological replicates). Scale bars: 50µm, unless indicated otherwise.
Supplementary Figure 5 Analysis of scRNA-seq data of tooth organoids driven into amelogenesis-resembling differentiation

a Projection of indicated genes on the integrated UMAP plot (see Figure 5a). b Expression levels (relative to GAPDH) of indicated genes in organoids cultured as specified (mean ± SEM; n=4 biological replicates). c Significant (FDR ≤ 0.05) DEG-based GO terms enriched in P4 versus P4-switch organoids. d DEG-based GSEA plots of the indicated hallmarks in P4-switch versus P4 organoids. Normalized enrichment score (NES-), and p- and FDR-values are listed. e Indicated regulons projected on the integrated UMAP plot. Dot plot of predicted HMGA2 regulon target genes in P4 and P4-switch organoids. f Pseudotime projected onto the integrated UMAP plot. Encircled area indicates a subcluster of potential transitional stage. g Indicated regulons projected on the integrated UMAP plot. h STRING protein-protein interaction network generated from the top 40 DEGs in P4-switch versus P4 organoids, predicting associations between proteins (nodes). The cluster analysis was subdivided in three colors by kmeans. Thickness of connecting line indicates confidence of interaction. Genes
specifically described in the text are highlighted in bold. Significant (FDR ≤ 0.05) DEG-based GO terms enriched in top 40 DEGs of P4-switch versus P4 organoids by Biological Process and KEGG Pathway analysis.
Supplementary Figure 6 Effect of TGFβ on differentiation in tooth organoids and assembloids

a Gene expression analysis of indicated TGFβ pathway components in organoids cultured as denoted. Expression is normalized to expression of GAPDH. Data are mean ± SEM of n=3 biological replicates. 

b Brightfield and fluorescent (eGFP) images of assembloids comprising dental epithelial (organoid-derived) cells and mesenchymal cells (DPSCs; marked by eGFP), cultured in TOM+αMEM. 

c Time-line of experimental set-up (d, day). Immunofluorescence staining for indicated markers of organoids cultured as denoted. DAPI (blue) was used to label the nuclei. Scale bar: 200µm, unless indicated otherwise. 

d-e Gene expression levels (relative to GAPDH) of indicated TGFβ pathway components in assembloids cultured in TOM+αMEM (mean ± SEM; n=3 biological replicates).
### SUPPLEMENTARY TABLES

**Supplementary Table 1 Tooth organoid medium (TOM)**

| Product             | Concentration | Supplier       | Catalogue Number |
|---------------------|---------------|----------------|------------------|
| Serum-free defined medium |               | Thermo Fisher Scientific | For composition, see Supplementary Table 4 |
| A83-01              | 0.5µM         | Sigma-Aldrich   | SML0788          |
| B27 (without vitamin A) | 2%            | Gibco          | 12587-010        |
| Cholera Toxin       | 100ng/ml      | Sigma-Aldrich   | c8052-.5mg       |
| FGF10               | 100ng/ml      | Peprotech      | 100-26           |
| FGF2 (= basic FGF)  | 20ng/ml       | R&D Systems    | 234-FSE-025      |
| FGF8                | 200ng/ml      | Peprotech      | AF-100-25        |
| L-glutamine         | 2mM           | Gibco          | 25030024         |
| IGF-1               | 100ng/ml      | Peprotech      | 100-11           |
| N2                  | 1%            | Gibco          | 17502-048        |
| N-acetyl L-cysteine | 1.25mM        | Sigma-Aldrich   | A7250            |
| Nicotinamide        | 10mM          | Sigma-Aldrich   | N0636            |
| Noggin              | 100ng/ml      | Peprotech      | 120-10C          |
| RSP01               | 200ng/ml      | Peprotech      | 120-38           |
| SB202190            | 10µM          | Biotechne (Tocris) | 1264          |
| SHH                 | 100ng/ml      | R&D Systems    | 464-SH-200       |
| WNT3A               | 200ng/ml      | R&D Systems    | 5036-WN-500      |
| Patient-derived organoid line | Age (year) | Sex  |
|------------------------------|-----------|------|
| Organoid_1                   | 18        | Female |
| Organoid_2                   | 15        | Female |
| Organoid_3                   | 16        | Female |
| Organoid_4\textsuperscript{a} | 18        | Male  |
| Organoid_5                   | 19        | Male  |
| Organoid_6                   | 18        | Female |
| Organoid_7\textsuperscript{a} | 15        | Female |
| Organoid_8                   | 16        | Male  |
| Organoid_9                   | 16        | Female |
| Organoid_10                  | 17        | Male  |
| Organoid_11                  | 14        | Female |
| Organoid_12                  | 15        | Male  |
| Organoid_13                  | 15        | Female |
| Organoid_14                  | 17        | Female |
| Organoid_15                  | 15        | Female |
| Organoid_16                  | 17        | Female |
| Organoid_17                  | 15        | Male  |
| Organoid_18                  | 15        | Female |
| Organoid_19                  | 19        | Female |
| Organoid_20\textsuperscript{b} | 15        | Male  |
| Organoid_21                  | 17        | Male  |
| Organoid_22\textsuperscript{b} | 18       | Female |
| Organoid_23                  | 15        | Female |
| Organoid_24                  | 15        | Female |
| Organoid_25                  | 19        | Male  |
| Organoid_26                  | 17        | Male  |
| Organoid_27                  | 17        | Male  |
| Organoid_28                  | 17        | Male  |
| Organoid_29                  | 17        | Female |
| Organoid_30                  | 15        | Female |

\textsuperscript{a}For in vivo transplantation

\textsuperscript{b}For scRNA-seq analysis
### Supplementary Table 3 Mineralization-inducing medium (MIM)

| Product                                           | Concentration | Supplier                      | Catalogue Number |
|---------------------------------------------------|---------------|-------------------------------|------------------|
| Keratinocyte serum-free medium (KBM-2)             |               | Lonza                         | CC-3103          |
| Calcium                                           | 0.09mM        | Thermo Fisher Scientific      | AC349610250      |
| EGF                                               | 1ng/ml        | R&D systems                   | 236-EG-200       |
| Bovine pituitary extract                          | 50µg/ml       | Thermo Fisher Scientific      | 13028014         |

### Supplementary Table 4 Serum-free defined medium (SFDM; pH 7.3)

| Name                                         | Concentration | Supplier            | Catalogue Number  |
|----------------------------------------------|---------------|---------------------|-------------------|
| Sterile H2O                                  |               |                     |                   |
| DMEM 1:1 F12 without Fe                      | 16.8 g/L      | Invitrogen          | 074-90715A        |
| Transferrin                                  | 5 mg/L        | Serva               | 36760.01          |
| Insulin from bovine pancreas                 | 5mg/L         | Sigma-Aldrich       | I6634             |
| Penicillin                                   | 35 mg/L       | Sigma-Aldrich       | P3032             |
| Streptomycin                                 | 50 mg/L       | Sigma-Aldrich       | S6501             |
| Ethanol absolute, ≥99.8% (EtOH)              | 600µL/L       | Fisher Chemical     | E/0650DF/15       |
| Catalase from bovine liver                   | 50µL/L        | Sigma-Aldrich       | C100              |
| NaHCO3                                       | 1 g/L         | Merck               | 106329            |
| Bovine serum albumin (BSA)                   | 5 g/L         | Serva               | 47330.03          |
Supplementary Table 5 Antibodies used for immunohistochemical/immunofluorescence staining

### Primary antibodies

| Antigen | Host | Supplier       | Clone or Catalogue Number | Dilution |
|---------|------|----------------|---------------------------|----------|
| AMELX   | mouse| Santa Cruz     | sc-365284                 | 1:100    |
| CC3     | rabbit| Sigma          | AB3623                    | 1:100    |
| CD44    | mouse| Abcam          | ab34485                   | 1:200    |
| CD90    | rabbit| Abcam          | ab133350                  | 1:100    |
| CK5     | rabbit| Bioledgend     | Poly19055                 | 1:100    |
| CK14    | mouse| Thermo Fisher Scientific | LL002 | 1:200 |
| CK19    | mouse| Dako           | M772                      | 1:50     |
| Ki67    | mouse| BD Bioscience  | 556003                    | 1:100    |
| ODAM    | rabbit| Proteintech    | 16509-1-AP                | 1:200    |
| P63     | rabbit| Abcam          | ab124762                  | 1:1000   |
| SOX2    | rabbit| Abcam          | ab92494                   | 1:2000   |
| VIM     | mouse| Dako           | V9                        | ready-to-use |

### Secondary antibodies

| Antigen | Host | Supplier       | Catalogue Number | Dilution |
|---------|------|----------------|------------------|----------|
| mouse IgG (Alexa 488) | donkey| Thermo Fisher Scientific | A-21202 | 1:1000 |
| mouse IgG (Alexa 555) | donkey| Thermo Fisher Scientific | A-31570 | 1:1000 |
| rabbit IgG (Alexa 488) | donkey| Thermo Fisher Scientific | A21206 | 1:1000 |
| rabbit IgG (Alexa 555) | donkey| Thermo Fisher Scientific | A-31572 | 1:1000 |
## Supplementary Table 6 Primers used for qPCR

| Gene   | Forward primer                  | Reverse primer                  |
|--------|--------------------------------|--------------------------------|
| AMBN   | CCTTGAGGAAGGAGAAGCTG           | CTGGGAGTGATGGACCTTGT            |
| AMTN   | AGCAGGAGGAGCAGGTGTA            | CCAAATTCAGGAGAGGTAG             |
| BMP4   | CTGGCTTGAGATCTCGAGCG           | TCACCTGCTCTCAGGACATGCT          |
| CALB2  | GATCTGCAAACCGCTGCC             | CGATGTAGCCTCTCTGCTG             |
| CASP6  | AAACCAGCAGATGTGCC             | CACCTCAGTTATGTTGCC             |
| COL3A1 | TGGCTGCAAGGATGCCGTGA          | TCTTCCCCGGAGACACCTC             |
| FN1    | CCATCGCAAACCGCTGCC            | AACACTTCTAGCTATGGGCT            |
| GAPDH  | GGTATCTGTGGAGGACCTAGC         | ATGCCAGTAGCTCTCCCTTCAGGG        |
| ITGA6  | GGCCTGTATGTGCCGAGTC           | AATCGCCCACACAAAAAAAACTG         |
| KLK4   | GGAACCTTTGCTCCTCGT            | AGCGGCTCAGAGCTTACTG             |
| LAMA3  | TGCTCAACTCGTCTGCTGCC          | TCCAGTCTCTGCTCCCCGTTC           |
| LAMB3  | GAGCAGCTACAAGACCTAGGCAGGGAGAG | CCAGGTCTTACCGAAGTCTG           |
| LAMC2  | TACAGAGCTGGAAGGAGGAGT          | GTTCTCTGGCTCTCACCCTG            |
| MDM2   | GGCAGGGGAGAGGTGATACAGAGAGAG  | GAAGCCATTCTACAGGAGGG            |
| MMP20  | TCCATCCTCATGGCGCTCAGCTGCTGACT| AGTGAAACCTGCCGCTCCAGAAA         |
| MSH2   | AGTCAGAGCCCCGTTAACCTCCCCCT    | GAGAGGCTGCTTACCCCTGG           |
| NCD    | CCTCTCTGGCGCTCTGGCATGAGAGAGAG| TAAGGATCTCAGGGCTCCG             |
| ODAM   | CAGGGAAGTTGATCCTCTTA          | GAGGTGTCTCCCAGGGTAG             |
| PITX2  | CAGCGGACCTCACCCTACCGA         | ATCTCTGAACCAAGACCCGGG           |
| POSTN  | TGCCAGCAGTGTGTCCTC            | CGTTTCCCTGCAAACCTCTA           |
| SNAI1  | TCGGAAGCTACTACAGGCTG          | AGATGAGCATTGCGAGAGGAAGG         |
| STIM1  | CACTGGTGCCACCTCCCGT           | CTGTACCTCGTACGTCGTTG           |
| TWIST1 | GACGGATCTAGCTGCCTTCT          | TCCATCCTCCAGACCGAGAAAGG         |
| ZEB1   | TTACACCTTGGCTACAGAACCC        | TTTACGATTACACCCAGACTG           |
Legends for Supplementary Datasets

Dataset 1. Cell type markers. Most discriminating genes per annotated cell type. Gene names (Gene), p values (p_val), average log fold change expression (avg_logFC), percentage of cells expressing the indicated gene in the cell type of interest (pct.1) and in all other cells together (pct.2), FDR-adjusted p value (p_val_adj) and cell type name are presented in the columns. DF, dental follicle; ERM, Epithelial Cell Rests of Malassez; NK, natural killer cells.

Dataset 2. Differentially expressed gene (DEG) analysis of ERM versus P1 organoids. The columns represent: gene names (Gene), p values (p_val), average log fold change expression (avg_logFC; positive values indicate higher and negative values lower expression in ERM versus P1 organoids), percentage of cells expressing the indicated gene in the ERM (pct.1) and in the P1 organoids (pct.2), and the FDR-adjusted p value (p_val_adj).

Dataset 3. Differentially expressed gene (DEG) analysis of ERM versus P4 organoids. The columns represent: gene names (Gene), p values (p_val), average log fold change expression (avg_logFC; positive values indicate higher and negative values lower expression in ERM versus P4 organoids), percentage of cells expressing the indicated gene in the ERM (pct.1) and in the P4 organoids (pct.2), and the FDR-adjusted p value (p_val_adj).

Dataset 4. Gene ontology (GO) analysis of DEGs between different cell type clusters. a GO analysis of genes upregulated in ERM versus P1 organoids. b GO analysis of genes upregulated in ERM versus P4 organoids. c GO analysis of genes upregulated in P1 organoids versus ERM. d GO analysis of genes upregulated in P4 organoids versus ERM. e GO analysis of genes upregulated in P4 organoids versus P4-switch organoids. f GO analysis of genes upregulated in P4-switch organoids versus P4 organoids. Columns represent: GO term (GO biological process), number of genes involved in specified GO term in the reference list (REFLIST), number of genes involved in specified GO term in uploaded DEG list (Count), number of genes expected in specified GO term based on the reference list (Expected), over-representation of the GO-term (Over-representation), fold enrichment of the number of genes observed in the uploaded DEG list (Fold Enrichment), p value, FDR and -log10(FDR).

Dataset 5. Differentially expressed gene (DEG) analysis in P1 organoids versus P4 organoids. The columns represent: gene names (Gene), p values (p_val), average log fold change expression (avg_logFC; positive values indicate higher and negative values lower expression in P1 organoids versus P4 organoids), percentage of cells expressing the indicated gene in the P1 organoids (pct.1) and in the P4 organoids (pct.2), and the FDR-adjusted p value (p_val_adj).
**Dataset 6.** Differentially expressed gene (DEG) analysis in P4 organoids versus P4-switch organoids. The columns represent: gene names (Gene), p values (p_val), average log fold change expression (avg_logFC; positive values indicate higher and negative values lower expression in P4 organoids versus P4-switch organoids), percentage of cells expressing the indicated gene in the P4 organoids (pct.1) and in the P4-switch organoids (pct.2), and the FDR-adjusted p value (p_val_adj).

**Dataset 7.** Protein-protein interactions of top 40 DEGs of P4-switch organoids versus P4 organoids analyzed by STRING. 

**a** Protein 1 (node1) interacts with protein 2 (node2), id of protein 1 (node1_string_id), id of protein 2 (node2_string_id), experimentally determined protein interaction (experimentally_determined_interaction), protein annotations (database_annotated), textmining evidence extracted from abstract of scientific literature (automated_textmining), combined protein-protein interaction score (combiner_score).

**b-c** GO analysis of Biological Processes and KEGG pathways of top 40 DEGs of P4-switch organoids versus P4 organoids. Columns represent: GO term ID, term description, observed gene count, background gene count, strength, FDR, matching proteins in the network (IDs and labels).