Molecular pathogenesis of fracture nonunion

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Abstract Fracture nonunion, a serious bone fracture complication, remains a challenge in clinical practice. Although the molecular pathogenesis of nonunion remains unclear, a better understanding may provide better approaches for its prevention, diagnosis and treatment at the molecular level. This review tries to summarise the progress made in studies of the pathogenesis of fracture nonunion. We discuss the evidence supporting the concept that the development of nonunion is related to genetic factors. The importance of several cytokines that regulate fracture healing in the pathogenesis of nonunion, such as tumour necrosis factor-α, interleukin-6, bone morphogenetic proteins, insulin-like growth factors, matrix metalloproteinas and vascular endothelial growth factor, has been proven in vitro, in animals and in humans. Nitric oxide and the Wnt signalling pathway also play important roles in the development of nonunion. We present potential strategies for the prevention, diagnosis and treatment of nonunion, and the interaction between genetic alteration and abnormal cytokine expression warrants further investigation.

The translational potential of this article: A better understanding of nonunion molecular pathogenesis may provide better approaches for its prevention, diagnosis and treatment in clinical practice.

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Introduction

Fracture healing is a complex but well-orchestrated process that results in the regeneration and functional restoration of bones. The US Food and Drug Administration has defined fracture nonunion as the absence of radiographic healing over 9 months with no visible healing progression in the last 3 months. As a serious fracture complication, nonunion has a significant effect on the quality of life and financial situation of patients and may be associated with
severe functional and psychological impairments. The latest research has demonstrated that the average risk of nonunion per fracture was 1.9%, and the rate of nonunion was up to 9% in specific fracture types (tibial and clavicular fractures) and in old patients [1]. According to a 2007 study, the cost of nonunion treatment is as high as US$25,000 per patient, more than twice that for normal fracture healing [2].

Many factors, including systemic, local and treatment factors, impair fracture healing and eventually result in nonunion. Systemic factors mainly include age, nutrition and systemic diseases, the most common of which are diabetes mellitus, anaemia and endocrine disorders. Local factors, such as the type of fracture, blood supply and infection, may also influence fracture healing. In addition, some evidence indicates that smoking and certain medications, including nonsteroidal antiinflammatory drugs (NSAIDs) and corticosteroids, might have side-effects that affect bone healing [3,4].

Although these environmental causes of nonunion at the individual (macro) level have been extensively studied, the specific signal pathways involved remain unclear. Under the influence of external risk factors, the systemic or local expression of specific cytokines and growth factors might be disturbed. In addition, some patients present a predisposition towards nonunion development because of genetic defects. For example, young patients without any systemic disease develop nonunion at every fracture site after multiple fractures, even when undergoing proper treatments. Genetic factors also lead to abnormal expression of cytokines and result in nonunion. Alterations of genes and cytokines, at the molecular level, are discussed in this review (Figure 1).

Three aspects involved in the molecular mechanism of nonunion development are discussed in this review: genetic factors, abnormal cytokine expression and other small molecules. A clear understanding of the molecular pathogenesis of nonunion can improve our knowledge of this complication and provide different approaches for its prevention, diagnosis and treatment at the molecular level.

Genetic factors

From a clinical perspective, it remains unknown why some patients without systemic or local risk factors present a predisposition towards nonunion development. Much evidence indicates that specific genetic variants and abnormal gene expression are the inherent causes of many diseases, which may also be true for fracture nonunion. Indeed, recent studies in this field have yielded valuable findings. The genetic factors related to nonunion that have been investigated to date are described below, and details of the related experiments are summarised in Table 1.

The first clinical study on the genetic predisposition towards nonunion was published in 2011 by Dimitriou et al. Fifteen single nucleotide polymorphisms (SNPs, which are a variation, such as a substitution, deletion or insertion, in a single nucleotide at a specific gene position) in four genes of the bone morphogenetic protein (BMP) pathway (BMP-2, BMP-7, Noggin and Smad6) were examined in 109 patients retrospectively. Two specific SNPs in Noggin and Smad6, both inhibitors of BMPs, appear to be responsible for the development of atrophic nonunion. It should be noted that this study did not exclude patients with other environmental risk factors, such as the type of fracture, smoking and NSAIDs use, because no significant difference in these factors was found between groups; however, the conclusion is weak because of the small size of the specific samples [5]. Thus, the correlation between these genes and nonunion needs to be further investigated. After excluding patients with other environmental factors, Zeeck et al. analysed SNPs in several cytokine genes that regulate fracture healing in patients with aseptic nonunion after femoral and tibia shaft fractures. Based on a comparison of the findings with those for patients with normal fracture healing, a platelet-derived growth factor (PDGF) haplotype was reported to be associated with aseptic nonunion [6]. In a study assessing the mutation frequency of genes that are crucial for the recognition and elimination of pathogens and fracture healing, the T and C/T alleles of the transforming growth factor-β (TGF-β) gene codon 10 and the mutated TLR4 gene W/1 were identified as possible risk factors for impaired recognition and elimination of bacteria, increasing the susceptibility of a fracture patient to develop septic nonunion [7].

Several recent studies have also suggested that genetic alterations significantly contribute as an etiological factor to the development of nonunion. Sathyendra et al. found that patients carrying five SNPs in four genes showed a significant association with atrophic nonunion [8]. Another study demonstrated that a T/G genotype at SNP rs3753793 in the CYR61 gene, which encodes an important signalling molecule that participates in many signalling pathways, may contribute to the development of nonunion [9]. The haplotype GTAA in BMP4 and C allele at rs13317 in FGFR1 are also associated with nonunion [10].

Some researchers have attempted to analyse the local gene expression at the fracture site and investigate different gene expression patterns between normal and impaired fracture healing. For instance, expression of eight genes in nonunion tissue was significantly increased compared with fresh callus tissue based on a cDNA array. Among these genes, CD01, COMP, FMOD and FN1 are important for the assembly and stabilisation of the extra-cellular matrix, CLU and TCS22 induce cell differentiation and proliferation and ACTA2 and PDE4DIP gene products, such as actin, participate in cytoskeletal organisation and maintenance. Moreover, overexpression of these genes in fracture tissue may impair the structure and function of bone healing—related cells, eventually leading to nonunion [11].

MicroRNAs (miRNAs) regulate gene expression related to many biological processes, such as cell proliferation, differentiation and organ development. Increasing evidence suggests that miRNAs play a key role in fracture healing and development of nonunion by regulating bone formation, resorption and remodelling. A comparison between fracture tissues with normal healing and those with impaired healing in mice showed that five miRNAs, miR-140-3p, miR-140-5p, miR-181a-5p, miR-181d-5p and miR-451a, are significantly upregulated in normally healing tissues [12]. Interestingly, a similar study in mice also reported that five different miRNAs, miR-31a-3p, miR-31a-5p, miR-146a-5p,
miR-146b-5p and miR-223-3p, are highly expressed in nonunion tissues [13], suggesting that miRNAs may contribute to the development of nonunion at the molecular level. Recently, investigators designed a new scoring system to assess the miRNA contribution to impaired fracture healing, and 11 miRNAs were identified to impairing fracture healing in aged female mice [14]. The expression of five miRNAs (miR-140-3p, miR-140-5p, miR-181a-1-3p, miR-210-3p and miR-222-3p) is altered in diabetic mice with impaired fracture healing when compared to normal mice [15]. As SPC3649, the first miRNA-targeting drug for the treatment of hepatitis C virus infection, is being investigated in clinical trials [16], we speculate that promoting or inhibiting miRNA function is a potential therapy for nonunion. Human studies need to be conducted to investigate the exact role of miRNAs in nonunion development.

This type of nonunion, which is caused by genetic alterations and abnormal gene expression, may account for refractory nonunion and cases of undetermined aetiology in clinical practice. New methods (e.g., genetic testing) can quickly assess the risk of developing nonunion for fracture patients, enabling prevention and timely intervention. If fracture patients with nonsurgical indications present a genetic predisposition for developing nonunion, then they may need to undergo a more aggressive treatment strategy, such as surgical treatment or adjuvant therapy, including electromagnetic field and ultrasound therapies. Additionally, if fracture patients with surgical indications present a genetic predisposition towards nonunion development, then bone-grafting or mesenchymal stem cells (MSCs) transplantation or the use of BMPs may be advised during the first surgical procedure to prevent nonunion. MSCs transplantation is a representative approach of cell therapy for promoting fracture healing [17,18]. Therapy selection for nonunion can also benefit from knowledge of genes related to nonunion. For example, the use of BMPs can prevent patients with genetic defects in the BMP pathway from developing nonunion and might be more effective for nonunion treatment in such patients than in those without such genetic defects. Because many studies have shown...

Figure 1  The pathogenesis of nonunion at molecular level. Both environmental risk factors and genetic factors lead to the abnormal expression of cytokines, which is the key point for nonunion development. Genetic factors and abnormal cytokine expression, at the molecular level, are discussed in this review.

BMP = bone morphogenetic protein; IGF = insulin-like growth factor; IL = interleukin; MMP = matrix metalloproteinase; SNP = single nucleotide polymorphism; TNF-α = tumour necrosis factor-α; VEGF = vascular endothelial growth factor.
Table 1  Summary of human studies that have investigated genes related to nonunion.

| Author and date          | Groups                              | Exclusion criteria                                                                 | Methods                  | Genes related to nonunion                                                                 |
|--------------------------|----------|-----------------------------------|--------------------------|-----------------------------------------------------------------------------------------|
| Dimitriou et al., 2011   | 62 patients with atrophic nonunion   | None                             | SNP analysis              | Risk factors of nonunion: G/G genotype of the rs1372857 SNP located within NOGGIN, T/T genotype of the rs2053423 SNP located within SMAD6 |
|                          | 47 patients with normal fracture healing |                                   |                          |                                                                                         |
| Zeckey et al., 2011      | 50 patients with femoral and tibial nonunion | Smoking, diabetes, bilateral fractures, use of corticoids and septic nonunion | SNP analysis              | Risk factors of nonunion: A PDGF haplotype (rs1800814, rs62433334, rs13309625; CCG)     |
| Grzegorz Szcześny et al., 2011 | 151 patients with nonunion 144 patients with normal fracture healing | Open fractures, massive contusion of soft tissues covering the fracture gap, trophic lesions of soft tissues, chronic inflammatory foci and diseases requiring medication with immunosuppressive drugs. | Mutation frequency found using gene analysis | Risk factors of nonunion: T/T and C/T genotype of TGF-β gene codon 10, mutated TLR4 gene W/1 |
| Sathyendra et al., 2014  | 33 patients with atrophic nonunion 29 patients with normal fracture healing | None                             | SNP analysis              | Risk factors of nonunion: (OR>1): C/T or T/T genotype at SNP rs2853550 within the IL1B gene, the C/T or T/T genotype at rs2297514 and the A/G or G/G genotype at rs2248814 within the NOS gene Protective factors (OR<1): G/G at rs3819089 within the MMP13 gene, G/G at rs270393 within the BMP6 gene |
| Sabir Ali et al., 2015   | 250 patients with nonunion 250 patients with normal fracture healing | Children and patients with a known systemic inflammatory disease, osteoporosis and other metabolic bone diseases, pathological fractures and subsequent nonunion and hypertrophic and infected nonunion | SNP analysis              | Risk factors of nonunion: T/G genotype at SNP rs3753793 within the CYR61 gene          |
| João Matheus Guimarães et al., 2013 | 66 patients with nonunion 101 patients with normal fracture healing | Patients presenting with pathological fractures, osteoporosis, other bone diseases that could interfere with calcium or phosphorus metabolism, hypertrophic and infected nonunion, pregnancy, and aged younger than 18 years | SNP analysis              | Risk factors of nonunion: A BMP4 haplotype (rs2761884, rs17563, rs2071047, rs762642; GTAA); C allele at rs13317 within the FGFRI gene Protective factors: G/T and G/G genotype at rs1342913 within the FAMSC gene |
| G. Zimmermann et al., 2012 | 8 patients with nonunion 7 patients with normal fracture healing | Renal insufficiency, liver disease, malignant tumours, collagenosis, inflammatory bowel disease, haematological diseases, long-term treatment with steroidal or nonsteroidal antiphlogistic drugs or other immunosuppressive agents, thrombophrophylactic agents, fluoroquinolones and tetracyclines, hormone substitution and smoking | cDNA array analysis       | Eight genes are overexpressed in the nonunion tissue: CD01, COMP, FMOD, FN1, CLU, TCS22, ACTA2 and PDE4DIP |

SNP = single nucleotide polymorphism.
that the delivery of BMP genes as well as other genes, including vascular endothelial growth factor (VEGF), PDGF, fibroblast growth factor (FGF), Osterix, Nell-1 and Runx-2, can enhance the generation of bone both in vivo and ex vivo [19–23], gene therapy might become applicable for nonunion in the foreseeable future and may represent the most useful treatment for this serious condition.

However, more studies with larger sample sizes and stricter screening criteria should be carried out to investigate more genes that encode cytokines involved in the fracture healing cascade and their expression patterns in the fracture region. More genes associated with nonunion remain to be found, thus offering greater possibilities and more alternatives to gene therapy for this type of nonunion. Notably, no study has shown an interaction or coordination between genetic factors and environmental risk factors, which both cause abnormal expression of cytokines and are equally essential for the development of nonunion. Therefore, these correlations await further study.

Abnormal cytokine expression

Fracture healing comprises four overlapping processes (the inflammatory phase, the cartilage formation and mineralisation phase, the cartilage resorption phase and the remodelling phase), each of which is regulated by expression of cytokines. The multiple cytokines that regulate the fracture healing cascade can be grouped into three categories: (1) proinflammatory cytokines, (2) members of the TGF-β superfamily and other growth factors and (3) metalloproteinases and angiogenic factors [24]. Under the influence of environmental risk factors, such as hyperglycaemia and using NSAIDs, the local and systemic expression of cytokines may be easily altered. Disruption of any of these cytokines can impair fracture healing and result in nonunion (Table 2). Based on the classification of the cytokines, we discuss three categories of cytokines which are related to fracture nonunion. Importantly, molecular targeted therapy is an effective, accurate and feasible approach for this type of nonunion.

Proinflammatory cytokines

Proinflammatory cytokines, including tumour necrosis factor-α (TNF-α) and, interleukin-6 (IL-6), help initiate the fracture healing cascade, and they may also play key roles in the remodelling phase [25,26].

Tumour necrosis factor-α

Local application of low-dose TNF-α within 24 h of injury, which corresponds to the inflammatory phase of fracture healing, promotes fracture healing by upregulating the innate immune response [27]. Based on research on the delayed resorption of cartilage during endochondral bone formation in TNF-α receptor–deficient mice, Gerstenfeld et al. concluded that TNF-α regulates cartilage resorption by inducing apoptosis in hypertrophic chondrocytes and recruiting osteoclasts [28]. Smoking impairs fracture healing, and nicotine is the main factor responsible for

| Biological process | Correlation between abnormal cytokine expression and nonunion |
|--------------------|---------------------------------------------------------------|
| Haematoma          | Interference of hypoxia increases ROS production, impairing BMP-2 expression |
| Hypoxia            | Alteration of IL-6/sIL-6Ra inhibits differentiation of MSCs to the osteogenic lineage |
| Inflammation       | Decreased BMP-2α impairs MSC differentiation and delays cartilage mineralisation (elevated MMP-7/MMP-12 degrades BMP-2; hypophosphate and NSAIDs impairs the BMP-2 pathway) |
| Recruitment of MSCs| IGF-Iα deficiency impairs cartilage mineralisation (caused by alcohol) |
| Cartilage resorption | Elevated TNF-α (caused by hyperglycaemia) accelerates cartilage resorption, decreased TNF-α (caused by smoking) delays cartilage resorption |
| Secondary bone formation | Deficiency of MMP-13 and MMP-9 impairs cartilage resorption by osteoclasts |
| Bone marrow established | Deficiency in MMP-2 impairs the remodelling of new bone in the callus |
| Mineralised bone matrix resorption by osteoclasts | Disturbance of NO and related amino acidsα can impair the remodelling phase |

| Table 2 | Abnormal cytokines related to nonunion at the stage of fracture healing. |

BMP = bone morphogenetic protein; IGF = insulin-like growth factor; MMP = matrix metalloproteinase; MSC = mesenchymal stem cell; NO = nitric oxide; NSAID = nonsteroidal antiinflammatory drug; ROS = reactive oxygen species; TNF-α = tumour necrosis factor-α. α Altered cytokine expression has been observed in patients with nonunion.
nonunion development in smokers [29,30]. Through the cholinergic antiinflammatory pathway, the secretion of TNF-α is inhibited by nicotine and the resorption of cartilage may be delayed [31].

Diabetes mellitus is another common risk factor for nonunion development. Cartilage resorption is accelerated in diabetic mice overexpressing TNF-α, which induces an increase in osteoclast numbers and apoptosis in chondrocytes [32,33]. Thus, applying TNF-α antagonists in a diabetic mouse model might reverse the accelerated resorption of cartilage caused by elevated TNF-α [33,34]. In addition, angiogenesis is impaired, and MSCs are significantly reduced in the fracture region in diabetic mice due to the cooperation of TNF-α and high glucose levels, which is also reversed by TNF-α antagonists [35,36]. Another study found that the serum concentration of TNF-α in 28 fracture patients with type 2 diabetes mellitus was significantly higher than that in 25 fracture patients without the disease [37]. However, in another study comparing 30 diabetic to 20 normoglycemic patients, no significant difference in the serum concentration of TNF-α was observed [38]. Because fracture patients with type 2 diabetes mellitus present a higher risk of developing nonunion, these studies indicate that the coordination between increased concentrations of TNF-α and glucose might alter the specific cell behaviours involved in cartilage resorption and angiogenesis, eventually resulting in nonunion. These findings indicate a potential approach to the prevention or treatment of fracture in patients with diabetes mellitus through TNF-α antagonists. Nonetheless, further studies are needed to prove that accelerated cartilage resorption and impaired angiogenesis are due solely to overexpression of TNF-α in diabetic patient.

Interleukin-6

IL-6, along with its soluble receptor sIL-6R, is another important proinflammatory cytokine that regulates recruitment and proliferation of MSCs and their differentiation to the osteogenic lineage [39,40]. Moreover, IL-6/sIL-6R can induce peripheral blood monocytes to differentiate into osteoclasts [41]. In IL-6 knockout mice, callus strength decreased and callus mineralisation and remodelling were delayed during early fracture healing because IL-6 increases nuclear factor kappa beta ligand production to promote osteoclastogenesis [42]. As other cytokines, such as macrophage colony-stimulating factor, also promote osteoclastogenesis, no difference in fracture healing was observed between IL-6−/−knockout and wild-type mice at 4–6 weeks postfracture [43]. It seems that the IL-6 mainly participates in the remodelling phase rather than the inflammatory phase. In another study, the investigators suppressed the activity of IL-6 in inflammatory tissue and found the fracture healing was unexpectedly promoted in fracture mice [44].

An in vitro experiment demonstrated that elevating the concentration of IL-6 alone does not promote MSCs differentiation into the osteogenic lineage because IL-6 and sIL-6R do not synergistically induce differentiation of MSCs under sIL-6R deficiency [45]. In fact, this study proved that low concentrations of sIL-6R inhibit MSCs differentiation. Cho et al. suggested that increasing concentrations of IL-6 have no influence on osteogenesis and can even inhibit the proliferation of primitive mesenchymal cells [46]. Interestingly, a recent study found significantly higher and lower serum concentrations of IL-6 and sIL-6R, respectively, in nonunion patients than in healthy individuals [47]. Thus, we can speculate that the alteration of IL-6/sIL-6R may account for nonunion development in some fracture patients. Indeed, raising the concentration of sIL-6R may promote the differentiation of MSCs and accelerate healing, especially in nonunion patients who have been transplanted with enriched MSCs. More recently however, researchers found the IL-6/sIL-6R, the IL-6 trans-signalling pathway, is not essential for fracture healing. Actually, the IL-6 classic signalling, IL-6 with membrane-anchored IL-6 receptor, plays a vital role in fracture healing [48]. As a result, distinguishing the exact role of the IL-6 trans-signalling pathway and IL-6 classic signalling in fracture healing is necessary to clarify the mechanism of IL-6 signalling for nonunion development.

Members of the TGF-β superfamily and other growth factors

Members of the TGF-β superfamily include BMPs, TGF-β, growth differentiation factors (GDFs), activins, inhibins and Müllerian-inhibiting substance; other important growth factors include insulin-like growth factors (IGFs), PDGF and FGFs [49]. All are important mediators of fracture healing. BMPs and IGFs, two types of cytokines that are related to nonunion, are discussed below.

Bone morphogenetic proteins

BMPs have been used clinically for the treatment of nonunion as an adjuvant therapy, and currently, BMP-2 and BMP-7 are the only commercially available BMPs [50,51]. However, a prospective study found that no benefit was observed when BMP-2 was added to autogenous bone graft in fracture patients with long bone nonunion [52]. Recently, a more convincing study that aimed to investigate the efficacy of BMP therapy in treating nonunion showed a similar result. The prospective, randomised, controlled cohort study demonstrated that no difference was seen when BMP-2 or BMP-7 was added to autogenous bone grafts in nonunion treatment [53]. The therapeutic efficacy of BMPs is limited in clinical practice—possibly because the use of BMPs locally may induce expression of BMP antagonists. Inhibition of BMPs antagonist seems to enhance the osteoinductive capacity of BMPs. However, this has only been proven in animal models [54]. Moreover, a study showed that the complications associated with the use of BMPs in scaphoid nonunion cannot be ignored [51]. These findings suggest us to re-evaluate the use of BMPs and find novel method of using BMPs in treating nonunion.

The expression levels of BMP-2, BMP-3, BMP-3B, BMP-6 and BMP-7; GDF-5 and GDF-7 and the BMP antagonists noggin, drm, sclerostin, BAMBI and follistatin are significantly lower within fibrous nonunion tissue when compared with a standard healing callus at several time points both in animal and human studies [55,56], and BMP-4 upregulation in nonunion tissue is proven in a prospective self-control
study [56], BMP-2, BMP-7 and BMP-14 expression levels are also decreased by a larger margin within the cartilage of human nonunion fractures [57,58]. As a result, the efficacy of other members of the BMP family in the treatment of nonunion need further study. Another study demonstrated no significant difference in circulating BMP levels between patients with nonunion and those with normal fracture healing [59], suggesting that BMP expression at the fracture site is not directly correlated with systemic BMPs. These findings provide evidence for the use of BMPs at fracture and nonunion sites and suggest that detecting the local concentration of BMPs might aid in the early diagnosis of nonunion.

Many environmental factors may lead to disturbance of the BMP pathway. NSAIDs, which are frequently used for postoperative pain control, may be a risk factor to develop nonunion [60]. Most NSAIDs inhibit the activity of cyclooxygenase-1 and cyclooxygenase-2 (COX-2), and thereby decrease the synthesis of prostaglandins. A study conducted by Daluiski et al. found that after treating osteoprogenitor cells with NSAIDs to inhibit the activity of COX-2, the response of osteoprogenitor cells to BMP decreases. As a result, the osteogenic potential of the cells is reduced [61]. Another study demonstrated that through upregulating the expression of COX-2, BMP-2 induces the phosphorylation of ATF4 in chondrocytes, which plays a critical role in skeletal development and maintenance [62]. It can therefore be speculated that by inhibiting the activity of COX-2, NSAIDs can impair fracture healing through the BMP pathway. Animal studies and clinical studies are needed to validate that NSAIDs impair fracture healing through the BMP pathway. BMP-2 expression of MSCs depends on hypoxic signals in the fracture tissue, which is mediated by reactive oxygen species. Interference of hypoxia during the early inflammatory phase of fracture healing leads to deficient scavenging and abnormal increase in reactive oxygen species production, thus impairing BMP-2 expression and cartilage ossification during endochondral bone formation, which leads to delayed union or nonunion [63].

Model mice with femoral fracture that received a phosphate-restricted diet exhibited BMP-2 resistance, resulting in impaired fracture healing exactly resembling that observed in the absence of BMP-2 signalling [64]. Moreover, phosphate restriction increased parathyroid hormone-related peptide expression, further attenuated chondrocyte differentiation and impaired hypertrophic chondrocyte apoptosis via the parathyroid hormone/parathyroid hormone-related peptide receptor [65]. Combined with phosphate, the use of BMP-2 in the treatment of nonunion may be more effective. However, as for acute fracture healing, a double-blind, randomised, controlled phase-II/III trial performed in 2013 on the efficacy and safety of recombinant human BMP-2/calcium phosphate matrix for treating closed tibial diaphyseal fracture suggested that the use of rhBMP-2/CPM did not significantly accelerate fracture healing after patients were treated with reamed intramedullary nailing [66]. Calcium phosphate bone substitute is a suitable alternative to the bone grafts used for clinical nonunion treatment. Regardless, further study is needed to determine whether increasing the local concentration of phosphate or combining BMP-2 with phosphate is effective for treating nonunion.

**Insulin-like growth factors**

IGFs, the most abundant growth factors in bone, comprise IGF-I and IGF-II. IGF-I is more potent than IGF-II. When bound to the IGF type 1 receptor (IGF1R), IGF-I activates autophosphorylation of the IGF1R cytoplasmic kinase domain, stimulating downstream signalling pathways through its interaction with various docking proteins, including insulin receptor substrate-1 (IRS-1) and Src homology/α-collagen [67]. IGF is a mediator of growth hormone, whereas the function of IGF is regulated by specific binding proteins, including IGF-binding proteins (IGFBPs; IGFBP-1 to IGFBP-6) and acid-labile subunit [68].

The knockout of IGF system—related genes impairs embryonic development and postnatal development of the skeleton in mice [69–71], and a recent study using an osteoblast-specific IGF1R conditional knockout mouse model also demonstrated impaired endochondral bone formation [72]. However, knockout of IGF-1 in osteocytes of mice augmented the healing of a fracture gap because loss of IGF-1 was compensated for by upregulation of BMP-2 and Wnt [73]. Significantly higher gene expression of IGF-I/II and IGFBP-6 has been reported in mice with nonunion, and IGFBP-5 expression in these mice is significantly lower than that in mice with normal fracture healing, thus exhibiting opposite trends [74]. Another prospective study showed that the time course of the serum concentrations of GH (growth hormone)/IGF-I axis components in patients with atrophic nonunion are significantly different from those of patients with normal fracture healing. Acid-labile subunit expression is lower during the early and late stages of fracture healing, while IGFBP-3 expression is lower during the late stage of fracture healing in nonunion [75]. Mathieu et al. also proved that the serum concentrations of IGF-I are lower in patients with nonunion [47]. The GH/IGF-I axis is easily affected by endocrine disorders; for example, in diabetic mice with fracture, the concentration of IGF-I is lower in both the serum and the fracture region [76]. Therefore, further studies in vivo are necessary to validate whether changes in the GH/IGF-I axis might initially prompt the development of nonunion before therapeutic targeting of GH/IGF-I axis can be considered.

The broken ends of fractures cannot connect in IRS-1 gene knockout-mice because IRS-1 deficiency cannot be compensated for by other signalling molecules, and transplating MSCs that express IGF-I into these mice can induce the transplanted MSCs to differentiate into the osteogenic lineage via autocrine and paracrine regenerative effects; as a result, the resulting increased bone volume and callus mineralisation rescue the failed fracture healing [77]. In addition, transplanting MSCs with systemically delivered IGF-I can also enhance fracture healing [78]. From the perspective of promoting fracture healing, IGF-I has shown great benefit in many experiments. Indeed, experiments with several animal models have shown that local administration of IGF-I can accelerate fracture healing [79–81]. Furthermore, the application of the recombinant human IGF-I/IGF binding protein-3 complex in osteoporotic patients with femoral fracture may be feasible and safe [82]. However, the potential therapeutic effects of IGF-I on
nonunion require further study. As the factor that potentially initiates the development of nonunion, the GH/IGF-I axis represents a possible target for the prevention and treatment of the condition. Notably, either transplanting MSCs expressing IGF-I or transplanting MSCs with systemically delivered IGF can fully implement the regenerative capacity of these cells to repair bone, suggesting a novel approach for improving the therapeutic efficacy of MSCs transplantation.

**Metalloproteinases and angiogenic factors**

**Matrix metalloproteinases**

During the endochondral ossification and remodelling phases, matrix metalloproteinases (MMPs) degrade the extracellular matrix of cartilage and bone, allowing vascular invasion into the newly generated bone. Imbalance among MMP family members and their regulators, tissue inhibitors of metalloproteinases, may also account for the molecular pathogenesis of nonunion. In MMP-13–knockout mice, chondroclast recruitment to the fracture callus is disturbed, thus slowing cartilage resorption [83]. A similar phenomenon was observed in MMP-9–knockout mice, whereby persistent cartilage hindered vascular penetration into the newly generated bone [84]. In contrast to the MMP-13– and MMP-9–knockout mice, MMP-2–knockout mice exhibit no defects in chondroclast recruitment or cartilage resorption [85]. These findings indicate that MMP-13 and MMP-9 affect both cartilage and bone remodelling, whereas MMP-2 delays only the remodelling of new bone in the callus. A prospective study showed that patients with nonunion had significantly higher serum concentrations of pro-MMP-1 and MMP-8 compared with those with normal fracture healing but that tissue inhibitors of metalloproteinase-1 was lower at several times points after fracture surgery [86]. Because the serum concentrations of enzymes may reflect local enzyme activity, detecting serum MMP levels might represent a novel approach for evaluating the risk of nonunion development in fracture patients. Interestingly, noninvasive examination of the levels of MMP-9 and MMP-13 in urine, which is more accurate than the serum levels, demonstrated that these molecules are potential biomarkers for fracture healing [87]. Moreover, MMP-7 and MMP-12 are significantly elevated in hypertrophic nonunion tissue, and these proteinases may bind to and degrade BMP-2 [88].

**Vascular endothelial growth factor**

The VEGF-dependent pathway modulates angiogenesis during fracture healing [89]. Fracture healing in rabbits could be delayed by anti-VEGF antibody [90]. During fracture healing, the expression of VEGF and vascularisation was higher in mice with impaired fracture healing when compared to mice with normal fracture healing [91]. Human studies showed a similar result: serum VEGF concentrations in patients with nonunion were higher compared to the patients with normal fracture healing during the 6-month observation after the fracture [92]. However, local application of VEGF can promote bone repair in established nonunion animal models [93,94]. It seems that hypervascularisation may impede fracture healing especially in the early phase of fracture healing whereas the treatment of an established nonunion can benefit from VEGF administration and vascularisation.

**Other molecules**

**Nitric oxide**

As a free radical gas, nitric oxide (NO) is a well-known inflammatory mediator in fracture healing [95,96]. NO-mediated vasodilation is essential for increasing blood flow into the fracture site in the inflammatory phase [97]. During the remodelling phase, NO mediates vascular reactivity, bone formation and resorption [98,99]. NO is produced through the conversion of arginine to citrulline, which is catalysed by nitric oxide synthases (NOSs). In another pathway, arginine can be converted to ornithine and urea by arginase as a precursor of collagens.

NOS-knockout mice exhibit disturbed arginine-NO metabolism and impaired fracture healing [100]. Moreover, a study in humans found lower concentrations of arginine, citrulline and ornithine in the fracture callus of patients with atrophic nonunion than in those with normal fracture. Compared to patients with normal fracture healing, arginine was significantly higher and ornithine was lower in the fracture callus in patients with hypertrophic nonunion [101]. We can conclude that amino acids metabolism related to NO is compromised in atrophic nonunion calluses, and the concentration of arginine and NO production increase in hypertrophic nonunion calluses. These findings suggest that NO is involved in the molecular pathogenesis of nonunion. Under stress conditions, such as wound healing and sepsis, arginine levels decrease, and arginine-NO metabolism can be disturbed; these changes may be the initial events in the development of nonunion, especially atrophic nonunion, in some patients [102–104]. Additionally, animal studies showed that oral l-arginine and oral products which can upregulate NOS expression promote fracture healing [105,106]. However, whether arginine or NOS supplementation can prevent and treat nonunion need to be investigated.

**Wnt signalling**

Wnts are a family of secreted glycoproteins involved in the fracture healing process. In the canonical Wnt signalling pathway, Wnts bind to the membrane receptor Fzd and one of the coreceptors, LRP5 and LRP6 (low-density lipoprotein receptor-related protein), and eventually the cytoplasmic level of β-catenin increases to regulate gene expression. The gene expressions involved in Wnt signalling pathway were upregulated during fracture healing in mice [107].

Acute alcohol exposure can disturb the Wnt signalling pathway by deregulating β-catenin expression in mice. Thus, fracture healing is impaired as a result of cartilage formation deficiency [108]. Applying a Wnt pathway activator can increase the level of β-catenin and reverse the impaired fracture healing in mice under alcohol exposure [109]. It is reported that the FOXO transcription factors, antagonists of the Wnt signalling pathway, may also be activated by alcohol [110].
The Wnt signalling pathway could be targeted to enhance fracture healing. Sclerostin and Dkk1 can inhibit the Wnt signalling pathway and impede fracture healing by binding to the LRP5/6 and other transmembrane protein. Sclerostin depletion and systemic administration of a sclerostin antibody with or without BMP-2 have shown the effect of promoting bone repair in mice [111–113]. Dkk1 antibodies also have the ability to enhance fracture healing in mice [114]. However, the therapeutic potential of Wnt antagonists for the nonunion treatment and prevention needs further researches.

Summary

Undoubtedly, some variations in genes, gene expression and cytokines related to fracture healing are associated with the development of fracture nonunion, though some aspects of the molecular pathogenesis remain unclear. A number of new approaches targeting different genes and cytokines might aid in the early identification of nonunion development risk in fracture patients and might be useful in preventing and treating nonunion. However, some cytokine alterations discussed may be caused by genetic factors rather than by external risk factors because both genetic factors and external risk factors contribute to abnormal cytokine expression. The current evidence needs to be validated in animal and human studies, and the interaction and coordination between genetic factors and environmental risk factors, as well as correlations among different cytokines, warrant further investigation.

Conflicts of interest statement

The authors have no conflicts of interest relevant to this article.

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