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Enhanced differentiation of dental pulp cells cultured on microtubular polymer scaffolds *in vitro*

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Abstract

Dental caries (tooth decay) is the most common chronic disease. Dental tissue engineering is a promising alternative approach to alleviate the shortcomings of the currently available restorative materials. Mimicking the natural extracellular matrix (ECM) could enhance the performance of tissue engineering scaffolds. In this study, we developed microtubular (~20 µm diameter) polymethyl methacrylate (PMMA) scaffolds resembling the tubular (~2.5 µm diameter) structure of dentin, the collagen-based mineralized tissue that forms the major portion of teeth, to study the effect of scaffold architecture on differentiation of mouse dental pulp cells *in vitro*. Flat (control), plasma-treated solid and microtubular PMMA scaffolds with densities of 240±15, 459±51 and 480±116 tubules/mm² were first characterized using scanning electron microscopy and contact angle measurements. Dental pulp cells were cultured on the surface of the scaffolds for up to 21 days and examined using various assays. Cell proliferation and mineralization were examined using Alamar Blue and Xylenol Orange (XO) staining assays, respectively. The differentiation of pulp cells into odontoblasts was examined by immunostaining for Nestin and by quantitative PCR analysis for *dentin matrix protein 1* (*Dmp1*), *dentin sialophosphoprotein* (*Dspp*) and *osteocalcin* (*Ocn*). Our results showed that the highest tubular density scaffolds significantly ($p<0.05$) enhanced differentiation of pulp cells into odontoblasts as compared to control flat scaffolds, as evidenced by increased expression of Nestin (5.4x). However, mineralization was suppressed on all surfaces, possibly due to low cell density. These results suggest that the microtubular architecture may be a desirable feature of scaffolds developed for clinical applications.

Lay Summary—Regenerative engineering of diseased or traumatized tooth structure could avoid the deficiencies of traditional dental restorative (filling) materials. Cells in the dental pulp have the potential to differentiate to dentin-producing odontoblast cells. Furthermore, cell-supporting scaffolds that mimic a natural extracellular matrix (ECM) are known to influence behavior of progenitor cells. Accordingly, we hypothesized that a dentin-like microtubular scaffold would enhance differentiation of dental pulp cells. The hypothesis was proven true and differentiation to odontoblasts increased with increasing density of the microtubules. However, mineralization was

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suppressed, possibly due to a low density of cells. The results demonstrate the potential benefits of a microtubular scaffold design to promote odontoblast cells for regeneration of dentin.

Keywords

dental pulp; tubular scaffold; tissue engineering; odontoblast

Introduction

Teeth are comprised of mineralized enamel, dentin and cementum tissues surrounding the dental pulp. Dentin comprises the bulk of the tooth and has a microtubular structure. The microtubules span the entire thickness of the dentin and form as a result of the mineralization mechanism of odontoblasts. The diameter and density of the microtubules vary within dentin from the pulp to the enamel, approximately 2.5 to 1.0 μm , respectively. Odontoblasts residing at the perimeter of the pulp extend cell processes into the microtubules [1]. Trauma to the tooth that damages the odontoblasts triggers recruitment of pulp progenitor cells to the site and differentiation to odontoblasts for repair [2]. Accordingly, understanding the effect of the microtubular architecture on differentiation of these progenitors is central to tooth repair/regeneration strategies.

Dental caries affecting the integrity of the mineralized tissues of teeth remains the most prevalent infectious disease worldwide [3]. Untreated, caries progresses through the outer enamel surface, into the dentin and through to the pulp. Current treatment involves removal of the affected dental tissue and replacement with synthetic materials, which fail to restore the biological function of the natural tissue and are prone to mechanical failure as well as penetration of bacteria and formation of secondary caries at the tooth-material interface [4]. These shortcomings of the current restorative materials [5, 6] stimulate development of improved materials, but also research in dental tissue engineering [7, 8].

Tissue engineering technology relies on the triad of stem/progenitor cells, highly porous biomaterial scaffolds and morphogenic signals to regenerate tissues. Previous efforts in dental tissue engineering have primarily focused on identifying suitable cells and odontogenic signals [9, 10]. However, the ultimate success of tissue engineering depends additionally on various scaffold characteristics that influence biomaterials-cell interactions. The influence of these characteristics, including surface chemistry, electrical charge, hydrophobicity/hydrophilicity, stiffness, topography, and architecture/structure of the scaffolds on cell response, is not yet fully understood [11–13].

Few investigators have studied the effect of different scaffold architectures, much yet tubular designs, on regeneration of dental tissue [14–16]. Wang *et al.* showed the superiority of nanofibrous scaffolds in odontogenic differentiation of human dental pulp stem cells (DPSCs) and mineralization as compared to solid wall scaffolds both *in vitro* and *in vivo* [17]. Similarly, Sumita *et al.* cultured primary cells from porcine molars on a 3D collagen sponge and showed its superiority in tooth regeneration over polyglycolic acid (PGA) fiber mesh scaffolds [14]. Tonomura *et al.* demonstrated that regeneration of a dentin-like tissue using primary porcine dental pulp cells *in vivo* was indirectly influenced by the scaffold architecture [15]. Scaffolds employed for dentin regeneration include natural treated dentin

matrices (TDM) [18–20], combinations of natural tooth and bio/synthetic polymers [21–23], or scaffolds made entirely from bio/synthetic materials [15, 16, 24–29]. However, the underlying mechanisms of interaction between primary dental pulp cells and polymeric scaffolds having a 3D microtubular dentin-like architecture have remained largely unexplored.

It has been shown that a microtubular architecture can influence differentiation of non-dental stem/progenitor cells into specific phenotypes. Oh *et al.* showed that adhesion and differentiation of human mesenchymal stem cells (hMSCs) cultured on nanotubular-shaped titanium oxide surface structures could solely be dictated by changing the nanotubule size in the absence of any morphogenic-inducing stimuli [30]. On nanotubular-shaped aluminum oxide structures with a pore size gradient, pore size influenced adhesion and osteogenic differentiation of hMSCs cells [31]. Dalby *et al.* showed that order/symmetry of the pores on a PMMA membrane stimulated hMSCs to produce bone mineral *in vitro*, even in the absence of osteogenic supplements [32]. Study of osteoblast-like cells cultured on polycarbonate membranes with different micropore sizes (2–8 μm) showed that large micropores promoted cell differentiation and matrix production [33]. Similarly, microtubular polymeric scaffolds enhanced differentiation of human bone marrow stromal cells (hBMSCs) into nerve cells as compared to planar control surfaces [34]. Bruckmann *et al.* studied the effect of a polystyrene membrane mimicking the structure of natural dentin on cell response of primary periodontal ligament fibroblasts (PDLF) or gingival fibroblasts (GF) and observed preferential deposition of the ECM matrix on the textured surface [24].

Accordingly, we hypothesized that a microtubular architecture would positively influence dental pulp cell behavior leading to increased dentinogenic differentiation. The hypothesis is based on the fact that natural dentin has a microtubular structure and imitating the characteristics of the natural extracellular matrix (ECM) could benefit scaffold performance [35]. In this study, dentin-like 3D microtubular PMMA scaffolds with tubule diameters of $\sim 20\text{ }\mu\text{m}$ and varying tubule densities were synthesized. The microtubules were straight and parallel. The effects of the tubular scaffolds and planar (flat, non-tubular) PMMA controls on proliferation, mineralization and dentinogenic differentiation of primary murine dental pulp cells were evaluated through *in vitro* cell culture studies.

Materials and Methods

Fabrication of scaffolds

Microtubular scaffolds with different tubule densities were fabricated by a fiber templating technique. Polyvinyl alcohol short-cut fibers (PVA-WN2, 51 mm, 1.3 denier, Engineered Fibers Technology), with a radius of $9.8\text{ }\mu\text{m}$, were packed inside 1 ml pipette tips (TipOne®). A 1 ml polymer solution composed of methyl methacrylate monomer (99%, Aldrich 55909), Luperox® A98 Benzoyl peroxide (Sigma-Aldrich) 1:400 (w:w) and ethylene glycol dimethacrylate (EGDM 98%, Aldrich 335681) 1:160 (v:v) was prepared and injected into the tips. The bottoms of the pipette tips were sealed with silicone (Factor II, Inc.) prior to addition of the polymer solution. To create scaffolds with different tubule densities, varying amounts of PVA fiber were loaded into the pipette tips. Scaffolds with 0.05, 0.1, and 0.2 g of sacrificial fiber were labeled low density (LD), medium density (MD)

and high density (HD) tubular scaffolds, respectively. To reduce fiber clumping when not close-packed, the fibers were manually combed to electrostatically charge them prior to packing into the pipette tips. The electrostatic charge dispersed the fibers more uniformly within the tip volume. The polymer solution-filled tips were placed inside a vacuum chamber for half an hour to remove air bubbles. The tips were placed inside an oven and polymerized at 50°C for 18 hrs, then at 90°C for an additional 21 hrs. The tips were then cut into 1 mm thick disks using a precision saw (Buehler Isomet® 1000). These PMMA disks were subsequently placed in distilled water and sonicated for about 2 hrs to completely dissolve the PVA fibers. Solid, planar PMMA disks, used as controls, were fabricated similarly, but without sacrificial fibers. To sterilize, the planar and tubular scaffolds were rinsed with DI and soaked in ethanol for an hour. Scaffolds were soaked in double distilled Millipore water afterwards for 48 hrs to allow for complete removal of ethanol and residual monomers. Scaffolds were dried in an oven at 60°C for 24 hrs and UV irradiated for an hour.

Plasma treatment

To improve adhesion of the pulp cells, planar and tubular PMMA scaffolds were plasma treated prior to cell culture using a plasma cleaner unit (Harrick Plasma PDC-32G) for 10 minutes in air at 0.27 mbar pressure with 18 W applied to the RF coil (high setting).

Characterization of the scaffolds

Scanning electron microscopy—The tubular scaffolds were sputter coated with gold and imaged using a scanning electron microscope, SEM (TM-1000 Hitachi, Japan). Image analysis of the SEM micrographs was accomplished with ImageJ software (NIH). The number and size distribution of the tubules were measured with the built-in “Analyze Particles” plugin and average spacing between tubules was estimated with the custom-written “ND” plugin [36] for ImageJ.

Contact angle measurement—Contact angles of non-treated and plasma-treated PMMA scaffolds were measured with a CAM-Plus (Tantec, Germany) sessile drop contact angle meter. A 10 µl droplet of deionized water was placed on each scaffold and the contact angle was measured using the Half Angle™ technique.

Cell culture—The PMMA scaffolds were glued to 12 mm microscope cover glasses (Fisher Scientific), with the tubules perpendicular to the surface of the cover glass, using a silicone medical adhesive (A-100, Factor II, Inc.) and placed in 24-well culture plates (Fisher Scientific). Primary dental pulp cultures were prepared from the coronal portions of pulps from unerupted first and second molars from 5–7-day old wild type mouse pups as previously described [37, 38]. All mice were maintained in the CD1 background. After isolation, cells were seeded on scaffolds at a density of 60,000 cells/cm² in Dulbecco's modified Eagle's medium (DMEM), 20% fetal bovine serum (FBS), 2 mM L-glutamine, 40 U/ml penicillin, 40 µg/ml streptomycin and 0.1 µg/ml Fungizone (Invitrogen). Scaffolds were then placed in an incubator for an hour before additional medium was added to 1 ml total volume in each well. Three days later, the medium was changed to DMEM containing 10% FBS. At day 7, the culture medium was replaced with differentiation medium containing Minimum Essential Medium alpha (αMEM), 10% FBS, with addition of 50

mg/ml fresh ascorbic acid and 4 mM β -glycerophosphate. Culture medium was changed every other day through the duration of the experiments.

Cell assays

Cell proliferation assay—The AlamarBlue assay, which is based on the detection of metabolic activity of the cultured cells, was used to assess cell proliferation of pulp cells on different scaffolds at days 3, 5, and 8. AlamarBlue dye (Invitrogen) was added to the culture dishes containing scaffolds at 1:10 (v/v) ratio and incubated for 2 hrs. Control samples were prepared by addition of the dye to wells containing base medium. Aliquots of 100 μ l of each medium were removed and added to 50 μ l of 3% sodium dodecyl sulfate (SDS) solution in 96-well plates (Costar™ black clear bottom plate, Fisher Scientific) to stop the reaction. The plates were subsequently read using a Gemini EM fluorescence microplate reader at the fluorescence wavelength of 570/610 nm.

Cell morphology—To examine morphology, cells cultured for 21 days on the scaffolds were fixed in 4% paraformaldehyde (PFA) for 45 min, rinsed with PBS and sequentially washed in gradients of 70, 90, and 100 % ethanol. Scaffolds were dried in a desiccator overnight and sputter coated with gold and examined using SEM (TM-1000 Hitachi, Japan).

RNA extraction and quantitative PCR (qPCR) analysis—Total RNA was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol and treated with RNase-free DNase to eliminate genomic DNA. Isolated RNA was reverse transcribed by Superscript II Reverse Transcriptase with Oligo(dT)_{12–18} primers (Life Technologies, Grand Island, NY). Gene expression was examined by qPCR analysis using the 2^{-CT} method as described previously [39–41]. For qPCR reactions, 9 ng of cDNA was combined with 5 μ l TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ), 2.5 μ l H₂O and 0.5 μ l TaqMan primers (total 10 μ l). TaqMan primers for *Dmp1* (Mm00803831_m1), *Dspp* (Mm00515666_m1), *Gapdh* (Mm99999915_g1) and *Ocn* (Mm03413826_mH) were purchased from Applied Biosystems. Amplification efficiency was determined using internal standard curves derived from a purified amplicon, diluted 2-fold (0.14 – 9.0 ng), and was close to 100% for all qPCR reactions. We defined the acceptable range of CT values representing gene expression to be between 10 and 35 cycles, according to manufacturer's recommendations (Applied Biosystems).

Immunostaining—To visualize actin cytoskeleton and nuclei of cells on different tubular scaffolds and controls, cells were cultured for 21 days and processed for immunostaining. Cells were fixed with 4% PFA in 4% sucrose containing PBS for 45 min followed by permeabilization with 0.2% Triton X-100 for 5 min, and were blocked with 0.2% gelatin in PBS for an hour. F-actin and cell nuclei were detected in fixed and permeabilized cells with 0.5 mM rhodamine/phalloidin (rho/pha) (Sigma-Aldrich, MO) and 1 μ g/ml 4',6-diamidino-2-phenylindole (DAPI), respectively.

To visualize the differentiation of dental pulp cells into odontoblast-like cells, immunostaining for nestin was performed using a mouse monoclonal antibody (2Q178, Santa Cruz Biotech, CA). Cells were incubated with the antibody (1:200) at room

temperature for 2 hrs followed by incubation with a secondary goat anti-mouse IgG labeled with Alexa-Fluor 488 antibody (1:300, Molecular Probes) for 45 min. Images were captured using a Zeiss LSM 780 confocal fluorescence microscope equipped with a diode laser 405 nm, an Argon laser with 458/488/514 nm lines, DPSS 561 nm and HeNe 633 nm lasers. Zen imaging software (Zeiss, Germany) was used to control illumination shutters, camera exposure and image acquisition.

Mineralization assay—Mineralization of cells cultured on different scaffolds and tissue culture plastic (TCP) controls was assessed by Xylenol Orange calcium staining at days 14, 18, and 21. Xylenol Orange powder (Sigma, St. Louis, MO) was dissolved in distilled water and filtered to make a 20 mM stock solution. Xylenol Orange at a final concentration of 20 μ M was added to the medium overnight. Cultures were given fresh medium without fluorochrome prior to imaging to avoid a non-specific fluorescent background. Xylenol Orange-stained mineralized nodules fluoresce red and can be visualized using a TRITC Red filter.

Statistical analysis

Results are presented as means $\pm$ standard deviation (SD). One-way ANOVA on ranks (Kruskal-Wallis test) and Dunn's pairwise comparison method were performed (SigmaPlot v. 11) to compare the cells cultured on controls and scaffolds with p -values of 0.05 indicating statistical significance. In calculating averages, at least three independent samples were tested unless otherwise mentioned.

Results

Characterization of scaffolds

Scanning electron microscopy—Fig. 1a shows SEM images of the cross sections of the fabricated microtubular PMMA scaffolds (planar, low density (LD), medium density (MD), and high density (HD)). The individual tubules were approximately circular with radii comparable to the sacrificial polyvinyl alcohol fibers, demonstrating that the templating process produced a faithful replica. The mean $\pm$ SD tubule cross-sectional area (μm^2) and density ($\#/\text{mm}^2$) for LD, MD, and HD tubular scaffolds are compared in Fig. 1b. The average tubule radius for the LD and MD scaffolds was $9.8 \pm 2.3 \mu\text{m}$, which is equal to the radius of the sacrificial fibers. As the packing density of sacrificial fibers increased, the wall thickness between neighboring tubules became smaller leading to the collapse of walls during dissolution of the fibers, combining of several neighboring tubules, and formation of larger tubules (see arrows). Accordingly, for the HD scaffolds, the average radius increased to $13 \pm 6.5 \mu\text{m}$. The combination of tubules in the HD scaffolds also explains why tubule density in MD and HD scaffolds were comparable. Tubules were not uniformly distributed across the scaffolds, however, there was less clustering than with fibers that were not electrostatically charged by combing (data not shown).

Contact angle measurement—To improve cell adhesion, fabricated PMMA scaffolds were plasma treated. To evaluate the change of surface energy as a result of plasma treatment, the contact angle of the fabricated scaffolds before and after plasma treatment was

measured (Fig. 2). The presence of tubules themselves, without plasma treatment, led to a significant increase in contact angle and accordingly surface energy relative to the planar control. Plasma treatment lowered the contact angle/surface energy of the planar and tubular PMMA scaffolds significantly on all surfaces. The highest reduction in contact angle was observed in the HD tubular scaffold where the post-treatment contact angle dropped to zero.

Cell assays

Cell proliferation—The propensity of cells to grow and spread on planar and microtubular scaffolds was assessed at days 3, 5, and 8 using the Alamar Blue assay (Fig. 3).

Fluorescence intensity is proportional to the cell density. While cell proliferation on HD scaffold was lower than on other surfaces at day 3, cells grew to reach comparable cell densities on all surfaces by day 8.

Cell morphology—To further assess the effect of different scaffolds on cell behavior, morphology of dental pulp cells cultured on planar, LD, MD, and HD tubular scaffolds were examined at day 21 using SEM (Fig. 4). Cells were confluent on all surfaces and formed more than a single monolayer in many areas. Areas with only a monolayer of cells are translucent to the electron beam, allowing visualization of the underlying surface. Additionally, opaque clusters of cells and matrix were formed on all scaffolds. On the surfaces of tubular scaffolds, cells bridged across the tubules. In areas where tubules are not entirely capped by cell bodies, cell processes can be seen that extend into the tubules (arrow). No special imaging with the SEM was necessary to view the areas of translucent monolayers of cells.

Immunostaining—To examine the effects of the tubular architecture on differentiation of dental pulp cells into odontoblast-like cells, cells were processed for immunostaining (green) for nestin, a differentiation marker for odontoblasts. The intensity of nestin expression by cells showed a gradual increase with scaffold tubule density (Fig. 5a and 5b). Moreover, cells expressing nestin displayed an elongated polarized morphology characteristic of odontoblasts (Fig. 5a).

Gene Expression—The effect of tubular architecture on mineralization and dentinogenesis in cells cultured on microtubular and planar scaffolds was further studied by examining the expression of *Ocn*, *Dmp1* and *Dspp* at day 21. The fold change was calculated relative to expression levels of cells cultured on standard tissue culture plastic. As shown in Fig. 6, there were increased levels of expression of all transcripts in cells cultured on LD, MD and HD tubular PMMA as compared to cells cultured on planar PMMA surfaces. Furthermore, there was increased expression of *Dspp* in cultures grown on tubular scaffolds as compared to the cultures grown on tissue culture plastic controls. On the other hand, there were no significant differences in the levels of expression of *Dmp1* in cultures grown on tubular scaffolds as compared to cultures grown on tissue culture plastic. There was a decrease in the expression level of *Ocn* on the tubular scaffolds as compared to the tissue culture plastic.

Cell mineralization—Mineralization of the dental pulp cells cultured on the planar and tubular PMMA scaffolds was examined using the XO assay. Fluorescence images from the XO staining (day 21) were overlaid on phase contrast images to simultaneously show mineralization and the underlying surface features, respectively (Fig. 7). The images showed that mineralization on both the planar and tubular PMMA scaffolds was weaker than on TCP. On TCP cells produced a mineralized matrix across almost the entire culture surface, while mineralization on the planar PMMA surface was only observed in a few small patches and barely grew in size with time. On the tubular scaffolds, limited staining was observed near some of the tubules. The fluorescence in this case might have been due to non-specific staining. Temporal evolution of mineralization nodules on the TCP controls (data not shown) was in agreement with previous reports [37, 38], where after addition of differentiation-inducing medium mineralized nodules started forming on day 10 and gradually grew larger in size.

Discussion

Dental caries is one of the most prevalent diseases throughout the world. Conventional restorative materials and techniques do not restore the physiological architecture and complete function of intact tissues [4]. In principle, regeneration of tooth structures could be achieved by a classical tissue engineering approach, using dental stem/progenitor cells, scaffold materials and relevant growth and differentiation factors. Vascularization would also need to be established [42], a topic that has been reviewed specifically for dental tissue engineering [43]. It is believed that the dental pulp contains pluripotent cells [44] that can differentiate into odontoblasts [45]. The exact mechanisms that induce differentiation of dental pulp cells into odontoblasts are unknown, and multiple factors may influence varying aspects of cell response on tissue engineering scaffolds. Conceptually, through seeding, migration and proliferation of dental pulp cells in or into the scaffold followed by differentiation into odontoblasts, a microtubular dentin resembling the native dentin would be formed [22, 27].

In the present work, microtubular scaffolds were fabricated with different tubule densities and the effect of mimicking the natural dentin structure on the differentiation of dental stem/progenitor cells into odontoblasts was evaluated. The diameter of human dentin tubules is in the range of 1–3 microns with densities varying from about 20,000/mm² at the dentin-enamel interface to 50,000/mm² at the pulpal wall [46]. Despite the resemblance of the fabricated tubular scaffolds to that of natural dentin, the fabricated scaffolds feature a larger diameter and lower density of tubules (Fig. 1). With the fiber template method employed, fiber diameter determines the size of tubules formed. There are two factors that limit the ability to fabricate scaffolds that exactly match the size feature of natural dentin, availability of fibers with diameters of a few microns, and the difficulty of dissolving or otherwise removing such small sacrificial fibers after polymerization. Furthermore, there is no documented method to rigorously control tubule density using this method. In this work, we demonstrated an improved control of tubule density by electrostatic charging and loading varying amounts of the sacrificial fibers into polymerization molds. This approach was most effective within a narrow range of fiber masses. In LD scaffolds, where the fibers were not very compact, the fibers tended to cluster, particularly in lines, due to the capillary effect of

the polymer solution. This caused a broad distribution of inter-tubule spacing and/or tubule wall thicknesses throughout the scaffold. In HD scaffolds, on the other hand, the close-packed structure of the fibers led to formation of very thin tubule walls. These thin walls subsequently collapsed leading to the combination of several neighbors and formation of larger tubules (Fig. 1). This caused a broad size distribution in tubule diameter. MD scaffolds made using an intermediate amount of sacrificial fiber proved to be more homogenous in terms of tubule diameter and spacing. Other common techniques that could be used to fabricate long aspect ratio microtubular scaffolds, such as phase separation or rapid prototyping, also cannot closely replicate the size features of the natural dentin [11–13]. Nonetheless, this study of dental pulp cell response to the scaffolds demonstrated that the synthetic larger microtubular structures influenced differentiation and supports use of the fiber templating model.

Cell adhesion is essential for any material used as a tissue engineering scaffold. The native PMMA surface offers very limited cell adhesion. Therefore, the fabricated scaffolds were plasma treated to improve cell adhesion. Our results show that dental pulp cells adhere, grow and reach confluence on the plasma-treated planar and microtubular scaffolds (Fig. 3 and 4). The presence of microtubules on the surface of the PMMA scaffolds led to an increase in the surface energy as evidenced by larger contact angles measured on these surfaces. The higher surface energy of the fabricated microtubular scaffold (more hydrophobic) surfaces exacerbated the already weak adhesion to the PMMA scaffolds. Few cells were observed on the surface of non-plasma treated tubular scaffolds after a 24-hr culture (data not shown). Furthermore, tubules seemed to be an obstacle towards cell proliferation at the early stages of cell growth (day 3, Fig. 3). The lower proliferation rate on the HD tubular scaffolds compared to planar, LD, or MD scaffolds suggests a threshold of tubule density at which initial cell proliferation is affected. Gradually, as the cells produced matrix the tubules were bridged (Fig. 4) creating a path for cell migration. Therefore, the ultimate cell densities on planar and tubular scaffolds were comparable (day 8).

In addition to suitable cell adhesion and proliferation, success of any tissue engineering approach to restore or regenerate tissues requires differentiation of stem/progenitor cells into specific phenotypes. Dentin is produced by odontoblasts [47] and differentiation of dental pulp cells into odontoblasts is key to regeneration of damaged dentin. We investigated the presence of odontoblast-like cells in the populations cultured on the fabricated planar and tubular scaffolds by assessing the amount of nestin, as well as markers for ECM proteins produced by odontoblasts. Type I collagen comprises nearly 90% of the proteins synthesized by odontoblasts. The non-collagenous dentin matrix proteins synthesized and excreted by odontoblasts include dentine sialophosphoprotein (*Dspp*) that subsequently cleaves into dentine sialoprotein (*Dsp*), dentine phosphoprotein (*Dpp*) and dentin matrix proteins 1, 2, and 3 (*Dmp1*, *Dmp2* and *Dmp3*, respectively). The non-collagenous proteins additionally include small amounts of osteopontin, bone sialoprotein, osteonectin and osteocalcin [48, 49]. Matrix proteins play an important role in directing the process of biomineralization. ECM molecule-derived mineralization accounts for the majority of dentin formation. ECM proteins control both the initiation and growth of the crystals formed by odontoblasts [1]. Nestin is expressed in preodontoblasts, as well as immature and mature odontoblasts [50].

Even though the gene expression profiles showed an upregulation of intercellular and matrix proteins produced by odontoblasts, barely any mineralization was detected on the fabricated PMMA scaffolds (Fig. 7). There are several reasons that could explain the poor mineralization of pulp cells on the PMMA scaffolds. First, it may be the result of lower cell numbers on the PMMA scaffolds compared to standard tissue culture plastic. Despite identical seeding densities, the proliferation assay of the pulp cultures showed that the final cell numbers on the planar and tubular PMMA scaffolds at the end of the proliferation stage (day 7) were significantly lower than that on TCP. The increased mineralization of the cultures grown on TCP in 12-well dishes (Fisher Scientific) could be due to the larger surface area (2 cm^2) available for cell proliferation compared with the surface area of the fabricated PMMA scaffolds which had a surface area of about 0.3 cm^2 . Cell number is known to be a determining factor in affecting cell signaling, gene expression and mineralization [51]. Additionally, mineralization is highly controlled by a number of ECM proteins and perhaps protein expressions were modified in a way that impeded biomineralization on the PMMA scaffolds. This suggests that while the microtubular architecture employed in this work upregulated differentiation of dental pulp cells into odontoblasts, there is a need for testing at higher cell densities and/or altering the chemistry of the scaffold. Additionally, the suitable scaffolds for regeneration of dentin should ideally be biodegradable to allow for gradual disappearance of the scaffold as the regeneration process proceeds and dentin is produced. The choice of PMMA, which is a non-degradable polymer, was based on its biocompatibility, ease of polymerization and previous reports [32]. Future efforts, however, must be focused on use of biodegradable alternatives to PMMA.

In this study we saw increased markers for odontoblasts with increasing microtubular density (Fig. 5 and 6), so one might ask if these effects would be further enhanced as the templated architecture more closely approximates the physiological density and diameter of dentin tubules. It is difficult to speculate, in part because changes in tubules density and diameter also affect contact angle, cell adhesion and likely proliferation. It has been shown that nano-sized titanium oxide tubes can, on their own, influence differentiation of human mesenchymal stem cells (hMSC) [30], but extrapolation to the effect of micron-size tubes on dental pulp cells would be speculative. Other investigators prepared $10\text{-}\mu\text{m}$ diameter tubular poly(ethyl methacrylate)-based, hydroxyapatite-coated scaffolds to mimic dentin and evaluated the biological response after implantation in the back of nude mice [28]. No quantitative information was generated, but histologically the dermal cells colonized the implants, their morphology was affected, and cell processes extended into the tubules. These and other previous studies, along with the present results demonstrate that tubular architectures can affect cell behavior. We might expect substrates with more dentin-like structures to have a stronger influence on dental pulp cells, but further such experimentation would be needed to confirm the effect.

The results reported here could have implications for other clinical areas. Tubular scaffolds are used for a range of tissue engineering applications as they provide the required properties of pore connectivity and nutrient transport very efficiently [52]. The unidirectional microtubular scaffolds fabricated in this work are particularly useful for guided tissue

engineering in highly oriented tissues such as nerve, blood vessel, ligament, tendon and cardiac muscle [53, 54].

Conclusion

Mimicking extracellular matrix is a strategy for enhancing control over progenitor cells in regenerative engineering applications. Here we tested the hypothesis that a microtubular dentin-like PMMA scaffold would influence differentiation of murine dental pulp cells. Scaffolds with different tubule densities (300–500 tubules/mm²) were successfully fabricated and cultured with murine dental pulp cells for up to 21 days. Surface energy of the fabricated scaffolds showed an increase on scaffolds with higher tubule densities, which necessitated the need to plasma-treat the scaffolds to facilitate cell adhesion and formation of confluent cell layers. The tubular scaffolds promoted differentiation of dental pulp cells into odontoblast-like cells as evidenced by an increased expression of nestin and *Dspp* on the microtubular scaffolds compared with the control flat surface. Despite the observed increase in the number of odontoblast-like cells on the tubular surfaces, poor mineralization was observed on flat and tubular scaffolds alike, which may be due to low cell density or an altered expression profile of ECM proteins on the PMMA scaffolds.

Acknowledgments

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References

1. Goldberg M, Kulkarni AB, Young M, Boskey A. Dentin: structure, composition and mineralization. *Front Biosci (Elite Ed)*. 2011; 3:711–35. [PubMed: 21196346]
2. Smith AJ, Smith JG, Shelton RM, Cooper PR. Harnessing the natural regenerative potential of the dental pulp. *Dent Clin North Am*. 2012; 56:589–601. [PubMed: 22835540]
3. Petersen PE. Global policy for improvement of oral health in the 21st century - implications to oral health research of World Health Assembly 2007, World Health Organization. *Community Dentistry and Oral Epidemiology*. 2009; 37:1–8. [PubMed: 19046331]
4. Spencer P, Ye Q, Misra A, et al. Proteins, pathogens, and failure at the composite-tooth interface. *J Dent Res*. 2014; 93:1243–9. [PubMed: 25190266]
5. Drummond JL. Degradation, fatigue, and failure of resin dental composite materials. *J Dent Res*. 2008; 87:710–9. [PubMed: 18650540]
6. Laske M, Opdam NJM, Bronkhorst EM, et al. Longevity of direct restorations in Dutch dental practices. Descriptive study out of a practice based research network. *J Dentistry*. 2016; 46:12–7.
7. Galler KM, D'Souza RN. Tissue engineering approaches for regenerative dentistry. *Regen Med*. 2011; 6:111–24. [PubMed: 21175291]
8. Yen AH, Yelick PC. Dental tissue regeneration - a mini-review. *Gerontology*. 2011; 57:85–94. [PubMed: 20453484]
9. Nakashima M, Akamine A. The application of tissue engineering to regeneration of pulp and dentin in endodontics. *J Endod*. 2005; 31:711–8. [PubMed: 16186748]
10. Nakashima M, Reddi AH. The application of bone morphogenetic proteins to dental tissue engineering. *Nat Biotechnol*. 2003; 21:1025–32. [PubMed: 12949568]
11. Chan BP, Leong KW. Scaffolding in tissue engineering: general approaches and tissue-specific considerations. *Eur Spine J*. 2008; 17(Suppl 4):467–79. [PubMed: 19005702]

12. O'Brien FJ. Biomaterials & scaffolds for tissue engineering. *Materials Today*. 2011; 14:88–95.
13. Yang S, Leong KF, Du Z, Chua CK. The design of scaffolds for use in tissue engineering. Part I. Traditional factors. *Tissue Eng*. 2001; 7:679–89. [PubMed: 11749726]
14. Sumita Y, Honda MJ, Ohara T, et al. Performance of collagen sponge as a 3-D scaffold for tooth-tissue engineering. *Biomaterials*. 2006; 27:3238–48. [PubMed: 16504285]
15. Tonomura A, Mizuno D, Hisada A, et al. Differential effect of scaffold shape on dentin regeneration. *Ann Biomed Eng*. 2010; 38:1664–71. [PubMed: 20087773]
16. Wang J, Ma H, Jin X, et al. The effect of scaffold architecture on odontogenic differentiation of human dental pulp stem cells. *Biomaterials*. 2011; 32:7822–30. [PubMed: 21663962]
17. Wang J, Liu X, Jin X, et al. The odontogenic differentiation of human dental pulp stem cells on nanofibrous poly(L-lactic acid) scaffolds in vitro and in vivo. *Acta Biomater*. 2010; 6:3856–63. [PubMed: 20406702]
18. Guo W, Gong K, Shi H, et al. Dental follicle cells and treated dentin matrix scaffold for tissue engineering the tooth root. *Biomaterials*. 2012; 33:1291–302. [PubMed: 22088889]
19. Jiao L, Xie L, Yang B, et al. Cryopreserved dentin matrix as a scaffold material for dentin-pulp tissue regeneration. *Biomaterials*. 2014; 35:4929–39. [PubMed: 24680189]
20. Li R, Guo W, Yang B, et al. Human treated dentin matrix as a natural scaffold for complete human dentin tissue regeneration. *Biomaterials*. 2011; 32:4525–38. [PubMed: 21458067]
21. Cavalcanti BN, Zeitlin BD, Nor JE. A hydrogel scaffold that maintains viability and supports differentiation of dental pulp stem cells. *Dent Mater*. 2013; 29:97–102. [PubMed: 22901827]
22. Cordeiro MM, Dong Z, Kaneko T, et al. Dental pulp tissue engineering with stem cells from exfoliated deciduous teeth. *J Endod*. 2008; 34:962–9. [PubMed: 18634928]
23. Huang GT, Shagranova K, Chan SW. Formation of odontoblast-like cells from cultured human dental pulp cells on dentin in vitro. *J Endod*. 2006; 32:1066–73. [PubMed: 17055908]
24. Bruckmann C, Walboomers XF, Matsuzaka K, Jansen JA. Periodontal ligament and gingival fibroblast adhesion to dentin-like textured surfaces. *Biomaterials*. 2005; 26:339–46. [PubMed: 15262476]
25. El-Backly RM, Massoud AG, El-Badry AM, et al. Regeneration of dentine/pulp-like tissue using a dental pulp stem cell/poly(lactic-co-glycolic) acid scaffold construct in New Zealand white rabbits. *Aust Endod J*. 2008; 34:52–67. [PubMed: 18666990]
26. Kim NR, Lee DH, Chung PH, Yang HC. Distinct differentiation properties of human dental pulp cells on collagen, gelatin, and chitosan scaffolds. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2009; 108:e94–100. [PubMed: 19836718]
27. Mao JJ, Kim SG, Zhou J, et al. Regenerative endodontics: barriers and strategies for clinical translation. *Dent Clin North Am*. 2012; 56:639–49. [PubMed: 22835543]
28. Valles-Lluch A, Novella-Maestre E, Sancho-Tello M, et al. Mimicking natural dentin using bioactive nanohybrid scaffolds for dentinal tissue engineering. *Tissue Eng Part A*. 2010; 16:2783–93. [PubMed: 20388038]
29. Zhang L, Morsi Y, Wang Y, et al. Review of scaffold design and stem cells for tooth regeneration. *Jpn Dent Sci Rev*. 2013; 49:14–26.
30. Oh S, Brammer KS, Li YS, et al. Stem cell fate dictated solely by altered nanotube dimension. *Proc Natl Acad Sci U S A*. 2009; 106:2130–5. [PubMed: 19179282]
31. Wang P-Y, Hyde L, Thissen H, et al. High-throughput characterisation of osteogenic differentiation of human mesenchymal stem cells using pore size gradients on porous alumina. *Biomaterials Science*. 2013; 1:889–1004.
32. Dalby MJ, Gadegaard N, Tare R, et al. The control of human mesenchymal cell differentiation using nanoscale symmetry and disorder. *Nat Mater*. 2007; 6:997–1003. [PubMed: 17891143]
33. Lee SJ, Choi JS, Park KS, et al. Response of MG63 osteoblast-like cells onto polycarbonate membrane surfaces with different micropore sizes. *Biomaterials*. 2004; 25:4699–707. [PubMed: 15120516]
34. Wang L, Wang ZH, Shen CY, et al. Differentiation of human bone marrow mesenchymal stem cells grown in terpolyesters of 3-hydroxyalkanoates scaffolds into nerve cells. *Biomaterials*. 2010; 31:1691–8. [PubMed: 19962755]

35. Ma PX. Biomimetic materials for tissue engineering. *Adv Drug Deliv Rev.* 2008; 60:184–98. [PubMed: 18045729]
36. Haeri M, Haeri M. ImageJ plugin for analysis of porous scaffolds used in tissue engineering. *JORS.* 2015; 3:e1.
37. Balic A, Aguila HL, Mina M. Identification of cells at early and late stages of polarization during odontoblast differentiation using pOBCol3.6GFP and pOBCol2. 3GFP transgenic mice. *Bone.* 2010; 47:948–58. [PubMed: 20728593]
38. Balic A, Rodgers B, Mina M. Mineralization and expression of Col1a1-3. 6GFP transgene in primary dental pulp culture. *Cells Tissues Organs.* 2009; 189:163–8. [PubMed: 18781059]
39. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{−(Delta Delta C(T))} Method. *Methods.* 2001; 25:402–8. [PubMed: 11846609]
40. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001; 29:e45. [PubMed: 11328886]
41. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc.* 2008; 3:1101–8. [PubMed: 18546601]
42. Rouwkema J, Khademhosseini A. Vascularization and Angiogenesis in Tissue Engineering: Beyond Creating Static Networks. *Trends Biotechnol.* 2016; 34:733–45. [PubMed: 27032730]
43. Demarco FF, Conde MC, Cavalcanti BN, et al. Dental pulp tissue engineering. *Braz Dent J.* 2011; 22:3–13. [PubMed: 21519641]
44. Nozaki T, Takeyasu M, Hirao A, et al. Differentiation of rat dental pulp-derived cells into an osteoblastic lineage. *Oral Sci Int.* 2005; 2:118–25.
45. Takeyasu M, Nozaki T, Watanabe M, et al. In vitro osteogenic differentiation potential of dental pulp stem cells. *J Oral Tissue Eng.* 2004; 2:25–30.
46. Marshall GW Jr, Marshall SJ, Kinney JH, Balooch M. The dentin substrate: structure and properties related to bonding. *J Dent.* 1997; 25:441–58. [PubMed: 9604576]
47. Arana-Chavez VE, Massa LF. Odontoblasts: the cells forming and maintaining dentine. *Int J Biochem Cell Biol.* 2004; 36:1367–73. [PubMed: 15147714]
48. Butler WT, Ritchie H. The nature and functional significance of dentin extracellular matrix proteins. *Int J Dev Biol.* 1995; 39:169–79. [PubMed: 7626404]
49. Papagerakis P, Berdal A, Mesbah M, et al. Investigation of osteocalcin, osteonectin, and dentin sialophosphoprotein in developing human teeth. *Bone.* 2002; 30:377–85. [PubMed: 11856645]
50. Quispe-Salcedo A, Ida-Yonemochi H, Nakatomi M, Ohshima H. Expression patterns of nestin and dentin sialoprotein during dentinogenesis in mice. *Biomed Res.* 2012; 33:119–32. [PubMed: 22572386]
51. Mauth, C., Huwig, A., Graf-Hausner, U., Roulet, JF. Restorative applications for dental pulp therapy. In: Ashammakhi, N.Reis, R., Chiellini, E., editors. *Topics in Tissue Engineering.* 2007.
52. Ma PX, Zhang R. Microtubular architecture of biodegradable polymer scaffolds. *J Biomed Mater Res.* 2001; 56:469–77. [PubMed: 11400124]
53. Lu Q, Simionescu A, Vyavahare N. Novel capillary channel fiber scaffolds for guided tissue engineering. *Acta Biomater.* 2005; 1:607–14. [PubMed: 16701841]
54. Madden LR, Mortisen DJ, Sussman EM, et al. Proangiogenic scaffolds as functional templates for cardiac tissue engineering. *Proc Natl Acad Sci U S A.* 2010; 107:15211–6. [PubMed: 20696917]

Fig. 1.

Architecture of the fabricated PMMA low density (LD), medium density (MD), high density (HD) tubular scaffolds, and planar PMMA control. **(a)** SEM micrographs. Note the presence of larger tubules formed due to collapse of tubule walls in regions where fibers were placed too close to each other (arrows). **(b)** Average projected surface area of tubules (black) and tubule density (gray). Areas of the LD and MD tubules were the same as the size of the sacrificial fibers used in the fabrication process. In HD scaffolds, the combination of several neighboring tubules due to collapse of their thin walls and formation of larger tubules led to a higher average tubule size as well as a broader size distribution (* $p < 0.05$ compared with the LD and MD scaffolds)

Fig. 2.

Surface contact angle of fabricated planar, LD, MD, and HD scaffolds before and after plasma treatment. The presence of tubules, without plasma treatment, raised the contact angle (* $p < 0.05$ compared with the planar control). Plasma treatment lowered the contact angle. Note that for the plasma treated HD scaffold the contact angle dropped to zero

Fig. 3.

Proliferation of dental pulp cells at day 3, 5, and 8 on LD, MD, HD tubular scaffolds and the planar control surface. Fluorescence intensity (arbitrary units) is proportional to cell density. Cell density on the HD scaffold was smaller at the earliest time point compared to the planar control ($*p < 0.05$), but later (day 5 and 8) there was no statistically significant difference among the different surfaces

Fig. 4. SEM micrographs of dental pulp cells cultured on LD, MD, HD tubular scaffolds, and the planar control surface (day 21). Pulp cells tended to bridge over the tubules. In a few cases where the tubules are not completely covered, extension of cell processes into tubules can be seen (arrow). (Scale bars: 20 μ m)

Fig. 5.

(a) Images of dental pulp cells cultured on LD, MD, HD tubular scaffolds and the planar control surface (day 21). Cells were stained to visualize actin cytoskeleton (red) and nestin (green). Nestin expression is observed in polarized elongated cells, which is the characteristic morphological feature of odontoblasts (Scale bar: 50 μ m). (b) Graph of nestin expression on LD, MD, HD tubular scaffolds and the planar control surface. Fluorescence intensity was normalized to the number of cells imaged. (* $p < 0.05$, $n = 1500-2000$)

Fig. 6.

Gene expression profile of dental pulp cells cultured on planar, LD, MD, and HD scaffolds for 21 days. Expression levels of *Dmp1*, *Dspp* and *Ocn* were normalized to those of *Gapdh*. The fold change was calculated relative to expression levels of cells cultured on standard tissue culture plastic. The RNA content of three independent samples for each gene were combined together to provide readable RNA content for the qPCR test

Fig. 7.

Detection of mineralization of dental pulp cells cultured on LD, MD, HD tubular scaffolds, and the planar and TCP control surfaces at 21 days. Fluorescence images from XO staining were overlaid on phase contrast images to reveal mineralization and features of the underlying surface, respectively. Dental pulp cells mineralized poorly on both planar and tubular PMMA scaffolds. On the planar PMMA controls, several clusters of mineralized nodules were observed, while on the tubular scaffolds a few small nodules were present, mainly at the tubules. On TCP, cells formed a mineralized matrix across almost the entire culture surface (Scale bar: 100 μ m)