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Bioactive nanofibers enable the identification of thrombospondin 2 as a key player in enamel regeneration

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ABSTRACT

Tissue regeneration and development involves highly synchronized signals both between cells and with the extracellular environment. Biomaterials can be tuned to mimic specific biological signals and control cell response(s). As a result, these materials can be used as tools to elucidate cell signaling pathways and candidate molecules involved with cellular processes. In this work, we explore enamel-forming cells, ameloblasts, which have a limited regenerative capacity. By exposing undifferentiated cells to a self-assembling matrix bearing RGDS epitopes, we elicited a regenerative signal at will that subsequently led to the identification of thrombospondin 2 (TSP2), an extracellular matrix protein that has not been previously recognized as a key player in enamel development and regeneration. Targeted disruption of the thrombospondin 2 gene (*Thbs2*) resulted in enamel formation with a disordered architecture that was highly susceptible to wear compared to their wild-type counterparts. To test the regenerative capacity, we injected the bioactive matrix into the enamel organ and discovered that the enamel organic epithelial cells in TSP-null mice failed to polarize on the surface of the artificial matrix, greatly reducing integrin β1 and Notch1 expression levels, which represent signaling pathways known to be associated with TSP2. These results suggest TSP2 plays an important role in regulating cell-matrix interactions during enamel formation. Exploiting the signaling pathways activated by biomaterials can provide insight into native signaling mechanisms crucial for tooth development and cell-based strategies for enamel regeneration.

1. Introduction

Unlike other mineralized tissues in the human body, tooth enamel cannot regenerate or repair itself because the enamel forming cells, ameloblasts, undergo apoptosis following tissue maturation. Tooth enamel is also formed during embryogenesis, making it difficult to evaluate biological signals in a concise, temporal manner. Biomaterials provide a unique approach to probe the developmental and regenerative signals for enamel, as they can be designed with peptide epitopes that are known to elicit specific cellular responses and downstream signaling, which may provide insight into biological processes.

Enamel is formed by ameloblast cells through epithelial–mesenchymal interactions mediated by specific cell-based receptors that coordinate the processes of morphogenesis and cytodifferentiation [1–5]. Enamel formation, or amelogenesis, is initiated when the ectoderm-derived inner enamel epithelial cells

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(IEE) exchange signals with neural ectomesenchyme-derived cells resulting in IEE cell elongation, polarization and differentiation to ameloblasts cells that synthesize and secrete the enamel matrix proteins. The two most abundant proteins in the enamel matrix, amelogenin [6,7] and ameloblastin [8], are intrinsically disordered proteins that undergo self-assembly to form a matrix that is guides formation of the carbonated hydroxyapatite (HAP) mineral phase [9–11]. Simultaneously with enamel matrix formation, the ameloblasts also direct enamel protein degradation and resorption resulting in almost complete replacement of the matrix by mineral. This biomineralization strategy produces a hierarchically organized bioceramic enamel tissue with both superior toughness and wear resistance [11–16].

Both cell–cell and cell-matrix signaling pathways act in concert to achieve the hierarchical structure of enamel. Cell surface receptors, particularly integrins, are necessary for mediating interactions between odontogenic epithelial cells and proteins located in the extracellular matrix (ECM) environment [3,17]. A major integrin-binding motif, Arg-Gly-Asp-Ser (RGDS, also referred to as RGD), was originally discovered in fibronectin, an ECM protein present in the basement membrane and essential for the growth and differentiation of enamel-secreting ameloblasts [18,19]. An RGDS-like domain has also been identified in the second most abundant enamel matrix protein, ameloblastin, which is involved in ameloblast cell adhesion to the forming enamel ECM [20–22].

Here we implement a synthetic, artificial ECM of self-assembling peptide amphiphiles displaying the integrin-binding RGDS epitope, which has been shown to provide the necessary signals to induce both differentiation of dental epithelial cells to ameloblasts with the deposition of enamel matrix and conversion to mineral [23,24]. Peptide amphiphiles are small molecules comprised of a hydrophobic alkyl segment covalently conjugated to a hydrophilic peptide head group [25] which, under physiological conditions, self-assemble to form nanofibers mimicking the extracellular matrix. These nanofibers display biological epitopes on their surfaces at high densities and act as a bioactive scaffold to induce a cellular response [26–29]. Previous studies demonstrated that naïve enamel organ epithelial (EOE) cells proliferate and differentiate into ameloblasts in response to an artificial matrix composed of branched RGDS peptide amphiphiles (bRGDS PA), with a pathway mediated through integrin receptor up-regulation [30,31]. Following bRGDS PA-induced differentiation, the cells synthesize and secrete an enamel extracellular matrix that mineralizes to form a regenerated enamel nodule *in vivo* at the site of injection of the bRGDS PA matrix [31]. Analysis of the EOE cell response using quantitative real-time-PCR array identified up-regulated thrombospondin 2 gene (*Thbs2*) expression suggesting that thrombospondin 2 (TSP2) is involved in the interactions with bRGDS PA matrix that promotes the induction of primary EOE cells to ameloblasts to achieve enamel regeneration [32]. Thrombospondin 2 (TSP2) has been found to mediate cell-to-ECM attachment and modulate various cell activities through recognizing growth factors and membrane-bound receptors such as integrins in other tissues [33–36], however its role in enamel development is not well known. In this study, we investigate the roles of TSP2 during enamel regeneration instructed by bRGDS PA artificial matrix and the functional properties of TSP2 during cell–matrix interactions associated with tooth enamel development and biomineralization.

2. Materials and methods

2.1. Peptide amphiphile synthesis

Standard 9-fluorenyl methoxy carbonyl (Fmoc) solid phase peptide synthesis chemistry was employed for the synthesis of branched RGDS peptide amphiphiles (bRGDS PA) as described in previous studies [25,31,37]. A 4-methyltrityl (Mtt) protecting group from the ε-amine of a lysine residue was removed and palmitic acid was then attached to the resulting free amine. Using a similar method, the RGDS sequence was coupled to the ε-amine of a lysine side chain to achieve the branched architecture [37]. Fmoc deprotection was performed using 30% piperidine in dimethylformamide (DMF) twice for 10 min each time. Amino acid and palmitic acid coupling reactions were performed with a mixture of 4 molar equivalents of protected amino acid or palmitic acid, 3.95 equivalents of 2-(1H-benzotriazol-1-yl)-1,1,2,2-tetramethyluronium hexafluorophosphate (HBTU) and 6 equivalents of diisopropylethylamine (DIEA) in a solvent mixture of 50% DMF, 25% dichloromethane (DCM) and 25% N-methyl pyrrolidine (NMP) for a minimum of 1 h. Kaiser tests were performed following amino acid and palmitic acid couplings to confirm a negative result for the presence of free amines. If necessary, the coupling was repeated until the test gave a negative result. Molecules were cleaved from the resin and protecting groups removed using a mixture of 92.5% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS), 2.5% 1-2 ethanedithiol (EDT) and 2.5% water for a minimum of 3 h. Excess TFA and scavengers were removed by rotary evaporation and the remaining solution was triturated with cold diethyl ether to form a white precipitate. MBHA Rink amide resin, Fmoc protected amino acids, Boc-Lys (Boc)-OH and Fmoc-Lys (Mtt)-OH and HBTU were purchased from EMD Chemicals, Inc.; all other reagents were purchased from VWR.

All molecules were purified using reversed-phase high-performance liquid chromatography (HPLC) on a Varian Prostar Model 210 preparative scale HPLC system equipped with a Phenomenex Jupiter Proteo column (C_{12} stationary phase, 10 μm, 90 Å, 30 × 150 mm). Purified fractions were characterized by electrospray ionization mass spectrometry (ESI-MS) using an Agilent 6510 Quadrupole Time-of-Flight (Q-Tof) instrument. The lyophilized molecules were stored at −20 °C before use. Scrambled bRGDS peptide amphiphiles (ScrRGDS PA) were synthesized and used as control nanofibers containing a non-signaling scrambled peptide epitope.

2.2. Real-time PCR

For detection of amelogenin (*Amelx*) and thrombospondin 2 gene (*Thbs2*) expression in response to bRGDS PA matrix, primary EOE cells from newborn wild-type mouse mandibular incisors were cultured on tissue culture plates or treated with 1% bRGDS PA matrix for 4 h. For characterizing the effects of *Thbs2 gene disruption on the expression of* amelogenin (*Amelx*), integrin β1 (*Itgb1*) and Notch1 (*Notch1*) *genes* in primary EOE cells in response to different substrates, EOE cells from newborn $Thbs2^{+/+}$or $Thsp2^{-/-}$ mouse mandibular incisors were separately cultured on tissue culture plates or 1% bRGDS PA matrix. Total RNA was extracted using the RNA-Bee reagent (TEL-TEST, Friendswood, TX). First strand cDNA was synthesized with 100 ng random oligodeoxynucleotide decamers (Ambion, Austin, TX). Real-time PCR was performed with a C-1000 thermal cycler CFX96 real-time PCR detection system (Bio-Rad). Primer sequences for each target gene were as follows: for *Amelx*, the forward primer was 5′-GGGGA CCTGG ATTTT GTTTG-3′, and the reverse primer was 5′-AACCA TAGGA AGGAT ACGGC TG-3′; for β-actin (*Actb*), used as an internal control, the forward primer was 5′-GGGAA ATCGT GCGTG ACATC-3′ and the reverse primer was 5′-GCGGC AGTGG CCATC TC-3′ [38,39]; for *Itgb1*, the forward primer was 5′-AGACT TCCGC ATTGG CTTTG -3′, and the reverse primer was 5′-GGCTG GTGCA GTTTT GTTCA-3’; for *Notch1*, the forward primer was 5′-GATGG CCTCA ATGGG TACAA G-3′, and the