POTENTIAL OF EXTRACELLULAR MATRIX MOLECULES IN PHARMACEUTICAL DEVELOPMENT

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POTENTIAL OF EXTRACELLULAR MATRIX MOLECULES IN PHARMACEUTICAL DEVELOPMENT

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EDITORIAL
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Editorial: Potential of Extracellular Matrix Molecules in Pharmaceutical Development

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Editorial on the Research Topic

Potential of Extracellular Matrix Molecules in Pharmaceutical Development

The extracellular matrix (ECM) is a complex cell-secreted network composed of a diverse array of fibrous proteins, proteoglycans, and proteolytic enzymes. Comprising approximately one-third of the human body, the ECM was initially considered an inactive, space-filling scaffold that solely provides structural support for cell growth. However, dysfunctions in the ECM have been associated with various human diseases. In this special issue of *Frontiers in Pharmacology*, Ahmad et al. contributed a minireview that manifests the current understanding of the major ECM components in muscle-associated conditions and their therapeutic potentials.

Meanwhile, the influence of some therapeutics on ECM is also broadly observed. For example, Tang et al. revealed that Anisodamine (ANI; also known as 7β-hydroxyhyoscyamine), a naturally occurring atropine derivative, not only effectively inhibited the apoptosis, senescence, and ECM degradation in the nucleus pulposus of intervertebral disc degeneration (IVDD) rats but also significantly promoted the synthesis of aggrecan and type II collagen. Considering the loss of ECM, particularly aggrecan and type II collagen, is a typical feature of IVDD, this study may provide intuitiveness for ANI's protective effects on cartilage, in addition to its known anti-inflammatory, analgesic, antipyretic, and platelet-inhibitory actions. In this special issue, Yang et al. also reported that long noncoding RNA (lncRNA) growth arrest specific transcript 5 (GAS5) bolsters the expression of growth arrest differentiation factor 5 (GDF5)—which plays an essential role in articular cartilage maintenance by inducing ECM and α5 integrin expression. Interestingly, they also found that GAS5 serves as an osteogenic regulator of periodontal ligament stem cells *via* GDF5 and p38/JNK signaling pathway, suggesting the critical function of GAS5 and GDF5 on ECM homeostasis in both bone and cartilage tissues.

Moreover, accumulating evidence currently demonstrates that ECM is a dynamic structure that directly interacts with the living cells. For instance, during the force-induced orthodontic tooth movement (OTM), a process of bone and periodontal ligament (PDL) remodeling, disorganized and compressed ECM architecture promotes osteoclastic bone resorption on the pressure side. At the same time, the stretching ECM structure stimulates osteoblastic bone formation. In this special issue, Li et al. accurately analyzed the ECM fiber remodeling and osteoclast recruitment corresponding to stress distribution. Hence, ECM has the capacity to modulate cell function and behavior. Undoubtedly, a comprehensive understanding of the mechanisms underlying these ECM-related disease pathologies and therapeutical benefits would help develop novel treatment strategies.

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Echo to these observations, some regenerative medicines targeting ECM components have been being developed, specifically since the new century. One exciting application of ECM-based-therapeutics is the use of decellularized ECM. By removing the cellular components, a decellularized ECM acts as a water-insoluble matrix and retains the physiological ECM properties that mimic the native microenvironment and support tissue regeneration. The intact biocompatibility, biodegradability, and bioinductivity of decellularized ECMs make them broadly applicable to various kinds of tissue regeneration. This strategy is particularly successful in bone repair applications. Thus, Lin et al. provided an overview of the function of multiple types of bone ECM and the applications of both ECM-modified and decellularized ECM scaffolds in bone repair and regeneration.

Furthermore, matricellular molecules that do not simply function as structural blocks are also critical components of the ECM. Compared with fiber-forming ECM elements, such as collagen, matricellular molecules may be more applicable in pharmaceutical development. Indeed, significant efforts have been devoted to developing matricellular protein-based pharmaceuticals, especially cancer therapeutics and fibrotic and inflammatory diseases. Particularly, proteoglycans have emerged as biomacromolecules with critical ECM remodeling, homeostasis, and signaling roles in the past 2 decades. Here, Walimbe and Panitch contributed a review on the recent preclinical efforts that open new avenues for developing new and exciting treatments with proteoglycan cores and mimetics in a broad range of regenerative medicine. Pang et al. conducted a review with a particular interest in the diverse functions of small leucine-rich proteoglycans (SLRPs), a group of ECM that exist in a wide range of connecting tissues, in skin wound healing. In this review, the most recent knowledge of SLRPs' anti-inflammatory, pro-angiogenic, pro-migratory, and pro-contractility, and signal transduction orchestrating effects, as well as their spatial-temporal expression in the skin, has been comprehensively summarized to pave the path for a new generation of pharmaceuticals discovery for patients suffering from skin wounds and their sequelae. In addition, by delineating the spatial-temporal distribution of fibromodulin (FMOD), an SLRP member, during cartilage and development, and FMOD's structural alteration in aging and arthritis progression, Li et al. suggest that FMOD holds the high potential as a new target of osteoarthritis management, pointing a novel direction of arthritis pharmaceutical development.

In summary, this special issue aims to give an overview of the most exciting progress in ECM-based strategies for pharmaceutic development, although it is inevitably incomplete in covering all aspects of ECM studies. Many promising projects are in advanced experimental stages and/or preliminary clinical trials. Given the exciting developments in the field, we believe that this special issue will provide specific insights into the establishment of novel ECM-based strategies that can pave the path for these emerging ECM-relevant therapies to improve human health.

AUTHOR CONTRIBUTIONS

ZZ conducted the initial draft and FC and XZ revised the manuscript.

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ORIGINAL RESEARCH
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Stress Distribution and Collagen Remodeling of Periodontal Ligament During Orthodontic Tooth Movement

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Periodontal ligament (PDL), as a mechanical connection between the alveolar bone and tooth, plays a pivotal role in force-induced orthodontic tooth movement (OTM). However, how mechanical force controls remodeling of PDL collagenous extracellular matrix (ECM) is largely unknown. Here, we aimed to evaluate the stress distribution and ECM fiber remodeling of PDL during the process of OTM. An experimental tooth movement model was built by ligating a coil spring between the left maxillary first molar and the central incisors. After activating the coil spring for 7 days, the distance of tooth movement was 0.324 ± 0.021 mm. The 3D finite element modeling showed that the PDL stress obviously concentrated at cervical margin of five roots and apical area of the mesial root, and the compression region was distributed at whole apical root and cervical margin of the medial side (normal stress < −0.05 MPa). After force induction, the ECM fibers were disordered and immature collagen III fibers significantly increased, especially in the apical region, which corresponds to the stress concentration and compression area. Furthermore, the osteoclasts and interleukin-1β expression were dramatically increased in the apical region of the force group. Taken together, orthodontic loading could change the stress distribution of PDL and induce a disordered arrangement and remodeling of ECM fibers. These findings provide orthodontists both mechanical and biological evidences that root resorption is prone to occur in the apical area during the process of OTM.

Keywords: extracellular matrix, collagen remodeling, stress distribution, finite element, periodontal ligament, orthodontic tooth movement (OTM)

INTRODUCTION

The process of orthodontic tooth movement (OTM) is characterized by collagenous extracellular matrix (ECM) remodeling of bone and periodontal ligament (PDL) mediated by an external mechanical force (Rangiani et al., 2016). After force induction, pressure and tension regions are generated in PDL (Yamaguchi et al., 2010). On the pressure side, disorganized and compressed ECM fibers induce osteoclastic bone resorption, whereas, on the tension side, stretching fibers stimulate osteoblastic bone formation (Oshiro et al., 2010; Ren et al., 2010). It has been shown that the biological response of PDL dependent on mechanical state regulates OTM efficiency (Odagaki

et al., 2018). Therefore, it is important to clarify the stress distribution and corresponding biological responses of PDL under an orthodontic force.

During the OTM process, PDL could respond to a mechanical force loading and present two main biological reactions: dynamic changes in collagen content and osteoclastogenesis (Feng et al., 2016). The ECM of PDL is the fibrous connective tissue joining the tooth to its surrounding bone. The collagen fibers with ordered arrangement are the main components. The collagenous ECM is mainly composed of type I (Col-I) and type III (Col-III) collagens, among which Col-I is dominant and mature (Xu et al., 2014). Functionally, Col-I fibers are response for strength and maintain stability of tooth position, whereas Col-III can relieve a tension force on the PDL during OTM (Li et al., 2010). It has been shown that the content of Col-III relative to Col-I increases in the early stage of collagen remodeling (de et al., 2009; Oryan et al., 2010). Another important reaction is osteoclast recruitment in PDL (Xie et al., 2010). It has been shown that PDL cells play crucial roles in osteoclastogenesis by expressing receptor activator of nuclear factor kappa. The recruited osteoclasts resorb and remodel the alveolar bone during OTM (Ha et al., 2003; Hasegawa, 2010).

Unlike bone tissue, the complex and tiny structure of PDL makes it difficult to directly measure the stress distribution in PDL. Lin et al. have predicted that the narrow region of PDL possesses a high strain by using CT images of tooth-PDL-bone specimen under compression (Lin et al., 2014). Recently, a more effective method of numerical simulation by 3D finite element (FE) is applied to directly reveal the stress condition of root (Yan et al., 2013). Here, we firstly used the FE method to evaluate the stress distribution of PDL based on micro-CT images of maxillary first molars under an orthodontic force, and then the biological response of PDL to different stress was analyzed histologically and immunohistochemically.

MATERIALS AND METHODS

Ethics Statement

The animal protocol was approved by the Peking University Ethical Committee (LA2013-92). All efforts were made to minimize animal number and suffering.

Animal Model of OTM

Five 6–8-week-old Sprague-Dawley rats were used for building an animal model of experimental tooth movement as previously described (He et al., 2015). Briefly, nickel-titanium coiled springs with 0.2-mm thickness, 1-mm diameter, and 5-mm length (Smart Technology, China) were ligated between the left maxillary first molar and the central incisors of rats and fixed to teeth with 0.2-mm stainless steel wires in the force group. A spring dynamometer device was used to standardize the orthodontic force, and the orthodontic force of the coil spring after activation was approximately 60 g in each rat (Dunn et al., 2007; He et al., 2015). The right side of the same rat was set as a control (**Figure 1A**). After 7 days, all the rats were sacrificed by overanesthesia, and the maxillae were obtained and fixed in 4% paraformaldehyde. A stereo microscope (SWZ1000, Nikon, Japan) was applied to record the occlusal view of each maxilla. The distance of tooth movement was measured between the midpoint of the distal-marginal ridge of the first molar and the midpoint of the mesial-marginal ridge of the second molar (Cao et al., 2014). Every measurement was repeated three times to get the mean value as the final measurement.

Finite Element Modeling

An accurate model of the maxillary first molar was constructed from the micro-CT images, which were acquired by a Skyscan 1174 micro-CT system (Bruker, Belgium) at resolution of 6.28 μm. The CT images were imported into Mimics software to segment the maxilla by Hounsfield values and manual mask segmentation. Three-dimensional geometry files of maxillae, teeth, and PDL were created for each mask and saved as stereolithographic (STL) files. A Computer Assisted Design software (Geomagic 12.0, Research Triangle Park, NC, USA) was applied to extract surfaces and solids from STL files. Triangle and intersection fixing techniques were performed and then Standard for the Exchange of Product model data files were created and exported separately into ANSYS 18.0 (Canonsburg, PA, USA). To simulate the experimental OTM, line pressure, which was in the position similar to the coil spring, was loaded on the distal surface of the maxillary first molar in the FE model.

Histological and Collagen Fiber Staining

After micro-CT scanning, the maxillae were demineralized in 10% ethylenediaminetetraacetic acid, dehydrated in ethanol, and embedded in paraffin. Serial longitudinal sections were obtained by vertical cutting of the first molars. Hematoxylin and eosin (HE) staining, Masson's trichrome staining, and picrocsirius red staining were applied to examine the histochemistry of the samples. HE, Masson's trichrome, and immunofluorescence staining were used to identify the collagen fiber arrangement. To assess collagen remodeling during OTM, the sections stained with picrosirius red were analyzed *via* a polarizing microscopy, in which collagen type I (Col-I) fibers were red, and collagen type III (Col-III) appeared green. For statistically analysis, we randomly selected three images from each cervical area and central area in the mesial or distal side. As for the apical area, we randomly selected three images from each mesial side and distal side. The proportion of Col-III in the PDL was calculated by an Image-Pro Plus 4.1 software (Media Cybernetics Inc. Silver Springs, MD).

Immunohistochemical Assay

Immunohistochemistry was performed with a two-step detection kit (Zhongshan Golden Bridge Biotechnology, Beijing, China) as before (Jin et al., 2019). Tissue sections in each group were subjected to antigen retrieval solution, blocked with 5% bovine serum albumin, and incubated overnight with

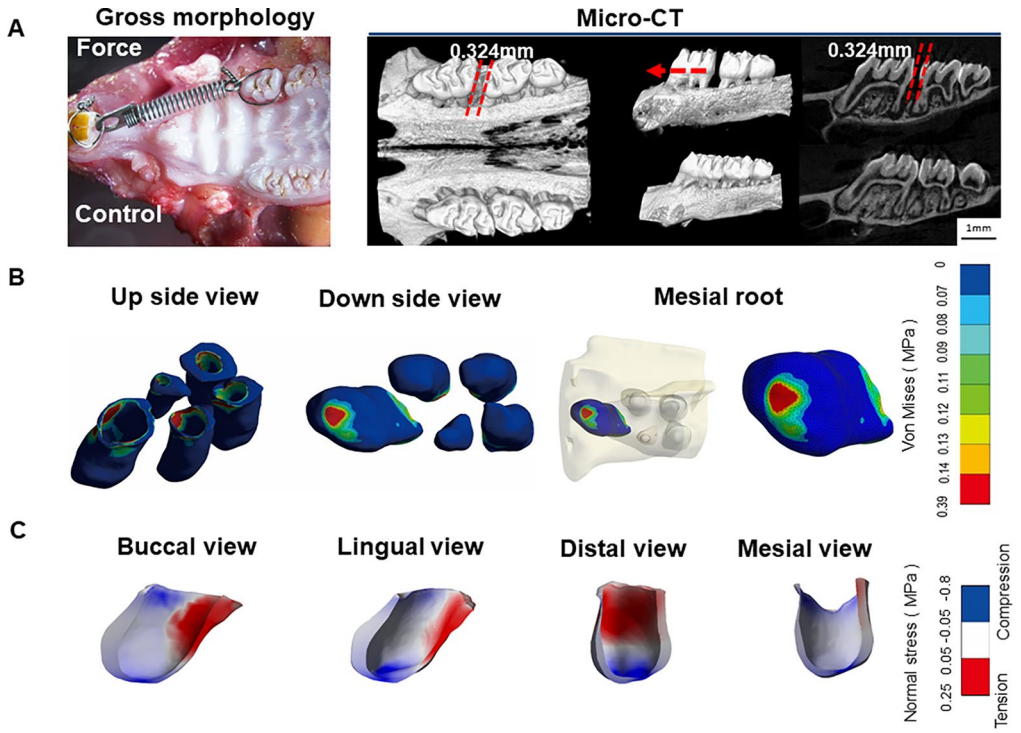

FIGURE 1 | (A) Micro-CT images of orthodontic tooth movement (OTM) after 7 days of force application. **(B)** Stress distribution in periodontal ligament (PDL) of maxillary first molars. Blue color represents low stress area (< 0.07 MPa), whereas Von Mises value above 0.07 MPa is set as stress concentration. Stress concentration was in PDL at apical area of the mesial root. **(C)** Tension-compression distribution in PDL of the mesial root. Blue color represents compression area with normal stress < -0.05 MPa while red color shows tension region with normal stress > 0.05 MPa.

antibodies against the Col-I (1:200; ab34710, Abcam), Col-III (1:200; ab7778, Abcam), and interleukin-1β (IL-1 β, 1:200; ab2105, Abcam). Samples were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies using diaminobenzidine (Zhongshan Golden Bridge Biotechnology, Beijing, China) as chromogen. Three different regions of each side were randomly chosen to count the number of positive cells for statistical analysis.

Tartrate-Resistant Acid Phosphatase Staining

The sections were deparaffinized to perform tartrate-resistant acid phosphatase (TRAP) test using a leukocyte acid phosphatase kit (387A, Sigma) according to the manufacturer's protocol. TRAP-positive multinucleated (> 3 nuclei) cells that are attached to the alveolar bone surface mesial to the distal buccal roots were counted.

Statistical Analysis

All the data were expressed as mean ± SD. All statistical analyses were performed with a GraphPad Prism 6 software (GraphPad Software, San Diego, CA, United States) and $P < 0.05$ was considered to be statistically significant. Furthermore, t-test was used to evaluate the difference between groups in different regions.

RESULTS

Stress Distribution in PDL Under an Orthodontic Force

Tooth movement through bone is induced by an appropriate mechanical force. Activation of coil springs could generate a light orthodontic force about 60 g, which made the left first molar move to the mesial about 0.324 ± 0.021 mm after 7 days. This distance is consistent with that reported in the literatures (Dunn et al., 2007; He et al., 2015). In contrast, the right first molar, which served as the control, did not move. The micro-CT images further confirmed the tooth movement without obvious root resorption under the light force (**Figure 1A**).

Based on the micro-CT images, a 3D FE model of five roots of the first molar during OTM was developed. This model could accurately reproduce the tooth-PDL-bone structure, which is generally assumed to be a simple geometry in previous FE analyses (Kamble et al., 2012; Zhang et al., 2017). In the FE analysis, we mainly focused on horizontal force by the line pressure, mimicking orthodontic tipping tooth movement. The PDL stress (> 0.07 MPa) obviously concentrated at cervical margin of the mesial side of five roots from the top and apical area of the mesial root from the bottom. In contrast, low stress (< 0.07 MPa) is distributed in the middle of five roots and the apical regions of the other four roots, except for the mesial root (**Figure 1B**).

Normal stress field was further applied to assess the tension-compression area. From the tension-compression analysis of the mesial root, the compression region was distributed at whole apical root and cervical margin of the medial side (normal stress < -0.05 MPa), whereas the tension zone was present at two thirds of crown of the distal side (normal stress > 0.05 MPa) (**Figure 1C**). This finding is different from the classical OTM theory that symmetric compression (mesial), and tension (distal) areas are present in PDL. However, a previous FE analysis also demonstrates that no distinct pressure and tension regions are detected for complex mechanical properties of PDL (Cattaneo et al., 2010). The stress concentration on the apical region might be closely correlated to high incidence of root resorption during OTM.

The Orthodontic Force Increased Col-III Expression in PDL

Collagen fibers in an ordered arrangement are the main components of ECM in PDL. Most fibers at the angle of 45° to the root are parallel with each other. Based on the FE results, we focused on the collagen remodeling of the mesial root. From HE and Masson stainings, the PDL fibers in the mesial area were irregular in shape and the cementum was discontinuous in the force group. Especially, the apical PDL was disoriented and even broken in the force group. In contrast, the periodontal space was well maintained between the root and alveolar bone, and the contour of the cementum was continuous in the control group (**Figure 2**). This finding was further confirmed by picrosirius red and immunofluorescence stainings (**Figure 3**).

Figure 3 illustrated that red-stained Col-I bundles were dominant in the control group. Compared to the apical area, there were much more green-stained Col-III fibers in the mesial and distal areas without loading. After force induction, the Col-III expression was significantly enhanced compared with the control group. This trend was more obvious in the apical region ($p < 0.001$) (**Figure 3B**). The immunofluorescence staining further revealed the disordered arrangement of collagen fibers in PDL in the force group. From immunohistochemistry, more Col-I positive staining cells were found in the control group compared with the force group. On the contrary, the opposite trend was observed in the Col-III positive staining cells. The ratio of Col-III positive cells was significantly increased in the force group ($p < 0.05$), especially in the apical region ($p < 0.001$) (**Figure 4**). Taken together, these results indicate that the immature collagen fibers increase during OTM.

Osteoclast Recruitment in PDL Under an Orthodontic Force

The sustained force could change the chemical environment by releasing inflammatory cytokines and, therefore, influence

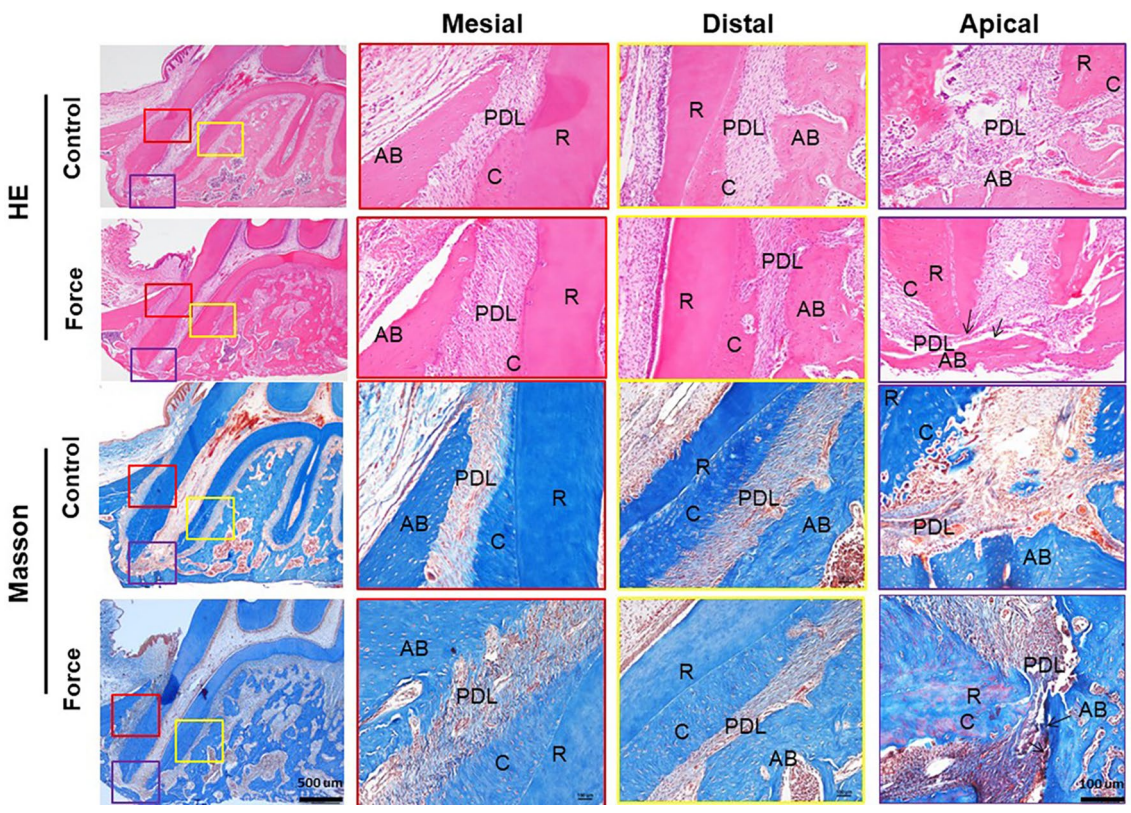

FIGURE 2 | HE and Masson stainings of PDL of the mesial root. The PDL fibers in the mesial area were irregular and the apical PDL was broken in the force group (arrows). AB, alveolar bone; R, root; PDL, periodontal ligament; C, cementum.

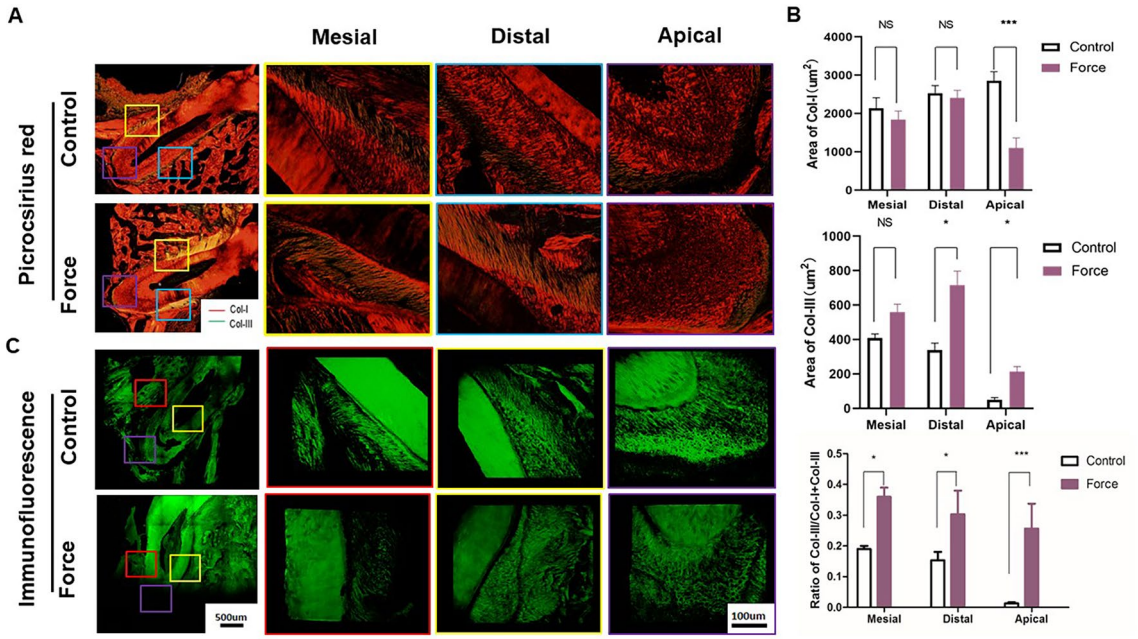

FIGURE 3 | (A) Picrocsirius red staining of periodontal ligament (PDL). Col-I fibers stain red, whereas Col-III fibers are green stained. **(B)** Semiquantification of Col-I and Col-III fiber area in **(A)**. There were more Col-III fibers in the distal and apical regions and less Col-I fibers in the apical regions in the force group (n = 18, mean ± SD). *: P < 0.05, ***: P < 0.001, NS, not significant. **(C)** Immunofluorescence staining of PDL showing disoriented fibers in the force group.

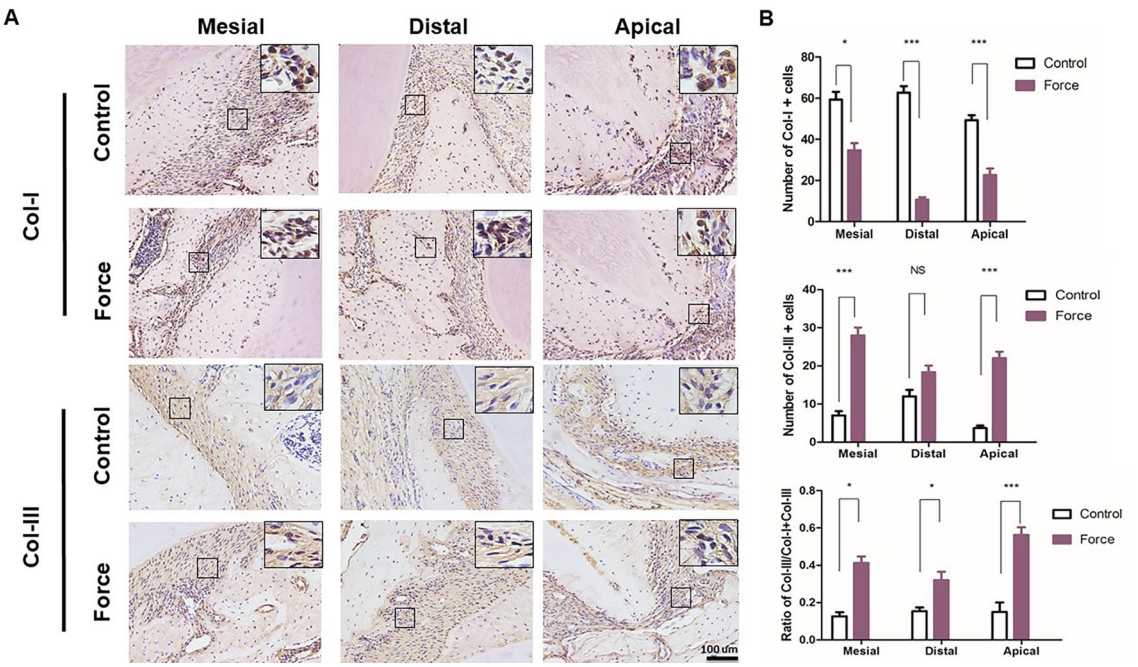

FIGURE 4 | (A) Immunohistochemical staining of Col-I and Col-III. **(B)** Semiquantification of Col-I$^+$ and Col-III$^+$ cells in **(A)**. There were more Col-III$^+$ and less Col-I$^+$ cells in the mesial and apical regions in the force group (n = 18, mean ± SD). *: $P < 0.05$, ***: $P < 0.001$, NS, not significant.

bone and root resorption. Immunohistochemical analysis demonstrated that an orthodontic force could dramatically enhance the IL-1β expression level especially in the apical region. The release of IL-1β could induce osteoclast differentiation and further promote root resorption (Kim et al., 2009; Baba et al., 2011) (**Figure 5A**). We next examined whether the force-induced osteoclastogenesis was influenced in different areas of the mesial root during the process of OTM. The TRAP staining showed that the number of osteoclasts was significantly increased in the apical region in the force group compared with the control group. No positive staining was found in the mesial and distal regions in both the control and the force groups (**Figure 5B**).

DISCUSSION

Orthodontic tooth movement is a synergistic result of physical phenomenon and biological responses of the tooth-alveolus complex to an externally applied force (Kalajzic et al., 2014). During the OTM process, PDL responds to mechanical force stimulation and provides a microenvironment for cellular reactions and tissue remodeling. The remodeling of collagenous ECM fiber in PDL corresponding to mechanical loading is largely unknown, although the bone remodeling process during OTM is well investigated (Rangiani et al., 2016). In the present study, we first accurately demonstrated PDL stress concentration regions by a 3D FE modeling and then particularly analyzed biological responses of PDL including collagen fiber remodeling and osteoclast recruitment corresponding to stress distribution. We found that a light orthodontic force temporarily broke collagen orderly arrangement and increased immature Col-III fiber number and inflammation of PDL, especially in the apical region, which corresponds to stress concentration area. Although orthodontic root resorption is a common and well-known phenomenon, the direct mechanical evidence is lacking. Here, a 3D FE modeling combined with histological analyses provides orthodontists both mechanical and biological evidences that root resorption is prone to occur in the apical area during the process of OTM.

Orthodontic treatment is highly related to collagen fiber remodeling in the ECM of PDL, which mainly consists of mature Col-III and immature Col-I fibers (Becker et al., 1991; Xu et al., 2017). Therefore, investigations on dynamic changes of collagen content would help us to clarify the biological response of PDL to mechanical loading. In our study, enhanced expression of Col-III in the tension areas of PDL, especially in the apical region, indicated active remodeling of PDL under orthodontic loading. In contrast, Col-I expression decreased significantly in the force group. This finding is consistent to a previous report that the Col-III/Col-I ratio is increased in the early phase of collagen remodeling, especially under a tension force. The accumulation of Col-III might contribute to relieving the tension force placed on PDL during OTM. During the late phase of PDL ECM remodeling, Col-III could be gradually replaced by Col-I until a normal Col-III/Col-I ratio is obtained. Stress from orthodontic loading is transmitted from PDL ECM *via* integrins, which induce a change in ECM synthesis, PDL remodeling, and ultimately tooth movement (Krishnan and Davidovitch, 2006;

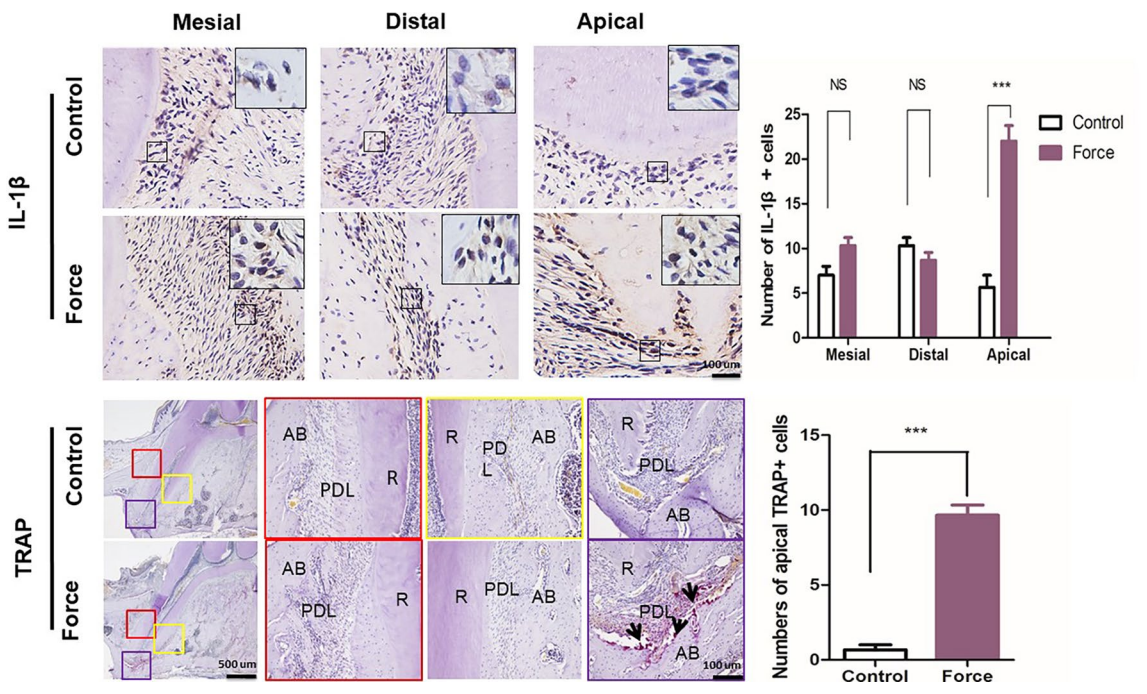

FIGURE 5 | (A) Immunohistochemistry and semiquantification of IL-1β positive cells (n = 18, mean ± SD). ***: $P < 0.001$, NS: not significant. **(B)** Tartrate-resistant acid phosphatase (TRAP) staining of the mesial root. Semiquantification of TRAP$^+$ cells indicated that the force group had much more TRAP$^+$ cells than the control group (n = 18, mean ± SD). ***: $P < 0.001$. R, root; PDL, periodontal ligament; AB, alveolar bone. Arrows, osteoclasts.

Ma et al., 2017). It has been shown that diabetes enhance the Col-III/Col-I ratio and prolong the PDL remodeling process under an orthodontic force (Li et al., 2010). Mechanistically, diabetes could elevate matrix metalloproteinase levels, which rapidly degrade collagen in PDL ECM, disturb fibroblast function, and finally complicate OTM (Chang et al., 2008).

Osteoclastogenesis in PDL is another key process during OTM, in which the recruited osteoclasts resorb and remodel the alveolar bone (Li et al., 2010; Hou et al., 2014). Osteoclasts are characterized by high expression levels of TRAP, osteoprotegerin, and Cathepsin K (Gerogianni et al., 2005; Rangiani et al., 2016). Here, we showed that a light orthodontic force enhanced the number of $TRAP^+$ osteoclasts in the apical region corresponding to stress concentration area. This suggests that bone or root resorption is prone to occur at the apical region, which is highly consistent with clinical phenomena (Ling et al., 2010). At the early stage of remodeling, no obvious bone or tooth resorption occurred under a continuous light force for 7 days in the study. According to the report of Wellington et al, osteoclasts induced by an orthodontic force originate by the fusion of recently recruited preosteoclasts from the marrow instead of from local PDL cells, although there are osteoclasts residing in the PDL space (Rody et al., 2001).

Mechanical forces cause capillary vasodilatation, followed by migration of leukocytes and the release of cytokines (Sasano et al., 2010). Several studies have provided experimental evidence to support a statement that cytokines regulate the bone and PDL remodeling processes during OTM (Norevall et al., 2010; Rangiani et al., 2016; Tsuge et al., 2016). Among the cytokines, IL-1β is thought to play a prominent role during OTM. Blocking IL-1β by a soluble receptor inhibits tooth movement (Lages et al., 2009; Baba et al., 2011). Various studies have shown that IL-1β stimulates bone resorption and inhibits bone formation *in vivo* (Nguyen et al., 1991; Baba et al., 2011; Diercke et al., 2012). The observation that transient IL-1β elevation in alveolar bone precedes the increase in osteoclasts population in several days suggests that recruitment of new preosteoclasts may be important in OTM.

As the highlight of our study, we investigated from the perspective of mechanics with modified 3D FE modeling and had directly proven that the stress distribution of apical region was more special compared with mesial and distal regions, which also provided orthodontists direct evidence that root resorption was prone to occur in the apical area.

CONCLUSION

Orthodontic loading could change the stress distribution of PDL and induce a disordered organization and remodeling of collagen fibers in the ECM of PDL. Immature collagen III fibers and inflammation increased during OTM, especially in the apical region, which corresponds to stress concentration area. Further research is needed to translate biological concepts into clinical practice.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/supplementary files.

ETHICS STATEMENT

The animal study was reviewed and approved by Peking University Ethical Committee (LA2013-92).

AUTHOR CONTRIBUTIONS

YL, YZ, and DH designed the study, analyzed the data, and revised the manuscript. ZL and MY performed the experiments, interpreted the data outcomes, and drafted the manuscript. SJ, YW, RL, BH, and DL contributed to the experimentation and data analysis. All authors reviewed and approved the final manuscript.

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Fibromodulin – A New Target of Osteoarthritis Management?

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INTRODUCTION

As a leading cause of disability among adults, osteoarthritis (OA) leads to serious public health and economic burdens. Currently, treatment options for OA are generally based on symptom severity and duration, with the goals of symptom alleviation and improvement in functional status (Taruc-Uy and Lynch, 2013). Nonpharmacologic and pharmacologic strategies are used initially, while a surgical approach to OA is reserved for chronic cases when these treatments failed. Unfortunately, the currently available clinical pharmacologic treatments for OA, such as analgesia, glucocorticoids, non-steroidal anti-inflammatory drugs, and disease-modifying antirheumatic drugs, are not adequately effective (Chevalier et al., 2009; Scott, 2010; Verbruggen et al., 2012; Chevalier et al., 2015; Appleton, 2018; Li and Zheng, 2018; Li et al., 2018), and generally associated with a diversity of adverse side-effects (Habib et al., 2010; Cooper et al., 2016; Compston, 2018). For instance, analgesia does not reduce inflammation and cartilage damage (Appleton, 2018), glucocorticoids have been reported to induce severe damages in the musculoskeletal, cardiovascular, and gastrointestinal systems (Cooper et al., 2016; Compston, 2018), and non-steroidal anti-inflammatory drugs do not actively control arthritis progression (Appleton, 2018). Accumulating evidence demonstrates that an ideal OA-combating agent should be able to reduce inflammation and promote cartilage regeneration safely, which has long been desired. In responding to this demand, the current strategy for disease-modifying osteoarthritis drug seeking has shifted to biologoical molecules that promote chondorgenic development and regenration.

To date, a diversity of well-known pro-chondrogenic growth factors, such as bone morphogenetic proteins (BMPs) and transforming growth factors (TGFs), have been examined for OA treatment. However, the results are not optimistic since intra-articular injection of these growth factors could even enhance the inflammatory infiltration in damaged joints (Allen et al., 1990; Fava et al., 1991; Hong et al., 2009). Meanwhile, multiple transcriptional factors that potentially suppress inflammation, such as nuclear factor of activated T cells 1 (NFATc1), NFATc2, and runt-related transcription factor 1 (RUNX1), have also be introduced in this arena against OA, while they do not hold much promise presently. For example, the function of NFATc proteins in arthritis is controversial (Yaykasli et al., 2009; Miclea et al., 2011; Greenblatt et al., 2013).

Another possibility for fighting OA is utilizing the extracellular matrix (ECM) molecules that naturally distribute in the articular cartilage. For example, fibromodulin (FMOD) is an ECM protein with multiple keratin sulfate side-chains that belongs to the small leucine-rich proteoglycan family (Plaas et al., 1990). It was first identified as a collagen-binding molecule broadly distributed in connective tissues, with particularly high expression in cartilage (Hedbom and Heinegard, 1989). In the past three

decades, in addition to the broad attention of its effects on collagen fibrillogenesis (Chen et al., 2010), muscle development (Lee et al., 2018a; Lee et al., 2018b), cell reprogramming (Zheng et al., 2012; Li et al., 2016; Zheng et al., 2019), angiogenesis (Jian et al., 2013; Zheng et al., 2014; Ao et al., 2017), wound healing (Zheng et al., 2017), and tumorigenesis (Pourhanifeh et al., 2019), the involvement of FMOD in cartilage development and maintenance as well as arthritis progression, especially in temporomandibular joint (TMJ) OA, has been investigated through world-wide collaboration. Here, we review the current research investigating FMOD and arthritis, and aim to provide novel insight into the potential use of FMOD for OA management.

SPATIOTEMPORAL DISTRIBUTION OF FMOD DURING CARTILAGE GROWTH AND DEVELOPMENT

An investigation that focused on mouse glenohumeral joints demonstrated that, at 12–13 days post-coitus when the limb buds are just condensing mesenchymal cells, FMOD was not detectable at the protein level (Murphy et al., 1999). Intense FMOD staining was first noticed at the surface of the scapular and humeral anlage intracellularly and pericellularly in the interzone at 14–15 days post-coitus (Murphy et al., 1999). Starting from 17 days post-coitus, a strong FMOD signal was found in the ECM surrounding the chondrocytes at the surface of the joints and proliferating chondrocytes in the epiphyses of the humerus and scapula (Murphy et al., 1999). Meanwhile, during postnatal maturation until adulthood, FMOD was detected throughout the ECM of the developing articular surface and the growth plate but was more abundant in articular cartilage (Murphy et al., 1999). Since FMOD was associated with prechondrocytic mesenchymal cells in the interzone before joint cavitation and with developing articular chondrocytes in the maturing and young adult limbs, it has been proposed that FMOD may function in the early genesis of articular cartilage (Murphy et al., 1999).

It is worth noting that FMOD shared a similar temporospatial transcriptional pattern with type II collagen in mouse knee joints during postnatal development, while *FMOD* gene expression reached the maximum level at 1 month old (Saamanen et al., 2001). *FMOD* transcription was restricted to chondrocytes and peaked in the proliferating zone and the early articular cartilage (Saamanen et al., 2001), which had been confirmed at the protein level by immunostaining (Murphy et al., 1999). In mature animals, *in situ* hybridization revealed that both pericellular and interterritorial cartilage at knee joints had high *FMOD* expression with the highest intensity in the middle and deep zones of the uncalcified cartilage (Saamanen et al., 2001). At 6 months old, FMOD staining decreased in the uncalcified cartilage but increased in the calcified cartilage (Saamanen et al., 2001). FMOD was also detected in the hypertrophic chondrocytes of the secondary ossification centers and growth plate of mice at 10 days old, and transcription of *FMOD* was diminished and finally disappeared with maturation and aging of the trabecular epiphyses (Saamanen et al., 2001).

STRUCTURAL ALTERATION OF FMOD IN AGING AND ARTHRITIS PROGRESSION

In addition to its spatiotemporal distribution, FMOD's structural heterogeneity was also noticed during articular cartilage growth and development. For instance, FMOD isolated from young articular cartilage carries neither α(2-6)-linked *N*-acetylneuraminic acid nor α(1-3)-linked fucose in the N-linked keratan sulfate chains (Lauder et al., 1996). Meanwhile, an age-related increase has been observed in the abundance of both α(2-6)-linked *N*-acetylneuraminic acid and α(1-3)-linked fucose, but not the levels of galactose sulfation (Lauder et al., 1998). Western blot showed FMOD-derived from fetal and neonatal articular cartilage (f/n-FMOD) as a diffused region with a relative molecular weight of 70–110 kDa (Cs-Szabo et al., 1995; Roughley et al., 1996), while FMOD-derived from mature adult (a-FMOD) was a more discrete component with a relative molecular weight of 67 kDa (Cs-Szabo et al., 1995; Roughley et al., 1996)—larger than the FMOD core protein without post-translational modifications (46 kDa). Interestingly, digesting f/n-FMOD with keratanase II or endo β-galactosidase reduces its molecular weight to a similar level of a-FMOD (Cs-Szabo et al., 1995). Thus, Roughley et al. argued that FMOD might predominantly exist in the proteoglycan form in juvenile cartilage tissues but is mainly in a glycoprotein form in the adult counterparts (Roughley et al., 1996).

Interestingly, FMOD is one of the small leucine-rich proteoglycans with the most significantly increased protein fragmentation in arthritis compared with macroscopically healthy articular cartilage from the age-matched donors (Melrose et al., 2008). In addition to the 59 kDa band, multiple small bands can be detected by Western blot when FMOD is isolated from articular cartilage of OA and rheumatoid arthritis patients (Cs-Szabo et al., 1995; Roughley et al., 1996; Melrose et al., 2008; Shu et al., 2019). Moreover, when using N-glycosidase to remove the sulfate chains from FMOD isolated from arthritic articular cartilage, several protein bands with the size of 43, 40, and 27 kDa were detected (Cs-Szabo et al., 1995). Therefore, arthritis progression may not only alter the degree and type of its carbohydrate substation but also lead to the breakage of the FMOD core protein.

Meanwhile, degradation of FMOD core protein was also observed in interleukin (IL)-1-challenged cartilage (Sztrolovics et al., 1999; Shu et al., 2019)—a representative model that elucidates the genetic and molecular pathogenesis of inflammation-related secondary OA (Kuyinu et al., 2016). The degradation of FMOD core protein was predominantly catalyzed by matrix metalloproteinases (MMPs) and ADAM metallopeptidases with thrombospondin type 1 motifs (ADAMTSs) (Kashiwagi et al., 2004; Shu et al., 2019). *In vitro* digestion of healthy human knee cartilage with MMP-13, ADAMTS-4, and ADAMTS-5 generated FMOD fragments of similar sizes as FMOD derived from OA cartilage without digestion (Shu et al., 2019). Notably, the fragmented FMOD is always detected by the antibody recognizing the N-terminal fragment of FMOD but not the one recognizing the C-terminal (Melrose et al., 2008; Shu et al., 2019). One possible explanation is that the C-terminus

is vulnerable to the fragmentation and not stably retained in the tissue, and substantially lost into the synovial fluid (Melrose et al., 2008). Importantly, MMP-13 degradation of FMOD resulted in a fragment of 30 kDa, which was also detected in moderately and severely fibrillated cartilage, instead of healthy or slightly fibrillated cartilage (Monfort et al., 2006). These phenomena may support the hypothesis that the sensitivity of FMOD protein fragmentation is increased along with the severity of cartilage degradation.

LESSONS FROM FMOD DEFICIENT MICE FOR OA INVESTIGATION

FMOD-null (*Fmod*$^{-/-}$) mice have distinct knee joints in comparison with their wildtype (WT) littermates at 36 weeks old (Gill et al., 2002), accompanied by a significantly higher histological arthritis score (Ameye et al., 2002). In addition, serial sections through FMOD-null mice knees showed degeneration and joint remodeling histologically. More severe incidences of degeneration occurred in the area of the tibial condyles that are uncovered by the menisci, as these sites experience the highest loading stress, resulting in considerable loss of cartilage and bone thickness (Gill et al., 2002). Moreover, the menisci of FMOD-null mice had a markedly less sharp profile with more rounded edges, similar to FMOD-null ligaments, which were also more likely to be damaged compared to WT ligaments. The area of tibial articular cartilage was even more exposed due to degenerated menisci compared to that of the WT littermates (Gill et al., 2002). Furthermore, knee joints of *Fmod*$^{-/-}$ mice at 80 weeks old displayed full-depth lesions of articular cartilage and clusters of cells that were not seen in the knee joints of WT littermates (Gill et al., 2002).

As biglycan (BGN) and FMOD have overlapping and possible compensatory functions in the joints (Shirakura et al., 2017), *BGN* and *FMOD* double-knockout (*Bgn*$^{-/0}$/*Fmod*$^{-/-}$) mice exhibit an earlier onset of OA than *Fmod*$^{-/-}$ mice. *Bgn*$^{-/0}$/*Fmod*$^{-/-}$ mice presented with an abnormal gait characterized by the decreased flexibility of knee and ankle joints (dragging leg), which was observed as early as 3 weeks old. Additionally, at 3 months old, the histological arthritis score of the *Bgn*$^{-/0}$/*Fmod*$^{-/-}$ knee joints was significantly higher than that of the WT knee joints. However, the abnormal gait phenomena were observed in neither *BGN* nor *FMOD* single knockout mice (Ameye et al., 2002).

Moreover, BGN and FMOD are also highly expressed in the disc and articular cartilage of the TMJ (Wadhwa et al., 2005a). *Bgn*$^{-/0}$/*Fmod*$^{-/-}$ mice developed accelerated OA accompanied by small vertical clefts in the condylar cartilage and partial disruption of the disc as compared to WT animals at 6 months old (Wadhwa et al., 2005b). At 18 months old, extensive cartilage erosion was visible in the *Bgn*$^{-/0}$/*Fmod*$^{-/-}$ mice TMJ (Wadhwa et al., 2005b).

POTENTIAL ROLES OF FMOD IN ARTHRITIS

There are several hypotheses about the possible roles of FMOD in arthritis. FMOD binds to collagens (Melching and Roughley, 1999), and fragmentation of FMOD during arthritis progression may destabilize collagen fibrils, rendering them more susceptible to tissue collagenases (Kashiwagi et al., 2004). However, such a difference between WT and FMOD-null mice may not necessarily have immediately visible effects at the ultrastructural level in adults (Ameye et al., 2002).

Alternatively, FMOD may sequester TGF-β/BMP superfamily members in the ECM and thereby prevent their binding to the cellular receptors (Wadhwa et al., 2005a). For example, when treating the TMJ with BMP2, both catabolic and anabolic markers were more profoundly upregulated in the *Bgn*$^{-/0}$/*Fmod*$^{-/-}$ mice than WT animals (Shirakura et al., 2017). This observation suggests that BGN and FMOD could protect the condyle from BMP2-induced matric turnover (Shirakura et al., 2017). Additionally, the sequestration of TGF-β1 in mandibular condylar chondrocyte ECM decreased in *Bgn*$^{-/0}$/*Fmod*$^{-/-}$ mice. The overactive TGF-β1 signal transduction in *Bgn*$^{-/0}$/*Fmod*$^{-/-}$ mice accelerated both production and degradation of type II collagen and aggrecan, and subsequently led to an overall imbalance in ECM turnover that favors cartilage degradation and the onset of OA (Embree et al., 2010).

FMOD may also function as a barrier preventing cell adhesion and subsequent cartilage damage. For example, FMOD administration dramatically prevents the adhesion of polymorphonuclear neutrophils and fibroblasts on articular cartilage surfaces (Noyori and Jasin, 1994; Mitani et al., 2001). This inhibition of cellular attachment may be attributed to the capability of FMOD to mask epitopes of cartilage collagen that face the joint cavity (Noyori and Jasin, 1994).

Furthermore, FMOD may participate in arthritis progression by directly manipulating inflammatory reactions. For instance, C1q and complement inhibitor factor H can directly bind to FMOD but in different regions (Akimoto et al., 2006). However, the deposition of the membrane attack complex and C5a release were lower in the presence of FMOD, presumably due to the formation of the FMOD-factor H complex (Akimoto et al., 2006). Interestingly, IL-1 only stimulates the binding of C1q, but not factor H, to the N-terminal fragment of FMOD in cartilage (Akimoto et al., 2006). Thus, FMOD may balance the activation of the classical complement pathway: when maintained in its intact form, FMOD silences the complement cascade by binding factor H; on the other hand, when FMOD is degraded or fragmented, as seen in OA (Melrose et al., 2008; Shu et al., 2019), the N-terminal FMOD segment binds to C1q and in turn activates the complement system to eliminate pathogens and damaged cells for tissue recovery and reconstruction.

FMOD has been used as an early marker of chondrogenesis (Barry et al., 2001). The expression level of FMOD is inversely correlated with the passage number of human chondrocytes in monolayer cultivation (Lin et al., 2008). In the TMJ cartilage of 3-month-old *Bgn*$^{-/0}$/*Fmod*$^{-/-}$ mice, fewer proliferative chondrocytes were noticed in comparison to that of their WT counterparts (Wadhwa et al., 2005b). Moreover, *Bgn*$^{-/0}$/*Fmod*$^{-/-}$ mice presented with more chondrocyte apoptosis in the articular cartilage than WT mice at the same developmental stage (Wadhwa et al., 2005a). A recent study even showed that microRNA-340-5p negatively regulated OA chondrocyte proliferation while stimulating apoptosis by reducing *FMOD*

expression (Zhang et al., 2018). Nevertheless, the exact function of FMOD in chondrogenesis has yet to be fully uncovered.

FURTHER DIRECTION

As aforementioned, FMOD is a critical ECM component involved in articular cartilage development, growth, aging, and arthritis; however, the exact functions of FMOD during arthritis are still unclear. Take advantage of the development of the Cre/Lox as well as CRISPR-Cas9 recombination system, the specific functions of FMOD during arthritis progression could be deciphered in detail with tissue-specific knockout animal models. Recently, it has been reported that FMOD can be successfully produced and purified from the cell culture supernatant of stable recombinant CHO-K1 cells transfected with a plasmid harboring the human *FMOD* gene (Zheng et al., 2012; Li et al., 2016; Pourhanifeh et al., 2019). Since FMOD whole protein is now easy to produce, further in-depth investigations are warranted to reveal the underlying mechanism of action of FMOD as a new generation disease-modifying osteoarthritis drug candidate. Last but not least, the plasmid- or virus-mediated expression, as well as directly synthesis, could be utilized to identify the functional sequence(s) of FMOD that regulate(s) cartilage development and pathology, which would further advance the pharmacology application of FMOD.

AUTHOR CONTRIBUTIONS

CL conceived the opinion. CL, PH, and ZZ wrote and revised the manuscript. WJ and CH edited and proofread the manuscript. CL, ZZ, and MZ supervised the writing process and approved the manuscript. All authors reviewed the final manuscript.

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Conflict of Interest: ZZ is an inventor on fibromodulin-related patents assigned to UCLA. ZZ is a founder of Scarless Laboratories Inc., which sublicenses fibromodulin-related patents from the UC Regents, who also hold equity in the company. ZZ is also a former officer of Scarless Laboratories Inc.

The handling editor is currently organizing a Research Topic with one of the authors ZZ and confirms the absence of any other collaboration.

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REVIEW
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Small Leucine-Rich Proteoglycans in Skin Wound Healing

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Healing of cutaneous wounds is a complex and well-coordinated process requiring cooperation among multiple cells from different lineages and delicately orchestrated signaling transduction of a diversity of growth factors, cytokines, and extracellular matrix (ECM) at the wound site. Most skin wound healing in adults is imperfect, characterized by scar formation which results in significant functional and psychological sequelae. Thus, the reconstruction of the damaged skin to its original state is of concern to doctors and scientists. Beyond the traditional treatments such as corticosteroid injection and radiation therapy, several growth factors or cytokines-based anti-scarring products are being or have been tested in clinical trials to optimize skin wound healing. Unfortunately, all have been unsatisfactory to date. Currently, accumulating evidence suggests that the ECM not only functions as the structural component of the tissue but also actively modulates signal transduction and regulates cellular behaviors, and thus, ECM should be considered as an alternative target for wound management pharmacotherapy. Of particular interest are small leucine-rich proteoglycans (SLRPs), a group of the ECM, which exist in a wide range of connecting tissues, including the skin. This manuscript summarizes the most current knowledge of SLRPs regarding their spatial-temporal expression in the skin, as well as lessons learned from the genetically modified animal models simulating human skin pathologies. In this review, particular focus is given on the diverse roles of SLRP in skin wound healing, such as anti-inflammation, pro-angiogenesis, pro-migration, pro-contraction, and orchestrate transforming growth factor (TGF)β signal transduction, since cumulative investigations have indicated their therapeutic potential on reducing scar formation in cutaneous wounds. By conducting this review, we intend to gain insight into the potential application of SLRPs in cutaneous wound healing management which may pave the way for the development of a new generation of pharmaceuticals to benefit the patients suffering from skin wounds and their sequelae.

Keywords: skin, skin wound healing, small leucine rich proteoglycans, extracelluar matrix, fibromodulin, decorin, biglycan, lumican

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INTRODUCTION

The skin, comprised of the epidermis, dermis, and deeper subcutaneous tissue, is the largest organ of the body, and functions as the first line of defense from external assaults (Proksch et al., 2008). Surgery and trauma in adults often result in wounds, which can cause the formation of refractory scars [i.e., hypertrophic scars and keloids, which are specific to humans (Baker et al., 2009)] with significant functional and psychological consequences (Bayat et al., 2003) that reduce the quality of life of individuals (Brown et al., 2008). Compared to the normal scars that can be much smaller than the original wound, keloids are defined as pathologic scars that extend beyond the area of the original wound, while hypertrophic scars are restricted to the wound borders (**Figure 1**) (Atiyeh et al., 2005; Baker et al., 2009; Naylor and Brissett, 2012). Consequently, annual spending on managing unwanted scarring exceeds $20 billion in the United States (Block et al., 2015). Local corticosteroid injection and radiation therapy are the current standards of care for patients who suffer from scar formation (Tziotzios et al., 2012); however, neither method shows consistent efficacy and often results in undesirable, sometimes severe, side effects (Gauglitz et al., 2011). For instance, local corticosteroids injection is known to cause reduced wound strength with increased risks of dehiscence, pigmentation changes, granulomas, and skin atrophy (Chang and Ries, 2001; Bayat et al., 2003); while radiation therapy is associated with growth inhibition, decreased wound strength, and increased long-term cancer risks (Haubner et al., 2012).

To date, several anti-scarring products targeting growth factors involved in the cutaneous wound healing process are being, or have been, tested in clinical trials for wound healing management. These include Juvista™ [recombinant transforming growth factor (TGF)β3, traditionally considered as an "antifibrotic TGFβ isoform"; clinicaltrials.gov: NCT00742443], interleukin (IL)10 (clinicaltrials.gov: NCT00984646) (Kieran et al., 2013; Kieran et al., 2014), DSC127 (NorLeu3-angiotensin; clinicaltrials.gov: NCT01830348), siRNA (RXI-109; clinicaltrials.gov: NCT02030275) and antisense oligonucleotides (EXC 001; clinicaltrials.gov: NCT01038297) that downregulate the expression of connective tissue growth factor (CTGF). However, most of these products failed to demonstrate efficacy in human trials. For instance, Juvista™ failed in phase III clinical trial in 2011 (McKee, 2011); Derma Sciences reported to stop all development work with DSC127 in scar reduction in 2015 (Levin, 2015); IL10 showed no efficacy of scar reduction in humans of African continental ancestral origin (Kieran et al., 2014); and clinical trials appear to have been halted for EXC001 (Pfizer, 2011) as there have been no public updates since 2012; Relatively, RXI-109 seems to have some benefits on the visual appearance of scar tissue in phase II clinical trials, but it requires multiple post-surgery injections, which bring higher therapeutic costs and increase the patient's suffering (Galiano, 2015). As a consequence, no drugs have been officially approved for the prevention and reduction of cutaneous scarring.

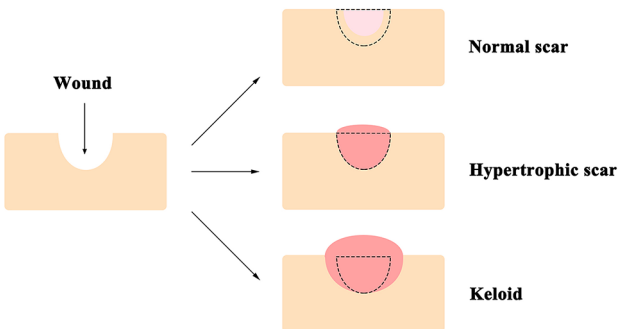

FIGURE 1 | A diagram of typical appearances of normal scar, hypertrophic scar, and keloid. Unlike that the normal scar is often smaller than the original wound, keloids extend beyond the edge of the original wound, while hypertrophic scars are restricted to the wound borders. The black dotted line demarcates the area of the original wound.

The extracellular matrix (ECM), composed of numerous macromolecules, not only functions as the critical structural components but also plays essential roles in modulating vital cellular processes, such as adhesion (Jian et al., 2013; Desseaux and Klok, 2015; Shih et al., 2016), migration (Estrach et al., 2011; Daley and Yamada, 2013; Jian et al., 2013; Scarpa and Mayor, 2016; Zheng et al., 2017), proliferation (Wight et al., 1992; Leiton et al., 2015; Cheng et al., 2016), differentiation (Jian et al., 2013; Hoshiba et al., 2016; Zheng et al., 2017), apoptosis (Ii et al., 2006; Oskarsson et al., 2015; Zhang et al., 2015), and cell fate determination (Bi et al., 2007; Zheng et al., 2012; Li et al., 2016; Zheng et al., 2019). Consequently, the ECM-based pharmacotherapeutics have been considered for treating fibrotic diseases (Ye et al., 2007), osteoarthritis (Clegg et al., 2006), osteoporosis (Stoch and Wagner, 2008), and malignancies (McKenzie, 2007). The most abundant ECM protein in connective tissues, collagen, forms the highly organized, three-dimensional macrostructure of the healthy skin (Ruszczak, 2003; Davison-Kotler et al., 2019). The initial formation and maintenance of normal, healthy collagenous matrix alignment require proteoglycans (PGs) (Chen and Birk, 2013), which are another broadly distributed component of the ECM in connective tissues to provide resilience, viscoelasticity, and a suitable environment for cellular function and development (Iozzo and Schaefer, 2015). Consisting of a core protein covalently attached with one or more glycosaminoglycan (GAG) chains, PGs play a pivotal role in the proper alignment of fibrous and elastic components in the skin and control the bioavailability of several growth factors in the ECM surrounding cells to stimulate the skin turnover and repair (Mary and James, 2015). Based on their structure, location, and properties, PGs can be divided into 4 classes: intracellular PGs, basement membrane PGs, cell-surface PGs, and extracellular PGs (Vynios, 2014; Iozzo and Schaefer, 2015). In this review we are primarily concerned with the extracellular PGs, which are known to play a role in skin wound healing. For instance, a large, aggregating and water-retaining extracellular PG, versican, is widely detected in the skin

(Carrino et al., 2011). Versican accumulation in the pericellular matrix leads to the fibroblast-myofibroblast transition in the dermis by knocking out a versican-degrading protease [ADAM metallopeptidase with thrombospondin type 1 motif (ADAMTS) 5] (Hattori et al., 2011). This indicates that versican accumulation may be beneficial for skin wound healing since the fibroblast-myofibroblast transition is pivotal for wound contraction. Aggrecan is another extracellular PG that was initially found in the cartilage and is absent in normal skin but accumulates in scar tissue (Velasco et al., 2011; Vynios, 2014; Mary and James, 2015). Aggrecan accumulation may hinder cell migration to the wound and prevent the transition of fibroblast progenitor cells to mature fibroblasts (Velasco et al., 2011). These studies suggest that aggrecan may be a potential target for reducing scar formation.

Besides versican and aggrecan, small leucine-rich proteoglycans (SLRPs), constitute another large family of extracellular PGs (Pietraszek-Gremplewicz et al., 2019) that play a pivotal role in collagen fibril growth, fibril organization, and ECM assembly in healthy skin (Merline et al., 2009; Chen and Birk, 2013). A typical SLRP has a core protein of 40–60 kDa with 10–12 leucine-rich repeat (LRR) motifs (Iozzo, 1999). Each LRR motif contains 20–29 amino acids, in which an 11-amino acid hallmark, LXXLXLXXNXL (X being any amino acid) can be identified (Iozzo, 1998; McEwan et al., 2006; Bella et al., 2008). Each LRR motif generally forms a curved conchoid structure in which LXXLXLXXNXL builds a β-strand, while β-strands from the LRRs assemble into a β-sheet that constitutes the concave surface of the entire SLRP core protein (**Figure 2**) (Scott et al., 2004).

The core proteins of SLRPs are thought to carry a two-fold biological function in the skin: (1) Regulating collagen fibrillogenesis, fibril organization, and ECM assembly to control tissue strength and biomechanics (Rada et al., 1993), which is prerequisite for skin development (Smith and Melrose, 2015); (2) modulating the bioactivities of a myriad of cytokines, chemokines, ligands, and receptors (Tillgren et al., 2009; Chen and Birk, 2013; Hultgardh-Nilsson et al., 2015) that orchestrate the wound healing process (Barrientos et al., 2008). Besides, members of the SLRP family generally obtain GAG modifications post-translationally. The multitude of substitution sites on the SLRP core protein, along with variable glycosylation states, result in a variety of SLRPs which can further facilitate their interactions with various cell surface receptors, chemokines, cytokines, and growth factors (Kram et al., 2017). Abnormalities in SLPR expression or structure often alter matrix integrity and lead to dysfunctional matrix assembly in the skin, like those found in human pathological situations and SLRP-deficient animal models (Chen and Birk, 2013). For example, expression and structural changes of some SLRPs were noticed during skin development (Carrino et al., 2011), which was summarized below. Taken together, SLRPs are not only important for structural establishment of the ECM but also crucial in a variety of biological and pathological processes, such as the remodeling of the ECM during cutaneous injury and repair (Kalamajski and Oldberg, 2010; Karsdal et al., 2013; Tracy et al., 2016; Karamanou et al., 2018).

This review aims at summarizing all relevant available information about the spatial-temporal expression pattern of

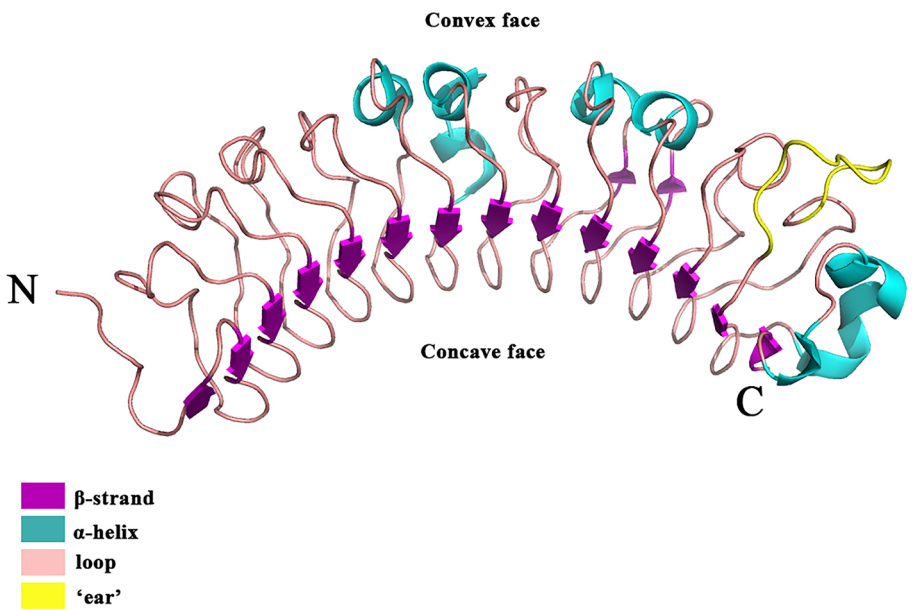

FIGURE 2 | The crystal structure of the DCN. DCN is the archetypal SLRP [the structure was retrieved from Protein Data Bank (PDB), ID: 1XKU] (Scott et al., 2004). DCN is a single-domain structure with a righthanded, curved solenoid fold characteristic of LRR proteins. The long β-sheet that forms the inner, concave face is comprised of 14 β-strands. The penultimate LRR that extends laterally from the main body of the molecule is referred to as the 'ear' (yellow) repeat, which is thought to be a distinctive feature of the SLRP family.

SLRPs in the skin, their relationship with human skin pathologies, and current understandings of their roles in skin wound healing to gain insights into their potentials as wound healing management pharmaceuticals.

THE SLRPS IN NORMAL AND DISEASED SKIN

Since decorin (DCN) was identified as the first SLRP (Krusius and Ruoslahti, 1986), many SLRP family members have been recognized in the last 30 years. Currently, 18 SLRPs have been divided into 5 classes based on the homologies at the genomic and protein level, the feature of the N-terminal cysteine residues with defined spacing, and chromosomal organization (Fisher et al., 1989; Henry et al., 2001; McEwan et al., 2006; Schaefer and Iozzo, 2008). For instance, SLRPs detected in the skin are predominantly Class I-III, which share a distinctive characteristic at the C-terminal, called an "ear" repeat (**Figure 2**). The "ear" repeat is the penultimate LRR that forms the most extended loop laterally from the convex face of the entire molecule (McEwan et al., 2006; Chen and Birk, 2011). The "ear" spreads from the first conserved C-terminal cysteine residue to the cysteine residue of the last LRR. Importantly, residues in the "ear" are not highly conserved among different SLRPs, which indicates a possible relationship to their specific functions. Consequently, the "ear" repeat is thought to maintain the configuration of the core protein and affect its ligand-binding ability (Chen and Birk, 2011; Chen and Birk, 2013). In comparison, Class IV and V SLRPs lack the "ear" repeat and are categorized as non-canonical classes of SLRPs (Schaefer and Iozzo, 2008).

Class I SLRPs

Five SLRP members are identified in this class, including DCN (Danielson et al., 1997), biglycan (BGN) (Corsi et al., 2002), asporin (ASPN) (Henry et al., 2001), extracellular matrix protein 2 (ECM2) (Nishiu et al., 1998), and extracellular matrix protein X (ECMX) (Iozzo and Schaefer, 2015). DCN, BGN, and ASPN all present in the skin with different transcriptional patterns, and ECM2 mRNA expression can also be detected in the skin (Maquart et al., 2010).

Decorin (DCN)

Containing a 36 kDa core protein with single chondroitin sulfate (CS) or dermatan sulfate (DS) chain (Roughley, 2006), DCN has been considered as the predominant interstitial PG in human skin (Carrino et al., 2011; Li et al., 2013). In the skin, DCN has been detected mostly in the reticular dermis, but absent from the papillary dermis. Minor DCN expression was also found in the epidermis (Fleischmajer et al., 1991; Lochner et al., 2007). Since DCN comprises most of the type I collagen-binding PGs in human skin (Li et al., 2013), it is thought to play a critical role in the regulation of fibril structure in the skin.

In fetal rat skin, transcription of DCN increases between embryonic days 16.5 (E16.5) and E18.5 (term, 21.5 days) which is correlated to the transition from fetal-type scarless healing to adult-type scarring period in the skin (Soo et al., 2000; Zheng et al., 2016). In human skin, the level of DCN accelerates with aging (Carrino et al., 2000; Carrino et al., 2003). For example, a clinical study showed that the transcriptional level of DCN in skin biopsies from older adult donors (61–68 years) was twofold greater than that of their younger counterparts (25–35 years) (Lochner et al., 2007). In addition to its elevated expression, the molecular weight of DCN in older human skin was found to be significantly smaller due to the shortened GAG chains (Li et al., 2013). Similar results have been replicated in rats (Ito et al., 2001; Nomura et al., 2003). Importantly, the cutaneous wound healing process of the elderly is much slower, while all healing phases differ from their younger counterparts, including delayed inflammatory response, delayed proliferative response, and much weaker remodeling phase (Gerstein et al., 1993; Gould et al., 2015). Therefore, the elevated expression and reduced weight of DCN in the skin of the aged population may be associated with their functional alterations, although the underlying mechanism is not fully elucidated and warrants further investigations. Moreover, altered expression of DCN has been detected in a number of human diseases with skin phenotypes, including decreased DCN in fibroblasts in patients with neonatal Marfan syndrome (Raghunath et al., 1993), increased DCN in fibroblasts from patients with localized scleroderma (Izumi et al., 1995), and increased DCN in fibroblasts from patients with systemic sclerosis (Westergren-Thorsson et al., 1996). Targeted disruption of DCN in mice results in abnormal collagen fibril morphology and skin fragility with markedly reduced tensile strength (Danielson et al., 1997). Another study showed that DCN and BGN double-knockout (KO) mice directly resemble the rare progeroid variant of human Ehlers-Danlos syndrome (EDS), in which skin fragility and progeroid changes in the skin (reduced hypodermis) are dramatically displayed (Corsi et al., 2002). Furthermore, in a progeroid patient carrying two point mutations in beta-1,4-galactosyltransferase 7 (B4GALT7), only 50% of the DCN exhibit GAG side-chain substitution on their core protein, which is thought to be a major mechanistic cause for the skin and wound healing defects observed in this patient with the progeroid form of EDS (Gotte and Kresse, 2005). These investigations indicate that DCN is crucial to the normal function of the skin and maybe a potential candidate for pharmacological development in the treatment of some skin diseases.

Biglycan (BGN)

Another well-studied Class I SLRP is BGN. BGN usually contains a 38 kDa core protein attached with two CS/DS chains; however, nonglycanated forms of BGN have also been detected in human intervertebral discs (Johnstone et al., 1993). While both BGN and DCN belong to Class I SLRPs and are able to bind with type I collagen fibrils directly, their spatial expression in skin is very different. For instance, DCN is mainly synthesized by interstitial fibroblasts, whereas BGN is secreted by both dermal and epidermal cells (Li et al., 2013). Besides, BGN is present in the connective tissue sheath of the hair follicle (Malgouries et al., 2008).

Similar to DCN, decreased BGN expression was also found in fibroblasts isolated from the skin of systemic sclerosis patients

(Westergren-Thorsson et al., 1996). Although BGN deficiency in mice also induces changes in collagen fibril morphology in the skin and leads to the mild cutaneous abnormalities with thinning of the dermis, skin fragility of BGN-deficient mice is not noticeably altered (Corsi et al., 2002). Corsi et al. claimed that the skin abnormalities in BGN-deficient mice were more subtle in comparison with DCN-deficient mice (Corsi et al., 2002), which in turn resulted in that research was not focused on BGN and its role in skin and wound healing.

Asporin (ASPN)

Unlike general SLPRs, ASPN contains a 43 kDa core protein, but lacks a GAG side chain (Lorenzo et al., 2001) and carries a polymorphic calcium-binding polyaspartate sequence (Kalamajski et al., 2009). ASPN has been found in dermis, perichondrium and periosteum, tendon, and eye sclera (Kou et al., 2007). ASPN-null mice exhibit an increased skin mechanical toughness due to the altered GAG composition and structure in the ECM (Maccarana et al., 2017). However, ASPN has not been studied in depth for its involvement in skin development and cutaneous wound healing.

Class II SLRPs

To date, this class contains 5 members that can be divided into 3 subgroups based on their protein homology. Subgroup A consists of fibromodulin (FMOD) (Velez-Delvalle et al., 2008) and lumican (LUM) (Yeh et al., 2010), subgroup B includes keratocan (KERA) (Corpuz et al., 1996) and proline and arginine rich end leucine rich repeat protein (PRELP) (Grover and Roughley, 2001), and subgroup C is comprised of osteomodulin (OMD) (Tasheva et al., 2002).

Fibromodulin (FMOD)

FMOD has a 42 kDa core protein with up to 4 N-linked keratan sulfate (KS) attached, which shares significant sequence homology with DCN and BGN (Antonsson et al., 1993). In the skin, FMOD is predominately secreted by dermal fibroblast and is also expressed by human epidermal keratinocytes *in vitro* and detected in the human epidermis *in vivo* (Velez-Delvalle et al., 2008).

Unlike other SLRPs, expression of FMOD significantly decreases during the transition from fetal-type scarless repair to adult-type repair with scaring in a fetal rat skin model (Soo et al., 2000; Zheng et al., 2016). Moreover, our recent study demonstrated that FMOD is essential for fetal-type scarless cutaneous wound healing by loss- and gain-of-function studies in mouse and rat models (Soo et al., 2000; Zheng et al., 2016).

Although FMOD-null mice showed no apparent defects in the unwounded skin (Chakravarti, 2002), a wider distribution of collagen fibril diameters accompanied with enlarged interfibrillar spaces between collagen fibrils was observed (Khorasani et al., 2011). Meanwhile, thinner collagen fibrils and abnormal fibers with increased deposition of LUM were also found in the tendons of FMOD-null mice (Svensson et al., 1999). As expected, FMOD and LUM double-deficient mice showed more obvious abnormalities, such as reduced body size, increased skin hyperextensibility, escalated gait abnormality, intensified joint laxity, and accelerated age-dependent osteoarthritis resembling EDS (Jepsen et al., 2002). These abnormal phenotypes may indicate a functional overlap between FMOD and LUM in modulating the ECM and cellular behavior in a broad range of tissues (Chakravarti, 2002; Jepsen et al., 2002). It is known that the re-organization of ECM is necessary during the healing process since pathological scarring occurs when the ECM is not appropriately reformed. Thus, the fact that FMOD is essential for regular collagen fibril organization in connective tissues suggests that FMOD may play a pivotal role in skin wound healing.

Lumican (LUM)

LUM was first isolated from the chicken cornea (Blochberger et al., 1992). LUM has a 38 kDa core protein with 4 N-linked sites within the LRR domain of the core protein that can be substituted by KS (Scott, 1996). It is expressed in the subepithelial dermis by dermal fibroblasts (Ying et al., 1997; Chakravarti et al., 1998). Interestingly, LUM is also secreted by melanoma cells but not normal melanocytes (Sifaki et al., 2006).

Unlike FMOD whose expression is reduced from early/mid-gestation when skin wounds heal scarlessly to late-gestation when skin wounds end up with adult-type scarring, LUM expression in fetal skin is upregulated during the same transition period, much like DCN (Zheng et al., 2016). On the contrary, a significant negative correlation between LUM transcriptional levels in human skin fibroblasts and donors' age was observed in a study involving 1-month- to 83-year-old participants (Vuillermoz et al., 2005). The steady decline in LUM expression accompanied by the upregulation of DCN expression with aging indicates that these changes may be contributing to the functional impairment of fibroblasts during aging, such as decreased fibroblast growth and survival (Campisi, 1998; Brown, 2004; Vuillermoz et al., 2005). Interestingly, similar to DCN-deficient mice, LUM-null mice display skin laxity and fragility resembling EDS (Chakravarti et al., 1998). It is worth noting that wounds in FMOD-null mice have delayed dermal fibroblast migration but accelerated epidermal migration accompanied by elevated LUM expression (Zheng et al., 2014b), indicating FMOD and LUM may predominately function on fibroblast and keratinocytes, respectively. Thus, in comparison with FMOD whose biopotency is mainly assessed on dermal functions (Zheng et al., 2014a; Zheng et al., 2014b; Zheng et al., 2016), the investigation of LUM is more focused on the cornea in which epidermal migration plays more essential roles during wound healing (Saika et al., 2000; Seomun and Joo, 2008; Frikeche et al., 2016).

Keratocan (KERA)

KERA is a 60–70 kDa KS substituted member of the SLRP family (Mary and James, 2015). It is mainly abundant in the cornea and detected in much lesser amount in the skin as a non-sulfated glycoprotein (Corpuz et al., 1996). The variety of the abundance and GAG structure of KERA found in different tissues suggests that its function be tissue-dependent. For example, in the cornea, KERA with long, highly sulfated KS chains has been thought to be essential for corneal transparency (Kao and Liu, 2002). Thus,

similar to LUM (Yamanaka et al., 2013), KERA is also a potential target for cornea healing therapies (Kao and Liu, 2002; Carlson et al., 2003; Liu et al., 2003; Chen et al., 2011). However, its role in the skin is still ambiguous for delineating.

Proline and Arginine Rich End Leucine Rich Repeat Protein (PRELP)

PRELP contains a 55-kDa core protein with no GAG and an N-terminal region which is highly unique, conserved, and rich in arginine and proline (Bengtsson et al., 2000). It functions as a molecule by anchoring basal membranes to the underlying connective tissue (Grover and Roughley, 1998; Grover and Roughley, 2001). For example, PRELP is expressed in the basement membrane between the epidermis and the dermis in the skin (Bengtsson et al., 2002). Overexpression of PRELP in mice leads to reduced collagen fiber bundle content and size in the dermis and decreases the thickness of the hypodermal fat layer in the skin (Grover et al., 2007), which somewhat resembles the symptoms of Hutchinson–Gilford progeria (Mounkes et al., 2003). In addition, PRELP can bind to perlecan (Bengtsson et al., 2002), which is thought to be essential for epidermal formation by regulating the survival of keratinocytes (Sher et al., 2006). This indicates that PRELP may participate in regulating the function of keratinocytes, but further studies are needed to elucidate it. However, a clear application of PRELP for wound healing is still lacking.

Interestingly, in comparison with Class I SLRPs, most Class II SLRPs seem to have a more executive function on epidermal keratinocytes. The one exception is FMOD, which has proven to be critical for maintaining the normal function of dermal fibroblasts (Zheng et al., 2016), as well as endothelial cells (Adini et al., 2014; Zheng et al., 2014a), like DCN and BGN. The response to different cell types may pave the fundamental for developing combination therapies of SLRPs to target both dermal and epidermal cells simultaneously to maximize their complementary biopotency and thus to optimize the skin wound healing outcome.

Class III SLRPs

To date, osteoglycin (OGN, also known as mimecan) (Tasheva et al., 2002), epiphycan (EPYC) (Johnson et al., 1997) and opticin (OPTC) (Reardon et al., 2000) constitute this class, which is characterized by a relatively low number of LRRs (7 LRRs) compared to the classic 10-12 LRRs of other classes.

OGN was first identified as a 25 kDa KS SLRP in the cornea, and a 36 kDa OGN protein without KS chains was also detected in other connective tissues including aorta, sclera, skin, cartilage, the vagus nerve, and in lesser amounts in the cerebellum, kidney, intestines, myocardium, and skeletal muscle (Funderburgh et al., 1997). As for its role in the skin, OGN-deficient mice display skin with moderately reduced tensile strength, which is correlated to the presence of thicker collagen fibrils that possess marked increases in collagen fibril diameter. OGN also plays a pivotal role in collagen fibrillogenesis in the skin (Tasheva et al., 2002). Although transcription of EPYC and OPTC have been detected in the skin (Reardon et al., 2000; Takanosu et al., 2001; Maquart et al., 2010), their biological function in the skin is unclear.

Class IV and Class V SLRPs

Class IV and V SLRPs are considered to be non-canonical classes of SLRPs. These include chondroadherin (CHAD) (Haglund et al., 2011), nyctalopin (NYX) (Bech-Hansen et al., 2000), tsukushi (TSKU) (Ohta et al., 2004), podocan (PODN) (Ross et al., 2003), and recently identified podocan like 1 (PODNL1) (Mochida et al., 2011). The function and spatial-temporal expression patterns of these SLRPs in the skin are rarely studied. A previous study detected CHAD mRNA in keratinocytes, and NYX mRNA in keratinocytes and skin fibroblasts (Maquart et al., 2010). Future studies, not necessarily limited to the skin, are required to reveal their biological functions.

The expression and distribution of known SLRPs in the skin, as well as the abnormalities observed in SLRP-deficient mice, are summarized in **Table 1**.

THE SLRPS IN SKIN WOUNDS AND WOUND HEALING

As a protective barrier shielding the human body from the environment, the skin plays a pivotal role in maintaining physiological homeostasis of the human body. Any lesion breaking the skin barrier will make the organism vulnerable to infections, thermal disorders, and fluid loss (Sorg et al., 2017). Skin wound healing is a dynamic, complex and tightly regulated process comprised of hemostasis, inflammation, proliferation, remodeling, and maturation phases, in which various cell types and mediators are recruited at the wound site, and complex interactions exist between different cells and the ECM (Martin, 1997; Diegelmann and Evans, 2004; Gibran et al., 2007; Artlett, 2013). During the process of wound healing, the ECM not only provides structural support for the tissues, but also serves as a platform for cells and mediators that regulates inter/intracellular signaling (Ghatak et al., 2015). As essential components of the ECMs, many SLRPs participate in a diversity of signaling pathways to regulate cellular activities during the wound healing process.

Inflammation

Following an injury, skin cells are exposed to acute inflammatory signals such as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (Takeuchi and Akira, 2010; Strbo et al., 2014). These patterns can be recognized by toll-like receptors (TLRs) to initiate inflammation. Leukocytes are attracted to the site of injury, accompanied by elevated levels of pro-inflammatory cytokines, and thus amplify the inflammatory response (Eming et al., 2014; Vestweber, 2015). Gradually, macrophages will display a transition from the M1 subset (phagocytic activity and production of pro-inflammatory cytokines) (Galli et al., 2011; Sindrilaru and Scharffetter-Kochanek, 2013) to the M2 subset (reparative activity with the synthesis of anti-inflammatory mediators and the production of the ECM) (Brancato and Albina, 2011; Sindrilaru and Scharffetter-Kochanek, 2013).

TABLE 1 | The expression and distribution of SLRPs and abnormalities of knock out and overexpression mice in the skin.

SLRP	Expression and distribution in the skin	Mice model	Abnormalities in the skin
Decorin	Most expression in the dermis and minor expression in the epidermis	Targeted disruption of decorin in exon2	Skin fragility with markedly reduced tensile strength
Biglycan	Expression in the dermis, the epidermis and the sheath of hair follicle	Targeted disruption of biglycan in exon2	Mild skin abnormalities with thinning of the dermis but without distinct skin fragility
Decorin and Biglycan	Not applicable	Decorin and biglycan double-knockout mice	Skin fragility and progeroid changes in the skin (reduced hypodermis)
Asporin	Expression in the dermis	Targeted disruption of asporin in exons 2–3	Increased skin mechanical toughness
Fibromodulin	Expression in the dermal fibroblast and human epidermal keratinocytes *in vitro* and the epidermis *in vivo*	Targeted disruption of fibromodulin in exon2	No overt defects in skin, but larger collagen fibrils and less orderly packed collagen fibrils with increased interfibrillar space
Lumican	Expression in the dermis	Targeted disruption of lumican in exon2	Skin laxity and fragility
Fibromodulin and Lumican	Not applicable	Fibromodulin and lumican double-knockout mice	Additional gross skin phenotypes including skin hyperextensibility
PRELP	Expression in the basement membrane between the epidermis and the dermis	Overexpression of PRELP transgenic mice	Decreased collagen fiber bundle content and size in the dermis, and the thinner hypodermal fat layer
Osteoglycin	Expression in the skin	Targeted disruption of osteoglycin in exon2	Reduction in the tensile strength of the skin, thicker collagen fibrils and a significant increase in collagen fibril diameter in the skin

This switch corresponds to the transition from the inflammation stage to the proliferation stage in the wound healing process.

Although there have been studies investigating the effects of SLRPs on the inflammatory response, most do not specifically focus on skin wound healing. Several studies have shown that DCN, BGN, and LUM can interact with TLR2 and/or TLR4 signaling pathways in innate immune responses to combat microbial pathogens. For instance, in mouse peritoneal macrophages, DCN induces tissue necrosis factor (TNF) and programmed cell death 4 (PDCD4) production through TLR2 and TLR4, which enhances the proinflammatory effects of lipopolysaccharides (LPS), a vital constituent of Gram-negative bacteria, which can trigger a robust immune response (Merline et al., 2011). BGN has also been proven to be a proinflammatory factor in mouse peritoneal macrophages by regulating the same signal pathways as DCN (Schaefer et al., 2005). Like DCN and BGN, LUM enhances host immune responses to LPS *via* TLR4 in mouse peritoneal macrophages (Wu et al., 2007). Unsurprisingly, LUM-null mice are hypo-responsive to LPS-induced septic shock with reduced pro-inflammatory cytokines production (Wu et al., 2007). Also, LUM has been shown to regulate inflammation in the development of colitis in mice (Lohr et al., 2012), and accelerate LPS-induced renal injury in mice *via* TLR4-nuclear factor κB (NFκB) pathway (Lu et al., 2015). Moreover, in LPS-induced wounds of the cornea, no induction of TNF or IL1β, and reduced infiltration of neutrophils and macrophages were found in LUM-null mice (Vij et al., 2005).

In contrast to their pro-inflammatory functions in the aforementioned infection scenario, SLRPs may act as anti-inflammatory factors to inhibit excessive inflammation during wound healing in the skin. For example, FMOD-null mice exhibit elevated and prolonged inflammatory infiltration in the skin wound area, accompanied by delayed reepithelialization (Zheng et al., 2014b). Similarly, LUM-deficient mice display an increased inflammatory macrophage density with delayed cutaneous wound healing (Yeh et al., 2010). Furthermore, TSKU has been detected in fibroblasts, myofibroblasts, and macrophages during skin wound healing in mice. Likewise, loss of TSKU causes increased TGFβ1 expression and excess inflammation (Niimori et al., 2014). Collectively, these studies suggest that SLRPs may play a diverse role in inflammatory response regulation, which may highly depend on the microenvironment.

Angiogenesis

The process of angiogenesis occurs accompanied by fibroblast proliferation when endothelial cells migrate to the wound site and provide the nutritive perfusion for fibroblasts and epithelial cells during the healing process (Martin, 1997; Demidova-Rice et al., 2012; Sorg et al., 2017). The involvement of SLRPs has been identified in the angiogenesis of wound healing, tumorigenesis, and other inflammatory processes. For instance, DCN exhibits antiangiogenic activities during cutaneous wound healing, while higher DCN expression was detected in human benign tumors *vs.* malignant vascular tumors (Jarvelainen et al., 2006; Salomaki et al., 2008). Meanwhile, impaired angiogenesis was found in the injured cornea of DCN-null mice (Schonherr et al., 2004), and repressed angiogenesis was also present in some tumors associated with reduced DCN expression (Nayak et al., 2013; Chui et al., 2014). These studies suggest that DCN can be either stimulatory or suppressive for angiogenesis, which may be related to the physiologic and pathologic conditions of tissues (Järveläinen et al., 2015).

BGN has been shown to have a proangiogenic effect in fracture healing (Berendsen et al., 2014; Myren et al., 2016), colon cancer (Xing et al., 2015), and tumor endothelial cells (Yamamoto et al., 2012), though its role in angiogenesis during skin wound healing is not clear.

FMOD was found to promote angiogenesis during cutaneous wound healing (Zheng et al., 2011; Zheng et al., 2014a). Particularly, FMOD was found to accelerate human umbilical vein endothelial cell adhesion and spreading, actin stress fiber

formation, and eventually tube-like structure network establishment *in vitro* (Jian et al., 2013). Furthermore, it has been confirmed that FMOD stimulates angiogenesis in various *in vivo* systems, such as neovascularization, wound healing and Matrigel™ plug assays. FMOD was also found to enhance vascular sprouting during normal retinal development (Adini et al., 2014). FMOD also promotes tumor angiogenesis of small cell lung cancer by upregulating angiogenic factor expression (Ao et al., 2017). Overall, these studies constitute evidence that FMOD displays angiogenic biopotency in numerous biological processes.

On the other hand, LUM was found to inhibit angiogenesis by interfering with integrin α2β1 activity and repressing matrix metalloproteinase (MMP)14 expression *in vivo* (Niewiarowska et al., 2011). LUM has also been identified as an inhibitor for tumor angiogenesis (Albig et al., 2007; Brezillon et al., 2009; Williams et al., 2011). Interestingly, angiogenesis was not altered in LUM-deficient mice in aortic ring assays, Matrigel™ plugs, or healing wound biopsies (Sharma et al., 2013). Thus, LUM is thought to exhibit an antiangiogenic effect in restricted circumstances, possibly only in some specific tumor microenvironments.

In summary, these studies paint the picture of SLRPs playing a wide range of roles in the angiogenesis of various biological processes. Specifically, FMOD is the only SLRP confirmed to enhance angiogenesis during skin wound healing, suggesting that it may have therapeutic potential in cutaneous healing of poorly vascularized wounds, such as in the scenarios of diabetic wounds.

Fibroblast Activities

Dermal fibroblasts are the predominant cellular component in the wound healing process. During the proliferation stage, fibroblasts migrate into the wound site, and gradually grow to produce a new provisional ECM through the production of collagen and fibronectin (Midwood et al., 2004). Wound contraction will occur when fibroblasts differentiate into myofibroblasts after reepithelialization. This process decreases the size of the wound and is followed by the removal of unneeded cells through apoptosis (Hinz, 2006). SLRPs are known to impact several of the critical functions of fibroblasts during wound healing, including migration, proliferation, differentiation, and collagen synthesis. As a result, abnormal expression of SLRPs can disrupt the wound healing process and possibly result in pathological scarring, as seen in keloids and hypertrophic scars.

TGFβ signaling has been thought to play a central role in both skin wound healing and scar formation (Faler et al., 2006; Penn et al., 2012; Pakyari et al., 2013), and DCN is known to bind to all three mammal TGFβ isoforms and represses their activity by sequestering the isoforms to the ECM and thus inhibiting their signal transduction (**Figure 3A**) (Droguett et al., 2006; Penn et al., 2012). DCN was also found to interact with CTGF-a downstream mediator of TGFβ1 signaling (**Figure 3B**) (Daniels et al., 2003; Shi-Wen et al., 2008; Vial et al., 2011). Moreover, DCN is able to activate epidermal growth factor receptor (EGFR)-mediated receptor auto-phosphorylation and downstream signaling pathways, such as the mitogen-activated protein kinase (MARK)1/3 pathway, to mobilize intracellular calcium, and activate other EGFR-dependent pathways in tumor cells to suppress cell growth (Moscatello et al., 1998; Patel et al., 1998). DCN displays similar cell growth suppression ability in dermal fibroblasts (Laine et al., 2000; Tran et al., 2004). Furthermore, a recent study revealed that DCN repressed corneal stromal fibroblasts migration *via* inducing EGFR degradation (**Figure 3C**) (Mohan et al., 2019), which has not been well investigated in the context of skin wounds, although low levels of DCN and increased activation of the MARK1/3 signaling have been observed in keloid tissues (Meenakshi et al., 2009). In addition to regulating growth factor signaling transduction, DCN serves as a stabilizer of the ECM tissue structure through binding of type I collagen and thus downregulates cellular proliferation and migration, as well as protein synthesis in a number of biological and pathologic processes (Iozzo, 1999; Tran et al., 2004). Keloid fibroblasts have less DCN expression than normal fibroblasts (Mukhopadhyay et al., 2010) while forcing DCN expression by adenovirus in keloid fibroblasts remarkably reduced their collagen synthesis and upregulated the transcriptional level of MMP1 and MMP3 (Lee et al., 2015). The expression of DCN in the fibroblasts isolated from the deep dermis was also lower than those derived from the superficial dermis (Honardoust et al., 2012b). This phenomenon indicates a possible relationship between lower DCN expression and hypertrophic scarring as deep dermal injuries often lead to hypertrophic scarring while superficial cutaneous wounds usually heal with minimal scarring (Wang et al., 2008; Marshall et al., 2018). In comparison to unwounded skin, post-burn hypertrophic scar tissue also has a lower level of DCN (Scott et al., 1995; Sayani et al., 2000). While DCN-deficient mice exhibit significantly postponed cutaneous wound healing (Jarvelainen et al., 2006). The lower DCN levels in keloids and hypertrophic scars may contribute to the unordered collagen arrangement and excess ECM production. Meanwhile, recombinant human DCN inhibits fibroblast proliferation and downregulates TGFβ1 production, and collagen synthesis in hypertrophic scar fibroblasts (Zhang et al., 2007). Moreover, DCN can inhibit the contraction of collagen gel encapsulation in normal or hypertrophic scar fibroblasts, which gives further indication that it may pose therapeutic potential in hypertrophic scarring (Zhang et al., 2009). Recent studies show that activation of Tuberoinfundibular peptide of 39 residues (TIP39)—Parathyroid Hormone 2 Receptor (PTH2R) signaling or blocking of microRNA 181b can induce DCN expression and promote wound repair (**Figure 3D**) (Kwan et al., 2015; Sato et al., 2017). Additionally, a collagen-binding peptidoglycan derived from DCN has been shown to inhibit MMP-mediated collagen degradation *in vitro* and reduce scar formation in mice (Stuart et al., 2011). Collectively, these results indicate that DCN may be a potential therapeutic agent for keloids and hypertrophic scars.

As a structurally homologous protein of DCN, BGN can also bind to TGFβ isoforms to attenuate its signal transduction (Hildebrand et al., 1994; Droguett et al., 2006; Penn et al., 2012). Expression of BGN was not altered in excisional skin wounds and hypercontracted/hyperpigmented scarring pig

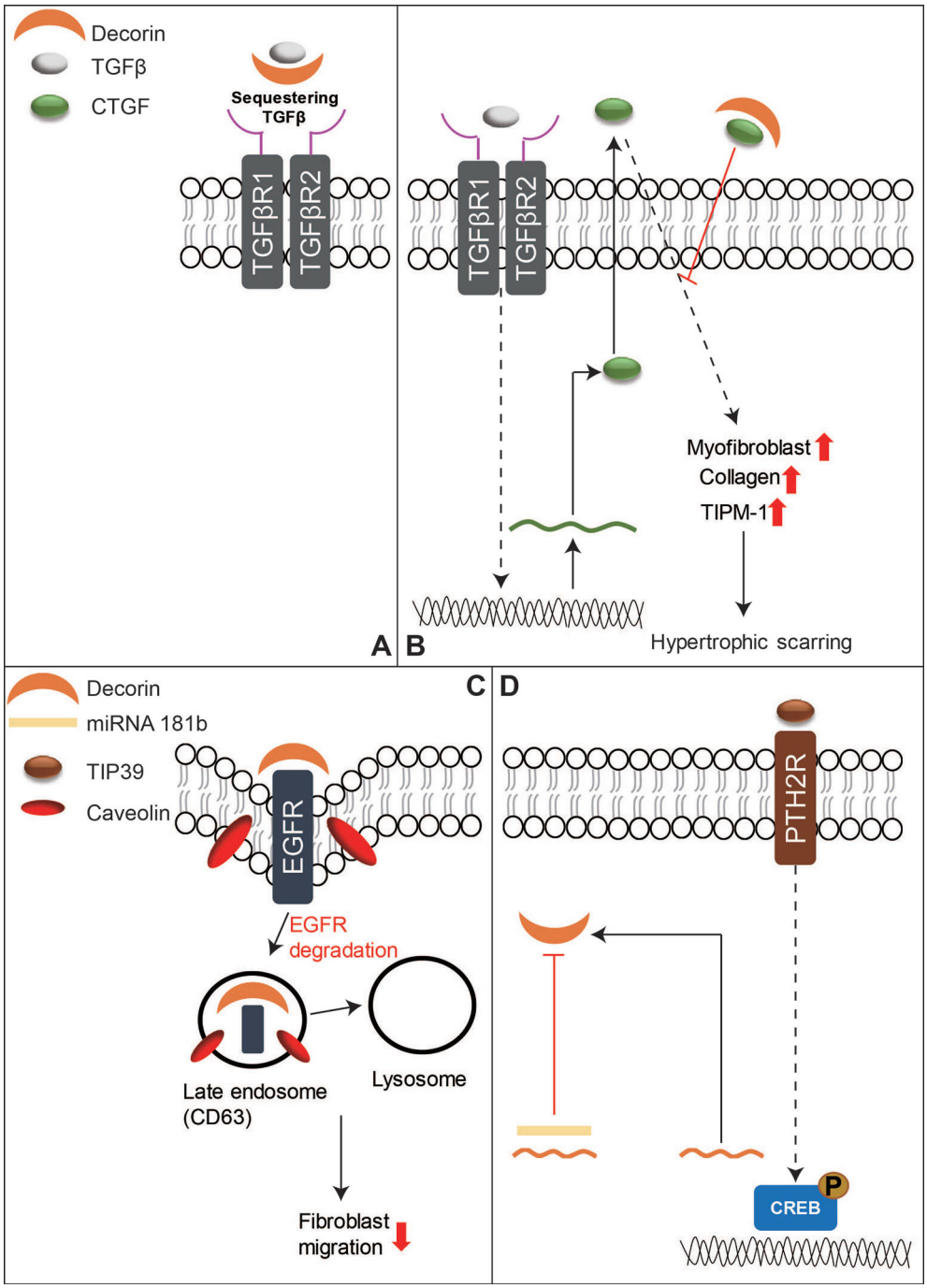

FIGURE 3 | A schematic diagram of the functions of DCN in skin wound healing. **(A)** DCN is known to bind to mammal TGFβ isoforms to sequester their signal transduction. **(B)** DCN can also bind to CTGF, which is a downstream mediator of TGFβ1 signaling, to reduce hypertrophic scarring. **(C)** In addition, DCN can repress fibroblasts migration by inducing EGFR degradation. **(D)** On the other hand, activation of TIP39-PTH2R signaling or blocking of microRNA 181b can induce DCN production, and thus benefit skin wound repair.

models (Olson et al., 2000; Gallant et al., 2004). However, the upregulation of BGN was observed in adult rat wound healing models (Soo et al., 2000). Elevated BGN expression was also observed in hypertrophic scars compared with that in normal scars (Armour et al., 2007; Honardoust et al., 2011). Deep dermal fibroblasts also have a higher BGN level than that of the superficial dermal fibroblasts (Honardoust et al., 2011; Honardoust et al., 2012a). Unfortunately, whether higher BGN

expression in deep dermal fibroblasts is relevant to the profibrotic or inflammatory response in deep dermal cutaneous injuries remains elusive. Although BGN has been shown to regulate proinflammatory cytokine expression and inflammatory response by TLR2 and TLR4 in the kidney, lung, and circulation (Babelova et al., 2009). Moreover, BGN transcription was up-regulated in keloid tissues (Hunzelmann et al., 1996). Interestingly, basic fibroblast growth factor (bFGF) can up-regulate BGN while suppressing DCN expression in keloid fibroblasts (Tan et al., 1993). Taken together, these results imply that BGN may be related to keloid and hypertrophic scarring. Further investigations are needed to confirm the involvement of BGN in cutaneous wound healing and elucidate the specific roles of BGN and DCN during the scar formation.

FMOD was down-regulated during adult rat wound healing with scar formation (Soo et al., 2000). Importantly, loss of FMOD can eliminate the ability of early-gestation fetal rodents to heal without scarring. Meanwhile, the administration of FMOD alone was capable of restoring scarless healing in late-gestation rat fetal wounds, which would naturally heal with scar (Zheng et al., 2016). In addition to restoring scarless wound healing in late-gestation fetal wounds, forcing FMOD elevation by adenovirus can also promote skin wound healing in adult rabbit full-thickness incisions (Stoff et al., 2007). Additionally, FMOD-deficient mice exhibit delayed wound closure and increased scar formation (Zheng et al., 2011; Zheng et al., 2014b).

Many review articles have already focused on the essential role of TGFβ signaling in wound healing (Faler et al., 2006; Penn et al., 2012; Pakyari et al., 2013; Lichtman et al., 2016). However, among SLRPs, only FMOD has been studied in detail about its interaction with TGFβ signaling to orchestrate the function of fibroblasts to enhance skin wound healing (Zheng et al., 2017). For example, the delayed cutaneous wound closure in FMOD-deficient mice may be attributed to the elevated local TGFβ3 levels (Zheng et al., 2011), since TGFβ3 selectively postpones dermal fibroblast proliferation and migration into the wound area (Bandyopadhyay et al., 2006; Han et al., 2012). Moreover, adult FMOD-null mouse wounds have higher expression of TGFβ receptors in comparison with their wild-type counterparts during the proliferative stage, but reduced expression of TGFβ ligands and receptors during the remodeling stage (Zheng et al., 2014b). Similar to DCN, FMOD is downregulated in postburn hypertrophic scars (Honardoust et al., 2011). Reduced fibromodulin in the deep dermis of the skin is thought to contribute to the development of hypertrophic scars after injuries (Honardoust et al., 2012a; Honardoust et al., 2012b). Interestingly, FMOD transcription was not altered following wound creation in an adult Yorkshire pig model, but exhibited a biphasic pattern of mRNA expression (initial increased at day 14, followed by decreased levels at days 28–42 and then a second peak by days 56–70) in an adult red Duroc pig model (Olson et al., 2000; Gallant et al., 2004). These observations are aligned with the previous hypothesis that the healing profile of the red Duroc pig wound model (which simulates hypertrophic healing in humans) (Harunari et al., 2006; Xie et al., 2007; Zhu et al., 2007) is inherently different from that of the Yorkshire pig wound model (which simulates normal scarring) (De Hemptinne et al., 2008; Seaton et al., 2015). Mechanically, like DCN and BGN, FMOD shows similar properties in its ability to bind to mammal TGFβ isoforms; however, it is a more effective competitor for TGFβ binding than DCN or BGN (Hildebrand et al., 1994). Traditionally, FMOD was considered an extracellular TGFβ reservoir (**Figure 4A**). Our recent studies have deeply explored the mechanisms by which FMOD orchestrates TGFβ1 signaling and subsequently reduces scar formation in adult skin wounds (Zheng et al., 2017): "(1) like fetal wounds (Larson et al., 2010), FMOD treatment to adult wounds causes reduced and more transient TGFβ1 expression; (2) like fetal wounds (Walraven et al., 2015), FMOD treatment induces high level of SMAD2 and SMAD3 phosphorylation, and low levels of several fibrosis-associated targets; (3) like fetal fibroblasts (Sandulache et al., 2007), FMOD treatment results in a more migratory and contractile phenotype; (4) like fetal fibroblasts (Colwell et al., 2006), FMOD treatment exhibits higher TGFβ1-stimulated CTGF expression levels for increased myofibroblast differentiation and contraction; and (5) much like fetal wounds (Cass et al., 1997), FMOD treatment results in more rapid myofibroblast clearance from the wound" (**Figure 4B**). Taken together, FMOD administration in adult wound models elicits a similar phenotype to fetal wounds at the molecular, cellular, and gross morphological levels (Zheng et al., 2017). Moreover, recent studies demonstrate that FMOD can directly reprogram human dermal fibroblasts into a multipotent stage, indicating its ability to regulate the intracellular signaling cascade and determine the cell fate (Zheng et al., 2012; Li et al., 2016; Zheng et al., 2019). Furthermore, from a translational aspect, recent studies have confirmed the biopotency of FMOD in reducing scar formation, accelerating wound tensile strength reestablishment, and improving dermal collagen architecture organization as well as gross wound appearance in multiple small and large preclinical animal models, and even within an excessive-mechanical-loading model (Zheng et al., 2017; Jiang et al., 2018), highlighting the enormous potentials of FMOD as a regenerative medicine for wound and scar therapies. Encouragingly, we have developed an FMOD-derived peptide (SLI-F06) which is being tested in a clinical trial for optimizing cutaneous wound healing (clinicaltrials.gov: NCT03880058).

LUM is the only known SLRP expressed by the epithelia during wound healing (Frikeche et al., 2016; Karamanou et al., 2018). During cornea wound healing, LUM is known to regulate collagen fibrillogenesis, keratinocyte phenotypes, corneal transparency modulation, angiogenesis, and extravasation of inflammatory cells (Park and Tseng, 2000; Vij et al., 2005; Hayashi et al., 2010; Chen et al., 2011; Karamanou et al., 2018). However, the role of LUM in skin wound healing has not been adequately assessed. Liu et al. reported that recombinant LUM protein promoted skin wound healing in adult mice by facilitating dermal fibroblast activation and contraction without promoting keratinocyte proliferation and migration (Liu et al., 2013). The increased fibroblast contractibility induced by LUM is regulated by integrin

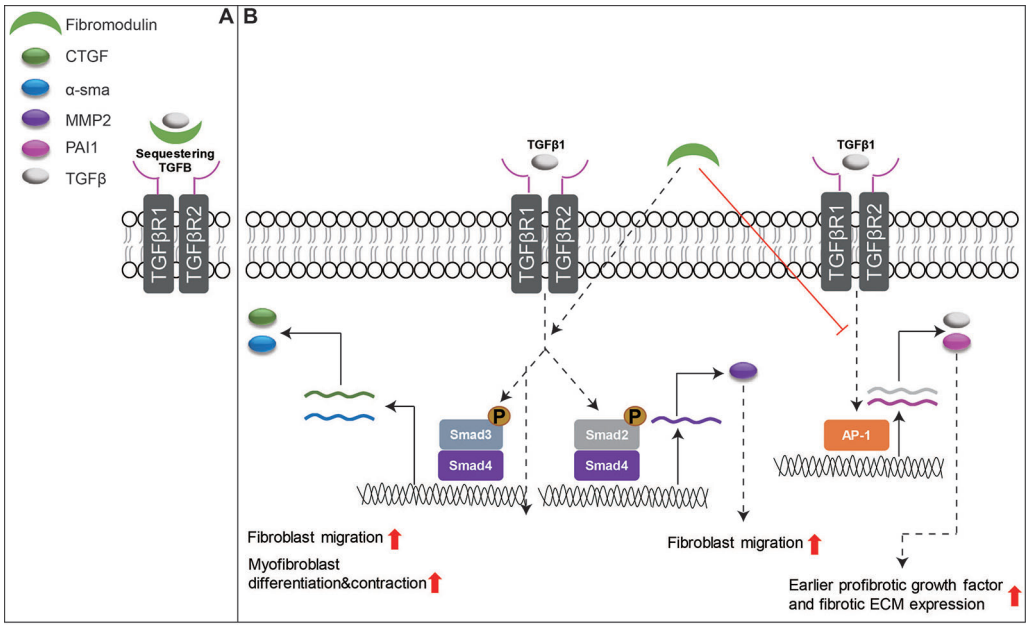

FIGURE 4 | Schematic depiction of the functions of FMOD in skin wound healing. **(A)** FMOD is able to bind to all three mammal TGFβ isoforms as an extracellular TGFβ reservoir. **(B)** Importantly, FMOD can selectively enhance SMAD3-mediated TGFβ1-responsive pro-migration and pro-contraction signaling, while reducing AP-1-mediated TGFβ1 auto-induction and fibrotic ECM accumulation during adult cutaneous wound healing.

subunit alpha (ITGA)-2 (Liu et al., 2013). Meanwhile, Zhao et al. reported that adenovirus-mediated LUM-overexpression suppressed excessive fibroblast proliferation and ECM production *in vitro via* inhibiting collagen - ITGA2 - protein tyrosine kinase (PTK)2 signaling through binding to the collagen receptor ITGA2 (**Figure 5**), which in turn to reduce scar formation by significantly inhibiting ECM deposition *in vivo* (Zhao et al., 2016). More interestingly, in comparison with normal skin-derived fibroblasts, hypertrophic scar-derived fibroblasts displayed reduced LUM expression (Zhao et al., 2016), while keloid-derived fibroblasts exhibited elevated LUM expression (Naitoh et al., 2005). Although LUM may be a potential pharmaceutical candidate for skin wound healing, its mechanism of action is far from clear.

ASPN is upregulated in keloid margin biopsy specimens compared with that from adjacent healthy skin, indicating it may serve as a potential biomarker for keloid disease (Shih et al., 2010). Likewise, comparative mass spectrometry-based proteomic analysis of keloids and healthy skin has shown that ASPN expression was significantly increased in keloid scars. This suggests that ASPN may be potentially used as a specific target for therapeutic intervention (Ong et al., 2010). In addition, upregulated OGN expression was also found in keloids by cDNA microarray analysis (Naitoh et al., 2005). However, except for DCN, BGN, FMOD, and LUM, investigations into other SLRPs for their potential benefits in skin wound healing are still very much in their infancy.

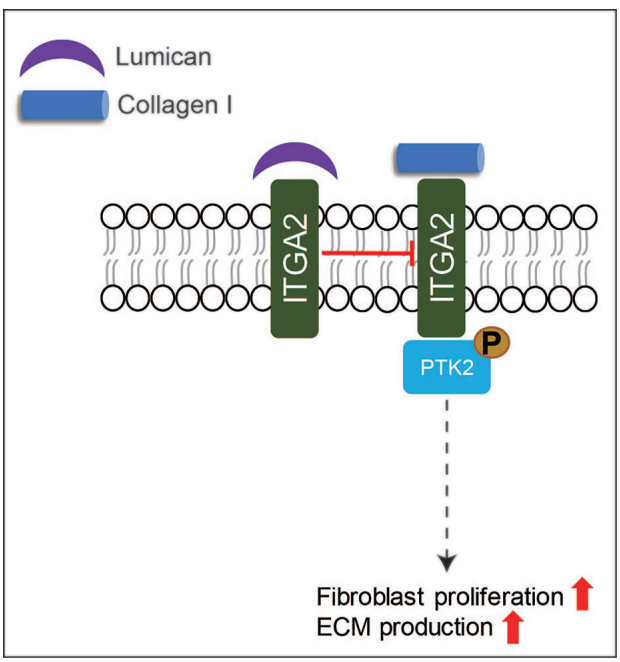

FIGURE 5 | A schematic description of the functions of LUM in skin wound healing. LUM can suppress excessive fibroblast proliferation and ECM production *via* inhibiting ITGA-PTK2 signaling through binding to ITGA2.

CONCLUSION AND PERSPECTIVES

SLRPs are important components of the ECM that play crucial roles in collagen fibril growth, fibril organization, and ECM assembly. They are also capable of modulating the function of a significant number of growth factors and cytokines and have even been thought to prevent fibrosis and organ dysfunction (Mecham, 2011; Schaefer, 2011; Klingberg et al., 2013). Thanks to worldwide collaboration over the last 30 years, much has been discovered in regard to the pivotal roles that SLRPs play in the different phases of the skin wound healing process, as well as the therapeutic potentials of SLRPs for reducing scar formation. However, the precise details concerning how each individual SLRP functions in the different phases of the healing process are still unclear. It is crucial to gain a specific understanding of the nature of the SLRP functional components, particularly in regard to their interactions with cell surface receptors, growth factors, and the ligands associated with molecular patterns of the skin wound healing process.

In conclusion, although there are still many obstacles that need to be surmounted before SLRPs can be applied in clinics for cutaneous wound healing management, a variety of SLRPs, such as DCN, BGN, FMOD, and LUM, exhibit great potential for future use in skin wound healing. For example, given the pro-angiogenic, pro-migratory, and pro-contraction potential of FMOD (Zheng et al., 2014a; Zheng et al., 2014b; Zheng et al., 2017), besides reducing scar formation, it may be used to accelerate the healing of wound with retarded closure such as seen in the diabetic patients (Falanga, 2005; Ekmektzoglou and Zografos, 2006). Considering LUM enhances the epithelial cell migration (Seomun and Joo, 2008), it could also be a candidate to address the major problem of delayed wound healing. In addition to the skin, LUM and KERA may also be beneficial for corneal wound healing (Saika et al., 2000; Carlson et al., 2005), such as in the scene of corneal wounds caused by refractive surgeries (Ljubimov and Saghizadeh, 2015). Nevertheless, the delivery system and administration regimen of these SLRPs are insurmountable issues to be optimized in the pursuit of the most promising outcomes.

AUTHOR CONTRIBUTIONS

ZZ conceived the presented idea. XP wrote the manuscript with support from ZZ. ND modified the grammar and improved the language. All authors contributed to the final manuscript.

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Conflict of Interest: ZZ is an inventor on fibromodulin-related patents assigned to UCLA, and he is also a founder and former officer of Scarless Laboratories, Inc., which sublicenses fibromodulin-related patents from the UC Regents, who also holds equity in the company.

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REVIEW
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Proteoglycans in Biomedicine: Resurgence of an Underexploited Class of ECM Molecules

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Proteoglycans have emerged as biomacromolecules with important roles in matrix remodeling, homeostasis, and signaling in the past two decades. Due to their negatively charged glycosaminoglycan chains as well as distinct core protein structures, they interact with a variety of molecules, including matrix proteins, growth factors, cytokines and chemokines, pathogens, and enzymes. This led to the dawn of glycan therapies in the 20th century, but this research was quickly overshadowed by readily available DNA and protein-based therapies. The recent development of recombinant technology and advances in our understanding of proteoglycan function have led to a resurgence of these molecules as potential therapeutics. This review focuses on the recent preclinical efforts that are bringing proteoglycan research and therapies back to the forefront. Examples of studies using proteoglycan cores and mimetics have also been included to give the readers a perspective on the wide-ranging and extensive applications of these versatile molecules. Collectively, these advances are opening new avenues for targeting diseases at a molecular level, and providing avenues for the development of new and exciting treatments in regenerative medicine.

Keywords: proteoglycans, small leucine rich proteoglycans, decorin, fibromodulin, chondroitin sulphate, dermatan sulphate, heparan sulphate, extracellular matrix

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INTRODUCTION

As researchers try to harness the therapeutic potential of biopolymers for new treatments, proteoglycans (PGs) and their glycosaminoglycan (GAG) side chains remain underexploited due to their complex nature and involvement in multiple biological processes. Glycosaminoglycans are linear long chains of anionic glycan molecules that comprise one of the three major biopolymers found in the body, other than nucleic acids and proteins. GAGs are primarily made up of monomers of either glucuronic or iduronic acid and N-acetylglucosamine. These glycan monomers are not directly encoded by the genome and have a high degree of heterogeneity in terms of their monomer sequences, chain lengths, and sulfation patterns due to posttranslational modifications regulated in the golgi apparatus, leading to a large structural diversity with no defined glycan code (Hudak and Bertozzi, 2014). Six major types of GAGs are currently identified in mammals—chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), heparan sulfate (HS), heparin (Hep), and hyaluronic acid (HA) (Köwitsch et al., 2018). Except for HA, all other GAGs are sulfated and

exist as anionic molecules conjugated to core proteins, making them a component of proteoglycans (PGs). In addition to direct conjugation to core proteins, GAGs interact with other proteins through electrostatic or hydrophobic interactions, as well as hydrogen bonds, further adding to their broad repertoire and complexity.

PGs are a heterogenous family of macromolecules with 43 members, differing in their core protein as well as the nature and number of GAG chains bound to the core (Iozzo and Schaefer, 2015), reaching an unprecedented level of sophistication. These intriguing molecules have been conserved through millions of years of evolution to reach new heights of functional significances. Iozzo and Schaefer (2015) proposed a comprehensive classification and nomenclature for PGs based on their location, genetic homology, and use of protein modules. They classified PGs into four major classes with distinct forms and functions: Class 1 consists of intracellular secretory granules, class 2 consists of cell surface PGs that are classified as either transmembrane or GPI-anchored, class 3 consists of pericellular basement membrane zone PGs, and class 4 consists of extracellular PGs classified as hyalectan-lectincan (HA binding and lectin binding), spock, and small leucine rich PGs (SLRPs).

Early studies conducted on PGs focused on one of the PGs of the vertebrate cartilage extracellular matrix, now known as Aggrecan. Cartilage extracellular matrix is uniquely made with the majority of the non fibrillar ECM composition consisting of PGs and HA (Sophia Fox et al., 2009). PGs as structural components are known to hydrate, protect, and lubricate cartilage tissue (Lohmander, 1988); leading to a vast majority of therapeutics targeted to treating osteoarthritis harnessing these properties. Further PG research revealed that all cells in the body are covered by a gel like glycocalyx (Luft, 1966), which consists of PGs and GAGs involved in a myriad of signaling and growth factor sequestering activities (Weinbaum et al., 2007). Healthy endothelial glycocalyx is the only known blood contacting surface that prevents blood clotting continuously, due to the PGs in it creating a barrier for protein adsorption and fibrin formation. Altering of the glycocalyx has been implicated in various disease conditions (Tarbell and Cancel, 2016; Liew et al., 2017), making it a key target for development of therapeutics. Multifaceted functions of PGs are now known to include growth factor sequestering (Gubbiotti et al., 2016), providing adhesive properties, inducing or inhibiting angiogenesis (Järveläinen et al., 2015; Poluzzi et al., 2016), modulating cell adhesion, proliferation, and regulation (Christensen et al., 2019), as well as interacting with other ECM molecules and controlling collagen fibrillogenesis (Weber et al., 1996; Chen et al., 2010; Kalamajski and Oldberg, 2010). Researchers began recognizing the ubiquitous nature and essential functions of PGs, discovering their role as essential bioactive components of the ECM with sophisticated functions in maintaining homeostasis. However, PGs remained largely untapped as a class of potential therapeutics in comparison to recombinant antibodies and DNA technologies until the last decade. This lag in harnessing the potential of PGs is partially due to the complexity inherent to the synthesis, regulation, and assembly of these molecules. However, advances in carbohydrate biopolymer synthesis, recombinant technology, and the recognition of the enormous potential of PGs as treatments have led to an exciting reemergence of PG engineered therapeutics. PGs represent the most complex and multifunctional class of molecules, making them one of the most versatile and exciting classes of therapeutic candidates.

The focus of this review is limited to recent advances and preclinical studies on naturally occurring proteoglycan molecules and proteoglycan mimetics as ECM based therapeutics. Built on decades of information about the complex signaling pathways and their downstream effectors, scientists are using PG core proteins, glycanated PGs, neo-PGs and PG mimetics to tackle human health and disease. To stay within the scope of the review, developments on GAGs alone as therapeutics, or detailed descriptions of the complex functions of all proteoglycans were not included. In order to provide context for harnessing the therapeutic value of PGs, structures of common proteoglycans are depicted in **Figure 1**. Readers are directed to read about the current developments in the use of GAGs alone as glycan therapeutics in Paderi and coworkers' recent review, which discusses the clinical relevance, applications and clinical stage pharmaceutical developments of these entities (Paderi et al., 2018). For in-depth information about the biological functions of PGs, readers are encouraged to read Izzo and Schaefer's recent review (Iozzo and Schaefer, 2015).

CELL SURFACE PGS

Glypicans

Glypicans are heparan sulfate proteoglycans (HSPGs) that are bound to the cell surface by glycophosphatidylinositol (GPI) anchors (see **Figure 1**). Glypican 1 nanoliposomes have been used to potentiate therapeutic angiogenesis for ischemic wound healing by the Baker group (Monteforte et al., 2016). Co-delivery of glypican-1 with FGF-2 markedly increased the recovery of perfusion and vessel formation in ischemic hind limbs of wild type and diabetic mice in comparison to mice treated with FGF-2 alone, proving that the proteoglycan played an important role in potentiating the activity of FGF-2. Han et al. (2016) showed that lower levels of glypican-3 were detected in patients with gastric cancer than in healthy gastric tissue, showing an inverse correlation between GP-3 levels and metastasis. Targeting glypican-3 or its downstream signaling pathways, or supplementation with adenoviral overexpression of glypican-3 in such cases might therefore, have the potential to suppress metastasis related to gastric cancer.

Syndecans

Syndecans are also HSPGs that act as transmembrane receptors capable of signaling independently or in combination with other receptors and integrins (Morgan et al., 2007; Elfenbein and Simons, 2013). Das and coworkers from the Baker research group have researched the use of syndecan 4 for the treatment

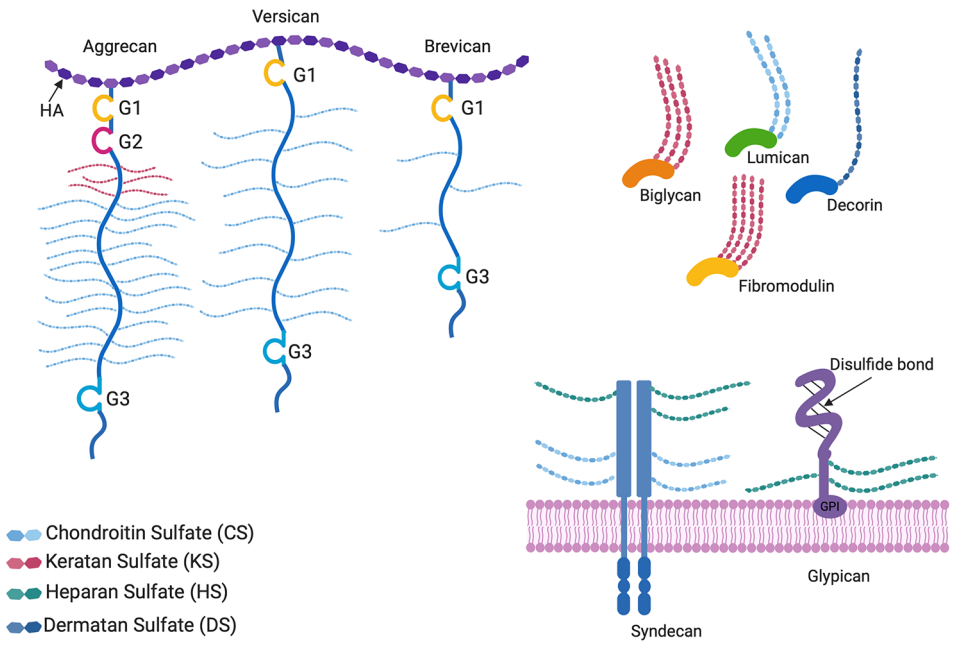

FIGURE 1 | Proteoglycan structures. The horseshoe shaped SLRPs (decorin, biglycan, lumican, and fibromodulin) and bottlebrush structured hyaluronan (HA) binding proteoglycans (aggrecan, versican, and brevican) are located in the extracellular matrix, whereas glypicans and syndecans are cell surface proteoglycans. G1, G2, and G3 are globular structural domains located at the N- and C-terminus of HA binding proteoglycan cores. Glypicans are bound to the cell surface by glycophosphatidylinositol (GPI) anchors. All proteoglycans differ in the GAG side chains attached to the core protein, as well as the lengths and sulfation patterns of the GAGs, thus adding to their complexity.

of diabetic wound healing because of FGF2 coreceptor activity of syndecans (Elfenbein and Simons, 2013). Syndecan 4 encapsulated in proteoliposomes as a delivery system showed promise in the treatment of diabetic ischemia in mice (Das et al., 2016a). Co-therapy of Syndecan with FGF-2 successfully enhanced therapeutic angiogenesis and sustained revascularization in the ischemic hind limb of diabetic, obese mice in comparison to the use of FGF2 alone (Das et al., 2016b). They also went on to test the effects of these proteoliposomes on PDGF-BB activity (Das et al., 2016b). Wounds treated with both syndecan-4 proteoliposomes and PDGF-BB had increased re-epithelization and angiogenesis in comparison to wounds treated with PDGF-BB alone. Moreover, the wounds treated with syndecan-4 proteoliposomes and PDGF-BB also had increased M2 macrophages and reduced M1 macrophages, suggesting syndecan-4 delivery induces immunomodulation within the healing wounds. These results demonstrate the promise of proteoglycans, in particular syndican-4, as a co-therapy for tissue regeneration and the treatment of nonhealing wounds.

Many glycan–protein interactions take place at the cellular interface, and cell surface PGs, especially syndicans, are heavily involved in growth factor interactions and cellular response to wound healing (Gallo et al., 1996; Brooks et al., 2012; Elfenbein and Simons, 2013). Given importance of membrane bound glycans including the syndicans and glypicans, future work to address key mechanistic queries such as whether the liposomes containing cell surface PGs fuse with cells, or is the mere presence of HS near the membrane surface a key factor in driving therapeutic potential at the cellular interface, could drive key developments in this burgeoning field.

LECTICANS

Aggrecan

Aggrecan is of great interest to many researchers due to its load bearing and water retaining ability to protect and hydrate cartilage tissue. Aggrecan exhibits a bottlebrush like structure in which chondroitin sulfate and keratan sulfate GAG chains are attached to a core protein consisting of 3 globular structural domains (see **Figure 1**) (Kiani et al., 2002). The Marcolongo group has done extensive characterization of bottle brush polymers using chondroitin sulfate and other synthetic polymers (instead of a core protein) as core-bristle aggrecan mimetics. By establishing a method to functionalize chondroitin-4-sulfate at the reducing end and incorporating it into either poly (acrylic acid) (PAA) or poly(acryloyl chloride), they were able to achieve large 1.6 MDa polymers with enhanced water uptake as compared to aggrecan alone. By modulating the size of the PAA and number of CS chains bound to it, they were able to successfully form polymers with tunable osmotic pressures for the treatment of osteoarthritis. These mimetics were also shown to diffuse through the cartilage matrix into the pericellular area,

and integrate into rabbit tissue before and after static loading, demonstrating the ability to engineer ECM on a molecular level (Sarkar et al., 2012; Prudnikova et al., 2017; Prudnikova et al., 2018; Phillips et al., 2019).

The Kipper research group designed graft copolymer nanoparticles of cationic polysaccharides such as chitosan with anionic GAGs like CS and Hep to form polyelectrolyte complex nanoparticles mimicking the size and chemistry of aggrecan. These nanoparticles were shown to maintain FGF-2 activity after 21 days of encapsulation and are superior to aggrecan alone as a delivery vehicle for growth factors (Place et al., 2014b). In a separate study to mimic PGs, they also generated copolymers using a heterobifunctional crosslinker to combine HA to the reducing ends of Hep/CS. They also reported successful grafting of these polymers to chitosan for the delivery of FGF2 (Place et al., 2014a).

Our lab has taken a different approach by mimicking the function of aggrecan, but not its structure. We have designed an aggrecan mimetic that is composed of chondroitin sulfate decorated with HA-binding peptides in an effort to mimic key aggrecan function for the treatment of osteoarthritis. These aggrecan mimetics have been shown to penetrate aggrecan-depleted cartilage, contribute to its overall compressive strength, and reduce catabolic activity in *in vivo* and *ex vivo* models of osteoarthritis (Bernhard and Panitch, 2012; Sharma et al., 2013; Sharma et al., 2016). Unlike hyaluronan and chondroitin sulfate, these mimetics were able to promote type II collagen synthesis and aggrecan expression when encapsulated with bovine chondrocytes in collagen hydrogels. The mimetic was also shown to be resistant to the enhanced proteolytic activity found in OA cartilage, since it lacks the known aggrecan cleavage sites.

From the above studies, two major approaches stand out in efforts to harness the biological activity of aggrecan—mimicking its structure, vs targeting GAGs to tissue locations; for example, by targeting HA binding to augment surrounding ECM. Both approaches have extensive potential to achieve improved tissue function and healing. Clinical applications for osteoarthritis treatment using these PG mimicking polymers appear on the near horizon as advanced synthesis and scale up techniques for protein conjugation and polymer synthesis become more readily available.

SMALL LEUCINE RICH PGS

By far the most widely researched class of PGs, SLRPs share structural similarities in their core protein of leucine rich tandem repeats flanked by cysteine rich repeats. The biological functions of SLRPs are too vast to be summarized in a single review, hence, we refer readers to recent comprehensive reviews focusing specifically on SLRPs (Iozzo, 1997; Iozzo, 1999; Schaefer and Iozzo, 2008; Chen and Birk, 2013; Hultgårdh-Nilsson et al., 2015; Nastase et al., 2018; Appunni et al., 2019). It is widely accepted that the horseshoe-shaped core protein of SLRPs is responsible for its binding to collagen, modulating collagen fibrillogenesis and protecting collagen from enzymatic cleavage (Karamanos et al., 2018). As new information comes to the forefront of SLRP research, researchers have discovered that the functionality of SLRPs changes based on whether the core protein is attached to its GAG chains, or as unmodified core protein (Yu et al., 2018). Multiple forms of these PGs are thus used as therapeutic candidates.

Decorin

Decorin is the archetypal, most extensively studied SLRP, and has been vastly characterized for its influence on collagen fibrillogenesis (Danielson et al., 1997) and involvement in scarless wound healing. Decorin is not just a structural entity, it plays a pivotal biological role in angiogenesis (Järveläinen et al., 2015), inflammation (Nastase et al., 2018), fibrosis (Ahmed et al., 2014), wound healing (Grisanti et al., 2005), oncosuppression (Sainio and Järveläinen, 2019), and endothelial cell health and autophagy (Neill et al., 2017) to name some. Due to this involvement in an enormous range of biological functions, decorin has aptly been termed as a "guardian from the matrix" (Neill et al., 2012). Decorin consists of a core protein with small leucine rich tandem repeats, with a dermatan sulfate or chondroitin sulfate GAG chain attached to it through the N terminus of the protein. Through its GAG side chain and core protein, it can bind to various growth factors such as TGFβ, as well as collagen and other ECM molecules, whereby it likely serves as a reservoir for TGFβ and stabilizes inter fibrillar organization of the collagen (Orgel et al., 2009).

Since the invention of human recombinant decorin core protein expressed in CHO cells, this PG has been manufactured using cGMP conditions and is being tested as a therapeutic for multiple disease indications, arguably bringing it closest to clinical implementation. Galacorin, the trademark name for the decorin drug produced through Catalent pharma, is being tested for the treatment of macular degeneration, diabetic retinopathy, and diabetic macular edema (Devore et al., 2010).

From a therapeutic research perspective, decorin has been investigated for its use in corneal wound healing. Grisanti et al. used decorin in an experimental glaucoma filtration surgery pilot study on rabbits (Grisanti et al., 2005). Postoperative results showed that rabbits treated with decorin had significantly less ECM deposition 14 days after surgery, as well as suppressed conjunctivital scarification. Hill et al. (2018) designed gellan based fluid gels for sustained delivery of human recombinant decorin through eye drops for corneal regeneration and found improved ocular function. Due to its ability to delay collagen fibrillogenesis, decorin is an attractive therapeutic candidate for anti-scarring treatments. It also acts as a TGF- β1/2 antagonist, and has been used as a treatment against spinal scarring. Ahmed et al. (2014) showed that treatment of acute and chronic dorsal funicular spinal cord lesions (DFL) in adult rats with decorin resulted in a reduction in wound cavity area, suppression of inflammatory fibrosis, and dissolution of mature scars due to decorin's fibrolytic activity and neutralization of TGF- β1/2. In an independent study, decorin treatment reduced hypertrophic

scarring through inhibition of the TGF-β1/Smad signaling pathway in a rat osteomyelitis model (Wang et al., 2016).

Decorin is also considered a potent oncosuppresor due to its ability to function as an endogenous pan-receptor tyrosine kinase inhibitor, a regulator of both autophagy and mitophagy, as well as a modulator of the immune system (Ahmed et al., 2014). Oncolytic adenovirus expressing decorin significantly inhibited the progression of bone metastases in MDA-MB-231 metastasis model of breast cancer (Yang et al., 2015). Adenovirus overexpression of IL-12 and decorin have demonstrated potent antitumor effects in a weakly immunogenic murine model of breast cancer (Oh et al., 2017a). Along similar lines, adenoviral overexpression of decorin and Granulocyte Macrophage Colony Stimulating Factor has shown anti-tumor potential in a model of murine colorectal cancer (Liu et al., 2017) (Wang et al., 2016). Shen and coworkers engineered a recombinant decorin fusion protein with an extended C-terminus comprised of a vascular homing peptide that recognizes inflamed blood vessels and penetrates deep into the vessel wall, known as CAR. In a study to evaluate its efficacy as a treatment for abdominal aortic aneurysm (AAA), they delivered the CAR-DCN molecule to mice with angiotensin-II induced AAAs, and found increased 28 day survival and reduced severity of AAA post treatment (Shen et al., 2017).

In addition to using the native decorin core protein and GAG-decorated molecules, synthetic mimetics of decorin have been developed. In an effort to mimic the collagen modulating function of decorin, our lab has designed a decorin mimetic made of collagen-binding peptides conjugated to a dermatan sulfate backbone (Paderi and Panitch, 2008). Similar to decorin, this molecule influences the fibril diameter of type I collagen on a nanoscale. Stuart et al. showed that these mimetics reduce dermal scarring in a rat linear incision model, due to their ability to mask existing collagen from matrix metalloprotease (MMP-1 and MMP-3) mediated proteolytic degradation while modulating collagen organization (Stuart et al., 2011). In addition, it was reported that similar to the anticoagulant, anti-thrombotic function of the glycocalyx, this mimetic was able to bind to exposed collagen in denuded arteries within minutes to suppress platelet binding and activation, and thus prevent resulting vascular intimal hyperplasia that would normally occur after percutaneous coronary intervention (PCI) sans mimetic (Scott and Panitch, 2014; Scott et al., 2017). The mimetic, termed DS-SILY, was able to reduce smooth muscle cell proliferation and migration, as well as reduce intimal hyperplasia *in vivo* in Ossabaw pigs by 60% as compared to controls (Paderi et al., 2011; Scott et al., 2013). After extensive *in vitro* and *in vivo* validation, DS-SILY is licensed through Symic bio, and is being tested in clinical trails for the treatment of peripheral vascular disease.

Lumican

Lumican has been extensively studied as a keratan sulfate proteoglycan responsible for corneal transparency and wound healing. Like other SLRPs, it is involved in modulating collagen fibrillogenesis and interacts with growth factors through its core protein (Rada et al., 1993). *Lum*(–/–) knockout mice have given way to an enormous amount of research diving into the unique functions of lumican in tendon and skin health, and corneal transparency (Chakravarti, 2002). The Chakravarti group has used these knockout mice to bring forth the importance of lumican in various indications such as bacterial phagocytosis, innate immunity, and corneal clearing. In a mouse corneal Lum (–/–) model infected with *Pseudomonas aeruginosa*, lumican was shown to be responsible for bacterial clearing and facilitation of an innate immune response. In *P. aeruginosa* lung infections, lumican-deficient Lum(–/–) mice failed to clear the bacterium from lung tissues, and showed poor survival rates (Shao et al., 2013a). Lumican modulates wound healing and innate immunity by interacting with receptors and immune cells such as macrophages (Shao et al., 2013b).

Soluble lumican core protein isolated from human amniotic membranes has been shown to effectively promote epithelial proliferation and migration in a study by Yeh et al. (2005). Lumican modulates fibroblast contact through the α2β1 integrin, a finding that has been exploited for therapeutic development. Recombinant lumican application on mice skin wounds showed enhanced wound healing in a study by Liu et al. (2013), possibly due to lumican promoting the contractility of fibroblasts through the α2β1 integrin. In an independent study, adenoviral overexpression of lumican in hypertrophic scarring rabbit models and fibroblasts effectively thinned the scar area and inhibited fibroblast proliferation, as well as successfully reduced focal adhesion kinase (FAK) phosphorylation as a result of binding to α2β1 integrin (Zhao et al., 2016).

Gesteira et al. (2017) designed a peptide mimicking the activity of lumican based on 13 C-terminal amino acids of lumican (LumC13). They showed that the peptide effectively forms a complex with type I receptor for TGFβ1 (ALK5) and promoted corneal wound healing in mice (Yamanaka et al., 2013). Lumican derived peptides–lumcorin, have been tested against melanoma and show therapeutic potential by inhibiting cell chemotaxis and melanoma growth through MMP-14 inhibition (Zeltz et al., 2009; Pietraszek et al., 2013). The vast array of studies showing the biological activity of lumican underscore the importance of proteogycans in homeostasis and disease and highlight the potential of targeting these ECM molecules to treat disease.

Biglycan

Biglycan shares structural similarities with decorin and comprises 12 leucine-rich repeats flanked by cysteine-rich domains. It is a major component of bone, cartilage, tendon and muscle. Biglycan has been studied as a potential therapeutic for musculoskeletal disorders, due to its involvement in modulating collagen fibrillogenesis as well as its role in modulating and maintaining musculoskeletal organization (Young and Fallon, 2012). Biglycan is predominantly expressed as a proteoglycan, but a mature form lacking GAG side chains, known as "nonglycanated" biglycan, has recently been shown to have specific functions in muscle and Wnt signaling (Amenta et al., 2011).

Duchenne muscular dystrophy (DMD) is caused by the loss of dystrophin in muscles, leading to membrane fragility and impaired signaling. Non-glycanated recombinant biglycan

delivered to dystrophic mice has been shown to recruit utrophin, an autosomal paralog of dystrophin, and a NOS-containing signaling complex to the muscle cell membrane to improve muscle health and function (Amenta et al., 2011). In an independent follow up study, Ito et al. hypothesized that biglycan expressed in a small number of muscle fibers was likely to have been secreted and anchored to the cell surface throughout the whole muscular fibers to improve motor function (Ito et al., 2017). An optimized version of the nonglycanated biglycan, "TVN-102", is under development as a candidate therapeutic for DMD (Fallon and McNally, 2018).

Fibromodulin

Fibromodulin (Fmod) has been widely investigated for its role in fetal-like scarless wound healing and angiogenesis. The Soo research group demonstrated that Fmod stimulated capillary infiltration into Matrigel plugs, enhanced angiogenesis in chick chorioallantoic membrane (CAM) assays, and restored the vascularity of *fmod*$^{-/-}$ mouse wounds (Jian et al., 2013; Zheng et al., 2014). These results suggest enhanced angiogenesis during cutaneous wound healing, proving that Fmod is an attractive therapeutic candidate for wound management especially in cases where angiogenesis is impaired, such as diabetic wounds. They also went on to use Fmod to reprogram fibroblasts into a multipotent cell type as a means to bypass mutation and malignancy risks associated with genetically modified iPS cells (Zheng et al., 2012; Li et al., 2016). Testing these reprogrammed cells *in vitro* and in a clinically relevant critical-sized calvarial defect model, they demonstrated strong osteogenic capacity of these cells without tumorigenesis, showing that Fmod reprogrammed cells present potential for bone regeneration.

Adenoviral transfection of fibromodulin (ad-Fmod) has gained popularity in the past decade, and multiple studies have utilized ad-Fmod to target wound healing and cancer. Jazi et al. (2016) probed the therapeutic effects of recombinant adenoviral vectors expressing Fmod for the treatment of diabetic nephropathy in streptozotocin induced diabetic rats. They found reduced expression of TGFβ1 in rats transfected with Fmod gene transfer, suggesting a mechanism of action for fibromodulin therapy. Given its potent role in promoting angiogenesis and wound healing, a study by Ranjzad et al. (2009) demonstrated significant reduction in neointimal thickness and area in an *ex vivo* human saphenous vein organ culture model following adenovirus mediated fibromodulin gene transfer. Delalande et al. (2015) used non-viral histidylated vectors for Fmod gene transfer and local Fmod expression to enhance achilles tendon healing; they demonstrated promising improvements in biomechanical and histological parameters in a rat achilles tendon injury model. Fmod has been shown to successfully inhibit the nuclear factor-κB (NF-κB) signaling and induce fibroblast apoptosis (Lee and Schiemann, 2011). Dawoody Nejad et al., 2017 demonstrated that recombinant Fmod was able to suppress TGFβ1 and NF- κB activity *in vitro* in a highly metastatic breast cancer cell line (Dawoody Nejad et al., 2017).

In summary, it is evident from the wide body of research reviewed above, that SLRP core proteins and their GAG components have important, and sometimes distinct, activities. The numerous approaches to use recombinant core proteins and functional mimetics highlight the diversity of strategies that can be employed in the use of SLRPs to enhance tissue regeneration and wound healing. To learn more about the recombinant production of PGs and their different domains, readers are encouraged to read Lord and Whitelock (2013) concise review. Further work in this field is warranted to better delineate the biological function of the core proteins, GAGs and synergies of the two to design therapies that focus on cell-ECM interactions, and are effective on a molecular level.

OTHER PGS

Proteoglycan 4/Lubricin

Proteoglycan 4 (PRG4) or lubricin is a mucin like proteoglycan/glycoprotein found in the synovial fluid of cartilage. It is responsible for lubricating the surface boundary of cartilage in synergy with HA. Interestingly, inflammation and osteoarthritis progression show an inverse relationship to lubricin expression, suggesting that it is directly involved in reducing inflammation and boundary friction levels (Iqbal et al., 2016).

Exploiting this information, the Schmidt and Tannin groups have extensively shown that PRG4 supplementation can restore normal cartilage boundary lubrication function to osteoarthritic SF (Schmidt et al., 2007; Ludwig et al., 2012). They have since, established a method for recombinant lubricin production, and are testing the functional effects of lubricin in other therapeutic areas such as intraabdominal lesions and contact lenses for ocular applications (Oh et al., 2017b; Samsom et al., 2018b). Lubris biopharma is a clinical stage start up company that is testing human recombinant lubricin for the treatment of dry eye (Lambiase et al., 2017) and osteoarthritis due to its role as a boundary lubricant. In an independent study by Larson et al. (2016), recombinant lubricin effectively reduced the coefficient of friction of bovine cartilage explants inflamed using IL1β. This further adds to the body of literature displaying the potential of lubricin in the treatment of osteoarthritis.

We and others have taken an approach that mimics the lubricating function of lubricin, but not its structural properties. We have designed a lubricin mimetic (mLub) by attaching type II collagen and HA binding peptides to a chondroitin sulfate backbone. Work done by Lawrence et al. (2015) demonstrated the ability of the mimetic to bind to articular cartilage and reduce the coefficient of friction on a macroscale. The Grinstaff lab designed anionic hydrophilic bottle-brush polymer lubricants using poly(7-oxanorbornene-2-carboxylate) as biolubricants for the treatment of osteoarthritis (Wathier et al., 2010). Synthesized *via* ring-opening metathesis polymerization, the polymer biolubricant showed promise in reducing friction and offering chondroprotection in *ex vivo* plug-on-plug and rat models of osteoarthritis (Wathier et al., 2013; Wathier et al., 2018). Further efforts to improve the polymer to make it better match the osmolarity of synovial fluid are being conducted by making it less anionic and

covalently conjugating pendent triethylene glycol (TEG) chains to it (Lakin et al., 2019). A note about lubricin—it is debated whether this molecule is a glycoprotein or a proteoglycan, since it is a glycosylated protein that does not have traditional glycans such as CS, DS, or heparin attached to a protein core, and the protein itself is glycosylated.

Perlecan (Heparan Sulfate Proteoglycan 2)

Perlecan is a large HSPG with a protein core composed of five distinct domains, which impart it with a wide range of functionalities to interact with other biological molecules (Douglass et al., 2015). The GAG-bearing domain I of Perlecan has been shown to promote chondrogenesis (French et al., 2002). Using this information, researchers have synthesized hydrogels containing the perlecan domain I along with HA (Jha et al., 2009) or type II collagen (Yang et al., 2006) to demonstrate enhanced binding and activity of bone morphogenic protein 2 (BMP2). BMP2 is considered a primary stimulant of chondrogenesis, and both studies showed robust stimulation of a cartilage specific ECM in comparison to controls not containing perlecan. Additionally, injectable microgels made up of HA and perlecan domain I showed enhanced activity of BMP2 in promoting cartilage matrix synthesis in a mouse early osteoarthritis model (Srinivasan et al., 2012). These results demonstrate that combining specific PG domains with hydrogels to drive growth factor activity may provide a higher level of control over cell fate and disease modulation.

Primarily considered a proangiogenic molecule, perlecan interacts with FGF2 and VEGF to regulate angiogenesis (Aviezer et al., 1994; Zoeller et al., 2009), making it an attractive potential therapy for wound healing where angiogenesis is impaired. Domain V (DV) of perlecan has been heavily investigated for its role in angiogenesis. The Bix group has investigated the potential of DV of perlecan to counteract the effects of amyloid-β (Aβ), which causes neurovascular dysfunction (Parham et al., 2014). Results from their studies showed improved endothelial proliferation, migration, and tubule formation despite treatment with Aβ by directly interfering with the α2 and α5 integrins (Clarke et al., 2012; Parham et al., 2016), thus promoting angiogenesis and supporting DV's potential as an anti-amyloid therapeutic. Among other neurovascular applications, DV has been suggested as a potential treatment for stroke and vascular dementia (Marcelo and Bix, 2015). A study by Lee et al. (2011) demonstrated enhanced post-stroke angiogenesis in rat and mouse models of stroke after DV treatment, suggesting it as a neuroprotective approach for stroke treatment. As a strategy to develop bioactive vascular grafts, Rnjak-Kovacina et al. (2016) functionalized silk with perlecan DV decorated with heparan sulfate and chondroitin sulfate chains to enhance endothelial cell adhesion and proliferation while inhibiting platelet binding effectively. These studies highlight the complicated balance between proteoglycan activity with and without their attached sidechains and emphasize the implications of these variations in therapeutic developments.

NeoPGs

The Godula research group synthesized HSPG neoPGs that completely circumvented the limitations of HSPG synthesis such as heterogeneity and batch to batch variability by incorporating disaccharides (diGAGs) generated by depolymerization of HS by bacterial heparinases into a poly (acrylamide) scaffold decorated with pendant *N*-methylaminooxy groups, which are reactive toward the hemiacetal functionality of the reducing glycans (Huang et al., 2014). By synthesizing a library of neoPGs and designing a microarray for testing binding to FGF-2, they were able to shortlist neoPGs with affinity to FGF2, which showed enhanced promotion of neural specification in embryonic stem cells deficient in HS biosynthesis.

The Hsieh-Wilson lab specializes in synthesis of glycomimetics to research the influence of GAG position and density on their avidity and specificity to interact with other proteins. Using end-functionalized ring-opening metathesis polymerization (ROMP) based polymers that mimic the native-like, multivalent architecture found on chondroitin sulfate (CS) proteoglycans, Lee et al. (2010) used norborene based backbones with biotin functionalized pendant sugars to create glycomimetics of various molecular weights and sulfation motifs. By controlling the sulfation patterns and display of these pendant sugars, novel mimetics for CS proteoglycans can be designed for targeted regeneration (Sotogaku et al., 2007; Miller and Hsieh-Wilson, 2015; Stopschinski et al., 2018).

The Pashkuleva group has designed mimimalistic PG mimetics by coassembly of aromatic peptides and carbohydrate amphiphiles. The amphiphiles Fmoc-glucosamine-6-sulfate (GlcN6S) and Fmoc-glucosamine-6-phosphate (Fmoc-GlcN6P) provided the functional element through the sulfate and phosphate groups, while fluorenylmethoxycarbonyl-diphenylalanine (Fmoc-FF) acted as a structural component, forming self-sustained macroscopic gels that are biocompatible and mimic the PG growth factor sequestering action, making these gels attractive for tissue engineering applications (Brito et al., 2019). In a separate study, Novoa-Carballal et al. (2018) synthesized star-like PG mimetics by grafting high molecular weight GAGs such as heparin and CS to hyperbranched synthetic cores like polyglycerol using oxime condensation. These mimetics showed enhanced binding to proteins by forming microfiber complexes instead of spherical nanocomplexes that form with linear GAGs, thus showing a larger degree of potential for modulating protein activity and presentation.

The Hudalla group is focused on creating self-assembling beta sheet nanofibers using synthetic glyco-peptides as supramolecular mimetics of glycoproteins. Hydrogels formed by these glycopeptides contain decorated n-acetylglucosamine and n-acetyllactosamine residues, which impart the gels with avidity to various proteins, especially galectins, a carbohydrate binding class of proteins involved in modulating cell proliferation, adhesion and apoptosis (Restuccia et al., 2015; Restuccia and Hudalla, 2018). By optimizing the content of

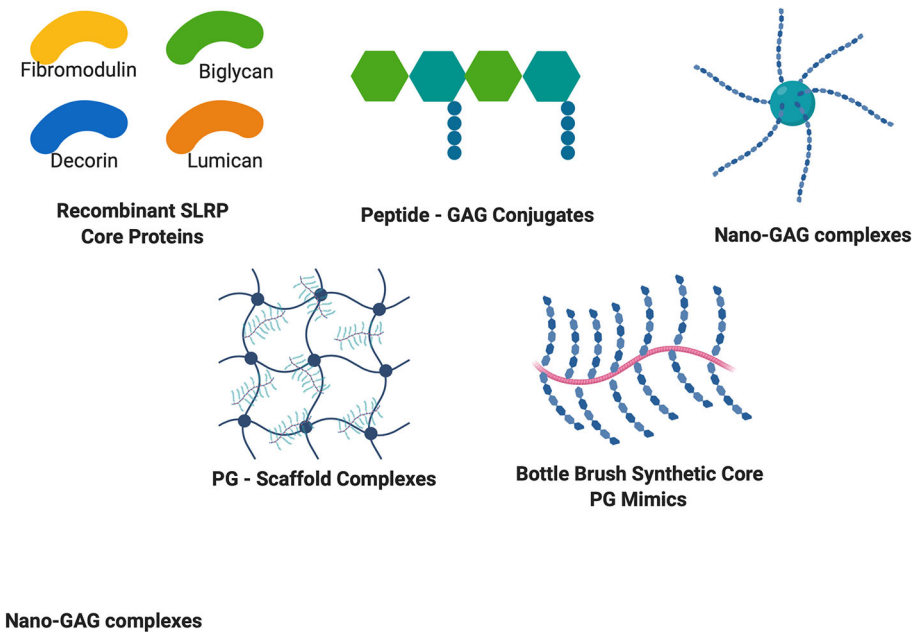

FIGURE 2 | Structures of recombinant PG cores and PG mimetics. Recombinant production of PG cores and variants in many cell lines has accelerated discovery of the therapeutic potential of PGs. Peptide-GAG conjugates and nano-GAG complexes are being used to mimic PG functions and binding ability. PG – Scaffold complexes are being explored as tissue engineering constructs for regenerative medicine, and bottle brush mimetics of PGs such as aggrecan are being explored to circumvent increased degradation of PGs to provide enhanced healing potential.

n-acetyllactosamine residues, they aim to inhibit protein-glycan interactions implicated in autoimmune and cancer disease progressions.

Overall, these synthetic approaches summarized in **Figure 2** bring new knowledge on structure function relationships as well as a powerful approach to design cell-ECM interactions to improve tissue function and healing.

CONCLUSIONS AND PERSPECTIVES

While pioneering researchers have been focused on the roles of GAGs and proteoglycans for years, it has only been within the last two decades that the staggering potential of PGs to modulate tissue environments has been more broadly appreciated. Their multifunctional biological processes, in particular, their ability to bind and sequester growth factors and interact with various ECM molecules and influence cellular signaling events, makes them extremely attractive drug conjugates for multiple disease indications. **Table 1** summarizes common proteoglycans and their therapeutic applications.. Clinical translation of these molecules, however, remains a challenge. Due to the advent of recombinant technology, adenoviral and non-viral gene transfers are attractive alternatives to purifying native PGs, a task that is considered extremely difficult and time intensive. However, while recombinant technology can synthesize core proteins of PGs fairly consistently, their post-translational GAG chain modifications remain a challenge. Some GAG chain structures require enzymes in the Golgi apparatus only found in mammals, and absent in single celled organisms used to synthesize recombinant PGs. Effectively conveying the mechanism of action of these drugs also remains a significant challenge, due to the diverse processes with which these molecules interact.

PG mimetics that convey similar bioactivity as their native counterparts are gaining popularity due to larger level of control over synthesis and optimization as well as cost effectiveness. Synthetic methodology, however, has its own challenging barriers toward manufacturing commercially relevant quantities. Rapid progress in synthetic GAG synthesis and sequencing, and current understanding of kinetics of PG binding interactions with growth factors are helping scientists create the next generation of PG therapies to control and target a variety of diseases. Key features of GAG length and sulfation alongside core protein interactions are being modulated to enhance binding interactions.

Focus on targeted and controlled release of these PGs is also gaining interest. Engineering PGs to sequester and control growth factor release are being explored for enhanced therapies. Approaches to target specific tissues, such as exploiting the binding ability of core proteins to collagen, or to HA, are being explored to create localized and functional treatments. Synthetic approaches to circumvent the heterogeneity of native PGs are being employed to control and tune specific sulfation patterns, binding potential, and specificity of mimetics to establish novel ways of modulating disease state.

TABLE 1 | Summary of proteoglycans and their therapeutic applications.

Proteoglycans	Predominant GAG	Therapeutic Application	References
Glypican 1-6	Heparan sulfate	Ischemic wound healing	(Monteforte et al., 2016)
		Suppressing metastasis in gastric cancer	(Han et al., 2016)
Syndecan 1-4	Heparan sulfate	Diabetic wound healing	(Das et al., 2016a; Das et al., 2016b; Das et al., 2016c)
Aggrecan	Chondroitin sulfate, keratan sulfate	Osteoarthritis	(Bernhard and Panitch, 2012; Place et al., 2014a; Phillips et al., 2019; Place et al., 2014b; Sarkar et al., 2012; Sharma et al., 2013; Sharma et al., 2016; Prudnikova et al., 2017; Prudnikova et al., 2018)
Decorin	Dermatan sulfate	Macular degeneration, diabetic retinopathy, diabetic macular edema	(Devore et al., 2010)
		Corneal wound healing	(Grisanti et al., 2005; Hill et al., 2018)
		Anti-scarring	(Stuart et al., 2011; Ahmed et al., 2014; Wang et al., 2016)
		Oncosupression	(Yang et al., 2015; Wang et al., 2016; Liu et al., 2017; Oh et al., 2017a)
		Abdominal aortic aneurysm	(Shen et al., 2017)
		Vascular neointimal hyperplasia	(Paderi et al., 2011; Scott and Panitch, 2014; Scott et al., 2017)
Lumican	Keratan sulfate	Corneal wound healing	(Chakravarti, 2002; Gesteira et al., 2017)
		Bacterial lung infections	(Shao et al., 2012; Shao et al., 2013a)
		Scarring	(Yeh et al., 2005; Liu et al., 2013; Yamanaka et al., 2013; Zhao et al., 2016)
		Melanoma	(Zeltz et al., 2009; Pietraszek et al., 2013)
Biglycan	Chondroitin sulfate	Duchenne muscular dystrophy	(Amenta et al., 2011; Ito et al., 2017; Fallon and McNally, 2018)
Fibromodulin	Keratan sulfate	Diabetic wounds and neuropathy	(Jian et al., 2013; Zheng et al., 2014; Jazi et al., 2016)
		Neointimal hyperplasia	(Ranjzad et al., 2009)
		Bone regeneration	(Zheng et al., 2012; Li et al., 2016)
		Tendon healing	(Delalande et al., 2015)
		Breast cancer metastasis	(Dawoody Nejad et al., 2017)
Lubricin	None	Osteoarthritis	(Iqbal et al., 2016; Lakin et al., 2019; Larson et al., 2016; Ludwig et al., 2012; Wathier et al., 2013; Lawrence et al., 2015)
		Ocular applications, dry eye	(Lambiase et al., 2017; Oh et al., 2017b; Samsom et al., 2018a)
Perlecan	Heparan sulfate	Cartilage regeneration	(French et al., 2002; Yang et al., 2006; Jha et al., 2009; Srinivasan et al., 2012)
		Ischemic wound healing	(Aviezer et al., 1994; Zoeller et al., 2009)
		Neurovascular dysfunction	(Clarke et al., 2012; Parham et al., 2016)
		Stroke and vascular dementia	(Lee et al., 2011; Marcelo and Bix, 2015)
		Neointimal hyperplasia	(Rnjak-Kovacina et al., 2016)

Furthermore, chemists and cell biologists are establishing novel mimetics that don't just necessarily mimic the structure of PGs, but also their function. There is still much to learn about the structure function relationships of PGs. Nevertheless, nascent preclinical developments have shown the promise of PG therapeutics to pioneer future treatments and breakthroughs in multiple disease indications such as wound healing, cancer, angiogenesis and hypertrophic scarring. Overall, advances in PG and GAG-based therapeutic development are putting a renewed focus on the importance of the ECM for tissue health and cell function, and opening the door for new classes of bioinspired and targeted drugs.

AUTHOR CONTRIBUTIONS

TW did the initial literature search and completed the first draft. AP suggested additional literature and added critical analysis and future directions.

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MINI REVIEW
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Cross-Talk Between Extracellular Matrix and Skeletal Muscle: Implications for Myopathies

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Skeletal muscle (SM) comprises around 40% of total body weight and is among the most important plastic tissues, as it supports skeletal development, controls body temperature, and manages glucose levels. Extracellular matrix (ECM) maintains the integrity of SM, enables biochemical signaling, provides structural support, and plays a vital role during myogenesis. Several human diseases are coupled with dysfunctions of the ECM, and several ECM components are involved in disease pathologies that affect almost all organ systems. Thus, mutations in ECM genes that encode proteins and their transmembrane receptors can result in diverse SM diseases, a large proportion of which are types of fibrosis and muscular dystrophy. In this review, we present major ECM components of SMs related to muscle-associated diseases, and discuss two major ECM myopathies, namely, collagen myopathy and laminin myopathies, and their therapeutic managements. A comprehensive understanding of the mechanisms underlying these ECM-related myopathies would undoubtedly aid the discovery of novel treatments for these devastating diseases.

Keywords: collagen, extracellular matrix, laminin, myopathy, skeletal muscle

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INTRODUCTION

Skeletal muscle (SM) is a contractile tissue primarily comprised of multinucleated myofibers. SM is one of the most important plastic tissues in the human body and accounts for around 40% of total body weight (Campbell and Stull, 2003; Frontera and Ochala, 2015; Kim et al., 2019). SMs contain multipotent precursor cells called muscle satellite cells (MSCs), which are localized below the basal lamina (BL) in myofibers, play vital roles in maintaining the integrity of SM, and participate in muscles regeneration *via* an organized myogenic program (Asakura et al., 2001). After injury, MSCs activate, proliferate, and fuse to form myofibers, which constitute the functional contractile parts of mature SM (Carlson and Faulkner, 1983; Bonilla et al., 1988; Stuelsatz et al., 2017).

Abbreviations: SM, Skeletal muscle; ECM, Extracellular matrix; MSCs, Muscle satellite cells; BL, Basal lamina; FN, Fibronectin; DGC, Dystrophin glycoprotein complex; UGC, Utrophin glycoprotein complex; DMD, Duchenne muscular dystrophy; BM, Basement membrane; UCMD, Ullrich congenital muscular dystrophy.

The developmental process of multinucleated myofibers with contractile capability from MSCs is termed myogenesis, and involves cell cycle arrest, cell fusion, increases in nuclear sizes, and the peripheral localization of nuclei (Charge and Rudnicki, 2004). Myogenesis is a decidedly regulated mechanism that is determined by the co-expressions of Pax3, Pax7, and myogenic-regulatory factors such as Myf5, Mrf4, MyoD, and myogenin in MSCs (Zammit and Beauchamp, 2001; Relaix et al., 2005; Baig et al., 2019; Kim et al., 2019; Lee et al., 2019).

SM supports skeletal development, aids skeletal movement, controls body temperature, and manages glucose uptake (Ahmad et al., 2018) and is composed of large numbers of long, multinucleated filaments, which are organized by extracellular matrix (ECM) (Davis et al., 2013). ECM plays important roles during wound healing, embryogenesis, and tissue repair and provides integrity and biochemical signals to cells (Govindan and Iovine, 2015; Ahmad et al., 2018) and is composed of glycoproteins like collagens, fibronectin (FN), and laminins (Frantz et al., 2010) (**Figure 1**). Furthermore, two major SM ECM proteins, that is, collagen and laminin, are known to be associated with myopathies. In previous studies, we explored the roles of a small number of ECM proteins like fibromodulin, matrix gla proteins, and dermatopontin during different stages of myogenesis (Lee et al., 2016; Ahmad et al., 2017; Lee et al., 2018; Kim et al., 2019) and found fibromodulin, as well as dermatopontin, is involved in the vigorous recruitment of MSCs at sites of injury, and thus, aids SM regeneration (Lee et al., 2018; Kim et al., 2019).

Secreted elements, such as diverse kinds of growth factors, are released during SM repair and are used to monitor muscle regeneration, but their roles and impacts on SM remodeling remain obscure (Karalaki et al., 2009). The transmembrane receptors send signals into cells from the ECM, and thus, initiate several cell functions, for instance survival, development, movement, and differentiation, which are important for sustaining the homeostasis (Theocharis et al., 2016). Notably, SM fibrosis arises in diabetes and muscular dystrophies and during immobilization and aging (Alnaqeeb et al., 1984; Williams and Goldspink, 1984; Berria et al., 2006; Gillies and Lieber, 2011).

In this review, we briefly describe SM and its major ECM components with emphasis on their roles in muscle-related diseases, which are generally fibrotic diseases or types of muscular dystrophy. We believe an in-depth understanding of the underlying mechanisms of ECM-related myopathies will aid the discovery of novel therapeutic options for the management of these devastating diseases.

ECM Role in the Development and Function of SM

ECM is a well-organized non-cellular environment that undergoes regular cycles of alterations, degradation, as well as reassembly. Cell-matrix communication contribute a vital role in cell adhesion and migration, and therefore, is critically important during embryonic and adult myogenesis (Goody et al., 2015). Various ECM proteins (e.g., dermatopontin, nidogen/entactin, periostin, and osteopontin) contribute to the regulations of cell-matrix communications and matrix assembly (Funanage et al., 1992; Norris et al., 2007; Kato et al., 2011; Kim et al., 2019).

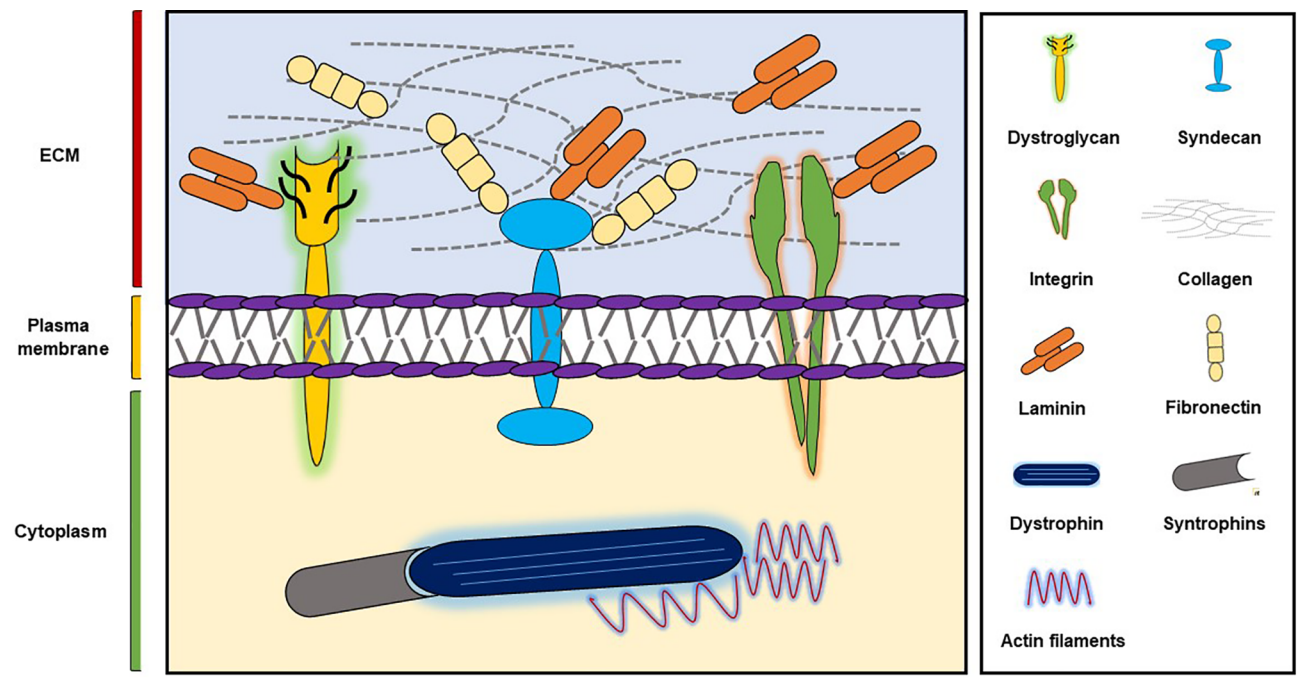

FIGURE 1 | ECM components and their major receptors.

Myoblasts fuse to form SM fibers during the fetal stage, and myofiber numbers remain constant throughout the postnatal period. However, during this period, myofiber sizes are increased by MSC fusion. SM is among the most adaptive of body tissues, and its regenerative ability after SM injury depends on MSCs, which though generally quiescent, turn on and start to proliferate, differentiate to myoblasts, and then fused with myofibers to reestablish the contractile nature of SM after SM or MSC injury (Griffin et al., 2010).

Cross-talk between MSCs and their microenvironments determines SM regeneration. According to the MSC niche concept, the fates of stem cells are determined by stimuli arising from surrounding environments. Furthermore, MSCs exist in specific niches, consisting of muscle myofibers, muscle residence cells, vascular systems and ECM (Wilschut et al., 2010), and their functions are profoundly influenced by different microenvironments.

MSCs are enclosed in laminin and are found between muscle fibers and BL, the latter of which is composed of collagen IV and laminin networks. MSCs bind to these components using integrin receptors (Sanes, 2003), which are heterodimeric transmembrane receptors that critically transform extracellular signals into intracellular responses and interact with ECM as directed by intracellular changes (Askari et al., 2009). BL also functions as a mechanical barrier that prevents MSC migratory loss from normal SM and it might also be involved in the inhibition of MSC differentiation in the absence of damage (Sanes, 2003).

Almost all myofibers in SM develop from somites, that is, from mesodermal structures that evolve in the early embryonic segmentation (Christ and Ordahl, 1995; Pourquie, 2001). FN and its communication with integrin play vital roles during somatic cell polarization and guidance (Ostrovsky et al., 1983; Lash et al., 1985). Furthermore, decorin has been found to participate in SM development by inhibiting myostatin activity, and thus, enhancing myogenic cell proliferation and differentiation (Kishioka et al., 2008).

Major Components of Muscle ECM

The ECM is comprised of various proteoglycans and fibrous proteins (e.g., collagens, elastins, FN, and laminins). Collagen is the main structural protein in SM ECM and holds 1 to 10% of SM dry weight (Dransfield, 1977; Gillies and Lieber, 2011). The two main ECM types are: 1) interstitial matrices—connective tissue matrices comprised of mixtures of collagens, elastins, FN, proteoglycans, and glycosaminoglycans (Florin et al., 2004), and 2) pericellular matrices—which interact with cells and have more diverse molecular compositions than surrounding interstitial matrix (Thorsteinsdottir et al., 2011).

ECM represents up to 10% of muscle weight and can be classified as endomysium, perimysium, or epimysium. The endomysium contains individual myofibril, whereas the perimysium partitions SMs into fascicles, and outer support to whole SM is provided by the epimysium (Kjaer, 2004). Type I collagen has been reported to be the predominant perimysial collagen, while type III collagen is dispersed between endomysium and epimysium (Light and Champion, 1984). BMs are predominantly comprised of laminins, collagen type IV, nidogen (entactin), and perlecan (Lebleu et al., 2007), whereas collagen types VI, XV, and XVIII are also present in the BMs of SM (Halfter et al., 1998). Reticular lamina, present beneath BM, is mostly comprised of collagen fibrils (types I, III and VI) and FN in a proteoglycan rich gel (Garg and Boppart, 2016).

Laminin, collagen IV, nidogen/entactin, agrin, biglycan, and perlecan form the BL that surrounds SM fibers (Gullberg et al., 1999; Tunggal et al., 2000; Jimenez-Mallebrera et al., 2005), and it has been proposed laminin in ECM stimulates myoblast proliferation and differentiation (Rooney et al., 2009). Laminin-211 is the predominent laminin isoform in BMs of adult SM (Ehrig et al., 1990; Sasaki et al., 2002), and integrins and non-integrins are two potential groups of laminin-211 receptors. Integrins are αβ heterodimeric transmembrane proteins with a huge number of functions that include adhesion, migration, and differentiation (Hynes, 2002). α7β1 is the major integrin of adult SM (Song et al., 1992; Burkin and Kaufman, 1999), and α-dystroglycan is the primary non-integrin cell surface receptor (Ahmad et al., 2018). The integrins α1β1, α2β2, α3β1, α6β1, α6β4, α7β1, α9β1, αvβ3, and αMβ2 are reported to bind laminin, and most recognize the globular domain of its long arm (Wondimu et al., 2004; Tzu and Marinkovich, 2008; Durbeej, 2010). However, only α3β1, α6β1, α6β4 and α7β1 integrin are considered highly selective laminin receptors (Humphries et al., 2006; Nishiuchi et al., 2006; Barczyk et al., 2010).

Proteoglycans in SM ECM predominantly belong to the small leucine-rich proteoglycan family, and the commonly found proteoglycans in SM ECM are chondroitin sulfate and dermatan sulfate glycosaminoglycans (Brandan and Inestrosa, 1987). Furthermore, interactions between proteoglycans and collagen sustain ECM structure and organization. Proteoglycans bind to collagen at particular positions (Pringle and Dodd, 1990), and thus, proteoglycan to collagen ratios vary in ECM. In addition, the leucine-rich repeats of decorin bind with collagen type I to determine the role of decorin as a regulator of collagen fibrillogenesis in SM (Gillies and Lieber, 2011).

Myopathies and ECM

Myopathy refers to muscle diseases in which muscle weakness due to muscle fiber dysfunction is the primary symptom. Other symptoms include muscle cramps, stiffness (myotonia), and spasm. Myopathies are broadly categorized as inherited and/or acquired (Stenzel and Schoser, 2017). Inherited myopathies predominantly affect SM tissues and are generally caused by mutations in the genes responsible for SM development, as exemplified by different types of non-dystrophic and dystrophic SM disorders, which manifest an extensive range of genetic and biochemical features. Muscular dystrophies, congenital metabolic myopathies, and myotonia are the most prevalent inherited myopathies (Cardamone et al., 2008; González-Jamett et al., 2018). Common muscle cramps are categorized as an acquired myopathy, for example; hypothyroid and hyperthyroid myopathies are caused by thyroid gland abnormalities (Ruff and Weissmann, 1988; Sindoni et al., 2016). Other systemic diseases (e.g., endocrine disorders, pituitary or adrenal dysfunction,

Cushing's disease, sarcoidosis, diabetes mellitus, mixed connective disease, and electrolyte imbalance) and toxic myopathies caused by medications are also examples of acquired myopathies (Chawla, 2011). Furthermore, SM myopathies have major effects on pathogenesis and clinical outcomes may result in cardiac arrest (Chapleau, 2014).

Several human diseases are associated with ECM abnormalities, and ECM components are implicated in the pathologies of disorders that affect almost every organ system. These ECM-linked diseases are generally attributable to factors ranging from abnormal signaling functions to inadequacies of the structural components of vital organs (Iozzo and Gubbiotti, 2018). Several SM-associated genetic disorders are typically caused by mutations in ECM elements and cell surface receptors. Interestingly, more than 150 ECM proteins have been reported to interact with integrin receptors (Zaidel-Bar et al., 2007; Ahmad et al., 2018).

The main ECM transmembrane receptor of SM is composed of dystrophin-glycoprotein complex (DGC), utrophin glycoprotein complex (UGC), and α7β1 integrin complex. These laminin-binding protein complexes can transform the progression of disease, and thus, are viewed as curative targets for disease intervention (Van Ry et al., 2017). α7β1 integrin is a laminin receptor found on the exterior surfaces of skeletal myoblasts and myofibers. In Duchenne muscular dystrophy (DMD), articulation of α7β1-mediated ECM binding may compensate for the absence of the dystrophin-mediated linkage. Collagen and laminin related dystrophies are typical of the diseases associated with these SM receptors (**Figure 2**).

In addition, the downregulation of integrin articulation may add to the progression of congenital laminin deficiencies (Burkin and Kaufman, 1999). Dystrophin is related to a muscle membrane (sarcolemmal) glycoprotein complex, which provides linkage with laminin. Interestingly, in the absence of dystrophin-related proteins (43DAG, 50DAG, 59DAP, 35DAG, and 156DAG) were markedly down-regulated in the sarcolemma of DMD patients and MDX mice (Matsumura et al., 1992).

Collagen Myopathy

Collagens are the most common and major component of ECM and are reported in almost all connective tissues. They fulfill a number of critical functions in SM including the transmission of forces to bones, tensile strength, and elasticity, and are also involved in the regulations of cell attachment and differentiation. Collagens also play critical roles in cell-to-ECM interactions *via* several transmembrane receptors. Collagen type VI (COL VI) is a component of SM ECM in terms of these interactions (Jobsis et al., 1996).

Collagen VI is consist of α1, α2, and α3 chains, which are encoded by COL6A1 and COL6A2 on chromosome 21q22 and by COL6A3 on 2q37, respectively (Allamand et al., 2011). Mutations in all three of these genes result in two main muscle disorders types, namely; 1) Ullrich congenital muscular dystrophy (UCMD) (severe phenotype) and 2) Bethlem myopathy (minor to moderate

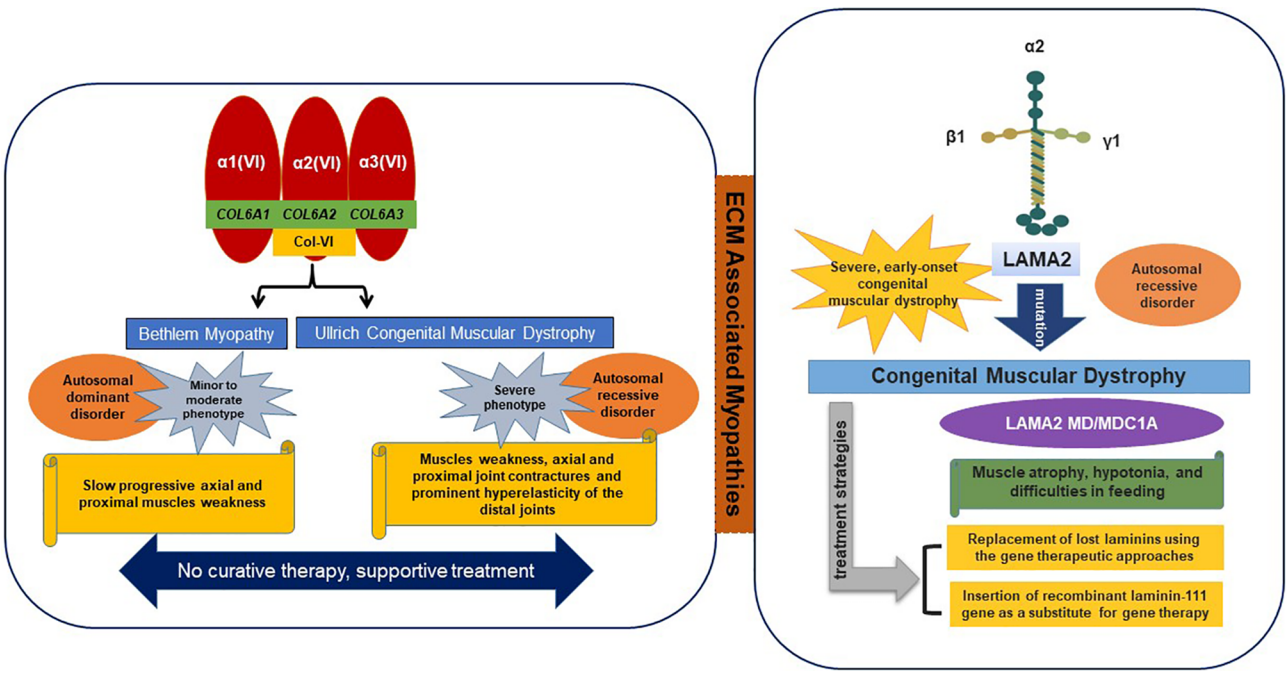

FIGURE 2 | ECM-associated myopathies.

phenotype). Recently, two additional phenotypes were found to be associated with mutations in the COL6A2 gene, that is, limb-girdle muscular dystrophy and myosclerosis (an autosomal recessive phenotype) (Bushby et al., 2014). In ECM, collagen VI interacts with various molecules (collagen II, IV, XIV, and decorin) and cell surface receptors (fibulin 2, hyaluronan, membrane-associated chondroitin sulfate proteoglycan 4, and biglycan) (Bonnemann, 2011).

Ullrich Congenital Muscular Dystrophy (UCMD)

UCMD is usually defined as an autosomal recessive condition that results in muscle weakness, contractures of proximal joints, and prominent hyperelasticity of distal joints (Lampe and Bushby, 2005). The UCMD phenotype is caused by loss-of-function mutations or dominant missense mutations involving glycine substitutions in the triple helical (Gly-Xaa-Yaa) motif or dominant exon-skipping mutations (Lampe and Bushby, 2005; Lampe et al., 2008). Mutations in the COL6A2 gene and more recently in the COL6A3 gene have been shown to cause UCMD (Demir et al., 2002). Usually, walking is delayed in affected children and they are unable to jump or run properly (Camacho Vanegas et al., 2001).

Bethlem Myopathy

Bethlem myopathy is a dominantly congenital, comparatively mild disease caused by mutations in the COL6A1, COL6A2, or COL6A3 genes characterized by progressive proximal muscle weakness and contractures (joint stiffness) of fingers, wrists, elbows, and ankles. Symptoms may be observed before birth due to reduced fetal movement, in early childhood due to late motor skill development, and in adults due to contractures of Achilles tendons or fingers (Lampe and Bushby, 2005; Baker et al., 2007).

The benchmark for the diagnosis of collagen myopathies is the identification of mutations in the COL6A1, COL6A2, or COL6A3 genes. Diagnoses of UCMD and Bethlem myopathy generally depend on distinctive clinical topographies in combination with mildly increased or normal levels of serum creatine kinase. Muscle biopsy is used to differentiate Bethlem myopathy and UCMD, for example, in Bethlem myopathy collagen VI immunolabeling of BL and endomysium are typically normal but in UCMD they are absent to obviously reduced (Lampe and Bushby, 2005). Treatments of Bethlem myopathy and UCMD involve supportive care following identical philosophies based on considerations of age at onset and observed symptom severity (Allamand et al., 2011).

Obstructive lung disease is common in patients with Bethlem myopathy and carries the risk of subsequent respiratory inadequacy (Bonnemann, 2011).

Laminin Myopathy

The laminin-α2 subunit is encoded by the LAMA2 gene and is primarily expressed in SM, and laminin-211 is the most abundant isoform found in BMs. Mutations in LAMA2 result in the most common types of congenital muscular dystrophies, that is, LAMA2 MD or MDC1A (Congenital type 1A), which are characterized by disruption of laminin-211 and account for 10–30% of reported cases (Sframeli et al., 2017; Mohassel et al., 2018). In general, the common symptoms of laminin myopathy are hypotonia, muscle weakness, and feeding difficulties, though joint contractures, including contractures of fingers and ankles, typically develop in later stage disease. The therapeutic strategies for LAMA 2 MD include replacement of lost laminins using gene therapeutic approaches or the administration of recombinant laminin-111 (Yurchenco et al., 2018). In addition to binding with typical receptors (integrins and dystroglycans), laminins bind with other ECM macromolecules such as nidogens and perlecan (Carmignac and Durbeej, 2012).

CONCLUDING REMARKS

SM is greatly influenced by ECM composition, and collagen is one of the main structural proteins in ECM. ECM myopathy is a group of genetic disorders caused by mutations in genes encoding proteins that provide critical associations between the ECM and muscle cells, and UCMD and Bethlem myopathy are the main SM diseases caused by mutation of collagen VI. Laminin largely composes the BL that surrounds muscle fibers, and laminin-211 plays a crucial role in SM function. LAMA2 MD and MDC1A are destructive muscular dystrophies caused by laminin α2 chain loss and have no cure. The discovery of additional information related to collagen VI and laminin 211 in human SM may prove to be decisive in terms of the developments of future therapies. The objective of this article was to improve understanding by exploring relations between ECM components and related diseases so as to aid the development of effective treatments. We recommend further research work be conducted to characterize ECM-related myopathies more precisely.

AUTHOR CONTRIBUTIONS

KA, SS, and SA wrote the initial draft of the paper and designed the figures. EL contributed to the systematic review of the literature. IC critically analyzed and approved the paper.

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Long Noncoding RNA GAS5 Promotes Osteogenic Differentiation of Human Periodontal Ligament Stem Cells by Regulating GDF5 and p38/JNK Signaling Pathway

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Both extracellular matrix (ECM) and stem cells contribute to the formation of bones. Accumulating evidence proved that the growth differentiation factor 5 (GDF5) plays a vital role in ECM osteogenesis regulation; the use of human periodontal ligament stem cells (hPDLSCs) may contribute to alveolar bone regeneration. Moreover, long noncoding RNAs (lncRNA) serves as a regulator in the growing process of cellular organisms including bone formation. Previous efforts has led us to the discovery that the expression of growth arrest specific transcript 5 (GAS5) changed in the osteogenic differentiation of hPDLSCs. Moreover, the expression of GAS5, as it turns out, is correlated to GDF5. This study attempts to investigate the inner workings of GAS5 in its regulation of osteoblastic differentiation of hPDLSCs. Cell transfection, Alkaline phosphatase (ALP) staining, Alizarin red S (ARS) staining, qRT-PCR, immunofluorescence staining analysis and western blotting were employed in this study. It came to our notice that GAS5 and GDF5 expression increased during osteogenesis induction of hPDLSCs. Knocking down of GAS5 inhibited the osteogenic differentiation of hPDLSCs, whereas overexpressing GAS5 promoted these effects. Molecular mechanism study further demonstrated that overexpressing GAS5 bolsters GDF5 expression and boosts the phosphorylation of JNK and p38 in hPDLSCs, with opposite effects in GAS5 knockdown group. To sum up, long noncoding RNA GAS5 serves to regulate the osteogenic differentiation of PDLSCs *via* GDF5 and p38/JNK signaling pathway. Our findings expand the theoretical understanding of the osteogenesis mechanism in hPDLSCs, providing new insights into the treatment of bone defects.

Keywords: human periodontal ligament stem cells, osteogenic differentiation, long noncoding RNA, growth arrest specific transcript 5, growth differentiation factor 5

INTRODUCTION

Alveolar bone loss has been posing perplexing challenges in the field of oral disease for decades. Scholars are committed in the search for more effective methods for bone regeneration. Bone is composed of calcified matrix—derived from extracellular matrix (ECM) and osteocytes (differentiated from osteoblasts). Extracellular matrix, a complicated complex of collagen, proteoglycans and glycoproteins, provides a specialized microenvironment for the proliferation, differentiation, aging and apoptosis of cells (Watt and Huck, 2013). Moreover, ECM, essential in the bone formation process, forms a microenvironment to regulate bioactivities of osteoblasts, providing signals for osteoblasts *via* diversified passages like Wnt and MAPK signaling pathways (Khatiwala et al., 2009; Lisignoli et al., 2017). MSCs are an important group of multipotent cells that can differentiate into a broad range of cell types including osteoblasts(Pittenger et al., 1999). Equipped with self-renewal and multi-differentiation capacity, MSCs are regarded as essential seeding cells in bone tissue engineering (Quarto et al., 2001; Rastegar et al., 2010).

Growth differentiation factor 5 (GDF5), as part of the bone morphogenic protein (BMP) family, is reported to serve an significant function in the tissue differentiation as well as repair of cartilage and bone (Mikic et al., 2004; Miyamoto et al., 2007). Previous study has demonstrated that implantation of GDF5 into ectopic sites in animal models induces the formation of neotendon/ligament-like tissue (Wolfman et al., 1997). GDF-5 is fundamental for articular cartilage maintenance by inducing ECM in articular cartilage and α5 integrin expression (Garciadiego-Cazares et al., 2015). Human cartilage ECM modulating proteins increased in response to GDF-5 protein treatment *via* Wnt signaling pathway (Enochson et al., 2014). Studies have fully investigated and proved the important function of GDF5 in the ECM osteogenic process.

Long noncoding RNAs (lncRNAs) refer to those RNAs with a length of more than 200 nucleotides (Esteller, 2011). In recent years, reports have suggested that lncRNAs serve a critical role in the regulation of the cell growth,differentiation and apoptosis (Guttman et al., 2011). Our research team conducted RNA-seq analysis on mRNA and lncRNA transcriptomes of osteogenically differentiated human periodontal ligament stem cells (hPDLSCs), one type of the mesenchymal stem cells derived from periodontal ligament tissue. And we found more than 200 lncRNAs were expressed differentially in the process of osteogenic induction (Zheng et al., 2018). Among them, lncRNA growth arrest specific transcript 5 (GAS5) showed significant change in expression between the undifferentiated and osteogenically differentiated hPDLSCs. Besides, we analyzed the expression pattern of lncRNAs during osteogenic differentiation of hPDLSCs, and formed the global co-expression networks to detect the genes that may participate in the osteoblast differentiation of hPDLSCs. In the network, we found that long noncoding RNA GAS5 showed strong correlation with GDF5 which is vital in osteoblast differentiation. It is interesting to explore whether GAS5 plays an important role in the process of osteogenic differentiation as GDF5 does. Located in chromosome 1q25.1, GAS5 is comprised of 12 exons with a short open reading frame that lack the ability to encode proteins (Schneider et al., 1988). Although GAS5 does not encode proteins, it is highly expressed in many tissues. The expression of GAS5 turns out to be even higher than many genes that encode proteins, which indicates that it may serve a functional role during the lifetime of the cell (Coccia et al., 1992). Besides, GAS5 is reported to participate in multiple stages of biological processes, like cell proliferation, apoptosis or migration (Pickard and Williams, 2016; Ding et al., 2018; Wang and Kong, 2018). Many studies treat GAS5 as a potent tumor suppressor as its deregulated expression has been linked with a legion of cancers (Ma et al., 2016; Xue et al., 2017)

However, researches on the role of GAS5 in osteogenesis of hPDLSCs are scarce. We attempt, therefore, to determine how GAS5 influences osteogenic induction process of hPDLSCs and explore the possible mechanism.

MATERIALS AND METHODS

Cell Cultivation and Induction

Healthy premolars were collected from three patients (16–20 years of age) in oral maxilla-facial surgery department. The periodontal ligament from the middle third of premolars was gently scraped and digested in trypsin (Gibco) for 5 min. The small pieces of tissue were then seeded onto a culture bottle and incubated in a growth medium (GM), which is composed of alpha minimum essential medium enriched with 10% fetal bovine serum (Gibco) and 1% penicillin and streptomycin in the presence of 5% CO_2 and a temperature of 37°C. The passages 3–6 of PDLSCs were utilized for subsequent experiments. These cells were identified and positive for mesenchymal stem cell markers CD73, CD105, and CD90 (Zheng et al., 2017). For the induction of differentiation in osteocytes, the hPDLSCs was cultured in osteogenic medium (OM), which is composed of GM supplemented with β-glycerophosphate (10 mM), dexamethasone (100 nM) and vitamin C (200 μM). The culture medium was changed every two days. The researchers obtained their ethical approval from the Ethics Committee (PKUSSIRB-201837096).

Cell Transfection

The siRNA control (si-NC) together with the small interfering RNAs (si-RNAs) against GAS5 (si-GAS5) and GDF5 (si-GDF5) were designed by Gene Pharma company (Shanghai, China). The si-RNA sequences were presented as following: si-GAS5, 5'-CUU GCCUGGACCAGCUUAATT-3'; si-GDF5, 5'-CCCAAGAAGG AUGAACCCATT-3'; si-NC, 5'-UUCUUCGAACGUGUCACG UTT-3'. When the cells have reached 70–80% of confluence, hPDLSCs were transfected by si-NC, si-GAS5 and si-GDF5 separately using Lipofectamine 3000 (Invitrogen) at 100 nM

and Opti-MEM every four days following the manufacturer's instructions. Recombinant lentivirus containing full-length GAS5 (GAS5) and the control (NC) was designed by Gene Pharma company (Shanghai, China). The cells were cultivated with medium containing specific lentivirus for 24 h and then exposed to medium containing puromycin (10 ng/ml) for cell selection.

Alkaline Phosphatase (ALP) Staining

Following the seven-day induction of osteogenesis, fixing was conducted to the cells using 4% paraformaldehyde for 10 min. Distilled water was then used for washing. Then an NBT/BCIP kit for staining (Co Win Biotech, Beijing, China) was used to conduct the ALP staining according to the protocol.

Alizarin Red S (ARS) Staining

On the fourteenth day following induction of osteogenesis, fixing of the cells was done in 4% paraformaldehyde for a duration of 10 min. Washing was done thrice using distilled water. After that, 1% Alizarin red S (Sigma-Aldrich St. Louis, MO) staining solution performed staining of the cells for 20 min to assess calcium deposition.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Invitrogen) was used to extract the total RNA from the PDLSCs as directed by the manufacturer's guidelines. The cDNA was then reverse transcribed by utilizing PrimeScriptTM RT Reagent Kit (Takara). The qRT-PCR was then done with the primers listed as follows: GAS5 forward primer: GTGTGGCTCTGGATAGCAC and reverse primer: AC CCAAGCAAGTCATCCATG; RUNX2 forward primer: ACT ACCAGCCACCGAGACCA and reverse primer: ACTGCTT GCAGCCTTAAATGACTCT; ALP forward primer: GAACG TGGTCACCTCCATCCT and reverse primer: TCTCGTGG TCACAATGC; OCN forward primer: CACTCCTCGCCCT ATTGGCGTG and reverse primer: CCCTCCTGCTTGGAC ACAAAGA; GDF forward primer: GCTGGGAGGTGTTCG ACATC and reverse primer: CACGGTCTTATCGTCCTGGC. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as normalization and the $2-\Delta\Delta CT$ method was used for calculations.

Western Blot Analysis

Collection and lysis of PDLSCs was conducted using RIPA Lysis Buffer which contains 1% protease inhibitor cocktail (Solarbio). Protein concentration was determined by a BCA kit (Thermo) and a total of 30 μg of protein was used for western blot analysis. The primary antibodies against RUNX2 (CST, #12556), GDF5 (Abcam, RRID: ab93855), p38 MAPK(Affinity, Cat#AF6456), phosphorylated p38 MAPK (Affinity, Cat#AF4001), JNK (Affinity, Cat#AF6318), phosphorylated JNK(Affinity, Cat#AF3318), ERK(Affinity, Cat#AF0155), phosphorylated ERK(Affinity, Cat#AF1015), and β-ACTIN (Abcam, RRID: ab8226) diluted at 1:1,000 overnight at 4°C. Three washes were done using TBST. Afterwards, incubation of the membranes was done with the anti-rabbit and anti-mouse secondary antibodies (ZB-2301 and ZB-2305, Zhongshan Golden Bridge Biotechnology, Beijing, China) which is diluted at 1:10,000 at room temperature for 1 h. Visualization of the bands was done by enhanced chemiluminescence using the Bio-Rad system for detection (ChemiDocTM MP Imaging System, USA). Intensity of the bands was measured using ImageJ. β-ACTIN internal control was used to ensure equal protein loading.

Immunofluorescence Staining

Cell plating was done onto sterile glass coverslips and cultured in GM or OM for seven days. Four percent paraformaldehyde was utilized to fix the cells for 20 min at room temperature. The cells were washed (0.01 M PBS) and permeabilized (1% Triton X-100), and then they were blocked with 5% goat serum (ZLI-9022, Zhongshan Golden Bridge Biotechnology, Beijing, China) for 1 h. Primary antibody OCN (Abcam, RRID: ab13418) and anti-rabbit secondary antibody (ZF-0511, Zhongshan Golden Bridge Biotechnology, Beijing, China) were used. DAPI staining was performed to stain nuclei and then the cells were observed and photographed using a confocal system for imaging (LSM 5 EXCITER, Carl Zeiss, Jena, Germany).

RNA Sequencing

The total RNA was extracted from GAS5 overexpressing and control group using TRIzol reagent (Invitrogen). cDNA libraries were constructed and samples were paired-end sequenced with an Illumina HiSeq 2000 platform. Whole transcriptome sequencing data were mapped to the human genome (hg38) using TopHat2. We used HTseq to count the genes and calculate the reads per kilobase transcriptome per million mapped reads (RPKM) to evaluate the gene expression level. Differentially expressed genes (DEGs) were defined based on fold changes greater than or equal to 2.0 and a false discovery rate of less than 0.05.

Statistical Analysis

All of the statistical analyses in this study were performed using SPSS 20.0 software (SPSS, Inc., Chicago, IL). Three repeated experiments were done. Results were presented as mean ± SD and analyzed employing Student's t test and one-way analysis of the variance.

RESULTS

The Expression of LncRNA GAS5 and GDF5 Is Upregulated During Osteoblast Differentiation of hPDLSCs

We cultured PDLSCs in OM for 0, 3, 7, and 14 days respectively to prove the effect of osteogenic induction. Quantitative RT-PCR results demonstrated that during the osteogenic differentiation of hPDLSCs the mRNA expression of RUNX2, ALP and OCN (osteogenic markers) significantly increased (**Figure 1A**). The intensity of ALP staining was gradually enhanced at days 0, 3, and 7 of osteogenic differentiation of hPDLSCs (**Figure 1B**). The ARS staining was also deepened progressively at days 0, 7, and 14 of osteogenic differentiation of hPDLSCs (**Figure 1C**). The

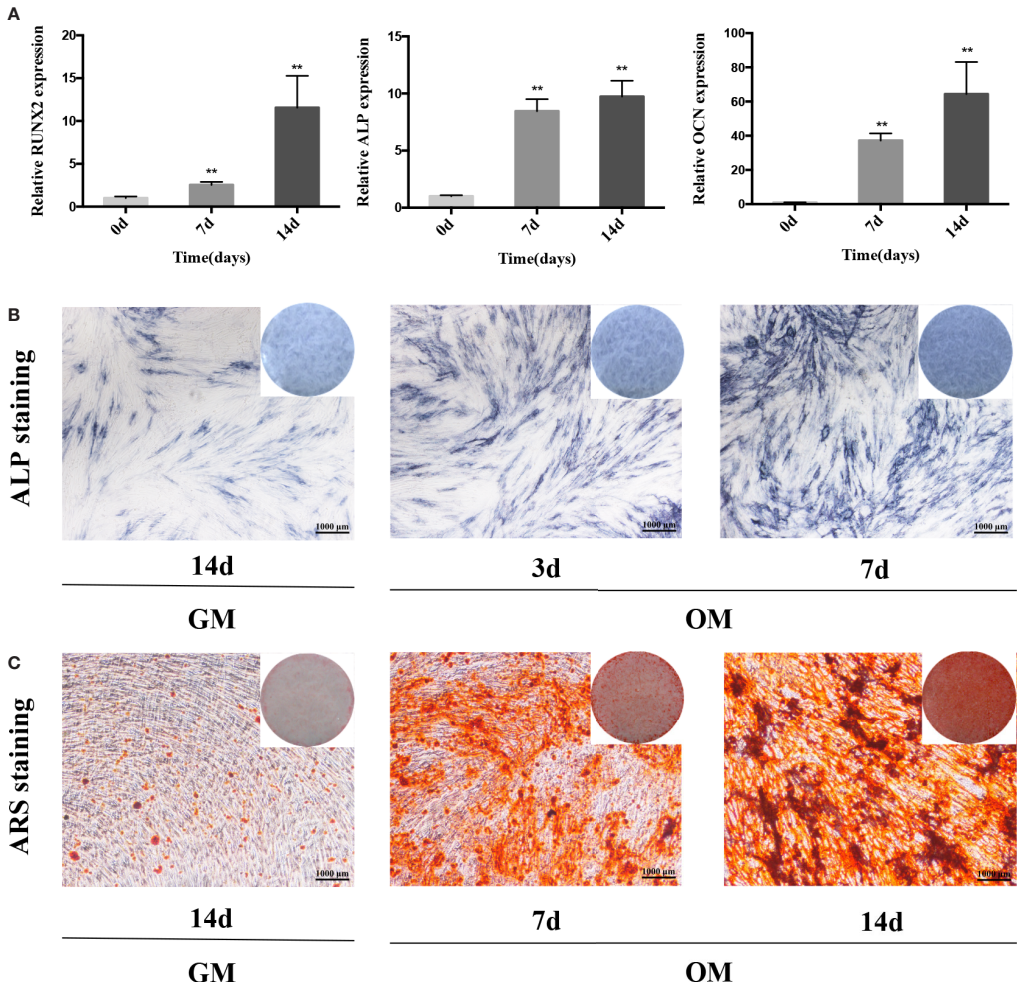

FIGURE 1 | The osteogenic induction of hPDLSCs. **(A)**: The relative expression of osteogenic makers namely RUNX2, ALP and OCN was determined by qRT-PCR during the osteogenesis of hPDLSCs for 0, 7 and 14 days. RNA expression at the above-mentioned time period was normalized to day 0. GAPDH was used as an internal control. **(B)**: The images of ALP staining in hPDLSCs cultivated in GM or OM for 3 and 7 days. **(C)**: The images of ARS staining in hPDLSCs cultivated in GM or OM for 7 and 14 days. **p < 0.01. hPDLSCs, human periodontal ligament stem cells; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RUNX2, runt-related transcription factor 2; ALP, alkaline phosphatase; OCN, osteocalcin; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; ARS, Alizarin red S; GM, growth medium; OM, osteogenic medium.

results indicated that the osteogenic induction was successful. To determine how GAS5 and GDF5 influences the osteogenic differentiation of PDLSCs, we conducted examination of its expression pattern, and found that the expression of GAS5 and GDF5 exhibited a gradual upregulation during the osteogenic differentiation for 14 days (**Figure 2**).

GAS5 Enhances Osteoblast Differentiation of hPDLSCs

To investigate the function of GAS5, we used si-GAS5 to knock down and lentivirus to overexpress GAS5 in hPDLSCs. The qRT-PCR results indicated that the expression of GAS5 declined nearly 80% using si-GAS5, and the efficiency of lentivirus transfection showed a more than 4-fold increase in comparison with the control group. Knocking down GAS5 caused a reduction in the mRNA levels of RUNX2, ALP and OCN compared to si-NC group, whereas overexpression of GAS5 upregulated those osteogenic related genes (**Figure 3A**). Western blot analysis further indicated that silencing GAS5 inhibited the expression of osteogenic related proteins RUNX2 and overexpressing GAS5 caused an increase in RUNX2 protein expression (**Figure 3B**). The staining of ALP decreased after seven-day osteogenic induction of GAS5 knockdown PDLSCs, whereas significantly enhanced in the GAS5 overexpression group in comparison with NC group (**Figure 3C**). Consistently, after the 14-day OM induction, matrix mineralization was inhibited in the GAS5 knockdown group and was promoted in the GAS5 overexpression group as revealed by ARS staining (**Figure 3D**). Besides, analysis by immunofluorescence revealed downregulated protein level of OCN in PDLSCs with si-GAS5 compared to that with si-NC at day 7 of osteogenic differentiation (**Figure 4D**).

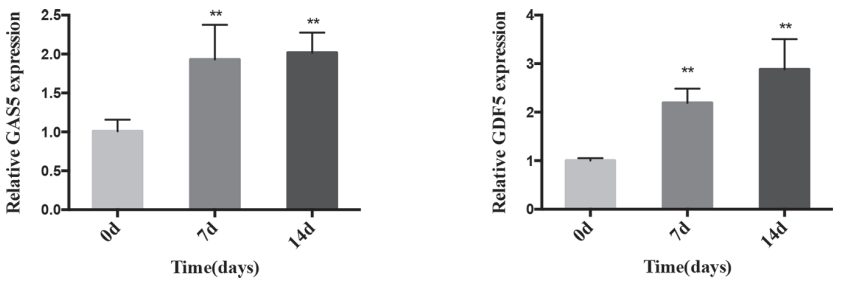

FIGURE 2 | Expression pattern of GAS5 and GDF5 during the osteoblast differentiation of hPDLSCs. The relative expression of GAS5 and GDF5 was determined by qRT-PCR during the osteogenesis of hPDLSCs for 0, 7 and 14 days. RNA expression at the above-mentioned time period was normalized to day 0. GAPDH was used as an internal control. **p <0.01. GAS5, lncRNA growth arrest specific transcript 5; GDF5, growth differentiation factor 5; hPDLSCs, human periodontal ligament stem cells; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; qRT-PCR, quantitative reverse-transcription polymerase chain reaction.

Downregulation of GDF5 Inhibits Osteoblast Differentiation of hPDLSCs

To help elucidate the role of GDF5 in osteogenic differentiation of hPDLSCs, similarly, we used si-GDF5 to realize the knockdown of GDF5 and the expression of GDF5 was reduced by 70–80%. Several osteogenic markers (RUNX2, ALP, OCN) was downregulated after knocking down GDF5 as tested by qRT-PCR (**Figure 4A**). The intensity of ALP staining decreased in the GDF5 knockdown group after the 7-day osteogenic differentiation (**Figure 4B**), and the ARS staining weakened consistently after the 14-day osteogenic differentiation (**Figure 4C**), indicating that downregulation of GDF5 inhibits osteoblast differentiation of hPDLSCs. In addition, immunofluorescence staining analysis further demonstrated that the negative effect of knocking down GDF5 on osteogenic differentiation of hPDLSCs (**Figure 4D**).

GAS5 Presents a Co-Expression Relationship With GDF5

In previous research, we analyzed the expression pattern of lncRNA during osteogenic differentiation of hPDLSCs, and visualized the global co-expression networks to find out the genes that may participate in the osteoblast differentiation of hPDLSCs. In the network, we found that GAS5 showed strong correlation with GDF5 that were found to play a vital role in osteoblast differentiation (**Figure 5A**). To confirm whether GDF5 is influenced by GAS5, we used RNA sequencing (RNA-seq) to identify differentially expressed genes in GAS5-overexpression PDLSCs compared to the control group (NC). And we investigated the potential regulatory roles played by differentially expressed genes *via* Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. Finally, we found 509 genes downregulated and 156 genes upregulated in the GAS5 overexpression group. Among these, the expression of GDF5 was increased to nearly 4-fold. The qRT-PCR results were consistent with the RNA-seq data. The mRNA expression of GDF5 was decreased after knocking down GAS5 and upregulated by overexpressing GAS5 (**Figure 5B**). Furthermore, western blot analysis demonstrated that GDF5 was decreased in si-GAS5 group and upregulated in the GAS5 overexpressing group (**Figure 5C**).

GAS5 Promotes and Knocking Down GDF5 Inhibits Osteogenic Differentiation of hPDLSCs Partly *via* Alleviating p38/JNK Phosphorylation

The relevant studies showed that p38 MAPK signaling pathway serve a significant role in the bone formation and inflammation, and it was activated by the TGF-β superfamily of proteins, including BMPs (Li et al., 2014). Furthermore, our group have confirmed that GDF5 can regulate osteogenic differentiation partially *via* phosphorylation of p38 and SMAD1/5/8 (Li X. et al., 2018). Thus, we investigated whether GAS5 and GDF5 can regulate the proteins in MAPK signaling pathway. To evaluate the levels of p38, phosphorylated p38 (p-p38), extracellular signal-regulated kinase 1/2 (ERK), phosphorylated ERK (p-ERK), c-Jun N-terminal kinase (JNK) and phosphorylated JNK (p-JNK) in MAPK pathway in the si-GAS5, si-GDF5, si-NC, GAS5 and NC treated hPDLSCs, western blot analysis was conducted. The results showed that the phosphorylation of p38 and JNK decreased in si-GAS5 group and there was no significant difference in the phosphorylation of ERK (**Figure 6**). The phosphorylation of p38 and JNK increased in GAS5 overexpressing group and there was no significant difference in the phosphorylation of ERK (**Figure 7**). The phosphorylation of p38, JNK and ERK all declined in si-GDF group (**Figure 8**).

DISCUSSION

GAS5 was overexpressed in growth arrest cells and was thus named growth arrest-specific 5 (Schneider et al., 1988). Ever since the discovery, researches related to GAS5 has been increasing dramatically. Its expression has been meticulously recorded in a large scope of tissues and its function has been thoroughly studied over different stages of development (Pickard

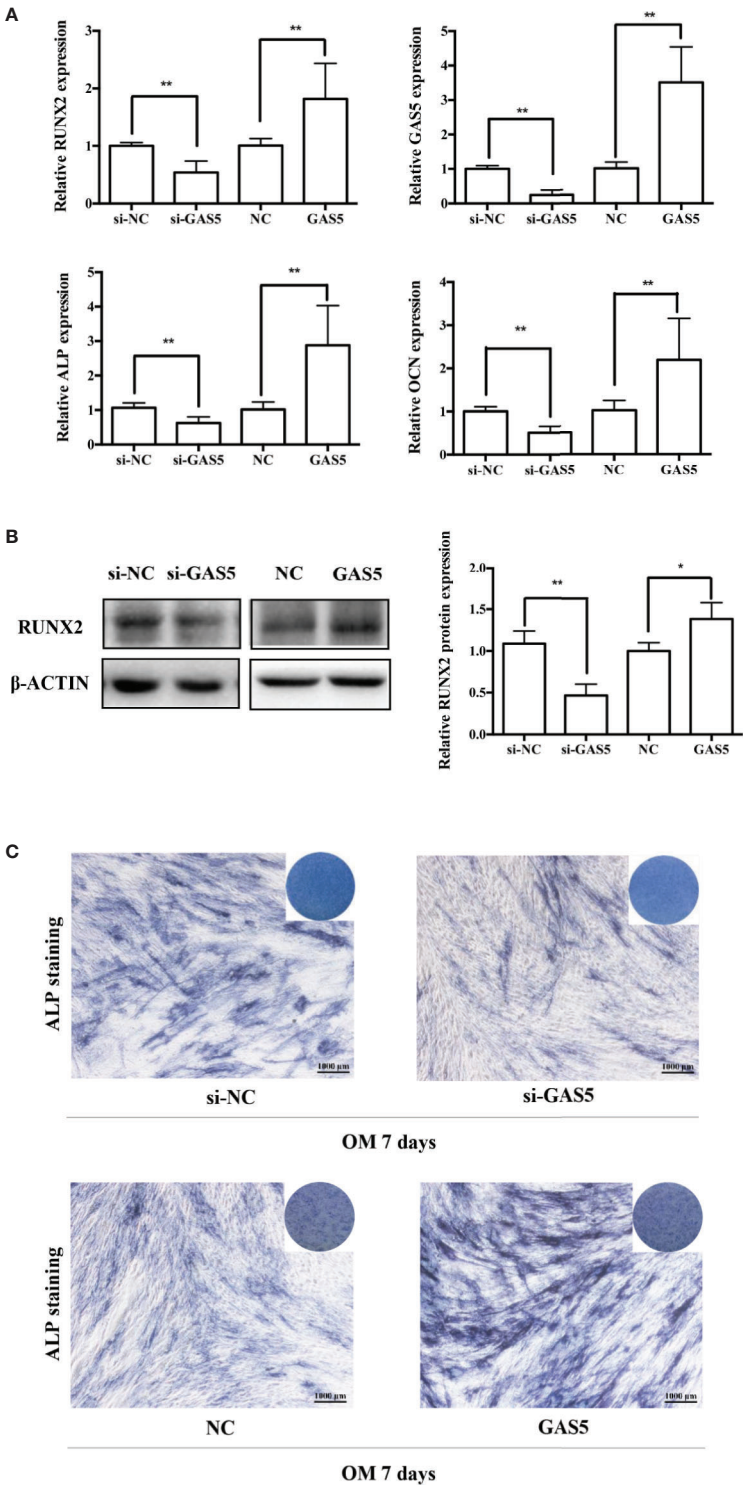

FIGURE 3 | Continued

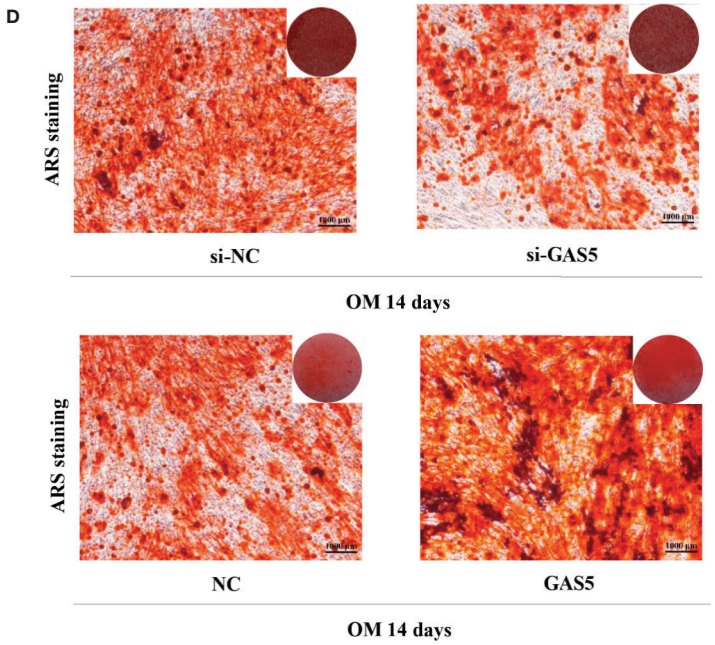

FIGURE 3 | GAS5 enhanced the osteoblast differentiation of hPDLSCs. **(A)**: The efficiency of transient transduction of si-GAS5 and lentivirus infection is measured by qRT-PCR. The mRNA expression of RUNX2, ALP, OCN was measured in si-NC, si-GAS5, NC and GAS5 group on the second day of osteogenic induction. GAPDH mRNA levels were employed for the process of normalization. **(B)**: Western blot analysis of the protein expression of RUNX2 and the internal control β-ACTIN on the third day of osteogenic induction. β-ACTIN was utilized for the normalization relative to si-NC groups. **(C)**: The images of ALP staining in si-NC, si-GAS5, NC and GAS5 group. Cells were cultured in GM or OM for 7 days. **(D)**: Images of ARS staining that stains for mineralized matrix in the si-NC, si-GAS5, NC and GAS5 group were also cultured in GM or OM for 14 days. *$p < 0.05$, **$p < 0.01$. hPDLSCs, human periodontal ligament stem cells; GDF5, growth differentiation factor 5; si-NC, small interfering RNA negative control; si-GAS5, the small interfering RNAs that target GAS5; NC, negative control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RUNX2, runt-related transcription factor 2; ALP, alkaline phosphatase; OCN, osteocalcin; GM, growth medium; OM, osteogenic medium; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; ARS, Alizarin red S.

and Williams, 2015). Its role as a tumor suppressor lncRNA attracted much attention when its low-expression was detected in cancers such as non-small cell lung cancer and colorectal carcinoma (Yang et al., 2017; Ding et al., 2018). Studies showed that GAS5 is essential in modulating the pluripotency and self-renew ability of mouse embryonic stem cells. Besides, it represses endodermal lineage differentiation and promotes induced pluripotent stem cells reprogramming (Tu et al., 2018). Also, its involvement in inflammation was studied with inconsistent results (Sun et al., 2017; Li F. et al., 2018). The function of GAS5 in osteogenesis, however, seems to be neglected by academia. To explore the uncharted waters, i.e. its role in osteogenic differentiation, we detected the expression pattern of GAS5 in osteogenic induction of hPDLSCs, and used si-GAS5 and GAS5 to confirm its regulatory function in mRNAs and proteins related to bone formation. We used RNA-seq to explore the co-expression network connections and found the relationship between GAS5 and GDF5, following that we found that GAS5 can promote osteogenesis of hPDLSCs by upregulating GDF5 *via* a p38/JNK signaling pathway.

Through complicated regular mechanisms, lncRNAs play an essential part throughout regulatory processes of genes. lncRNAs can mediate epigenetic regulation *via* binding to proteins, such as chromatin regulatory complexes before the transcription processes (Wutz and Gribnau, 2007). During the transcription process, lncRNAs can interact with transcriptional factors and affect its activity (Feng et al., 2006). Besides, the post-transcriptional processes is also influenced by lncRNAs, mostly through competing endogenous RNA (ceRNA) mechanism (Poliseno et al., 2010). As for GAS5, the specific regulatory mechanism remains unclear. It is reported that GAS5 can specifically unite with DNA segments in the glucocorticoid receptor (GR), inhibiting the bonding between the GR and glucocorticoid response elements in target genes (Kino et al., 2010). Besides, GAS5 can integrate with eukaryotic translation initiation factor-4E or act as competing endogenous RNA (ceRNA), such as miR-21 (Hu et al., 2014; Song et al., 2014). The relationship between GAS5 and GDF5 is uncovered by the network analysis in our previous work. Then we found that GAS5 can regulate the expression of GDF5 in the osteogenic differentiation of PDLSCs. It is reported that miR-21 promoted the chondrogenesis of osteoarthritis by directly targeting GDF5 (Zhang et al., 2014). We propose that there might be a lncRNA-miRNA regulatory loop between GAS5 and GDF5; miR-21 might potentially serve as one of the links. The specific path in which GAS5 interacts with GDF5, however, remains to be investigated.

Mitogen-activated protein kinases (MAPKs) are a family of evolutionarily conserved serine/threonine kinases that can help

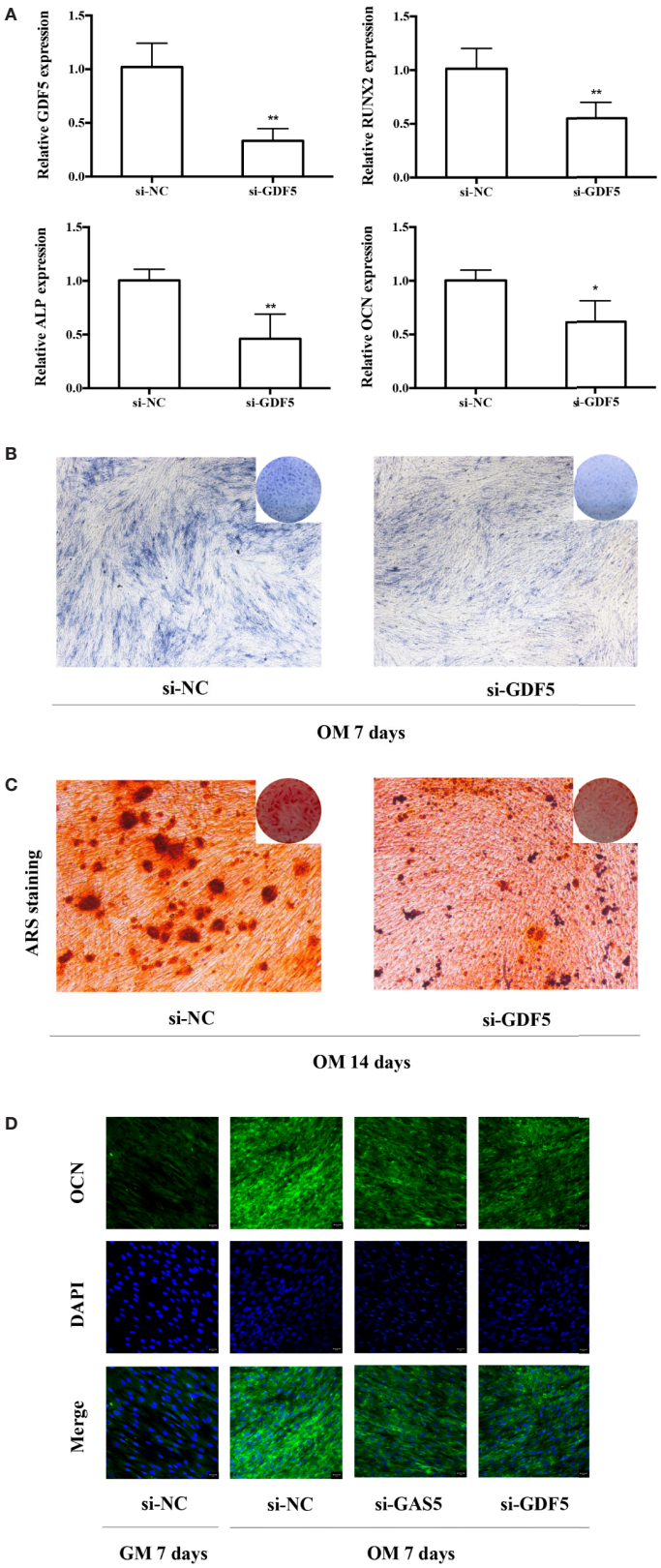

FIGURE 4 | Continued

FIGURE 4 | Knockdown of GDF5 inhibits osteoblast differentiation of hPDLSCs. **(A)**: The efficiency of transient transduction of si-GDF5 is measured by qRT-PCR. The mRNA expression of RUNX2, ALP, OCN was measured in si-NC and si-GDF5 group on the second day of osteogenic induction. GAPDH was used for the normalization process relative to si-NC groups. **(B)**: Images of ALP in the si-GDF5 and si-NC groups. Cells were cultured in OM for 7 days. **(C)**: Images of ARS which stains for mineralized matrix in the si-GDF5 and si-NC groups. Cells were cultured in OM for 14 days. **(D)**: Immunofluorescence staining analysis of OCN protein expression at si-NC, si-GAS5 and si-GDF5 groups. Cells were cultured in GM or OM for 7 days. *p < 0.05, **p < 0.01. hPDLSCs, human periodontal ligament stem cells; si-NC, small interfering RNA negative control; si-GDF5, the small interfering RNAs that target GDF5; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RUNX2, runt-related transcription factor 2; ALP, alkaline phosphatase; OCN, osteocalcin; GM, growth medium; OM, osteogenic medium; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; ARS, Alizarin red S.

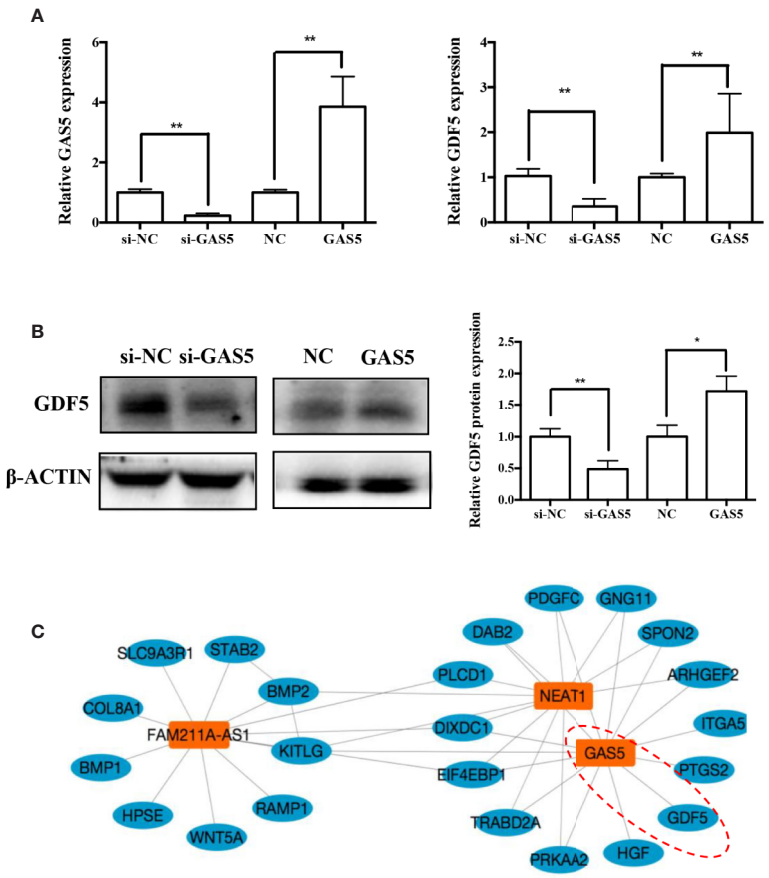

FIGURE 5 | GAS5 regulated the expression of GDF5. **(A)**: The mRNA expression of GDF5 as is detected by qRT-PCR in si-NC, si-GAS5, NC and GAS5 group. GAPDH was employed for the normalization. **(B)**: Western blot analysis of the protein expression of GDF5 and the internal control β-ACTIN in si-NC, si-GAS5, NC and GAS5 group. The histogram demonstrates the quantification of the band intensities. **(C)**: The co-expression network connections of the module, containing GAS5 and NEAT1. *p < 0.05, **p < 0.01. GAS5, lncRNA growth arrest specific transcript 5; GDF5, growth differentiation factor 5; si-NC, small interfering RNA negative control; si-GAS5, the small interfering RNAs that target GAS5; NC, negative control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; qRT-PCR, quantitative reverse-transcription polymerase chain reaction.

to transduce extracellular stimuli into cells and nuclei, participating in multiple biological processes such as cellular proliferation, differentiation, and apoptosis (Bluthgen and Legewie, 2008; Kim and Choi, 2015). MAPKs have three main subfamilies, namely, extracellular signal-regulated kinase (ERK), c jun N-terminal kinase (JNK), and p38. Previous studies have demonstrated that MAPK serves a vital role in the regulation of bone mass *via* control of osteoblast differentiation (Higuchi et al., 2002; Greenblatt et al., 2010). To further investigate the mechanism by which GAS5 promoted the osteogenic differentiation of PDLSCs, we detected the protein levels of JNK, p38, ERK, and their phosphorylated forms. The results indicated that GAS5 promoted the phosphorylated levels of JNK, and p38, whereas not significantly changed the expression levels of ERK.

Studies have indicated that the JNK and p38 phosphorylation is involved in the osteogenesis of PDLSCs. It is demonstrated that cannabinoid receptor 1 enhanced the osteo/dentinogenic

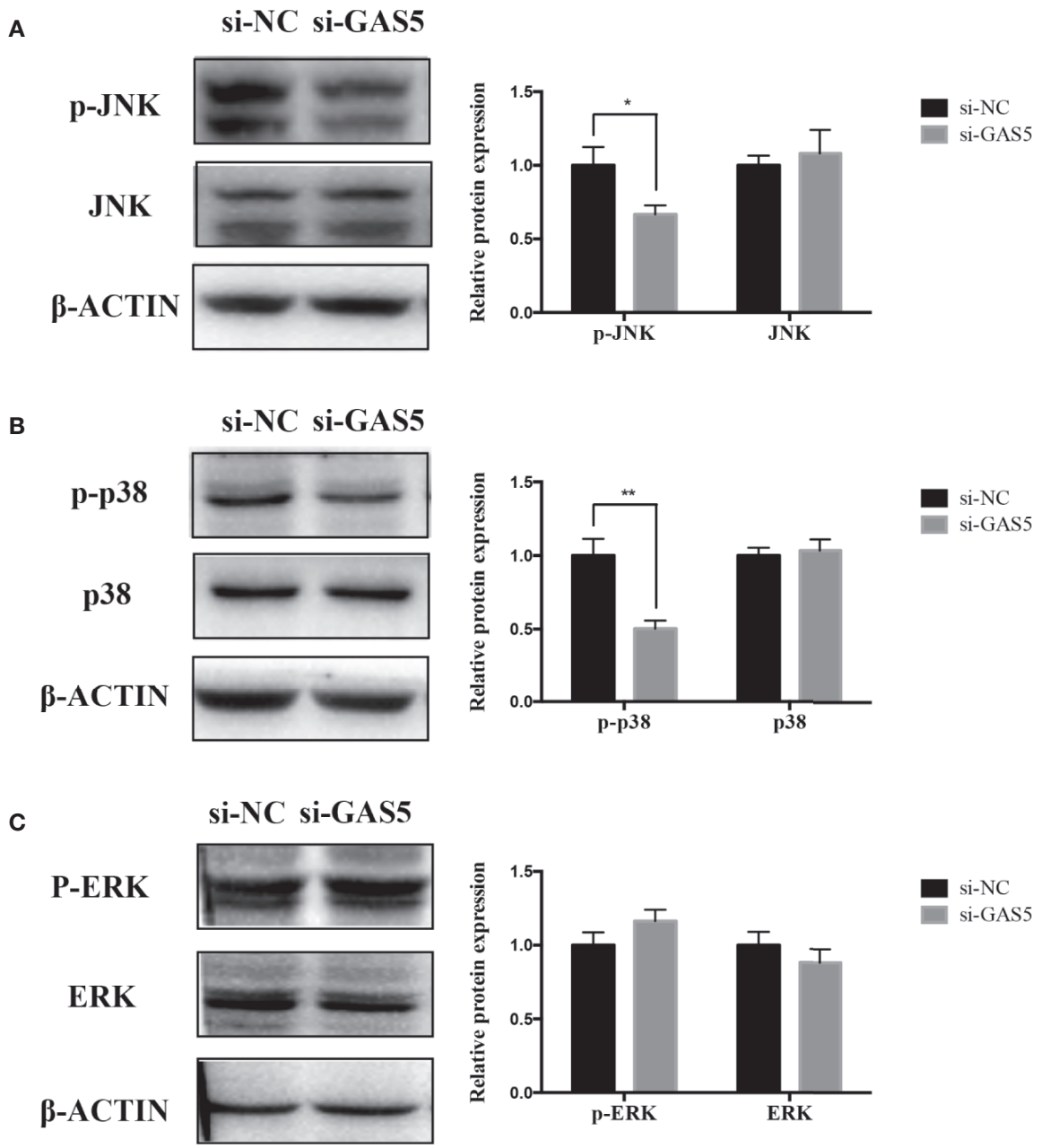

FIGURE 6 | Knockdown of GAS5 weakened the phosphorylation of JNK and p38 in hPDLSCs. **(A)**: Western blot analysis of phosphorylated JNK (p-JNK), JNK and β-ACTIN in hPDLSCs transfected with si-NC or si-GAS5. Histogram showed the quantification of the band intensities. **(B)**: Western blot analysis of phosphorylated p38 (p-p38), p38 mitogen-activated protein kinase (p38), and β-ACTIN in hPDLSCs transfected with si-NC or si-GAS5. Histogram showed the quantification of the band intensities. **(C)**: Western blot analysis of phosphorylated ERK (p-ERK), ERK and β-ACTIN in hPDLSCs transfected with si-NC or si-GAS5. Histogram showed the quantification of the band intensities. *$p < 0.05$, **$p < 0.01$. hPDLSCs, human periodontal ligament stem cells; GAS5, lncRNA growth arrest specific transcript 5; si-NC: small interfering RNA negative control; si-GAS5: the small interfering RNAs that target GAS5; NC, negative control; JNK, c-Jun N-terminal kinase; ERK, intracellular signal-regulated kinase 1/2.

differentiation ability of periodontal ligament stem cells *via* p38 MAPK and JNK in an inflammatory environment (Yan et al., 2019). Bone morphogenetic protein-9 induces PDLSCs osteogenic differentiation through the ERK and p38 signal pathways (Ye et al., 2014). In the present study, GAS5 increased the phosphorylated levels of JNK and p38, which indicated GAS5 enhanced osteogenic differentiation of PDLSCs possibly through activation of p38 MAPK and JNK signaling pathway. In addition to the MAPK pathway, other pathway like Wnt/β-catenin, BMP/TGF-β pathways have also been reported

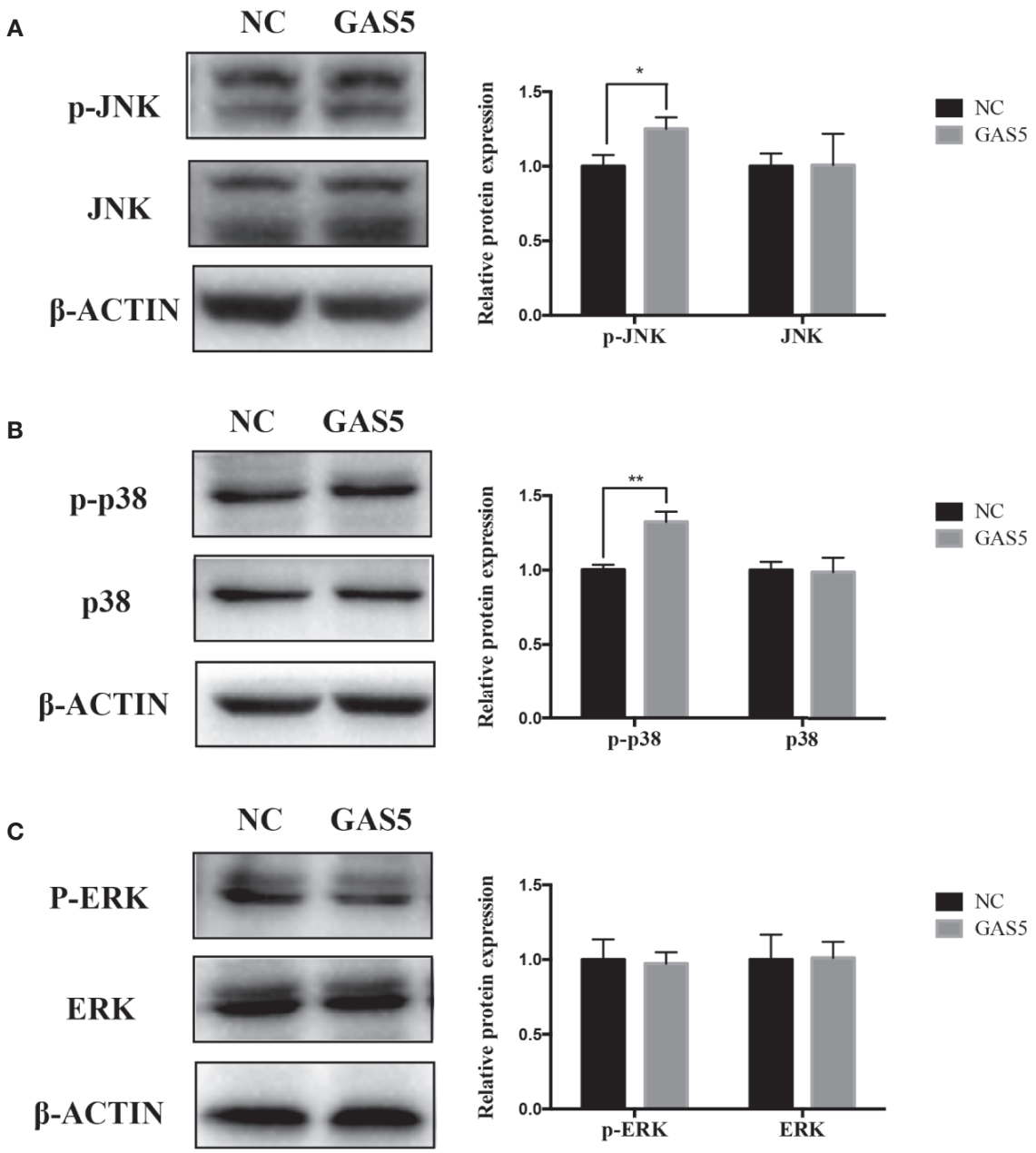

FIGURE 7 | Overexpressing GAS5 enhanced the phosphorylation of JNK and p38 in hPDLSCs. **(A)**: Western blot analysis of phosphorylated JNK (p-JNK), JNK and β-ACTIN in hPDLSCs transfected with NC or GAS5. Histogram showed the quantification of the band intensities. **(B)**: Western blot analysis of phosphorylated p38 (p-p38), p38 mitogen-activated protein kinase (p38), and β-ACTIN in hPDLSCs transfected with NC or GAS5. Histogram showed the quantification of the band intensities. **(C)**: Western blot analysis of phosphorylated ERK (p-ERK), ERK and β-ACTIN in hPDLSCs transfected with NC or GAS5. Histogram showed the quantification of the band intensities. *$p < 0.05$, **$p < 0.01$. hPDLSCs, human periodontal ligament stem cells; GAS5, lncRNA growth arrest specific transcript 5; NC, negative control; JNK, c-Jun N-terminal kinase; ERK, intracellular signal-regulated kinase 1/2.

to be involved in the osteogenesis of PDLSCs (Cao et al., 2017; Kim et al., 2018). There are intricate cross-talks between the pathways, forming a complex regulatory network; all pathways have not been exhaustively studied. More experiments are expected for a better understanding of the perplexing picture of regulatory network.

Loss of homeostasis in ECM can contribute to cartilage defect and bone disorders, such as osteoarthritis and osteoporosis (Paiva and Granjeiro, 2017; Rahmati et al., 2017). Our findings attempt to offer new perspectives into the ability of GAS5 to promote hPDLSCs osteogenic differentiation partially *via* regulating GDF5 and participating in p38/JNK pathway. Our results shed

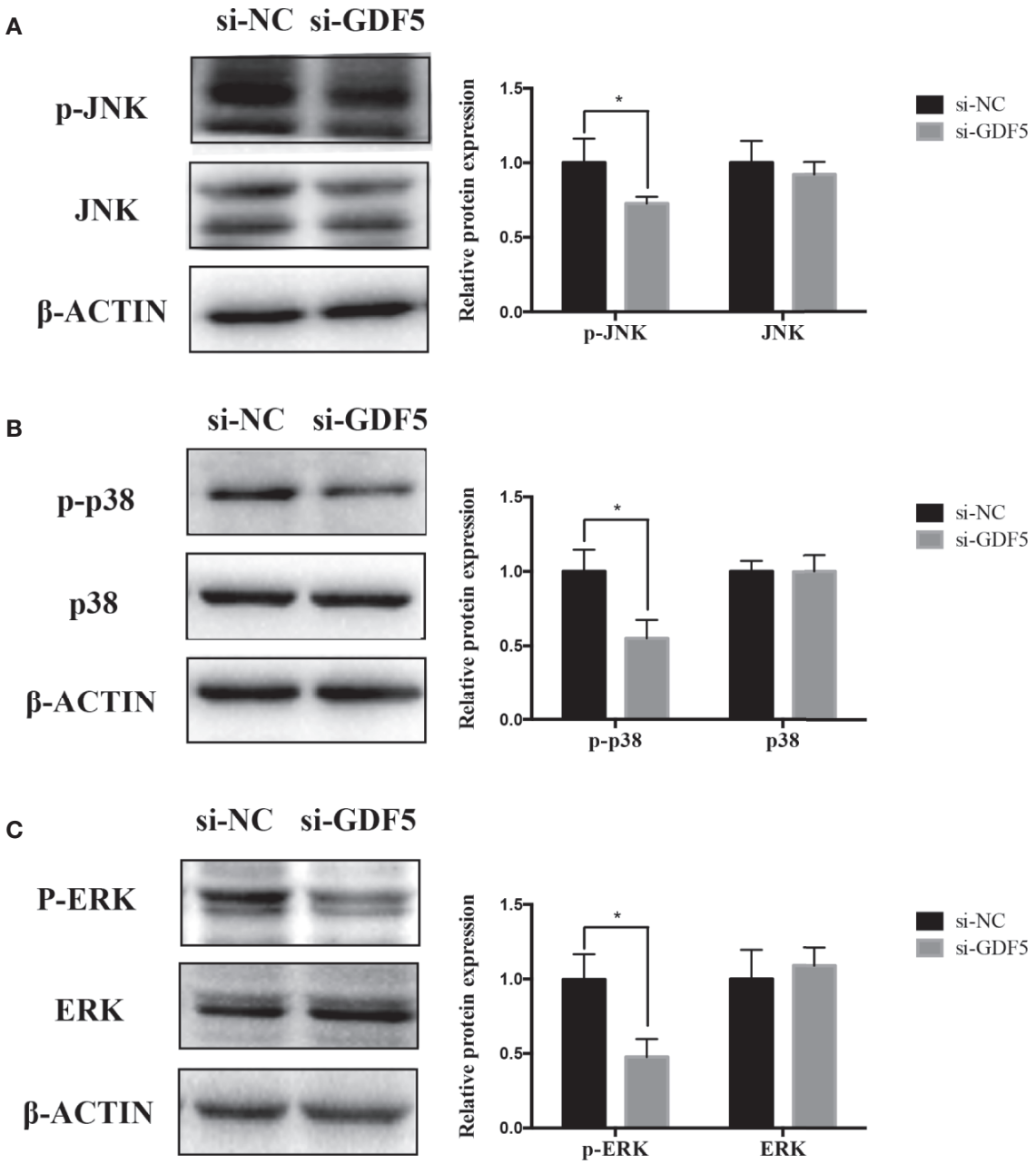

FIGURE 8 | Knockdown of GDF5 weakened the phosphorylation of JNK and p38 in hPDLSCs. **(A)**: Western blot analysis of phosphorylated JNK (p-JNK), JNK and β-ACTIN in hPDLSCs transfected with si-NC or si-GDF5. Histogram showed the quantification of the band intensities. **(B)**: Western blot analyses of phosphorylated p38 (p-p38), p38 mitogen-activated protein kinase (p38), and β-ACTIN in hPDLSCs transfected with si-NC or si-GDF5. Histogram showed the quantification of the band intensities. **(C)**: Western blot analyses of phosphorylated ERK (p-ERK), ERK and β-ACTIN in hPDLSCs transfected with si-NC or si-GDF5. Histogram showed the quantification of the band intensities. *p <0.05. hPDLSCs, human periodontal ligament stem cells; GDF5: growth differentiation factor 5; si-NC: small interfering RNA negative control; si-GDF5: the small interfering RNAs that target GDF5; NC, negative control; JNK, c-Jun N-terminal kinase; ERK, intracellular signal-regulated kinase 1/2.

lights on the application of hPDLSCs combined with GAS5, which could be a potentially useful approach in stimulating osteogenesis in bone tissue engineering. And its regulatory network may cast some light to the mechanism of bone disorders.

DATA AVAILABILITY STATEMENT

RNA sequence data has been uploaded into NCBI Sequence Read Archive with accession numbers "SRR11093247" and "SRR11093246".

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Peking University School of Stomatology, Beijing, People's Republic of China (PKUSSIRB—2011007). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

QY contributed to cell experiment, collection of data and analysis, statistical analysis, and writing of the manuscript. YHa contributed to cell experiment. PL, XL, and YHu contributed to the data collection and statistical analysis. WL, YZ, and LJ contributed to the design of this study as well as the revision of the manuscript. All authors have read and approved the final article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00701/full#supplementary-material

DATA SHEET 1 | Raw data.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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The Bone Extracellular Matrix in Bone Formation and Regeneration

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Bone regeneration repairs bone tissue lost due to trauma, fractures, and tumors, or absent due to congenital disorders. The extracellular matrix (ECM) is an intricate dynamic bio-environment with precisely regulated mechanical and biochemical properties. In bone, ECMs are involved in regulating cell adhesion, proliferation, and responses to growth factors, differentiation, and ultimately, the functional characteristics of the mature bone. Bone ECM can induce the production of new bone by osteoblast-lineage cells, such as MSCs, osteoblasts, and osteocytes and the absorption of bone by osteoclasts. With the rapid development of bone regenerative medicine, the osteoinductive, osteoconductive, and osteogenic potential of ECM-based scaffolds has attracted increasing attention. ECM-based scaffolds for bone tissue engineering can be divided into two types, that is, ECM-modified biomaterial scaffold and decellularized ECM scaffold. Tissue engineering strategies that utilize the functional ECM are superior at guiding the formation of specific tissues at the implantation site. In this review, we provide an overview of the function of various types of bone ECMs in bone tissue and their regulation roles in the behaviors of osteoblast-lineage cells and osteoclasts. We also summarize the application of bone ECM in bone repair and regeneration. A better understanding of the role of bone ECM in guiding cellular behavior and tissue function is essential for its future applications in bone repair and regenerative medicine.

Keywords: ECM, bone formation, bone tissue engineering, bone repair, bone cells

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INTRODUCTION

Trauma, fractures, congenital disease, or tumors can cause bone defects that are challenging to heal. This is especially true for large bones, where the missing tissue is larger than the spontaneous healing ability of osteoblasts (El-Rashidy et al., 2017; Fabris et al., 2018). For small defects, autologous bone grafts remain the gold standard. This approach relies on bone tissue harvested from a patient's own donor site, which is transplanted into the same patient's damaged area. Because the grafts contain the native bone matrix, osteoblasts, and growth factors, they intrinsically possess osteoinductivity and osteoconductivity (Garcia-Gareta et al., 2015). However, this approach is limited by the available sources of grafts and secondary damage at the donor site. By contrast, while having similar biological characteristics and mechanical properties as autogenous bone, allogeneic

bone carries the risk of transmission of infectious diseases and the possibility of immune rejection (Hinsenkamp et al., 2012).

In recent years, tissue engineering technology has enabled the production of artificial bone in large quantities. The resulting materials have the potential advantages of excellent biocompatibility, osteoinductivity, and osteoconductivity, providing a promising new method for bone repair. The manufacture of superior tissue-engineering constructs depends on three basic elements: appropriate scaffolds to support tissue-cell regeneration, cytokines, and appropriate seed cells. As the physical basis of artificial grafts, scaffold materials play a key role in the construction of artificial bone (Noori et al., 2017). Ideally, the scaffold material should mimic the characteristics of natural bone, providing a suitable biochemical environment and biomechanical support for the adhesion, migration, proliferation, osteogenic differentiation, and angiogenesis of seed cells on the scaffold. Finally, it must allow the gradual integration into the host tissue during the healing process, allowing it to bear normal loads (Mishra et al., 2016; Roseti et al., 2017). During bone regeneration, the homing of mesenchymal stem cells (MSCs), the formation of osteoblasts, extracellular matrix (ECM) and osteoid mineralization, and the formation of terminally differentiated osteocytes play an important role in bone formation (Wang et al., 2013).

The ECM is a non-cellular three-dimensional structure secreted by cells into the extracellular space. It is composed of specific proteins and polysaccharides. The ECM of each tissue type has a unique composition and topology during development (Frantz et al., 2010). The ECM provides the tissue with integrity and elasticity, and it is constantly being reformed due to changes in the abundance of receptors, growth factors, and the pH of the local environment to control the development, function, and homeostasis of tissues and organ (Bonnans et al., 2014; Mouw et al., 2014). The ECM is considered to represent the fourth element in the development of bone tissue engineering (Ravindran et al., 2012). The bone matrix comprises organic (40%) and inorganic compounds (60%). Moreover, its exact composition differs based on sex, age, and health conditions. The main inorganic components of the ECM are calcium-deficient apatite and trace elements. By contrast, the organic ECM is significantly more complex consists mainly of collagen type I (90%), and noncollagenous proteins (10%). It is mainly synthesized by osteoblasts before the mineralization process (Mansour et al., 2017). The non-collagenous proteins can be classified into four groups: γ-carboxyglutamate-containing proteins, proteoglycans, glycoproteins, and small integrin-binding ligands N-linked glycoproteins (SIBLINs) (Paiva and Granjeiro, 2017). Bone ECM dynamically interacts with osteoblast-lineage cells and osteoclasts to regulate the formation of new bone during regeneration.

In this review, we briefly introduce the inorganic and organic ECM of bone tissue (**Table 1**), including collagenous and non-collagenous proteins, and summarize the effects of the ECM on osteoblast-lineage cells, including MSCs, osteoblasts, and osteocytes, and osteoclasts. Finally, the application of ECM-based scaffold for bone regeneration in bone tissue engineering is reviewed.

MAJOR COMPONENTS OF BONE ECM

Organic ECM

Collagenous Proteins

The collagen type I, III, and V are the most abundant constituents of the organic ECM in bones. The main function of collagens is mechanical support and to act as a scaffold for bone cells (Saito and Marumo, 2015). Type I collagen accounts for 90% of the total collagen in bone tissue and forms triple helices of polypeptides which form the collagen fibrils. These fibrils interact with other collagenous and noncollagenous proteins to assemble the higher-order fibril bundles and fibers (Varma et al., 2016). Collagen types III and V regulate the fiber diameter and fibrillogenesis of type I collagen and are present in smaller amounts (Garnero, 2015). The inter- and intra-chain crosslinks of collagen are key to its mechanical properties, which maintain the polypeptide chains in a tightly organized fibril structure. Collagen plays an important role in determining bone strength. The lack of type I collagen or mutation of collagen structure results in changes in the ECM, and thus significantly increases fracture risk (Fonseca et al., 2014).

Noncollagenous Proteins

Proteoglycans

Proteoglycans are characterized by the presence of glycosaminoglycan (GAG) residues covalently bound to the protein core. The six types of GAG residues found in proteoglycans include keratan sulfate, chondroitin sulfate, heparan sulfate, hyaluronic acid, and dermatan sulfate (Kjellen and Lindahl, 1991). Small leucine-rich proteoglycans (SLRPs), such as biglycan, decorin, keratocan, and asporin, are important proteoglycans family in the bone. SLRPs are secreted extracellular proteins that interact with cell surface receptors and cytokines to regulate both normal and pathological cellular behaviors. During bone formation, SLRPs participate in all stages including cell proliferation, osteogenesis, mineral deposition, and bone remodeling (Kirby and Young, 2018). In addition, SLRPs regulate the process of collagen fibrillogenesis, the dysregulation of which leads to defects in the organization and production of collagen, culminating in fibrosis due to either orthopedic injuries or genetic deficiencies (Moorehead et al., 2019). Biglycan and decorin are class I SLRPs that contain either dermatan or chondroitin sulfate GAG chains. Biglycan is expressed during the process of cell proliferation and mineralization, while Decorin is continuously expressed starting from bone matrix deposition. Keratocan is mainly expressed in osteoblasts and involved in regulating bone formation and mineral deposition rates (Coulson-Thomas et al., 2015). Asporin, another member of SLRP, has been shown to bind with type I collagen to promote collagen mineralization (Kalamajski et al., 2009). Therefore, SLRPs play an essential role to maintain bone homeostasis.

γ-Carboxyglutamic Acid-Containing Proteins

One important group of bone ECM proteins contains γ-carboxyglutamic acid (Gla), a specific modified glutamic acid

TABLE 1 | The list of bone ECM components and their role in bone formation.

Bone ECM	Expressed from	Function in bone tissue	Reference
Organic ECM			
Collagenous protein			
Type I collagen	Osteoblast	–Scaffold for bone cells –Maintain bone strength	(Saito and Marumo, 2015)
		–Promote bone formation –Regulate collagen fibrillogenesis	(Fonseca et al., 2014)
Types III and V collagen	Bone	–Promote bone	(Garnero, 2015)
Noncollagenous protein			
Proteoglycans			
Biglycan	Osteoblast	–Promote collagen fibrillogenesis –Promote bone formation	(Moorehead et al., 2019)
Decorin	Osteoblast	–Promote collagen fibrillogenesis –Promote bone formation	(Coulson-Thomas et al., 2015)
Keratocan	Osteoblast	–Promote mineral deposition rates	
Asporin	Articular cartilage or periodontal tissue	–Promote collagen mineralization	(Kalamajski et al., 2009)
γ-carboxyglutamic acid-containing proteins			
Osteocalcin	Osteoblast	–Regulate calcium metabolism –Indicate bone formation	(Mizokami et al., 2017)
Matrix Gla Protein *(MGP)*	Osteoblast, osteocyte, and chondrocyte	–Inhibit bone formation and mineralization	(Kaipatur et al., 2008)
Periostin	Osteoblast and precursor cells	–Regulate collagen fibrillogenesis -Maintain bone strength	(Wen et al., 2018)
Glycoproteins			
Osteonectin	Osteoblast	-Promote bone formation and mineralization -Regulate collagen fibrillogenesis	(Rosset and Bradshaw, 2016)
		-Maintain biomechanical properties	(Delany et al., 2000)
Thrombospondins	Osteoblast	-Promote bone formation -Regulate collagen fibrillogenesis	(Delany and Hankenson, 2009)
R-spondins	Bone	-Promoter Wnt/β-catenin signaling -Regulate bone development	(Shi et al., 2017)
Small integrin-binding ligand *N*-linked glycoproteins/SIBLINGs			
BSP	Mineralized tissues	–Promote bone formation and mineralization	(Marinovich et al., 2016)
OPN	Osteoblast, odontoblast and osteocyte	–Promote bone formation and mineralization –Regulate bone remodeling	(Singh et al., 2018)
DMP1	Osteocyte and dentin	–Regulate phosphate metabolism –Promote bone mineralization	(Jani et al., 2016)
MEPE	Osteocyte and dentin	–Regulate phosphate metabolism –Promote bone mineralization	(Zelenchuk et al., 2015)
Inorganic ECM			
Hydroxyapatite	Bone	–Biomineralization	(Tavafoghi and Cerruti, 2016)

produced by a vitamin K-dependent post-translational modification. These proteins are mainly present in the serum, bone matrix, dentin, and other calcified tissues (Finkelman and Butler, 1985). The main Gla-containing proteins in the bone are osteocalcin (OCN), matrix Gla protein (MGP), and periostin (Wen et al., 2018). OCN is specifically expressed by bone-forming osteoblasts and contains three Gla residues, which give OCN the ability to bind calcium to modulate calcium metabolism by mediating its association with hydroxyapatite. The bone resorption process reduces OCN's affinity for hydroxyapatite, thereby enhance the release of OCN into circulation. Circulating OCN not only acts as a hormone that regulates glucose and energy metabolism, but its concentration in serum can be used as a biochemical indicator of bone formation (Mizokami et al., 2017). MGP is a 14-kDa extracellular protein that synthesized by osteoblasts, osteocytes, and chondrocytes in the bone. MGP-deficient mice have reportedly exhibited premature bone mineralization, while mice with MGP overexpression in osteoblasts showed reduced mineralization of intramembranous bone and hypomineralized tooth dentin and cementum (Luo et al., 1997; Kaipatur et al., 2008). Obviously, MGP is responsible for disrupting bone formation and inhibiting mineralization.

Except for OCN and MGP, periostin is another abundantly expressed Gla-containing protein in bone. Periostin is mainly secreted by osteoblasts and their precursor cells in long bones and is also found in other organs, such as the heart (Wen et al., 2018). Structurally, periostin comprises four domains, a signal sequence, a cysteine-rich emilin-like (EMI) domain, four repetitive and conserved FAS-1 domains, and a variable hydrophilic C-terminal domain, each of which provides different functions, such as FAS-1 providing cell adhesion ability (Merle and Garnero, 2012). As an adhesion molecule, periostin promotes aggregation, adhesion, proliferation, and

differentiation of osteoblasts by binding to cell surface receptors. Moreover, periostin participates in collagen folding and fibrillogenesis, which is essential for matrix assembly and further maintains bone strength (Wen et al., 2018).

Glycoproteins

Glycoproteins contain covalently attached carbohydrate molecules on the protein chain in various combinations and positions. Of glycoprotein in the bone matrix, osteonectin, also known as secreted protein acidic and rich in cysteine (SPARC), is a common representative. It is present in mineralized tissues and highly expressed in osteoblasts of bone. Osteonectin is a vital regulator of the calcium release by binding collagen and HA crystals, thereby influencing the mineralization of collagen during bone formation (Rosset and Bradshaw, 2016). With experiments *in vivo*, Delany et al. (2000) demonstrates that osteonectin-null mice had lower total collagen I content, bone mineral density and numbers of osteoblasts and osteoclasts in bone, and exhibited reduced biomechanical properties. Thus, osteonectin takes part in regulating bone remodeling and maintaining bone mass. Thrombospondins (TSPs), which are classified as TSP1 through TSP5, are present in developing skeleton and bone and is expressed by osteoblasts. In mice, global knockout of TSP-1, -3, and -5 can cause severe abnormalities in skeletal development (Delany and Hankenson, 2009). Moreover, TSP1-null mice show the increased bone mass and cortical bone size, and the differentiation of osteoblast is promoted, which is partly by activating latent TGF-β (Amend et al., 2015). TSP2-null mice have enhanced cortical bone density and osteoprogenitor numbers, combined with the abnormality of collagen fibrillogenesis (Hankenson et al., 2000). These indicate that TSPs play a critical role in bone cell differentiation and maintaining bone mass. R-spondins (roof plate-specific spondin) are a group of four secreted homologous glycoproteins (Rspo1-4) that belong to thrombospondin repeat containing matricellular protein family. They are widely expressed at different stages of skeletal tissue and act as a reinforcer of the Wnt/β-catenin signaling pathway through leucine-rich repeat-containing G-protein-coupled receptors 4, 5, and 6 (Lgr4/5/6). In bone tissue, R-spondins are identified as regulators of the skeleton that control embryonic bone development and adult bone remodeling (Shi et al., 2017).

Small Integrin-Binding Ligand N-Linked Glycoproteins/ SIBLINGs

SIBLINGs are a family of glycophosphoproteins that includes bone sialoprotein (BSP), osteopontin (OPN), dentin matrix protein-1 (DMP1), dentin sialophosphoprotein (DSPP), and matrix extracellular phosphoglycoprotein (MEPE). These proteins are predominantly found in mature, mineralized tissues, such as dentin and bone (Bellahcene et al., 2008).

BSP is a highly glycosylated noncollagenous phosphoprotein, that is expressed at the beginning of hard connective tissue mineralization. As a result of the deletion of BSP in mice, cementum deposition is significantly reduced, and long bone length and cortical thickness, the rate of bone formation are also reduced. Thus, BSP is vital in the regulation of osteoblast differentiation and initiation of matrix mineralization in bone tissue (Marinovich et al., 2016). Like BSP, OPN is a major regulator of bone formation, mineralization, especially in bone turnover. It is highly expressed by osteoblasts, odontoblasts, and osteocytes. OPN is abundant in serine-, acidic, and aspartate-rich motif, which are potential phosphorylation sites involved in inhibiting mineralization. In bone remodeling, OPN regulates osteoclastogenesis and osteoclast activity, which contributes to bone formation and resorption (Singh et al., 2018).

DMP1 and MEPE are mainly produced by fully differentiated osteoblasts in bone, and also expressed by pulp cells and odontoblasts. DSPP is important for the mineralization of tooth dentin, and is consequently abundant in dentin tissue (Bouleftour et al., 2019). Mice lacking DMP1 show severe bone defects, displaying increased serum fibroblast growth factor 23 (FGF23) and decreased serum phosphorus, as well as deformed and low-mineralized bone (Jani et al., 2016). Knockout of MEPE in mice increases bone mass and trabecular density and shows abnormal cancellous bone. Moreover, MEPE interacts with DMP1 and PHEX to affect FGF23 expression, thereby regulating phosphate, mineralization, and bone turnover (Zelenchuk et al., 2015). DMP1 and MEPE, thus, appear as key regulators of matrix mineralization and phosphate metabolism.

Inorganic ECM

The main inorganic constituent of hard tissues, such as bone and dentine, is hydroxyapatite (HA, $Ca_5(PO_4)_3OH$) (Ramesh et al., 2018). The deposition of HA occurs through the process called biomineralization. Interactions between minerals and matrix in teeth and bones, such as amino acids present in non-collagenous proteins, control HA formation. Collagen is produced during the mineralization of tissue and acts as a template for the deposition of HA (Tavafoghi and Cerruti, 2016). Due to the significant chemical and physical resemblance of HA to the mineral constituents of human bones and teeth, it is both biocompatible and osteoconductive. Consequently, HA is widely used for coatings on metallic implants, bone fillings, and injectable bone substitutes (Ramesh et al., 2018).

FUNCTION OF THE BONE ECM IN OSTEOBLAST-LINEAGE BIOLOGY

Osteoblast-lineage cells are bone-forming cells in bone remodeling. Osteoblasts develop from multipotent mesenchymal stem cells (MSCs), which can be isolated from the bone marrow or other tissues. The osteogenic differentiation of MSCs can be divided into four steps: (i) the commitment step produces lineage-specific progenitor cells; (ii) the proliferative phase of osteoprogenitors, in which genes associated with the cell cycle and histone signals are expressed; (iii) the phase of ECM secretion and morphological changes of immature osteoblasts; (iv) osteoid mineralization initiated by mature osteoblasts, which become terminally differentiated osteocytes (Paiva and Granjeiro, 2017). MSCs, osteoblasts, and osteocytes sense mechanical and biochemical signals from the ECM and

respond to these signals by regulating their fate (Assis-Ribas et al., 2018).

Regulation of BMSCs by the ECM

BMSCs are capable of migration, proliferation, differentiation, and cell-cell communication. Moreover, they can synthesize copious amounts of extracellular matrix proteins such as collagen type IIIα1 and Vα1, α5 and β5 integrin chains, fibronectin, connective tissue growth factor, and transforming growth factor beta I (TGFβI) (Ren et al., 2011). These are considered to be important for MSC homing and fate determination, such as adhesion, expansion, and spreading, through integrin receptors.

As an osteoblastic agent, TGFβ is coupled to the bone ECMs and moderately regulates the differentiation of early BMSCs into matrix-producing osteoblasts and osteocyte. Biglycan is can regulate the biological activity of TGF-β as well as matrix organization by binding to collagen type I. It has been reported that BMSCs isolated from biglycan-KO mice produced low amounts of collagen type I and showed a reduced response to TGF-β. Moreover, the deficiency of biglycan disrupts the ability to produce BMSCs, and also attenuates it's normal metabolic activity. In addition, biglycan-KO mice show the low activity of alkaline phosphatase (ALP)-positive MSCs, possibly due to apoptosis, which leads to a decrease of proliferation (Chen et al., 2002). In mice lacking biglycan and decorin (another member of the SLRP family), high concentrations of TGF-β activate downstream signaling pathways that stop the proliferation and induce the apoptosis of BMSCs. Therefore, decorin and biglycan mediate the proper sequestration of TGF-β and play a vital role in regulating the survival and growth of BMSCs (Bi et al., 2005).

Besides proteoglycans, glycoprotein TSP1 is also a major regulator of TGF-β activation and critical for regulation of the behaviors of MSCs inside the adult bone marrow niche microenvironment. In MSCs, TSP1 inhibits MSCs osteogenesis with decreased expression of Runx2 and ALP expression. This inhibition is due to latent TGF-β activation in MSCs, since anti-TGF-β antibody increased ALP activity in the presence of TSP1 (Bailey Dubose et al., 2012). Furthermore, the TSP1 effect on MSC proliferation has been reported to be mediated by activation of endogenous TGFβ in a dose-dependent manner. By contrast, the proliferation of MSC is not affected by TSP2, which can't activate TGFβ (Belotti et al., 2016). Therefore, TGFβ acts as an intermediary of TSP1 activity on MSCs.

Type I collagen fibrils in bone ECM also modulate osteogenesis by binding with integrins of osteoblast progenitors, which leads to initiated osteoblast differentiation cascade through Runx2 transcriptional activation (Elango et al., 2019). Fibrillogenesis starts from the interaction between type I and type V collagen, and then forms linear fibril. SLRP and thrombospondins can regulate collagen assembly by interacting with collagen fibrils. In mice, deletion of TSP2 results in increased number and proliferation ability of MSC, and also characterized by delayed osteogenesis and increased adipogenesis (Hankenson et al., 2000). Deficiency of TSP2 inhibits the differentiation of primary MSCs into osteoblasts, accompanied by decreased matrix collagen content and disrupted type I collagen assemble process (Alford et al., 2013). These results suggest that, unlike TSP1, TSP2 may act as an inhibitor of MSCs proliferation and a promoter of differentiation by regulating the mechanism of collagen fibrillogenesis.

Other ECM molecules, such as OPN, OCN, and DMP1, can regulate the proliferation of MSCs and osteogenesis. OPN increases the proliferation capacity of MSCs in a dose-dependent manner. On the other hand, OCN promotes the differentiation of MSCs into osteoblasts, with the increase of extracellular calcium levels, ALP activity, and the mRNA expression of OPN and OCN (Carvalho et al., 2019a). Numerous studies find that cytoskeleton and chromatin organization can affect cell migration. Liu and colleagues indicate that F-actin cytoskeleton and chromatin structure organized by EZH2-mediated H3K27me3 involves OPN-induced MSCs migration (Liu et al., 2018; Liu et al., 2019). In addition to stimulating the maturation of osteoblasts and osteocytes, DMP1 can also affect the pluripotency of MSCs. When DMP1 is removed, MSCs increasingly differentiate into osteogenic cells and bone mass, suggesting that it is a negative regulator of MSC differentiation (Zhang S. F. et al., 2018). Taken together, ECM that participates in bone formation and mineralization also significantly contributes to the growth, survival, and differentiation of MSCs (**Table 2**).

Regulation of Osteoblasts by the ECM

Immature and mature osteoblasts are the intermediate cells during MSCs osteogenesis. It continues the process of differentiation, along with the secretion of ECM and osteoid mineralization. Osteoblasts require a surface to synthesize new matrix, which is provided by collagen. If there is no substrate, osteoblasts synthesize a matrix that is only organized in the short range. Thus, this organized surface is used by osteoblasts to deposit mechanically stable and correctly structured bone tissue (Kerschnitzki et al., 2011). Different structures composed of type I collagen have different effects on the behavior of osteoblasts. In contrast to soluble and fibrillar forms, denatured forms of type I collagen inhibit the proliferation of osteoblast-like cells and can stimulate osteoblastic differentiation (Tsai et al., 2010). A small amount of type III collagen is also found in collagen fibrils of bone. Type III collagen null mice show affected osteoblast differentiation, consistent with decreased ALP activity, reduced osteogenic markers (OCN and BSP), and mineralization capacity (Volk et al., 2014). Therefore, collagen acts as a tissue scaffold, providing a matrix for anchoring cells and regulating the growth and osteogenic properties of osteoblasts.

Part of ECM protein not only regulates collagen fibrillogenesis but is required for osteoblast lineage progression, which ultimately affects mineralization. The contributions of osteonectin, keratocan, TSP1, and TSP2 to collagen fibrillogenesis have been extensively reported. In terms of influencing the maturation and function of osteoblasts, osteonectin and keratocan-null mice show fewer osteoblasts and decreased mineralized nodules in mutant cells (Igwe et al., 2011; Rosset and Bradshaw, 2016). TSP1 inhibits the mineralization of osteoblast *in vitro* and *in vivo* (Ueno et al.,

TABLE 2 | Function of the bone ECM in MSCs.

Bone ECM	Functions in MSCs	Mechanism	Cell/Mice model	Reference
Biglycan	BMSCs production and proliferation (+)	Regulate amounts of collagen type I and response to TGF-β	Biglycan$^{-/-}$ mice	(Chen et al., 2002)
Biglycan and Decorin	BMSCs survival and growth (+)	Regulate response to TGF-β	Biglycan Decorin DKO mice	(Bi et al., 2005)
TSP1	MSC osteogenesis (–); MSC proliferation (+)	Latent TGF-β activation	MSCs	(Bailey Dubose et al., 2012; Belotti et al., 2016)
TSP2	MSC number and proliferation ability (–); MSC osteogenesis (+)	Regulate collagen fibrillogenesis	TSP2$^{-/-}$ mice	(Hankenson et al., 2000; Alford et al., 2013)
OPN	MSC proliferation capacity (+); MSCs migration (+)	Regulate F-actin cytoskeleton and chromatin structure	MSCs	(Carvalho et al., 2019a; Liu et al., 2019)
OCN	MSC osteogenesis (+)	Increase extracellular calcium and ALP	MSCs	(Carvalho et al., 2019a)
DMP1	MSCs pluripotency (+); MSC osteogenesis (–)	–	*Prx1-cre*; *DMP1$^{fl/fl}$* mice	(Zhang S.F. et al., 2018)

DKO, double knockout.

2006). However, TSP2 promotes osteoblast mineralization by promoting the organization of osteoblast-derived ECM (Alford et al., 2010). Collectively, those proteins mediate the mineralization of osteoblasts through regulating collagen fibrillogenesis to some extent.

ECM molecules BSP and OPN are two SIBLINGs that contribute to the regulation of osteoblasts. BSP is crucial for the synthesis of the ECM and HA nucleation activity. It can promote osteoblast differentiation and enhance early bone mineralization to produce new bone *in vivo*. Especially the RGD sequence of BSP, which mediates the osteoblast behaviors by FAK and other extracellular kinases (Holm et al., 2015). By contrast, OPN can inhibit the process of osteoblast osteogenesis through inhibition of BMP-2, and act as a mineralization inhibitor of osteoblast in a phosphate-dependent manner (Huang et al., 2004; Singh et al., 2018). Consistent with that of OPN, OCN, which is produced by osteoblast, is considered as an inhibitor of bone mineralization. Osteocalcin null mice show larger HA crystal size, suggesting that osteocalcin may regulate the maturation rate of minerals (Zoch et al., 2016).

The Wnt pathway is an important regulatory for bone formation. Three ECM molecules, MGP, R-spondin2, and periostin, have been identified to modulate the mineralization of osteoblast through Wnt signaling. Knockdown of MGP inhibits the differentiation and mineralization of osteoblasts *via* up-regulating Wnt/β-catenin signaling pathway. Consistent with the results of *in vivo* experiment that overexpression of MGP inhibits the decreased bone mineral density induced by ovariectomy (Zhang J. et al., 2019). As a wnt agonist, R-spondin2 is abundantly expressed in pre-osteoblasts stimulated by Wnt. R-spondin2 promotes osteoblastogenesis *in vitro* and bone mass *in vivo*, supporting its vital role in osteoblastogenesis and bone development (Knight et al., 2018). Sclerostin is an important inhibitor of WNT/β-catenin signaling and regulates osteoblast matrix generation. It has been reported that periostin may interact directly with sclerostin and promotes Wnt signaling inhibited by sclerostin (Bonnet et al., 2016). Moreover, periostin can also affect osteoblast differentiation and bone formation, suggesting that periostin is involved in bone anabolism by regulating Wnt/β-catenin signaling (Merle and Garnero, 2012) (**Table 3**).

Regulation of Osteocytes by the ECM

Osteocytes are the terminally differentiated immobilized cells in the bone matrix. Although embedded in the bone matrix, osteocytes form contacts with each other and with bone lining cells, which aid bone growth and repair.

The bone matrix present around the intricate lacuno-canalicular network of osteocytes is continuously being resorbed and deposited in a process called perilacunar/canalicular remodeling (Dole et al., 2017). Changes in the overall formation rate of the canalicular network increase osteoblast activity and bone formation. Recently, it is demonstrated that the process by which osteocytes push type I collagen fibers outward from the center of the formed lacuna mediates osteocytes lacunae formation, which is accompanied by increased collagen deposition and collagen-fiber network compaction surround the lacunae. Therefore, the dynamic assembly of bone collagen contributes greatly to the encapsulation and mineralization of osteocytes in bone matrix (Shiflett et al., 2019).

Osteocytes can sense and respond to external mechanical cues. The stiffness of the surrounding matrix is one of the most important signals that regulate osteocyte behaviors, and changes in the stiffness of the ECM induce alterations in the cytoskeleton and cell morphology, as well as fibronectin, which leads to changes in paxillin and in turn affects the elongation of osteocyte gap junctions (Zhang D. M. et al., 2018). As osteocytes begin to expand processes and start mineralizing the neighboring matrix, the expression of DMP1 and MEPE is upregulated. The stiffness of the ECM, and especially that of the collagen-based substrates, affects DMP1 expression. The levels of DMP1 and Sclerostin are greatly increased on collagen-based substrates with low stiffness, indicating enhanced osteocyte differentiation compared to ECM substrates with high stiffness (Mullen et al., 2013). Changes of DMP1 levels mediate the sensing of mechanical stimuli by osteocytes, which may increase the attachment of osteocytes

TABLE 3 | Function of the bone ECM in osteoblasts.

Bone ECM	Functions in osteoblasts	Mechanism	Cell/Mice model	Reference
Type I collagen	Osteoblast proliferation (–) Osteogenesis (+)	Denatured forms of collagen	MG63 cells	(Tsai et al., 2010)
Type III collagen	Osteogenesis and mineralization (+)	Regulate type I collagen, BSP, and OCN	$Col3^{-/-}$ mice	(Volk et al., 2014)
Osteonectin	Osteoblast number and differentiation (+); bone formation (+)	Regulate collagen fibrillogenesis	$Osteonectin^{-/-}$ mice	(Rosset and Bradshaw, 2016)
Keratocan	Osteoblast number and differentiation (+); bone formation (+)	Regulate collagen fibrillogenesis	$Keratocan^{-/-}$ mice	(Igwe et al., 2011)
TSP1	Osteoblast mineralization (–)	Regulate collagen fibrillogenesis	MC3T3-E1 cells	(Ueno et al., 2006)
TSP2	Osteoblast mineralization (+)	Organization of osteoblast-derived ECM	MC3T3-E1 cells	(Alford et al., 2010)
BSP	Osteoblast differentiation and early bone mineralization (+)	FAK and other extracellular kinases	$BSP^{-/-}$ mice	(Holm et al., 2015)
OPN	Osteoblast osteogenesis and mineralization (–)	BMP-2, phosphate-dependent manner	MC3T3-E1 cells	(Huang et al., 2004; Singh et al., 2018)
OCN	Osteoblast mineralization (–)	–	$OCN^{-/-}$ mice	(Zoch et al., 2016)
MGP	Osteoblast differentiation and mineralization (+)	Wnt/β-catenin signaling pathway	MG63 cells	(Zhang J. et al., 2019)
R-spondin2	Osteoblast differentiation (+)	Wnt/β-catenin signaling pathway	*Ocn-Cre*; $Rspo2^{fl/fl}$ mice	(Knight et al., 2018)
Periostin	Osteoblast differentiation and bone formation (+)	Wnt/β-catenin signaling pathway		(Merle and Garnero, 2012)

and remodeling of the matrix present inside the local microenvironment (Gluhak-Heinrich et al., 2003). In addition, DMP1 also inhibits the apoptosis of osteocytes, enhances bone mineralization, and prevents the disintegration of the osteocyte network (Dussold et al., 2019). MEPE is synchronized with DMP1 and differentially regulates bone remodeling by mechanical loading. MEPE knockout mice show increased bone mass, accompanied by suppressed mineralization, suggesting that both DMP1 and MEPE can regulate the mineralization in osteocytes and lacunar wall (Gluhak-Heinrich et al., 2007) (**Table 4**).

FUNCTION OF THE BONE ECM IN OSTEOCLASTS

Osteoclasts, are multinucleated cells formed from the fusion and differentiation of monocyte/macrophage precursors, involve in bone resorption. The formation and activity of osteoclasts activated by macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor κB (NF-κB) ligand (RANKL), which are derived from osteoblasts (Lin et al., 2019).

Upon osteoclast formation, TSP1, TSP2, MGP, and biglycan regulate osteoclast differentiation and resorption activity in different regulatory mechanisms. Both TSP1 and TSP2 are key positive regulators in osteoclast differentiation. TSP1 functions in the early stage of osteoclastogenesis, and TSP1 deficiency mice show decreased differentiation and activity of osteoclast. This is caused by increased inducible nitric oxide synthase (iNOS) (Amend et al., 2015). However, TSP2 induces osteoclastogenesis through NFATc1, which is a RANKL-dependent pathway, accompanied by an increased RANKL/OPG ratio (Wang et al., 2019). In contrast, MGP suppresses the nuclear translocation of NFATc1 and intracellular Ca^{2+} flux in osteoclasts, which in turns attenuate the differentiation and bone resorption. MGP also inhibits bone formation and MGP-null mice exhibit an osteopenic phenotype, suggesting that MGP plays a stronger role in bone absorption than in bone formation (Zhang Y. et al., 2019). With the same regulation mechanism as MGP, type I collagen can also act as an inhibitor of bone development by osteoclasts. The formation of osteoclasts can be suppressed by full length or 30–75 kDa fragments of type I collagen, which binds with the collagen receptor LAIR-1 and thereby maintaining bone strength (Boraschi-Diaz et al., 2018). TNFα has been shown to regulate osteoclast differentiation and survival in a RANKL-independent manner. In biglycan and fibromodulin double knockout mice, osteoclasts possess higher differentiation potential and surround with increased TNFα and RANKL cytokine. Exogenous biglycan or fibromodulin weakens the ability of osteoclast precursors to form TRAP-positive multinucleated cells. Therefore, biglycan alone or coupled with

TABLE 4 | Function of the bone ECM in osteocytes.

Bone ECM	Functions in osteocytes	Mechanism	Cell/Mice model	Reference
Type I collagen	Osteocyte mineralization (+)	Collagen deposition and collagen-fiber network compaction	GFP-$col^{+/-}$/Dmp1-$Cre^{+/-}$/tdTomato$^{+/-}$ mice	(Shiflett et al., 2019)
DMP1	Osteocyte attachment (+); Osteocyte apoptosis (–)	External mechanical force	$Col4a3^{-/-}$ mice	(Gluhak-Heinrich et al., 2003; Dussold et al., 2019)
MEPE	Osteocyte mineralization (+)	External mechanical force	$MEPE^{-/-}$ mice	(Gluhak-Heinrich et al., 2007)

fibromodulin regulates osteclastogenesis through TNFα and/or RANKL to control bone mass (Kram et al., 2017).

The RGD sequence of OPN and BSP interact with αvβ3 integrin initiate osteoclast adhesion to bone matrix and formation of actin ring of polarized osteoclasts, which is crucial for bone development. Integrin-matrix combination is vital for podosome formation on osteoclasts. Thus, OPN plays a major role in osteoclast activity and sealing zone formation of osteoclasts (Singh et al., 2018). Moreover, OPN can be secreted by human osteoclasts in addition to osteoblast during bone resorption, which can be used as a chemokine for subsequent bone formation and resorption (Luukkonen et al., 2019). In addition, osteoclast surfaces and the number of osteoclasts are decreased in BSP knockout mice. BSP can promote bone resorption, and the migration of preosteoclast and mature osteoclasts is impaired in the absence of BSP (Boudiffa et al., 2010). OPN and BSP can act as a network to coordinate the function of osteoclasts. Osteoclasts derived from OPN and BSP double knockout mice exhibit higher number and resorption activity. The interaction between OPN/BSP and αVβ3 integrin may participate in determining osteoclast adhesion to bone matrix surface and subsequent resorption (Bouleftour et al., 2019) (**Table 5**).

APPLICATION OF THE ECM FOR BONE TISSUE ENGINEERING

Tissue engineering utilizes the basic principles and methods of life sciences and engineering to create functional tissue substitutes *in vitro*, which can be used to repair tissue defects and replace the partial or total loss of organ function (Shafiee and Atala, 2017). Tissue-engineering strategies rely on three basic elements—seed cells, scaffolds, and cytokines—which interact to produce engineered tissue constructs (Hu, 1992). Most tissue engineering approaches rely on renewable seed cells, such as stem cells, to restore damaged sites. The production of large amounts of growth factors and ECM components during the proliferation of seed cells increases the flexibility of the scaffold and promotes the proliferation and differentiation of autologous progenitor cells, thereby further enhancing tissue repair. Furthermore, cytokines bind to receptors on the cell surface, which transmit extracellular signals to the cell interior to regulate cell proliferation and differentiation, or enhance the formation of the ECM (Zhang et al., 2016). The scaffold provides an appropriate three-dimensional (3D) structure that guides the growth of seed cells to achieve correct tissue remodeling. Ideal scaffolds must have good biocompatibility, biodegradability, biomechanical properties, permeability, surface characteristics, and must not promote immune rejection (Yi et al., 2017).

In recent years, bone tissue engineering has developed rapidly, providing a promising new approach for bone repair. However, due to the complex anatomical structure of bone and the high mechanical stress that the engineered tissue must withstand *in vivo*, bone tissue regeneration remains one of the major challenges of tissue engineering (Vieira et al., 2017). Bone grafts can be used to stimulate or increase the formation of new bone around fractures or surgical implants, as well as to regenerate or replace the bone lost due to infection, trauma, or disease (Polo-Corrales et al., 2014). The ideal scaffold should also promote the attachment, increase the viability and proliferation, as well as induce osteogenic differentiation and angiogenesis. Finally, the material must be able to gradually integrate with the host tissue and bear the same load (Roseti et al., 2017). Bone scaffolds are usually made of biodegradable materials that are porous and effectively integrate seed cells, growth factors, and drugs, as well as provide mechanical support during the repair and regeneration of the damaged bone (Bose et al., 2012).

With the rapid development of regenerative medicine, the ECM has gained attention as the fourth element in the development of bone tissue engineering (Ravindran et al., 2012) (**Figure 1**). The ECM acts as a physical scaffold and substrate for cell adhesion, delivering biochemical and biomechanical signals for cells to initiate migration, differentiation, morphogenesis, and homeostasis (Yi et al., 2017).

TABLE 5 | Function of the bone ECM in osteoclasts.

Bone ECM	Functions in osteoclasts	Mechanism	Cell/Mice model	Reference
TSP1	Osteoclast differentiation and activity (+)	Decrease inducible nitric oxide synthase (iNOS)	$TSP1^{-/-}$ mice	(Amend et al., 2015)
TSP2	Osteoclastogenesis (+)	Transactivation of NFATc1; Increase RANKL/OPG ratio	RAW 264.7 cells	(Wang et al., 2019)
MGP	Osteoclast differentiation and bone resorption (–)	Suppress the nuclear translocation of NFATc1 and intracellular Ca^{2+} flux	$MGP^{-/-}$ mice	(Zhang Y. et al., 2019)
Type I collagen	Osteoclast formation (–)	Bind with the collagen receptor LAIR-1	Primary BMMs	(Boraschi-Diaz et al., 2018)
Biglycan	Osteoclast precursors differentiation (–)	Decrease TNFα and RANKL cytokine	Biglycan Fibromodulin DKO mice	(Kram et al., 2017)
OPN	Osteoclast activity and sealing zone formation (+)	RGD sequence interact with αvβ3 integrin	Primary BMMs	(Singh et al., 2018)
BSP	Osteoclast surface, number, migration and bone resorption (+)	RGD sequence interact with αvβ3 integrin	$BSP^{-/-}$ mice $BSP^{-/-}$ preosteoclast	(Boudiffa et al., 2010)
OPN and BSP	Osteoclast number and bone resorption (+)	RGD sequence interact with αvβ3 integrin	OPN BSP DKO mice	(Bouleftour et al., 2019)

DKO, double knockout.

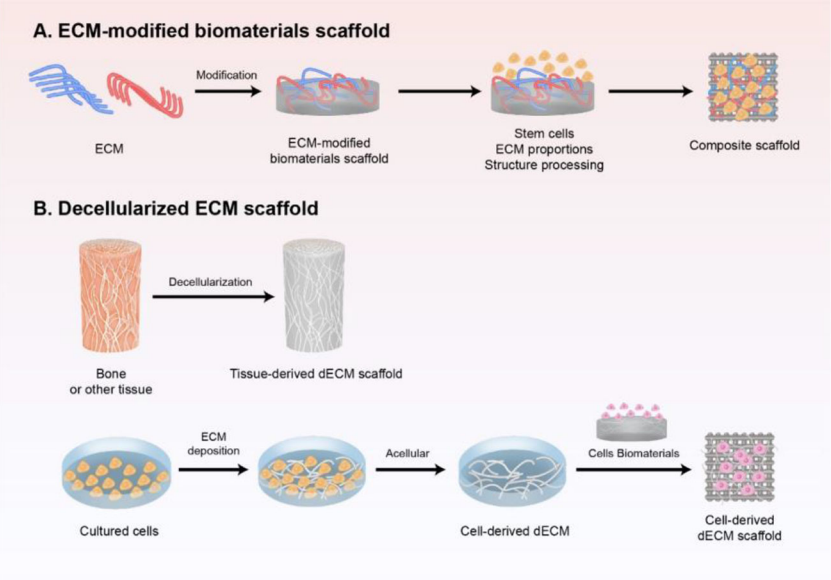

FIGURE 1 | Schematic preparation of ECM-based scaffold in bone regeneration. **(A)** ECM-modified biomaterials scaffold. Different components and contents of ECM modified with biomaterial-based scaffold, and further modified with stem cells and structure processing to mimic the natural biomaterials. **(B)** decellularized ECM scaffold obtained either from tissue *in vivo* or cultured cells *in vitro* by decellularization, which is a promising strategy to induce bone regeneration and has good clinical performance.

ECM-Modified Biomaterial Scaffold

Extracellular matrix components such as collagen, HA, and fibronectin are commonly used as natural biomaterials for the preparation of scaffolds. ECM itself or modified with biomaterial-based scaffold is used in biological scaffolds to mimic the natural biomaterials. Because a single bone ECM component cannot generally simulate the complex osteogenic microenvironment, two or more materials are used to generate a composite that can produce a synergistic effect.

ECM act as a surface coating material on absorbable polymers and is increasingly being used to manufacture biodegradable scaffolds for bone reconstruction materials. Rentsch et al. constructed polycaprolactone-co-lactide (PCL) scaffolds coated with 3D collagen I/chondroitin sulfate (Coll I/CS) to repair rabbit calvarial bone defects. Compared with PCL scaffolds, more new bone was formed in the central defect of the Coll/CS coated PCL group, and it was more evenly distributed in the scaffolds after 6 months following implantation (Rentsch et al., 2014). In addition, titanium (Ti) was coated with Col1 and implanted into the femoral condyles of osteopenic rats to evaluate the osteointegration, the total bone ingrowth of the TiColl material following ovariectomy increased significantly from 4 to 12 weeks after implantation, compared with Ti alone (Sartori et al., 2015). Interestingly, the osteogenic potential of hydroxyapatite/β-tricalcium phosphate (HA/β-TCP) was improved by surface immobilization of MEPE peptide. The HA/β-TCP with the MEPE peptide stimulated bone regeneration in a mouse calvarial defect model compared to unmodified HA/β-TCP. Newly formed bones undergo physiological remodeling mediated by osteoclasts (Acharya et al., 2012). Therefore, due to the special structure and function of ECM, it might be beneficial for the biopolymer scaffold to perform signal connection and conduction with cells, improve the osteoconduction and osteointegration, and guide cell growth and tissue remodeling.

As an important ECM component of natural bone tissue, HA has also been used in materials for bone regeneration and bone repairs, such as bone fillings and injectable bone substitutes. A HA modified PCL/HA composite had better biocompatibility for hMSCs cells with higher proliferation and osteogenic potential, compared to neat PCL. Whereby the efficiency of attachment between hMSCs and the PCL/HA scaffold was improved with a higher HA content of 5% to 10% and in a HA concentration-dependent manner (Kumar et al., 2017). This means that in addition to the different components of modified ECM to affect the cell behaviors in bone regeneration, different ECM contents also play different roles.

In bone tissue engineering, biological scaffolds are required not only to have components similar to natural bone, but also to have similar structural properties. A collagen-apatite (Col-Ap) nanocomposite that emulates bone-like subfibrillar nanostructures was constructed to mimic natural bone. The Col-Ap nanocomposite scaffold was able to activate bone-forming cells, promote inward vascularization, as well as induce the synthesis of the ECM mediated by increased TGFβ1. (Liu et al., 2016). In addition, Haj et al. demonstrated that nanofibrous HA/chitosan (nHAp/CTS) scaffolds seeded with MSCs were superior to membranous HAp/CTS in a rat

model of cranial bone defect regeneration. The MSCs in the nanofibrous scaffold activated the integrin-BMP/Smad signaling, leading to higher proliferation and ALP activity (Liu et al., 2013). Similar to nanofibrous HA scaffold, Shamaz et al. obtained electrospun microfibrous sheets by combining layers of a microfibrous mat composed of electrospun poly(L-lactic acid) (PLLA), gelatin–nanoHA matrix (GHA), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide called GHA-MF_E. When human adipose-derived MSCs (hADMSCs) were grown on this GHA-MF_E scaffold, they displayed higher ALP activity *in vitro*. Moreover, the GHA-MF_E fiber scaffolds significantly increased the rate of new bone formation in rabbit femoral cortical bone defect after 4 weeks of implantation compared with commercial Surgiwear™ (Shamaz et al., 2015). Obviously, the surface morphology and overall topology of ECM in scaffolds are significantly involved in determining their capacity for cell loading and growth in bone tissue engineering.

Stem cells are receiving increasing attention in regenerative medicine, including bone regeneration. Because of their good proliferation ability and capacity for osteogenic differentiation. On the other hand, stem cells are capable of synthesizing an ECM that can accelerate calcification and repair, thereby restoring the function of damaged bones (Clough et al., 2015; Gao et al., 2017). Chamieh et al. treated critical-size calvarial defects in rats using human dental pulp stem cells (DPSCs) seeded onto collagen gel scaffolds. Compared to untreated defects, the scaffolds containing DPSCs significantly promoted the formation of correctly structured new bone and increased the volume of fibrous connective tissue and mineralized tissue, which was accompanied by the increased expression of osteogenic ALP and type I collagen (Chamieh et al., 2016). When MSCs on laminated HA nanoparticle (nHA)/poly-hydroxybutyrate (PHB) (nHA/PHB) were co-implanted, it resulted in improved promoted the formation of osteoid tissue and ECM, with ingrowth of blood vessels into the graft two months after subcutaneous implantation on the dorsal site of mice model (Chen et al., 2017). Moreover, MSCs derived from induced pluripotent stem cells (iPSC-MSCs) combined with HAp/Col/CTS nanofibers also had a good bone regeneration ability in mice cranial defects, with almost 2-fold higher bone density than either TCP, CTS or HAp/CTS scaffolds. This might due to increased secretion of Alp and Col (Xie et al., 2016). On account of the synergistic effect of stem cells and ECM, the stem cells/ECM composite scaffolds are more conducive to bone remodeling than ECM modified scaffolds. Besides stem cells, endothelial cells (ECs) that contribute to vascularization can provide adequate nutritional support for the scaffold. Osteogenic differentiated MSCs (OMSCs) and ECs were seeded into a nano-HA/polyurethane (n-HA/PU) scaffold at a ratio of 0.5/1.5, was more effective for bone repair in rat condylar femoral defects than OMSC scaffold and scaffold alone. Therefore, ECs in OMSC/EC-scaffold plays an important role in bone formation and vascularization (Li et al., 2019).

In the clinical study, the absorbable collagen sponge scaffold contains bone-stimulating agents, such as rhBMP-2, rhBMP-7, and PRP, to treat long bone defects and fracture of the patient. The patients showed bony healing and new bone formation in the defect site (Govender et al., 2002; Calori et al., 2008). Except for collagen, controlled proportions of HA together with modified calcium phosphate, TCP, and ionic species to form Bonelike®, which can be used in non-critical bone defects treatment. Bonelike® has a similar chemical and structural composition of human bone. Bonelike® itself or combined with MSCs improved bone regeneration by promoting bone growth and vascularization in bone defect patients (Campos et al., 2019). Moreover, eggshell-derived nano-hydroxyapatite for bone transplantation has strong safety and can obtain good bone regeneration performance. In the third month after implantation in patients, bone graft showed increased bone density and complete healing (Kattimani et al., 2019). Therefore, the use of ECM-modified scaffold in bone regeneration is significantly better than standard treatment by reducing the frequency of secondary intervention, while reducing the infection rate in patients with an open bone defect.

Above all, different types, proportions, structures of ECM, and even different implanted cells can all affect the bone regeneration performance of the ECM-modified biomaterial scaffold, suggesting that there may be a set of elements of ECM that work in concert to guide bone regeneration. Moreover, it remains unknown how much each of these factors or the combination of these factors contributes to ECM in the scaffold. Further studies are still needed to fully reveal the multiple functions of ECM in the ECM-modified biomaterial scaffold during bone repair.

Decellularized ECM Scaffold

Although the ECM-modified biomaterial scaffold based on different compositions and ratios of bone ECM can improve bone defect repair, the complex matrix components and activities cannot be completely stimulated in biomimetic bone tissue. In addition, these artificial scaffolds lack specific cell niche and anatomical structures of target tissues, and cannot guarantee good integration of cellular and molecular cues (Zhang et al., 2016). Therefore, decellularized ECM scaffold obtained either from tissue *in vivo* or cultured cells *in vitro* is a promising strategy to induce bone regeneration and has a good clinical performance. It has the advantage of maintaining ECM components, providing the original geometry and flexibility of the tissue, while also offering inherently low immunogenicity (Hoshiba et al., 2016). The decellularized ECM provides mechanical support for the regenerating cells and affects both their migration and cell fate decision (Gallie et al., 1989).

Tissue-Derived Decellularized ECM Scaffold

Bone-derived decellularized ECM (dECM) can provide a native microenvironment containing ECM proteins, type I collagen, and growth factors including bone morphogenetic proteins. Kim et al. used dECM from porcine bone to form 3D-printed PCL/β-TCP/bone dECM scaffolds, which promoted more new bone regeneration 6 weeks after repair of a rabbit calvarial defect *in vivo*. Importantly, bone tissue developed into the interior of the scaffold. By contrast, bone tissue formed only at the edge of the

scaffold without dECM (Kim et al., 2018). A dECM derived from the porous growth plate (GP) was fabricated to repair critical-sized rat cranial defects. Higher levels of mineralized tissue and increased vascular volume were observed 8 weeks after implantation, which might be caused by reduced production of IL-1β and IL-8 and superior osteogenic capacity compared to native GP (Cunniffe et al., 2017). In addition, 3D ECM scaffold produced from decellularized periosteum promoted bone mineralization by controlling the size and direction of mineral crystals in rabbit bone defect regeneration, suggesting the crucial role of periosteum ECM in efficient healing of fractures and bone regeneration (Lin et al., 2018). In clinical, decellularized bone ECM from bovine trabecular bone discs with patient autogenous MSCs could treat distal tibia fracture. After 6 months, active bone formation can be detected in both callus and graft of the patient (Hesse et al., 2010). This means that native decellularized bone transplantation has a broad application prospect in orthopedic surgery.

A dECM produced from non-bone tissue can also be used in bone regeneration. Mohiuddin et al. demonstrated that a combination of decellularized adipose tissue (DAT) with adipose-derived stromal/stem cells (ASCs) is effective in the regenerative bone repair of mice critical-size femur defects. The group treated with the DAT hydrogel showed a higher deposition of OPN and collagen I as well as a higher bone area than the untreated group (Mohiuddin et al., 2019). Beyond that, porcine small intestinal submucosa (SIS) ECM was combined with true bone ceramic (TBC) and mineralized, to fabricate the tissue-derived ECM scaffold mSIS/TBC. This scaffold promoted the viability, proliferation, and osteogenesis of rat MSCs through the ERK1/2 and Smad1/5/8 signal pathways *in vitro*. Most importantly, bone formation in a rat critical size cranial defect model was greatly improved by the mSIS/TBC scaffold compared to a pure TBC scaffold (Sun et al., 2018). Taken together, the abundance of multiple ECM components in dECM from the tissue is an ideal biomaterial for bone tissue engineering.

Cell-Derived Decellularized ECM Scaffold

Autologous cells grown aseptically *in vitro* can be used to produce a cell-derived decellularized ECM avoiding the disadvantages of a tissue-derived decellularized ECM. ECM scaffolds derived from stem cells and bone cells can potentially better mimic the native bone microenvironment, thereby inducing bone regeneration (Sun et al., 2018). In vitro, adipose-derived stem cells (ASCs) on hMSCs derived decellularized ECM showed more osteogenic colonies, accompanied by increased expression of osteogenic markers (Zhang et al., 2015). dECM derived from co-cultured MSCs and HUVECs promoted the osteogenic and angiogenic potential of BMSCs. Moreover, the 1/3 ratio of MSCs/HUVECs has the best angiogenic effect on MSCs (Carvalho et al., 2019b). Cell-derived dECM, rich in collagen, matrix macromolecules, and growth factors, has good biocompatibility and biodegradability, making it beneficial for the proliferation and osteogenic differentiation of MSCs, and can be used as cell culture matrix for bone regeneration medicine.

In bone repair applications, cell-derived dECM combined with inorganic material to composite hybrid scaffolds, providing stronger osteoinductive properties and mechanical support. The implantation of osteogenic ECM sheets (OECMS) that retain the native collagen I and growth factors, together with HA, enhanced bone regeneration in a rat model of femoral non-union at 5 and 8 weeks. The OECMS contained TGF-β and BMP2, leading to increased osteoinduction and osteoconduction (Onishi et al., 2018). When a dECM derived from MG63 cells was deposited on a CS/PCL scaffold, hMSCs exhibited enhanced attachment, proliferation, and osteogenic differentiation, and the scaffold showed anti-inflammatory features *in vitro*. Moreover, the dECM-coated CS/PCL demonstrated a good bone regeneration ability after *in vivo* implantation in rat calvarial defects, which was associated with increased mineralized tissue (Wu et al., 2019). According to the characteristics of different biomaterials and the good osteoinduction of ECM, tissue-engineered grafts can be customized to overcome the limitations of autograft and allograft.

Beyond that, dECM scaffolds for bone repair can also be obtained from other, non-bone cells. A PLGA/PLA scaffold was coated with dECM form human lung fibroblasts (hFDM) in bone defect repair by delivering BMP-2. The dECM/PLGA/PLA scaffold significantly promoted new bone formation in a rat model of a calvarial bone defect. Notably, the addition of BMP-2 led to almost complete healing of bone defects (Kim et al., 2015). Mesenchymal stromal cells derived from human nasal inferior turbinate tissue (hTMSCs) were combined with a 3D-printed PCL/poly(lactic-co-glycolic acid) (PLGA)/β-TCP scaffold to form a mineralized ECM scaffold. The corresponding implants improved bone formation in ectopic and orthotopic rat models compared to the bare scaffold, in accord with the increased osteogenic differentiation of hTMSCs on 3D-printed hybrid scaffolds *in vitro* (Pati et al., 2015). Further development of 3D printing technology in ECM-based scaffolds is beneficial to the field of bone tissue engineering and regenerative medicine.

CONCLUSIONS AND PROSPECTS

Although natural bone grafts from autologous or allogeneic sources are the best choice for bone defect repair, their clinical applications are limited due to complications during surgery related to their sourcing. With the development of tissue engineering technology, biomaterials manufactured using materials engineering, nanotechnology, and 3D printing been used to develop novel implants for bone regeneration. However, many such novel materials suffer from shortcomings such as poor biocompatibility, low osteoinductivity, and high immunogenicity. ECM scaffolds have unique advantages in all these areas. Because they can better simulate the composition, distribution, and biochemical signals of various matrix components in native bone tissue, they can emulate the natural bone microenvironment. Consequently, such materials can effectively support bone regeneration and guide tissue reconstruction. Common ECM-modified scaffold designs use a

single or a combination of components of the ECM or apply a coating combined with biomaterials to produce scaffolds. Even when using decellularized preparations of autologous or allogeneic tissue or cells cultured *in vitro*, the integrity and mechanical properties of the matrix components are preserved, while achieving low immunogenicity by removing cell-bound antigens. Bone ECM has been demonstrated to enhance bone regeneration. Therefore, the application of the ECM-modified biomaterial scaffold and decellularized ECM scaffold has become a new frontier in tissue engineering and regenerative medicine.

Nevertheless, the clinical application of ECM-modified biomaterial scaffold or decellularized ECM scaffold in bone repair still faces many problems, such as the preservation of growth factors and biochemical signals in the ECM during decellularization, modification of the ECM, design, and processing of ECM scaffolds, and standardization and mass production for clinical studies. There are decellularization methods that retain the characteristics and functions of the ECM. However, due to the complexity and dynamics of its components, there has been no systematic analysis of the components of the ECM secreted by cells or tissues, and it is not clear if decellularized ECM can completely match the biochemical imprint of the native bone ECM. Therefore, the components and composition of decellularized ECM scaffolds, as well as the dynamic changes of ECM under different culture conditions should be further studied to make it more similar to the natural ECM composition. Additionally, it is difficult to precisely control the ECM components secreted by cells, so that they can be standardized and unified in mass production. Cells can be genetically modified to express specific products in a timely and quantitative manner, and appropriate bioreactors can be used to monitor cell growth and product secretion. Consequently, ECM release standards can be established to improve the quality of the graft. Finally, the ECM can be modified by adding growth factors and bioactive molecules during the preparation of ECM scaffolds to improve the effectiveness of bone defect repair. Therefore, the types and amounts of bioactive molecules need to be further studied. While additives can enhance the bone regeneration ability of the defect site, they must not affect the growth of other adjacent tissues at the graft site, hence avoiding inflammation and hyperplasia. In addition, ECM scaffolds can be combined with autologous pluripotent stem cells or organ-specific progenitor cells for a better therapeutic effect. Finally, the design and processing of ECM scaffolds can make them fill the defect site more accurately, offering better mechanical support and functional bionics. With the development of 3D printing technology in recent years, the ECM can be processed through biological printing to obtain scaffolds with various topology, such as porous and lamellar, or even scaffolds with a shape that exactly matches the defect site. Thus, the implant can be designed for improved bionic mechanical properties and stronger bone regeneration ability.

In conclusion, the application of ECM in bone formation and bone regeneration is full of opportunities and challenges. In the future, further studies on the cellular and molecular mechanisms the mediate the effects of the ECM on bone cells and bone repair will contribute to the further development of ECM-based scaffolds in bone tissue engineering.

AUTHOR CONTRIBUTIONS

XL and SP drafted the manuscript. AQ and Y-GG designed the project.

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Anisodamine Maintains the Stability of Intervertebral Disc Tissue by Inhibiting the Senescence of Nucleus Pulposus Cells and Degradation of Extracellular Matrix via Interleukin-6/Janus Kinases/Signal Transducer and Activator of Transcription 3 Pathway

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Objectives: Anisodamine (ANI) has been used to treat a variety of diseases. However, the study of ANI in intervertebral disc degeneration (IVDD) is unclear. This study investigated the effects of ANI on degenerative nucleus pulposus cells (NPCs) and IVDD rats, and its possible mechanisms.

Methods: Human nucleus pulposus cells (HNPCs) were treated with IL-1β (20 ng/ml) to simulate IVDD, and an IVDD rat model was constructed. IL-1β-induced HNPCs were treated with different concentrations (10, 20, or 40 μM) of ANI, and IVDD rats were also treated with ANI (1 mg/kg).

Results: ANI treatment significantly reduced the apoptosis, caspase-3 and SA-β-gal activities, and p53 and p21 proteins expression, while promoted telomerase activity and aggrecan and collagen II synthesis in IL-1β-induced HNPCs. Moreover, the introduction of ANI inhibited the expression of IL-6, phosphorylation of JAK and STAT3, and nuclear translocation of p-STAT3 in Degenerated HNPCs. Additionally, the application of ANI abolished the effects of IL-6 on apoptosis, SA-β-gal and telomerase activity, and the expression of p53, p21, aggrecan and collagen II proteins in degenerated HNPCs. Simultaneously, ANI treatment enhanced the effects of AG490 (inhibitor of JAK/STAT3 pathway) on IL-1β-induced apoptosis, senescence and ECM degradation in HNPCs. Furthermore, ANI treatment markedly inhibited the apoptosis and senescence in the nucleus pulposus of IVDD rats, while promoted the synthesis of aggrecan and collagen II. ANI treatment obviously inhibited JAK and STAT3 phosphorylation and inhibited nuclear translocation of p-STAT3 in IVDD rats.

Conclusion: ANI inhibited the senescence and ECM degradation of NPCs by regulating the IL-6/JAK/STAT3 pathway to improve the function of NPCs in IVDD, which may provide new ideas for the treatment of IVDD.

Keywords: anisodamine, intervertebral disc degeneration, senescence, extracellular matrix, interleukin-6/janus kinases/signal transducer and activator of transcription 3 pathway

INTRODUCTION

Intervertebral disc degeneration (IVDD) is the most common cause of lower back pain and is the basis of spinal degenerative diseases (Deng et al., 2017a). Due to the limited tissue regeneration ability of the lumbar intervertebral disc, it is difficult to be reversed after degeneration. Nucleus pulposus cells (NPCs) are the only constituent cells of the nucleus pulposus of intervertebral disc that the abnormal function of NPCs seriously affects the occurrence and development of IVDD (Rosenzweig et al., 2017). The main manifestations of IVDD are a decrease in the number of NPCs and a decrease in the synthesis of extracellular matrix (ECM). It is currently believed that IVDD is a pathological process involving multiple factors, which is related to genetic susceptibility, mechanical load, inflammatory cytokines, extracellular matrix degradation, cellular senescence and apoptosis.

Since IVDD is age-related, the senescence of various stress-induced NPCs plays a crucial role in the progression of IVDD (Gao et al., 2018). Senescence limits the division of NPCs and ultimately leads to apoptosis. Besides, senescence cells can also produce a large number of matrix degrading enzymes (MMPs) and inflammatory factors, further worsening the living environment of cells, which is also the pathological basis of IVDD. There is increasing evidence that signaling pathways play an important role in regulating the onset and persistence of senescence (Wang et al., 2006; Zirkel et al., 2018). After cellular senescence, a variety of kinases and transcription factors are activated, a process involving a variety of intracellular signal transduction pathways.

STAT3 is an important signal transduction molecule in cells that plays an important role in cell survival, apoptosis and senescence (Chipuk et al., 2010). STAT3 can be phosphorylated by a variety of kinases to form homologs or form heterodimers with other members of the STAT family, which in turn enter the nucleus from the cytoplasm to initiate transcription of downstream genes. The kinase that phosphorylates STAT3 includes JAK, proto-oncogene tyrosine-protein kinase, and the like. JAK/STAT3 can be activated by a variety of cytokines (such as IL-6) and growth factors (such as EGF). IL-6/JAK/STAT3 signaling pathway is the most important signaling pathway and its abnormal activation is related to various pathophysiological processes including inflammation, apoptosis and senescence (Liu et al., 2014; Johnson et al., 2018). As a multifunctional cytokine, IL-6 promotes the accumulation of inflammatory cells and stimulates the release of inflammatory mediators, aggravating the inflammatory response of IVDD (Deng et al., 2016). Additionally, IL-6 can directly participate in the regulation of intervertebral disc cell proliferation, apoptosis and ECM synthesis and decomposition imbalance, which in turn causes IVDD (Zhou et al., 2017). Moreover, IL-6 binds to its receptor and induces cellular senescence by activating the JAK/STAT3 pathway. Targeting silencing of the IL-6/STAT3 pathway in human intervertebral disc NPCs can delay the development of IDD by inhibiting ECM degradation to inhibit MMP-2 production (Ji et al., 2016).

Anisodamine (ANI), an alkaloid extracted from the root of *Anisodus tanguticus*, is an M-cholinergic receptor blocker. Studies have confirmed that ANI has the effect of relieving microvascular spasm and improving microcirculation (Poupko et al., 2007). ANI inhibits inflammatory response and apoptosis of renal tubular cells by reducing the expression of endoplasmic reticulum stress markers (IRE-1α, CHOP), NLRP3 inflammasome and inflammatory factors (IL-1α, IL-β and IL-18) (Yuan et al., 2017), and also reduces cardiomyocyte apoptosis by inhibiting oxidative stress, down-regulating the expression of caspase-3 and Bax (Yao et al., 2018). Furthermore, Luo and Zhou (2009) showed that ANI inhibits the deposition of ECM of experimental hepatic fibrosis by inhibiting MMP-2 expression. However, the role of ANI in IVDD is currently rarely studied.

As such, the present study was to investigate the effects of ANI on apoptosis, senescence and ECM degradation of degenerative human nucleus pulposus cells (HNPCs), and to explore its potential mechanism. Moreover, we also investigated the effect of ANI on IVDD *in vivo* by preparing a rat model of IVDD. We hope to provide new ideas for the treatment of IVDD.

MATERIALS AND METHODS

Cell Culture

Human nucleus pulposus cells (HNPCs) were obtained from ScienCell Research Laboratories (Carlsbad, CA, United States) and cultured in Nucleus Pulposus Cell Medium (NPCM, ScienCell Research Laboratories) in a 37°C, 5% CO_2 incubator. The NPCM was changed every 3 days. When fused to 80%, the cells were trypsinized and subcultured at a ratio of 3:1.

Experimental Design

HNPCs were treated with 20 ng/ml of IL-1β for 48 h to induce a degenerated NPCs model (Deng et al., 2017b). The dose of ANI was determined by treating HNPCs with ANI (0–200 μM). Subsequently, cells were divided into four groups to analyze the effects of ANI on the apoptosis, senescence and ECM degradation of HNPCs: control group, IL-1β group, IL-1β+ANI-10 group, IL-1β+ANI-20 group and IL-1β+ANI-40 group. Moreover, rescue experiments were also performed by dividing the cells into five groups: control group, IL-1β group, IL-1β+ANI-40 group, IL-1β+IL-6 group, and IL-1β+IL-6+ANI-40 group. All treatments were performed for 48 h. Among them, the IL-1β+ANI + IL-6 group was first treated with IL-6 (10 mg/L) for 12 h, while IL-61β+ANI + AG490 group was first treated with AG490 (10 μM) for 12 h; then 40 μM ANI was added for the remaining time of 48 h.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay

HPNCs cells were seeded into 96-well plates (1×10^6 cells/well), incubated overnight at 37°C, 5%CO_2 incubator. Then, HPNCs were exposed to IL-1β (0–100 ng/ml) or ANI (0–200 μM) for 48 h. After treatments, cells were washed with fresh medium and added to MTT solution (Sigma-Aldrich, United States) and incubated for 4 h at room temperature. After aspirating the MTT solution, the cells were incubated with 100 μl DMSO for 5 min at room temperature. The optical density values of the plate were measured at 490 nm on a Tecan Sunrise Absorbance Microplate Reader (Tecan Group, Switzerland).

Lactate Dehydrogenase (LDH) Assay

The cytotoxicity of ANI-treated HNPCs was evaluated by measuring the release of LDH using a CytoTox96 Non-Radioactive Cytotoxicity Assay kit (Beyotime, China). All procedures were performed in strict accordance with the kit instructions.

Caspase-3 Activity

The activity of caspase-3 was measured using a caspase-3 activity assay kit (Beyotime, China). In brief, the treated HNPCs were incubated with 300 μl of lysis buffer for 15 min at 4°C. Subsequently, the supernatant was collected by centrifugation at 15,000 g for 15 min. 10 μl protein of cell lysate per sample in 80 μl reaction buffer were incubated for 4 h at 37°C and then placed on the auto microplate reader (Molecular Devices, Sunnyvale, CA, United States). The activity of caspase-3 was determined by measuring the absorbance at 405 nm.

Senescence-Associated β-galactosidase Activity

Freshly prepared SA-β-gal staining fixative was added to the treated HNPCs according to the kit instructions (Beyotime, China) and incubated at 37°C for 3 h. After washing three times with 0.01 mol/L of PBS, the SA-β-gal staining solution was added dropwise, and placed in a humid box at 37°C overnight in the dark. The staining was observed under a light microscope. Five high power fields were randomly selected, and the senescence process of HNPCs was determined by calculating the ratio of SA-β-gal positive cells to the total number of cells (Wang et al., 2015).

Telomerase Activity

After the treated HNPCs were lyzed and centrifuged, the supernatant was collected. Telomerase activity (IU/L) was measured using a telomerase enzyme-linked immunosorbent assay (ELISA) kit (MIbio, China) according to the manufacturer's instructions.

Western Blot

Nuclear protein and total protein were extracted from IVDD rats' nucleus pulposus tissues and nucleus pulposus cells using Nuclear/Cytoplasmic protein Extraction Kit and Total protein Extraction Kit (AmyJet Scientific, Wuhan, China), respectively, according to the manufacturer's instructions. Protein concentration was determined using the BCA method. The protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked in Tris buffer containing 5% skim milk for 2 h. After washing with PBS, the membrane was incubated with anti-Bax antibody, anti-Bcl-2 antibody, anti-p53 antibody, anti-p21 antibody, anti-collagen II antibody, anti-aggrecan antibody, anti-IL-6 antibody, anti-JAK antibody, anti-Phospho-JAK antibody, anti-STAT3 antibody, anti-Phospho-STAT3 (Tyr-705) antibody, anti-Lamin B antibody, or anti-GAPDH antibody overnight at 4°C. After washing with PBS, the membrane was incubated with secondary antibody (1:1,000; Abcam) for 2 h at room temperature. Development was carried out using an enhanced chemiluminescent reagent. Protein was detected with Image Acquisition using Image Quant LAS 4000 (GE Healthcare Life Sciences, Marlborough, MA, United States).

Hoechst 33258 Staining

NPCs were seeded into 6-well plates embedded with aseptically treated coverslips and cells were cultured to 80% confluence. The medium in the 6-well plate was aspirated, and the medium containing different concentrations of ANI was added, and cultured in a 37°C, 5% CO_2 incubator for 36 h. Subsequently, the liquid in the well was aspirated and rinsed three times with PBS.4% paraformaldehyde was added to the well plate for 20 min at room temperature, followed by rinsing three times with PBS. Hoechst 33258 working solution was added to the well plate and stained for 20 min in the dark at room temperature, and then rinsed three times with PBS. Finally, the slide was sealed with an anti-fluorescence quenching solution (glycerol: PBS = 1:9), and observed under a fluorescence microscope and photographed.

Immunofluorescence (IFC) Staining

NPCs were seeded into 24-well plates embedded with aseptically treated coverslips and cells were cultured to 80% confluence. The medium was removed and washed twice with PBS, and then the cells were fixed in 3.5% formaldehyde for 30 min at room temperature. After washing the cells three times with PBS, they were treated with 0.1% Triton X-100 in PBS for 20 min. Subsequently, the cells were incubated with 3% BSA and 0.05% Tween for 30 min at 37°C. Subsequently, the cells were incubated with rabbit monoclonal anti-p-STAT3 (1:1,000; Abcam) overnight at 4°C. After washing, cells were treated with fluorescent anti-rabbit secondary antibody (1:500; Abcam) for 2 h at room temperature. The nuclei were treated with 4,6-diamidino-2-phenylindole (DAPI). Fluorescence images were acquired under a co-aggregation microscope (Leica, Mannheim, Germany).

Animal Model and Treatment

Male Sprague-Dawley rats (6 weeks) were provided by Institute of Life Sciences (Beijing, China). Rats were housed in standard cages with an ambient temperature of 23 ± 2°C, a humidity of 55% ± 10%, and a light and dark period of 12 h. The experiment was

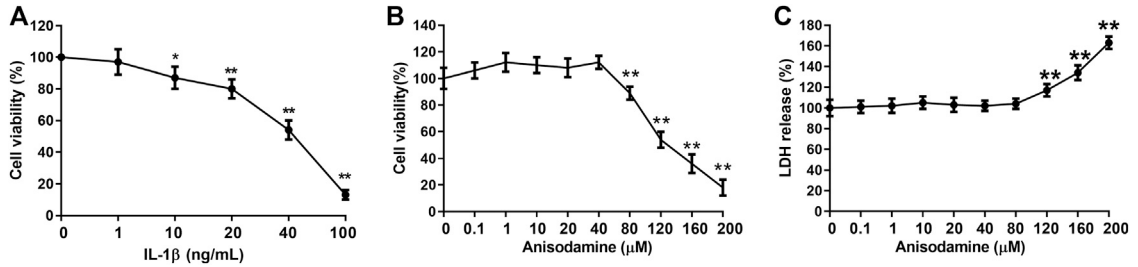

FIGURE 1 | Cytotoxicity experiments. **(A)** The effect of IL-1β on the activity of HNPCs was analyzed by MTT assay. **(B)** The effect of ANI on the activity of HNPCs was analyzed by MTT assay. **(C)** The toxic effects of ANI on HNPCs were analyzed by LDH assay. $^{*}p < 0.05$, $^{**}p < 0.01$.

carried out after 1 week of adaptive feeding in rats. The IVDD rat model was prepared by fiber loop puncture (Jeong et al., 2010). In brief, the rat tail disc (Co4-5) was located on the coccygeal vertebrae. A 26G puncture needle was used to pierce the entire annulus of the fiber through the skin of the tail. The needle was held in the disc for 1 min. Immediate after the operation, ANI was intraperitoneally injected at a dose of 1 mg/kg per day (Ge et al., 2018). Rats in the control group and the IVDD group were injected with the same dose of physiological saline. Four weeks after surgery, rats were sacrificed by overdosing 0.1% sodium pentobarbital, tails were harvested and Co4-5 intervertebral disc samples were collected. All surgical interventions, treatments and postoperative animal care procedures were performed in strict accordance with the Animal Care and Use Committee of Peking Union Medical College Hospital.

Immunochemistry

Immunohistochemistry was used to analyze the expression of p53, collagen II and p-STAT3 (phospho Y705) protein in rat tail intervertebral disc samples. After the sample was fixed with 4% paraformaldehyde for 24 h, decalcification, dehydration, waxing, and embedding treatment were performed to prepare paraffin sections (6 μm). Paraffin sections were dewaxed, hydrated, and incubated in freshly prepared 3% H_2O_2 for 10 min at room temperature. After the sections were repaired by microwave antigen for 20 min, goat serum was added and blocked at room temperature for 10 min. Sections were incubated with p53, collagen II and p-STAT3 (phospho Y705) antibody overnight at 4°C. After washing, biotinylated secondary antibodies were added to the sections for 4 h. Subsequently, freshly prepared DAB chromogenic solution was added to the sections for color development, and was terminated at the appropriate time under the microscope. The samples were counterstained with hematoxylin dye solution and rinsed with distilled water. After dehydration by gradient ethanol, the samples were sealed with a neutral gum. Five areas were selected under an optical microscope for photographing and preservation.

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) Assay

Frozen sections (5 μm) were prepared by taking 4% paraformaldehyde-fixed nucleus pulposus tissue and dehydrating in 20% sucrose overnight. Subsequently, the tail disc samples were subjected to an *in situ* TUNEL reaction using an ApopTag InSitu apoptosis detection kit (Millipore, Billerica, United States) according to the manufacturer's protocol. Apoptotic cells were imaged under a light microscope (magnification, ×400). Among them, red fluorescence was used to label apoptotic nucleus pulposus nuclei, and blue DAPI was used to label all nuclei. Red and blue were superimposed on apoptotic cells.

Statistical Analysis

All statistical analyses were performed using SPSS 20.0 software (IBM Corp., Armonk, NY, United States), and graphs were generated using GraphPad Prism 5 Software (Graph Pad Software, Inc., La Jolla, CA, United States). Student's t test was used to analyze proteins expression. An ANOVA was also performed comparing more than two groups. p (two-tailed) <0.05 were considered statistically significant.

RESULTS

Cytotoxicity Test

IL-1β can be used to establish IVDD *in vitro* (Deng et al., 2017). In order to study the effect of ANI on IVDD, IL-1β was used to treat HNPCs to induce IVDD *in vitro*. We found that when the concentration of IL-1β was greater than 10 ng/ml, the activity of HNPCs was delayed, and the inhibition was enhanced with increasing concentration (**Figure 1A**). Hence, we used 20 ng/ml of IL-1β for subsequent experiments. Additionally, the cytotoxicity of ANI to HNPCs was examined by treating HNPCs with different concentrations (0–200 μM) of ANI for 48 h. We found that ANI did not promote or inhibit the proliferation of HNPCs at concentrations ranging from 0 to 40 μM (**Figure 1B**). However, when the concentration was higher than 80 μM, the viability of cells was obviously decreased (**Figure 1B**), and cytotoxicity was also detected by the LDH assay (**Figure 1C**). Therefore, we selected ANI at concentrations of 10, 20, and 40 μM for subsequent experiments.

ANI Inhibited Apoptosis, Senescence and ECM Degradation of HNPCs Induced by IL-1β

Next, we used different concentrations (10, 20, 40 μM) of ANI to treat IL-1β-induced HNPCs to analyze the effect of ANI on IVDD

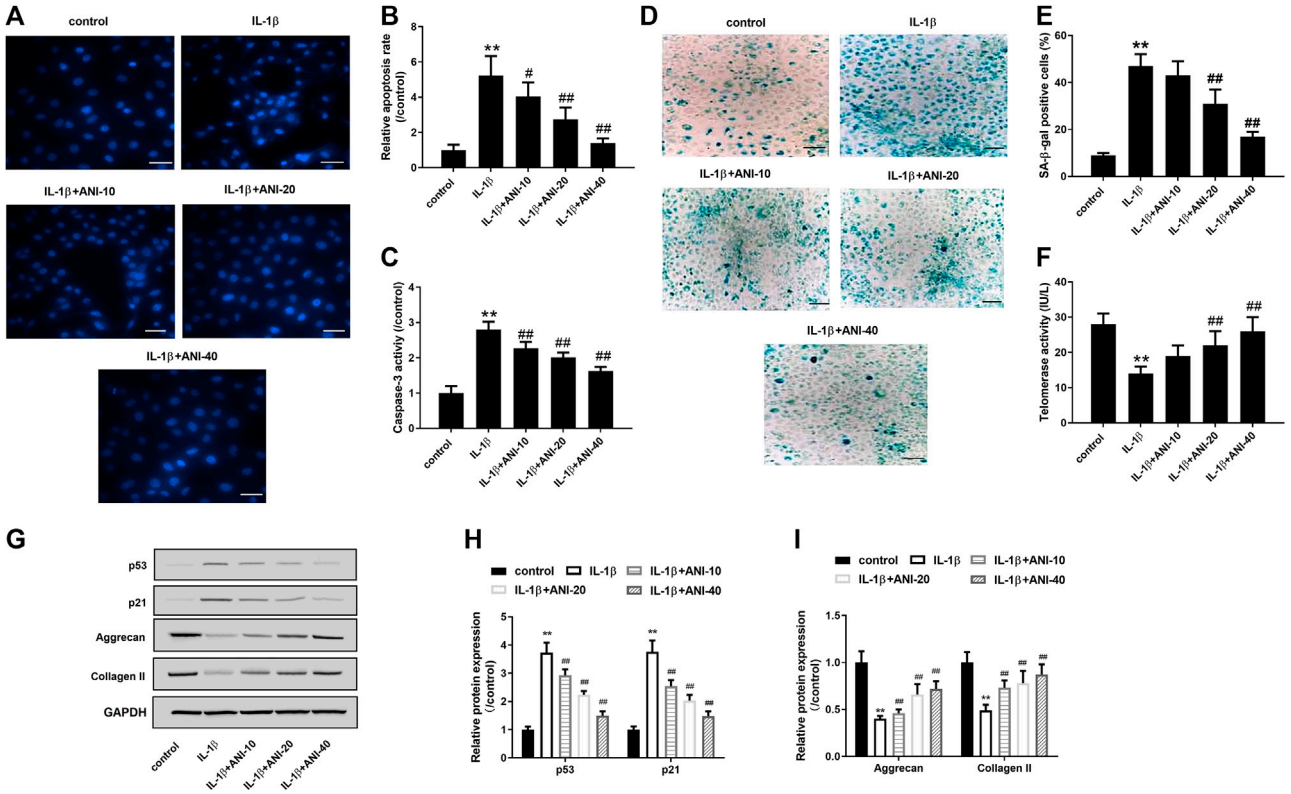

FIGURE 2 | ANI inhibited apoptosis, senescence and ECM degradation of HNPCs induced by IL-1β. **(A,B)** Hoechst 33258 staining was used to analysis the apoptosis of HNPCs. Bar = 50 μm. **(C)** The activity of caspase-3 in HNPCs was detected by a caspase-3 activity kit showed that ANI inhibited caspase-3 activity of HNPCs induced by IL-1β. **(D,E)** SA-β-gal staining was used to analyze senescence of HNPCs. Bar = 100 μm. **(F)** Telomerase activity assay was used to analyze senescence of HNPCs. **(G–I)** The expression of p53, p21, aggrecan and collagen II in HNPCs treated with or without ANI was analyzed by western blot assay. All experiments were performed as means ± SD of three times in duplicates. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. IL-1β group.

in vitro. Hoechst33258 staining showed that the apoptosis of HNPCs after IL-1β treatment was obvious, and the apoptosis rate of IL-1β group was obviously higher than that of control group ($p < 0.05$), while ANI treatment could markedly reduce the apoptosis of HNPCs induced by IL-1β ($p < 0.05$, **Figures 2A**, **B**). Further analysis of the activity of the apoptotic initiation marker protein caspase-3 revealed that the activity of caspase-3 in the IL-1β group was evidently higher than that in the control group ($p < 0.05$), while ANI treatment inhibited the activation of caspase-3 in degenerating HNPCs in a dose-dependent manner ($p < 0.05$, **Figure 2C**).

Senescence associated β-galactosidase (SA-β-gal) activation is a hallmark feature of cellular senescence (Strzeszewska et al., 2018), and p53 is the most important senescence regulatory protein that promotes cellular senescence (Kim et al., 2009). A decrease in telomerase activity reflects an increase in cellular senescence (Li et al., 2019). In the present study, SA-β-gal staining showed that the proportion of cells stained with SA-β-gal positively in IL-1β-induced HNPCs was markedly increased, while treatment with 20 and 40 μM of ANI markedly reduced the proportion of SA-β-gal positive cells (**Figures 2D,E**). Telomerase activity assay further confirmed that ANI treatment significantly improved IL-1β-induced senescence of HNPCs (**Figure 2F**). What is more, western blot analysis showed that IL-1β could induce the increase of p53 and p21 protein expression in HNPCs, while ANI treatment could inhibit the expression of p53 and p21 proteins in degenerative HNPCs ($p < 0.05$, **Figures 2G,H**).

HNPCs mainly secrete aggrecan and collagen II, which are also the main components of ECM. We next observed the effect of ANI on the extracellular matrix. The results showed that collagen II and aggrecan proteins expression in IL-1β-induced HNPCs was significantly decreased, while ANI could promote the synthesis of collagen II and aggrecan ($p < 0.05$, **Figures 2G,I**). These results suggested that ANI may mitigate disc degeneration by reducing apoptosis, senescence and ECM degradation of HNPCs.

ANI Inhibited Activation of Interleukin-6/Janus Kinases/Signal Transducer and Activator of Transcription3 Pathway in Human nucleus pulposus cells Induced by IL-1β

To investigate the possible mechanism by which ANI improves IVDD, we analyzed changes in IL-6/JAK/STAT3 signaling pathways in degenerative NPCs. Western blot analysis showed that IL-1β treatment significantly increased IL-6 expression in

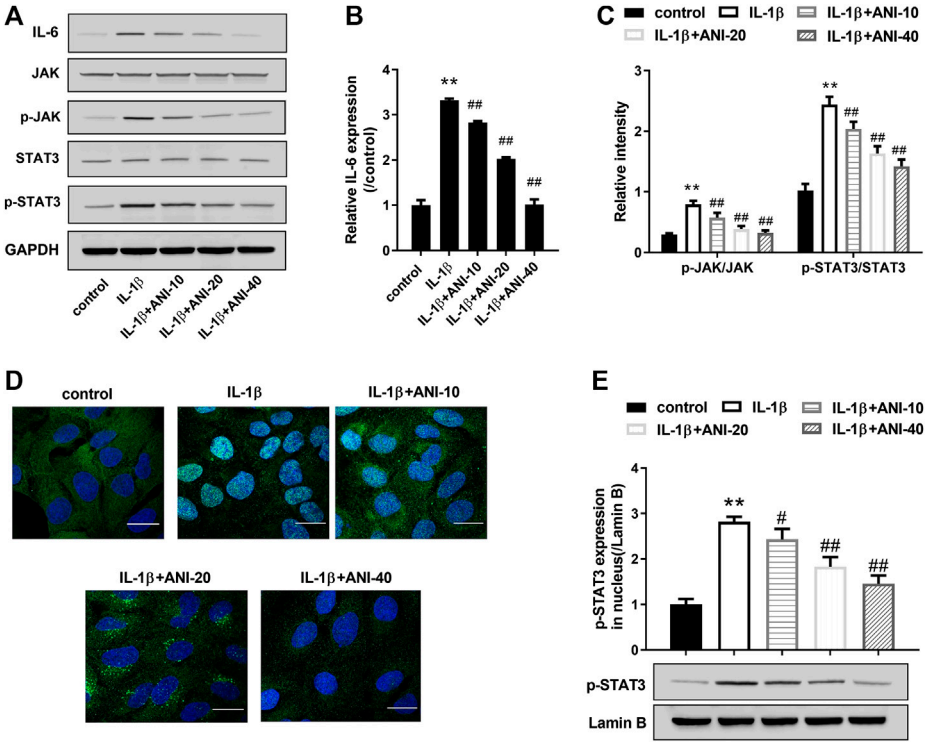

FIGURE 3 | ANI inhibited IL-1β-induced activation of IL-6/JAK/STAT3 pathway in HNPCs. **(A–C)** The expression of IL-6, JAK, *p*-JAK, STAT3 and p-STAT3 in HNPCs was analyzed by western blot assay. **(D)** Immunofluorescence staining was used to analyze nuclear translocation of p-STAT3 in HNPCs. Bar = 25 μm. **(E)** The expression of p-STAT3 in nucleus was analyzed by western blot assay. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control group; $^{\#}p < 0.05$ vs IL-1β group.

HNPCs and promoted phosphorylation of STAT3 and JAK compared with the control group (**Figures 3A–C**). Moreover, IGF staining also observed that IL-1β treatment induced nuclear translocation of p-STAT3 in HNPCs (**Figure 3D**), and increased the expression of p-STAT3 in the nucleus (**Figure 3E**). Compared with the IL-1β group, ANI treatment inhibited IL-6 expression, phosphorylation of JAK and STAT3, and nuclear translocation of p-STAT3 in HNPCs. These results suggested that ANI treatment could inhibit the activation of the IL-6/JAK/STAT3 pathway in IL-1β-induced HNPCs.

ANI Inhibited IL-1β-Induced Apoptosis, Senescence and ECM Degradation of HNPCs by Regulating IL-6/JAK/STAT3 Signaling Pathway

To analyze whether ANI suppresses HNPCs degradation by inhibiting the IL-6/JAK/STAT3 signaling pathway, we performed a rescue experiment on IL-1β-induced HNPCs with IL-6 (10 mg/L) or PI3K/AKT inhibitor AG490 (10 μM) prior to treatment with ANI (40 μM). As expected, IL-6 treatment could enhance the activation of IL-1β stimulation on the IL-6/JAK/STAT3 pathway, which was reflected by increased IL-6 expression and increased phosphorylation levels of JAK and STAT3, while ANI treatment could eliminate this effect to a certain extent (**Figure 4A**). Moreover, IL-6 treatment could eliminate the activation of IL-6/JAK/STAT3 pathway stimulated by IL-1β, and ANI treatment enhanced this effect (**Figure 4B**). The results showed that IL-6 treatment could promote apoptosis (**Figure 4C**), caspase-3 activity (**Figure 4D**), SA-β-gal activity (**Figure 4E**), and p53 and p21 proteins expression (**Figures 4G,H**), while decreased telomerase activity (**Figure 4F**), and aggrecan and collagen II synthesis (**Figures 4G,I**). More importantly, the apoptotic rate and caspase-3 activity in the IL-1β+IL-6+ANI-40 group were significantly lower than those in the IL-1β+IL-6 group (**Figures 4C,D**). Furthermore, ANI abolished the effects of IL-6 on SA-β-gal activity (**Figure 4C**), telomerase activity (**Figure 4F**), and p53 and p21 proteins expression (**Figures 4G,H**). Moreover, ANI reversed the inhibitory effect of IL-6 on the synthesis of aggrecan and collagen II (**Figures 4G,I**) in IL-1β-induced HNPCs. Moreover, ANI treatment enhanced the effects of AG490 on IL-1β-induced apoptosis, senescence and ECM degradation in HNPCs (**Figures 4C–I**). These results revealed that ANI may play a protective role in degenerative HNPCs by regulating the IL-6/JAK/STAT3 pathway.

ANI Inhibited Apoptosis, Senescence and ECM Degradation of Nucleus Pulposus Tissue by Inhibiting IL-6/JAK/STAT3 Signaling Pathway *In Vivo*

We treated IVDD rats for 4 weeks by intraperitoneal injection of ANI to analyze the effect of ANI on IVDD *in vivo*. The TUNEL assay showed a significant decrease in apoptosis in the nucleus pulposus of the IVDD + ANI group (**Figure 5A**). Consistently, ANI increased Bcl-2 expression while decreased Bax expresson of IVDD rats (**Figure 5B**). Western blot analysis (**Figures 5C,D**) showed that ANI treatment markedly inhibited the expression of p53 and p21proteins in the nucleus pulposus of IVDD rats, whereas promoted the expression of aggrecan and collagen II (**Figure 5E**). Additionally, immunohistochemical analysis also showed that ANI

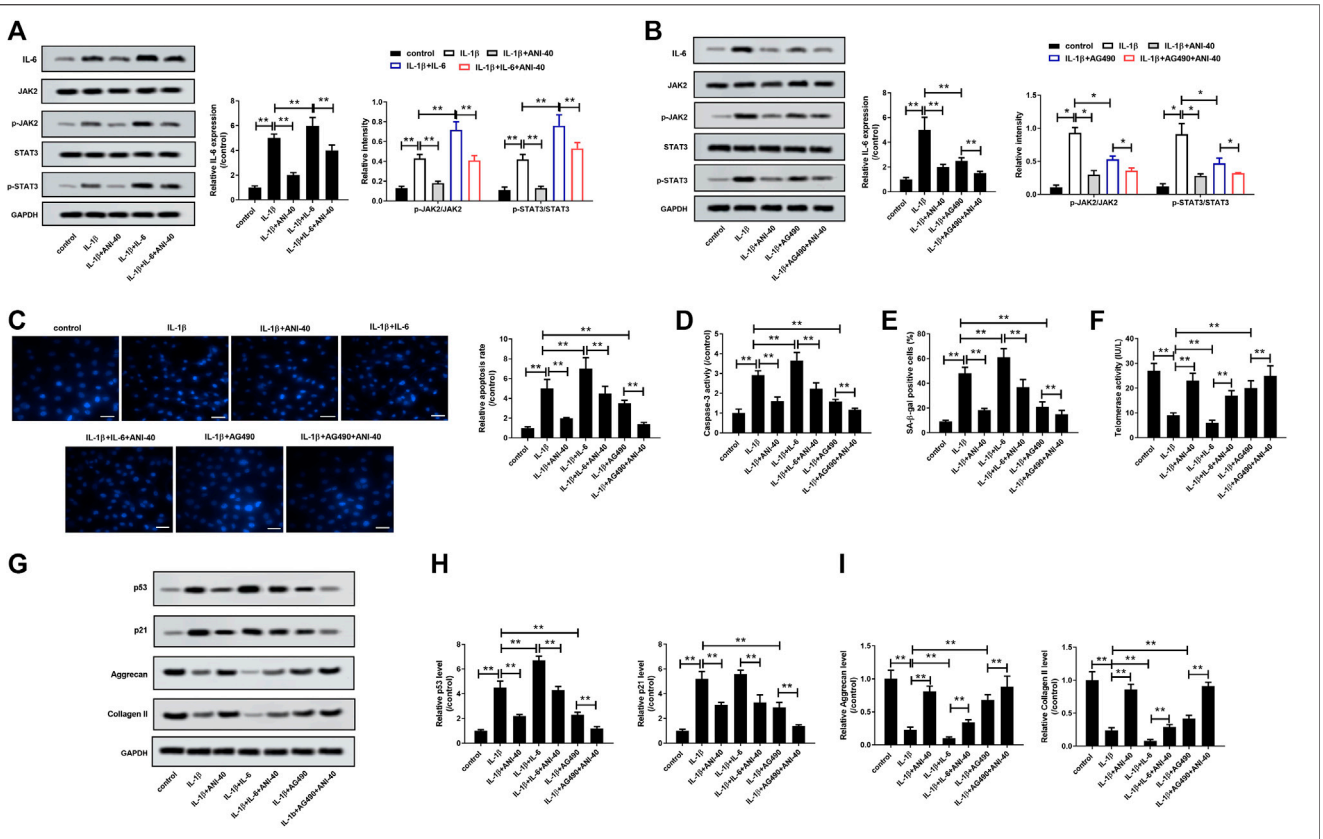

FIGURE 4 | ANI inhibited IL-1β-induced apoptosis, senescence, and ECM degradation of HNPCs by modulating the IL-6/JAK/STAT3 signaling pathway. **(A,B)** The expression of IL6, JAK, *p*-JAK, STAT3 and p-STAT3 in HNPCs was analyzed by western blot assay. **(C)** Hoechst 33258 staining was used to analyze the apoptosis of HNPCs. Bar = 50 μm. **(D)** Detection of caspase-3 activity. SA-β-gal staining **(E)** and telomerase activity assay **(F)** were used to analyze senescence of HNPCs. Bar = 100 μm. **(G–I)** The expression of p53, p21, aggrecan and collagen II in HNPCs was analyzed by western blot assay. $^{*}p < 0.05$, $^{**}p < 0.$

treatment increased the expression of p53 and collagen II in the nucleus pulposus of IVDD rats (**Figure 5F**). What is more, ANI treatment significantly inhibited the expression of IL-6 expression, phosphorylation of STAT3 and JAK, and nuclear translocation of p-STAT3 in nucleus pulposus of IVDD rats (**Figures 5G–J**). Moreover, ANI treatment reduced the localization of p-STAT3 in the nucleus pulposus of IVDD rats (**Figure 5K**).

DISCUSSION

As an M receptor blocker, ANI has proven to be a relatively safe and effective drug that has been used in the treatment of various diseases including rheumatoid arthritis, ischemia-reperfusion injury, and respiratory diseases (Poupko et al., 2007; Zhou et al., 2014). However, the role of ANI in IVDD is unclear. This study found that ANI could inhibit IL-1β-induced apoptosis and senescence of HNPCs, and promote collagen II and synthesize *in vitro*. Consistent with *in vitro* study, ANI could improve the degeneration of intervertebral disc degeneration in IVDD rats by inhibiting the apoptosis and senescence of NPCs and inhibiting ECM degradation in the nucleus pulposus tissue. More importantly, we found that the improvement in IVDD by ANI may be related to its inhibition of IL-6/JAK/STAT3 signaling pathway.

Cellular senescence is a proliferative cell that continues to suffer from exogenous and endogenous stress and damage, entering a perpetual cell cycle arrest state (Yousefzadeh et al., 2019). Adult intervertebral discs are the largest avascular tissue in the body. Ischemia, hypoxia, and nutrient deprivation in the intervertebral disc increase the stress factors such as high lactate metabolism, high osmotic pressure, and oxidative damage, which induce premature senescence of NPCs (Feng et al., 2016). Apoptosis is the final link in the senescence of nucleus pulposus cells (Jung et al., 2019). We found that the apoptotic rate of degenerative HNPCs increased significantly, and the activity of caspase-3, a key executive molecule and the main effector molecule of apoptosis, was also significantly increased in IL-1β-induced HNPCs, suggesting the occurrence of apoptosis in degenerative HNPCs. SA-β-gal is a relatively specific cell senescence marker (Oh et al., 2018) that is derived from lysosome β-galactosidase that reflects increased expression of lysosomal β-galactosidase protein in senescent cells (Bo et al., 2006). Roberts et al. (2006) and Gruber et al. (2007) showed an increase in SA-β-gal staining-positive cells in degenerated intervertebral discs and nucleus pulposus compared with undegenerated discs. Two signaling pathways alone or synergistically induce cellular senescence: the p53/p21 and p16INK4A/pRB pathways, in which the p53/p21 pathway is

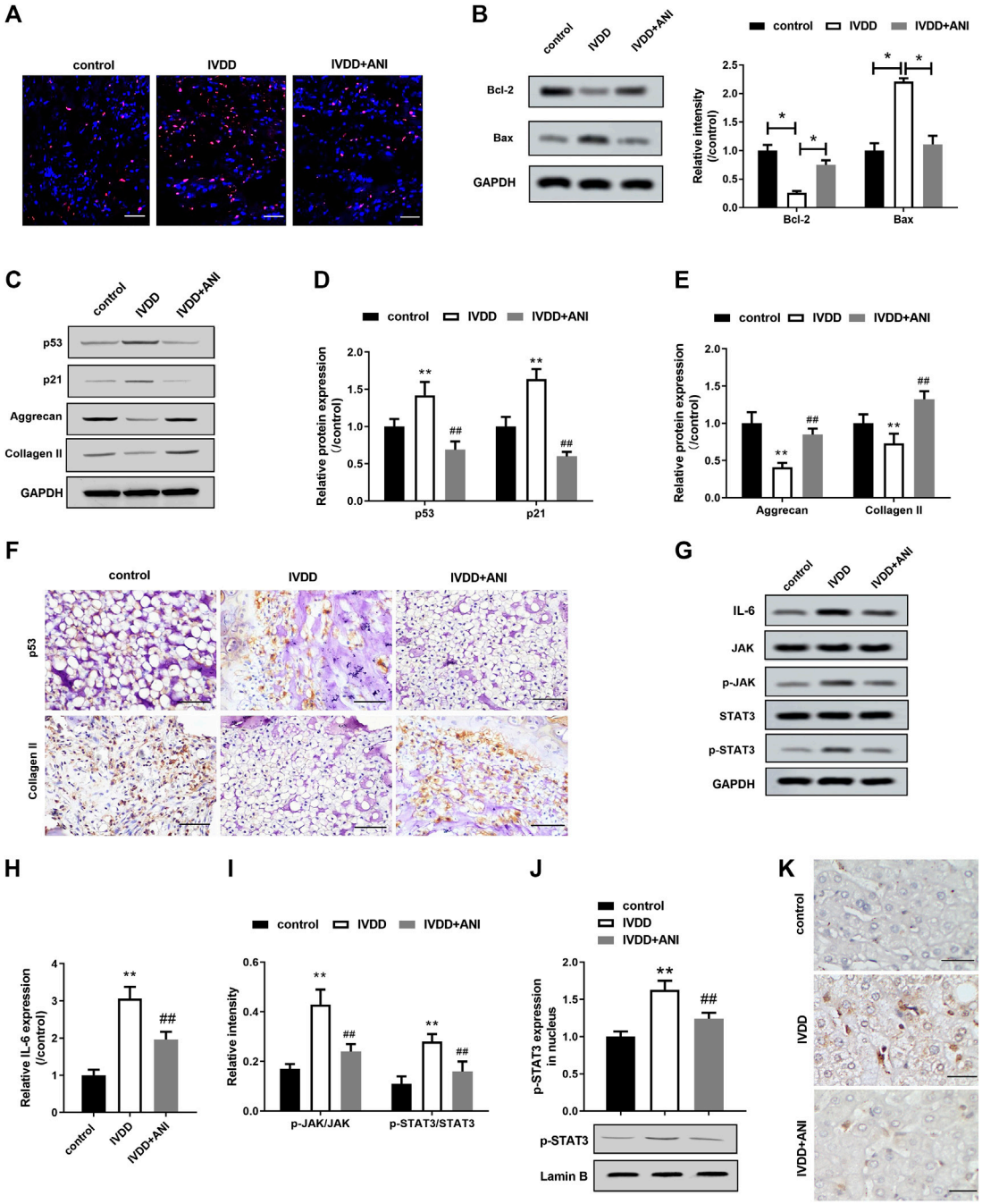

FIGURE 5 | ANI inhibited apoptosis, senescence and ECM degradation in nucleus pulposus tissue by inhibiting IL-6/JAK/STAT3 signaling pathway. **(A)** TUNEL assay was used to analyze apoptosis in the nucleus pulposus tissue of IVDD rats with or without ANI treatment. Bar = 100 μm. Red was the apoptotic nucleus pulposus nucleus, and blue was the nucleus. **(B)** Western blot assay was used to analyze the expression of Bcl-2 and Bax proteins in the nucleus pulposus of IVDD rats with or without ANI treatment. **(C–E)** Western blot assay was used to analyze the expression of p53, p21, aggrecan and collagen II proteins in nucleus pulposus tissue of IVDD rats with or without ANI treatment. **(F)** Immunohistochemistry was used to analyze the expression of p53 and aggrecan proteins in nucleus pulposus tissues. Bar = 100 μm. **(G–I)** Western blot analysis was used to analyze the expression of *p*-JAK and p-STAT3 in the nucleus pulposus of IVDD rats with or without ANI treatment. **(J)** Western blot assay was used to analyze the expression of p-STAT3 protein in the nucleus pulposus tissue. **(K)** The localization of p-STAT3 in nucleus pulposus tissues was analyzed by immunohistochemistry. Bar = 50 μm. $N = 6$. $^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. IVDD.

dominant in IVDD (Kim et al., 2009). Progressive shortening of telomeres activates the expression of the tumor suppressor gene p53 by mimicking DNA damage signals, which in turn activates its downstream gene p21. Activation of p21 inhibits cyclin-dependent kinase two and cyclin E, which in turn reduces the degree of phosphorylation of retinoblastoma (RB) protein. The

hypophosphorylated RB protein binds to and inactivates the nuclear transcription factor E2F, eventually causing senescence (Jeyapalan and Sedivy, 2008). Moreover, the p53 pathway can also be directly caused by cellular DNA damage induced by stress. Studies have shown that hyperosmolar states can activate this pathway by damaging the DNA of NPCs, and inactivation of p53 reverses this process (Mavrogonatou and Kletsas, 2009). We also observed an increase in activity of SA-β-gal, as well as p53 and p21 protein expression in IL-1β-induced HNPCs, and a decrease in telomerase activity, suggesting senescence in degenerative intervertebral discs. Senescence not only reduces the cell viability of NPCs, but also causes phenotypic changes that lead to the degradation of ECM (Gruber et al., 2007). ECM not only maintains the integrity of the intervertebral disc, but also regulates the survival, morphology and differentiation of NPCs by providing mechanical and biochemical pathways to NPCs. A typical feature of IVDD is the loss of ECM components. Aggrecan and collagen II are the most important components of nucleus pulposus ECM. We also observed a decrease in the expression of aggrecan and collagen II in IL-1β-induced HNPCs, suggesting degradation of ECM in HNPCs. These results demonstrated that IL-1β could induce degeneration of HNPCs *in vitro* by inducing apoptosis, senescence, and ECM degradation, thereby inducing IVDD. Consistent with *in vitro* studies, we also observed senescence, apoptosis, and ECM degradation in degenerated intervertebral disc nucleus tissues in IVDD rats.

Delaying the senescence of NPCs and promoting the synthesis of ECM components to inhibit the initiation of IVDD is expected to be the key to the treatment of IVDD (Cai et al., 2016; Chen et al., 2018). ANI has been shown to have anti-inflammatory, anti-apoptotic and anti-oxidative effects. The present study showed that ANI inhibited apoptosis and SA-β-gal activity, whereas increased telomere activity and collagen II and aggrecan synthesis in IL-1β-induced HNPCs. Simultaneously, the results also showed in the IVDD rat model that ANI treatment could increase Bcl-2 expression while reduce Bax expression in nucleus pulposus tissue, which are key regulators of apoptosis (Chipuk et al., 2010; Delbridge et al., 2016), inhibited the expression of p53 and p21, and promoted the synthesis of collagen II and aggrecan. Zhang et al. (2019) also showed that moxibustion treatment may be beneficial to IVDD by reducing apoptosis, which is manifested in the up-regulation of Bcl-2 expression and down-regulation of Bax expression. Collectively, these results revealed that ANI may extenuate IVDD by inhibiting apoptosis, senescence, and ECM degradation of NPCs.

As one of the pro-inflammatory cytokines, IL-6 has been shown to accelerate IVDD (Risbud and Shapiro, 2014). IL-6 forms a complex IL-6/IL6R/gp130 by binding to its receptor IL-6R, and dimerization of the gp130 molecule results in phosphorylation with JAK. Activated JAK further phosphorylates STAT3, which forms a dimer and translocates to the nucleus to activate transcription and expression of the corresponding target gene, such as Bcl-xL, Bcl-2, CyclinD1, Fas, VEGF, MMP-2, etc. (Wang and Sun, 2014). Blocking the IL-6/JAK/STAT3 pathway has become a new strategy for the treatment of disease (Johnson et al., 2018). For example, IL-6 and its receptor IL-6R monoclonal antibody have been used in the treatment of rheumatoid arthritis (Pelechas et al., 2017) and uveitis (Elkinson and McCormack, 2013), and JAK small molecule inhibitors have also been used in the treatment of rheumatoid arthritis and myelofibrosis (Mascarenhas and Hoffman, 2012; Traynor, 2012). We found that ANI inhibited the expression of IL-6 and the phosphorylation of JAK and STAT3 in IL-1β-induced HNPCs, and also inhibited nuclear translocation of p-STAT3 *in vitro*. Additionally, IL-6 reversed the inhibitory effect of ANI on IL-6/JAK/STAT3 pathway activation *in vitro*. And, IL-6 could eliminate the anti-apoptosis, anti-aging and anti-ECM degradation effects of ANI on degenerative HNPCs to some extent. More importantly, ANI abolished the effects of IL-6 on apoptosis, senescence and ECM degradation of degenerative HNPCs. Additionally, ANI treatment enhanced the effects of AG490 (inhibitor of JAK/STAT3 pathway) on IL-1β-induced apoptosis, senescence and ECM degradation in HNPCs. Moreover, ANI treatment was also observed in IVDD rats to inhibit the activation of the IL-6/JAK/STAT3 pathway in nucleus pulposus tissues. Kojima et al. (2012) found that IL-6 and soluble IL-6R stimulation induce cellular senescence by causing DNA damage response and p53 accumulation, a process that uses STAT3 as a major trigger and enhancer component. Inhibition of IL-6/JAK/STAT3 pathway activation can delay IVDD by inhibiting apoptosis of NPCs and degradation of ECM (Suzuki et al., 2017; Zhou et al., 2017). Based on these studies, we hypothesized that ANI may play a protective role in IVDD by inhibiting the activation of IL-6/JAK/STAT3 pathway to suppress apoptosis, senescence and ECM degradation of NPCs.

CONCLUSION

Taken together, ANI could improve the function of NPCs in IVDD by inhibiting the apoptosis, senescence and ECM degradation via negatively regulating IL-6/JAK/STAT3 signaling pathway. This study may provide insight for further study into the protective effects of ANI. However, further studies are needed to demonstrate the effect of ANI in IVDD to promote the clinical treatment of IVDD.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of Peking Union Medical College Hospital.

AUTHOR CONTRIBUTIONS

NT and HZ designed the research; NT and YD performed the research; CC analyzed the data and wrote the paper; and HZ conceived the idea and supervised the whole project. All authors discussed the results and commented on the manuscript.

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