Odontogenic Induction of Dental Stem Cells by Extracellular Matrix-Inspired Three-Dimensional Scaffold

Sriram Ravindran, PhD, Youbin Zhang, PhD, Chun-Chieh Huang, MS, and Anne George, PhD

Currently, root canal therapy is the only clinical treatment available to treat damaged or necrotic dental pulp tissue arising from caries. This treatment results in the loss of tooth vitality. Somatic dental stem cell-based tissue engineering approaches can alleviate this problem by preserving tooth vitality. Dental stem cells are multipotent and under appropriate conditions could be used for dental pulp tissue engineering. Successful use of these cells in pulp repair requires a combination of growth factors and appropriate scaffolds to induce cell differentiation. In this study, we demonstrate the odontogenic differentiation of human dental pulp stem cells (DPSCs) and the human periodontal ligament stem cells when cultured on a decellularized 3D extracellular matrix (ECM) scaffold without the need for exogenous addition of growth factors. Subcutaneous implantation of the ECM scaffolds containing DPSCs showed the formation of dental pulp-like tissue with cells expressing dentin sialoprotein (DSP) and dentin phosphophoryn (DPP). Additionally, we also show that the ECM scaffold can be exploited as a tool to study the extracellular function of multifunctional proteins. These promising results demonstrate the feasibility of developing these biomimetic scaffolds for treatment of dental caries.

Introduction

DENTAL CARIES is one of the most prevalent dental disease and a chronic disease in children aged 5–17.¹ According to the World Health Organization (WHO) report from 2003, approximately 90% of the world's population has experienced dental caries. Apart from poor oral hygiene, treatments like chemotherapy and radiation therapy also contribute toward formation of caries. The conventional treatment for pulp inflammation due to dental caries is root canal therapy. Approximately 24 million root canals are performed annually in the United States alone. This therapy involves the complete removal of the infected pulp followed by disinfection and filling up of the chamber with a trioxide component such as mineral trioxide culminating in capping up with a crown. The problem with this approach lies in the removal of the pulp. The pulp tissue offers vitality, sensitivity, and regenerative ability to the tooth. All of these properties are lost as a result of root canal therapy, which in many cases leads to secondary infections. As tooth sensitivity is lost, the secondary infections go unnoticed, until the infection spreads to the surrounding tissues. This condition can, in some cases, lead to sepsis and other serious complications leading to a significant reduction in the quality of life of the patient.

Tissue engineering is a promising therapy to regenerate dental tissues and can provide an excellent replacement for root canal therapy. Two types of approaches exist in the

current regenerative dental research to restore normal function to damaged tissue or missing teeth. One is the engineering of individual components such as the dental pulp,^{2,3} the periodontal ligament⁴ and alveolar bone⁵ and the other is engineering the entire functional tooth.⁶ In the current study, we have focused on dental pulp tissue engineering using somatic dental stem cells.

Several different dental stem cell sources have been investigated for regenerating the dental pulp. Of these, the most common ones are dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), and stem cells from human exfoliated deciduous teeth (SHED).⁷ Although SHED have shown promise for banking and to be useful for tissue engineering applications,^{8–12} in terms of immediate clinical relevance, the DPSCs and PDLSCs are well suited for engineering the dental pulp as they can be isolated from an adult patient. The multipotent ability of these stem cells has been well documented.^{13–15} However, to trigger the differentiation of these stem cells into the required lineage for tissue engineering purposes, a combination of growth factors and differentiation agents is required.

In vivo, the extracellular matrix (ECM) dictates cellular proliferation, migration, and differentiation.¹⁶ Mimicking the complexity of the ECM will result in cellular environments favorable for lineage-specific differentiation of stem cells.^{17–22} In the present study, the possibility of utilizing odontoblast ECM embedded type I collagen/chitosan scaffolds for dental pulp tissue engineering using DPSCs and PDLSCs was

investigated. Type I collagen is the most abundant protein in the human body and it forms a major ECM component of the dental pulp tissue. Additionally, hydroxyapatite nucleation occurs on the type I collagen framework. For these reasons, we chose type I collagen as one of the components of the starting scaffold matrix. On the other hand, chitosan is a naturally occurring biocompatible biopolymer that has antimicrobial properties.²³ We have discussed the use of type I collagen and chitosan as biomaterials in our published review article.²⁴ We have shown that this blend is suitable for mesenchymal stem cell culture and for the generation of ECM integrated scaffolds.^{18,25} Finally, we have investigated the ability of odontogenic ECM scaffolds to be used as a tool to decipher the extracellular roles of multifunctional proteins.

Materials and Methods

Cell culture

The human DPSCs and the human PDLSCs were used in this study. Both cell types were kindly provided by Dr. Songtao Shi (University of Southern California). Both cell types were cultured in the α MEM (GIBCO) with 20% FBS (GIBCO), 1% L-glutamine (GIBCO), and 1% antibiotic and antimycotic solution (anti-anti; GIBCO).

Generation of ECM scaffolds

The ECM scaffolds were generated using previously published protocols.¹⁸ Briefly, DPSCs (2 million cells/mL) were cultured in a 1:1 collagen/chitosan hydrogel for 2 weeks in the presence of differentiation media. After this, the cells were lysed followed by DNA digestion resulting in an ECM embedded collagen/chitosan scaffold.

In vitro differentiation of DPSCs and PDLSCs

DPSCs and PDLSCs (1 million cells/scaffold) were cultured on the ECM scaffolds in the presence of growth media without any additional supplements for a period of 2 weeks or 4 weeks. RNA from the cells was isolated according to published protocol¹⁸ followed by cDNA synthesis. The osteogenesis real-time PCR array (Qiagen) was used to analyze changes in the expression levels involved in odontogenic differentiation of stem cells. When required, the ECM scaffolds were incubated overnight at 4°C in a 500 μ L solution of DPP antibody (1/100 dilution). Before cell seeding and gene expression analysis, the scaffolds were washed extensively to remove nonspecifically bound antibodies. Scaffolds incubated similarly with preimmune serum served as controls.

Statistical analysis

The data were expressed as fold change in gene expression in cells cultured in the ECM scaffold with respect to the control scaffold. Statistical significance was calculated using the software package provided by the manufacturer and is expressed as a *p* value obtained from the Student's *t*-test.

In vivo implantation experiments

All experiments were performed according to approved UIC animal care protocols (Assurance No: A3460-01). Triplicate control (collagen/chitosan scaffolds without the ECM) and ECM scaffolds were implanted subcutaneously on

the back of 1-month-old male nude mice (Charles River laboratories) for 2 weeks. Each mouse contained one control scaffold and one ECM scaffold on either side of the spine. After 2 weeks, the animals were euthanized followed by extraction of the scaffolds. The scaffolds were fixed in formalin, paraffin embedded, and sectioned into 5- μ m-thick sections for histology and immunohistochemistry according to published protocol.²⁵

Histology

Tissue sections were deparaffinized in xylene, hydrated in graded ethanol solutions, and stained with hematoxylin and eosin (H&E) stains and Alizarin Red as per standard published protocols.²⁵ The stained sections were imaged using a Zeiss Axiovert inverted microscope equipped with the axiovision imaging software. To observe the autofluorescence from red blood corpuscles (RBCs), the sections were imaged using TRITC red filter sets. Collagen autofluorescence was observed by imaging the samples using the green fluorescent protein (GFP) filter sets.

Immunohistochemistry

All sections were deparaffinized in xylene, hydrated in graded ethanol solutions, and immunostained according to published protocols.^{18,25} The following antibodies were used: mouse anti-Tubulin antibody (1/1000; Sigma), rabbit anti-Fibronectin antibody (1/200; Sigma), mouse anti-bone morphogenetic protein 2 (BMP2) antibody (1/100; BD Biosciences), rabbit anti-transforming growth factor β (TGF β) antibody (1/100; Santacruz biotechnology), rabbit anti-VEGF antibody (1/100; Santacruz biotechnology), mouse anti-PEDF antibody (1/100; Millipore), rabbit anti-MMP2 antibody (1/100; Santacruz), rabbit anti-MMP9 antibody (1/100; Santacruz), mouse anti-phospho serine and phosphor tyrosine antibodies (1/100; Sigma), mouse anti-C-DMP1 antibody (1/2000; gift from Dr. Qin, University of Texas), mouse anti-Thrombospondin antibody (1/100; Sigma), rabbit anti-dentin sialoprotein (DSP) and anti-dentin phosphophoryn (DPP) antibodies (1/250; characterized in our laboratory,²⁶), and mouse anti-von Willebrand factor antibody (1/100; Santacruz biotechnology). For all immunohistochemistry experiments with fluorescent secondary antibody labeling, anti-mouse FITC (1/100; Sigma) and anti-rabbit TRITC (1/100; Sigma) antibodies were used. All fluorescently immunostained sections were imaged using a Zeiss LSM 710 confocal microscope equipped with Zen image analysis software.

In vitro nucleation

In vitro nucleation was performed as per previously published protocol.¹⁸ The nucleated samples were dehydrated, coated with osmium tetroxide, and imaged using a LEO Gemini 1525 scanning electron microscope. Energy dispersive X-ray (EDX) analysis was also performed using the same instrument to obtain the relative amounts of calcium and phosphorus in the samples. Transmission electron microscopy (TEM) was used to perform electron diffraction analysis on the nucleated samples. Briefly, the samples were dried, crushed, loaded onto TEM grids, and imaged using a Joel 3010EX transmission electron microscope.

Results

Characterization of the ECM scaffold

Fluorescent immunohistochemical analysis for several ECM proteins was performed to characterize the scaffold. Figure 1 shows representative confocal images of ECM scaffold sections stained with various antibodies. Tubulin served as a negative control for the intracellular protein (Fig. 1B) and fibronectin served as the positive control for the ECM protein (Fig. 1A). DAPI nuclear staining was performed to ensure the absence of residual DNA from lysed cells (Fig. 1C). Sections stained with the FITC-labeled mouse secondary antibody and TRITC-labeled rabbit secondary antibody alone served as

controls for secondary antibody nonspecific binding (Fig. 1D). The ECM scaffold stained positive for growth factors such as BMP2, TGF β , VEGF, and PEDF (Fig. 1E–H), metalloproteases such as MMP2 and MMP9 (Fig. 1I, J), phosphorylated proteins (Fig. 1K, L), noncollagenous proteins such as DMP1, thrombospondin (Fig. 1M, N), and pulp-specific noncollagenous proteins DSP and DPP (Fig. 1O, P).

In vitro hydroxyapatite nucleation

Dental pulp cells have the ability to differentiate into odontoblasts and form reparative dentin. This function implies that the secreted matrix possesses that ability to

FIG. 1. Expression of extracellular matrix (ECM) proteins: (A–P) are representative confocal micrographs showing the expression of various ECM proteins in the ECM scaffold sections. Fibronectin (A) was used as a positive control and Tubulin (B) was used as a negative control for intracellular protein. DAPI staining (C) was performed to rule out the presence of DNA material in the ECM scaffold. (D) shows the absence of rabbit and mouse nonspecific secondary antibodies. BMP2, bone morphogenetic protein 2; TGF β , transforming growth factor beta; VEGF, vascular endothelial growth factor; MMP, matrix metalloprotease. Color images available online at www.liebertpub.com/tea

FIG. 2. *In vitro* nucleation: (A) represents nucleation of amorphous calcium phosphate polymorphs in the control collagen/chitosan scaffold. (A1) shows the energy dispersive X-ray (EDX) analysis indicating the presence of calcium and phosphorus ions (arrows point to calcium and phosphorus peaks) along with the Ca to P ratio. (A2) shows electron diffraction data from the nucleated control scaffold indicating the presence of amorphous calcium phosphate. (B) represents nucleation of crystalline hydroxyapatite in the ECM scaffold. (B1) represents the corresponding EDX analysis showing the presence of calcium and phosphorus ions (arrows point to calcium and phosphorus peaks) and the Ca to P ratio. (B2) shows the electron diffraction data from the nucleated ECM scaffold indicating the presence of crystalline hydroxyapatite. Color images available online at www.liebertpub.com/tea

nucleate hydroxyapatite. Results from *in vitro* nucleation assays showed that the ECM was able to nucleate crystalline hydroxyapatite (Fig. 2B). The control scaffold (devoid of embedded ECM) only nucleated amorphous calcium phosphate (Fig. 2A). EDX analysis indicated that the ratio of calcium to phosphate in the nucleated ECM scaffolds was 1.59 ($n=5$, SEM=0.069) indicating the presence of crystalline hydroxyapatite (Fig. 2B1). This observation was confirmed using electron diffraction analysis that showed the presence of crystalline hydroxyapatite (Fig. 2B2). On the other hand, the ratio of calcium to phosphate in the nucleated control scaffolds was 1.31 ($n=5$, SEM 0.013) indicating the presence of amorphous calcium phosphate (Fig. 2A1, A2).

Differentiation of DPSCs and PDLSCs on the ECM scaffolds

DPSCs and PDLSCs were cultured on the ECM scaffold and the control scaffold for a period of 2 weeks in the presence of standard growth media. Real-time PCR was performed to analyze gene expression profiles of key genes

involved in odontoblastic differentiation of stem cells. Table 1 provides a list of genes significantly regulated in DPSCs after 2 weeks of culture. They have been categorized according to functionality and the data show change in gene expression on the ECM scaffold compared to the control scaffold. It is evident from the table that several growth factors, transcription factors, ECM proteins, and receptors were significantly regulated as a result of culture on the ECM scaffold. Tables 2 and 3 provide a similar list of genes significantly altered with respect to the control scaffold when PDLSCs were cultured within the ECM scaffold for a period of 2 and 4 weeks, respectively. The PDLSCs showed significant positive regulation of odontoblast-specific genes.

The ECM scaffold can be used to identify the extracellular function of multifunctional proteins

There are several proteins involved in the cellular machinery that perform multiple functions both intra- and extracellularly depending on the biological scenario. One such protein is dentin phosphophoryn. The extracellular role of

TABLE 1. SHOWING A LIST OF GENES REGULATED WHEN DPSCs WERE CULTURED FOR 2 WEEKS IN GROWTH MEDIA IN THE ECM SCAFFOLDS WITH OR WITHOUT DPP BLOCKING AS OPPOSED TO THE CONTROL SCAFFOLD

Genes	Description	Fold change (p-value from t-test)	
		ECM	ECM+pp block
	Growth factors		
<u>BMP2</u>	Bone morphogenetic protein 2	3.2 (0.019)	2.48 (0.065)
<u>BMP4</u>	Bone morphogenetic protein 4	9.48 (0.001)	5.24 (0.03)
<u>BMP5</u>	Bone morphogenetic protein 5	19.89 (0.0002)	9.86 (0.092)
<u>FGF1</u>	Fibroblast growth factor 1	-3.84 (0.0001)	-3.84 (0.0004)
<u>FGF2</u>	Fibroblast growth factor 2	-1.96 (0.003)	-1.63 (0.017)
<u>IGF2</u>	Insulin-like growth factor 2	22.11 (0.017)	9.90 (0.003)
<u>TGFB1</u>	Transforming growth factor beta 1	1.57 (0.02)	0.98
<u>TGFB2</u>	Transforming growth factor beta 2	-1.91 (0.001)	-1.56 (0.001)
<u>VEGFA</u>	Vascular endothelial growth factor A	5.45 (0.0007)	5.63 (0.002)
<u>VEGFB</u>	Vascular endothelial growth factor B	2.30 (0.002)	1.67 (0.065)
	Transcription factors		
<u>RUNX2</u>	Runt-related transcription factor 2	1.53 (0.07)	1.08
<u>NFKB1</u>	Nuclear factor kappa of B-cells 1	-2.25 (0.001)	-2.32 (0.001)
<u>SMAD2</u>	SMAD family member 2	-5.5 (0.001)	-4.9 (0.002)
<u>SMAD4</u>	SMAD family member 4	-1.51 (0.04)	-1.73 (0.01)
<u>TWIST1</u>	Twist homolog 1	2.9 (0.003)	2.24 (0.003)
	ECM proteins		
<u>BGN</u>	Biglycan	10.75 (0.000023)	3.53 (0.23)
<u>COL10A1</u>	Type X collagen	-1.85 (0.008)	-1.02 (0.94)
<u>COL1A1</u>	Type I collagen	5.45 (0.05)	2.6 (0.29)
	Proteases		
<u>BMP1</u>	Bone morphogenetic protein 1	-3.83 (0.0007)	-5.12 (0.0006)
<u>MMP10</u>	Matrix metalloproteinase 10	30.65 (0.0001)	20.68 (0.016)
<u>MMP2</u>	Matrix metalloproteinase 2	4.34 (0.005)	3.78 (0.018)
	Adhesion molecules		
<u>CDH11</u>	Cadherin 11	1.71 (0.008)	1.02
<u>ITGA1</u>	Integrin alpha 1	-2.78 (0.0003)	-3.29 (0.0002)
<u>ITGA2</u>	Integrin alpha 2	-16.23 (0.0001)	-8.36 (0.0005)
<u>ITGB1</u>	Integrin beta 1	-8.33 (0.0001)	-7.57 (0.0002)
<u>VCAM1</u>	Vascular cell adhesion molecule 1	-1.5 (0.05)	-2.01 (0.03)
	Receptors		
<u>IGF1R</u>	Insulin-like growth factor 1 receptor	-1.84 (0.006)	-2.6 (0.005)
	Other		
<u>ACTB</u>	β Actin	3.6 (0.00008)	2.19 (0.09)

Negative sign under fold change represents downregulation. *p*-Value was calculated using the Student's *t*-test. Underlined genes show differential expression with DPP blocking.

DPSCs, dental pulp stem cells; ECM, extracellular matrix.

DPP in nucleating hydroxyapatite is well established.²⁷ However, the extracellular signaling function of DPP is not well defined. We attempted to isolate the extracellular signaling role of DPP by blocking DPP in the ECM scaffold by means of antibody treatment. The last column in Table 1 gives the list of genes that were regulated during differentiation of DPSCs on the ECM scaffolds that were blocked with the DPP antibody. The underlined genes in Table 1 were the ones that were significantly affected as a result of DPP blocking. It is evident from the table that DPP blocking resulted in reduced growth factor production, downregulation of Runx2, and reduced type I collagen synthesis. No significant change was observed when the scaffolds were blocked with rabbit IgG control.

ECM scaffold permits neovascularization in vivo

The control and ECM scaffolds were implanted subcutaneously in male nude mice for a period of 2 weeks. Neovascularization was evident only in the ECM scaffolds. The

red fluorescent images of H&E stained sections showing non-nucleated RBCs (Fig. 3A, B) demonstrate this clearly. The green fluorescent images in Figure 3A and B are from collagen autofluorescence in the green channel. A stronger fluorescence signal from the ECM scaffold suggests increased collagen synthesis. Immunohistochemistry was performed with the anti-von Willebrand factor antibody to look for the presence of endothelial cells within the control and ECM scaffolds. Results presented in Figure 3A and B show the presence of endothelial cells within the ECM scaffolds forming a capillary network (black arrows, last panel in Fig. 3B). However, in the control scaffolds, endothelial cells were observed only in the periphery (black arrow, last panel in Fig. 3A). No capillary-like structures were observed in these scaffolds.

ECM scaffold induces increased calcium deposition and polarization of collagen fibrils in vivo

Sections from the implanted control and ECM scaffolds were stained with Alizarin Red to look for calcium deposition.

TABLE 2. SHOWING A LIST OF GENES REGULATED WHEN PDLSCs WERE CULTURED FOR 2 WEEKS IN GROWTH MEDIA IN THE ECM SCAFFOLDS

Gene symbol	Description	Fold change	p-Value (t-test)
	Growth factors		
BMP2	Bone morphogenetic protein 2	10.11	0.064
BMP5	Bone morphogenetic protein 5	1.94	0.096
IGF1	Insulin-like growth factor 1	2.71	0.0146
PDGFA	Platelet-derived growth factor A	1.84	0.0728
TGFβ1	Transforming growth factor beta 1	2.15	0.0091
TGFβ2	Transforming growth factor beta 2	1.41	0.0674
TGFβ3	Transforming growth factor beta 3	1.97	0.0065
VEGFA	Vascular endothelial growth factor A	2.05	0.0115
	Transcription factors		
RUNX 2	Runt-related transcription factor 2	1.87	0.0575
SOX 9	(Sex determining region Y)-box 9	4.60	0.0123
VDR	Vitamin D receptor	2.96	0.0135
	ECM proteins		
AMELY	Amelogenin	3.11	0.0022
BGN	Biglycan	2.91	0.061
COL5A1	Type V collagen	2.50	0.0407
COMP	Cartilage oligomeric matrix protein	3.92	0.0519
FN1	Fibronectin	1.76	0.0665
	Adhesion molecules		
ICAM1	Intercellular adhesion molecule 1	4.02	0.0461
ITGA2	Integrin alpha 2	2.36	0.0010
ITGA3	Integrin alpha 3	1.97	0.0019
CDH11	Cadherin 11	2.06	0.0127
	Phosphatases		
MMP2	Matrix metalloprotease 2	2.89	0.0095
	Other		
TFIP11	Tuftelin interactin protein 11	1.72	0.0618

p-Value was calculated using the Student's *t*-test.
PDLSC, periodontal ligament stem cells.

Results indicated that the ECM scaffold triggered increased extracellular calcium deposition (Fig. 4C) when compared with the control scaffold (Fig. 4A). Collagen fibril orientation serves as a template for mineral deposition. When the sections were viewed under polarized light to visualize collagen fibril orientation, we could observe polarized collagen fibrils on the edges of the ECM scaffold (Fig. 4D). Only randomly oriented collagen fibrils were observed in the control scaffold (Fig. 4A). This result indicated that the differentiating DPSCs in the ECM scaffold triggered collagen synthesis and organization.

In vivo expression of DMP1, DSP, and DPP

We analyzed the expression pattern of DMP1, DSP, and DPP in the control and ECM implant sections. Immunohistochemical data showed a slight increase in the expression of DMP1 (Fig. 5A, B). However, a marked increase in the expression of DSP and DPP was observed in the ECM scaffold compared to the control scaffold (Fig. 5A, B). Tubulin levels remained constant in both scaffolds and served as a positive control (Fig. 4A, B). No staining was observed in the sections treated with the TRITC rabbit and FITC mouse secondary antibodies alone and served as a negative control (Fig. 4C). The laser intensities and imaging parameters were maintained constant.

Discussion

ECM-based scaffolds mimic the composition of the native tissue. In the present study, we have developed an odonto-

blast-specific ECM scaffold to promote odontogenic differentiation of two different multipotent adult dental stem cells, namely, DPSCs and PDLSCs.^{13,15} These cell-generated ECM-based scaffolds were first decellularized before analysis. Characterization of the ECM scaffolds by immunostaining showed that it contained a rich source of matrix proteins and matrix metalloproteases (MMPs), growth factors such as BMP2, TGF β, VEGF, and PEDF. Immunostaining with anti-p serine and p-tyrosine antibodies demonstrated the presence of several phosphorylated proteins in the ECM scaffold. It is well established that phosphorylated proteins play a key role in the mineralization process and serve as phosphorus donors for the nucleation of calcium phosphate polymorphs.²⁸ Therefore, this scaffold could mimic the *in vivo* scenario of odontogenic cells and initiate calcium phosphate nucleation.

Three major noncollagenous proteins synthesized by the odontoblasts are DMP1, DSP, and DPP. The presence of these three proteins in the scaffold was essential to provide bioactive cues for early and terminal differentiation of the dental stem cells. Other matrix proteins that were identified were thrombospondin and fibronectin. Although this is not an exhaustive list of proteins present in the ECM, it provides glimpses into the rich bioactive composition of the ECM scaffold. We had published earlier on the composition of the osteogenic scaffold and shown that DPP and DSP were not present in detectable amounts.¹⁸ This observation confirmed published reports on the amount of DSP and DPP synthesized by osteoblasts.²⁹ Other noted differences in the

TABLE 3. SHOWING A LIST OF GENES REGULATED WHEN PDLSCs WERE CULTURED FOR 4 WEEKS IN GROWTH MEDIA IN THE ECM SCAFFOLDS

Gene symbol	Description	Fold change	p-Value (t-test)
	Growth factors		
BMP2	Bone morphogenetic protein 2	2.64	0.102
BMP5	Bone morphogenetic protein 5	2.69	0.032
CSF3	Colony stimulating factor 3	2.03	0.073
FGF2	Fibroblast growth factor 2	-2.23	0.004
IGF2	Insulin-like growth factor 2	-3.4	0.043
TGF β 2	Transforming growth factor beta 2	1.72	0.001
TGF β 3	Transforming growth factor beta 3	2.09	0.051
VEGFA	Vascular endothelial growth factor A	1.65	0.162
	Transcription factors		
RUNX 2	Runt-related transcription factor 2	1.92	0.0051
SMAD1	SMAD family member 1	1.33	0.011
SMAD2	SMAD family member 2	1.22	0.0033
SMAD3	SMAD family member 3	1.43	0.075
SMAD4	SMAD family member 4	1.54	0.002
SOX9	Sex determining region Y)-box 9	1.87	0.25
VDR	Vitamin D receptor	1.7	0.0204
	ECM proteins		
COL12A1	Type XII Collagen	1.72	0.0065
COL14A1	Type XIV Collagen	1.72	0.0005
COL1A1	Type I Collagen	1.81	0.17
COL5A1	Type V Collagen	1.62	0.090
COMP	Cartilage oligomeric matrix protein	2.27	0.14
DSPP	Dentin sialo phosphoprotein	3.32	0.011
FN1	Fibronectin	1.55	0.043
	Adhesion molecules		
ITGA1	Integrin alpha 1	1.53	0.042
ITGA2	Integrin alpha 2	1.57	0.009
ITGAM	Integrin alpha M	3.32	0.034
CDH11	Cadherin 11	1.81	0.019
	Phosphatases		
MMP10	Matrix metalloprotease 10	3.14	0.005
MMP8	Matrix metalloprotease 8	2.37	0.2221
	Receptors		
CD36	CD36 molecule	-1.91	0.03

p-Value was calculated using the Student's *t*-test.

composition of the ECM scaffold were the levels of TGF β . Low levels of TGF β were present in the odontogenic matrix when compared with the osteogenic matrix. These differences show that although osteoblasts and odontoblasts synthesize a calcifying organic matrix, their compositions are markedly different. More detailed studies are required to quantitatively assess the difference in the levels of ECM proteins synthesized by the osteoblasts and odontoblasts and our future efforts will be dedicated to explore this possibility.

One of the important functions of the dental pulp is to provide regenerative potential to compensate for lost dentin by enabling the differentiation of pulp cells into odontoblasts capable of synthesizing a calcifying matrix. The odontogenic ECM scaffold used in this study had the potential to induce nucleation of hydroxyapatite crystals. Under the same conditions, the control collagen/chitosan scaffold could only induce deposition of amorphous calcium phosphate polymorphs. When implanted subcutaneously in mice, the proteins in the ECM scaffold could trigger the deposition of oriented collagen fibers. The capability of the ECM scaffolds to dictate deposition of oriented collagen fibers and hydroxyapatite suggests the possibility of using such scaffolds

for the formation of a reparative dentin bridge in clinical settings.

To understand the mechanism by which the ECM scaffold facilitated the odontogenic differentiation of DPSCs, gene expression analysis was performed. Results from the osteogenic PCR array show that several growth factors, transcription factors, MMPs, and cell surface receptors, which are required for odontogenic differentiation, were upregulated, while others were downregulated when DPSCs were cultured within the 3D ECM scaffolds. Analysis of the scaffolds after subcutaneous implantation showed the formation of dental pulp-like tissue with cells expressing DSP and DPP. Additionally, the ECM scaffold promoted neovascularization and increased collagen deposition. Differentiation of PDLSCs on the odontogenic scaffold was also interesting. PDLSCs have to be potential to differentiate into osteogenic, odontogenic, or cementogenic lineage depending on the biological cues. Results from the PCR array showed that the PDLSCs could differentiate into odontogenic lineage when cultured on the 3D ECM scaffold. Two genes of interest that were upregulated and served as markers for odontogenic differentiation were amelogenin and *DSPP*. *DSPP* is a well-known

FIG. 3. H&E staining and von Willebrand factor immunohistochemistry (IHC) of subcutaneous implant sections: **(A)** is a representative micrograph of an H&E stained section from the control scaffold after 2 weeks of subcutaneous implantation in nude mice imaged under brightfield, red fluorescence (autofluorescence from the red blood corpuscles (RBCs) and eosin stained areas) and green fluorescence (autofluorescence from the collagen) channels. The last panel in **(A)** shows the presence of endothelial cells stained with the von Willebrand factor antibody present in the periphery of the scaffold (black arrow). **(B)** is a similar imaging of an H&E stained section from the ECM scaffold. The arrows in **(B)** represent RBCs from the blood vessels. Also, note the increased signal in the green channel indicating increased collagen presence. The last panel in **(B)** shows the von Willebrand factor stained endothelial cells within the ECM scaffold forming capillary-like structures. All imaging parameters were maintained constant. Color images available online at www.liebertpub.com/tea

marker of odontogenic differentiation and published reports show that amelogenin is expressed by odontoblasts and pulp cells.^{30–33} Expression of DSPP and amelogenin were not observed during osteogenic differentiation of mesenchymal stem cells.¹⁸ These results conclusively demonstrate the odontogenic potential of the ECM scaffold to drive lineage-specific differentiation of somatic dental stem cells.

Apart from its ability to induce lineage-specific differentiation and serve as a scaffold for tissue regeneration, the ECM scaffold can function as a biomimetic platform to analyze the extracellular function of multifunctional proteins. The cell produces and secretes several proteins that possess both intracellular and extracellular functions. Over the years, several such proteins have been identified. Many of the

FIG. 4. Alizarin Red staining and collagen orientation in implant sections: **(A, C)** show representative images of Alizarin Red stained sections from control **(A)** and ECM scaffold **(C)** implant sections. Note the increased calcium deposition in **(C)**. The arrow in **(C)** represents increased staining observed at the periphery. **(B, D)** show representative images of collagen fibril orientation in control **(B)** and ECM scaffold **(D)** implant sections. Note the presence of polarized collagen at the periphery of the scaffold in **(D)** (arrow in 4D) and its absence in **(B)**. Color images available online at www.liebertpub.com/tea

FIG. 5. Immunofluorescent staining of sections from subcutaneous implants: **(A, B)** are representative confocal micrographs showing the expression of DMP1, DSP, Tubulin, and DPP in the control **(A)** and ECM scaffolds **(B)** implanted with DPSCs subcutaneously in nude mice. Note the markedly increased expression of DSP and DPP in the ECM scaffold sections. **(C)** shows negative staining from the FITC-conjugated mouse and TRITC-conjugated rabbit secondary antibodies. All imaging parameters were maintained constant. DSP, dentin sialoprotein; DPP, dentin phosphophoryn. Color images available online at www.liebertpub.com/tea

NCPs, such as DMP1 and DPP, are multifunctional.^{34–38} Recently, we identified that the endoplasmic reticulum chaperone protein GRP-78 plays a role in mineralized matrix formation.^{39,40} As proof of concept, we sought to identify the extracellular function of DPP during odontogenic differentiation of DPSCs. Thus far, all of the studies performed to analyze the role of DPP in odontoblast differentiation have been performed on 2D cultures and isolated systems where there is minimal involvement from other ECM proteins. We used a loss of function approach by blocking the extracellular DPP in the ECM scaffold using the anti-DPP antibody followed by differentiating DPSCs within the ECM scaffold. Fold change in gene expression with respect to control was calculated for the unblocked and blocked ECM scaffolds. Results indicated that the expression of some genes was significantly influenced as a result of DPP blocking. Specifically, a significant reduction in the expression levels of growth factors *BMP4*, *5*, *IGF1*, and *TGFβ1* was observed. This could be attributed to the downregulation of *RUNX2* and ECM protein biglycan and ultimately reduction in type I collagen synthesis all of which are evident in the gene expression data. Highlights from this analysis indicate that DPP influenced BMP and TGFβ signaling pathways and had minimum influence on FGF signaling and expression of *VEGF* and its downstream effectors. This data are novel, physiologically relevant, and cannot be obtained using conventional methods. Our future efforts will be directed toward understanding this process in a more detailed manner to better understand the functional complexities of ECM-mediated signaling enabling better biomimetic designs for tissue engineering purposes.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Anne George, PhD

Department of Oral Biology

College of Dentistry

University of Illinois at Chicago

Chicago, IL 60612

E-mail: anneg@uic.edu

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