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REVIEW

Fibroblast Insights into the Pathogenesis of Ankylosing Spondylitis

Zhenhua Liu[1,*], Mingxi Cai[2,*], Haoteng Ke[2,*], Huazong Deng[2], Weijia Ye[2], Tao Wang[1], Qifan Chen[1], Shuizhong Cen[1]

[1]Department of Spinal Surgery, Orthopedic Medical Center, Zhujiang Hospital, Southern Medical University, Guangzhou, 510280, People's Republic of China; [2]The Second Clinical School, Zhujiang Hospital, Southern Medical University, Guangzhou, 510280, People's Republic of China

*These authors contributed equally to this work

Correspondence: Shuizhong Cen, Department of Spinal Surgery, Orthopedic Medical Center, Zhujiang Hospital, Southern Medical University, Guangzhou, People's Republic of China, Tel +86 13480297160, Email censhzh3@mail2.sysu.edu.cn

Purpose of the Review: Emerging evidence has shown that ankylosing spondylitis fibroblasts (ASFs) act as crucial participants in inflammation and abnormal ossification in ankylosing spondylitis (AS). This review examines the investigations into ASFs and their pathological behavior, which contributes to inflammatory microenvironments and abnormal bone formation. The review spans the period from 2000 to 2023, with a primary focus on the most recent decade. Additionally, the review provides an in-depth discussion on studies on ASF ossification at the cellular level.

Recent Findings: ASFs organize immune functions by recruiting immune cells and influencing their differentiation and activation, thus mediate the inflammatory response in the early phase of disease. ASFs promote joint destruction at sites of cartilage and actively promote abnormal ossification by recruiting osteoblasts, differentiation into myofibroblasts or ossification directly. Many signaling pathways and cytokines such as Wnt signaling and BMP/TGF-β signaling are involved in ASF ossification.

Summary: ASFs play a key role in AS inflammation and osteogenesis. Further studies are required to elucidate molecular mechanisms behind that and provide new targets and directions for AS diagnosis and treatment from a new perspective of fibroblasts.

Keywords: ankylosing spondylitis, fibroblast, inflammation, ossification

Introduction

Ankylosing spondylitis (AS), a chronic inflammatory arthritis that mainly affects the spine and sacroiliac joints, leads to structural and functional impairments and progressive ankylosis of the axial skeleton.[1] As a special inflammatory disease, inflammation in AS has a widespread influence. Axial inflammation, including sacroiliac arthritis, leads to irreversible structural damage and restrict spinal mobility. Peripheral manifestations such as arthritis in lower extremities and enthesitis at the insertion of the Achilles tendon and plantar fascia are also commonly observed.[2] Moreover, patients may concurrently exhibit extra-musculoskeletal manifestations, specifically uveitis, Inflammatory Bowel Disease (IBD), and psoriasis.[3] And unlike other systemic autoimmune diseases, AS is characterized by a dominant role of the innate immune system. This is marked by aberrant activity of innate and innate-like immune cells, which leads to their unique inflammatory conditions.[4] Then, as MRI results showed, abnormal bone formation is more likely to occur in sites with previous inflammation.[5] When osteophytes span the entire joint cavity, this results in the immobilization of the affected joint, a condition known as bony ankylosis. This can potentially lead to restricted spinal motion and even result in permanent disability.[6] The clinical criteria for AS include Sacroiliitis on imaging plus one or more spondyloarthritis (SpA) features. Or, being HLA-B27 positive plus two or more other SpA features. The histologic SpA features of AS always include enthesis, sacroiliitis, syndesmophyte formation, ectopic ossification, and so on, all of which contribute to the progression of the disease.[7] Intriguingly, fibroblasts were frequently observed among the above lesions, participating in early sacroiliitis,[8,9] mediating the invasion of the subchondral bone,[8,10,11] and even contributing to adipocyte

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accumulation.[12] This suggests that ankylosing spondylitis fibroblasts (ASFs) may play a crucial role in the etiology of AS. In vitro, the feature displayed in ASF culture also supports this and relates ASF to structural damage and bony ankylosis in AS.[13–16]

Fibroblasts exist in all organizations of the body and maintain the structural integrity and tissue health of connective tissue by secreting collagen and extracellular matrix. In recent years, an increasing number of researchers have realized that fibroblasts are involved in inflammation, tissue damage, bone erosion, and the destruction of articular cartilage in autoimmune diseases. For example, fibroblasts are considered to be a reservoir that can provide specialized activated fibroblasts that lead to a pathological process in RA,[17] which would lead to inflammatory and joint erosion by forming pannus and other factors.[18] Similarly, fibroblasts, as possible target cells in the pathology of AS, have attracted increasing attention from many researchers in recent years, while there have been no systematic reviews on ASFs to lead to further research.

Thus, to obtain a further understanding of the special inflammation conditions and bony ankylosis in AS, we focus on the recent investigation of ASFs and their pathological behaviour that contribute to inflammatory microenvironments and abnormal bone formation. Additionally, the review provides an in-depth discussion on studies on ASF ossification at the cellular level.

Fibroblasts in AS

Previously, fibroblasts were considered to be a homogeneous cell population, while emerging evidence indicates that fibroblasts can serve as a reservoir that can provide tissue-specialized fibroblasts and pathological fibroblasts in disease.[17] In AS, with THY1 (also known as CD90),[19] vimentin,[20] alpha smooth muscle actin, prolyl 4-hydroxylase β,[21] S100A4, etc., used as markers in recent studies, ASFs were reported to mainly regulate the inflammatory environment and mediate abnormal ossification.[22]

First, similar to rheumatoid arthritis synovial fibroblasts (RASFs) in RA, ASFs can cause certain pathological responses in diseases by regulating factor secretion, coordinating inflammatory responses, regulating tissue homeostasis, and mediating joint remodeling. Currently, fibroblast research in RA is well-established,[23] with key findings in fibroblast subpopulations, fibroblast interactions,[18] and fibroblast-targeted therapies,[24,25] among others. AS and RA are autoimmune diseases with significant differences but certain similarities, especially in inflammation. Leveraging the successful practices from RA research may help us elucidate the specific mechanisms of ASFs, particularly in the context of AS inflammation.

As both the major cells in joint remodeling, osteoclasts are terminally differentiated myeloid with distinct morphological and phenotypic characteristics,[26] while osteoblasts differentiated from mesenchymal stromal cells (MSCs).[27] Though with significant difference in differentiation, cellular communication between them is essential for bone remodeling, which maintains bone homeostasis.[28] Meanwhile, fibroblasts derived from the same source of bone marrow MSCs as osteoblasts,[29] with considerable evidence showing significant expression of osteogenic markers, were observed in ASFs, suggesting that ASFs may also play a key role in the abnormal bone proliferation of AS.

According to the series of studies that are discussed in this review, it is clear that fibroblasts are inextricably linked to the different stages of structural damage progress in AS: inflammation → erosive destruction → syndesmophyte formation→ ankylosis.[11]

Histological Evidence of ASFs

Emerging histological evidence has revealed that ASFs from subchondral bone marrow may play a role in the aetiology of AS by recruiting immune cells to regulate the inflammatory environment and bone metabolism in joint remodeling (Figure 1).

ASFs Mediate the Inflammatory Response

In earlier disease stages of AS, morning stiffness and inflammatory back pain are major symptoms. Radiographs of the sacroiliac joints could appear normal in the early phase of disease many years before structural changes become apparent.[1] In this disease state, fibroblasts have been reported to act as dynamic participants in immune processes.[30]

Emerging histological evidence has shown that subchondral ASF-rich tissue is infiltrated with inflammatory cells in early sacroiliitis without definite structural damage.[8,9] The inflammatory cells reported include CD45+ lymphocytes that seem to invade a degenerate cartilaginous area[31] and cell populations of CD4 and CD8 T cells that infiltrate the cartilage from the subchondral bone marrow along with osteoclast-mediated resorption of the bone end plate. Remarkably, it

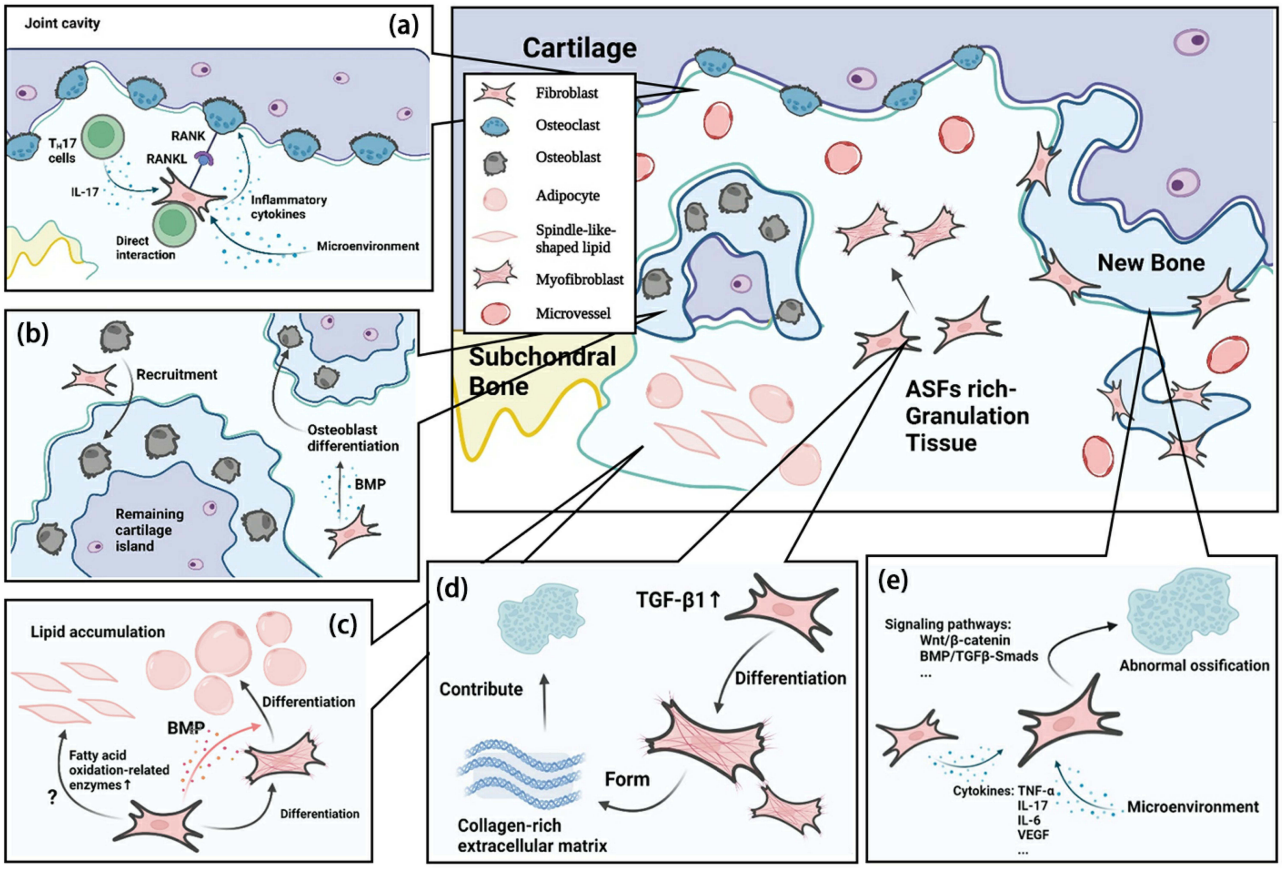

Figure 1 ASFs in AS joint remodeling. ASFs promote joint destruction at sites of cartilage and actively promote abnormal ossification by recruiting osteoblasts, differentiation into myofibroblasts or ossification directly. (**a**) ASFs affect osteoclasts to promote bone erosion. ASFs recruit a cohort of osteoclasts towards the joint surface and secret RANKL and inflammatory cytokines to induce the function of osteoclasts with the effect of Th17. The inflammatory environment will be the key to lead to this phenomenon. (**b**) ASFs affect osteoblasts to promote bone formation. Evidence suggested that ASFs recruit osteoblasts into proximity of the remaining cartilage islands and lead to bone formation. The overexpression of BMP in ASFs will facilitate the cellular differentiation of osteoblasts. (**c**) ASFs contribute to fat enrichment. Evidence shows that the lipid accumulation in ASF-rich tissue occasionally in the form of spindle-like–shaped lipid accumulation directly within the ASFs. ASFs also activate as myofibroblasts and differentiate into adipocyte induced by BMPs. (**d**) ASFs activate as myofibroblasts to form collagen-rich extracellular matrix independent of inflammation, which contribute to abnormal bone formation. (**e**) Direct transformation of ASFs into new bone. Considerable cell-level studies suggest that ASFs have the potential for osteogenic differentiation themselves with some signaling pathways and cytokines involved. Created with Biorender.com.

seems that the infiltration of subchondral T-cell substantially declined simultaneously with the cartilage destruction during disease progression, which suggests its correlation with the existence of cartilage at the joint surface.

This result indicated that ASFs' potential to enhance the bone resorption of osteoclasts by participating in pro-inflammatory milieu formation through secreting inflammatory factors such as Tumour Necrosis Factor (TNF),[32] a mechanism that might resemble animal models of arthritis in which mice overexpressing TNF develop destructive arthritis caused by activated osteoclasts.[33] Based on clinical trials, anti-TNF therapy has been proven to improve clinical symptoms in AS.[34] So far adalimumab, etanercept, golimumab, and infliximab have been used for the treatment of AS,[35] and among them Infliximab showed a relatively better effect.[36] However, there remains a subset of patients who do not respond to this treatment.[37] Additionally, a significant proportion of patients experience a reduction in efficacy after an initial period of response, intolerance, or adverse reactions, and might need a switch to an alternative treatment regimen.[38] Therefore, in recent years, researchers have been making continuous efforts to improve and try other targets such as IL-17[39] and Janus kinase (JAK).[40] By and large, a series of further researches in ASFs involved TNF secretion may provide a fibroblast new insight into AS anti-TNF treatment.

Appel et al observed a significantly higher number of Th17 cells present in the bone marrow of subchondral ASF-rich tissue-affected facet joints.[41] In the past few years, the cytokine Interleukin-17A (IL-17A) has been shown to play an important role in the pathogenesis of human chronic inflammatory diseases. Based on clinical trials performed with IL-17

blockade in AS, IL-17 inhibition has been supported to be an effective treatment for AS.[42] Inflammatory factors such as TNFα, IL-1 and IL-6 synergize with IL-17 to induce further proinflammatory cytokine and chemokine secretion by fibroblasts, amplifying inflammatory reactions and increasing the aggregation of inflammatory cells.[18,43] These effects have been proven in RA,[18] and we suggest determining the exact relationship among ASFs, IL-17 and inflammation. In addition, IL-17 is thought to enhance the function of osteoclasts via ASFs, which will be discussed below.

Dense CD163+ macrophages were also reported to infiltrate subchondral ASF-rich tissue.[9,32,44] However, in AS patients of long-standing, bone marrow macrophages appear to have a minor effect on inflammation or repair due to the fact that the number of bone marrow macrophages shows no significant differences with non-AS controls.[45]

In addition, there are CD20+ B-cell infiltrates in subchondral ASF-rich tissue, especially in AS patients with persistent inflammation in the spine.[45] B-cells have been overlooked in the discussion of AS pathogenesis for so long, mainly due to the fact that autoantibodies seem to have a minor role in AS. However, the resistance of B cells after anti–tumour necrosis factor α (anti-TNFα) therapy[46] might explain why relapses occur soon after TNF-α therapy has been discontinued. In addition, a few small clinical trials used rituximab in AS and achieved successful treatment response,[47] again pointing to a possible role of B cells in the immunopathology of the disease.[45] An inhibition of B cell antigen-presenting function might be a possible explanation. It is reported that B cells are able to mediate osteoclastogenesis through regulating the level of osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL),[48] which could be a possible mechanism to relate B cells and ASFs.

Fibroblasts are now thought to organize immune functions by recruiting immune cells and influencing their differentiation and activation. Specific mechanisms may include cytokine and chemokine secretion and behavioural modulation through extracellular matrix remodeling. In addition, the transfer of extracellular matrix microenvironments was recently described.[30] The research of Cambré et al described another interesting mechanism: it showed that biomechanical forces in joints would cause fibroblasts to secrete C–C motif ligand 2 (CCL2) among other chemokines responsible for the attraction of inflammatory monocytes towards biomechanically exposed sites and lead to the differentiation of these cells into osteoclasts.[49]

In rheumatoid arthritis (RA), the relationship between RASFs and inflammation has been reviewed in detail. In RA, the destructive properties of RASFs can be activated by IL-6, TNF-α and IL-1b, which are elevated in AS inflammation as well. The activated RASFs then further amplify the inflammatory cycle by producing inflammatory factors themselves.[23,50] Furthermore, RASFs can enhance their activation and cytokine release through direct contact with inflammatory cells, such as T cells and macrophages. The factors above, in the meantime, recruit, activate, and promote the differentiation of other, engaged in the inflammatory cycle in RA, cell types.[18] Given the similar relationship between inflammation and fibroblasts in AS and RA, studies on RA may support us in determining the specific mechanism by which ASFs relate to inflammation.

With the subchondral ASF-rich tissue forming up with infiltrated immune cells, a high density of CD34+ microvessels was also observed in AS subchondral bone. It was reported to increase only when there was still cartilage at the surface of the femoral heads.[32] Growth factors such as vascular endothelial growth factor (VEGF) released by ASFs may support angiogenesis, which is supposed to allow inflammatory factors and immune cells to access and participate in the AS inflammatory environment and even lead to bone erosion.[8,51,52] Another report suggested that microvascular aggregation was observed not only near lymphocytic infiltration but also at sites of new bone formation, where osteoblasts lined the trabecular bone.[45] It implied that the formation of microvessels had a different role in AS, beyond just contributing to inflammation. This role could be pivotal for bony ankylosis in AS, potentially due to increased nutritional needs. However, the exact mechanism behind this process still necessitates further exploration (Figure 2).

ASFs Promote Joint Remodeling

According to recent studies discussed in this review, it is clear that fibroblasts are inextricably linked to the different stages of structural damage progress in AS:

1. Inflammation: The disease begins with inflammation, typically in the sacroiliac joints and spreads to the spine in most patients. 2. Erosive destruction: These refer to the wearing away of the bone, which are considered minor signs of

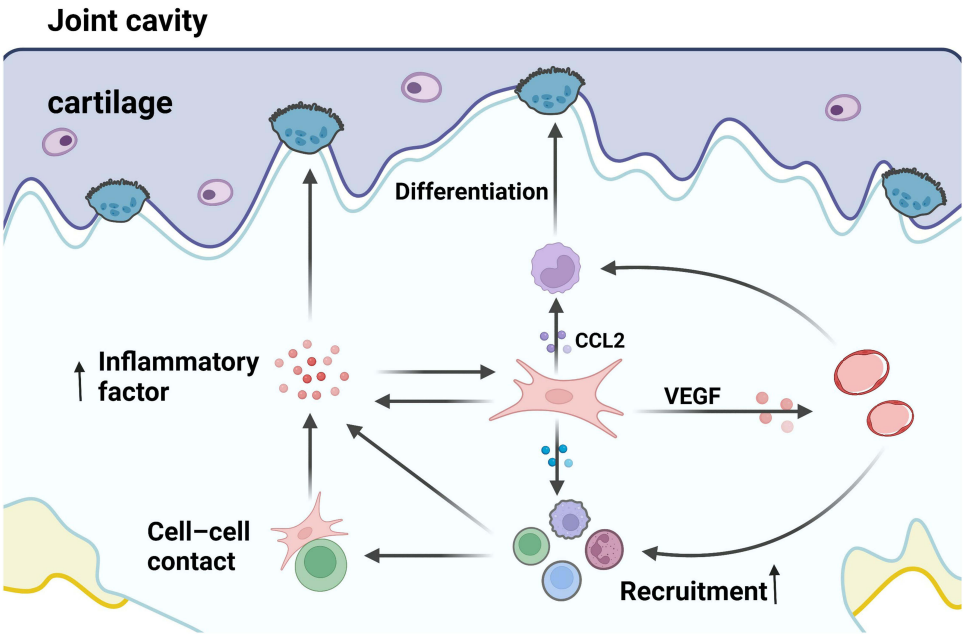

Figure 2 ASFs mediate the inflammatory response. Subchondral ASF-rich tissue is infiltrated with inflammatory cells in the early phase of disease. Vascular endothelial growth factor (VEGF) `released by ASFs may support angiogenesis, which is supposed to allow inflammatory factors and immune cells to access and participate in the inflammatory environment. Biomechanical forces in joints would cause fibroblasts to secrete CCL2 among other chemokines responsible for the attraction of inflammatory monocytes towards biomechanically exposed sites and lead to the differentiation of these cells into osteoclasts. Inflammatory factors may enhance the destructive properties of ASFs, including tumour necrosis factor (TNF)-α, IL-6 and IL-1b. In turn, activated ASFs further enhance the inflammatory cycle by producing inflammatory factors themselves. Moreover, cell–cell contact between ASFs and inflammatory cells enhances the activation of ASFs, increasing the release of inflammatory cytokines. These factors, in turn, recruit, activate and promote the differentiation of multiple cell types that contribute to the inflammatory cycle, which may promote the inflammatory response. Created with Biorender.com.

progression. 3. Syndesmophyte formation: syndesmophytes are bony growths inside the ligaments of the spine. They are a characteristic feature of AS and can lead to reduced physical function and quality of life.[53] 4. Ankylosis: This is the final stage where the vertebrae fuse together due to the growth of syndesmophytes, leading to a rigid and inflexible spine.[11] Unlike Ossification of the Posterior Longitudinal Ligament (OPLL), whose most common location happens in the cervical spine region and can compress the spinal cord and cause neurological deficits,[54,55] symptoms like pain and stiffness caused by AS always start from the lower back. Radiographs of sacroiliac arthritis, one of the most common causes of low back pain, are the hallmarks of AS structural damage progress, which is presented as ASF-related subchondral sclerosis, uniform joint space narrowing and erosions in early and ankylosis in later progress.[56–58]

ASFs Affect Osteoclasts to Promote Bone Erosion

In AS-mediated joint destruction, especially erosion of the subchondral bone plate of the joint, ASFs may promote osteoclast function or initiate their recruitment to promote bone erosion (Figure 1a). Some pathological studies have shown that ASFs can mediate the invasion of the subchondral bone plate by forming a cohort of osteoclasts towards the joint surface.[8,10,11] In monocyte lineage, osteoclasts express receptor activator of nuclear factor (NF)-κB (RANK), whereas cells of the mesenchymal lineage, such as osteoblasts, fibroblasts, and synoviocytes, express RANKL.[59] As a key mediator of osteoclast formation, RANKL directly induces osteoclast development and bone resorption when binding to RANK.[60,61] The mechanism by which ASFs mediate osteoclast enhancement may be the secretion of RANKL as mesenchymal cells, which enhances osteoclast function.[42]

Based on histological evidence, the presence of osteoclasts seems to be related to inflammation since they always increase and then decrease simultaneously.[11,32,62] Moreover, studies have shown that inflammatory cytokines such as TNF, interleukin-1 (IL-1), and IL-17 stimulate RANKL expression on mesenchymal cells while also increasing RANK's action on osteoclasts by CD40 ligand, as well as by Toll-like receptor 2 (TLR-2) and TLR-4 ligands in vitro, thus inducing bone destruction and causing a downregulation of osteoblast function.[63–65] Both of the above results led us to the conclusion that inflammation preceding bone formation would enhance osteoclast function by upregulating RANKL secretion. However,

the AS expression of RANKL, osteoprotegerin (OPG), and RANK in another study appeared to be largely independent of the levels of systemic and local inflammation.[65] Results that seemed contradictory require further study.

ASFs Affect Osteoblasts to Promote Bone Formation

Depending on some evidence, ASFs with osteoclasts towards the joint surface might bring osteoblasts closer to the cartilage by creating a channel through the subchondral bone plate. It is noteworthy that this hypothesis corresponds to the irregular ankylosis observed on radiographs of AS joints (Figure 1b). This suggests that the remaining cartilage islands in joints with broken cartilage are transformed into bone by the invading groups of ASFs and other functional cells mediated by them.[9,12] However, the specific mechanism is still unclear, although the possibility is that TNF and IL-17 can cause bony proliferation when there is no contact between osteoblasts and osteoclasts by upregulating receptor activator of nuclear factor-κ B Ligand (RANKL) secretion of mesenchymal lineages, such as osteoblasts, fibroblasts, and synoviocytes.[42,63] In addition, osteogenic differentiation characteristics that have been proven to be overexpressed by ASFs, such as bone morphogenetic protein (BMP), may facilitate cellular differentiation of osteoblasts,[66] and the function of Cx43 discussed in a later section may provide another potential mechanism.

Therefore, based on histological evidence and the close relationship between fibroblasts and osteoblasts,[29] it would be important to understand AS aetiology to demonstrate how ASFs affect osteoblasts through specific molecular mechanisms.

ASFs Contribute to Fat Enrichment

Several MRI studies have demonstrated that "inflammation → fat deposition → new bone formation" can be observed in MRI and X-ray findings during follow-up of AS patients over time.[67] An obvious correlation was found between new fat deposition and new bone formation. Once AS patients have developed fat deposits, new bone formation continues to occur more frequently in both the clinical trial and the observational cohort.[68,69] Taken together, the above results suggest that fat deposition plays an important role in new bone formation in AS.

Previous studies have suggested that fat deposition is mainly composed of adipocytes. However, adipocytes alone could not explain the "increased local fat deposition but lower body fat level in AS"; this opposite phenomenon suggests that other cells may be involved in fat accumulation.[70] Recent studies have shown that ASFs also play a role in fat deposition in two ways (Figure 1c).

In the first place, Bleil et al found evidence of adipocyte accumulation in ASF-rich tissue, manifested either as cells containing a large fat vacuole resembling adipocyte or as spindle-shaped direct lipid accumulation within the ASFs occasionally.[12]

In addition, some researchers observed the conversion of myofibroblasts to adipocytes, which could be a theoretical basis for the hypothesis that lipid accumulation occurs within the extracellular matrix or within ASFs. Interestingly, BMP signaling is necessary for this process.[71] Overexpression of BMP signaling in ASFs strengthens the belief that ASFs may be involved in fat deposition.

ASFs Activate as Myofibroblasts to Form Extracellular Matrix Independent of Inflammation

Currently, the results from multiple randomized clinical trials showed that new bone formation or radiographic progression is not inhibited by the usage of various TNF-α blockers for 2–4 years,[72–75] suggesting that bone formation in AS is at least partly dissociated from inflammation. The study of Yeremenko et al suggested that ASFs' participation in bone remodeling is mostly independent from the inflammatory environment and instead seems to be caused by an intrinsic transcriptional signature.[21] Their analysis of AS biopsy samples showed that a large majority of the genes that were overexpressed in AS synovium were related to muscle–myocyte–myofibroblast biology, and α-smooth muscle actin (α-SMA) showed substantial colocalization with CD146 and CD90, which are markers of myofibroblasts, pericytes, and mesenchymal stem cells, accompanied by the expression of the fibroblast markers vimentin and prolyl 4-hydroxylase β.[21] In addition, the levels of Transforming Growth Factor Beta-1 (TGF-β1), which is perhaps the most impactful morphogen in mediating pathological fibroblasts in myofibroblast differentiation,[76] were increased in ligamentum flava and paraspinal muscle tissues of AS patients.[77] Therefore, it is natural to hypothesize that ASFs may play a role by activating myofibroblasts and hypothetically forming collagen-rich extracellular matrix adjacent to the articular bone (Figure 1d).

Calcification of these extracellular matrices, when Ca2+ and Pi present at physiologic concentrations,[78] might result in syndesmophyte formation and progressive ankylosis. Beyer and JHW Distler thought the findings above provide a new idea and a basic dataset to build upon in new research projects in AS. In their hypothesis, the pathogenetic feature of fibrotic diseases may relate to AS, and thus, the disease mechanisms from fibrotic disease may be translated to the pathogenesis of AS. If this hypothesis could be proven by future studies, blocking extracellular matrix formation and calcification, and thus blocking syndesmophyte formation and ankylosis in AS,[78–80] may be more readily achieved by using the therapeutic toolbox of fibrotic disease to target fibroblasts.[79,80] Surprisingly, Stougaard et al demonstrated that the antifibrotic drug pirfenidone could inhibit fibroblast-like synoviocyte-related osteoblast mineralization in spondyloarthritis.[81]

Direct Transformation of ASFs into New Bone

Progressive ankylosis, the most unique phenomenon in AS, is due to abnormal bone formation. Recent studies have shown that ASFs have the potential for osteogenic differentiation themselves, either by observation at the tissue level[9] or by isolated cultures of fibroblasts from patients[13–15] (Figure 1e). Osteogenic markers are markedly upregulated in ASF cultures.[16] There is no doubt that there is a theoretical basis since fibroblasts are of the same origin as osteoblasts.[29] In this review, we will report the results of cell-level studies in detail.

Cell-Level Studies of ASFs

Signaling Pathways Mainly Involved in ASF Ossification

Wnt/β-Catenin

Having been historically subdivided into three main branches, Wnt signaling contains the canonical Wnt pathway, the Wnt/PCP pathway, and the Wnt/Ca2+ pathway. The fact that canonical pathways are a major component of Wnt signaling in bone cells has led to a relatively clear understanding of the mechanisms by which Wnt affects bone.[82] We focus on how Wnt signaling relates to the ossification of ASFs.

Some target molecules have been reported to affect ASFs via Wnt signaling (Figure 3).

First, a recent study showed that a low expression of miR-124 enhanced GSK-3β expression and in turn weakened Wnt/β-catenin pathway activity, leading to the inhibition of osteogenic differentiation of ligament ASFs.[83] In the absence of Wnt, the destruction complex containing Gsk-3β phosphorylated β-catenin, leading to the proteasomal degradation of β-catenin. In addition, PGE-2 activated the trimetric G-protein by binding to its EP2 receptor and then activated PI3-kinase, which in turn activated Akt. Then, Akt phosphorylated GSK-3β, leading to the inhibition of its phosphorylation of β-catenin. Based on this, celastrol (an active compound isolated from Tripterygium wilfordii) was found to inhibit PGE-2-induced osteogenic differentiation of ASFs in vitro.[84] Second, DVL-2 prevented β-catenin by titrating GSK-3β from Axin complex degradation and was identified as the target of miR-495 and highly expressed in AS. Du et al found that miR-495 and si-DVL-2 upregulated the expression of β-catenin and downregulated the p-β-catenin level in synovial ASFs.[85] Third, the canonical Wnt signaling pathway can be antagonized by secreted proteins from the Dickkopf (Dkk) family, which bind with high affinity to lipoprotein receptor-related protein 5/6 (LRP5/6) and thereby directly prevent Wnt binding.[86] Several recent studies have also found that the level of DKK-1 bound to LRP-6 is lower in AS patients than in healthy controls. Downregulation of DKK-1 enhances the proliferation and osteogenic potential of ASFs via the Wnt/β-catenin signaling pathway in vitro.[87] MiR-17-5p and miR-146a have been proven to affect the proliferation and osteogenic potential of ASFs by regulating DKK-1 expression.[20,88] In addition, some other factors involved in Wnt signaling directly bind extracellular Wnt, such as sFRP3/FRZB (members of the sFRP family). It has been reported that IL-22 has the capacity to increase the expression of sFRP3/FRZB and thereby inhibit Wnt signaling in fibroblasts in both in vitro and ex vivo models.[89]

In addition, some factors have been proven to affect ASFs' proliferation and ossification by Wnt signaling through an exact mechanism. C-X-C chemokine receptor type 4 (CXCR4) was upregulated in AS and led to increasing ossification and growth rates of ASFs.[90,91] In contrast, inorganic pyrophosphate transport regulator Gene (ANKH) overexpression inhibited viability, mineralization, and ossification in ASFs.[92] Both of the above findings may indicate a new way to study ASFs via Wnt signaling.

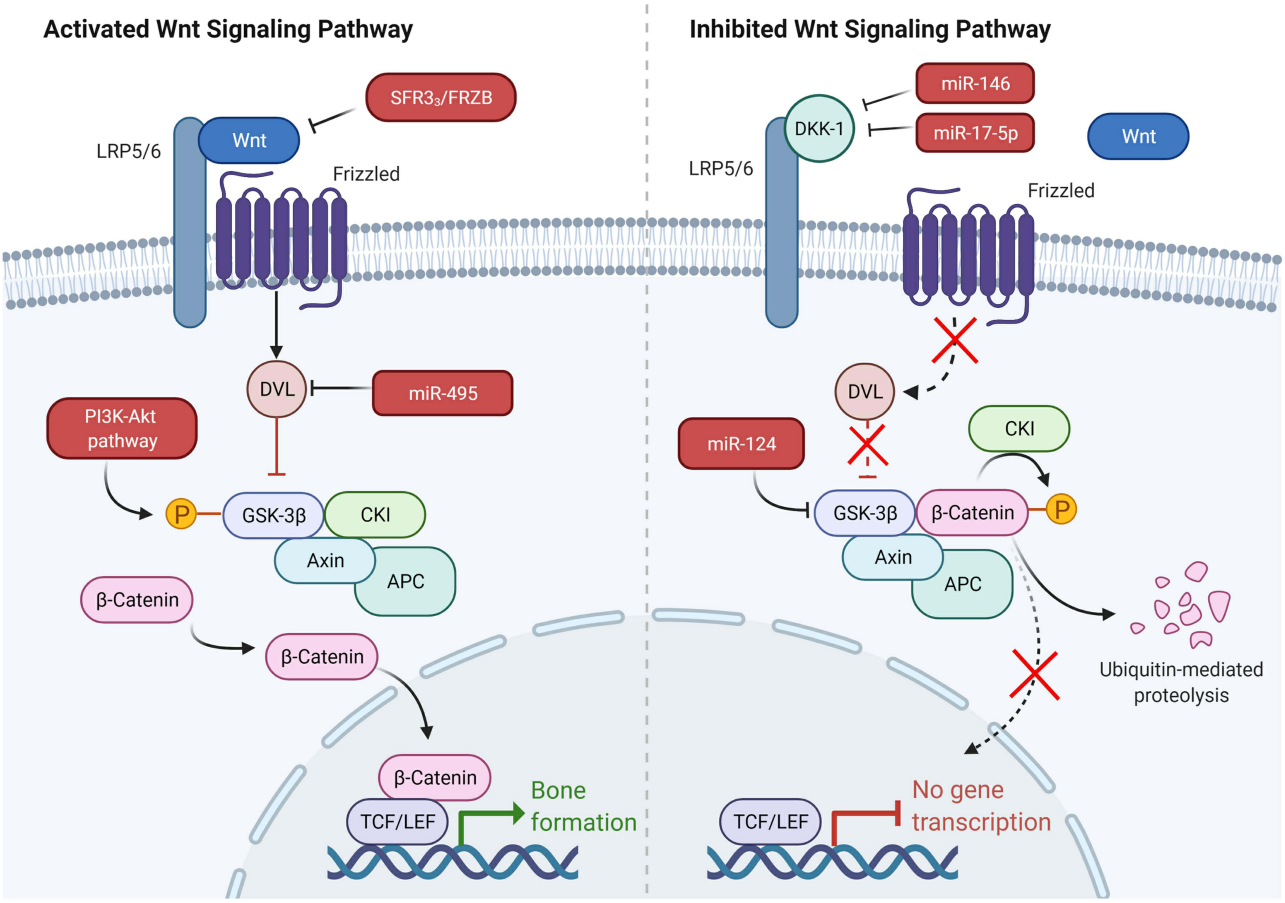

Figure 3 Some target molecules that affect ASFs via Wnt signaling. Downregulation of miR-124 expression enhanced GSK-3β expression, weakened Wnt/β-catenin pathway activity. PGE-2 activated the trimeric G-protein by binding to its EP2 receptor and then activated PI3-kinase, which in turn activated Akt. Then, Akt phosphorylated GSK-3β, leading to the inhibition of its phosphorylation of β-catenin. DVL-2 prevented β-catenin by titrating GSK-3β from Axin complex degradation and was identified as the target of miR-495 and highly expressed in AS. MiR-495 and si-DVL-2 upregulated the expression of β-catenin and downregulated the p-β-catenin level in synovial ASFs. Dickkopf (Dkk) family bind with high affinity to lipoprotein receptor related protein 5/6 (LRP5/6) and thereby directly prevent Wnt binding. MiR-17-5p and miR-146a affect the proliferation and osteogenic potential of ASFs by regulating DKK-1 expression. sFRP3/FRZB (members of the sFRP family) directly bind extracellular Wnt. IL-22 has the capacity to increase the expression of sFRP3/FRZB and thereby inhibit Wnt signaling. Created with Biorender.com.

BMP/TGFβ-Smads

The TGF-β superfamily comprises a group of polypeptide factors with similar structures, including TGF-β, BMPs, and over 30 other members, that play indispensable roles in many cellular functions, such as bone reconstruction. Both TGF-β1 and BMP2 are thought to be involved in bone formation as important osteogenic mediators.[93] Recent studies have suggested that BMP/TGF-β signaling contributes to the progression of AS and emerging evidence has shown high expression of the BMP/TGF-β signaling pathway in ASFs.[94]

BMP receptors contain type I and type II receptors. Activated through binding with a ligand to induce autophosphorylation, the type II receptor activates the type I receptor, which in turn forms a complex receptor and finally leads to the BMP activation.[95] Core-binding factor a1 (Cbfa1)/runt-related transcription factor 2 (Runx2), which is an essential transcription factor for osteoblastic differentiation and osteogenesis, is reported to be dependent on Smad1/Smad5 activation caused by BMP-2 stimulation.[96] mRNA expression of Cbfa1 was induced by continuous activation of Smad1 or Smad5. In contrast, transfecting the cells with dominant-negative Smad1, Smad4, Smad5, or Smad6 significantly reduced the BMP-2-induced expression of Cbfa1.

Cbfa1/Runx2, bone morphogenetic protein receptors (BMPR-I/II), and Smad family protein receptor-regulated Smads (Smad1/5/8), common-partner Smad (Smad4), and phosphorylated Smads (pSmad1/5/8) were found to be overexpressed in ASFs in multiple studies. TGF-β1 and p-Smad2/3 were also found to be overexpressed by Zhang et al.[97] These data,

together with the findings summarized in Table 1,[16,77,97–104] confirm that ASFs are target cells of BMP/TGF-β signaling and indicate that a highly activated BMP/TGF-β signal transduction pathway exists in ASFs. Concurrently, based on the evidence that the Smad6 expression is lower in ASFs than normal, it reveals the lack of self-regulation and inhibition in the BMP/TGF-β signaling of ASFs.

β-proteoglycan (TβRIII), originally defined as an auxiliary receptor of the TGF-β superfamily, was an abundant membrane-anchoring protein. A recent study has reported that TβRIII plays a critical role in TGF-β/Smad signaling. Another report suggested that using a biosensor method, BMP2, BMP4, and BMP7 can bind to TβRIII. BMP2 activity was significantly downregulated due to the loss of TβRIII.[106] TβRIII binds all three TGF-β ligands and BMP2, and Zhang et al further elaborated that the expression of TGF-β1, BMP2, and TβRIII was markedly increased in ASFs. Moreover, TGF-β1 combined with BMP2 significantly upregulated the expression of TβRIII but not TβRI or TβRII, further suggesting that TβRIII upregulation may participate in the osteogenic differentiation of ASFs.[97]

Matrix metalloproteinase (MMP)-2 has recently been demonstrated to be associated with AS, the mRNA expression level of which in the ASFs group was approximately four times higher than that in the control group. Moreover, the expression of Cbfa-1 was significantly downregulated by MMP-2 gene silencing and in turn inhibited the activation of the BMP/Smad signaling pathway. The results revealed that MMP-2 gene silencing may reduce the osteogenesis of ASFs

Table 1 BMP/TGF-β Signaling in ASFs

The TGF-β Signal Transduction Pathway	Osteogenic Marks and Others	Finding	Ref.
Cbfa1, BMPR-I and BMPR-II, Smad1, Smad5, Smad4, pSmad1, pSmad5, Smad6(-)	ALP activity, collagen, osteocalcin	The fibroblasts of hip joint capsules in patients with AS cultured in vitro have biologic characteristics of osteogenic differentiation and may be important target cells of AS ossification. The Activated BMP/Smads signalling pathway could potentially be a mechanism relating to fibroblasts differentiating into osteoblasts and an ossification mechanism for AS.	[16]
Cbfa1	ALP activity, collagen, osteocalcin	Icariin can inhibit the promotion effect of cytokine BMP-2 on the expression of AS fibroblast specific transcription factor Cbfa1 or Osx, so as to achieve the purpose of inhibiting fibroblast ossification.	[98]
Smad1, Smad4, pSmad1, RUNX2	–	Ele could have a hand in anti-osteogenic differentiation of AS fibroblasts by inhibiting the BMP/SMADs signal pathway and subsequently blocking expression of ossification marker genes RUNX2 that initiate the osteogenic differentiation.	[99]
Cbfα1	Cx43/pCx43	BQZ is able to decrease the protein levels of Cx43/pCx43 and Cbfα1 in fibroblasts in the presence or absence of rhBMP-2.	[100]
TGF-β1	MMP-3	Increased levels of MMP-3 and TGF-β1 may contribute to the supraspinous ligament degeneration and fibrosis in ligamentum flava and paraspinal muscles, respectively, during the progression of AS.	[105]
Cbfa1, pSmad1, pSmad5, Smad4	–	BSQJ can inhibit osteogenic differentiation of AS fifibroblasts in vitro by suppressing the activation of the BMP/Smads signal pathway.	[101]
BMPRII, Cbfal pSmad1, pSmad5 Smad1, Smad4, Smad5 and Smad6(-)	NF-α, IL-1β, IL-6	Triptolide may be used to treat AS, the mechanism of which may be through the BMP/Smad pathway.	[102]
Cbfa-1, BMP-2, Smad1, Smad4, Smad1/5/8	MMP-2	MMP-2 gene silencing may reduce the osteogenesis of fibroblasts in AS by inhibiting the activation of the BMP/Smad signalling pathway.	[103]
Runx2, BMPR-II, p-Smad5, Smad5	ALP activity, collagen-1, osteocalcin	miR-214-3p could inhibit osteogenic differentiation of AS fibroblasts by targeting BMP2 and blocking the BMP–TGFβ axis	[104]
TβRIII, TGF-β1, BMP2, p-Smad2/3, Smad4, p-Smad1, RUNX2	S100A4	TGF-β1 combined with BMP2 may participate in the osteogenic differentiation of AS-SLFs by acting on up-regulated TβRIII, thus resulting in excessive activation of both TGF-β1/Smad and BMP2/BMPR1A/Smad/RUNX2 signalling.	[97]

by inhibiting the activation of the BMP/Smad signaling pathway.[103] Connexin43 (Cx43) is a major gap junction protein in bone and has been shown to play a critical role in osteoblast differentiation in recent studies. A study suggests that cell communication mediated by gap junctions is indispensable for osteoblast differentiation induced by BMP-2.[107] Fibroblasts from AS and Ossification of the Posterior Longitudinal Ligament (OPLL) patients were reported to have osteogenic characteristics that were upregulated by Cx43.[100,108] These studies indicated that Cx43 may be a potential target to explain how ASFs affect osteoblasts.

Pi3k/Akt and AMPK

Regarding the inflammatory factors that are overexpressed in ASFs, Qin et al reported that as a kind of specific agonist for adenosine 5'-monophosphate activated protein kinase (AMPK), which was blocked in AS, metformin could decrease these inflammatory factors and inhibit ASF ossification.[109] This finding indicates the potential of the Pi3k/Akt and AMPK pathways to mediate inflammation and bone formation in AS. In addition, rapamycin blockade of mammalian target of rapamycin (mTOR) in AS also ameliorated ASF-mediated pathological progression independent of IL-17A and TNF-α cytokines,[110] which also confirmed the role of the Pi3k/Akt and AMPK pathways in ASF ossification.

MAPK-ERK

In ASFs, the high expression of Annexin A2 upregulated by IL-6 drew the attention of Li et al. They performed further research and observed that silencing of Annexin A2 ameliorated the ASF ossification induced by IL-6. They also proved that Annexin A2 might activate extracellular signal-regulated kinase (ERK) signaling and induce ASF ossification by mitogen-activated protein kinase (MEK) inhibitor experiments.[111] In addition, mitogen-activated protein kinase 1 (MAPK1) was also observed to increase in synovial ASFs.[91]

Other Cytokines in ASFs

Apart from investigating signaling pathways, many other researchers have attempted to explore and identify the correlations between ASFs and inflammatory and bone ankylosing processes in AS by studying microRNAs[112,113] (summarized in Table 2 [20,83,85,88,103,104,114–118]) and other cytokines (summarized in Table 3). The results are obviously based on the fact that with cytokine secretion and the function of mediating the inflammatory microenvironment, ASFs have been proven to have apparent osteogenic potential. Thus, it is natural to hypothesize that ASFs

Table 2 Micro RNA in ASFs

Micro RNA	Function	Finding	Ref.
hsa-miR-20a, hsa-miR-300, hsa-miR-185, hsa-miR-30d, hsa-miR-320a, hsamiR-130b, hsa-miR-33a, hsa-miR-155, hsa-miR-222	Osteogenic differentiation of human ligament fibroblasts	Osteoclasts might induce the osteogenic differentiation of fibroblasts in vitro and that miRNA may play an important role in regulation of the cell-cell interaction between osteoclasts and fibroblasts.	[114]
miR-17-5p, miR-27b-3p	Increase the osteogenic differentiation potentials of ligament fibroblasts	Provided comprehensive lncRNA, miRNA, and mRNA profiles for AS hip joint ligaments. Bioinformatics approaches were used to predict the potential functions of DE mRNAs and initially explore their roles in the pathogenesis of AS. The cell experiment indicated that miRNAs might participate in ossification.	[115]
miR-146a	Enhanced proliferation and osteogenic potential of ASFs by inhibiting DKK1 expression	MiR-146a knockdown hindered AS progression partially by regulating target DKK1 expression, offering a potential therapy application for AS patients.	[88]

(*Continued*)

Table 2 (Continued).

Micro RNA	Function	Finding	Ref.
miR-124	Enhanced the differentiation of ligament ASFs into osteoblasts by inhibiting GSK-3β expression	MiR-495 depressed inflammatory response and promoted bone differentiation of HFLS cells, and this was accompanied by mediating wnt/β-catenin /Runx-2 pathway by targeting DVL-2.	[83]
miR-495	Depressed inflammatory response and promoted bone differentiation of synovial ASFs	MiR-495 depressed inflammatory response and promoted bone differentiation of HFLS cells, and this was accompanied by mediating wnt/β-catenin /Runx-2 pathway by targeting DVL-2.	[85]
miR-17-5p	Regulated osteogenic differentiation of ASFs by targeting the 3' UTR of ankylosis protein homolog (ANKH)	Reveal a role of the miR-17-5p-ANKH axis in the regulation of heterotopic ossification, which is essential for therapeutic intervention in heterotopic ossification in AS.	[20]
miR-214-3p	Prevent ASFs osteogenic differentiation by targeting BMP2 and blocking BMP-TGF axis	MiR-214-3p could prevent AS fibroblast osteogenic differentiation by targeting BMP2 and blocking BMP-TGFβ axis.	[104]
miR-204-5p	Inhibited the osteogenic differentiation of ligament ASFs by targeting Notch2	MiR-204-5p may negatively regulate Notch2 expression and may be a potential therapeutic target for AS.	[103]
miR-21	Contributed to new bone formation and significantly elevated expressions of STAT3, JAK2, and IL-17	MiR-21 may act as a potential mediator between new bone formation and inflammation in AS.	[116]
miR-204	Target and inhibit GSDMD protein expression, inhibited the pyroptosis rate and Caspase-1/PI double-positive cells and reduced [Ca2+], ROS, NLRP3, Caspase-1 and Caspase-11 levels in ASFs	TGF-β1 combined with BMP2 may participate in the osteogenic differentiation of AS-SLFs by acting on up-regulated TβRIII, thus resulting in excessive activation of both TGF-β1/Smad and BMP2/ BMPR1A/Smad/RUNX2 signalling.	[117]
miR-1290	Negatively regulated CDK6 expression to enhance cell proliferation	Hsa_circ_0056558 and CDK6 suppressed cell proliferation and differentiation while enhanced cell apoptosis by competitive binding to miR-1290 in AS, which might be possibly achieved by PI3K/AKT/ NF-κB pathway	[118]

Table 3 Several Cytokine and Cell Types Involved in as Bone Homeostasis

Cytokines	Cell Type	Role in AS Bone Destruction	Ref.
RANK	Osteoclasts	As a key mediator of osteoclast formation, RANKL directly induces osteoclast development and bone resorption when binding to RANK.	[60,61]
RANKL	Mesenchymal cells (ASFs, osteoblasts, synoviocytes)	The mechanism by which ASFs mediate osteoclast enhancement may be the secretion of RANKL as mesenchymal cells, which enhances osteoclast function.	[42]
TNF, IL-1, IL-17	Mesenchymal cells (ASFs, osteoblasts, synoviocytes)	Inflammatory cytokines stimulate RANKL expression.	[63–65]
CD40 ligand, TLR-2 and TLR-4 ligands	Osteoclasts	Inflammatory cytokines increase RANK's action on osteoclasts.	[63–65]
VEGF	ASFs	Facilitates ASF ossification in synergy with TNF-α, IL-17, IL-6.	[105]
MPO	ASFs	Abnormal increase may induce high expression of inflammatory factors in ASFs through the phagosome pathway.	[119]
CCL2, TNC	ASFs	Overexpressed by ASFs in response to mechanical stress, leading to pathological progression.	[49]
MAPK-ERK	ASFs	MAPK1 was observed to be increased in synovial ASFs. Annexin A2 might activate ERK signaling and induce ASF ossification by MEK inhibitor experiments.	[91,111]

would be a bridge between inflammatory and abnormal bone formation in AS by secreting cytokines or being affected by other cytokines.

The overexpression of TNF-α, IL-17, IL-6 and other cytokines is believed to play an important role in the abnormal pathological changes in ASFs.[110,120] These cytokines synergized with VEGF to facilitate ASF ossification, and the mRNA levels were markedly positively correlated with the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI).[105] Concurrently, Yu et al supposed that the abnormal increase in myeloperoxidase (MPO) may induce the high expression of inflammatory factors in ASFs through the phagosome pathway.[119]

Recently, based on anatomical characteristics and mechanobiology in AS-affected areas, such as sacroiliac joints and entheses, researchers have started to search for a correlation between mechanical force and AS pathogenesis.[121] Emerging evidence has shown that in response to mechanical stress, ASFs overexpress some cytokines, such as CCL2[49] and Tenascin-C (TNC),[122,123] leading to pathological progression. The activation of the Hippo/YAP signaling pathway would be a novel mechanism, although more research is required to confirm this hypothesis.

Concluding Remarks

As the pathological mechanism of AS has been further investigated, there is a growing awareness of the important role that ASFs may play in the regulation of the AS-specific inflammatory environment and in mediating the subsequent progression of ankylosing bone proliferation. This review looks at previous studies and for the first time summarizes the role of fibroblasts in AS pathology, suggesting that ASFs play a crucial role in AS. We also attempt to theoretically elucidate the unspecified issues of "bone metabolism", "inflammation and osteogenesis" in AS at the fibroblast level by reviewing the literature.

In conclusion, ASFs, as a group of frequently observed cells among the histologic AS features,[8,9] organize immune functions by recruiting immune cells and influencing their differentiation and activation, thus mediate the inflammatory response in the early phase of disease. ASFs promote joint destruction at sites of cartilage and actively promote abnormal ossification by recruiting osteoblasts, differentiation into myofibroblasts or ossification directly. Besides, evidence of adipocyte accumulation in ASF-rich tissue also relates ASFs to fat deposition in AS.[12] A growing body of evidence has pointed towards the involvement of many signaling pathways and cytokines. The mediation of bone erosion is illustrated by ASFs' secretion of RANKL as mesenchymal cells,[42] which directly induces osteoclast development and bone resorption when binding to RANK.[60,61] When it comes to in vitro ASF ossification research, Wnt signaling and[90,91] BMP/TGF-β signaling[16,98] are proved to be the major factors. In addition to being involved in ossification, TGF-β is also an impactful factor in ASFs' myofibroblast differentiation.[21,76] Among the molecular mechanism study of ASFs in AS inflammation and osteogenesis, TNF and IL-17 are active factors that draw our attention,[42,63] which may even be the link that connects them.

However, most of the current studies on ASFs are limited to the cellular level or histology, and the candidate targets and gene expression modifications discussed above are primarily obtained from in vitro cell studies. The vast discrepancy between the in vitro cell culture and the in vivo complex pathological microenvironment may lead to large differences in the relevant studies and thus limits further understanding. Besides, a number of the in vitro studies above lack good data and stringent experimental conditions including proper controls, which makes them only offer some reference value that still requires further study to confirm. Another major limitation may be that the distinction between pathogenic subtypes of ASFs is unclear. It is still unknown which ASF subtypes exerting pathological effects may present optimal targets. In particular, when fibroblasts are used as therapeutic targets, identifying such pathological subtypes will also contribute to avoiding the impact on normal fibroblasts. Encouragingly, rapid advances in RNA sequencing, tissue single-cell profiling, and spatial transcriptomic techniques would enable the exact identification of ASFs.[43]

With increasing attention given to ASF investigations, elucidating the key roles and molecular mechanisms of ASFs in AS inflammation and osteogenesis would provide new targets and directions for AS diagnosis and treatment from a new perspective of fibroblasts.

Abbreviations

ASFs, ankylosing spondylitis fibroblasts; AS, ankylosing spondylitis; SpA, spondyloarthritis; RASFs, rheumatoid arthritis synovial fibroblasts; MSCs, mesenchymal stromal cells; TNF, Tumour Necrosis Factor; IL-17A, Interleukin-17A; CCL2, C–C motif ligand 2; VEGF, vascular endothelial growth factor; BMP, Bone morphogenetic protein; RANKL, receptor activator of nuclear factor-κ B Ligand; IL-1, interleukin-1; TLR-2, Toll-like receptor 2; OPG, Osteoprotegerin; LRP5/6, lipoprotein receptor-related protein 5/6; CXCR4, C-X-C chemokine receptor type 4; ANKH, inorganic pyrophosphate transport regulator Gene; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase; MMP, Matrix metalloproteinase; OPLL, Ossification of the Posterior Longitudinal Ligament; TGF-β1, Transforming Growth Factor Beta-1; AMPK, adenosine 5'-monophosphate activated protein kinase; mTOR, mammalian target of rapamycin; TNC, Tenascin-C.

Data Sharing Statement

The data that support the findings of this study are available. The authors will supply the relevant data in response to reasonable requests.

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Disclosure

The authors declare that they have no competing interests in this work.

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