Nonmulberry Silk Braids Direct Terminal Osteocytic Differentiation through Activation of Wnt-Signaling

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ABSTRACT: Silk polymers can regulate osteogenesis by mimicking some features of the extracellular matrix of bone and facilitate mineralized deposition on their surface by cultured osteoprogenitors. However, terminal differentiation of these mineralizing osteoblasts into osteocytic phenotypes has not yet been demonstrated on silk. Therefore, in this study we test the hypothesis that flat braids of natively (nonregenerated) spun nonmulberry silk A. mylitta, possessing mechanical stiffness in the range of trabecular bone, can regulate osteocytic differentiation within their 3D microenvironment. We seeded human preosteoblasts onto these braids and cultured them under varied temperatures (33.5 and 39 °C), soluble factors (dexamethasone, ascorbic acid, and β-glycerophosphate), and cytokine (TGF-β1). After 1 week, cell dendrites were conspicuously evident, confirming osteocyte differentiation, especially, in the presence of osteogenic factors and TGF-β1 expressing all characteristic osteocyte markers (podoplanin, DMP-1, and sclerostin). A. mylitta silk braids alone were sufficient to induce this differentiation, albeit only transiently. Therefore, we believe that the combinatorial effect of A. mylitta silk (surface chemistry, braid rigidity, and topography), osteogenic differentiation factors, and TGF-β1 were critical in stabilizing the mature osteocytic phenotype. Interestingly, Wnt signaling promoted osteocytic differentiation as evidenced by the upregulated expression of β-catenin in the presence of osteogenic factors and growth factor. This study highlights the role of nonmulberry silk braids in regulating stable osteocytic differentiation. Future studies could benefit from this understanding of the signaling mechanisms associated with silk-based matrices in order to develop 3D in vitro bone model systems.

KEYWORDS: nonmulberry silk, Wnt/β-catenin signaling, osteogenic differentiation, textile braid, osteocyte

1. INTRODUCTION

Tailoring the 3D architecture, surface chemistry, amino acid sequences, or secondary conformation of silk fibers has emerged as an effective strategy for the fabrication of biomimetic silk-based materials for bone tissue engineering. Silk fibroin, the primary component of silk fibers, comprises unique amino acid sequences (GAGAGS and GAGAGY) in repeating units which enables the tight packaging of protein chains into antiparallel β-sheets and renders strength and resilience to the structures, akin to native bone matrix. Of the two widely known varieties of silkworm silk, Bombyx mori (mulberry) and Antheraea mylitta (nonmulberry), the latter has shown exceptional potential in bone regeneration and mineralization both in vitro and in vivo. This is mostly attributed to the presence of an additional Arg-Gly-Asp (RGD) peptide-motif in A. mylitta. However, as the sequence of A. mylitta is only partially known, the existence and exact role of this sequence is still debatable. Moreover, silk-based tissue engineering mostly relies upon tailoring scaffolds by reconstitution of dissolved silk fibers into various 3D morphologies. Since A. mylitta silk is difficult to dissolve using standard laboratory solvents, their application has largely been limited.

Apart from the exceptional mechanical properties, silk fibroin protein is considered a potential matrix for bone tissue engineering due to its striking resemblance to collagen type I. As proof of concept, previously we used silk fibroin from B.mori silkworm to investigate the role of material properties (chemical, structural, and mechanical) in regulating hydroxyapatite deposition on silk using simulated body fluid. Interestingly, we noticed that B. mori braids, constituting of native silk fibers, regulated hydroxyapatite deposition along the c-axis (002), oriented longitudinally parallel to silk fibers, typically found in native bone. This was a very significant finding as the mechanism was found to be exactly similar to the biomineralization of collagen type I in bone. On the contrary, the regenerated 3D porous silk scaffold lacked this potential, most likely due to the disruption of amino acid sequences as a result of dissolution and reconstitution of original protein assembly. This was followed by a comparative in vivo investigation on the osteogenic potential of 3D porous B. mori with its nonmulberry counterpart A. mylitta in critical size

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calvarial defects of rats. Radiographic and histological evidence revealed complete osseo-integration of implanted defects within 6 months, albeit only in A. mylitta scaffolds. On the basis of these results, we hypothesized that natively spun fibers of nonmulberry silk A. mylitta will provide a suitable matrix for bone regeneration studies.

In native bone, osteoblasts initiate the synthesis and deposition of osteoid matrix wherein these cells get entrapped and start transitioning into dendritic osteocytes, characterized by genotypic expression of dentin matrix protein-1 (Dmp-1) and sclerostin (Sost). Existing strategies to establish such osteocyte models include the use of commercial cell lines in 2D monolayer or animal model testing. However, the signaling cues provided by 3D architectures (biochemical, topographical, and mechanotransduction) that direct cellular differentiation toward a particular lineage cannot be replicated in conventional monolayers. Animal testing, on the other hand, is expensive, is plagued by ethical concerns, and often fails to correlate with human physiology. To circumvent these issues, 3D culture systems of polymers and proteins are becoming popular. To date, in vitro studies on silk reported mineralized matrix deposition using osteoblast cultures, but we could not find any report on complete cellular differentiation and transition into terminally differentiated osteocytes. Therefore, with an aim to develop a 3D bone model on A. mylitta braids ex vivo by reproducing the phenotypic and genotypic transition of preosteoblasts to dendritic osteocytes, akin to native bone, we will address the following key points: (i) examining cellular responses toward precisely fabricated hierarchical complexities of such nonmulberry braided structures, (ii) the role of substrate properties in regulating osteocytic differentiation on silk matrices; and (iii) major signaling pathways directing osteogenic signaling on nonmulberry silk matrices.

Osteogenic signaling on silk scaffolds has been poorly understood so far. Jung et al. studied the gene expression of rat bone marrow cells on B. mori silk fibroin proteins which demonstrated suppressed Notch-activated genes while upregulating the expression of osteogenic markers, thus indicating a critical role of silk fibroin proteins in suppressing Notch signaling. Little is known about the role of silk in driving the key osteogenic signaling mechanisms. Wnt signaling, one of the main bone signaling pathways, is regulated via β-catenin, a transcription factor known to express in the presence of transforming growth factor-β1 (TGF-β1). TGF-β1, the most abundant cytokine in human bone (200 mg/kg), plays a central role in bone matrix turnover, storage of minerals, and the generation of hematopoietic cell lineage. Varying the concentration of TGF-β resulted in varied outcomes in in vitro and in vivo studies. This poor correlation between the existing literature on in vitro and in vivo based experiments further emphasizes the inadequacies of the current in vitro 3D culture systems for bone tissue engineering and demands a reliable, long-term sustainable in vitro model to accurately capture the physiology of native bone tissue microenvironments. Such 3D in vitro models will not only facilitate an improved understanding of the fabrication of advanced materials but also help in investigating pathological signaling mechanisms and screening of drug candidates for therapeutic applications.

Considering the applications of this in vitro model in bone differentiation, hFOBs 1.19, a preosteoblastic cell line with surface markers similar to osteogenic progenitors served as an excellent choice for the study. While the use of cell lines is often
plagued with concerns regarding genetic manipulation and misrepresentation of human physiology, an interesting trait about this cell line is that it is immortalized but non-transformed, transfected stably with temperature sensitive SV40 T-antigen. Gene expression analysis showed that at 39 °C, hFOBs expressed upregulated levels of bone-related markers: Cbfal, parathyroid hormone receptor, and osteocalcin. Differentiation of hFOBs into mature osteoblasts synthesizing mineralized matrix in 3D culture systems is known, but no study has so far reported the transition of these cells into osteocytic phenotype on any material. Keogh et al. cultured hFOBs on collagen-glycosaminoglycan scaffolds and demonstrated early evidence of mineralization only after 35 days in vitro.

Therefore, the underlying mechanisms that trigger terminal differentiation of preosteoblast cells to osteocytes, especially as a function of the underlying substrate, still remains a question.

In this study, we test the hypothesis that nonregenerated, natively spun A. mylitta silk fibers fabricated into a braided morphology can regulate extracellular matrix synthesis and osteocytic transition of cultured cells within the 3D micro-environment. The effect of biomechanical, topographical, and biological cues were investigated by culturing human preosteoblast cells in the presence or absence of bone inducing factors and TGF-J1. Osteogenic differentiation into mature osteoblasts/osteocytic phenotype was investigated by real-time gene expression profiling from early osteoblasts to terminal osteocytes, monitoring cell morphology and metabolic activity and protein synthesis by confocal microscopy. To the best of our knowledge, this is the first study reported on nonmulberry silk braids that maps the entire process of bone differentiation from preosteoblasts to stable osteocytes on A. mylitta braids. Beyond the conventional scope of clinical bone graft substitutes, this nonmulberry silk braid has immense therapeutic potential for being used as a 3D in vitro model system of bone differentiation for screening the efficacy of drugs targeted toward bone disorders.

2. MATERIALS AND METHODS

2.1. Source of Silk Yarn. A. mylitta silk fibers of 60 N m (16.7 tex) count were procured from Starling Silk Mills Pvt. Ltd., Malda, Bengal such that silk double yarn (obtained by twisting two single yarns together) was produced with a resulting count of 30 N m (33.3 tex) to develop braided structures, so that the yarns could withstand forces during the braiding process.

2.1.1. Fabrication of A. mylitta Braids. Flat braids of silk were produced by passing a fixed number of yarns in a diagonally opposite direction to the subsequent layer such that each bundle alternates over and under another bundle of yarn. Then, the structure is oriented at an angle to the circumferential axis of the resulting braid on a tube and under another bundle of yarn. Then, the structure is oriented at an angle to the circumferential axis of the resulting braid on a tube. Braid angle was measured from macroscopic images obtained using an inverted optical microscope (Leica DM2500P, Germany) equipped with a high resolution digital microscope camera system (Leica DFC425C, Germany). A total of 12 observations were taken across random regions in the braided sample.

2.1.2.3. Packing Fraction. Packing fraction is the fraction of yarn volume in a fabric structure that is occupied by a collective bunch of fibers. Packing fraction of silk scaffolds was measured as described elsewhere.

packing fraction = total cross-sectional area of the fibers /cross-sectional area of yarn = volume of fiber in yarn /volume of yarn

2.1.2.4. Twist Per Inch (tpi). For measuring the twist density, the untwist-retwist method was used on a EY06 type Eureka Precision Instrument. Yarn length of 20 cm (n = 5) was used to measure the tpi.

2.1.2.5. Tensile Strength. The tensile tests of A. mylitta braids (n = 3) were carried out by a HSKS Tensile Strength Tester testing machine under ambient conditions, i.e., 28 °C and 70 ± 5% relative humidity. The gauge length used was 75 mm with 300 mm/min cross-head speed.

2.1.2.6. Compression. The compression test was performed using a ZwickRoell LTM 1000 machine on braids (n = 3) measuring 10 mm × 4 mm. For measuring the silk braid compression, the Essidal Thickness Gauge was used where different levels of load from 50 gf/cm² to 2000 gf/cm² were applied, and the consequential change in material thickness was recorded.

2.2. In Vitro Cell Culture. 2.2.1. Cell culture. Human fetal osteoblast cell line (hFOB 1.19 ATCC, CRL-11372) was purchased from Promochem, Bangalore, India. According to ATCC guidelines, hFOBs cultured at 33.5 °C undergo rapid cell division, whereas cell differentiation is observed at the temperature of 39 °C. The cells were expanded in standard medium comprising Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 Ham, 1:1 mixture (cat. no. AL187A, Himedia) with 10% FBS (Biological Industries, cat. no. 04-121-1A), and 3 μg/mL Geneticin (cat. no. 10131035, Invitrogen) at 33.5 °C in 95% humidity and 5% CO₂.

2.2.2. Cell Seeding on Braids. Scaffolds (4 mm × 4 mm × 0.6 mm) were sterilized in 70% ethanol followed by prewetting in complete medium overnight followed by cell seeding with 1.72 × 10⁵ cells per cm² of the braided construct. After 2 days of incubation in standard media, half of the cell-seeded constructs were shifted to different conditions. Differentiation media contained standard media with 10 nM dexamethasone (Sigma-Aldrich, USA), 0.01 M β-glycerol phosphate (Sigma-Aldrich, USA), and 50 μg/mL ascorbic acid-2-phosphate (Sigma-Aldrich, USA) to promote cell differentiation. Media were changed twice a week. All cell culture condition sets were carried out at both 33.5 and 39 °C.

2.3. Analyzing Cell Behavior. 2.3.1. Quantification of Cell Attachment on Braids. The braids (n = 3) seeded with 2 × 10⁵ hFOBs were placed in a 24-well plate with wells precoated with 2% poly(2-hydroxyethyl methacrylate) (Sigma, USA, cat. no. P3932) in order to avoid cell attachment to the wells. Nonattached cells were collected by centrifugation, stained with trypan blue, and counted using a Neubauer chamber to determine % adhesion.

2.3.2. Determining Metabolic Activity. The cellular metabolic activity was determined using the standard MTT assay. After 1 and 7 days, hFOB-laden braids (n = 3, experiment repeated twice) were stained with tetrazolium MTT salt (1:10 ratio in media) at 37 °C for 4 h followed by dissolution using dimethyl sulfoxide. The absorbance was measured at 560 nm, using an iMark microplate absorbance reader (Biorad). Cell-free braids served as a control for this procedure.

2.3.3. Scanning Electron Microscopy. After 7 days, cell-laden braids were fixed, dehydrated with gradient alcohol series, gold coated (EMITECH K550X, UK), and visualized using SEM (model EVO 50, Zeiss, UK). To compute mean pore size, 20 pores were measured across 3 images per sample using ImageJ software (NIH, USA).

2.4.4. Real Time-Polymerase Chain Reaction (RT-PCR). For the experiment, the total RNA was isolated at day 1, 7, and 14 from cells
cultured on braids using an RNeasy minikit (Qiagen) as per the manufacturer’s protocol. RNA concentration was determined using a Nanodrop 2000C (Thermo Scientific, Wilmington, USA) spectrophotometer, and cDNA was synthesized using a first strand cDNA Synthesis Kit (ThermoScientific, cat. no. K1612). Real-time quantitative PCR was conducted using SYBR Green Master Mix (Quantitect, cat. no. 204074) and a rotorgene Q thermocycler (Qiagen). QuantiTect primers (Qiagen) used for gene expression analysis included alkaline phosphatase (ALP; cat. no. QT00211582), collagen type 1 alpha (COL1A1; cat. no. QT00037793), Runx-related transcription factor (RUNX2; cat. no. QT00020517), osteonectin (SPARC; cat. no. QT00018620), osteopontin (SPP1; cat. no. QT01008798), osteocalcin (BGLAP; cat. no. QT00232771), podoplanin (cat. no. QT01015084), DMP1 (cat. no. QT00022078), SOST (cat. no. QT00077882), Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, cat. no. QT00079247) was used as the house keeping gene. hFOBs cultured in the form of a 2D monolayer served as a control. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, cat. no. QT00079247) was used as the house keeping gene. hFOBs cultured in the form of a 2D monolayer served as a control. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, cat. no. QT00079247) was used as the house keeping gene. hFOBs cultured in the form of a 2D monolayer served as a control. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, cat. no. QT00079247) was used as the house keeping gene. hFOBs cultured in the form of a 2D monolayer served as a control. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, cat. no. QT00079247) was used as the house keeping gene. hFOBs cultured in the form of a 2D monolayer served as a control. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, cat. no. QT00079247) was used as the housekeeping gene. hFOBs cultured in the form of a 2D monolayer served as a control. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, cat. no. QT00079247) was used as the housekeeping gene. hFOBs cultured in the form of a 2D monolayer served as a control. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, cat. no. QT00079247) was used as the housekeeping gene. hFOBs cultured in the form of a 2D monolayer served as a control.

2.4.5. Immunoﬂuorescence Studies. For immunoﬂuorescence analysis, hFOB-laden braids were harvested at day 7, ﬁxed, permeabilized, and blocked.17 Staining was performed using antioestopentin (10 μg/mL, Millipore) followed by secondary staining with goat anti-mouse IgG antibody-FITC conjugate (1:200, Millipore) for 1 h at RT. Actin staining was performed for 30 min at RT using rhodamine phalloidin (Sigma, cat. no. P1951), followed by Alexafluor 546 (cat. no. A11003, Millipore, MA, USA). Nuclear staining was performed with DAPI (Sigma-Aldrich, USA, cat. no. 32670). For image capturing, a Leica TCS SP5 (Leica Microsystems) inverted confocal laser scanning microscope was used.

2.5. Statistical Analysis. Data are presented as the mean ± standard deviation, where n is the number of experimental repeats conducted. To determine statistical signiﬁcance of data, Student’s t test was conducted, and probability values in the range of p < 0.05 were noted as signiﬁcant.

3. RESULTS

3.1. Structural Hierarchy of Braids. The tpi value of the yarn was carefully optimized to 6.12 in order to achieve the desired mechanical properties required to withstand braiding and aid in compaction of the braided structure. The physical characterization of the ﬁbers, yarn (Table 1), and braids (Table 2) was performed. Moreover, the braid depicted a ﬂattened morphology comprising several layers of hierarchy (Figure 1B). The measured thickness of the total braid was 0.61 mm. The criss-cross braid structure (Figure 1C) comprised two ply yarns (Figure 1D), with 34 yarns intertwined to make a single layer of the braided scaffold. Each yarn (two ply) was further made up of 66 ﬁbers: building blocks of the 3D braided architecture (Figure 1E). Quantitative analysis revealed a network of uniformly oriented multilayer stack of ﬁbers with a thickness of 21.9 μm ± 0.2 μm each. Further to this, considering the dimensions of a human osteon (223 μm),19 we decided to use A. mylitta ﬁbers in the 3D braided structure with average pore sizes close to this value (i.e., 196 μm) as determined with SEM micrographs. In addition, a constant braiding angle in the range of 35°–45° with respect to the subsequent layer (Figure 1A) resulted in the compaction of the structure due to the packing of a large number of such ﬁbers which was possibly a major reason for the resulting lower packing factor and pore size of the braided fabric. Moreover, some silk ﬁbers appeared loose in the textile braid structure possibly due to the degree of physical wear during the braid fabrication and/or degumming process (Figure 1B). This made the material surface relatively rough. The surface roughness of yarn as evaluated by AFM (Rq) corresponded to 77 nm (Figure 2). This increased surface roughness of the braids due to the interconnected porous structures aids in the formation of neo-bone tissue, allowing adequate integration of host tissue/cells and channels for nutrient/metabolic waste diffusion.20 Moreover, it is well-known that increasing roughness and stiffness of scaffolds leads to superior differentiation of osteogenic progenitors.21 Particularly, human bone marrow stem cells differentiating into mature osteoblasts on stiffer matrices have been extensively researched.3 Also, the fact that the braid was fabricated directly from native silk ﬁbers without the usual dissolution process further contributed toward the increased stability of the protein chains and resultant rigidity of the braided construct (Table 2).

3.2. Cell Behavior on Braids. 3.2.1. Initial Cell Attachment. Cell counting data revealed that approximately 59000 + 23 out of the total 2 × 10^6 hFOBs seeded were found floating/dead in culture wells on day 1, resulting in 70.5 + 6.7% of cell attachment (Table 3).

3.2.2. Cell Growth Kinetics. Surprisingly, at the 33.5 °C proliferation temperature of the cell line,41 no signiﬁcant increase in metabolic activity of hFOB-laden braids was observed (p < 0.05), both with and without differentiation factors (Figure 3). However, when the temperature was raised to 39 °C (differentiation temperature),43 the metabolic activity of hFOBs declined by 2.4-fold (p < 0.05) in the absence of osteogenic differentiation factors, while the value increased signiﬁcantly following treatment with osteogenic differentiation factors. This increase in hFOB activity at 39 °C, which has not been witnessed earlier, emphasizes the interaction of the osteogenic differentiation factors with the hFOB-seeded A. mylitta braids.

3.2.3. Morphological Analysis. By day 1, a conspicuous sheath of cells along with extracellular matrix components (which starts synthesizing as early as 4 h post-cell seeding)42 covered the majority of the braided surface, encapsulating the underlying pores and interyarn spaces (Figure 4A), as cell attachment was signiﬁcantly high (Table 3). On higher magniﬁcation (2500×), the individual cells adhered onto the braids (Figure 4B; white arrow) and attained an elongated morphology along the long axis of the ﬁbers (Figure 4C). This was possible as the diameter of the silk ﬁbers was similar to that of hFOBs and hence ensured proper wrapping of the cells across the surface of the ﬁbers in a 3D orientation.

Table 1. Characterization of A. mylitta Two Ply Yarn

<table>
<thead>
<tr>
<th>Characterization</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>yarn diameter (μm)</td>
<td>274</td>
</tr>
<tr>
<td>linear density (tex)</td>
<td>33.33</td>
</tr>
<tr>
<td>packing fraction (%)</td>
<td>42</td>
</tr>
<tr>
<td>elongation (%)</td>
<td>9.6</td>
</tr>
</tbody>
</table>

Table 2. Mechanical Properties of the A. mylitta Braid

<table>
<thead>
<tr>
<th>Test Type</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tensile Testing</td>
<td></td>
</tr>
<tr>
<td>ultimate tensile stress (MPa)</td>
<td>42.86</td>
</tr>
<tr>
<td>ultimate tensile strain (%)</td>
<td>30.32</td>
</tr>
<tr>
<td>initial modulus (N/tex)</td>
<td>13.88</td>
</tr>
<tr>
<td>elongation (%)</td>
<td>9.6</td>
</tr>
<tr>
<td>Compression Testing</td>
<td></td>
</tr>
<tr>
<td>compression % at 50 g/cm²</td>
<td>17.91</td>
</tr>
<tr>
<td>Young’s modulus (MPa)</td>
<td>1.41</td>
</tr>
</tbody>
</table>
After 7 days of incubation at 33.5 °C without the presence of any differentiation factors (Figure 4D−F), a confluent sheath of cellular matrix was evident (Figure 4D). In regions where the cells were directly in contact with the braid, cell morphology appeared more rounded (Figure 4E, boxed regions), out of which few assumed long dendritic processes resembling osteocyte-like morphology (Figure 4E, inset) suggesting the role of the A. mylitta surface in accelerating differentiation. On the other hand, hFOBs associated with the cell-synthesized matrix were more flattened (Figure 4F). In the presence of osteogenic factors, the cellular matrix was more prominent (Figure 4G). Because of this extensively spread sheath on the braided surface, most of the cells were entrapped within the matrix region spread across yarns and pores of the braids (Figure 4H and I).

Table 3. Summary of Quantitation Data from Cell Adhesion

<table>
<thead>
<tr>
<th>matrix</th>
<th>hFOBs seeded</th>
<th>no. of floating cells</th>
<th>% floating cells</th>
<th>no. of attached cells</th>
<th>% attachment</th>
</tr>
</thead>
<tbody>
<tr>
<td>braid (3D)</td>
<td>2 × 10^5</td>
<td>5.9 × 10^4</td>
<td>29.5</td>
<td>1.41 × 10^5</td>
<td>70.5</td>
</tr>
<tr>
<td>Petri dish (2D)</td>
<td>2 × 10^5</td>
<td>1.6 × 10^4</td>
<td>8</td>
<td>1.84 × 10^5</td>
<td>92</td>
</tr>
</tbody>
</table>

After 7 days of incubation at 33.5 °C without the presence of any differentiation factors (Figure 4D−F), a confluent sheath of cellular matrix was evident (Figure 4D). In regions where the cells were directly in contact with the braid, cell morphology appeared more rounded (Figure 4E, boxed regions), out of which few assumed long dendritic processes resembling osteocyte-like morphology (Figure 4E, inset) suggesting the role of the A. mylitta surface in accelerating differentiation. On the other hand, hFOBs associated with the cell-synthesized matrix were more flattened (Figure 4F). In the presence of osteogenic factors, the cellular matrix was more prominent (Figure 4G). Because of this extensively spread sheath on the braided surface, most of the cells were entrapped within the matrix region spread across yarns and pores of the braids (Figure 4H and I).

Figure 2. Surface roughness of silk yarn (n = 3) by atomic force microscopy displaying the height sensor, phase, and 3D micrograph.

Figure 3. MTT of hFOB-laden A. mylitta braids measured at different temperatures. * represents statistically significant data where p < 0.05 and n = 3/group.
When cultured at 39 °C, a confluent cell sheath, observed in all other groups, was not evident in hFOB-laden braids cultured in the absence of differentiation factors (Figure 4J), also evident from lower MTT values (Figure 3). Cell morphology was highly irregular and distorted (Figure 4K, asterisk) with traces of debris/fragments (Figure 4L, arrowheads), which were possibly an outcome of cellular degradation due to apoptosis. On the contrary, in the presence of differentiation factors, a continuous sheath of cellular matrix (data not shown) invaded by distinct cellular morphologies later indicated different stages of osteogenic differentiation (Figure 4M–O): (i) a rounded hFOB with high contrast particle deposition on the surface (asterisk *) indicative of mineral-like deposition (Figure 4M) and (ii) typical osteocyte-like morphology with prominent dendritic protrusions (Figure 4N and O) spread across multiple fibers (Figure 4O). This coexistence of osteoblasts and osteocytes on A. mylitta matrix may facilitate a bidirectional communication between the two cell types and generate important signals which may influence the differentiation of osteoblasts toward osteocytes; however, this is only speculation at this stage and would require a controlled series of experimentation to be validated.

3.2.4. Effect of Varying Differentiation Conditions on Osteogenic Gene Expression. The expression of osteogenic-specific markers for hFOBs cultured either on braids (Figure 5) or 2D monolayers (Figure S1) with and without differentiation factors at 39 °C only. hFOBs cultured at 33.5 °C (which is the proliferative temperature), demonstrated nominal expression of
all differentiation markers throughout the culture period (data not shown).

3.2.4.1. Osteoblast Markers. The expression of ALP in hFOB-laden braids was increased with time in all the conditions, corresponding to 1.1-fold upregulation with differentiation factors and 30.6-fold upregulation without factors from 7 to 14 days (Figure 5). The addition of differentiation factors rapidly upregulated the expression by 6.4-fold as early as day 1 ($p < 0.05$). Addition of TGF-$\beta_1$, drastically downregulated ($p < 0.05$) the ALP transcript levels at all time points in hFOB-laden braids (Figure 5) as well as the 2D monolayer (Figure S1). Moreover, as expected, the 2D monolayer demonstrated 1.8-fold upregulation of ALP in the presence of differentiation factors over standard media conditions by day 7; however, the expression was significantly lower ($p < 0.05$) than that of cell-laden 3D braids (Figure S1).

The expression of COL1A1, an important extracellular matrix (ECM) protein of the bone, was significantly upregulated in hFOB-laden braids by day 7 ($p < 0.05$) and declined by 14 days for both conditions (Figure 5); indicating a temporal pattern of osteogenic differentiation. Negligible expression of COL1A1 ($\leq 1$ fold change) was detected in the presence of TGF-$\beta_1$, both with and without differentiation factors. For 2D monolayer control (Figure S1), COL1A1 transcript levels were nominal for all conditions tested.

Similarly, the expression of Runx2, the master gene for bone differentiation, demonstrated drastic upregulation by day 7 in hFOB-laden braids cultured only in the presence of differentiation factors ($p < 0.05$) and subsequently declined by 62.2-fold by day 14, typically following the pattern of osteogenic differentiation. A similar pattern of expression was also observed for the 2D monolayer cultured with differentiation factors (Figure S1); however, the expression levels were significantly lower than those in braids. In comparison, hFOB-laden braids cultured without differentiation factors depicted significantly lower expression of RUNX2 at all time points ($p < 0.05$). Upon addition of TGF-$\beta_1$, the Runx2 transcript levels were found to be significantly downregulated by day 14 ($p < 0.05$) for both conditions as compared to groups where no TGF-$\beta_1$ was added.

Figure 5. Gene expression analysis of hFOB-laden A. mylitta braids ($n = 3$ group) cultured at 39 °C in different experimental conditions up to day 14. Abbreviations: w/o, without osteogenic factors/standard media conditions; w/o + TGF, without osteogenic factors, with 10 ng/mL TGF-$\beta_1$; with, with osteogenic factors; with + TGF, with osteogenic factors, with 10 ng/mL TGF-$\beta_1$.  

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The expression of SPARC, a midstage ECM marker, in hFOB-laden braids depicted an expression pattern similar to that of Runx2, wherein drastic upregulation was observed by day 7 only in the presence of differentiation factors, which significantly declined by day 14 (52.4-fold decrease). Similarly, in the presence of TGF-β1, nominal expression of SPARC was observed in both experimental groups in braids (Figure 5) as well as monolayer (Figure S1) cultures.

For SPP1 or osteopontin, a reverse trend could be identified compared to that of the above groups (Figure 5) with significant upregulation of SPP1 expression in hFOB-laden braids without differentiation factors at day 7 (3.8 fold) and day 14 (4.6 fold) compared to that in differentiation culture media. Moreover, the transcript levels were drastically upregulated \( (p < 0.05) \) in the presence of TGF-β1 by day 14. This pattern of differentiation was also observed for monolayer cultures (Figure S1).

The expression of BGLAP, indicative of later stages of osteoblast differentiation involving the deposition of calcified matrix, was also analyzed. While the expression was significantly upregulated \( (p < 0.05) \) in the presence of differentiated factors by day 7, the transcript levels subsequently declined by day 14. An interesting trend that was observed in this particular gene was the drastically upregulated activity of BGLAP in the presence of TGF-β1 with \( (p < 0.05) \). Similarly, in 2D monolayer culture, the expression of BGLAP was maximal in the case of differentiation factors (26-fold) and minimal (2-fold) by day 7. The expression was either similar (differentiation conditions) or upregulated (standard media conditions) in the presence of TGF-β1.

3.2.4.2. Osteocytic Markers. Podoplanin, an early osteocytic marker, was drastically upregulated in hFOB-laden braids cultured both with (826-fold) and without differentiation factors (333.1-fold) by day 7, and the subsequent expression differed significantly between the two groups (Figure 5). While the expression significantly declined to 129.8-fold in the presence of differentiation factors, an upregulation by 5.1 times was noticed without differentiation factors by day 14. The addition of TGF-β1 triggered the podoplanin expression by 1.5 times, albeit only in the hFOB-laden braids cultured with differentiation media. In comparison, the 2D monolayer culture demonstrated negligible expression of podoplanin by day 7 suggesting no evidence of transformation to the osteocytic phenotype in monolayer conditions.

DMP1, an important regulator of matrix mineralization, demonstrated maximal expression in the case of hFOB-laden braids cultured in the presence of TGF-β1 with \( (123.6 \text{-fold}) \) and without \( (98.4 \text{-fold}) \) differentiation factors as compared to their non-TGF-β1 counterparts by day 14. hFOB-laden braids cultured in standard culture media without differentiation factors demonstrated nominal expression throughout the culture period. As DMP1 expresses in the later osteocytic stages of osteoblast to osteocyte transition, its expression was not evident in the early days of osteoblast differentiation (i.e., up to day 7) in all groups. Similar to podoplanin (Figure 5), the expression of DMP1 was negligible in the 2D monolayer culture.
SOST, suggesting the terminal osteocytic phenotype, showed a trend similar to that of DMP1 (Figure 5) transcript levels. The hFOB-laden braids cultured without differentiation factors showed negligible expression throughout the culture period; however, the expression got significantly ($p < 0.05$) upregulated in the presence of TGF-$\beta$1. However, in the presence of differentiation factors, the expression levels of SOST were more or less comparable, indicating no significant contribution of the presence of the TGF-$\beta$1 monolayer culture (Figure S1) showed negligible expression of this osteocytic marker.

That fact that osteocytic expression was only evident in hFOB-laden braids and not the monolayer culture emphasizes on the role of the 3D environment and silk surface chemistry.
for providing essential biomechanical, topographical, and chemical cues for silk matrix-mediated cell signaling.

3.2.5. Dose Dependent Response of TGF-β1. A biphasic response of TGF-β1 was observed on hFOB-laden A. mylitta braids (Figure 6A), as reported earlier. The expression of β-catenin showed a steep increase at day 7, albeit only in TGF-β1 concentrations ranging from 2 to 6 ng/mL. By day 14, β-catenin expression declined in these groups showing significantly reduced expression (p < 0.05). However, in concentrations >6 ng/mL, a slightly different trend was observed. A gradual increase in β-catenin expression was noticed demonstrating 22-fold and 59.7-fold upregulation at 8 ng/mL (Figure 6A) and 10 ng/mL (Figure 6B), respectively, after 14 days. On the basis of these results, the desired concentration of 10 ng/mL was selected. Controls, consisting of hFOB-laden braids cultured in standard culture media without additional differentiation factors or TGF-β1, demonstrated a pattern similar to that of groups containing lower dosages of TGF-β1 (<6 ng/mL), albeit with lower expression levels (maximal 5.8-fold increase by day 7) (Figure 6C). As expected, the 2D monolayer culture depicted nominal expression of β-catenin after 7 days (Figure 6C).

3.2.5.1. hFOB-Laden A. mylitta Braids Regulate Osteogenesis through Wnt/β-Catenin Signaling. As reported earlier, we found that TGF-β1 demonstrated a dose dependent response on the expression of β-catenin (Figure 6A; section 3.2.5), hence validating its involvement in regulating Wnt/β-catenin signaling on hFOB-laden braids. As evident in later stages of differentiation, β-catenin demonstrated maximal upregulation in TGF-β1 supplemented groups (59.7- and 100.4-fold with and without differentiation factors, respectively) by day 14 (p < 0.05) which would in turn dictate the dependency of hFOBs to follow osteogenic signaling via Wnt/β-catenin signaling in the presence of TGF-β1. Although, a drastic increase in β-catenin expression was also noted by day 7 (121.7-fold) even without TGF-β1 addition, the expression decreased by day 14, suggesting the requirement of additional factors in promoting the end-term differentiation of cells on A. mylitta braids. In contrast, negligible expression of β-catenin was observed in the 2D monolayer culture after 14 days (Figure 6C).

3.2.6. Fluorescence Microscopy. In the absence of differentiation factors, cell morphology was more rounded (Figure 7A), and actin stress fibers were not very evident by day 7, as observed in SEM micrographs (Figure 4A–L). On the contrary, hFOBs cultured in the presence of differentiation factors demonstrated extensively elongated stress fibers (Figure 7B, yellow arrows) resembling dendritic processes associated with transition to the osteocytic phenotype, as also evident in SEM (Figure 4M–O) and RT-PCR (Figure 5). Further, the synthesis of osteopontin was localized to a majority of hFOBs with discrete patches of staining evident within the hFOB-laden braids in both differentiation conditions, albeit expression was relatively higher in the absence of differentiation factors by day 7 (Figure 7C) as also evident in the gene expression analysis (Figure 5; SPP1). On the contrary, the synthesis of osteonectin by day 7 was mostly localized to hFOB-laden braids cultured in the presence of differentiation factors (Figure 7E and F) similar to gene expression data (Figure 5; SPARC). However, one limitation with the immunofluorescence of silk is the inherent autofluorescence arising from the aromatic amino acids (tyrosine and tryptophan), as seen by the blue staining of silk fibers.

4. DISCUSSION

To our knowledge, this is the first report on the development of a braided hierarchy, constituted of native (nonregenerated) nonmulberry silk fibers, as a potential scaffold for bone tissue engineering. The braids fabricated in this study mitigate the lack of adequate osteoconductivity associated with regenerated silk scaffolds while avoiding the fabrication challenges associated with most tissue engineered scaffolds, as the dissolution of this variety of silk is a difficult task to achieve. These native fibers of nonmulberry silk would be more osteogenic compared to regenerated silk scaffolds, as the protein assembly has not been disrupted during manufacturing. Other reported studies on A. mylitta scaffolds, such as with conventional freeze-drying, have reconstituted dissolved silk fibers that unfortunately result in decreased apatite deposition due to the disrupted protein chains as a result of processing parameters. Moreover, the method of textile braiding is advantageous due to its ease of fabrication, customizability, and cost-effectiveness.

We extensively investigated the mechanics of this braided architecture of A. mylitta silk fibers, as mechanical properties can act as a potential limiting factor in the context of bone engineering scaffolds, especially because bone is a load-bearing tissue. The suitability of A. mylitta as a potential scaffold for bone partially arises from its exceptional mechanical properties (stiffness of 1.41 MPa) in the range of cancellous bone (0.5–14.6 MPa). As reported in our previous study, the rate of degradation of A. mylitta silk is incomparably higher than that of the mulberry varieties (B. mori) both in vitro and in vivo. Moreover, the size of the pores, a critical feature of osteoconductive materials, is comparable to that of a human osteon (223 μm), while still retaining appropriate moduli. The tailored braided angle, which was in the range of 35°–40°, also aided in sufficient compaction of the braided structure, hence adding to the mechanical properties. Taken together, these data clearly indicate that the said mechanical properties of the braid used here are favorable for implantation studies.

Subsequently, we examined important aspects of cell-matrix interactions between human preosteoblasts and the A. mylitta braids taking into consideration cell adhesion and distribution that influence subsequent cell behavior, metabolic activity, cell morphology, gene expression analysis, and protein synthesis. The SEM and AFM results on pore size and surface roughness, respectively, indicated the topographical relevance of the A. mylitta fibers that could enhance cell adhesion, orientation, and infiltration at different orders of magnitude; fiber assembly is shown at the micrometer level by SEM and surface roughness as demonstrated by AFM in the order of nanometers. After analyzing the cellular distribution profile on a braided surface, we found that osteoblasts were localized both within the pore spaces as well as also being oriented parallel to the longitudinal axis of the fibers. Overall, from the cell distribution data, it is logical to speculate these two important aspects of bone formation: bone formation occurring from the infiltrated osteoblasts within pores which can regenerate bone from the scaffold’s interior, as well as cellular alignment along the fibers as they orient along the periphery of fibers forming bone from outside. However, this hypothesis warrants in vivo investigations to provide a clearer picture on the mechanism of bone formation as a function of braided geometry.

Moreover, the data demonstrate relatively high cell adhesion (lower than that in the 2D Petri dish) and orientation of seeded
osteoblasts in compliance with the surface chemistry and braid morphology. However, the values depicted for cell attachment (Table 3) include both initial adhesion of hFOBs on braids as well as proliferation up to 1 day; nevertheless, the fact that native silk fibrin consisting of a specific sequence of amino acids leads to enhanced cellular responses, along with other factors including surface roughness, rigidity, surface chemistry, or all of these combined, remains true. Another interesting revelation made by a recent study was that mechanical stiffness plays a crucial role in determining the fate of osteoprogenitors.23 While softer matrices (0.58 kPa) facilitated osteocytic differentiation of seeded MC3T3-E1 progenitors on gelatin-based 3D hydrogels, stiffer matrices (1.47 kPa), on the contrary, retained the osteoblastic phenotype until 56 days in culture. However, it will be difficult to compare results as the material and cell line used are entirely different.

The gene expression data clearly demonstrate that A. mylitta braids are innately osteoconductive and enable rapid differentiation of cultured preosteoblasts to mature osteocytes within 2 weeks. This was also evident from the extensively elongated dendritic cellular extensions as shown by SEM and confocal imaging data, a characteristic feature of the mature osteocytic phenotype. The Ct (copy number) values reported are normalized to the expression in the 2D monolayer, cultured under similar conditions. As expected, gene expression of cell-laden braids cultured in the presence of pro-osteogenic factors increased, compared to that in A. mylitta braids alone or in the 2D monolayer. Interestingly, transient expression of osteocytic differentiation was evident in cell-laden A. mylitta braids alone despite the absence of pro-osteogenic differentiation conditions. The observation that A. mylitta structures correlate closely with previously reported in vivo studies where 3D porous scaffolds of A. mylitta successfully regenerated critical sized defects of rat calvariae,7 an effect which might be more enhanced in the natively spun braids, demonstrates the osteogenic ability of the material in directing cellular differentiation since no differentiation was evident in 2D monolayer cultures. Moreover, significant changes in the gene expression profile of cell-laden A. mylitta braids following supplementation with TGF-β1 further produced a series of questions about the role of underlying signaling mechanisms involved. Note that to be able to identify the exact signaling pathway involved, the cell behavior on braids was extensively analyzed under varied experimental parameters including temperature, pro-osteogenic factors, and the presence or absence of nonmulberry braids (2D vs 3D), which is discussed in detail below.

4.1. Wnt/β-Catenin Signaling in Regulating Mineralized Bone Differentiation on A. mylitta Braids. An interesting observation was the rapid transition of hFOBs on A. mylitta braids into the osteocytic phenotype within 2 weeks of culture, a phenomenon not yet reported for hFOBs even under extended culture periods.14 While the early osteocyte gene continued to increase (podoplanin), we found a nominal expression of Dmp1 (which participates in the process of matrix mineralization)28 at all time points studied. A possible explanation could be the reduced production of associated cytokines/growth factors involved in mineralization, which was also validated from the nominal osteocalcin expression. However, within the commercial setup, this rapid transition of preosteoblasts to transitory osteocytes may not serve as an ideal model of bone differentiation. Therefore, standard osteogenic reagents (dexamethasone, ascorbic acid, and β-glycerophosphate) were added to improve the extent of osteogenesis in cell-laden braids. The addition of factors convincingly upregulated the expression of osteogenic markers over A. mylitta braids alone.

Another major reason for the suppressed activity of mineralized bone apatite could be the upregulated expression of Sost gene. It is known that sclerostin, secreted by mature osteocytes, binds to Lrp5 to antagonize the action of Wnt hence inhibiting Wnt/β-catenin signaling.29,30 Literature suggests that deletion of the Sost gene in mice resulted in increased bone mass and strength,31 whereas overexpression of SOST demonstrated lower bone mass.32 Similarly, loss of SOST expression in humans is often associated with bone disorders associated with high bone mass such as Van Buchem’s disease33 and osteoarthritis.34 Hence, it was logical to believe that osteocyte secreted SOST caused the inhibition of mineralized bone tissue by blocking Wnt/Lrp signaling in osteoblasts.28,29 This mechanism resulted in the lack of apatite deposition on nonmulberry silk braids.

Under physiological conditions of bone differentiation, accumulation of β-catenin starts in the cytoplasm subsequently translocating into the cell nucleus wherein it interacts with the Tcf/Lef family of transcription factors. This interaction further regulates the activation of several important genes associated with proliferation, differentiation, and apoptosis of osteogenic cells.35 Therefore, to examine if this arrest of osteoblast differentiation observed at the later stages is possibly due to compromised Wnt/β-catenin signaling,36,37 we supplemented the cultures with TGF-β1. It is well-known that activation of the Wnt/β-catenin signaling pathway results in a decrease of apoptosis in osteogenic cells.38 In this context, the first indicator for the enhanced activity of Wnt/β-catenin signaling in the case of TGF-β1 supplemented braids was the increased metabolic activity found in hFOB-laden braids even when cultured at 39 °C, a restrictive temperature of the cell line. hFOB-laden braids cultured in TGF-β1 were found to restore the antagonizing action of SOST, thus validating the direct involvement of Wnt/β-catenin signaling in hFOBs cultured on nonmulberry silk braids. The expression of both mineralization associated genes, BGLAP and DMP1, was found to be restored, suggesting a natural process of bone differentiation. Moreover, the significant relationship between SOST and β-catenin was established by the reciprocal levels of expression observed in hFOB-laden braids cultured without any differentiation factors. With a 8.5-fold decline in the SOST expression level, the corresponding increase in β-catenin was roughly 23-fold after 14 days in culture. However, the addition of TGF-β1 successfully upregulated the expression of β-catenin, hence triggering Wnt/β-catenin signaling in regulating osteogenesis in hFOB-laden A. mylitta braids in vitro, with increased SOST expression suggesting a more stable phenotypic transition.
Furthermore, it is often contemplated that a cross-talk with the prostaglandin pathway activates the Wnt/β-catenin pathway in osteocytes under mechanical stimulus subsequently resulting in the downregulation of Sost. Since no mechanical load was applied to the cell-laden braids in our case, the SOST levels were constantly upregulated while simultaneously inhibiting mineralized bone differentiation. The compelling evidence indicates that supplementation of TGF-β1 increases mineral bone in hFOB-laden A. mylitta braids in vitro while simultaneously upregulating SOST to ensure the transition of preosteoblasts into phenotypically stable osteocytes within 2 weeks of the culture period.

Though beyond the scope of this study, extensive research may be needed in determining how osteocytes perceive and enable signal transduction in response to such fibrous hierarchies. Moreover, mediation of the physical attributes of the textile braid such as the number of fibers per yarn, thickness of yarns, pore size, braid angle, rigidity as well as surface chemistry (comparison of different silk species) and their impact on cellular differentiation may provide a more comprehensive overview for controlling osteocyte differentiation for long-term sustainable cultures. Also, application of a mechanical stimulus and/or a bioreactor system for facilitating proper nutrient diffusion and cellular infiltration under load bearing conditions will aid in providing a biequivalent model replicating the in vivo microenvironment more closely. Furthermore, the coexistence of osteoblasts and osteocytes, as observed in A. mylitta braids cultured with osteogenic factors, their relevance and cross-talk in facilitating cell—cell communication for osteogenic differentiation needs to be established. As evident, targeting the Wnt/β-catenin pathway because of its critical role in controlling osteocyte differentiation factors significantly improves the expression of osteogenic-related markers marking a more discrete process of bone differentiation. However, the combinatorial effect of osteogenic factors with TGF-β1 and the nonmulberry braided matrix resulted in completely establishing the process of osteogenic differentiation from preosteoblasts to terminal osteocytes expressing functional sclerostin regulated via Wnt/β-catenin signaling. This makes A. mylitta braided constructs an ideal model system for studying in vitro bone differentiation.

5. CONCLUSION

Recently, much attention is being given to osteocyte-related biology as its significance in regulating the structural and functional mechanisms of bone hierarchy is being realized. Having said that, there still remain several obstacles which need to be addressed to fully untap the potential of this field of research. In conclusion, hFOB-laden A. mylitta braids display all of the characteristics of an in vitro 3D osteogenic microenvironment including the extensive dendritic processes, lacunocanalicular structure, distinct genetic expression, and responses to growth factors (TGF-β1), all indicating the development of a mineralized bone-like microenvironment. These cells on A. mylitta braids alone rapidly induce the differentiation of preosteoblasts into osteocytes, albeit this transition was only transient. The additional of osteogenic differentiation factors significantly improved the expression of osteogenic-related markers marking a more discrete process of bone differentiation. However, the combinatorial effect of osteogenic factors with TGF-β1 and the nonmulberry braided matrix resulted in completely establishing the process of osteogenic differentiation from preosteoblasts to terminal osteocytes expressing functional sclerostin regulated via Wnt/β-catenin signaling. This makes A. mylitta braided constructs an ideal model system for studying in vitro bone differentiation.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomater-ials.7b00006.

Gene expression analysis of a 2D monolayer (PDF)

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Notes

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REFERENCES


(12) Yen, M. L.; Chien, C. C.; Chiu, I. M.; Huang, H. I.; Chen, Y. C.; Hu, H. I.; Yen, B. L. Multilineage differentiation and characterization of the human fetal osteoblastic 1.19 cell line: a possible in vitro model...


