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Cellular Proliferation, Self-Assembly, and Modulation of Signaling Pathways in Silk Fibroin Gelatin-Based 3D Bioprinted Constructs

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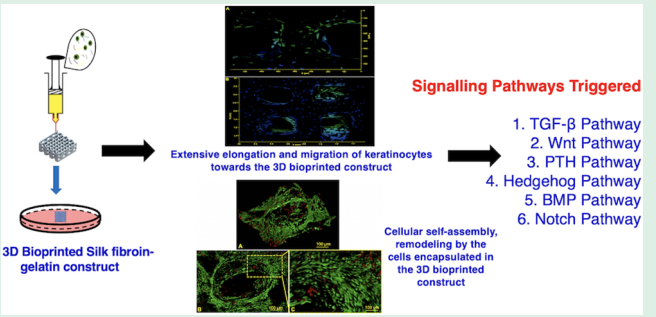
ABSTRACT: Three-dimensional (3D) bioprinting is a highly innovative and promising technology to render precise positioning of biologics together with living cells and extracellular matrix (ECM) constituents. In spite of such enthralling potential, the fabrication of a clinically relevant engineered tissue is quite challenging. A constellation of factors simulating the complex architecture of the native tissue, selection of the "ideal bioink", optimization of the biochemical, mechanical, and topographical functions of the cell-laden printed construct, cellular differentiation, their self-assembly, and remodeling into the desired lineage postprinting present major complications. Keeping this in view, we have attempted to highlight the use of silk fibroin (SF) protein from *Bombyx mori* silkworm as a promising biomaterial of choice for the formulation of bioink owing to its distinct characteristics involving rheology behavior, self-supporting filamentous extrusion, and a suitable biomaterial to achieve resolution printing. Further, we have elaborated on how SF gelatin bioink can in specific regulate the cellular differentiation pathway of progenitor cells, the mechanism of cellular self-assembly, cell migration, matrix remodeling, and self-orientation, leading to the desired tissue-specific construct. How features of bioink and fabrication design aspects can induce *in vitro* tissue patterning and anatomically relevant tissue organization have also been explored in this review. Importantly, we have tried to shift the understanding of bioprinted tissue regeneration from a cell-proliferation-centric and gene-expression-centric point of view to the complex role of the microenvironment present within the bioprinted constructs. We believe that shedding light on these factors would help in achieving the so-called "ideal 3D bioprinted construct" to meet the shortages of high-quality donor tissues for the regeneration of the damaged and diseased ones.



KEYWORDS: *3D bioprinting, silk fibroin, bioink, remodeling, ECM, morphogenesis, scaffold, Wnt signaling*

1. INTRODUCTION

Fabrication of a coveted tissue construct using three-dimensional (3D) bioprinting has become a rapidly evolving technology. Bioprinting enables one to recapitulate the desired tissue architecture by the strategic deposition of biological materials, living cells, and biochemical cues, particularly in a layer-by-layer fashion.[1] Traditional tissue engineering methods failed to develop complex biomimetic assembly, resulting in an oversimplified tissue construct, which offers poor relevance to human physiology (in terms of the architecture, orientation) as well as improper cellular microenvironments (in terms of chemistry and mechanical stiffness).[2] The ability to attain spatiotemporal directional positioning of different cells has made 3D bioprinting the ideal strategy to fabricate anatomically relevant living cell-laden 3D structures *in vitro*.[3] Despite such alluring potential, obtaining clinically relevant engineered tissues via bioprinting is still a challenging proposition.[4]

There are still many unmet needs in the field of bioprinting. A bioprintable substance or the hydrogel material that is used for encapsulation of cells in 3D bioprinting is popularly known as "bioink", which plays a key role in defining cellular microenvironments. Although several biomaterials have been explored as the bioink of choice for the regeneration of the impaired or diseased tissue, there is no universal biomaterial that can fulfill the essential requirements for all types of tissues. Moreover, many of them are incompatible with the current bioprinting technology.[5] An ideal bioink should display appropriate rheological, mechanical, and biological properties of the target tissue, which are crucial to warrant the precise

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functionality of the bioprinted organ and tissues.[6] Several factors must be taken care of to achieve good printability that includes rheology of the bioink, viscosity, time of gelation, filament stability, along with cytocompatibility.[7,8] Besides the diameter of the nozzle, the shape, length, and extrusion pressure must be assessed to guarantee that the shear pressure should have minimal influence on the cell survival.[9,10]

Other than the optimization of bioink, 3D bioprinting is associated with several other challenges. This encompasses (a) achievement of instant cell attachment and preservation of the cell viability for the long term.[11] (b) A proper understanding of how these bioink materials govern the long-lasting cellular functionality is still lacking. Whether the encapsulated naive/progenitor cells can self-assemble during/after differentiation and remodel themselves into the desired specific lineage within the printed construct and how such constructs might expedite tissue regeneration remain elusive. (c) Another principal feature of resolution is the positioning of the cells spatially within the construct such that after migration whether the cells orient themselves randomly or into a particular tissue type is a big question. For example, when angiogenic vessel segments were printed in a parallel orientation, instead of following the same direction of the microvasculature so-formed post-implantation, cells migrated randomly and did not maintain this prepatterned topology.[12] Hence, how to induce neovascularization and achieve optimal blood flow is still an unsolved issue. (d) Most of the bioprinting studies have advanced, employing a single-cell type to produce a homocellular tissue construct; however, native tissues are heterocellular and made up of multiple cell types, anatomically patterned in highly complex structures. The involvement of multiple cell types becomes crucial while fabricating functional tissue components. As a result, bioprinting multiple cell types necessities in-depth knowledge of the ideal culture conditions encompassing the correct medium as well as reagents to assist their growth and behavior.[13]

Given this, we believe that silk fibroin (SF) protein from the *Bombyx mori* silkworm[14] can serve as a potential candidate for the preparation of the so-called "ideal bioink", due to its inherent spinnability and cytocompatibility and ability to modulate certain cellular signaling pathways.[15,16] The reason behind the use of SF as a bioink of choice can be attributed to the fact that the shear force can be transmitted in regenerated silk by applying pneumatic pressure, which alters the secondary conformations (from random coil to crystalline β-sheet) such that the sol−gel transition can be imparted. That is a major advantage of SF bioink over others, as it evades the need for toxic organic solvents and high temperatures which may affect the cell viability.[17] Apart from the shear stress, various other methods such as freezing treatment,[18] cross-linker and salt addition,[19] alcohol solution,[20] and pH value adjustment[21] can also modify the secondary conformations in silk protein. The amphiphilic nature of silk with the surface tension of 0.04−0.07 N m^{-1} helps to generate the flowability of ink under shear stress.[22] The degree of β-sheet content[23] of silk bioink can regulate *in vivo* degradability and mechanical properties of the construct.

There are several recent reviews on 3D printing[24,25] and 3D bioprinting,[1,26] using different biomaterials of choice. Most of them focus on the processing methods, optimization of rheology, and print fidelity, employing a variety of bioink. So far, much emphasis has been devoted to enhancing cell growth and *in vitro* maturation, in terms of gene expression, within the bioprinted constructs. However, efforts to understand the mechanism of cellular self-assembly within the bioprinted construct remain uncontrolled. How features of bioink and fabrication design aspects can induce *in vitro* tissue patterning and anatomically relevant tissue organization have not yet been explored. There is a need to shift the understanding of bioprinted tissue regeneration from a cell proliferation-centric and gene expression-centric point of view to the complex role of the microenvironment present within the bioprinted constructs. Therefore, in this review, we have attempted to highlight the challenges involved in 3D bioprinting and the use of SF bioink from *Bombyx mori* as a suitable biomaterial to achieve high-resolution printing. Further, we have elaborated how SF gelatin bioink can regulate cell migration, matrix remodeling, self-orientation, and signaling pathways of progenitor cells, leading to a desired tissue-specific construct. We believe that shedding light on these factors would help in achieving the so-called "ideal 3D bioprinted construct" to meet the shortages of high-quality donor tissues for the regeneration of the damaged and diseased ones.

2. CHALLENGES IN BIOPRINTING WITH REGENERATED SILK SOLUTION AND SILK BIOINK RHEOLOGY

Regenerated silk alone as bioink might not meet all the necessities of 3D bioprinting. Some of the challenges encountered while using regenerated silk for bioprinting are summarized below:

- Shear-thinning properties of silk fibroin solution have been observed at lower concentrations <20% which is not optimal for printing. On the other hand, at concentration >20%, it shows Newtonian properties[16] even after increasing the shear rate. It is due to high entanglement and limited mobility at a high concentration which reduces their ability of SF polymer chains to self-align.
- The SF chain when exposed to shear force >100 s^{-1} during printing results in nozzle clogging, restricting the smooth flow due to the transition from the random coil to the β-sheet by shear-induced crystallization.[27] To overcome the instant solidification at the nozzle, a low shear force can be applied, followed by deposition in an 80−90% methanol/ethanol coagulating bath to support the solidification immediately after extrusion and fabricating the complex structure. However, this cannot be used for bioink as cells cannot survive in an alcohol bath.[16]
- SF protein does not contain putative cell attachment motifs, which can be compensated by using natural ECM components, gelatin or peptides, or other biofunctionalized components to enhance the cell attachment and differentiation.[27]
- Removing sericin from silk fibroin brings about the random degradation of the fibroin chain, depending on the temperature and extent of degumming[28] which further affects the viscosity of the ink.[29,30] The low viscosity of bioink requires cross-linkers, which in turn may affect the embedded cells.[12] This problem could be overcome by using native silk protein directly from the silk gland,[31] but their availability is limited and might cause ethical concerns.

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Optimizing the intrinsic physical properties (surface tension, viscosity, and rheology) along with the material properties (swelling ratio) is the major challenge in 3D bioprinting with SF protein bioink. Hence, for achieving high printing accuracy and resolution, all these inherent limitations of silk bioink must be considered as they may critically affect the printability, porosity, and geometry of the printed construct. Silk rheology depends on various factors, and one of them is the source of silk protein.[32] During reverse engineering (degumming, dissolution of cocoons, and reconstitution by removal of lithium bromide) of silk fiber, the silk solution undergoes a β-sheet transformation by disrupting the water of hydration through shear force,[33] subjecting to temperature[34] or pH,[21] the presence of ions[35] or macromolecular crowders,[36] application of sonication/electric field,[37] etc. Instant gelation occurred by immersion in alcohol,[27] whereas slow gelation happened by annealing water vapor.[34]

Although modifications of the physiological environment and silk rheology within the silk gland are well understood, very little is known about the effect of extensional flow fields on the native silk protein.[38] During 3D bioprinting, extensive stretching is not applicable, but only gelation kinetics controls the flowability and solidification of the construct. Hence, the absence of elongational viscosity might cause localized disruption of flow. Two other factors that play a major role in governing the rheology of bioink are the storage modulus G' and loss modulus G''. Loss factor tan δ is another critical factor, which is defined by G''/G'.[39] The hydrogel behaves like a solid when $G' > G''$ or tan $\delta < 1$, and it flows like a liquid when $G' < G''$ or tan $\delta > 1$. In general, in 3D printing, just before and during extrusion, G'' should be higher than G'. After extrusion, G' should exceed G'' by self-aggregation or the cross-linking method. At higher frequencies, the viscoelasticity is higher in native silk solution, while at lower frequencies $G' < G''$[40] (also known as crossover point of the modulus). This crossover was found to occur at angular frequencies between 1 and 10 rad s^{-1}.[41,42] The fibroin polymer chains may get stretched while injecting through the micronozzle tube, resulting in extrudate swell (due to release of stored elastic energy). Extrudate swell must be optimized to encourage smooth flow and to prevent dripping.

We employed SF gelatin (SF-G) bioink, which not only is directly printable but also could easily be cross-linked *in situ* with no cytotoxic effect.[43] The advantage of using gelatin with SF was that it exhibited thermoresponsive behavior even without cross-linking,[43] having a sol-to-gel transition at 22.5 °C[44] essential for extrusion-based bioprinting in fabricating 3D structures. However, cross-linking of the printed scaffold is required to achieve stability under physiological conditions.[43] Noteworthy, at a broad concentration range, SF-G blends exhibited shear thinning behavior, particularly due to the emergence of an interpenetrating gel network via physical cross-linking. Besides, differences among silk and gelatin concerning the isoelectric point could be another probable reason for bringing about the formation of physical gel.[45] In another study, SF-G was used with polyol (such as glycerol, 1,3-propanediol, 1,2,6-hexane triol, adonitol, or erythritol) in a 3:1 ratio to affect the degree of β-sheet optimization of the mechanical properties. It was observed that 1% of glycerol increased the gelation of the SF-G blend bioink.[46]

Another less addressed aspect of bioprinting is how the addition of cells can drastically affect the rheological behavior of bioink. At varying concentrations, viscosity differs due to contrasting cell-to-cell and cell-to-biopolymer interactions.[47] We observed a sudden alteration in viscosity in the SF-G bioink both prior to and post encapsulation of mesenchymal stem cells at the time of flow that affected the print fidelity (data unpublished). One must consider the thixotropic nature of the cellular cytoskeleton and cytoplasm[48] (time-dependent reduction in viscosity with enhanced shear rate) which may affect the resultant rheological properties of bioink.

Taken together, we have highlighted the overall perception of the requirement of 3D printing using SF bioink. The primary understanding of the supramolecular functionality of the bioink can not only induce *in vitro* tissue patterning but also help in achieving the fabrication of anatomically relevant tissue organization via resolution printing. Hence, we believe that SF bioink can fulfill the unmet demand for a novel bioink for the fabrication of tissue analogues.

3. CELL MIGRATION AND MATRIX REMODELING POSTPRINTING

To obtain biomimetic and functional tissue-like constructs, it is vital to take into consideration the various developmental stages the bioprinted construct needs to undergo that include cell viability, cellular functionality *in vitro*, construct implantation, integration, as well as remodeling *in vivo*. However, postprinting tissue maturation is a time-dependent and important initial aspect in the extent of cell proliferation. Random, uncontrolled cell migration within the bioprinted construct can destroy preplanned design aspects.

In human tissues, cell proliferation is majorly guided by ECM composition and orientation. Hence, it may be anticipated that the matrix component derived from the decellularized tissues might be a potential bioink and may dispense a rich biomimetic environment crucial for vital cellular activities such as cell proliferation.[49] However, during the decellularization process, whether the decellularized matrix induces any alterations in the secondary conformations of fibrous ECM, even though the entire ECM architecture appears to be preserved histologically, is a big question.[50] Any conformational changes in the collagen may expose certain hidden antigenic motifs which may evoke an immune response. Also, the presence of an α-gal epitope in the decellularized construct may lead to calcification and degradation, ultimately resulting in graft rejection.

During migration, chemotaxis (gradient of chemical compositions), durotaxis (gradient of stiffness),[51] and haptotaxis (gradient of cellular adhesion sites by integrins or focal adhesion points) govern whether cells will migrate randomly or in a specific direction.[52] During chemotaxis-induced migration, cells release matrix-degrading enzymes (e.g., matrix metalloproteinases or "aggrecanases" a disintegrin and metalloproteinase with thrombospondin motifs) or endocytose these factors by cellular receptors. During haptotactic migration, cells release cytokines and chemokines, which ionically immobilize on the ECM. Then the migratory cells remodel the pericellular ECM and form gradients of adhesion sites to migrate.

Silk-based bioinks are known to play a key role in supporting cell proliferation of the bioprinted construct. Its degradation rate can be precisely controlled, varying from weeks to years following implantation *in vivo*, which in turn depends on the scaffold type, the cross-linking agent, crystallization, and other factors involved.[53,54] Initially, when cells are encapsulated within the bioink, they are rounded ("amoeboid"), but with

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time cells may assume elongated ("mesenchymal") morphology and start to migrate (Figures 1 and 2). Rho GTPase

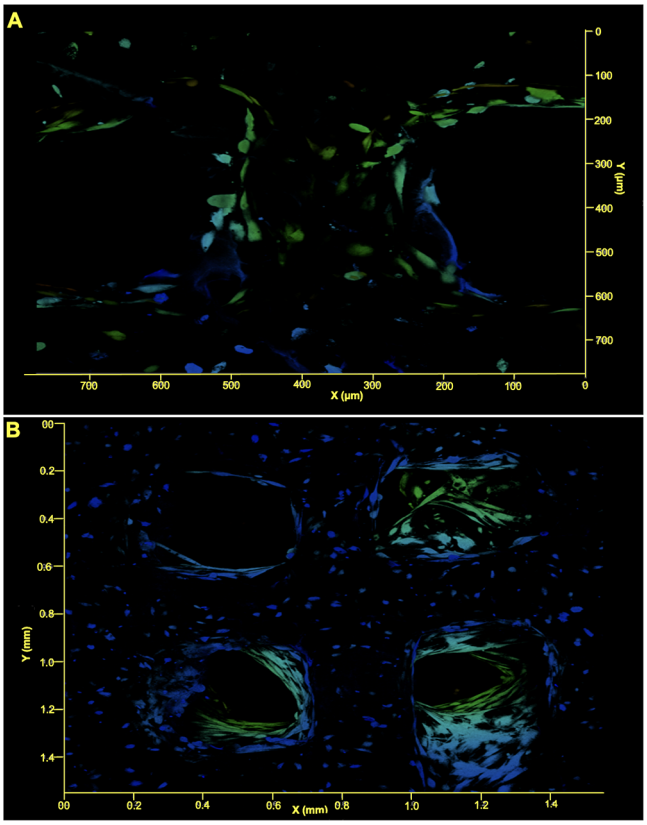

Figure 1. After an injury to human skin, keratinocytes at the wound periphery get activated and retrogress from terminal differentiation. Then they display migratory ability, change cellular polarity, reorganize the cytoskeleton, and facilitate wound closure. (A) Uniform distribution of keratinocytes can be seen in the silk-gelatin hydrogel bioink in a bioprinted full-thickness skin construct at day 3. (B) On day 14, extensive elongation and migration of keratinocytes were noticed toward the pores of the bioprinted construct, resulting in almost entirely covered pores. Substantial extension of the cytoskeleton and gradual closing of pores are akin to the keratinocyte migration during the re-epithelialization step in injured skin. Proteomic analysis reveals the notable similarity of the bioprinted construct to the native human skin, suggesting the pathways involved in skin development.[58]

controls cell contraction and rounded morphology,[55] whereas Rac GTPase drives cell protrusion, elongation, and migration due to increased actin polymerization. For example, in one of our studies, we reported that RhoA expression in only silk constructs was upregulated by 3.5-fold in 2 weeks, while there was a decrease in the expression in the fourth week. SF-G constructs displayed a 2-fold increase by 2 weeks that remained persistent until the fourth week. Contrastingly, Rac1 did not follow this trend, and there was no expression until the second week. Importantly, an upregulated expression of 2.2-fold of Rac1 was observed in SF-G constructs in comparison to only SF constructs.[27]

The most important aspect of cell migration is the capability of the cell to polarize within the hydrogel bioink. During migration, cell polarization can happen at a single cell level or a cluster of cells migrating together. Cell encapsulation in hydrogel has several constraints in proliferation, which include

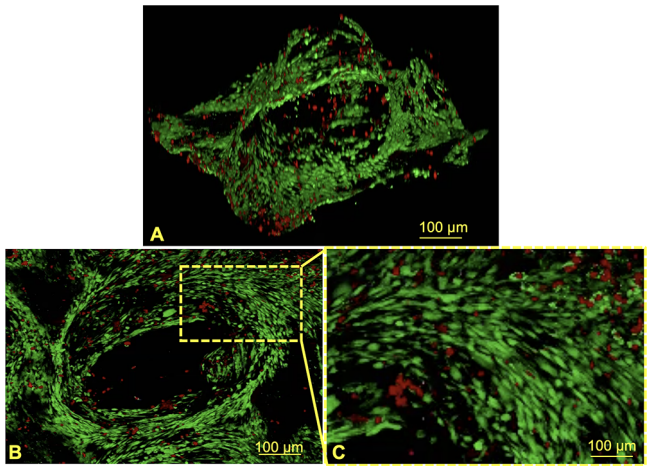

Figure 2. Live/dead images of bone marrow stem cells encapsulated in silk-gelatin-based 3D bioprinted constructs, cultured in proliferation media. (A) A complex structure of the entire 3D bioprinted construct. (B) Initially all the encapsulated cells were dispersed. After 2 weeks, cells found to get self-assembled remodeled their pericellular matrix and showed cell polarity. (C) Magnified image of (B) where spindle-shaped cells can be observed that orient parallel in a particular direction.[73]

constricted cell interactions, due to the entrapment of the cells within the hydrogel. As a result, there may be an inefficiency of the cells to stretch or spread or of migration, colonization, and proliferation of the immobilized cells, specifically at elevated concentrations of the hydrogel.[56] Therefore, care must be taken to provide an ideal environment for the optimum development of the bioink appropriate for the cell deposition by circumventing any secondary components or the parameters postprocessing that may result in cellular impairment. Directional migration *in vivo* is coordinated by extracellular cues that must be incorporated in the bioprinted construct. For example, the orientation of a bioprinted construct can significantly affect cell alignment, directionality, and polarization.[57] In full-thickness 3D-bioprinted skin tissue constructs using SF-G bioink laden with fibroblast and keratinocytes, certain design aspects were introduced to recapitulate undulated morphology between the dermis and epidermis and regional cellular orientation.[58] Although the fibroblasts remain encapsulated within the SF-G bioink, the keratinocytes gradually exhibited front-rear polarity, whereas many of them started to move out of the construct as early as Day 7. However, on Day 14 there was intensive migration of the keratinocyte toward the pore which continued even until Day 21, thus covering the pores almost entirely (Figure 1). On the other hand, cells in alginate-based constructs did not support cell adhesion, whereas the SF-G-based matrices aided in the cell adhesion, migration, as well as multilineage differentiation of encapsulated stem cells.[43] Apart from this, cellular mobility can be regulated by protease secreted by the resident cells to drive this remodeling process which commences biomaterial degradation, as well as invadopodia or actin-driven migration mechanisms. The absence of any toxic byproduct of degradation is desirable since it can further influence the remodeling process. Hence, the physical, chemical, and mechanical degradation properties of the scaffolds should be optimized before printing.[59] Despite tremendous advancement in the field of matrix stabilization and its properties employed in 3D bioprinting, only a handful could qualify until the

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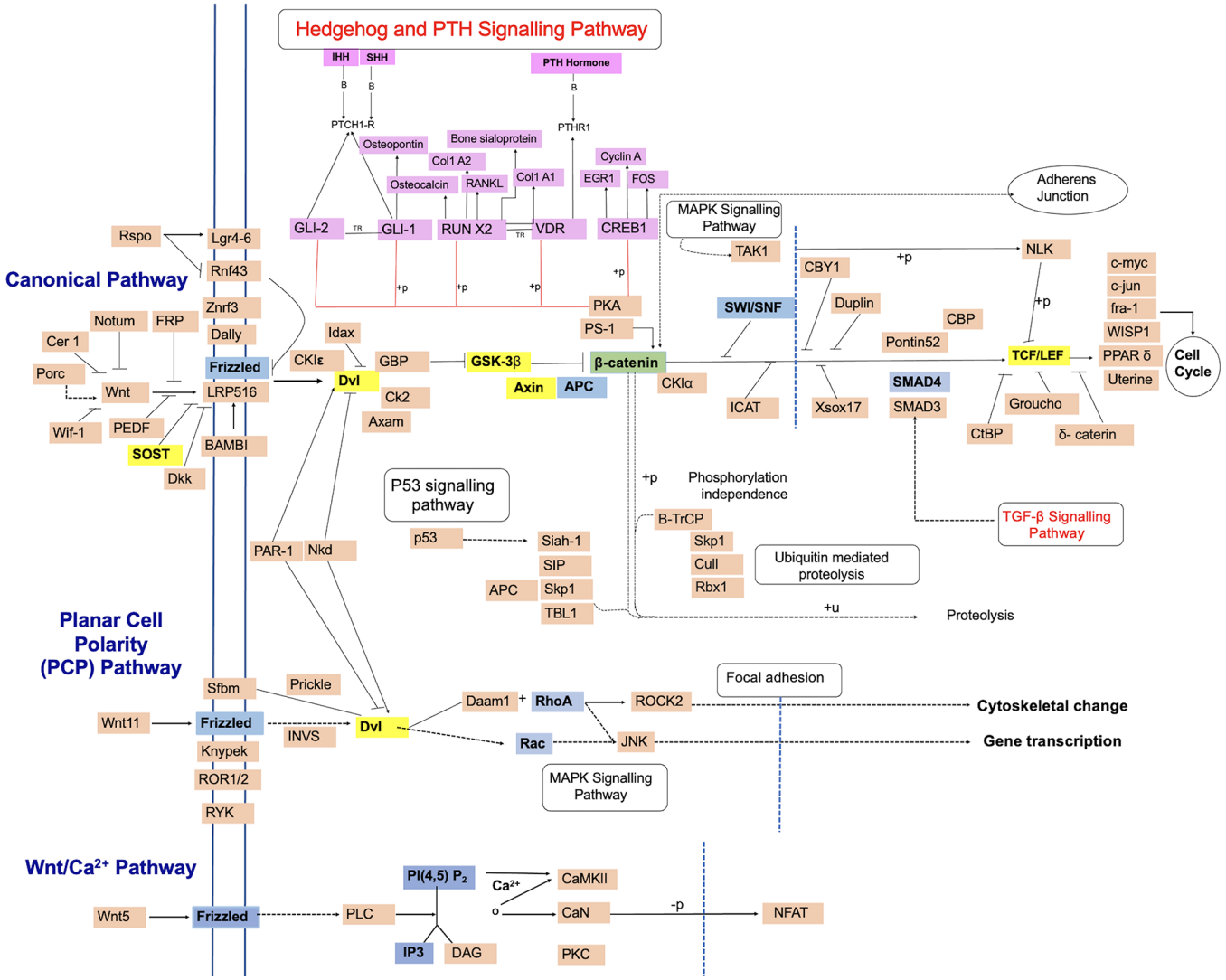

Figure 3. Overview of canonical and noncanonical Wnt signaling pathways responsible for regulating differential chondrogenesis and osteogenesis in *Bombyx mori* (BM) silk-based matrices. Blue boxes represent the genes involved in chondrogenesis. Those involved in osteogenesis are shown in yellow. Pink boxes indicate the genes involved in Hedgehog and PTH signaling. Green is used for both cartilage and bone formation based on the shreds of evidence we generated so far.[66,71] Upregulated expression of β-catenin, frizzled receptors points toward the activation of canonical signaling,[92] whereas that of PI(4,5) P_2(4,5-bisphosphatephospho diesterase β-3 and 4 triggers the noncanonical pathway. Upregulated expression of adenomatous polyposis coli (APC) protein acts downstream of Wnt/β-catenin signaling, whereas increased Rho, Rac expression in BM matrices controls the function of chondrocytes during differentiation.[27] Contrarily, downregulation in the expression of the SWI/SNF complex stimulates β-catenin protein levels and increases its nuclear accumulation in the Wnt/β-catenin signaling pathway.[93] Glycogen synthase kinase 3 beta (GSK-3 β) engages dishevelled (Dvl) proteins to the plasma membrane such that they get polymerized and activated, resulting in osteoblast differentiation and bone mineralization, and are involved in the early stage of bone development along with Axin and β-catenin.[23] Accumulation of β-catenin during bone differentiation results in its interaction with the LEF (lymphoid enhancer factor) and TCF (T-cell factor) family of transcription factors, resulting in bone differentiation and apoptosis of osteogenic cells in silk matrices.[71] The ubiquitination of β-catenin is inhibited by PKA (Protein kinase A), causing the accumulation of β-catenin and activation of the Wnt signaling pathway. PKA, in turn, acts as the crosstalk and activates the Hedgehog and PTH signaling pathway responsible for regulating osteogenesis in BM scaffolds,[94] where + p is phosphorylation, + u ubiquitinylation, and TR transcriptional regulation.

preclinical testing phase. Hence, further experiments are needed to have a piece of deep knowledge regarding the SF-G bioink and others coupled with this.

Taken together, if a 3D bioprinted construct is to give rise to new functional tissue, cells encapsulated within the bioink must proliferate and gradually replace the printed material components with a newly synthesized ECM simulating the native cell niches and pathological tissue morphologies. This was carried out under precisely controlled conditions, especially in bioreactors, so that optimized alteration of the stimuli can be provided to precisely regulate the cell migration process. This would thus fulfill the achievement of the desired architecture of the native ECM and would provide proper cell encapsulation, support, and protection.

4. CELLULAR SELF-ASSEMBLY

3D bioprinting holds great promise over the traditional tissue engineering practices due to its potential to be incorporated into a variety of biochemical, structural, and mechanical cues spatially for guiding the cellular orientation, regulation of cell−cell adhesion, and warranting a highly ordered and complex

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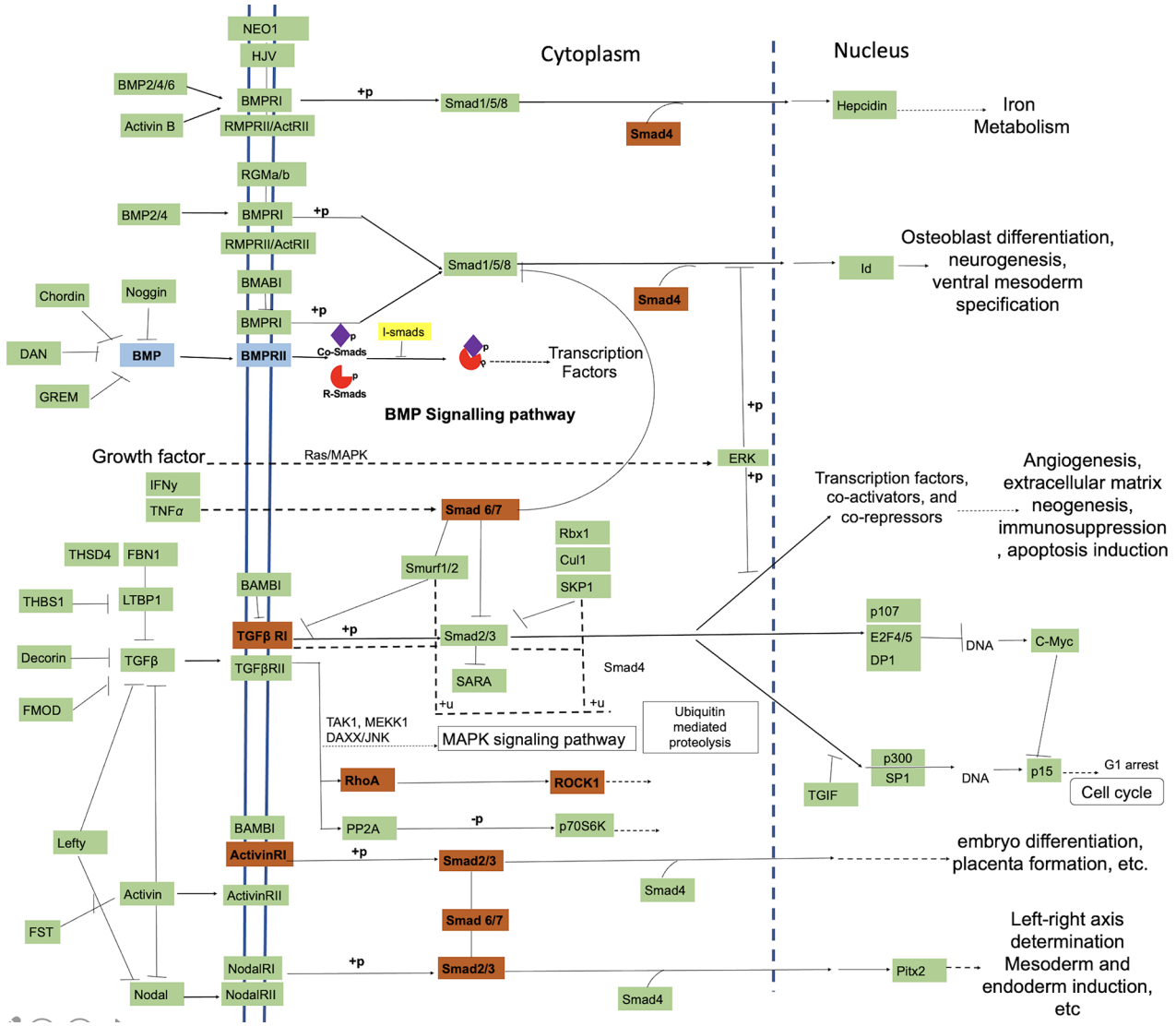

Figure 4. Overview of the TGF β-signaling pathway for regulating differential chondrogenesis and osteogenesis in *Bombyx mori* (BM) silk-based matrices. The orange box represents the genes involved in chondrogenesis, whereas blue boxes represent the genes involved in both chondrogenesis and differential osteogenesis based on the pieces of evidence we generated so far.[78,2] GDF6 (growth differentiation factor 6) is a member of the BMP family. Decreased expression of this in BM matrices was observed, whereas an increase in its expression was obtained upon addition of TGF β3, resulting in the repression of hypertrophy and stimulating chondrogenesis in silk matrices (data unpublished). Overexpression of RhoA/Rock suppresses alkaline phosphatase and mineralization[15] in BM matrices. The presence of gelatin in 3D bioprinted constructs induces cell migration (Rho).[15] BMP acts as the crosstalk between the TGF beta and BMP signaling pathway, resulting in enhanced osteogenesis in silk-based matrices.[23] SMAD7 induces inhibition of the TGFβ pathway via multiple mechanisms, which also involves moderating degradation of the type I receptor. In general, SMAD 7 interferes with both TGFβ and BMP signaling,[95] whereas overexpression of both SMAD6 and 7 inhibits BMP-mediated osteoblast and chondrocyte differentiation,[96] where + p is phosphorylation and + u ubiquitinylation.

network system to form anatomically relevant tissues.[60] Several developmental mechanisms enable a higher level of cellular development which involves alterations in cellular behavior such as apoptosis, mitosis, cell adhesion differentiation, etc., which play a crucial role in the emergence of the specific organization of cells in a three-dimensional state known as a "pattern".[61] For example, while printing a tissue, we mix the cells and bioink in a specific proportion, and every cell encapsulated within the bioink initially remains separated from the other. To recapitulate the pattern during development, they remodel their surrounding matrix and undergo morphogenesis and patterned cellular orientation (Figure 2). The specific pattern of developing human embryonic tissue takes place by two main mechanisms: (a) due to release of morphogens, which affects the responding cells and activates differential expression of genes in those cells, and (b) every cell during its developmental phase experiences a series of changes in their transcriptional state until an entire repertoire of cell types becomes specified.[62] These phenotypically identical cells undergo self-assembly to develop a specific domain. This is followed by the development of stable boundaries among those domains, which is crucial for biological functionality. Many times both mechanisms of tissue patterning act together.[63] It is still a major challenge in how one can recapitulate such complex and tightly regulated mechanisms by strategically encapsulating cells within the tailor-made hydrogel bioink, which would permit patterning of cells, nevertheless ensuing ECM formation, the hydrogel matrix digestion, and degrada-

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tion. An often-ignored part in bioprinting is how overall structural design cues of the construct, porosity, and pore geometry of the scaffold and nature of the bioink hydrogel play a critical role in stimulating the formation of tissue self-organization or cellular self-assembly and patterning.

To develop a full-thickness skin tissue model,[58] the most striking questions were whether our developed 3D printed architecture would be successful in recapitulating the undulated morphology of the rete-ridges. On the other hand, uncontrolled cellular self-assembly may destroy the prede-signed structure and the morphogenesis required to form a native skin-tissue-like construct. Confocal microscopic images of cells stained with calcein within the bioprinted construct revealed the conservation of the undulated morphology during the entire duration of the culture.[58] The morphological transition of the fibroblast to spindle shape from round in the dermal layer was an indication of the proliferation of the embedded fibroblast without any hindrance.[64] The unique fibrillary deposition of fibronectin, which plays an important role in morphogenesis,[65] was noticeable around the cells at Day 14 of the 3D bioprinted construct, similar to the one found in the native human skin. Immunofluorescence study with cytokeratin 1, a marker for modulating inflammatory networks and skin barrier functioning, suggested terminal differentiation and maturation of the cultured keratinocytes, thus resulting in the growth of the cornified epidermal layer, stratum corneum, at the air−liquid interface. Significant expression of genes involved in ECM development, as well as the differentiation proteins, corroborates that the embedded cells are functional in the 3D bioprinted construct and are capable of skin development akin to the native skin tissue. This study offers an example of the formation of a so-called "ideal tissue", where SF bioink not only supported the cell migration and proliferation but also promoted the process of cellular self-assembly to recapitulate the morphogenesis pattern taking place *in vivo*.

5. MODULATION OF OSTEOGENIC AND CHONDROGENIC SIGNALING PATHWAYS

Lately, a wide variety of materials are being explored as a bioink for printing different types of tissues. It becomes crucial to apprehend how a variety of biomaterials can regulate the cellular signaling pathway to accelerate tissue regeneration. For the first time, we have illustrated that SF can inherently upregulate the Wnt pathway in mesenchymal progenitor cells and/or primary chondrocytes.[66,15] The Wnt signaling pathway, one of the vital signal transduction pathways, is an essential transcriptional element that manifests in the presence of transforming growth factor-β1 (TGF-β1) (Figure 3). As a result, for tissues and organs in which the Wnt signaling pathway is upregulated *in vivo* during embryonic developmental stages, the use of SF protein would be rational/justified for tissue engineering of those tissues. Exceptional results have been obtained by using silk in various tissues such as skin,[58] cartilage,[66] intestines,[67] etc. Wnt signaling pathways play a crucial role in the development of various tissues as well as immune response. Wnt signaling pathways can be divided into two categories, based on the downstream events and availability of the ligands—the canonical (β-catenin dependent) and noncanonical Wnt pathways (β-catenin independent). Activation of the canonical Wnt pathway results in the determination of body axis, induction of proliferation and differentiation of cells, and morphogenetic signaling. The noncanonical Wnt signaling pathway is classified into two categories, Wnt/planar cell polarity (PCP) and Wnt/Ca^{2+} pathways, which regulate polarization of cells and assembly of ECM (particularly fibrillin and fibronectin) (Figure 3).

Several signaling pathways such as the bone morphogenetic protein (BMP)/transforming growth factor-beta (TGF-β) (Figure 4), notch, Wnt, insulin-like growth factor (IGF), and platelet-derived growth factor (PDGF) are responsible for bone formation and remodeling. Noteworthy, some of these are associated with controlling osteogenesis on SF-based scaffolds. For example, osteoblast differentiation is negatively regulated by the Notch pathway and also regulates the differentiation of the osteoclasts negatively.[68,69] Silk fibroin peptides having low molecular weight ensued upregulated expression of ALP and RUNX2 mRNA by inducing a remarkable downregulation in the Notch signaling in bone marrow cells.[70,23] On the other hand, the Wnt/β-catenin pathway was found to regulate terminal osteogenic differentiation in both textile braids and 3D bioprinted matrices by employing preosteoblasts and BMSCs, respectively.[71] Besides, the PTH (parathyroid hormone) and hedgehog signaling pathway are reported to play a key role in regulating differential osteogenesis in silk-based scaffolds[72] (Figure 3).

To translate these insights into the modulation of signaling pathways in the context of bioprinting, we exploited the use of SF-G bioink laden with mesenchymal stem cells (TVA-BMSC) for the development of bone tissue equivalents.[73] Here we focused on the prospects of developmental biology to induce osteogenic potentiality of the 3D bioprinted SF-G constructs *in vitro*. Gene expression of RUNX2, COL 1, early differentiation markers, and ALP, ON, and OPN, genes involved in the osteogenic differentiation of mid- and mid-to-late stage, and the terminal osteocytic genes in the BMSC-laden bioprinted construct closely mimic the gene expression pattern *in vivo*. It is worth mentioning that our bone marrow mesenchymal cell (BMSC) laden SF-G bioink could trigger the indian hedgehog (IHH) and the canonical Wnt/β-catenin pathway during the differentiation of TVA-BMSC osteogenically (Figure 3). Moreover, further supplementation with T3 (triiodothyronine) and mimicking the route of endochondral ossification (3 weeks of the chondrogenic differentiation, followed by 2 weeks of osteogenic differentiation) can lead to the activation of PTH, Wnt/β-catenin, and IHH pathways, thus, in turn, enhancing the mineralization and osteogenic differentiation capability of the BMSC. Taken together, our study was the first to report the successive signaling cascades occurring during the transdifferentiation of osteoblasts from chondrocytes following endochondral ossification.

To further ameliorate our understanding about modulation of signaling pathways, we coupled Ca^{2+} ions with SF-G bioink for inducing osteogenesis of human bone marrow cells harvested from healthy donors and compared the effect of the local cellular availability of calcium ions in the construct vs that added in the media by evaluating the gene expression pattern of osteogenic markers.[74] This study reported strikingly valuable knowledge regarding the importance of the SF-G-$CaCl_2$ bioink in regulating the expression of a large number of proteins and signaling pathways such as the BMP signaling pathway, β-catenin, and PTH signaling pathway, which are critical in bone regeneration. The sustained calcium release at a defined dose from the bioink linked by ionic and covalent interaction and the optimized concentration manifests it to be appropriate for 3D bioprinting of an osteogenic construct.

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Table 1. Comparative Analysis of Silk Fibroin Protein Bioink Based 3D Bioprintied Construct versus Other Polymeric Bioinks

S. no.	type	cell migration	cellular self assembly	regulation of signaling pathways	challenges
1	silk fibroin	a. When mixed with gelatin, upregulated expression of Rac1 of around 2.2-fold was observed in SF-G constructs in comparison to only SF constructs, which is responsible for cellular migration[75]	a. Substantial migration of cultured keratinocytes occurred at Day 14, which carried out until Day 21 with enhanced longitudinal tensions, resulting in almost covered pores[58]	PTH,[72] hedgehog,[72] Wnt,[66,77] TGF-β,[78,2] BMP,[23] and notch[68] signaling pathway found to be drastically modulated in presence of fibroin protein	a. Optimizing the intrinsic physical properties (surface tension, viscosity, rheology) along with the material properties (swelling ratio)
		b. Enhanced cell attachment, proliferation, and migration were observed when dermal fibroblast cells and umbilical vein endothelial cells were cultured on micro/nanofibrous networks into porous SF scaffolds[76]	b. BMSC encapsulated in SF-G constructs developed new cell clusters by cellular self-assembly via modification of cell polarity, cell-ECM, and cell−cell interaction resulting in morphogenic features akin to native cartilage[15]		b. Frequent nozzle clogging during printing at a shear force >100 s^{-1}
					c. SF protein does not contain putative cell attachment motifs
					d. Mixing with another polymer required to obtain specific biofunctionality
2	agarose	a. Agarose is not so cell-friendly like SF, due to the poor cell adhesion, which in turn causes limited rate of cell proliferation and biosynthesis[79]	a. Instead of a spread morphology, cells retained a round morphology[80]	Not investigated	a. Difficult for inkjet printing due to its viscous nature and temperature requirement, which may result in nozzle clogging[81]
		b. Insignificant cellular adhesion and spreading indicate it to not suitable for cell culture, but it serves well for a 3D culture of cellular aggregates in the form of mold material[80]			
3	alginate	a. Increased gene expression levels with Col2 and Sox9 was observed in both alginate sulfate tyramine and alginate tyramine hydrogels encapsulated with chondrocytes in comparison to the chondrocytes grown in 2D[82]	a. On Day 1 the bioprinted chondrocytes manifested round morphology, whereas they became elongated by Day 28 of culture when amine-presenting silica nanoparticles were added to oxidized alginate bioink[83]	NF-$\kappa\beta$,[84,85] MAPK[86] signaling pathway	Cellular adhesion is low, with slow degradation properties which may result in poor cellular differentiation and proliferation[87]
4	collagen	a. Majorly, cell migration into the collagen matrix is brought about by angiogenic invasion and sprouting[88]	a. Smooth muscle cells seeded in collagen pattern by printing exhibited cell attachment to the boundary initially followed by proliferation in the pattern under static conditions[89]	Studies focused on modulation of signaling pathways are limited[90]	a. Requires other biomaterial along with it since collagen is very weak for scaffold fabrication[91]
		b. Extensive studies have been reported for single-cell migration as well as clustered cell migration in collagen gel (particularly cancer cell or dermal cell migration)	b. The human umbilical vein endothelial cells resulted in the formation of tube-like structure on Days 8−9, followed by the increase in density of the capillary network and formation of more branches within the lumen[88]		b. Structural modification may occur during sterilization[1]

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Until now, knowledge of the chondrogenic signaling pathways on silk-based scaffolds has been inadequate. Due to this lack of understanding, over the past few decades efforts to develop fully functional cartilage tissue construct had limited success for the restoration of the structural−functional properties of the degenerated or injured cartilage tissue.[2] A major constraint which still exists is the inefficacy of the tissue engineers to differentiate the fabricated cartilage tissue equivalents concerning distinct articular and transient cartilage. As a result, most of the time tissue engineers developed transient instead of stable articular cartilage, which fails to withstand the load. In that respect, we exploited the use of human BMSCs encapsulated in SF-G bioink to produce a phenotypically stable 3D bioprinted cartilaginous tissue construct and to evaluate their differentiation perspective.[15] The study reported minimized hypertrophy in the construct developed with the SF-G bioink coupled with BMSC in comparison to the SF-G bioink laden with chondrocytes. From the high level of expression of HIF1A (hypoxia-inducible factor 1-alpha) and HDAC4 (histone deacetylase 4), the genes involved in the regulation of hypoxia, we assumed that these led to upregulated expression of the chondrogenic markers like COMP1 and ACAN (aggrecan). Thus, we anticipated that our SF-G bioink played a key role in regulating hypoxia; further, the immobilization of growth factors resulted in the increased chondrogenic ability of BMSCs laden in the 3D-bioprinted construct in comparison to the 3D aggregates. However, the underlying signaling pathways involved in this remained unexplained.

As a subsequent step in this direction, we encapsulated TVA-BMSCs in SF-G bioink to study the reduced hypertrophic differentiation and signaling pathways procured by the SF-G bioink.[66] Our lab was the first to inquire about the definitive chondrocyte markers, such as autotaxin and proteoglycan 4, involved in cartilage differentiation. We demonstrated that in the absence of TGF-β1 the BMSCs encapsulated in the SF-G bioink bypass hypertrophic differentiation and differentiate into phenotypically stable articular cartilage. This helped us to generate deep mechanistic insights about the indian hedgehog (IHH) and canonical Wnt signaling pathways involved in the regulation of hypertrophy. Overall, this study showed that we introspect that amalgamating the principle of developmental biology and the 3D bioprinting technique could help generate adequate instructive cues to trigger patient-specific signaling pathways.

One limitation of the above-mentioned study[66] was the lack of validation in large-scale proteomics analysis. Hence, we tried to shed light on the inherent ability of SF in regulating chondrogenic-specific signaling pathways (Figures 3 and 4) in the presence and absence of TGF-β3 (in communication) by gene ontological analysis and KEGG pathway analysis until 28 days. Even early time point interaction with SF-G bioink could activate three major anabolic signaling pathways like Wnt, Notch, and HIF-1 in human BMSC. At the same time, the Wnt/Ca^{2+} pathway was found to antagonize the canonical Wnt/β-catenin pathway. As the culture time progressed, simultaneous upregulation and a downregulation that is feedback-loop-based of Wnt/β-catenin via activation Wnt/Ca^{2+} signaling led to inhibition of hypertrophic differentiation of hBMSC. This is a significant insight into how signaling pathways can be specifically modulated during chondrogenic differentiation in a bioprinted construct.

6. CONCLUSION

Taken together, we contemplate that the utmost attention should be given in comprehending how a bioink/scaffold can regulate the cellular signaling cascades, particularly which regulate tissue regeneration, homeostasis, and immune response. Besides, optimization of an array of factors such as the chemistry of the bioink matrix, anatomically inspired architecture of the printed construct, mechanical properties, and morphogenic signals would guide activation of the specific signaling cascade in bioprinted constructs. Such mechanistic insights in amalgamation with 3D bioprinting can be employed and expanded further to simulate the cellular signaling at the developmental level to fabricate anatomically and physiologically pertinent 3D bioprinted construct equivalents for clinical translational research.

In comparison to the other hydrogel used, SF bioink from *Bombyx mori* can overcome the major challenges encountered in 3D Bioprinting (Table 1). The choice of suitable biomaterial like SF and improving the printing resolution can develop structures with intricate architectures. An optimized blend of the SF-G bioink has immense potential for obtaining 3D bioprinted functional tissue analogues which can closely replicate the native tissue with not only long-term viability but also high reproducibility. Further, its inherent ability to activate tissue-specific signaling pathways broadens the areas of study and hence can be employed for the development of *in vitro* diseased tissue models as well as 3D bioprinted constructs for transplantation prospects. Organs are complex structures, and the dire need for custom-made tissue replacements on demand for mimicking the anatomical complexity of the human tissues is challenging.

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Notes

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