Differential Regulation of Hedgehog and Parathyroid Signaling in Mulberry and Nonmulberry Silk Fibroin Textile Braids

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ABSTRACT: Even after several decades of research, the most optimal source of silk for promoting osteogenesis in situ is still a subject of debate. A major gap in existing knowledge is role of underlying signaling mechanisms in both the mulberry and nonmulberry silk species that leads to the development of differential levels of osteogenesis. In our previous study, we elucidated the role of Wnt/β-catenin signaling for promoting superior osteogenic differentiation in nonmulberry silk braids in the presence of TGF-β and pro-osteogenic supplements. Here, we provide a comparative osteogenic analysis of the two most popular silk species (mulberry and nonmulberry silk), in the form of silk braids prepared from natively spun fibers, by conducting detailed gene expression profiling using 25 different osteogenic markers, followed by further validation by immunohistochemistry. Our study provides novel insights into the direct regulatory role of nonmulberry silk fibroin braids on hedgehog and parathyroid signaling pathways in controlling osteogenic differentiation of cultured human fetal osteoblasts (hFOBs), a phenomenon not very evident in the mulberry silk textile braids. Although both silk braids enabled adequate cellular attachment, proliferation, and extracellular collagen matrix formation, superior expression of osteogenic markers (ALP, VDR, Runx2), matrix proteins (Col1A2, OPN), and signaling molecules (GLI1, GLI2, Shh) with characteristic terminal osteocytic phenotype could only be observed in nonmulberry silk. Therefore, our study provided detailed insights into the development of engineered bone to be a prospective tissue equivalent with potential to provide the essential instructive elements for activating physiological pathways of bone differentiation. Such engineered constructs have potential for use as an in vitro model for drug testing and as scaffolds for bone regeneration strategies.

KEYWORDS: mulberry and nonmulberry silk, hedgehog/parathyroid signaling, osteogenesis, in vitro model

1. INTRODUCTION

Even after decades of research within the realm of bone tissue engineering, the fabrication of an anatomically relevant sized, clinically competent, patient-specific bone equivalent is not yet developed. The majority of current studies seed osteogenic progenitors on 3D porous scaffolds and provide them with growth factors/cytokines to induce osteogenic differentiation, but our understanding about underlying molecular dynamics is still rather limited. Undoubtedly, the role of scaffold surface chemistry, stiffness, and geometry is imperative in directing cellular responses toward osteogenic differentiation by providing adequate instructive cues, mechano-transduction and spatiotemporal guidance for proper cell adhesion, orientation, growth, differentiation, and morphogenesis. 1 Silk fibroin protein, as a biomaterial for bone tissue engineering applications, has benefits over other contemporary materials in ways more than one. 2,3 In our previous study we demonstrated that during biomineralization of silk matrix, the deposited hydroxyapatite nanoparticles interact closely with the amino acid motifs of native silk fibroin polymer chains, so that the c-axis of the hydroxyapatite crystals were aligned along the long axis of the silk fiber due to alignment of proteins originated from the elongational flow during the natural silk spinning process. In addition, the calcium:phosphorus ratio was found to be similar to native bone matrix. 4

Therefore, our current focus is to optimize the osteogenic differentiation on silk-based matrices to direct cellular mechanisms of osteo-progenitor cells toward the osteogenic lineage using (i) appropriate combinations of bioactive molecules and signaling molecules (such as TGF-β); 5 (ii) optimal cell sources (human preosteoblasts); 5 and/or, (iii) appropriate silk source (mulberry silk Bombyx mori, Bm or nonmulberry silk Antheraea mylitta, Am) and scaffold geometry. 6 Ideally, by putting together an appropriate combination of the above parameters, the progenitor cells should be coax to undergo terminal differentiation toward osteogenic lineage in vitro by providing conditions akin to native bone development and should follow the molecular changes observed in vivo ultimately culminating in the formation of a tissue that is molecularly indistinguishable from physiological bone. A useful finding will be to target transcription factors specific to the primary osteogenic signaling
pathways in response to different materials (which is Am versus Bm silk fibroin in our case). This knowledge can then be utilized to generate deep insights about the scaffold properties and how they can be modified for developing clinically conformant bone constructs.

Hedgehog and parathyroid (PTH) signaling pathways play important role in skeletal development and postnatal skeleton homeostasis, by crosstalking with multiple bone signaling pathways, such as Wnt/β-catenin, Notch and BMP. Both signaling pathways take part in bone and cartilage development and in maintaining homeostasis. Parathyroid hormone-like hormone (PTHrP) stimulates osteogenic cell proliferation and terminal differentiation into osteocytic phenotype; Sonic hedgehog homologue (SHH) cause hypertrophic differentiation of chondrocytes and osteoprogenitors into bone-forming osteoblasts. Disruption of these closely interacting signaling pathways, mostly resulting from traumatic injuries, genetic diseases or hormonal imbalances, often results in skeleton bone deformities such as osteoarthritis, osteoporosis, Myhre syndrome etc. But, a huge disparity between the in vitro and in vivo studies often leads to speculations for reliability of results for applicability of engineered bone to clinical scenario. In conventional tissue engineering, constructs often undergo heterogeneous and/or uncontrolled extent of differentiation suggesting that the engineered constructs may often be highly unreliable and cannot be trusted for intended tissue replacement in clinical setup. Therefore, generating insights about the underlying signaling pathways with respect to the materials can develop controlled cellular responses toward the target tissue.

A few comparative studies have been conducted on the two silk types (mulberry silk Bm vs nonmulberry silk Am) to investigate the most optimal source of silk in terms of promoting adequate bone formation and remodelling. In our previous study, we compared osteogenic differentiation of primary rat mesenchymal progenitor cells (MSCs) on silk film of both Bm and Am origin. Interestingly, 33 colonies of MSCs were visible on Am silk film as compared to only 12 colonies observed in their Bm mulberry counterpart. Increased terminal differentiation may arise due to several procedural aspects; (i) treatment with protease XIV in vitro. However, such varied outcomes may arise due to several procedural aspects; (i) different methods of isolation (Am fibroin was extracted using an anionic surfactant sodium dodecyl sulfate while LiBr method was used for Bm), (ii) protein concentration of the two may vary depending upon isolation protocols and β-sheet content. Naskar et al. immobilized titanium surfaces with either variety of silk (mulberry and nonmulberry) and found the expression of osteogenic markers (ALP and osteocalcin) as early as day 7 in Am modified surfaces. They attributed better osteogenic tendency of Am scaffolds to the presence of cell adhesive Arg-Gly-Asp (RGD) motifs, however the percentage of this sequence in Am silk fibroin may be rather nominal. With incorporated RGD in Bm silk fibroin by genetic modifications, researchers showed a 6 fold increase in cell adhesion over native Bm films. However, one limitation with this technique is that the stabilization process of silk postfabrication might lead to entrapment of these extrinsically added RGD sequences, hence making them inaccessible for cellular interactions. In another contradicting result, both the silk varieties tested for their osteogenic and adipogenic potential using rat MSCs in vitro demonstrated comparable extent of cell differentiation. Some of these studies may also be debatable, as the fibroin protein was isolated directly from the silk glands of Am silkworms, but for Bm silk cocoons were dissolved. However, as the amino acid sequences of both the species varies to a large extent, further cellular signaling cascade studies are required to establish which variety of silk, mulberry or nonmulberry fibroin, is better for bone tissue engineering. Surprisingly, in spite of the striking variations in the results obtained in the two silk species, very limited literature is available on the underlying regulatory mechanisms that trigger such responses.

Therefore, in this study, we test the hypothesis that nonregenerated, natively spun fibers of both Bm and Am fibroin fabricated into a specialized 3D architecture (by textile braiding technique) can regulate ECM synthesis and mature phenotypic transition of cultured osteogenic progenitors under the influence of specific signaling cascades. We performed a study on native Am silk braids which demonstrated terminal osteocytic differentiation of cultured human preosteoblasts within 2 weeks of culture demonstrating the effect of biomechanical, topographical, and biological cues in the presence or absence of bone inducing factors and TGF-β1 via Wnt/β-catenin signaling. Here, we provide a detailed comparative analysis of the two silk species for osteogenic differentiation into mature osteoblasts/osteocytic phenotype by investigating the role of different silk chemistries (Am versus Bm) in triggering differential osteogenesis. To the best of our knowledge, this is the first study reported on bone differentiation on silk braids that maps the entire cascade of signaling-related molecules associated with hedgehog and PTH pathways regulating the differentiation of osteogenic progenitors to stable and mature phenotype.

2. EXPERIMENTAL SECTION

2.1. Materials. Am and Bm silk fibers of 60 N m (16.7 tex) counts were procured from Starling Silk Mills Pvt. Ltd., Malda, India.

2.2. Methods. 2.2.1. Textile Braid Fabrication and Characterization. Two single silk yarns were twisted to obtain a 2-ply yarn, which were in turn twisted together using a 17 spindle flat braiding machine to develop the braid fabric structure. Next, braids were degummed in 0.02 M Na2CO3 solution for 2 consecutive cycles of 15 min each followed by extensive washing with deionized water. After drying, braids were cut into smaller uniform dimensions (4 mm × 4 mm) and autoclaved for subsequent experimentation.

The thickness of the braids was measured using a Digital thickness gauge (Mitutoyo, South Asia Pvt Ltd., India). Braided morphology was viewed on samples precoated with gold–palladium using SEM JEOL 5610LV (JEOL, Japan). The porosity of both Am and Bm (n = 3 per group) was measured using liquid displacement method. Surface roughness (Rq) was measured using Digital Instruments Nanoscope in contact mode over three randomly chosen areas (10 μm × 10 μm dimension). The diameter of each fiber and yarn (n = 150 each) was measured using a projection microscope (WeswoxOptik microscope, MP-385A, India) at different magnifications. Braid angle was measured from optical micrographs (Leica DM2500P, Germany) across 12 random regions in the braided sample. Packing fraction was attained by dividing the cross-sectional area of yarn by the total cross-sectional area of the fibers. Twist per inch (tpi) was determined using the untwist-retwist method on a EY06 type Eureka Precision Instrument using yarn length of 20 cm (n = 5 per group). The tensile strength of...
Am and Bm braids (n = 3 per group) was deduced using HSKS Tensile Strength Tester materials testing machine using 75 mm gauge length with 300 mm/min cross-head speed. Compression of braids with the dimensions of 10 mm × 4 mm (n = 3 per group) was determined using a ZwickRoell LTM 1000 machine and Essdial Thickness Gauge with different levels (50 µm/cm² to 2000 µm/cm²), and the consequential change in material thickness was recorded.

2.2.2. Cell Culture on Braids. Human fetal osteoblast cell line (hFOB 1.19, CRL-11372) was procured from ATCC. The cells were expanded in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 Ham, 1:1 mixture (Himedia, cat. no. 04-121-1A), and 3 µg/mL Geneticin (Invitrogen, cat. no. 10131035) at 33.5 °C in 95% humidity and 5% CO₂. Sterilized braids were prewetted in complete medium overnight followed by seeding with 2 × 10⁵ hFOBs per cm² of the scaffold in a 24 well plate. After 2 days of incubating the hFOB-seeded braids in expansion medium, differentiation media was added, as optimized previously containing TGF-β1 (10 ng/mL) and pro-ostrogenic supplements [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)] and incubated at 39.5 °C to allow differentiation of cells.

2.2.3. Biological Characterization of Cell-Seeded Braids.

2.2.3.1. Metabolic Activity. The metabolic activity of hFOB-seeded braids (n = 3 per group), experiment repeated twice was determined after 7, 14, and 21 days using the standard MTT assay. Briefly, MTT solution (1:10 ratio in media) added to braided constructs at 37 °C for 4 h was dissolved using dimethyl sulfoxide and absorbance was measured at 560 nm using an iMark microplate absorbance reader (BioRAD). Acellular braids acted as controls.

2.2.3.2. Total DNA Content. Total DNA of hFOB-seeded braids (n = 3 per group), experiment repeated twice was harvested after 1, 7, 14, and 21 days using the standard MTT assay. Briefly, MTT solution (1:10 ratio in media) added to braided constructs at 37 °C for 4 h was dissolved using dimethyl sulfoxide and absorbance was measured at 560 nm using an iMark microplate absorbance reader (BioRAD). Acellular braids acted as controls.

2.2.3.3. Total Collagen Estimation. The total collagen content normalized to DNA was estimated in hFOB-seeded braids (n = 3 per group), experiment repeated twice using Hydroxyproline assay after 7 and 14 days.

2.2.3.4. SEM/EDX. The morphology of hFOBs was monitored on braided constructs after 7 and 14 days of culturing. Briefly, cell-seeded braids were fixed, dehydrated with ethanol gradient, gold coated (EMITECH K550X, UK), and visualized using SEM. EDX was performed to confirm the elemental composition of mineral-like deposition on carbon coated braids (n = 3 per group) using Zeiss EVO 50 high definition SEM and the respective Ca/P ratios were computed.

2.2.3.5. Gene Expression Profiling. After 14 days, the total RNA was isolated from hFOB-seeded braids using RNAasy minikit (Qiagen) as per the manufacturer’s protocol. RNA concentration was determined using a Nanodrop 2000C (Thermo Scientific, Wilmington, USA). The total RNA was reverse transcribed into cDNA using the High Capacity cDNA Synthesis Kit (Life Technologies, Grand Island, USA). The mRNA expression of the target genes was analyzed using an ABI 7500 Fast PCR system (Applied Biosystems, Foster City, USA) according to the manufacturer’s protocol. The mRNA expression of the target genes was normalized using GAPDH as housekeeping gene.

2.2.3.6. Immunohistochemistry. To validate the gene expression data, immunohistochemistry of the Am and Bm constructs was performed on day 14 samples. Briefly, fixed braids were permeabilized, blocked and stained. Dual staining was performed using primary antibodies: antistreptavidin (1:50, Millipore), anti-Col I (1:200, Invitrogen), antiparathyroid-related (1:200, Invitrogen), anti-MMP13 (1:200, Invitrogen) and anti-GLI1 (1:100, Invitrogen) followed by 1 h incubation with secondary antibody cocktails; Alexa Fluor 546 goat antioimmunoglobulin G antibody (1:200, Millipore) and Alexa Fluor 488 goat antioimmunoglobulin G antibody (1:200, Millipore) at RT. Actin staining was performed for 30 min at RT using rhodamine phallolidin (Sigma, cat. no. P1951). 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.

2.2.3.7. 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, we conducted Student’s t test, and probability values in the range of p < 0.05 were noted as significant.

3. RESULTS

3.1. Physical characteristics of Braids. Structurally, the SEM micrographs depicted a flat braided morphology (Figure 1A, B), with each, Am and Bm, consisting of 34 two ply yarns intertwined to make a braided fabric scaffold (Figure 1C, D). The braid angle in both braids was in the range of 35–40 degrees, hence making the structure compact and resulting in lower packing fraction. The twist per inch (tpi) was optimized for both Am and Bm yarns (Table 1) in order to render appropriate mechanical properties for braid fabrication. The fiber diameter in braids varied between the two species, with
Am fibers depicting ~2 times larger diameter (or flat filaments) over Bm fibers. Pore sizes of both (Am and Bm) braids measured using ImageJ were close to that of a human osteon (223 μm). Degummed Am fibroin fibers in braids possessed significantly lower surface roughness (Rq) over Bm (Figure 1E, F), hence possibly rendering a more adhesive surface for osteogenic cells. As the braid was fabricated directly using native silk fibers, but not by dissolution of cocoon and regeneration process, the fibroin protein composition, secondary/tertiary conformations, orientation of β-sheet crystallines were preserved. The resultant rigidity of the braided construct was hence preserved (Table 1), with the values of Young’s modulus considerably close, as reported previously. Tensile moduli of both braids were several orders of magnitude higher than the compression moduli.

3.2. Cell Proliferation and ECM Synthesis. MTT data revealed no significant differences (p > 0.05) in the metabolic activity of hFOBs cultured on the Am and Bm braids throughout the 21 days culture period (Figure 2A). Both the braids depicted a declining pattern in the cell metabolic activity over time. While the decrease in metabolic activity was significant within the first 7 days (~1.5 fold for both Am and Bm), the values became consistent over days 14 and 21. This decrease in the relative levels of metabolic activity of cultured hFOBs with time (Figure 2A) is suggestive of either apoptosis or onset of differentiation.

In terms of cell proliferation, quantitative evidence was established by measuring the DNA content of the hFOB-seeded braids (Figure 2B). However, the pattern of cell proliferation appeared to be reciprocal to the rate of cell metabolic activity. Both the constructs (Am and Bm) depicted comparable DNA values within the initial 7 days of culture. However, by day 14, maximal DNA content was observed in Bm braids, which again declined by ~1.3 fold by day 21, albeit the values were significantly higher (p < 0.05) as compared to Am braids. Although the increased proliferation over time (Figure 2B) exceeded the possibility of occurrence of apoptosis in hFOB-seeded braids, detailed gene and protein expressions conducted further will determine their cell differentiation activity over time.

The total collagen content was estimated in order to measure the difference in the extent of ECM production by the cultured hFOBs on braided constructs (Figure 2C). In Am braids, the value for normalized total collagen content was comparable over the 14 day time period (p < 0.05). Similarly, slight decrease (p < 0.05) corresponding to 1.8 fold from day 7 to 14 was observed in the Bm braids.

3.3. Morphological Analysis of hFOB-Seeded Silk Braids. By day 7, a conspicuous sheath of hFOBs along with ECM components covered majority of the braided surface in both Am (Figure 3A, B) and Bm (Figure 3C, D). Both hFOB-seeded braids depicted conspicuous evidence of high contrast mineral-like deposition (red arrows) suggestive of cell-synthesized hydroxyapatite deposition. Their composition was further confirmed by EDX analysis, with Am braids depicting Ca/P ratio (1.6) (Figure 3G), which is close to the range of native bone mineral (1.67) as compared to Bm with Ca/P of 1.4 (Figure 3H). In Am braids, individual cells adhered onto the braids attained long dendritic processes resembling osteocyte-like morphology (Figure 3A; subset) suggesting the role of the Am surface in accelerating osteocytic phenotype, as seen in our previous study. These osteocyte-like cells were in the range of 10–15 μm, characteristic dimensions of osteocytic morphology. On the contrary, no such cellular morphology was visible on the surface of Bm braids after 7 days of hFOB culture. After 14 days of culture, cells were directly in contact with the Am braids, with cellular morphology typical of osteocytic phenotype, as observed in SEM (Figure 3C) as well as actin-
phalloidin staining (Figure 3E). On the other hand, hFOBs associated with the Bm braids were mostly encapsulated within the cell-synthesized matrix and appeared more elongated (Figure 3D, F; arrowheads). The presence of cells as well as ECM matrix was more prominent, by day 14 extensively spread on the braided surface, covering the underlying yarns and pores of the Bm braids, as also validated by increased proliferation (Figure 2B) and total collagen content by day 14 in Bm braids (Figure 2C).

3.4. Involvement of Hedgehog and PTH Signaling in Regulating Differential Osteogenesis on Silk Braids after 14 Days. 3.4.1. Signaling-Related Regulatory Genes. Protein patched homologue (PTCH) and Smoothed homologue (Smo) (Table 2) are both mediators of hedgehog signaling which operate via activation of Gli family. Once hedgehog binds to PTCH, Smo (which is repressed by PTCH) activates Gli family. Activated Gli1 and Gli2 receptors then initiate Ihh-dependent osteoblast differentiation in osteoprogenitors. Since expression of these aforementioned markers (Shh, Gli1, Gli2, Smo, and PTCH; Figure 4A) was found to be significantly upregulated in the Am-seeded braids by day 14 (Table 2), this indicated a direct involvement of hedgehog-dependent signaling in regulating osteogenesis on Am matrix. Discrete patches of Gli1 were also prominent in the respective
confocal micrographs of Am braids (Figure 4C) hence validating the enhanced protein synthesis in Am braids as compared to Bm braids (Figure 4D). Another hedgehog family member, Sonic hedgehog (Shh), known to regulate bone formation and osteoblastogenesis in cooperation with bone morphogenetic proteins (BMPs), also showed marked upregulation in Am braids as compared to Bm (Figure 4A).

In parathyroid signaling, PTH (parathyroid hormone) and PTHLH (parathyroid hormone like hormone) induce concentration-dependent anabolic and catabolic effects on osteoblasts via PTH1R. The 25-fold upregulation in PTH gene found at the transcriptional and translational level (Figure 4B) in the case of Am braids, as opposed to no expression in Bm, indicated strong influence of parathyroid signaling in regulating osteogenic differentiation of hFOBs on Am. The immunofluorescence analysis further validated the upregulated expression of PTH in Am braids (Figure 4E) as compared to negligible expression in Bm braids (Figure 4F).

### Table 2. Summary of 25 Genes Involved in Hedgehog/PTH Signaling

<table>
<thead>
<tr>
<th>symbol</th>
<th>gene name</th>
<th>stage of differentiation / function</th>
<th>Am</th>
<th>Bm</th>
<th>ratio</th>
<th>ref</th>
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<tr>
<td>1</td>
<td>Runx2</td>
<td>runt-related transcription factor 2</td>
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<td>↑</td>
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<td>54</td>
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<td>↑</td>
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<td>55</td>
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<td>↓</td>
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<td>3</td>
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<tr>
<td>5</td>
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<td>↓</td>
<td>232.3</td>
<td>3,19</td>
</tr>
<tr>
<td>6</td>
<td>IBSP</td>
<td>integrin binding sialoprotein</td>
<td>↓</td>
<td>↓</td>
<td>6.3</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td>PTHLH</td>
<td>parathyroid hormone like hormone</td>
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<td>↓</td>
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<td>56</td>
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<td>8</td>
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<td>↓</td>
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<td>3</td>
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<tr>
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<td>FBJ murine osteosarcoma viral oncogene homolog</td>
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<td>↓</td>
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<td>57</td>
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<td>11</td>
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<td>GLI family zinc finger 2</td>
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<td>↓</td>
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<td>26</td>
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<tr>
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<td>sonic hedgehog</td>
<td>↓</td>
<td>↓</td>
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<td>64</td>
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<td>↓</td>
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<td>65</td>
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<td>↓</td>
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<td>61</td>
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*Down arrow, downregulated; up arrow, upregulated; blank space, indicates no expression. Nonsignificant expression: where fold change of ≤1 was obtained.*

### 3.4.2. Early to Mid Osteogenic Markers

While the expression of all the important transcription factors mediating the process of osteoblastogenesis in hFOB-seeded braids (Figure 5A) showed marked increase in Am by day 14, the expression of the corresponding osteogenic genes was significantly low (Figure 5A). This is probably due to the 14 day time point considered, wherein the expression of late osteoblast/osteocyte markers predominate the osteoblast differentiation cycle as seen in our previous study, while suppressing the expression of early markers concomitantly.

For this reason, vitamin D receptor (VDR) and matrix metalloproteinase 13 (MMP13) markers, both upregulated by Osterix; an osteoblast-specific transcription factor which activates a repertoire of genes during differentiation of preosteoblasts into mature osteoblasts, showed significant upregulation in Bm braids by day 14. Interestingly, MMP13 demonstrated maximal upregulation corresponding to 500 fold increase in Am braids over Bm, which was also evident in the conspicuously stained Am braids with MMP13 antibody.
The expression of Col1A2, an ECM marker, also depicted relatively upregulated activity in Bm braids (Figure 5A), further validated by immunofluorescence analysis (Figure 5B, C), which was in accordance with the measured collagen content at day 14 (Figure 2C). On the contrary, the expression of SPP1 or osteopontin, a phosphorylated glycoprotein secreted to the mineralizing extracellular matrix by osteoblasts, was 9-fold upregulated in Am braids as compared to Bm after 14 days of culture (Figure 5A), as further validated by the immunofluorescence images (Figure 5D, E).

3.4.3. Late Osteogenic Markers. The expression of late (integrin binding sialoprotein (IBSP), PTHLH, osteocalcin (BGLAP)) and postmineralization (FOS) markers, was significantly upregulated in Am braids as compared to Bm by day 14 (Figure 6A). The expression of genes associated with bone calcification; BGLAP and IBSP, corresponded to 3 and 7 fold upregulation respectively in Am braids over Bm braids (Figure 6A). The concrete staining of BGLAP in Am braids (Figure 6B, C) further established the fact that late-osteogenic markers were predominantly evident on Am braids. This data further corroborated with the occurrence of osteocytic phenotype in SEM (Figure 3C, D). FOS, a cell growth regulated gene, which is known to precede terminal differentiation of osteoblasts, was upregulated by 1.1 fold in Am constructs (p < 0.05) as compared to Bm constructs.

Overall, Table 2 summarizes all the genes involved in the hedgehog and PTH signaling pathways and their corresponding activity in the Am and Bm braids cultured with hFOBs over the 14 day time period.

4. DISCUSSION

Bone tissue undergoes constant process of remodeling by a complex interplay of signaling mechanisms (Wnt/β-catenin, BMP, Ihh, PTH) that regulate the process of cellular differentiation. So far, several studies have documented culturing osteoblasts or mesenchymal stem cells on scaffolds in the presence of a constellation of differentiation factors mostly by trial-and-error method instead of rationally simulating the underlying molecular dynamics. As a result,
many of these studies generated rather poor reproducibility due to either donor-to-donor variability, uncontrolled differentiation of cells into phenotypically unstable populations or nonhomogenous chemical, architectural, and textural cues provided by the underlying materials. Moreover, none of the studies attempted to explore the role of the underlying key signaling mechanisms responsible for triggering differentiation toward bone-specific lineage.

Our research focus has been to reveal key osteogenic signaling mechanisms associated with silk-based matrices. Most of the research laboratories either dissolve silk cocoons, or directly isolate from the gland of silkworm to fabricate scaffolds. However, the process of dissolution of silkworm cocoons and

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**Figure 5.** (A) Comparative gene expression analysis of early osteogenic differentiation markers on Am and Bm braids at day 14 (n = 3 per group, p < 0.05 is statistically significant represented by ‘*’). (B−G) Confocal micrographs of Am and Bm braids showing discrete patches of Col1A2 (red), SPP1 (red), MMP13 (green) staining at day 14. DAPI (blue) was used for nuclear staining. The braided fibers stained in blue is due to autofluorescence of silk.
regeneration of fibroin protein can denature some amino acid sequences, or impart irreversible modulation in secondary/tertiary conformations of silk fibroin protein, orientation of crystallites in the direction of silk fiber axis. We reported the deposition of hydroxyapatite crystals with the crystallographic c-axis (002) longitudinally aligned parallel to fibroin fibers long axis, a mechanism similar to biomineralisation of collagen type I in native bone tissue.4 Interestingly, this was only evident in the natively spun textile braids and not 3D porous scaffolds developed from regenerated silk.4 That is why in this study we decided to use braids using natively spun silk fibers.

We have previously reported the involvement of Wnt/β-catenin signaling in regulating terminal osteogenic differentiation of native Am-based textile braids5 and 3D bioprinted Bm silk-based matrices3 using preosteoblasts (hFOBs) and BMSCs, respectively. In this study, we attempted to further explore osteogenic differentiation in silk-based braids by investigating: (i) the role of other signaling pathways that are involved in the process of osteogenic differentiation on silk braids, (ii) material properties of silk braids by comparative analysis between the two popular silk species, Am versus Bm, and their effect on cellular behavior. In summary, our data suggests that culturing hFOBs, in the presence of TGF-β1 and pro-osteogenic supplements, within the native Am silk braid offers long-term cell survival, osteogenic differentiation via hedgehog and PTH signaling3 and terminal differentiation into mature osteocytic phenotype.

We evaluated the expression of cyclins in the constructs to elucidate the progress in cell growth cycle over cultured braids. During progression of the cell cycle, modulation in cyclins expression governs whether the cell would undergo division, apoptosis or senescence, or differentiation. Cyclin D1 (CCND1) acts in the G1 phase of the cell cycle to ensure progression of the cycle.36 Though nonsignificant (<1 fold; Table 2), CCND1 expression was slightly upregulated in Bm braids as compared to Am. This difference was also visible in the proliferation data wherein hFOB-seeded Bm braids showed higher DNA content as compared to Am (Figure 2B). To further elucidate the role of signaling pathways on braided constructs, day 14 time point was chosen as maximal expression of osteogenic-related markers was evident by this time.5 Although the sequence of these events for activating the signaling mechanisms in skeletal bone development are known,3 their role in the context of silk fibroin scaffold matrix is novel. Figure 7 depicts a detailed representation of the two signaling mechanisms (hedgehog and PTH) and their relative expression in the two silk species (Am vs Bm) under evaluation.

Figure 6. (A) Comparative gene expression analysis of late/post mineralization markers in Am and Bm braids at day 14 (n = 3 per group p < 0.05 is statistically significant represented by ‘*’). (B, C) Confocal micrographs of Am and Bm braids showing discrete patches of BGLAP (osteocalcin; red) staining at day 14. DAPI (blue) was used for nuclear staining. The braided fibers stained in blue is due to autofluorescence of silk.

Figure 6.
expressed higher levels of PTH1R, indicating the direct role of type of silk scaffolds in regulating its expression. Gli2, an important mediator for hedgehog-dependent osteoblast differentiation governs the Runx2 expression during osteoblastogenesis. Therefore, the upregulated activity of the master gene (Runx2), then regulates the activity of subsequent osteogenic genes responsible for matrix synthesis, mineralization and terminal differentiation. Until this point, although with relatively lower expression, hFOB-seeded Bm braids depicted favorable levels of osteogenic upregulation. However, the Bm surface did not seem to favor mature and/or terminal differentiation of cultured hFOBs (or at least until the 14 day reported period). This was evident from the nominal expression of PTH and PTHLH genes in Bm braids (Figure 4B).

PTH and PTHrP/PTHLH possess very similar structures for binding and activating their common PTH1R receptor, commonly possessed by PTH/PTHrP target cells including osteoblasts. PTHrP circulates as a paracrine regulator expressed by various tissues (skin, bone, blood vessels, etc.) while PTH is secreted from the parathyroid gland and both play a crucial role in regulating bone formation by promoting osteoblast proliferation, survival, and differentiation. However, their response mostly relies upon the cell source, species, experimental conditions and stage of osteoblast differentiation. Studies have reported that PTHrP aids in cell survival of immature MSCs, however, for mature cells, it is responsible for apoptosis. This may also be a contributing factor toward the decrease in DNA content for Am braids over the 21 day culture period. Because increased expression of PTHLH/PTHrP was observed in Am braids as compared to Bm, this may account for a decrease in DNA content indicative of cell death in Am constructs. Majority of studies have reported PTHrP expression in late osteoblasts and osteocytes, with very low expression in the case of preosteoblasts. Hence, marked increase ($p < 0.05$) of PTHrP in the Am constructs (7.5 fold) over Bm constructs. This was further supported by the immunofluorescence data showing typical osteocytic phenotype in the case of Am braids (Figure 3). Once terminally differentiated, osteoblasts transition into osteocytes and express PTH1R.

Figure 7. Schematic representation of hedgehog and PTH Signaling pathways responsible for regulating differential osteogenesis on Am and Bm scaffolds in vitro after 14 days in culture.
extensive diversification among different species of silkworm silk. In the native mulberry silk (Bm), glycine, alanine, and serine constitute about 82% of the total amino acids, whereas, in nonmulberry Am silk it is about 73%. Therefore, the hydrophilic to hydrophobic amino acid ratio for Am (9.06–9.85) is higher than Bm (5.29–6.22). Matrix wettability strongly governs the adhesion efficiency of hFOBs, with a high rate of cell attachment on relatively hydrophilic surfaces and low attachment rates on hydrophobic surfaces. This cell attachment phase on a given surface occurs within few hours of cell seeding (<2 h). On day 1, metabolic activity and DNA content of Am and Bm constructs was comparable (Figure 2), inspite of different braid properties (Table 1). This may be due to the degumming process of silk also. Degumming process causes harsh surface modifications to silk surface which eventually modify the surface texture (surface roughness, morphology, wettability) and tensile strength of silk fibers. Surface roughness exerts strong influence on cell adhesion. In a study conducted by Setzer et al., cell proliferation in hFOBs was reduced on rough titanium as well as on rough zirconia surfaces. However, differences in cell source and passages, materials, varied fabrication strategies, etc., provide variable results and therefore it may not be appropriate to compare directly with our data.

Contradicting to existing literature, the more hydrophobic and less stiff (Table 1) Bm surface showed significantly increased proliferation by day 14 in our case (Figure 2). Lim et al. compared the expression of osteopontin and type I collagen to compare ECM protein production on hydrophilic (silane-treated quartz) and hydrophobic (silane-treated glass) surfaces. After 6 days, they found that hFOBs on hydrophobic surfaces expressed significantly lower levels of ECM markers over hydrophilic surfaces; however, this difference became negligible after 6 days. This is completely in accordance with our data wherein the day 14 expression of osteogenic markers Runx2, Col1A2, VDR showed relative upregulation in Bm braids over Am braids, albeit with a delayed time period. Now, it is well-known that osteoblasts proliferate more on stiffer matrices than softer matrices. Interestingly, by day 14, due to recovered osteogenic activity on Bm constructs, significantly upregulated levels of cell-synthesized Col1A2 matrix was observed (Figures 3 and 5). Apparently, hFOBs cultured on Bm braids produced their own ECM, which was likely to reinforce the stiffness of the Bm braid due to increased local matrix density and collagen assembly consequently establishing a new mechanical and biochemical microenvironment which lead to increase in cell proliferation day 14 onward in Bm braids (Figure 2). A proof of concept is that this proliferative phase is typically concurrent with the expression of Runx2 and Col markers, which were also significantly higher in Bm braids by day 14 (Figure 5). Because Am braids transitioned into mature osteoblastic/osteocytic phenotype by day 14 (Figures 4 and 5), no significant increase in proliferation was observed (Figure 2).

The phenotypic shift from osteoblast to osteocyte are strongly dictated by the stiffness of the ECM (both material and cell synthesized). Am braids demonstrated moderately higher value of Young’s modulus than Bm braids which may be attributing to enhanced osteoblast differentiation in Am constructs (Figure 6). Although most studies have reported osteoblastic differentiation with respect to different materials, very few have monitored osteocyte differentiation as a function of ECM stiffness. For example, Mullen et al. showed that early osteocytic differentiation (marked by DMP-1 expression) was governed by softer matrices (<300 kPa). However, they used collagen substrates and cultured murine preosteoblasts and therefore cannot be compared to our study.

Within the realm of silk scaffold-based tissue engineering, there are several unexplained issues that might play critical roles in promoting osteogenic responses. For instance, the presence of RGD sequences in nonmulberry silk often have been attributed to superior tissue regeneration by several researchers without convincing evidence. Although the percentage of this RGD peptide sequence in the Am silk fibroin is still debatable, it underlines the need for complete peptide mapping/protein/acid sequence for confirming the quantitative estimation of RGD in Am silk fibroin. Hence instead of stressing the role of RGD sequences only, there is a need to explore other amino acid sequences in Am silk fibroin that might be critically important to trigger osteogenic differentiation. Hence findings of this study may pave the way to identify the underlying reasons behind the differential activation of signaling pathways of silk fibroin-based biomaterials.

On the one hand, we have attempted to develop understanding about how two different silk scaffold material characteristics (chemistry, roughness, stiffness, porosity, orientation, design) generate biological signals and govern cell behavior, proliferation, and differentiation. At the same time, there is unprecedented progress in the use of genetic tools in synthetic biology to reprogram artificial genetic signaling pathways. Combination of these two strategies can further augment transient or permanent activation or deactivation of specific cellular pathways ensuring patient-specific tissue regeneration. Overall, this understanding will not only be beneficial for providing a clinical bone graft substitute using improvised silk-based biomaterials but the knowledge obtained could also be utilized in developing in vitro bone disease models. Such models aid in generating insights into the functioning of developmental pathways and disease progression and possess immense therapeutic potential for screening the efficacy of drugs targeted toward bone disorders.

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**Notes**

The authors declare no competing financial interest.

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