Xenogeneic dentin matrix as a scaffold for biomineralization and induced odontogenesis

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Abstract
Commonly recognized mechanisms of the xenogeneic-extracellular matrix-based regenerative medicine include timely degradation, release of bioactive molecules, induced differentiation of stem cells, and well-controlled inflammation. This process is most feasible for stromal tissue reconstruction, yet unsuitable for non-degradable scaffold and prefabricated-shaped tissue regeneration, like odontogenesis. Treated dentin matrix (TDM) has been identified as a bioactive scaffold for dentin regeneration. This study explored xenogeneic porcine TDM (pTDM) for induced odontogenesis. The biological characteristics of pTDM were compared with human TDM (hTDM). To investigate its bioinductive capacities on allogeneic dental follicle cells (DFCs) in the inflammation microenvironment, pTDM populated with human DFCs were cocultured with human peripheral blood mononuclear cells (hPBMCs), and pTDM populated with rat DFCs were transplanted into rat subcutaneous model. The results showed pTDM possessed similar mineral phases and bioactive molecules with hTDM. hDFCs, under the induction of pTDM and hTDM, expressed similar col-I, osteopontin and alkaline phosphatase (ALP) (all expressed by odontoblasts). Whereas, the expression of col-I, dentin matrix protein-1 (DMP-1) and bone sialoprotein (BSP) were down-regulated when cocultured with hPBMCs. The xenogeneic implants inevitably initiated Th1 inflammation (up-regulated CD8, TNF-α, IL-1β, etc) in vivo. However, the biomineralization of pre-dentin and cementum were still processed, and collagen fibrils, odontoblast-like cells, fibroblasts contributed to odontogenesis. Although partially absorbed at 3 weeks, the implants were positively expressed odontogenesis-related proteins like col-I and DMP-1. Taken together, xenogeneic TDM conserved ultrastructure and molecules for introducing allogeneic DFCs to odontogenic differentiation, and promoting odontogenesis and biomineralization in vivo. Yet effective immunomodulation methods warrant further explorations.

1. Introduction
The extracellular matrix (ECM) usually presents as a complex milieu of ultrastructure and molecules maintained by inherent tissues or organs. Apart from the original purpose of serving as a three-dimensional (3D) scaffold, ECM has been identified as a reservoir of information in the forms of molecular signaling and mechanical cues [1]. In the former, ECM components, such as collagens, fibronectin, laminin ligands, growth factors, and cytokines, regulate specific functions of stemcells or neighboring cells. Yet shared common proteins, different tissuespecific ECM composites regulate cell differentiation and display a unique signature for the regeneration of targeted tissues [2, 3]. In the latter, the mechanical properties of ECM are mainly dependent on three constituents: elastic fibers, fibrillar collagens, and glycosaminoglycans (GAGs). The neighboring cells often sense the ECM through integrins, with the
actomyosin cytoskeleton allowing them to interact with fibers so that to establish mechanical homeostasis. The mechanical quantities of ECM, including stress, strain, and stiffness, might also introduce the differentiation of stem cells towards distinct lineages [4]. This mechanotransduction could be observed in the remodeling of tendons, skin, and heart tissues [5]. Therefore, despite diverse commercial ECM synthesis, the natural ECM derived from inherent tissues favor the direction of stem cells towards distinct tissue regeneration.

Major components of ECM are identified to be well-conserved across mammalian species. This phenomenon lays the fundamental for commercial use of xenogeneic ECM as effective substitutes [6], such as Porcine small intestinal submucosa (SIS) for intestinal grafts, Bovine pericardium and Porcine heart valve for myocardial patches, Porcine dermis for skin grafts, etc [7, 8]. Not surprisingly, biomechanical and bioinductive properties of these xenogeneic ECM are insufficient to promote a positive constructive process, since the released bioactive molecules also incite inflammation and trigger a complex immune-microenvironment [9, 10]. For example, type I collagen (col-I) accounts for the major structural protein in various ECM and manages the biomechanical behavior in tendinous and ligamentous structures. However, both porcine and human col-I are demonstrated to elicit CTL T cell proliferation and Th1 inflammation [11]. Except for studies focused upon modification of host-ECM interaction towards an anti-inflammatory and remodeling state, the intended differentiation of populated stem/progenitor cells at situ immune-microenvironment is little discussed [12].

Yet the constructive remodeling mechanism of ECM is not fully understood, the rapid degradation, release of active molecules, stem cell differentiation, and arrangement of new ECM structure are primary events during the remodeling of ECM [12] such as SIS. Given the degradation of implanted ECM and neo-deposition of ECM components, mechanical and structural properties of tissue would be in a dynamic state [5, 9]. Therefore, an intact 3D structural ECM with inductive and biomechanical properties would be an ideal scaffold for hard or high-mineralized parenchymal tissue repair. In the tooth organ, dentin is characterized by hydroxyapatite crystals within a rich organic matrix [13, 14]. Like ECM components, the collagens, primary col-I, occupy the major structural proteins in the dentin matrix. Other tissue-specific proteins, such as dentin sialophosphoprotein (DSPP) and dentin matrix protein-1 (DMP-1), contribute to odontogenesis and re-mineralization [15]. Processed by decellularization and demineralization, the treated dentin matrix (TDM) is monitored as a reservoir of bioactive molecules for induced odontogenesis, and providing support strength for masticatory force [16, 17]. Consisted of dentino-pulp complex and cementum-periodontal ligament (PDL)-alveolar bone complex, tooth organ could be selected as a typical model for research on intact parenchymal and stromal tissue regeneration [18].

Our preliminary study illustrated hydroxyapatite and organic functional groups of porcine TDM (pTDM), and found a combination of pTDM with allogeneic dental follicle cells (DFCs) could initiate a constructive process characterized by M1-to-M2 macrophage polarization [19]. However, before translational research of pTDM for induced odontogenesis, it is essential to compare the properties of pTDM with hTDM. The present study thoroughly evaluated and compared the molecular and structural properties of pTDM with hTDM, with the aspects of morphological and physiological features, the release of bioactive molecules, and bioinductive functions. Since a positive tissue-engineering construction of targeted tissues also depends upon the differentiation of re-populated tissue-specific cells, bioinductive functions of pTDM at situ immune-microenvironment were thoroughly investigated in vitro and in vivo. The normal hard connective tissue, such as bone and teeth, is formed through reinforcement of organic fibrils matrices by calcium phosphate crystals. The in vivo analysis mainly monitored the gradient collagen deposition and odontogenesis of the implanted complex (the combination of pTDM with allogeneic DFCs). Altogether detected the feasibility of xenogeneic acellular dentin matrix for induced odontogenesis.

2. Materials and methods

All experiments were conducted under the protocol approved by the West China Hospital of Stomatology Institutional Review Board, Sichuan University.

2.1. Scaffold preparation and characteristics

2.1.1. Preparation of treated dentin matrix (TDM)

Processed by decellularization and demineralization as described previously [16, 17, 19], human TDM (hTDM) and pTDM were fabricated. Briefly, human premolar teeth were extracted for clinical reasons. Porcine deciduous incisor teeth were obtained from executed pigs in the daily morning. For all collected teeth, the dental pulp tissue, pre-dentin and periodontal tissue were completely scraped away with a curette. The resulting dentin was formed into a root-shaped matrix, with a length of 10 mm and a thickness of 1.0 mm approximately [16]. The matrices were mechanically cleaned in deionized water by an ultrasonic cleaner, and then sequentially treated with ethylene diamine tetra-acetic acid (EDTA, Sigma-Aldrich, St. Louis, MO, USA). Respectively, hTDM was exposed to 17% EDTA for 5 min, 10% EDTA for 5 min, and 5% EDTA for 10 min, while pTDM was incubated in 17% EDTA for 20 min, 10% EDTA for 18 min, and 5% EDTA for 15 min. After washed
in deionized water using an ultrasonic cleaner, both hTDM and pTDM were obtained, and then maintained in sterile PBS with 100 units ml$^{-1}$ penicillin (HyClone, USA), and 100 mg ml$^{-1}$ streptomycin (HyClone, USA) for 72 h, and finally stored in $\alpha$-minimal essential medium ( $\alpha$-MEM) at 4 $^\circ$C. Morphological and histological observations of TDM were performed using scanning electron microscopy (SEM, Inspect F, FEI, Eindhoven, Netherlands) and HE staining.

2.1.2. Comparison of physicochemical properties
The phase structure of hTDM and pTDM were examined by x-ray diffraction (XRD, PANalytical EMPYREAN, Almelo, Netherlands). The XRD parameters were set as a generator voltage of 40 kV using a radiation source of copper (Cu), at room temperature. The step scan was set at 0.0263$^\circ$ with a $2\theta$ range of 10$^\circ$–70$^\circ$. Then the crystalline phase was observed regarding the standard JCPDS cards.

hTDM and pTDM were formed intofreeze-dried powders. X-ray photoemission spectroscopy (KRATOS, XSAM 800) was applied to evaluate chemical compositions of C, Ca, P, and O of matrix powders. The x-ray source was set at 150 w, using K-alpha XPS with monochromatic Al-K$\alpha$ x-rays (beam energy = 1000 eV). Constant pass energy of 200 eV was set, with a binding energy range of 0–1200 eV (1.0 eV step$^{-1}$). Corrected to the C 1s peak at 284.6 eV, high-resolution scans were acquired at constant pass energy of 50 eV (0.1 eV step$^{-1}$) with binding energy regions of the selected elements. The energy of emitted electrons was detected using a hemispherical energy analyzer with a pass energy range of 50 (for narrow scans) to 200 eV (for the survey).

2.1.3. Comparison of released bioactive molecules
Liquid extraction samples of the hTDM and pTDM were collected according to the protocol of the International Standardization Organization (ISO 10993), with a ratio of 20 g scaffold per 100 ml $\alpha$-MEM, as previously described [16, 20]. Incubated at 37 $^\circ$C for 1 week, the resulting protein concentrations were measured using ELISA (R&D Systems, Minneapolis, Minn., USA), including type I collagen (COL-I), transforming growth factor-beta1 (TGF- $\beta_1$), DSPP, dentin matrix protein-1 (DMP-1) and Biglycan.

2.2. Induced odontogenesis by pTDM in vitro
2.2.1. Isolation, culture, and identification of DFCs
Our preliminary study demonstrated that bioengineered tooth root constructed of pTDM seeded with allogeneic DFCs could serve as a feasible approach for PDL-like tissue reconstruction [19]. For analysis in vitro, hDFCs were isolated and cultured as described previously [16]. Briefly, human dental follicles were obtained from impacted third molars of healthy young individuals under 18 years old. Then the dental follicles were digested with collagenase and trypsin and incubated in $\alpha$-MEM (HyClone, Logan, UT, USA) supplemented with fetal bovine serum (FBS, HyClone, Logan, UT, USA), 100 U ml$^{-1}$ penicillin and 100 $\mu$g ml$^{-1}$ streptomycin in a humidified 5% CO$_2$ incubator at 37 $^\circ$C. Passage 2–4 were used for the experiments.

The expression of mesenchymal stem cell-associated surface antigens was evaluated on passage three hDFCs. A total of 1 x 10$^6$ hDFCs were collected and washed twice with PBS containing 2% FBS. Then cells were incubated at 4 $^\circ$C for 30 min with the respective antibodies in PBS with 2% FBS of a final 100 $\mu$l. The following antibodies were used for evaluation: mouse anti-human CD34-FITC, CD44-FITC, CD45-FITC, CD90-FITC, CD29-PE, CD105-PE, CD166-PE (BD Biosciences, Heidelberg, Germany). The labeled cells were washed twice in 1 ml of PBS with 2% FBS. The samples were measured using BD Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA) and analyzed by FlowJo software (TreeStar, San Carlos, CA, USA).

2.2.2. Comparison of the bioinductive capacities of pTDM with hTDM
The hDFCs were seeded onto the hTDM or pTDM at a density of 1 x 10$^5$ cells per well in six-well plates and then incubated for 1 week. All differentiated hDFCs per well were collected. The influence of the pTDM-derived microenvironment on the biological behavior of hDFCs was investigated by real-time PCR, and compared with those effects induced by the hTDM.

For real-time PCR analysis, expression of the following genes was evaluated: COL-I, alkaline phosphatase (ALP), bone sialoprotein (BSP), osteopontin (OPN), and DSPP. Total RNA was isolated from cells using RNAiso Plus according to the manufacturer’s instructions (TaKaRa Biotechnology, Dalian, China). The RNA was reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Pittsburgh, PA, USA). Real-time PCR was performed using the Eco™ Real-Time PCR System (Illumina, San Diego, USA) with SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa Biotechnology, Dalian, China) according to the manufacturer’s instructions. The mRNA expression levels were normalized to that of GAPDH (mean ± SEM; n = 3). The primer sequences used are listed in table 1.
microenvironment mostly, six-well plates (Thermo Fisher Scientific, USA) were coated with a suboptimal dose of monoclonal anti-CD3 (anti-CD3 antibody, Biolegend, USA) to obtain a basal stimulation of immune cells, which does not induce T cell proliferation or merely low proliferation rates [11]. 800 μl of anti-CD3 antibody at 0.025 μg ml⁻¹ was placed in a six-well plate and was incubated for approximately 14 h at 4 °C and washed three times with PBS.

The hDFCs were seeded onto the pTDM as described above, resulting in a xenogeneic bioengineered tooth root complex of pTDM/hDFCs. To analyze the inductive capacities of pTDM in the inflammation microenvironment in vitro, the complexes were taken out to be co-cultured with hPBMCs. The hPBMCs (1.5 × 10⁶) were taken out to be co-cultured with hPBMCs. Listed in table ± expression levels were normalized to that of GAPDH.

DMP-1 were analyzed by real-time PCR. The mRNA was isolated using RNAiso Plus as described above. pTDM/hDFCs complexes (0.8 g pTDM) were transferred into a T-25 culture flask, and cultured in α-MEM medium consisting of 20% FBS, 100 U ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomycin at 37 °C in a 5% CO₂ incubator. The cells reached confluent within 15–20 days and were passaged conventionally. rDFCs of 3rd passage were used for the following experiment.

For analysis in vivo, allogeneic rDFCs were seeded upon pTDM, and the resulted xenogeneic bioengineered root complexes of pTDM/rDFCs were transplanted into rats subcutaneously. Rats, ages of 10–12 weeks old, were obtained from the Laboratory Animal Research Centre of Sichuan University. Rats were carefully kept with regular feed and sterile water. All animal experiments were conducted in accordance with the committee guidelines of Sichuan University.

2.3.2. Acute inflammation analysis

Eight Sprague-Dawley (SD) rats were randomly divided into two groups. The test group: 0.5–1 cm incision was made along the midline of the abdomen of SD rat after anesthetized, then tissues were dissected, and pTDM/rDFCs were transplanted subcutaneously. A sham-operated group was included as a control: tissues were closely sutured without any implantations after dissection. To evaluate the host inflammatory response, the tissues infiltrating into pTDM/rDFCs complexes at 3 days, insides and outsides, were collected immediately. The total RNA of infiltrated tissues were isolated using RNAiso Plus as described above. Quadruple quantitative RT-PCR reactions were performed on a QuantStudio™ 6 Flex Real-Time PCR System, using TaqMan® Gene Expression assays containing TaqMan Gene Expression probes (Thermo Scientific, formerly Applied Biosystems, Foster City, CA). All genes were normalized to GAPDH and Actb. Relative gene expression (fold change) was determined by the comparative CT method.

Table 1. Primer sequence for real-time PCR.

| Gene   | Forward (5’-3’)                      | Reverse (5’-3’)                      | Product size Accession |
|--------|--------------------------------------|--------------------------------------|------------------------|
| COL-1  | AACATGGGAGACTGTTGAGACCT              | CGGCCATACCTGAACTGGAATC               | 145 NM_00088.3         |
| ALP    | TAAACGACATCCGCACACGCCT               | TTCTTCCAGGTTCAAGCAGGTT              | 170 NM_004784.4        |
| OPN    | CAGTTGTCACCCACAGATTAGAC              | GTGATGTCCTCTGCTGTAGCATC              | 127 XM_017008564.1     |
| DSPP   | AGTGGGGCAGATGAGGAGGGA                 | GTCGCCATCCGCAGGCCCTCAA              | 86 NM_014208.3         |
| BSP    | CGACAAAGCGGTAACAGGCACAG              | TTCTCATTGTCCTCTCCGTCTGCT            | 82 NM_004967.3         |
| TGFB-1 | GTGGAACATCAACGGGCTTACACT             | GTGGAAGCTGAAGCAATAGTTGG             | 167 NM_000660.5        |
| DMP-1  | CCCGCCACCTCAGCCCCACTCA               | TGGCTTTCCTGCTGTCAGACTCTCT           | 180 U89012.1           |
| GAPDH  | CTTTGTGATCGTGGAAGAGCT                | GTAGAGGGCAGGGATGATGTTCT             | 132 NM_001289746.1    |

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2.3.3. Histology analysis
Another 16 SD rats were randomly divided into four separate groups. pTDM/rDFCs complexes were transplanted as described above. The rats were sacrificed and the xenogeneic complexes were harvested at 3 days, 1 week, 1 month, and 3 months. Four rats per time-point were served as a group. All harvested specimens were fixed in 4% neutral buffered paraformaldehyde. Then all samples were demineralized for 3 months, embedded, and sectioned into 5 µm thick sections for Hematoxylin and Eosin (H&E) stain, Masson’s Trichrome stain according to the manufacturer’s instructions.

Immunohistochemistry was performed to evaluate the induced odontogenesis of xenogeneic bioengineered root complex (pTDM/rDFCs). The primary antibodies used were mouse anti-rat DMP-1 (Abcam, Cambridge, MA, UK), mouse anti-rat COL-1, (Abcam, Cambridge, MA, UK). Sections were deparaffinized and rehydrated through a graded ethanol process. Then the slides were under antigen retrieval and incubated for 1 h with goat serum working fluid to prevent nonspecific antibody binding at room temperature. Thereafter, slides were decanted and incubated with primary antibodies overnight at 4°C. Then, the slides were incubated with 3% hydrogen peroxide for 30 min at room temperature to inhibit endogenous peroxidase activity. Finally, the slides were washed by PBS and incubated with the secondary antibody for 30 min at room temperature, followed by DAB staining. A light microscope (Olympus BX43, Tokyo, Japan) was applied to observe the resulted images.

2.4. Statistical analysis
Statistical analysis was performed using GraphPad Prism (Graphpad Software, San Diego, CA, USA). Data were presented as mean values ± SEM (standard error of the mean) and statistically analyzed utilizing the t-test. Two-tailed analysis of variance was used in the statistical analysis. The difference was considered significant at *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

3. Results

3.1. Comparison of physicochemical properties of pTDM with hTDM
The obtained human and porcine dentin matrices (figure 1(A), (i–ii)) were processed into the TDM, resulting in a cone-shape scaffold like a tooth root (figure 1(A), (iii–iv)). H&E images showed no remaining cells and confirmed regularly distributed dentin tubules in both pTDM and hTDM (figure 1(A), (v–vi)). By sequential treatment with EDTA, the sufficiently exposed dentinal tubules were observed both in pTDM and in hTDM, and were confirmed by SEM analysis (figure 1(B)). The software Image-Pro Plus 6.0 was used to measure the diameter of dentinal tubules, with an AOI area of 60 × 55 µm. The mean values of dentinal tubule diameters showed no difference between pTDM and hTDM (figure 1(B)). These exposed tubules would guarantee the effective release of odontogenesis-related proteins.

Powers of pTDM and hTDM were investigated by XRD and XPS. XRD revealed that pTDM showed similar peaks to hTDM approximately at the 2θ of 25.526°, 31–32° (figure 2(A)). And the major diffraction peak of pTDM or hTDM were also consistent with the Joint Committee on Powder Diffraction Standards card (JCPDS, No.09-0432) of hydroxyapatite, with the most prominent peaks between 31.4° and 32.5° (figure 2(A)). Parallelled to hTDM, XPS spectrum of pTDM displayed similar major binding energy of C 1s at 284.5 eV (figure 2(C)), Ca 2p at 346.8 eV (figure 2(D)), O 1s at 530.6 eV (figure 2(E)) and P 2p at 132.5 eV (figure 2(F)). Other spectra of elements, such as Ca 2s, Ca 3s, Ca 3p, P 2s, Mg, were also close between pTDM and hTDM (figure 2(B)). These similar crystal phases and components confirmed chemical and physical similarities identification of pTDM to hTDM.

3.2. Comparison of molecular information of pTDM with hTDM
The quantification by ELISA kit further detected the concentrations of odontogenesis-related proteins in the liquid extracts from pTDM and hTDM (figure 3 [16, 20]). As to the major components of Col-I, there was no significant difference between the two groups. However, pTDM released fewer growth factors, like TGF-β1, than that of hTDM. The results also indicated that the concentration of some dentin-specific proteins, such as DMP-1, contained in the pTDM liquid extract showed no statistical difference with that of hTDM, while pTDM released more DSPP than that of hTDM. We also demonstrated a higher concentration of biglycan, the predominant proteoglycans (PGs) present in dentin, in the pTDM liquid extract. Even though the lower level of TGF-β1, col-I, and TGF-β1 remained to be high concentration units of ng/ml in pTDM liquid extract. These structural proteins, growth factors, and dentin-specific proteins released from pTDM revealed its potential capacities for induced odontogenesis.

3.3. Comparison of bioinductive capacities of pTDM with hTDM
Allogeneic human DFCs (hDFCs) were obtained at the 3rd passage as previously described. The cell surface markers of hDFCs were characterized by flow cytometric analysis (figure 4(A)). The hDFCs were strongly positive for CD29 (adhesion molecules), CD44, CD90 (cell surface markers associated with mesenchymal stromal cells), CD105 and CD166 (cell surface markers associated with mesenchymal stromal cells and endothelial cells), and negative
for CD33 (myeloid cells associated markers), CD34, and CD45 (hemopoietic lineages associated makers). These surface antigen results were consistent with the identification of hDFCs as mesenchymal stem cells rather than lymphatic or hemopoietic lineages.

The bioinductive effects of pTDM and hTDM on allogeneic hDFCs were also investigated. After 7 days of induction, hDFCs were growing normally and showed chemotaxis towards the two matrices under light microscopy (figure 4(B)). Cell morphology was also examined by SEM (figure 4(C)), hDFCs spread over and covered both hTDM and pTDM surface at high density. Obviously, hDFCs on the surface of both hTDM and pTDM surface showed a pattern of proliferation in a regular direction. This cellular polarity might suggest differentiation potential. Then, the biological behavior of the hDFCs was investigated by real-time PCR (figure 4(D)). The gene expression of col-I, ALP, OPN in hDFCs showed no statistical differences between the induction of pTDM and hTDM. This result indicated that, compared with hTDM, pTDM could also serve as an inductive microenvironment for structural protein deposition and bone formation. In pTDM group, relatively lower expression of DSPP and BSP were detected, which revealed weakened capacities of xenogeneic TDM in odontogenesis in comparison to allogeneic TDM.

3.4. The effects of inflammation on bioinductive capacities of pTDM in vitro

Porcine matrix was demonstrated to induce immune cell proliferation in co-action with low-dose anti-CD3 stimulation when co-cultured with human PBMCs [11]. Further investigations were carried out to capture whether the bioinductive capacities of pTDM were affected in this inflammation microenvironment. After 5 days of co-culture, hDFCs covered upon pTDM were collected, and the odontogenesis-related proteins expressed in these hDFCs were analyzed by real-time PCR (figure 5). Down-regulated gene expression of col-I and TGF-β1 of hDFCs were detected in contrast to non-coculture groups, indicating dampened matrix deposition in an inflammation environment. Meanwhile, the inflammation environment also impaired the gene expression of dentin-specific proteins in hDFCs, such as DMP-1, BSP, even
Figure 2. Comparison of physicochemical properties of pTDM with hTDM. (A) The XRD revealed crystal microstructure of pTDM and hTDM, consistent with the JCPDS of hydroxyapatite. (B) XPS spectra of elements conserved in pTDM and hTDM, which displays similar major binding energy of C 1s at 284.5 eV (C), Ca 2p at 346.8 eV (D), O 1s at 530.6 eV (E) and P 2p at 132.5 eV (F).

Figure 3. Comparison of molecular information of pTDM with hTDM. Odontogenesis-related proteins in the liquid extracts from pTDM and hTDM were quantified by ELISA kits, such as type I collagen (col-I), transforming growth factor-beta1 (TGF-β1), dentin sialophosphoprotein (DSPP), dentin matrix protein-1 (DMP-1) and Biglycan. Data are means ± SEM (n ≥ 3). *p < 0.05, **p < 0.001, ***p < 0.0001; NS, no significance.

though a higher expression of DSPP was observed.

Taken together, the xenogeneic TDM could not only provide bio-inductive information but also trigger inflammation, the resulted complex microenvironment might in reverse affect the induced odontogenesis of hDFCs.

3.5. Induced odontogenesis of pTDM in the inflammation microenvironment in vivo

The host-to-xenograft microenvironment was simulated when the combination of pTDM and allo- geneic rat DFGs (pTDM/rDFGs) was implanted in a rat subcutaneous model. Several studies have
Figure 4. Comparison of bioinductive capacities of pTDM with hTDM. (A) Flow-cytometric analysis showed that the DFCs positively expressed CD29, CD44, CD90, CD105, CD166, and negatively for CD33, CD34, CD45. (B) After 7 days of induction, hDFCs were growing normally and showed chemotaxis towards the two matrices under light microscopy. Scale bars, 100 μm. (C) After 7 days of induction, hDFCs spread over and covered both hTDM and pTDM surface at high density. Scale bars, 50 μm. (D) Under induction of pTDM, the gene expression of col-I, alkaline phosphatase (ALP), osteopontin (OPN) in hDFCs showed no statistical differences to that of hTDM, while lower expression of DSPP and bone sialoprotein (BSP) were detected than that of hTDM. The data were normalized to those of GAPDH mRNA and are presented relative to those of hDFCs under hTDM induction, set as 1. Data are means ± SEM (n ≥ 3). *p < 0.05; NS, no significance.
Figure 5. The effects of inflammation on bioinductive capacities of pTDM in vitro. After co-culture of pTDM/hDFCs with hPBMCs for 5 days, odontogenesis-related proteins expressed in hDFCs were partially impaired, including down-regulated col-I, TGF-β1, DMP-1, BSP, and higher expression of DSPP. The data were normalized to those of GAPDH mRNA and are presented relative to those of hDFCs without co-culture, set as 1. Data are means ± SEM (n ≥ 3). ∗∗p < 0.01, ∗∗∗p < 0.001. NS, no significance.

Figure 6. Th1/Th2 inflammation analysis after 3 days transplantation of pTDM/rDFCs in a rat subcutaneous model. The up-regulated CD4, CD8, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-12b and decreased IL-6 were detected at the implanted sites, indicating a Th1-type inflammation. A sham-operated group was included as a control. The data were normalized to those of GAPDH mRNA and are presented relative to the control group, set as 1. Data are means ± SEM (n ≥ 3). ∗∗p < 0.01, ∗∗∗p < 0.001, ∗∗∗∗p < 0.0001; NS, no significance.

demonstrated the role of Th1 and Th2 lymphocytes in cell-mediated immune responses to xenografts. Compared with the sham-operated group, a Th1 type inflammation was activated by implanted pTDM/rDFCs after 3 days transplantation, such as increased tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-12b (figure 6). Whereas the Th2-related cytokine, such as IL-6, were decreased at the infiltrating tissues upon pTDM/rDFCs. The collective pro-inflammatory cytokines could lead to the stimulation of complement-fixing antibody isotypes and differentiation of CD8+ cells to a cytotoxic phenotype. Accordingly, the up-regulated gene expression of CD4 and CD8 were detected at the implanted sites, indicating the inflammation microenvironment initiated by implanted pTDM/rDFCs at the acute phase.

Histology examination monitored the gradient tissue infiltration and odontogenesis at the inside situ of implanted pTDM/rDFCs complex (figure 7). Compared with the phenomenon that a few cells distributed at the inner layer of the complex at 3 days (figures 7(A), (a), and (e)), elevated tissue deposition
Figure 7. Odontogenic differentiation of pTDM/rDFCs in the inflammation microenvironment in vivo (histology examination at the inside situ). (A) Gradient tissue infiltration was observed from 3 days to 3 months ((a)–(d): H&E analysis, (e)–(h): Masson staining), including fibroblasts (b) and fibrils (f) at 1 week, and predentin, blood vessels, and collagen fibrils (c, g) at 1 month, as well as polarizing odontoblast-like cells at 3 months (d, h). (Black arrows: fibroblast. Yellow stars: blood vessels. Red arrows: fibrils. P: predentin. odl: odontoblast-like cells.) (B) Immunohistochemistry showed the positively stained col-I and DMP-1 proteins in the predentin, dentin, and pulp-like tissues. Scale bars, 100 µm.

At the inside situ was shown by H&E and Masson staining at 1 week implantation. Structures such as fibroblasts (figures 7(A), (b): black arrows) and newly formed fibrils (figures 7(A) and (f)) were initiated and involved at this stage. At 1 month of implantation, the newly formed structures such as predentin, blood vessels, and collagen fibrils were observed. Until 3 months, polarizing odontoblast-like cells participated in typical dentino-pulp structures reconstruction. Immunohistochemistry (figure 7(B)) further showed the positively stained Col-I and DMP-1 proteins in the predentin, dentin, and pulp-like tissues.

Accordingly, the processed tissue reconstruction at the outside situ of implanted pTDM/rDFCs was also examined (figure 8(A)). As is shown by H&E and Masson staining, a few cells and collagen fibers were wrapped around the pTDM/rDFCs complex at 3 days implantation (figures 8(A), (a) and (e)), while enriched matrix deposition at the outside situ of pTDM/rDFCs was displayed at 1 week implantation. Respectively, large numbers of fibroblasts (figures 8(A), (b): black arrows), as well as newly formed collagen fibrils and blood vessels (figures 8(A) and (f)), were recruited at the outside situ at the 1 week stage. Until 1 month of implantation, the mature fibrils and cementum-like structures were integrated into periodontal-like tissue reconstruction (figures 8(A), (c) and (g)). However, some absorptions were observed at the surface layer of pTDM after 3 month transplantation, resulting in a partially-impaired dentin matrix both in H&E and Masson images (figures 8(A), (d) and (h)). Even though degraded dentin matrix, the implants were strongly stained by dentin-specific proteins like col-I and DMP-1 at 3 month transplantation (figure 8(B)).

Collectively, both typical dentino-pulp structures and periodontal-like tissues were detected during the processed tissue reconstruction of implanted pTDM/rDFCs complex. However, the dentin-specific protein DSPP was negatively stained until 3 months implantation (data not shown). The activated Th1 inflammation might be responsible for the partially
Figure 8. Odontogenic differentiation of pTDM/rDFCs in the inflammation microenvironment in vivo (histology examination at the outside situ). (A) Gradient tissue infiltration was observed from 3 days to 3 months ((a)−(d): H&E analysis, (e)−(h): Masson staining), including fibroblasts (b), fibrils and blood vessels (f) at 1 week, and cementum-like tissues at 1 month (c), (g), as well as partially-impaired dentin matrix at 3 months (d, h). (Black arrows: fibroblast. Yellow stars: blood vessels. Ce: cementum-like tissues. Red arrows: impaired dentin matrix.) (B) Immunohistochemistry showed the positively stained col-I and DMP-1 proteins in the dentin tissues. Scale bars, 100 µm.

weakened odontogenesis and outer layer absorption of pTDM.

4. Discussion

A promising way to circumvent the limitations of the current biological scaffold is through the usage of xenogeneic ECM [7, 21], especially porcine source. Like allogeneic ECM, xenogeneic ECM also functions by immediate degradation and timely release of bioactive constituents [22]. The rapid degradation in vivo of xenogeneic ECM minimizes the host-to-graft immune response and even prompts host response towards a constructive phenotype [23, 24]. When concerned about the mechanical strength of these ECM, the common state is short-time diminishment and quickly followed by reconstructed biomechanical stress along with newly matrix deposition [12]. Therefore, most commonly xenogeneic ECM was applied for soft/stromal tissue remodeling [25], a nondegradable ECM explored for hard/mineralized-parenchymal tissue reconstruction and constant mechanical loading remains elusive. The tooth is identified as a typical organ consisted of stromal and mineralized-parenchymal tissues [18, 26]. Biologically, stromal tissues function as nourishment supply and injured repair, while mineralized-parenchymal tissues provide loading support. Therefore, for the ideal repair of tooth loss, the implanted scaffold is expected to not only behave tooth morphology but also serve as loading support [27]. Our prior studies have suggested that the TDM could be an ideal alternative, and a combination of xenogeneic TDM with allogeneic DFCs could be regarded as a feasible approach for the induced constructive process [19, 28]. This study further compared the biological characteristics of pTDM with hTDM, with the aspects of morphological and physiological features, the release of bioactive molecules, and bioinductive functions. All these works would make compositional efforts for xenogeneic-matrix-based tissue regeneration.
Previous studies recommended a three-step gradient protocol for human dentin, resulting in sufficiently exposing dentin tubules [16]. However, this incubation time treating with EDTA was not effectively applied for porcine dentin since species-specific differences. In our study, gradient 17%, 10%, and 5% EDTA treatment protocol were taken, with prolonged incubation time, to treat the porcine dentin matrix. The normal hard connective tissue, such as bone and teeth, is formed through reinforcement of organic fibrils matrices by calcium phosphate crystals [29]. These mineral crystallites are located in or around fibrils, namely intrafibrillar mineralization or extrafibrillar mineralization [30]. Demineralization of the dentin matrix is the primary step for the release of bioactive molecules [31]. Selectively extrafibrillar demineralization and preservation of intrafibrillar mineralization are paramount in maintaining fibril topography and the mechanical properties (such as stiffness) of dentin matrix [32, 33]. It was reported that dentin exposed to alkaline materials, such as Ca(OH)₂ or tricalcium silicate cement, released growth factors, such as TGF-β1 via collagen degradation and exhibited a significant reduction in mechanical strength [34]. Although longer treatment time of porcine dentin than that of human dentin, this study showed an intact hydroxyapatite phase and preserved necessary minerals ions of both pTDM and hTDM. Our previous study also reconstructed a 3D biomechanical model based on pTDM, which exhibited positive biomechanical properties for the dissipation of masticatory forces [19, 27]. Therefore, through partial-demineralization by gradient EDTA concentrations, pTDM displayed similar mineral phases to hTDM and maintained mechanical properties, while rendering the release of bioactive molecular extraction and introducing the odontogenic differentiation of DFCs. The mechanism needs further exploration.

The dentin formation is believed to be a concordant interaction between ECM molecules and mineral crystals. Ca²⁺ ions combine with PO₄³⁻ to initiate the nucleation process, while collagen fibrils provide a spatial template to induce mineral crystals deposit [35]. Our results showed the most amount of type I collagen released from hTDM and pTDM. Through self-assembly into collagen fibrils and the lateral aggregation of collagen fibrils, collagen fibrils were strengthened and migrating toward mineralization fronts [36]. In dentin, type I collagen accounts for ~56% of minerals in its pores of collagen fibrils, while the non-collagenous proteins (NCPs) act as promotors, stabilizers, and even inhibitors of this biomineralization process [15, 35]. For example, DMP-1 behaves a great affinity to calcium and collagen fibrils, initiates nucleation of calcium-phosphate crystals, and modulates crystal growth [37]. DSPP is immuno-localized in the peritubular dentin and might be downregulated by TGF-β1[38]. Parallely, our results found that less TGF-β1 and more DSPP were detected in pTDM liquid extracts than those in hTDM liquid extracts. DSP, the N-terminal part of DSPP, mainly regulates matrix formation, while DPP, the C-terminal region of DSP, is involved in intrafibrillar mineralization and maturation of mineralized dentin [39]. Other ECM molecules account for proportion in dentin matrix, for example, PGs represent less than 3% by volume. PGs are recognized to modulate the structural organization of ECM in skeleton connective tissues [40]. Decorin and biglycan are the two most abundant PGs in dentin, function by binding to collagen molecules, interacting with soluble growth factors, and orienting collagen fibrils for matrix organization [41]. Consequently, col-I and NCPs released from pTDM could effectively contribute to biomineralization and induced odontogenesis.

When transplant hDFCs/hTDM complex into immunodeficiency mice subcutaneously, the previous study used an anti-human mitochondria antibody, which only reacts with human cells, to identify that the implanted hDFCs provided the source for induced odontogenesis [20]. Although odontoblasts are implicated in the synthesis and mineralization of dentin ECM, only ~10.1% of the TDM proteome was shared with the odontoblast proteome [42]. Therefore, odontogenic differentiation of implanted dental cells plays a major role in tissue-engineered dentin regeneration [43, 44]. hDFCs, under pTDM induction, expressed parallel col-I, ALP, OPN to that of hTDM. Whereas the odontogenic differentiation of implanted dental cells may play a major role in tissue-engineered dentin regeneration. Down-regulated TGF-β1 might reversely account for the non-inhibited expression of DSPP [38]. Even though inflammatory cytokines were also detected in vivo, a combination of pTDM and allogeneic rat DFCs still induced a constructive process initially.

Fibroblasts were recruited to the inside and outside situ of the implanted rDFCs/pTDM complex at 1 week. Newly blood vessels were observed at inside situ (1 month) and outside situ (1 week). More importantly, a layer of cell nucleus was aligned at the inner of predentin, which was responsible for secreting collagen and NCPs as well as the formation of predentin. In the proximal predentin, secreted type I collagen together with formed collagen fibrillation were implicated [45]. Most NCPs are secreted in the mature dentin, near the mineralization front [14]. In contrast to thin fibrils in xTDM (pTDM) at 3 days, biomineralization together with dense dentin tubules were prosperous until 3 months, leading to accordant morphology of dentino-pulp complex. A layer of cementum-like tissue was remineralized and formed to connect dentin with peripheral tissues at 1 month, yet it was unfortunately degraded until 3 months. The
remodeling process of cementum might be influenced by biomechanical factors since cementum serves to attach the tooth to the alveolar bone via PDL [46]. Occasional inflammation also occurs in the clinical application of SIS for musculotendinous soft tissue reconstruction, and the reconstructed tendon cannot withstand load without primal weight-bearing [47]. Orthotopic implantation in alveolar bone might be optimal for periodontal tissue reconstruction because of native masticatory force.

In conclusion, although impaired odontogenic differentiation of allogenic DFCs was observed in vitro and in vivo inflammation microenvironment, xenogeneic pTDM, consisting of necessary mineral ions and ECM molecules, could provide bioinductive microenvironment for dentin regeneration. In contrast to bone tissues, there is little or no remodeling in dentin. Consequently, odontogenesis provides an excellent model to study the biomimORIZATION and regeneration of hard/mineralized-parenchymal tissues. What is a promising way to reconstruct the integrity of hard-soft tissues? Recently, modulation from pro-inflammatory M1 macrophage to anti-inflammatory/tissue-regenerating M2 phenotype is suggested to be crucial for the fate of implanted scaffolds [15, 48], it is unclear whether it would be feasible for a non-degradable scaffold. More information is required before these questions can be answered [49].

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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Author contributions

R Y and W T designed the experiments and wrote the paper. H L performed the experiments, interpreted the data and the statistical analysis, and co-edited the paper. H L and B M fabricated dentin matrix and incubated cells. H Y performed animal experiments. J Q performed co-culture experiments in vitro.

Conflict of interest

The authors declare that they have no competing interests.

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References

[1] Taylor D A, Sampaio L C, Ferdous Z, Gobin A S and Taite L J 2018 Decellularized matrices in regenerative medicine Acta Biomater. 74 74–89
[2] Ragelle H, Naba A, Larson B L, Zhou F, Prijic M, Whittaker C A, Del Rosario A, Langer R, Hynes R O and Anderson D G 2017 Comprehensive proteomic characterization of stem cell-derived extracellular matrices Biomaterials 128 147–59
[3] Yin Z, Chen X, Zhu T, Hu J J, Song H X, Shen W L, Jiang L Y, Heng B C, Ji J F and Ouyang H W 2013 The effect of decellularized matrices on human tendon stem/progenitor cell differentiation and tendon repair Acta Biomater. 9 9317–29
[4] Padhi A and Nain A S 2020 ECM in differentiation: a review Ann. Biomed. Eng. 48 10871–89
[5] Humphrey J D, Dufresne E R and Schwartz M A 2014 Mechanotransduction and extracellular matrix homeostasis Nat. Rev. Mol. Cell Biol. 15 802–12
[6] Dua K S, Hogan W J, Aadam A A and Gasparri M 2016 In vivo osseophagel regeneration in a human being by use of a non-biological scaffold and extracellular matrix Lancet 388 55–61
[7] Badyak S F 2004 Xenogenic extracellular matrix as a scaffold for tissue reconstruction Transpl. Immunol. 12 367–77
[8] Lin T et al 2018 Hydrogel derived from porcine decellularized nerve tissue as a promising biomaterial for repairing peripheral nerve defects Acta Biomater. 73 326–38
[9] Wong M L, Wong J L, Vapniarsky N and Griffiths L G 2016 In vivo xenogenic scaffold fate is determined by residual antigenicity and extracellular matrix preservation Biomaterials 92 1–12
[10] Grainger D W 2013 All charged up about implanted biomaterials Nat. Biotechnol. 31 307–9
[11] Bayrak A, Tyrralla M, Ladhoff J, Schleicher M, Stock U A, Volk H D and Seifert M 2010 Human immune responses to porcine xenogeneic matrices and their extracellular matrix constituents in vitro Biomaterials 31 3793–803
[12] Cramer M C and Badyak S F 2020 Extracellular matrix-based biomaterials and their influence upon cell behavior Ann. Biomed. Eng. 48 2132–53
[13] Butler W T 1995 Dentin matrix proteins and dentinogenesis Connect. Tissue Res. 33 59–65
[14] Linde A 1983 The extracellular matrix of the dental pulp and dentin J. Dent. Res. 64 523–9
[15] Jin S S et al 2019 A biomimetic hierarchical nanointerface orchestrates macrophage polarization and mesenchymal stem cell recruitment to promote endogenous bone regeneration ACS Nano 13 6581–95
[16] Li R et al 2011 Human treated dentin matrix as a natural scaffold for complete human dentin tissue regeneration Biomaterials 32 4525–38
[17] Guo W et al 2009 The use of dentin matrix scaffold and dental follicle cells for dentin regeneration Biomaterials 30 6708–23
[18] He L et al 2019 Parenchymal and stromal tissue regeneration of tooth organ by pivotal signals reinstated in decellularized matrix Nat. Mater. 18 627–37
[19] Li H et al 2017 Xenogeneic bio-root prompts the constructive process characterized by macrophage phenotype polarization in rodents and nonhuman primates Adv. Healthcare Mater. 6 1601112
[20] Jiao L, Xie L, Yang B, Yu M, Jiang Z, Feng L, Guo W and Tian W 2014 Cryopreserved dentin matrix as a scaffold
material for dentin–pulp tissue regeneration Biomaterials 35 4929–39
[21] Cooper D K 2003 Clinical xenotransplantation–how close are we? Lancet 362 557–9
[22] Patzer S S, Fatehi Hassanabad A and Fedak P W M 2019 Accellular extracellular matrix bioscaffolds for cardiac repair and regeneration Front. Cell Dev. Biol. 7 63
[23] Wong M L and Griffiths I G 2014 Immunogenicity in xenogenic scaffold generation: antigen removal vs. decellularization Acta Biomater. 10 1806–16
[24] Keane T J and Badylak S F 2015 The host response to allogeneic and xenogeneic biological scaffold materials J. Tissue Eng. Regen Med. 9 504–11
[25] Freytes D O, Badylak S F, Webster T J, Geddes I A and Randell A E 2004 Biaxial strength of multilaminated extracellular matrix scaffolds Biomaterials 25 2553–61
[26] Thesleff I and Tummers M 2008 Tooth Organogenesis and Regeneration (Cambridge, MA: StemBook)
[27] Luo X, Yang B, Sheng L, Chen J, Li H, Xie L, Chen G, Yu M, Guo W and Tian W 2015 CAD based design sensitivity analysis and shape optimization of scaffolds for bio-root regeneration in swine Biomaterials 57 59–72
[28] Sun J, Li J, Li H, Yang H, Chen J, Yang B, Huo F, Guo W and Tian W 2017 tBHQ suppresses osteoclastic resorption in xenogeneic-treated dentin matrix-based scaffolds Adv. Healthcare Mater. 6 1700127
[29] Thrivikraman G et al 2019 Rapid fabrication of vascularized and innervated cell-laden bone models with biomimetic intrabifilar collagen mineralization Nat. Commun. 10 5320
[30] Xu Z, Yang Y, Zhao W, Wang Z, Landis W J, Cui Q and Sahal N 2015 Molecular mechanisms for intrabifilar collagen mineralization in skeletal tissues Biomaterials 39 59–66
[31] Salehi S, Cooper P, Smith A and Ferracane J 2016 Dentin matrix components extracted with phosphoric acid enhance cell proliferation and mineralization Dent. Mater. 32 334–42
[32] Kinney J H, Habelitz S, Marshall S J and Marshall G W 2003 The importance of intrabifilar mineralization of collagen on the mechanical properties of dentin J. Dent. Res. 82 597–61
[33] Gu L S, Cai X, Guo J M, Pashley D H, Breschi L, Xu H H K, Wang X Y, Tay F R and Niu L N 2019 Chitosan-based extrabifilar demineralization for dentin bonding J. Dent. Res. 98 186–93
[34] Huang X Q, Camba J, Gu L S, Bergeron B E, Ricucci D, Pashley D H, Tay F R and Niu L N 2018 Mechanism of bioactive molecular extraction from mineralized dentin by calcium hydroxide and tricalcium silicate cement Dent. Mater. 34 317–30
[35] Abou Neel E A, Aljabo A, Strange A, Ibrahim S, Coathup M, Young A M, Bozec L and Mudera V 2016 Demineralization-remineralization dynamics in teeth and bone Int. J. Nanomed. 11 4743–63