REVIEW

Nanomedicine-Driven Approaches for Kartogenin Delivery: Advancing Chondrogenic Differentiation and Cartilage Regeneration in Tissue Engineering

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Abstract: Articular cartilage degradation and osteocartilage defects are the most prevalent concerns that vary from localized to more systemic forms of cartilage disease. However, regulating chondrogenic differentiation within the joints remains a significant challenge. Kartogenin, a small heterocyclic compound, has recently garnered considerable attention as a potential therapeutic agent, owing to both chondrogenic and chondroprotective properties for intra-articular therapy. Initially, it was created for osteoarthritis; it has also been used to address various diseased conditions, such as the regeneration of disc and bone-tendon junctions. On top of that, it preserves the equilibrium between cartilage catabolism and anabolism, while also mitigating inflammation and alleviating pain by preventing damage induced by cytokines. To modulate tissue function and cellular behaviour, it is crucial to have sustained release of ketogenic through an appropriate delivery system. A multitude of biomaterial-based carriers have been developed for the prolonged release of kartogenin. Moreover, many biological mechanisms of action of kartogenin have been identified. The most critical molecular mechanism among them is the dissociation of filamin A from core-binding factor (CBF)-β induced by kartogenin. Filamin A subsequently translocates to the nucleus, where it engages with RUNX-1 to transcribe genes implicated in the chondrogenesis of mesenchymal stem cells. This review focuses on the development of biomaterials functionalized with kartogenin, including their structure, design, physicochemical properties, biological roles, molecular mechanisms of action, and applications in tissue engineering and regenerative medicine. In conclusion, we discussed the future possibilities and challenges posed by recent advancements in kartogenin research and their potential applications in tissue regeneration.

Keywords: Kartogenin, cartilage regeneration, osteoarthritis, TGF-β, chondrogenesis

Introduction

Regenerative medicine is the branch of medicine that endeavours to restore normal functions by replacing or regenerating human cells, tissues, and organs.[1] Osteoarthritis (OA) is a degenerative disease characterized by degeneration of articular cartilage, ligamentous injury, and loss of extracellular matrix (ECM), leading to pain, inflammation, and impaired joint functions.[2] It has been stated as the most prevalent form of arthritis among adults, impacting approximately 32.5 million Americans, causing disability among the older population.[3,4] It is challenging to successfully repeal the development of OA due to the avascular nature of cartilage and the low density of the dispersed chondrocytes, which are the limited resident cells in cartilage.[5] The therapeutic strategies for OA are focused on alleviating pain and functional impairment, including non-pharmacological and pharmacological interventions.[6] Non-pharmacological interventions might not be sufficient for patients developing symptomatic OA. The pharmacological interventions for OA include medications, such as oral non-steroidal anti-inflammatory drugs (NSAIDs) and acetaminophen. Yet, serious concerns are raised by the fact that NSAIDs might cause adverse effects in the gastrointestinal, renal, and cardiovascular systems[7] and are therefore restricted. Recently, there has been increasing interest in focused small-molecule therapies for the treatment of OA over

palliative medications, using intra-articular (IA) injections. However, these methods always come with several problems, such as donor site morbidity, immunogenic rejection, limited availability, and disease transmission.[8]

In regenerative medicine, the most effective approach for addressing OA includes promoting chondrogenic differentiation of mesenchymal stem cells (MSCs) and the generation of new cartilage tissue. The ultimate objective of this strategy is to overcome the paucity of chondrocyte availability to repair injured cartilage.[1] To solve this problem, in 2012, Kristen Johnson et al, after screening 22,000 drug-like compounds, discovered that Kartogenin (KGN) is a small non-protein molecule that promotes the regeneration of cartilage by stimulating the specific differentiation of mesenchymal stem cells (MSCs) into chondrocytes. It is a chondroprotective and chondrogenic agent.[9] As compared to other growth factors, it has greater potential for cartilage regeneration.[10] Growth factors, such as cytokines, which include bone morphogenetic protein (BMP), and protein growth factors like transforming growth factors-β (TGF-β), Insulin-like growth factor 1 (IGF-1), are crucial for inducing stem cells to differentiate into chondrogenic cells.[11] Among them, TGF-β has been suggested as a possible therapeutic agent to improve MSC-based articular cartilage regeneration.

Furthermore, to achieve rapid cartilage regeneration, a substantial intra-articular (IA) dosage of TGF-β is required, which may result in osteophyte production, synovial fibrosis, joint swelling, synovitis, and deterioration of articular cartilage. However, the utilization of TGF-β and other growth factors might be restricted by their instability, short half-life, and low immunogenicity in vivo.[12] The bone morphogenetic proteins (BMPs) were identified to promote chondrogenic differentiation of MSCs both in vitro and in vivo. However, BMPs might induce bone development after ectopic implantation, indicating that the management and modulation of BMPs are essential for optimum cartilage tissue engineering approaches. By regulating proteoglycan formation and breakdown in chondrocytes, IGF-1 may protect cartilage homeostasis and increase survival and proliferation. However, OA inflammation lowers IGF-1 levels and chondrocyte responsiveness to IGF-1, preventing functional and structural integrity.[13–15] Meanwhile, KGN is a small heterocyclic stable molecule that can be stored and transported at room temperature. These advantages of KGN to peptide growth factors render it a promising candidate for cartilage regeneration. Therefore, it is attracting significant interest as an emerging chondrogenic candidate for IA treatment.

Even though KGN was initially established to cure OA, it has been utilized to treat other diseased conditions as well as promote disc and cartilage repair through bone-tendon regeneration.[16] The cartilage nodules that develop in the presence of KGN comprise collagen II (COLII), aggrecan (ACAN), and proteoglycans, which are structurally fundamental elements of hyaline cartilage. While ACAN provides resistance to stress or compression in the cartilage region, COL II controls the metabolic balance.[3] Furthermore, under pathophysiological conditions, KGN was able to drastically lower the release of pro-inflammatory nitric oxide (NO) and cytokine-induced glycosaminoglycans (GAGs) breakdown in cartilage tissues. The study revealed that IA injection of KGN can support cartilage regeneration as evidenced by a decrease in fibrillations in the superficial and mid-zone of articular cartilage using the collagenase VII–induced chronic joint injury model and the acute ligament ligation model.[17] On the other hand, Patients with OA have defective chondrocytes that produce an excess of degrading enzymes such as matrix metalloproteinase 13 (MMP13) and a disintegrin metalloproteinase with thrombospondin motifs 5 (ADAMTS5), which exacerbates articular cartilage destruction.[18] In this regard, KGN treatment has shown a dose-dependent rise in the production of COL II and ACAN in chondrocytes exposed to IL-1β.[19] This impact is observed only in inflammatory conditions, whereas KGN seems to have little to no impact on ACAN and COL II levels in healthy chondrocytes. Furthermore, KGN functions to inhibit MMP13 and ADAMTS5, hence protecting the cartilage extracellular matrix and ameliorating inflammation.[18]

Johnson et al investigated the mechanism through which KGN functions by interacting with the actin-binding protein filamin A and disrupting its equilibrium with the transcription factor CBFβ. CBFβ enters the nucleus, where it interacts with RUNX-1 and forms a CBFβ-RUNX-1 complex that promotes the transcription of proteins associated with chondrogenesis and improves the production of ECM in cartilage.[9] Additionally, a recent study revealed that KGN improved chondrogenesis by raising the levels of chondrogenic marker genes, including ACAN, COL II, and Sox9.[20,21] It has been observed that RUNX-1 interacts with Sox5, Sox9, and Sox6, which are members of the SOX family, to stimulate chondrogenic differentiation and the development of cartilage matrix. It is well-established that they work by interacting with SOX DNA motifs that are located in enhancer regions. These regions are primarily associated with cartilage-specific genes, and as a result, they regulate the expression of a variety of genes that are involved in

chondrogenesis, including those that encode ECM proteins like proteoglycans and collagens.[22] In another studied mechanism, KGN primarily activates the JNK-RUNX-1 pathway, which causes it to have pro-chondrogenic effects, and it suppresses the β-catenin-RUNX-2 pathway, providing anti-osteogenic effects.[23,24] It has been demonstrated that KGN stimulates the chondrogenic differentiation of stem cells, promotes tendon-bone junction regeneration in injured rabbits, and increases the proliferation of bone marrow-derived mesenchymal stem cells (BMSCs).[25] However, KGN promotes the proliferation of BMCs and human adipose-derived stem cells by activating the AMPK-SIRT1 signalling pathway. In summary, KGN might be a potential molecule that can be implicated as an endogenous stem cell-based treatment for OA patients. KGN in vivo application was investigated in two OA mice models. Both models showed an enhanced new articular cartilage formation, a reduced level of cartilage breakdown products in the serum, and enhanced load-bearing capacity. KGN promotes cartilage nodule formation during mouse limb development by upregulating genes encoding hedgehog and TGF-β signalling.[26]

The chemical and physical properties of KGN include limited bioavailability, long-term efficacy, short retention time, and hydrophobicity, often constraining its effectiveness. The implementation of nanomedicine in bone and cartilage healing may enhance treatment outcomes by substituting damaged osteochondral tissue with healthy biological tissue, possibly reinstating joint function. Furthermore, advancements in nanomaterials have made it easier for the development of biomimetic scaffolds featuring nanostructures that improve cell proliferation and migration, thereby promoting the regeneration of damaged tissues.[27] Likewise, nanoparticles (NPs) and extracellular vesicles serve as drug delivery systems that facilitate targeted treatment for different osteochondral lesions while mitigating drug adverse effects.[28] Therefore, the advancement of nanomedicine has markedly improved the diagnostics and therapies for cartilage and bone disorders. Notably, the structure and properties of KGN, especially its hydrophobicity and carboxyl groups, make it facile to incorporate into a wide range of biomaterials. KGN used with other drugs may have synergistic effects. Tengfei et al synthesized two new conjugated KGN formulations and compared their drug release to free KGN to create a cartilage-targeting delivery strategy. However, KGN conjugated formulations showed that a single dosage of free KGN was ineffective. This conjugated charge-based cartilage-targeted delivery formulation reduces medication dosage with prolonged release.[29]

A study by Kang et al revealed that in vivo nano KGN conjugated chitosan IA injection resulted in much fewer degenerative defects in rats than in unconjugated KGN injected rats.[30] IA injection of nano KGN conjugated materials has gained significant attention in drug delivery systems for cartilage repair, which was discussed later in this review.

Nano KGN-enriched biomaterials show potential for directing tissue regeneration, which is a practical approach. Regardless of the increasing understanding of KGN in cartilage regeneration, there are still several limitations to its clinical implementation. These include concerns about metabolic pathways, long-term safety, as well as other potential drug interactions. However, recently, an analogue of KGN called KA3475 has been developed after significant chemical modification, which substantially enhanced the chemical stability and potency of KGN. KA3475 finished a clinical trial (NCT03133676) in 60 OA patients. The results have not been released because of the safety and effectiveness of the drug.[31,32] These potential improvements employ sustained-release formulations to avoid repeated injections, which create pain in local injection sites, inflammation, and issues. Biomaterial scientists, biologists, and clinicians must work together to solve these problems and progress KGN therapy. These teams may enhance KGN treatment by improving drug distribution and creating patient-specific solutions. This partnership speeds the development of new OA medicines and ensures their scientific efficacy and clinical viability, increasing patient outcomes. This cooperation will accelerate drug development and ensure scientific efficacy and clinical feasibility, improving OA patient outcomes.

This study thoroughly researched the physicochemical properties and biological functions of KGN. Furthermore, we provide a comprehensive analysis of cutting-edge functionalized biomaterials, emphasizing their design, structure, function, application, and mechanism of action. Moreover, we examine the essential characteristics for subsequent clinical applications in various biomaterials. Furthermore, Figure 1 illustrated the process of chondrocyte differentiation and cartilage formation. To achieve successful cartilage formation, it is important to understand the intricate molecular events and various pathways, which plays a significant role in cartilage formation.[33]
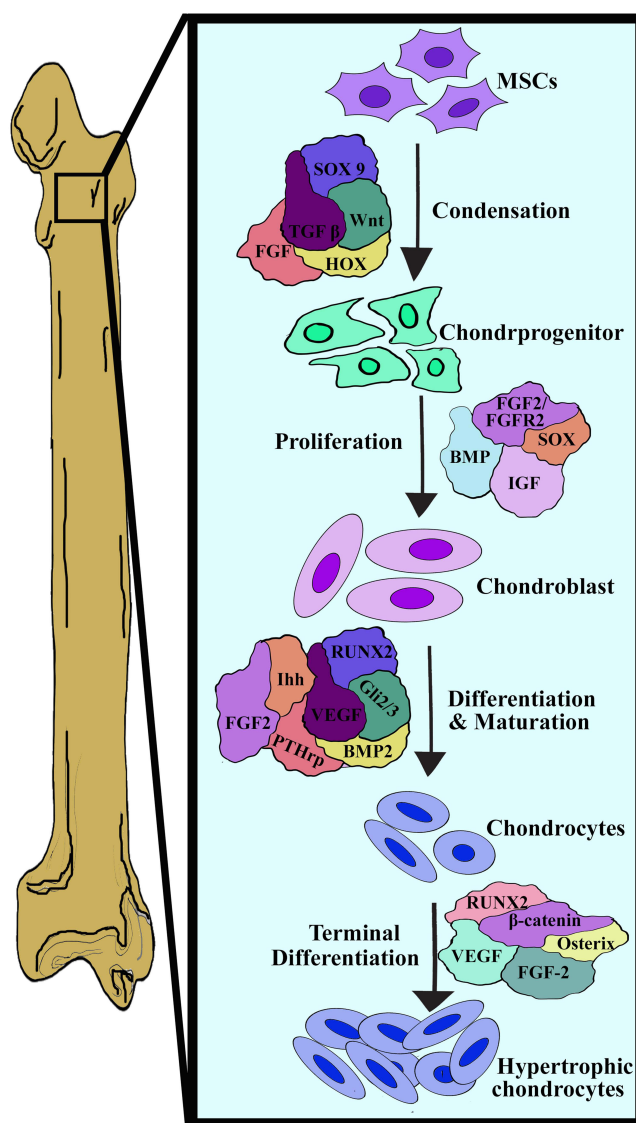
Figure 1 Mechanism of chondrocytes proliferation and differentiation. Data from Park et al.[33]

Structural Properties OF KGN

With a molecular weight of 317.3 g/mol, KGN is structurally composed of phthalic acid (PA) and 4-aminobiphenyl (4-ABP) linked together by an amide bond. The 4-ABP serves as a potent inducer of chondrogenic differentiation compared to KGN (Figure 2).[34,35] Though the amide bond is stable, it is often hydrolysed by several enzymes, including alkaline ceramidases, allantiasis, metalloproteases, serine proteases, aldehyde oxidase, cysteine proteases, amidases, and peptidases, by releasing its hydrolysates, phthalic acid and 4-ABP.[36] The structure of KGN can be confirmed by utilizing various analytical techniques, such as Fourier transform infrared spectroscopy (FTIR) and hydrogen nuclear magnetic resonance.(^{1}HNMR)[37] Other compounds, such as the new organic nitrate RS-7897, are shown to exhibit hydrolysis of an amide link that may be a more active molecule compared to the parent molecule.[38] However, amide bonds are often utilized in drug design as a linker to bind the drugs to a specific peptide target. KGN has excellent biocompatibility, functions in a concentration-dependent manner, and possesses a prolonged half-life. Although their effects on various cell types vary, MSCs optimally respond to a concentration of 10 μM, whereas tendon stem cells (TSCs) respond best to a concentration of 100 μM.[39] On the other hand, KGN hydrophobicity leads to poor solubility in water but is soluble in
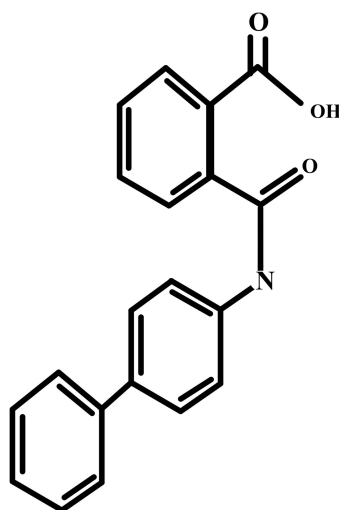
Kartogenin

Figure 2 The chemical structure of KGN.[40]

dimethyl sulfoxide, which affects its bioavailability and the efficacy of drug administration. Moreover, it demonstrates stable storage properties at room temperature.[9]

In addition, KGN has two hydrogen bond donor sites along with three hydrogen bond acceptor sites. This property allows the implementation of polysaccharides to provide a reversible and defective attachment of KGN to the matrix structure by hydrogen bonding during the processing of the scaffold.[35] Therefore, expensive or time-consuming binding techniques are not necessary. Moreover, the carboxyl group of KGN allows covalent bonding like esterification or amidation with hydroxyl and amine groups, respectively, which supports chemical cross-linking with other materials. Junqi Dai et al effectively linked KGN to HAMA by an esterification reaction, resulting in HAMA-KGN, which, when combined with GelMA and evaluated using a microfluidic system, yielded esterase-responsive GHKM. These microspheres demonstrate excellent biocompatibility, minimum invasiveness for injection, and sustained drug release properties.[41] To let amidation reaction, Qing H et al prepared nanocarriers KGN conjugated to the surface of PAMAM and the end group of polyethylene glycol (PEG), using amide reaction between the carboxyl group of KGN amino group of PAMAM-PEG to obtain PEG-PAMAM-KGN (PPK) and KGN-PEG-PAMAM (KPP) conjugate. The resulting nanocarriers enhanced the chondrogenic differentiation, retention time in joints, and sustained drug release.[42] Similarly, Wenshuai Fan et al synthesised PN-KGN conjugated nanospheres by reaction between the carboxyl group of KGN and the amine group of PN to form amide bonds, where PN-KGN facilitated the sustained release of KGN and provided prolonged cartilage preservation with minimal intra-articular injections.[43] The chemical structure of KGN is depicted in Figure 2.[40] In view of these chemical and physical properties, KGN may be used in biomedical engineering by chemical conjugation or physical encapsulation.

Biosynthesis and Mechanism of Action

The biosynthesis of KGN involves a mixture of 4-ABP and PA. This compound can be obtained by combining phenylboronic acid with either 4-iodoaniline or 4-bromoaniline. On the contrary, amide bond-breaking substances such as peptidases and amidases can hydrolyze KGN, resulting in the release of its hydrolysis products PA and 4-ABP. Interestingly, 4-ABP exhibited a greater ability to ameliorate the OA injury by promoting chondrogenic differentiation and MSC proliferation than KGN.[4] Several different mechanisms of action have been proposed to provide evidence in favour of the chondrogenic effects of KGN.

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## Filamin A/CBFβ Signalling Pathway for Chondrogenesis

KGN promotes chondrogenic development in MSCs by interacting with an actin-binding protein, which is filamin A. KGN binds to the FC-1 segment of FLNA by displacing CBFβ from its binding site in the cytoplasm. Afterwards, CBFβ enters the nucleus and binds to the RUNX-1 factors that control the production of proteins and genes linked to chondrogenesis through a CBFβ-RUNX-1 transcription program (Figure 3). RUNX-1 induces osteoblast differentiation and chondrocyte proliferation, contributing to the repression of OA.[34,44] Based on prior knowledge, the CBFβ-RUNX-1 transcription process may also preserve chondrogenesis function by keeping RUNX-2 at a low level.[45] Despite this, KGN has been found to increase the levels of COLII, ACAN, and MMP inhibitors, indicating that it functions in both preserving the chondrocyte phenotype and safeguarding the cartilage matrix from degeneration. The schematic diagram representing the molecular mechanism of action of KGN by Filamin A/CBFβ/RUNX-1 Pathway has been demonstrated in Figure 3.[46]

Hippo/TAOK1 Signalling Pathway for Chondrogenesis

The Hippo signalling pathway is yet another well-known mechanism that controls cell differentiation and development. According to studies conducted by Jing et al small extracellular vesicles developed from KGN preconditioned hUCMSCs
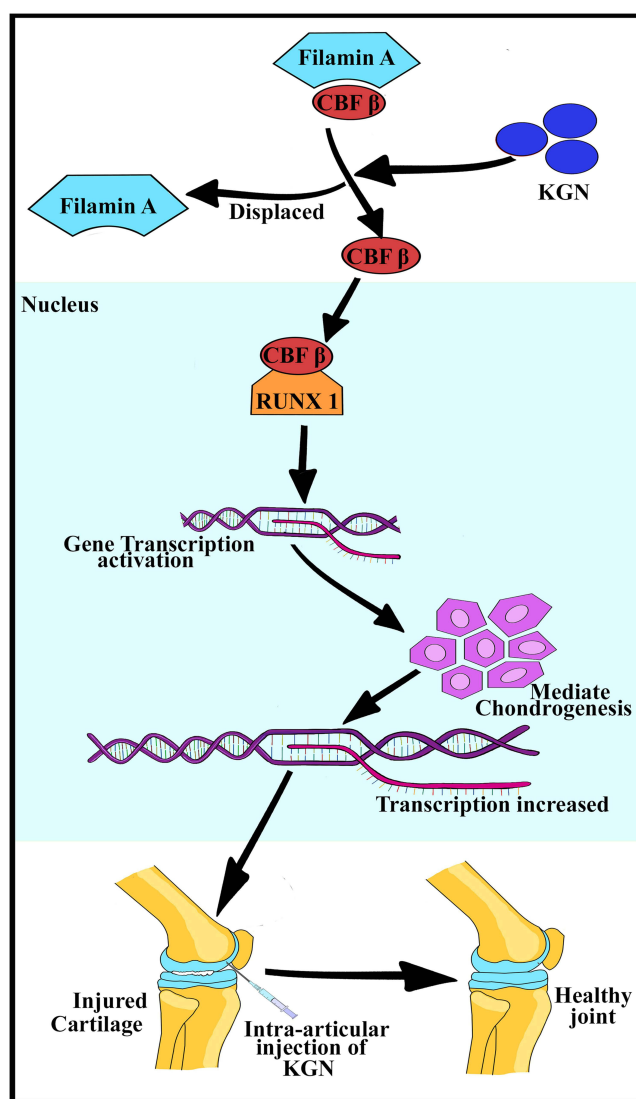
Figure 3 Schematic diagram of KGN molecular mechanism of action by the Filamin A/CBFβ/RUNX-1 Pathway. Data from Marini et al.[46]
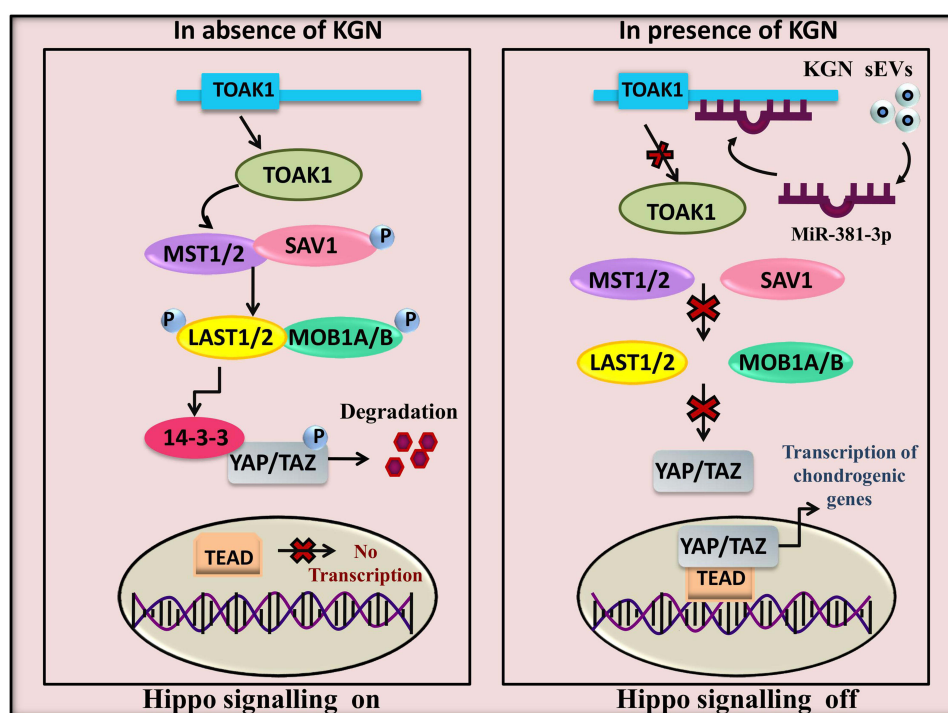
Figure 4 Molecular mechanism of KGN induced Hippo/TAOK1 Signalling Pathway. Adapted from Fu M, Hu Y, Lan T, Guan KL, Luo T, Luo M. The Hippo signalling pathway and its implications in human health and diseases. Signal Transduct Target Ther. 2022;7(1):376. Copyright © 2022, The Author(s). This article is licensed under a Creative Commons Attribution 4.0 International License.[48]

may transport miR-381-3p, target TAOK1, and stimulate chondrogenic differentiation of native MSCs. A possible target of miR-381-3p was selected to be TAOK1, an upstream regulator of the hippo signalling pathway (Figure 4). The dual luciferase reporter studies demonstrated that miR-381-3p enhanced chondrogenesis by directly targeting TAOK1 through limiting the 3′ untranslated region that blocked the Hippo signalling pathway.[47,48]

The AKT/P13K Signalling Pathway for Chondrogenesis

Besides the aforementioned pathways, a recent study revealed the role of KGN in the chondrogenic process regulated by the AKT/P13K pathway. It demonstrated that the lytic product of KGN, which is 4-ABP, promotes chondrogenic differentiation of MSC. 4-ABP can stimulate the PI3K-Akt pathway, leading to the proliferation of cartilage-derived stem/progenitor cells (CSPC) and MSCs in cartilage. PI3Ks include a family of lipid kinases that regulate a broad range of physiological activities, including cell proliferation, survival, trafficking, and migration.[44] The PI3K-dependent signalling also promotes pluripotent embryonic stem cell self-renewal. The PI3K-Akt pathway plays an important role in governing chondrocyte proliferation and differentiation as well as terminal chondrocyte differentiation in both adult and embryonic chondrogenesis (Figure 5a). c-Jun N-terminal kinases are an essential enzyme in the PI3K-Akt pathway, involved in actin cytoskeleton reorganization, which is required for MSC chondrogenesis.[49–52] The 4-ABP activates the PI3K-Akt pathway and increases the expression of a downstream molecule, which is the cell cycle protein CDK2. In addition, it suggested that the 4-ABP mechanism of action relies on RSK-3, which is a key regulator of cell differentiation and proliferation. RSK-3 promotes the proliferation of CSPC.[44]

The Smad/TGF-β Pathway for Chondrogenesis

Another significant regulatory system for skeletogenesis and joint formation is the TGF-β signalling pathway. Chondrogenesis and lubricin expression are both regulated by this mechanism. In contrast to TGF-β stimulation, which might increase Smad 1/5/8 phosphorylation in MSCs, Decker et al discovered that KGN had no impact on this process.[53] The receptor-activated Smads are classified into two groups: Smad1/5/8 and Smad2/3. These Smads are again activated by distinct molecular

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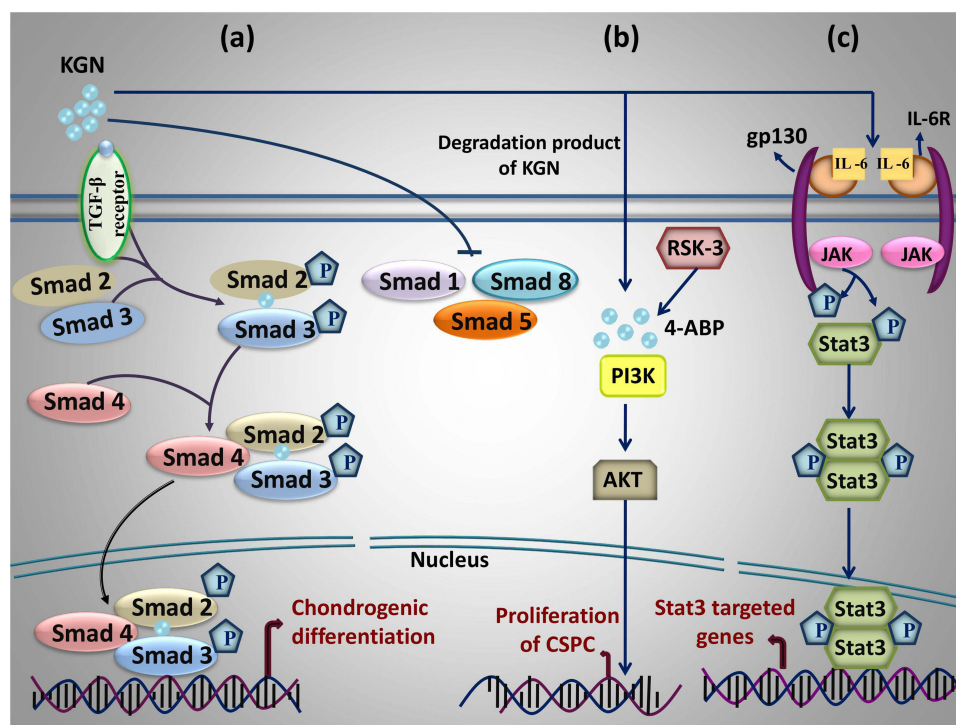
Figure 5 (**a**) AKT/PI3K Signalling Pathway, (**b**) The molecular mechanism of KGN induced Smad/TGF-β Pathway, and (**c**) IL-6/Stat3 Pathway.[51,52]

compounds. Smad1/5/8 induces chondrocyte hypertrophy and OA pathogenesis, whereas Smad2/3 protects against chondrocyte hypertrophy.[54] It has been shown that KGN influences the TGF-β/Smad pathway by enhancing phosphorylation and subsequently activating Smads (Figure 5b).[51,52] The distinct function of KGN consists of its ability to stimulate Smad2/3, which leads to a chondroprotective effect. It has been shown that chondrogenic development of MSCs obtained from synovial fluid is more successfully stimulated by combining KGN and TGF-β3 than by using either factor separately. This effect is achieved by enhancing the Smad2/3 pathway, whereas KGN inhibits TGF-β3-induced hypertrophy via the Smad1/5/8 pathway.[55,56] On the other hand, Wang et al discovered that KGN may stimulate type I collagen production in dermal fibroblasts via the TGF-β/Smad4/5 pathway. KGN enhances the p-Smad5 protein, which then entirely enters the nucleus.[57,58] Likewise, the Smad4 protein is overexpressed and translocated to the nucleus. The p-Smad4 and p-Smad5 proteins consequently activate subsequent genes. This suggests that KGN may function via several routes, depending on the cell type.[57]

The IL-6/Stat3 Pathway for Chondrogenesis

Tao et al evidenced that KGN can stimulate mitosis in CSPCs, resulting in an increase in both the percentage and number of G2-M phase cells. The group treated with KGN resulted in a 10% higher proportion of G2-M phase cells, which was almost twice as high as in the control group. The transcriptomic analysis of rat CSPCs showed substantial differences between KGN-treated and control samples. Compared to the control group, the KGN-treated group had significantly greater levels of IL-6 as well as its co-receptor Gp130 gene expression. KGN stimulation improved the phosphorylation of Stat3, an IL-6 downstream protein (Figure 5c).[51,52] Increased articular cartilage thickness was seen in the destabilization of the medial meniscus (DMM) animal model after IA KGN injection. Following IA KGN injection, IHC labelling also showed the increased distribution of CD44+/CD105+ cells in cartilage and elevation of Stat3 phosphorylation. The findings, therefore, indicated that KGN stimulated IL-6/Stat3-dependent proliferation, at least in part, to enhance cartilage regeneration.[59] According to recent research, IL-6 has a role in maintaining MSCs in their undifferentiated form and is associated with MSC development via JAK/STAT3 signalling.[60] Various factors, including periostin, TGF-β, mechanical stimulation, and platelet-rich plasma(PRP), have been shown to positively impact both of these cellular processes, hence promoting cartilage regeneration.[59]

The Indian Hedgehog (Ihh) Pathway for Chondrogenesis

The Indian hedgehog (Ihh) pathway is crucial for cartilage tissue formation and maintenance. As a crucial signalling molecule, it controls the differentiation, proliferation, and osteoblast differentiation of chondrocytes and is secreted by pre-hypertrophic chondrocytes in the growth plate. The absence of this protein inhibits the development of synovial joints in embryos.[61] Ihh is produced in the growth plate by chondrocytes. When it binds to its trimeric receptor Patched-1 (Ptch1) in vertebrates, inhibition of Smoothened (Smo) would be relieved (Figure 6).[62] Consequently, Smo activated and translocated to the cilium, where it recruited the suppressor of fused homologues, the Sufu-Gli complex. As discussed above, the active Smo dissociates the bonding between Sufu and Gli, enabling activated Gli2/3 to reach the nucleus and stimulate the production of Ihh target genes likeGli1 and Ptch1.[63,64] Furthermore, it was also possible to inhibit KGN- Ihh signalling by treating KGN with cyclopamine (CPN), a drug that blocks hedgehogs.[65] Another study by Chen et al suggests that Ihh and Sonic Hedgehog (Shh) regulate the activation of early cartilaginous differentiation in vivo and in vitro. It may have a synergistic impact on chondrogenic differentiation and chondrogenesis in the rotary cell culture system (RCCS) and in cartilage repair.[66]
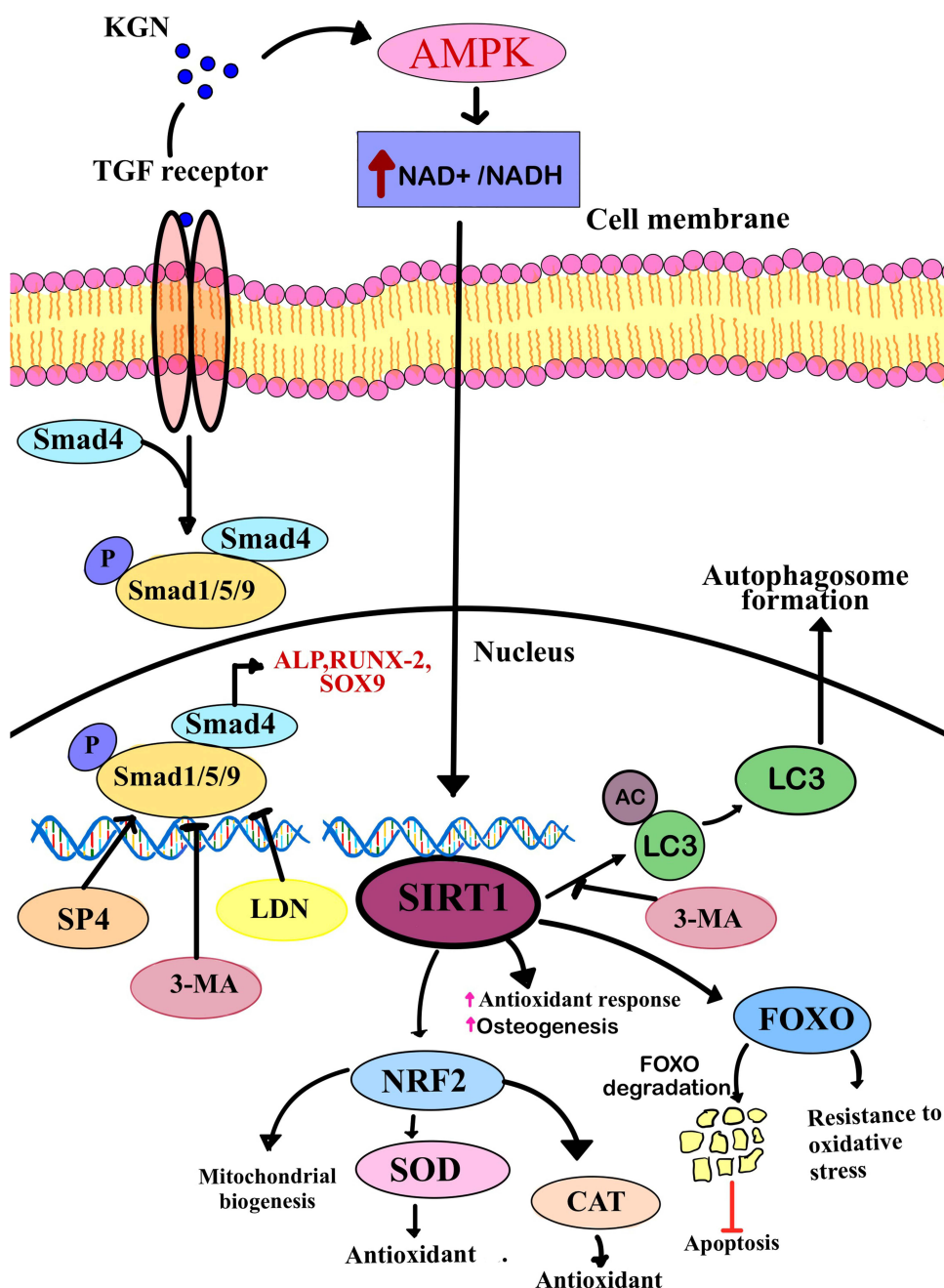
The AMPK-SIRT1/Antioxidant Signalling Pathway for Osteogenesis

It has been observed that KGN promoted the osteogenic differentiation of BMSCs, increased the level of production of antioxidant enzymes, and reduced intracellular reactive oxygen species (ROS).[67] KGN increased BMSC levels of antioxidant enzymes and osteogenic differentiation in a dose-dependent manner. By suppressing the expression of p16INK4α, Silent Information Regulator Type 1 (SIRT1) prevented oxidative stress-induced premature senescence in MSCs and encouraged multi-lineage differentiation in MSCs.[68] Furthermore, SIRT1 has been linked to the mediation of antioxidant activities via the reduction of intracellular ROS generation and the upregulation of the expression of Nuclear factor erythroid 2-related factor 2 (NRF2), a pivotal transcription factor that modulates over 200 cytoprotective genes. It has been shown to translocate to the nucleus in response to oxidative stress and transcribe many intracellular antioxidant enzymes, including superoxide dismutase 2 (SOD2) and catalase (CAT).[69] Nonetheless, it has been shown that excessive
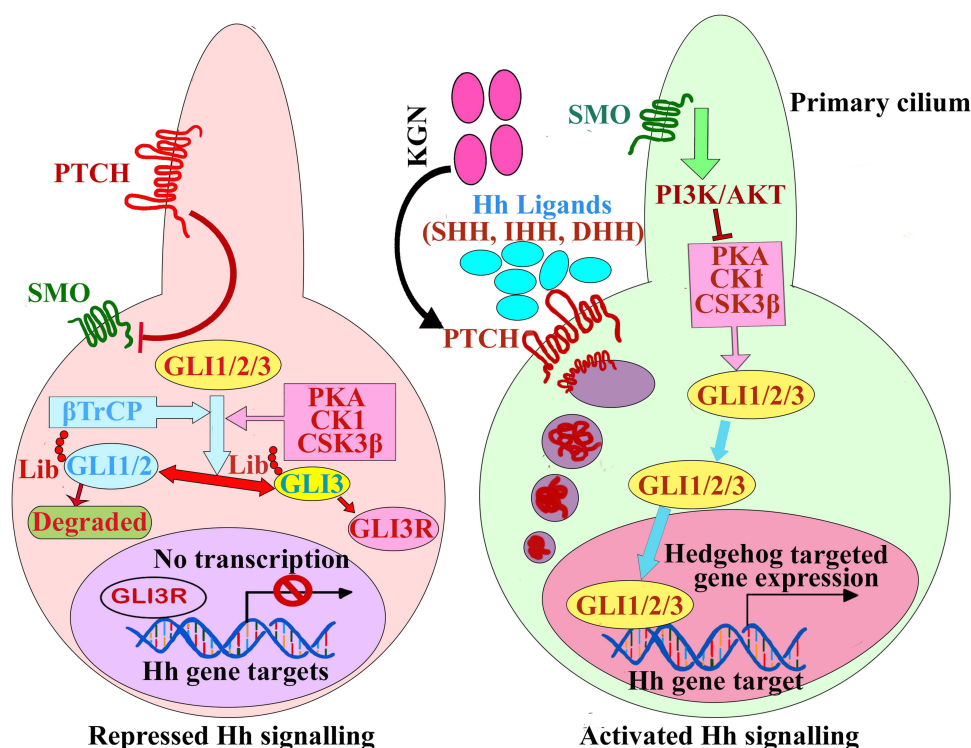
Figure 6 Molecular representation of repressed (left), in absence of hh ligands and activated (right) KGN induced hh signalling pathway, in presence of hh ligands. Adapted from Ok CY, Singh RR, Vega F. Aberrant activation of the hedgehog signaling pathway in malignant hematological neoplasms. Am J Med. 2012;180(1):2–11. Copyright © 2012 American Society for Investigative Pathology. Published by Elsevier Inc. All rights reserved.[62]

Figure 7 Molecular mechanism of KGN induced AMPK-SIRT1/Antioxidant signalling pathway.[71,72]

ROS generation aids in cartilage damage and the advancement of OA. Apart from the AMPK-SIRT1 signalling pathway, it has been proposed that KGN therapy enhances the expression of TGF-β1 and other members of the transforming TGF-β superfamily genes. KGN has no impact on the mitogen-activated protein kinase (MAPK) signalling pathway, but it may potently trigger the phosphorylation of smad4/smad5 in the TGF-β signalling pathway (Figure 7).[70] Therefore, KGN may hold promise for the therapeutic use of MSCs in bone tissue engineering in addition to articular cartilage repair.

The Autophagy-Induced Response for Osteogenesis

KGN enhances BMSCs osteogenesis by increasing the Smad1/5/9 pathway and autophagy activity. Autophagy is a conserved biological mechanism which promotes cell survival under stress and maintains homeostasis.[73,74] Previous studies have shown

that autophagy holds an indispensable mechanism for regulating bone regeneration. It has also been established that autophagy can improve osteogenesis by supporting osteoblast mineralization and differentiation, which affects autophagosomes in calcium extracellular transportation. Autophagic activity plays a significant role in determining the potential for MSC differentiation through its regulation of osteoblastic or adipogenic lineages.[75,76] Furthermore, autophagic levels may affect the survival rate of bone-related cells in adverse conditions. Several autophagy marker genes, including (LC3), Atg7 and Beclin1, have been proposed for regulating autophagy. LC3 is available in two forms: LC3-I, which is unconjugated to lipids and distributed in the cytoplasm. Whereas LC3-II is conjugated to phosphatidyl ethanolamine and utilised as a specific autophagosome marker to access autophagic flux.[77] In addition, the involvement of autophagy during KGN treatment was confirmed by (3-MA), an inhibitor of autophagy. The action of 3-MA to inhibit autophagy resulted in a notable decrease in the mRNA and protein expression levels of alkaline phosphatase (ALP), as well as Runt related transcription factor-2 (RUNX-2), in BMSCs treated with KGN.[78] As a result, the activity of osteogenic differentiation was significantly reduced. According to Decker et al investigation, they suggested that KGN might stimulate the phosphorylation of Smad2/ 3 of E11.5 limb bud mesenchymal cells, which would generally stimulate the development of mice embryonic limbs.[53] Furthermore, bone marrow-MSCs (BM-MSC) differentiation potential may be suppressed by LDN, a specific inhibitor of the Smad signalling pathway, whereas 3-MA's inhibitory effects on osteogenic differentiation in BMSCs can be reduced by the activator SB4.[79] The findings suggested that KGN stimulated BMMSCs autophagic activity and improved their potential for osteogenic differentiation via increasing Smad1/5/9 phosphorylation. Thus, autophagy and Smad1/5/9 play an important role in osteogenic differentiation in BMSCs as well as fibroblasts and endothelial cells (Figure 7).[70–72,80]

The Anti-Osteogenic Mechanism of KGN

A recent study has revealed the anti-osteogenic effect of KGN. KGN preconditioning may have two advantageous effects on chondrogenic differentiation triggered by TGF-β3. It committed MSCs to a pre-cartilaginous stage accompanied by reduced β-catenin expression enhanced JNK phosphorylation (Figure 8).[3,81] These anti-ossific and pro-chondrogenic effects may be linked with the inhibition of β-catenin/RUNX-2 pathways and the activation of JNK/RUNX-1 signalling
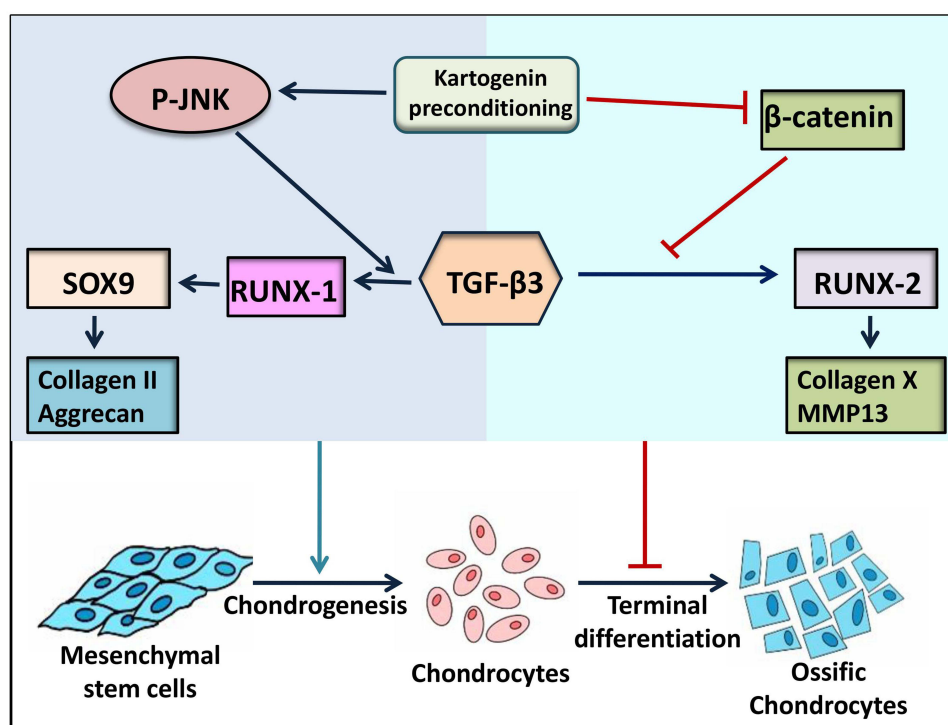
Figure 8 The molecular mechanism of KGN induced JNK/RUNX-1 signalling pathway. Reproduced from Jing H, Zhang X, Gao M, et al. Kartogenin preconditioning commits mesenchymal stem cells to a precartilaginous stage with enhanced chondrogenic potential by modulating JNK and β-catenin–related pathways. FASEB J. 2019;33(4):5641–5653. © FASEB.[3]

pathways. JNK functions through the remodelling of the actin cytoskeleton, requisite for chondrogenic differentiation.[50] On the other hand, β-catenin related signalling pathways, including the PI3K/Akt/GSK-3b/β-catenin pathways and canonical Wnt/β-catenin, play a major role in MSC osteogenic development.[50] The in vivo findings revealed that tracheal defects in a rabbit model might be restored using tissue-engineered patches made of KGN-preconditioned hUC-MSCs along with electrospun nanofilms encapsulating TGF-β3. Re-epithelization was also observed, demonstrating a promising strategy for developing tissue-engineered trachea.[50]

The mechanism of action of KGN has been increasingly elucidated by an increasing number of investigations. The mechanism behind the actions of KGN, initially discovered by Johnson et al is filamin A/CBFβ/RUNX1, which is the most predominant and generally established mechanism involved in KGN activity. Apart from this, the Ihh and TGFβ/ Smad pathways are quite widespread. However, research fails to define the number of mechanisms by which KGN addresses cartilage defects. Consequently, comprehensive study is required to investigate the complex interactions between these pathways and their wider significance in cartilage regeneration and MSC development.

The Application of Biomaterial-Mediated Drug Delivery of KGN in The Biomedical Field

KGN has been employed in many tissue engineering fields as well as in regenerative fields, including cartilage protection and regeneration, tendon-bone healing, wound healing, scaffold engineering, and limb development. Regenerative medicine is the process of replacing old or damaged cells with genetically identical new and functioning cells to repair or establish normal function of cells. KGN could be helpful in cartilage repair, stimulate collagen synthesis for wound healing, encourage the establishment of a cartilage-like transition zone in tendon-bone junctions, and govern limb growth in a coordinated manner. Owing to its bioactivity, KGN may be incorporated into nanoparticles, nanofibers, hydrogel, liposomes, nanovesicles, and dendrimers - as nanodrug delivery systems (Figure 9). In order to efficiently target bone or cartilage regeneration, some essential applications of KGN are as discussed below:

Scaffold Engineering

A scaffold is a three-dimensional (3D) matrix structure that promotes cell survival and holds significant promise for tissue regeneration. An ideal scaffold should have several properties, such as a porous structure, an effective mode of action, a low immunogenicity, excellent biocompatibility, an appropriate degradation rate, and excellent mechanical strength.[82] Grafted scaffolds need to be effectively rigid to withstand multidirectional compressive loads.[83] Shi et al employed a quick cross-linkable, ultraviolet-reactive scaffold loaded with KGN NPs to treat full-thickness cartilage lesions in an in vivo model. The NPs-incorporated scaffolds were loaded into the defect sites and then exposed to ultraviolet (UV) light. The scaffold that was combined with KGN had a major beneficial effect on cartilage regeneration.[84,85] Similarly, Hong et al developed a unique multi-dimensional combination treatment that promoted cartilage regeneration. The scaffold was composed of soluble poly-L lysine/KGN (L−K) NPs and poly (lactic-co-glycolic acid) PLGA/methacrylate hyaluronic acid (PLHA). The porous PLHA scaffold was developed by combining UV and co-precipitation crosslinking. Through in vitro cytotoxicity tests, the synthesized scaffold was revealed to be biocompatible with adipose-derived stem cells (ADSCs) and to enhance the secretion of COL II by ADSCs in a dosage-dependent manner, as shown by immune fluorescence assay. It demonstrated that the PLHA scaffold degraded and finally differentiated into cartilage in vivo, while the nascent tissue subsequently regenerated.[86,87]

Furthermore, Xuan et al created scaffolds made up of poly (1,3-propylene sebacate) (PPS), poly (glycerol sebacate) (PGS), and bioactive KGN. The scaffolds supported enhanced chondrogenic differentiation and cartilage repair in full-thickness lesions of rat femoro-patellar groove after 12 weeks and suppressed osteogenic differentiation of BMSCs in a dosage-dependent manner.[88] Similarly, Li et al designed a KGN-filled PLGA-PEG-PLGA thermo gel to fix cartilage damage. Based on the in vitro results, 42.4% of KGN was released after 196 hours. BMSCs and KGN both were combined in the thermo gel. The in vivo result reported that the scaffold-encapsulated drug can help cartilage tissue regeneration. The findings showed that the newly developed cartilage had high content of COLII and GAGs, a fully smooth surface, along with decreased cartilage matrix degradation.[89] In another study, Liu et al created a layer-specific biomimetic biphasic
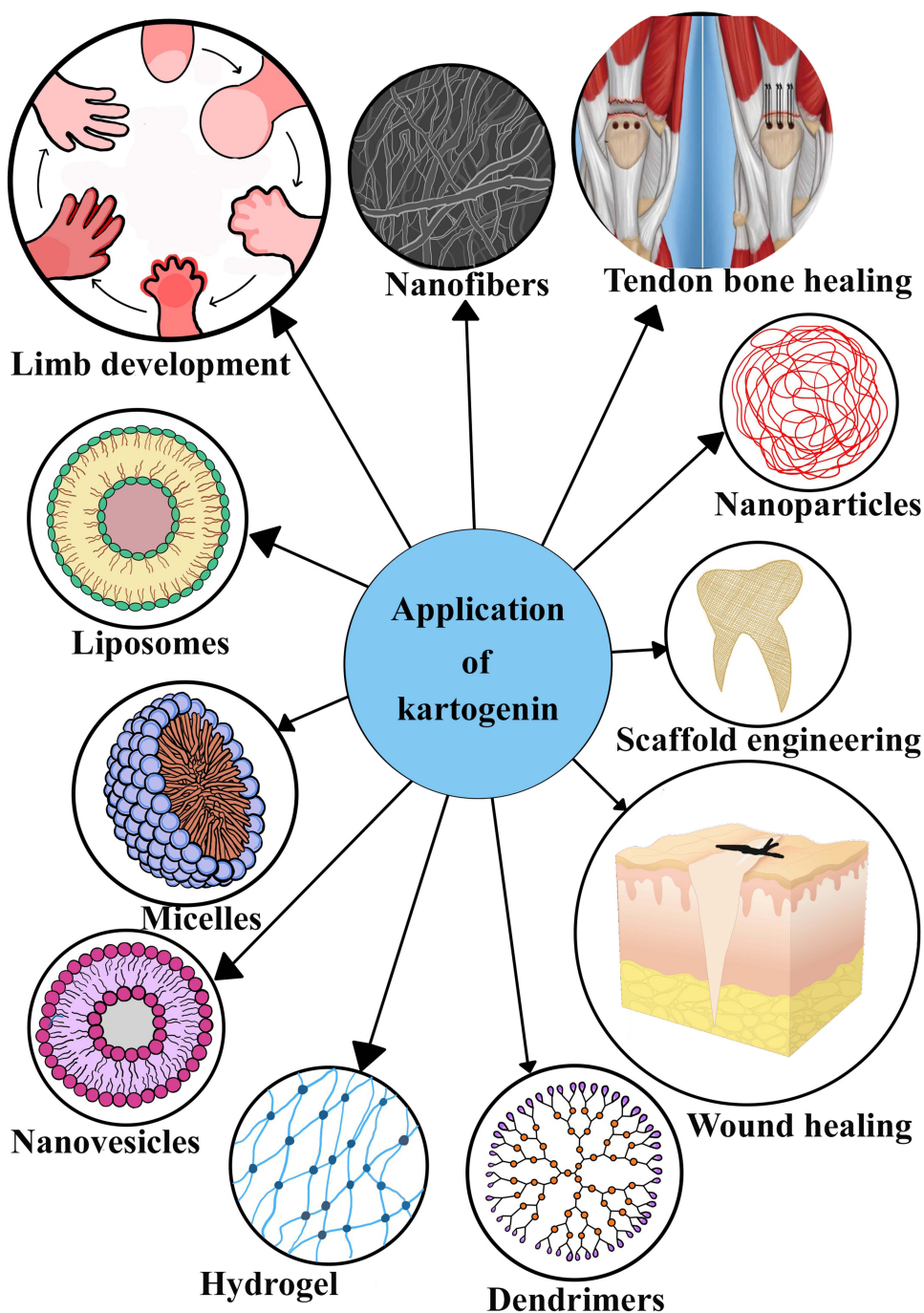
Figure 9 Schematic diagram of KGN delivery system and application as biomaterials.

osteochondral scaffold (BBOS) with stem cell differentiation inducer. Additionally, the BBOS cartilage regeneration layer (cartilage scaffold, CS) had a hyaluronic acid that looks like cartilage that is mechanically enhanced by host-guest supramolecular units, regulating the release of KGN. Furthermore, the bone regeneration layer (bone scaffold, BS) is a 3D-printed hydroxyapatite (HA) scaffold which releases alendronate (ALN).[90] The two semi-immersion-bound layers may control the stem cell hierarchical targeted differentiation activity. In comparison to the drug-free scaffold, it was possible to encourage the MSCs in the BBOS to develop into osteoblasts and chondrocytes. The in vivo findings showed that the regeneration of bone or cartilage is strongly promoted in the corresponding layers. This BBOS with layered specific inducer

release is predicted to be developed into a novel osteochondral regeneration technique.[91,92] Likewise, Zhao et al developed a biocompatible scaffold containing poly(lactic-co-glycolic acid) (PLGA) and chondrocyte extracellular matrix (CECM) microspheres. This scaffold can mimic the regenerative microenvironment and promote chondrogenic differentiation of BMSCs both in vivo and in vitro.[93] In contrast, Sepahdar et al reported an effective composite composed of polyvinyl alcohol- polyurethane (PVA-PU) hydrogel with KGN-incorporated PLGA NPs for cartilage tissue regeneration isolated from MSC. Increasing the concentration of KGN increased the cytotoxicity of the examined NP scaffold. Their findings revealed that the KGN-loaded NP Scaffold assisted MSC differentiation into chondrocytes.[78]

These investigations explained how porous scaffolds can be used as drug delivery systems for KGN in tissue regeneration applications. Utilizing a variety of methods, including 3D printing, conjugation with microspheres, nanoparticles, or hydrogels and reversible attachment, offers scaffold designing sustained drug delivery strategies. However, their method of preparation and implantation are more intricate, elevating both costs, invasiveness for the patients, and they may be more appropriate for long-term repair conditions, particularly in cartilage regeneration that demands robust mechanical support in weight-bearing joints. There is potential for improving cartilage regeneration and the management of diseases related to cartilage through more research and development in this area, as scaffold can mimic the extracellular matrix to promote cell attachment and tissue formation. Drug-containing scaffolds greatly improved MSC chondrogenic and osteogenic development in comparison to drug-free scaffolds.

Nanoscale Applications of KGN

NPs have been broadly used in various fields, including gene and drug delivery, bioimaging, anti-microbial and antitumor targeting because of their wide range of functions and diverse forms.[94] These particles can preserve bioactive substances, reduce their adverse effects, control their release profiles, maximizing their therapeutic benefits, and effectively distribute them to target cells.[95] It is worth mentioning that NPs can be produced with exceptional precision in terms of their shape, surface characteristics, and size, which can substantially improve their solubility, cellular uptake, and immuno-compatibility.[96] In addition, the utilization of NPs to encapsulate active molecules provides several benefits, such as improved solubility, protection against degradation in biological fluids, as well as facilitating controlled, sustained release across space and time.[97] Particle size is a key aspect of the therapeutic effects because it influences penetration as well as retention time in the cartilage matrix.[98] However, the exact size of the drug formulation for IA delivery is still under debate.[99] Rothenfluh et al proposed that NPs that self-assemble with a size of less than 60 nm could easily pass through the heavy collagen network found in the cartilage extracellular matrix. This would transform the matrix of the cartilage from a barrier into a reservoir for the NPs. To improve bioavailability in cartilage, they engineered NPs with a regular and spherical shape with an average size of 25 nm that should be able to efficiently penetrate the matrix of cartilage under the dynamic compression of normal transportation. This would allow an intra-tissue release of nano KGN rather than just an IA release with enhanced bioavailability.[100,101]

Almeida et al examined how surface chemistry affects the properties of KGN-loaded nanoparticles and how they interact with hematopoietic MSCs by investigating the KGN-loaded PLGA, PLGA–PEG–HA, and PLGA–PEGNPs. The PLGA–PEG–HA and PLGA–PEG NPs released more KGN than the PLGA NPs. This suggested that KGN interacts more with the hydrophobic PLGA than with the hydrophilic HA or PEG at the surface of the NPs. The larger PLGA–PEG and PLGA–PEG–HA particles may have larger pores, as they released the most KGN. Over short periods of time (approximately 7 days), MSCs that were formulated with nano KGN displayed a high rise in sulphated GAGs, which was an indication of chondrogenic differentiation in comparison to non-KGN formulations.[101] Similarly, Kang et al utilized chitosan, a chitin derivative distinguished by its amino group and polycationic properties, conjugated with KGN through an amide bond involving 1-ethyl-3-(3-dimethyl aminopropyl) carbamide along with N-hydroxy succinimide (EDC/NHS), to produce NPs using ionic gelation method. This involved the tripolyphosphate anion interaction with the cationic chitosan through electrostatic forces. The result showed that the chitosan NPs conjugated with nano KGN enhanced the biocompatibility and aqueous solubility of hydrophobic KGN. Furthermore, these NPs exhibited sustained in vitro release for a period of seven weeks. These polymer-drug conjugates showed a promising technique for delivering drugs to treat OA.[102] Additionally, they created thermo-responsive polymeric NPs based on chitosan. KGN was covalently cross-linked with dual drug-release NP outside and revealed that changes in temperature can control the release of each drug separately.[103] According to a study by Fan et al nano KGN-conjugated polyurethane NPs (PN-KGN) were successfully

developed by synthesizing amphiphilic PN with pendant amino groups. The amine group of PN and the carboxyl group of KGN then reacted to form amide bonds. Additionally, an IA injection of PN neither caused any joint swelling nor accelerated the progression of OA compared to the control group. This deduced that PN was biocompatible in vivo. Their findings revealed that PN-KGNNPs can protect joint cartilage better than KGN and stop the progression of OA.[43] Recently there was a study stated that injecting NPs into mice joints caused severe inflammation.[104]

In addition to the size of particle, the charge of particles also determines their retention and penetration into the cartilage matrix. The ECM inside cartilage is negatively charged, it offers an exceptional opportunity of employing electrostatic interactions to improve drug carriers' movement, uptake, and binding. Drug-carriers that were positively charged exhibited greater ingestion as well as faster penetration than their neutral counterparts of the same size.[105,106] Furthermore, studies have documented that cationic polymeric NPs formed electrostatic clusters with endogenous hyaluronic acid following IA injection, which resulted in an extended-release profile and increased retention time in joints.[107,108] A prior study indicated that pendant amino groups ($-NH_2$) can enhance the cationic properties of PU.[109] Hence, it suggested that the amino groups could extend retention time in the cartilage matrix and facilitate electrostatic interactions with anionic proteoglycans.

On the other hand, Silva et al prepared nano KGN-incorporated coaxial poly (glycerol sebacate)/PCL aligned nanofibers using the chemical cross-linking method. These fibres released KGN over time and caused MSCs to differentiate into chondrogenic cells.[110] A flexible nanofiber structure was created to heal wounds at the interface tissue.[111] In another study, nano KGN was linked to the polydopamine layer on the silk fibroin nanofiber surface. Using the core-shell nanofiber structure helped to interconnect tendons with bones.[112]

It is possible to manage the loading and release of KGN, the stability of suspension, as well as the cellular uptake of nanoparticles, by adjusting their composition, structure, and biological activity. This will ultimately affect the functional efficacy of the particles. Combining nanoparticles with hydrogels to create composite biomaterial systems for controlled drug delivery is becoming more common to further improve therapeutic efficacy, particularly for localized applications.

KGN-Loaded Exosomes

Exosomes are attracting significant interest because of their distinctive biological properties, multidisciplinary functions and potential therapeutic applications. According to reports, MSCs have a therapeutic impact that involves stimulating tissue receptor cell activity via paracrine mechanisms, as opposed to directly replacing cells.[113] When MSCs are in an active or resting state, they release a membrane secretory system called exosomes into the extracellular matrix. They have several advantages, including targeted distribution, stable chemical properties, simple preservation, selective assembly, efficient tissue regeneration, and the ability to retain the active components of MSCs.[114] When compared to direct cell transplantation, exosome therapy shows an improved homing effect, increased flexibility, and increased immune modulatory activity.[115] The paracrine system secretes extracellular vesicles that attach to target cells and release a range of cytokines. Following that, these cytokines control tissue regeneration. They have a diameter of 40–120 nm and are packed with proteins, lipids, nucleic acids, and other substances released by cells.[116,117] According to Xu et al exosomes containing the surface-displayed E7 peptide (E7-Exo) may target synovial fluid-derived MSCs (SF-MSCs), which can be produced by fusing the peptide E7 with Lamp 2b (the exosomal membrane protein). In an OA rat model, IA injection of SF-MSCs along with E7-Exo/KGN co-administration at the knee joints exhibited more marked therapeutic advantages compared to KGN alone and KGN injected by exosomes without E7.[118] As per Wang et al secretory elements derived from UC-MSCs possessed the ability to control MSC differentiation.[57] Following this, Zhang et al observed that exosomes derived from embryonic stem cells (ESCs) could stimulate osteochondral regeneration.[119] Similarly, Cosenza et al observed that exosomes extracted from BM-MSCs could protect the bone and cartilage from deterioration in OA patients.[120] In a recent study, Huang et al postulated that MSC-derived exosomes might serve as an alternative therapy for cartilage repair in an approach called cell-based tissue engineering.[121,122] In a later investigation, Tao et al discovered that exosomes derived from synovial mesenchymal stem cells (SMSCs) had a noteworthy capacity to prevent OA and that the effectiveness of this method might be markedly augmented by the upregulation of miR-140-5p in SMSCs.[123] Wu et al proposed that IPFP-MSC-EXOs rich in miR-100-5p might suppress mTOR in OA and so preserve articular cartilage.[124] Qi et al found that BMSC exosomes may block the p38, ERK, and Akt pathways that cause mitochondrial dysfunction-induced apoptosis in chondrocytes.[125] As a result, several studies have shown the benefits of MSC-EXOs for OA prevention and cartilage

regeneration. Exosome components and functions, however, are very diverse and may change depending on whether they are taken from other cell types or else the same cell types under various circumstances.[126]

In their study, Kato et al demonstrated that exosome function can be controlled by pre-treatment. They employed to activate synovial fibroblasts and reported that exosomes isolated from IL-1 cells induced a greater number of osteoarthritic alterations in articular chondrocytes than those without IL-1 stimulation.[127,128] Similarly, Shao et al observed that exosomes produced by IPFP-MSCs might increase the chondrocytes production by increasing its anabolic effects, decrease the catabolic effects by lowering MMP expression, and raise the COL-II, ACAN, and SOX9 expression. In addition, compared to exosomes produced from IPFP-MSCs without pre-treatment, exosomes extracted from IPFP-MSCs treated with KGN may considerably increase anabolic effects and decrease catabolic effects.[2] In another report, Liu et al, found that exosomes obtained from nano KGN preconditioned BMSCs (KGN-BMSC-Exos) outperformed exosomes derived from BMSCs. The chondral matrix development and degradation of BMSC-Exos were both enhanced by nano KGN preconditioning.[129] However, there are numerous challenges for using MSCs clinically to treat cartilage damages, such as ethical clearance and policy issues. Because of these, we need to keep looking for a cell-free method to support cartilage repair that might be helpful to put into practice. Thus, exosomes provide a potential cell-free approach to stimulate cartilage regeneration based on MSCs.

The use of exosomes increased the effective concentration of KGN within cells and considerably facilitated the chondrogenesis of MSCs both in vitro and in vivo. Despite the increasing number of studies on the impacts of exosomes, there are still some shortcomings in the area of drug delivery systems. Native exosomes, for example, are not naturally made to deliver specific cargo. Instead, cargo is delivered to different types of cells in vivo without any preference. However, with an enhanced comprehension of exosomes, it is anticipated that superior techniques would be produced to maximize their design and functionality.

KGN for Wound Healing

Wound healing is a complicated process that involves several cell lineages and tissues. The preliminary goals of wound healing are to ensure fast wound closure while leaving a biologically similar and functionally appealing scar. The collagen-rich granulation tissue created by fibroblasts is thought to be crucial for wound healing.[130,131] KGN promotes lubricin accumulation through the ADAMTS5 and c-Myc pathways as well as reducing intervertebral disk degeneration and alleviating pain. Wang et al discovered that KGN promotes collagen I production by activating Smad4/Smad5/TGF-β pathway, indicating its potential application in wound healing.[132] Furthermore, KGN had no obvious adverse impacts on fibroblast viability, morphology, or survival. In vivo, experiments have shown that KGN may significantly enhance collagen production in the skin and improve wound healing in mice. The group treated with KGN had a significantly faster wound healing time than the control group (12.8±2.3 days vs 16.4±3.4 days).[133]

Chondrogenic Potential of KGN: Tendon-Bone Healing

The rising risk of tendon-bone junction (TBJ) injuries is high, particularly in the field of sports. TBJ injuries heal slowly and often result in the formation of scar tissue that impairs normal function. The unique structure of TBJ consists of three components, including a tendon, a zone of transitional fibrocartilage (both calcified and uncalcified), and bone.[134,135] The fibrocartilage structure acts like a shock absorber and minimizes the level of stress that is transferred to tendons. Since tendons allow complicated movements in many aspects, TBJs are subjected to high-stress levels, which increase their risk of acute or chronic injuries, especially in athletes who engage in intensely competitive sports. KGN chondrogenic potential was used to promote the fibro-cartilaginous zone during TBJ repair.[136] Zhang et al studied the in vitro nano KGN effect on chondrogenic differentiation and proliferation of rabbit BMSCs as well as patellar tendon stem/progenitor cells (PTSCs). Nano KGN stimulated chondrogenic differentiation of stem cells and concentration-dependently increased cell proliferation in both cell type, as indicated by the upregulation of chondrogenic markers Sox9, COL II, and ACAN expression. In vivo, nano KGN stimulated the development of cartilage- tissues in rat patellar tendons and improved wound healing at damaged rat Achilles TBJs.[137]

KGN Mediated Limb Development

Besides the KGN chondrogenic effect, the biological effects on other different tissues have been investigated. Limb development requires the coordinated development of several tissue types, including cartilage, bones, ligaments, tendons, and joints. The joint formation is critical in vertebrate limb development, allowing animal limbs to adapt successfully to an array of ecological niches.[138] The preliminary sign of joint development is the formation of an interzone, which occurs when extremely flat and condensed mesenchymal cells arise. The interzone is composed of three layers: a dense middle cell layer and two outer cell layers. The middle interzone layer is directly linked with the formation of articular chondrocytes, while the outer layers support the expansion of long bone anlagen through appositional growth.[139] Decker et al investigated whether KGN controls limb developmental processes by examining its effects on complete limb explants while committing pre-skeletal mesenchymal cells from limb buds in mouse embryos. KGN not only increased cartilage nodule production but also increased tendon maturation, digit cartilaginous anlage elongation, synovial joint creation, and interzone compaction.[52] KGN worked through comprehensive, central processes that ordinarily encourage, direct, and coordinate total limb development. KGN increased gene expression for TGF-β (particularly TFG-β1) and hedgehog superfamily members. Exogenous TGF-β1 induced cartilage nodule development like KGN, where both TGF-β1 and KGN significantly increased the expression of SZP/lubricin/PRG4 in articular superficial zone cells. Overall, their findings indicated that KGN might be an effective tool for tissue repair and limb regeneration strategies.[52,140]

KGN-Loaded Liposomes

Liposomes are lipid bi-layered membrane structures that effectively encapsulate hydrophilic and hydrophobic drugs, respectively, inside the core and in the lipid bilayers. Besides this, liposomes have significant benefits in drug delivery systems (for example, high biocompatibility, effectively controlled drug release, and passive targeting capabilities).[141,142] Yang et al presented a simple and successful technique for producing mono-disperse liposomes employing microfluidics technology, a photo-cross-linkable gelatine methacryloyl (GelMA) matrix combined with KGN-loaded liposomes. Further, to enhance liposome stability and alleviate drug release, GelMA micro-gels were used to immobilize liposomes in the matrix structure by non-covalent interaction and physical network obstruction. They deduced that KGN-loaded GelMA@Lipomicrogels (GelMA@Lipo@KGN) may release prolonged KGN for more than three weeks and significantly increase chondrocyte differentiation of BMSCs in vitro when compared to KGN-loaded liposomes (Lipo@KGN). Furthermore, an in vivo study showed that injecting GelMA@Lipo@KGN into the joints of rats with surgically induced OA prevented cartilage degeneration, osteophyte burden, and subchondral bone changes while improving joint residence over five weeks.[143]

The Liposome-based delivery system acts as dynamic carriers that improved the biocompatibility and controlled release of KGN. By integrating liposomes within scaffolds, this hybrid system can enhance the mechanical support without changing its therapeutic efficacy.

KGN-Loaded Dendrimers

Dendrimers are molecules that are about nanometers in size and have a radially symmetrical shape that is uniform and monodisperse. Their structure appears to be a large, hyper-branched molecules deliberately engineered such that their end groups, or those extending to the outside, might be functionalized to alter their biological or physicochemical properties. They have various applications in supra molecular chemistry, especially in host-guest interactions and self-assembly. Their distinctive characteristics make them potential candidates for many different purposes.[144] Qing et al utilized a partially PEGylated polyamidoamine (PAMAM) dendrimer as a nanocarrier to transport nano KGN into the cytoplasm of MSCs and stimulate chondrogenic differentiation. In that case, PEG-PAMAM-KGN (PPK) and KGN-PEG-PAMAM (KPP) conjugates were created by linking nano KGN to the surface of PAMAM and the end group of polyethylene glycol, respectively. In comparison to PPK and free KGN, the KPP increased the expression of chondrogenic markers by increasing the level of CBFβ nuclear localization. Both OA and healthy rats possessed fluorescein-labeled PPK that could persist in the joint space for a long time. To treat OA, PPK might be a good nanocarrier for IA medication delivery system.[42]

The branching architecture of dendrimers enables targeted administration and high drug loading capacity, which may enhance KGN localization at damaged sites.

KGN in Hydrogels

For many years, hydrogel has been utilized in 3D cell culture research applications. The capacity to retain high water and elasticity makes them mimic native tissues. The absorption of water causes hydrogels to swell.[145] Water gets absorbed by the functional groups attached to the polymeric backbone and prevents it from dissolving.[146] Since the formation of inter-fibrillar or intermolecular cross-link structures between the network chains of fibrillar proteins or polymer molecules, respectively.[147,148] Hydrogels comprise desirable and controlled properties that are essential for tissue engineering. These include tunable mechanical strength and stiffness, intrinsic adaptability and biocompatibility, swellability, as well as biodegradability. It has been utilized to repair damaged tissue since they have a complex structure and function that are reminiscent of the ECM.[149] In 2017, Zhu et al prepared an injectable hydrogel comprising HA and chitosan in conjugation with KGN, which released KGN continuously. This hydrogel successfully stimulated cell differentiation in the nucleus pulposus of an intervertebral disk. In view of its excellent mechanical characteristics, KGN-conjugated hydrogel was selected as the best gel for enhancing cell proliferation and differentiation.[150] To achieve thermo-sensitivity and KGN, Dorsa Dehghan-Baniani chemically modified chitosan using β-Glycerophosphate (β-GP), N-(β-maleimidopropyloxy) succinimide ester BMPs and KGN to create in situ formation of chitosan hydrogels with an improved shear modulus, in order to achieve thermo-sensitivity. A large range of shear moduli, from 50 to 250 kPa, was covered by the hydrogen with a shear modulus of 78 ± 5 kPa.[151] It takes just a few minutes for it to gel at 37 °C after being injected non-invasively into the defect site. The thermo-sensitive hydrogel that was produced had several intriguing properties for cartilage tissue engineering, including an improved shear modulus, gelation behaviour, long-term drug release and injectability.[152] In a later investigation, Yuan worked on a thermos-sensitive dual drug-loaded hydroxypropyl chitin hydrogel (HPCH) system that could release stromal-derived factor-1α-like polypeptides (SDFP) and KGN for stem cell recruitment and chondrogenic differentiation. The network structure of the hydrogel allowed for the exchange of nutrients and enhanced cell growth. However, it worked effectively by fixing defects with temperature-sensitive properties. The system demonstrated superior biocompatibility in vitro as well as promoted chondrogenic differentiation and stem-cell recruitment. Additionally, it downregulated chondrocyte catabolism in inflammatory conditions. Experimental evidence suggests that the dual-drug hydrogel systems may stimulate articular cartilage regeneration in rats. This research provided strong evidence that the HPCH system, including KGN along with SDFP, is a promising approach for treating articular cartilage defects.[153]

The incorporation of nanomaterials within hydrogels has improved drug delivery.[154] For instance, Diaet al studied nano KGN-loaded PLGA MPs in a collagen-based hydrogel using the emulsification evaporation method.[155] Fan et al also used the EDC/NHS condensation process to attach KGN to polyurethane nanoparticles, which led to a 14% loading efficiency.[156] Houreh et al developed a multi-layered scaffold using a polycaprolactone (PCL) mat and CS hydrogel to improve the stability, integrity, and mechanical properties of CS, specifically for developing in vivo transplantation. The conjugated nano KGN in the matrix structure stimulated MSC chondrogenesis with its slow release. Furthermore, the gene expression study revealed that in the presence of KGN, the expression levels of COLL2, SOX9, and ACAN were higher. However, the expression of COLLX was downregulated, suggesting a synergistic impact of TGF-β and hypertrophy. The study confirmed that the multilayer structure system of KGN-loaded SDFP and HPCH not only effectively repaired articular cartilage defects but also demonstrated a successful treatment strategy.[157,158] Zhang et al synthesized a metal-ion-collagen hydrogel that was supra-molecularly wrapped with the chondro-inductive factor KGN using cyclodextrin (CD). Furthermore, the hydrogel was able to greatly increase the production of cartilage matrix components (ACAN as well as type II collagen) in articular chondrocytes without having any effect on cell proliferation or migration in vitro. The protective effects were based on improving intracellular antioxidant properties and mitochondrial activity in chondrocytes. In vivo, hydrogel injection enhanced cartilage regenerating capability and subchondral bone restoration after full-thickness lesions. This work highlighted the potential of a dynamic chondrogenic hydrogel as a new approach to treat refractory cartilage injury by facilitating healing processes via mitochondrial reinforcement.[159] An additional investigation by Mohsenifard et al designed an interpenetrating polymer network (IPN) hydrogel

containing beta-cyclodextrin (β-CD)-modified alginate/cartilage ECM, KGN, and 3D-printed poly (ε-caprolactone) (PCL)/starch microfiber network. This prototype demonstrated promise as a treatment for articular cartilage defects.[160] Massaro et al created a new halloysite nanotube (HNT)-based carrier system for the possible intra-articular administration of KGN using laponite (Lap) hydrogel (HNT/KGN/Lap), which displayed no harmful effects.[161]

Yuan et al employed Schiff base cross-linking reaction to develop an injectable CMC-OCS hydrogel incorporating MPs, and the PME method was utilized to fabricate PLGA MPs@KGN. When compared to control group hydrogels, the procured CMC-OCS@MPs@KGN resulted in a reduced gelation time along with a slower rate of weight loss. It was helpful for the cartilage healing process since the hydrogel@MPs had a significantly increased compressive elastic modulus.[162,163] Fan et al prepared a cell-free hydrogel containing TGF-β3 and polyurethane NPs (PN-KGN) to promote cartilage repair by simultaneously captivating endogenous MSCs and stimulating chondrogenesis in the recruited cells. The results revealed that the chondrogenesis of MSCs was improved by a combination of nano KGN and TGF-β3. It was also found that KGN promoted MSC chondrogenesis by reducing the degradation of RUNX-1, a protein that physically interacts with p-Smad3 in MSC nuclei.[156] Furthermore, Zere et al created two distinct configurations of laminated composite scaffolds using PCL: Gelatin electrospun mat, alginate sulphate hydrogel, and nano KGN. The prepared composite scaffold exhibited several advantages, including enhanced mechanical properties, reduced damage potential (less energy wasted), interconnected pores of fibre and hydrogel, a good swelling ratio, and properly controlled biodegradability.[164,165] In another study, Mungal et al reported the successful preparation of self-assembled PEGylated KGN (PEG/KGN) micelles through covalent crosslinking of OH-PEG-NH$_2$ along with 3-hydroxypropanoic acid-grafted KGN using carbodiimide chemistry. Over a period of five days in vitro, the HA/PEG/KGN hydrogels showed continuous KGN release. In vivo studies demonstrated that, in comparison to free HA hydrogel, HA/PEG/KGN hydrogel considerably reduced OA development in rats.[166] Chen et al designed a biocompatible hydrogel scaffold system for cartilage regeneration loaded nano KGN and with synthetic melanin nanoparticles (SMNP). The matrix was composed of cellulose nanocrystals, HAMA (hyaluronic acid-methacrylate anhydride) and GelMA (gelatin-methacrylate anhydride). The SMNP-KGN/Gel exhibited a suitable degradation rate, favourable mechanical properties, adequate MRI contrast enhancement and prolonged KGN release. The multi-parametric MRI could be utilized to evaluate hydrogel scaffold functionalization and degradation rate longitudinally and non-invasively in vivo. The SMNP-KGN/Gel demonstrated better thermal stability, excellent mechanical properties, and detectable MRI contrast enhancement. At the same time, both in vitro as well as in vivo studies revealed that nano KGN prolonged release encouraged BMSCs chondrocyte differentiation and proliferation, which in turn facilitated cartilage repair.[167,168] Liu et al developed a functional and convenient hydrogel-based CM-KGN@GelMA, which consists of TGF-β1, imitating CM-10 peptide and KGN encapsulation in liposomes. Since CM-10 was covalently crosslinked with GelMA, it had preserved its bioactivity to control BMSC chondrogenesis persistently to avoid the drawbacks of TGF-β1. A combination of KGN@Lipo and CM-10 stimulated chondrogenic differentiation of BMSCs by upregulating the expression of SOX9 and RUNX-1 in vitro.[169,170]

Hydrogel exhibits a biocompatible environment that is extremely hydrophilic, facilitating cell growth and differentiation, which can also release KGN in a controlled manner, thus boosting its therapeutic efficiency. Furthermore, the amalgamation of microspheres and hydrogel can simultaneously leverage the benefits of both. Numerous natural hydrogel materials degrade too quickly, hindering the process of prolonged drug release. Moreover, hydrogels generally exhibit low mechanical strength, rendering them inadequate for bearing the stresses of heavily loaded joint sites. Consequently, hydrogels may have more potent drug for early intervention in cartilage injuries or conditions necessitating injectable therapies, particularly treatments that do not demand long-term mechanical support but require sustained drug release. Future advancements in this area should result in joint repair options that are more personalized, efficient, and long-lasting.

Summary

In summary, KGN is unequivocally a pathway for cartilage regeneration. It has shown potential as a small chemical for stem cell-mediated cartilage repair. The biosafety, chondroprotective properties, and capacity to induce chondrogenesis have all been established. Irrespective of its prospective applications, there are scientific challenges and difficulties that need to be resolved. Particular aspects include the exact mechanism of action that needs more investigation. The results

of KGN need the enhancement of its long-term stability in water environments. Nonetheless, the brief retention and rapid elimination of KGN are notable limitations for clinical use, limiting its potential for long-term therapeutic advantages. The intra-articular injection of KGN has been found to enhance cartilage regeneration. However, oral administration presents a more straightforward and non-invasive application method that requires further studies, especially when combined with other drugs. Moreover, there is a paucity of research about the actual in vivo drug dose or its metabolites, despite KGN's use in the development and functionalization of various materials and the investigation of in vitro release profiles. Before proceeding with clinical applications, it is crucial to evaluate the safety and efficacy of KGN and its degradation products. When combined with biomaterials, it has enhanced synergistic actions that modify the physico-chemical and surface properties. Over the last decade, the biofunctions and applications of KGN have significantly advanced, enhancing our understanding and augmenting its therapeutic potential. Hydrogels were used for drug delivery and regulated release of medications to enhance retention duration in joints. Conversely, there are contradictory reports on the efficacy of KGN works. It is stated that the chondrogenic induction of KGN is much inferior to that of TGF, indicating its constraints for drug delivery in cartilage regeneration. Despite the potential of biomaterial-based KGN delivery, issues persist in maintaining its stability and bioactivity. In order to enhance the targeting and efficacy of delivery systems, investigation on innovative biomaterials, such as, nanoscale 3D composites and stimuli responsive materials (eg, pH, light, temperature) have become mandatory. The ultimate goal of advance fabrications strategies is to create functionalized carriers that overcome the challenges of conventional treatments. There are various advantages of using different delivery systems for long term therapy which are supported by polymeric microspheres and for short term therapy exosomes provide rapid release for efficient delivery of drugs. Additionally, novel biomaterials must be investigated for the incorporation, transport, and release of KGN.

Abbreviations

4-ABP, 4-aminobiphenyl; ACAN, Aggrecan; ADAMTS5, ADAM metallopeptidase with thrombospondin type 1 motif 5; ALP, Alkaline phosphatase; BMP, Bone morphogenetic protein; BMSC, Bone marrow-derived mesenchymal stem cell; CBFβ, Core binding factorβ; CECM, Chondrocyte extracellular matrix; COLII, collagen II; CPN, Cyclopamine; CSPC, Cartilage derived stem/progenitor cells; DMM, Destabilization of the medial meniscus; ECM, extracellular matrix; EVs, Extracellular vesicles; FTIR, Fourier transform infrared spectroscopy; GAG, Glycosaminoglycans; HA, Hydroxyapatite; HNMR, Hydrogen nuclear magnetic resonance; IA, Intra articular; IGF-1, Insulin-like Growth Factor-1; IHH, Indian hedgehog; KGN, Kartogenin; LC3, Light chain-3; MAPK, Mitogen activated protein kinase; MMP13, Matrix metallo-proteinase-13; MSC, Mesenchymal stem cells; NO, Nitric oxide; NP, Nanoparticle; NRF2, Nuclear factor erythroid 2-related factor 2; NSAIDS, Nonsteroidal anti-inflammatory drugs; OA, Osteoarthritis; PA, Phthalic acid; PRP, Platelet-rich plasma; Ptch1, Patched-1; PTSCSs, Patellar tendon stem/progenitor cells; RCCS, Rotary cell culture system; ROS, Reactive oxygen species; RUNX-1, Runt-related transcription factor 1; RUNX-2, Runt-related transcription factor 2; SHH, Sonic Hedgehog; SIRT1, Silent information regulator Type 1; SMNP, Synthetic melanin nanoparticles; Smo, Smoothened; SOX9, SRY-box transcription factor 9; TBJ, Tendon bone junction; TGF-β, Transforming growth factor- β; TSC, Tendon stem cells.

Disclosure

The authors report no conflicts of interest in this work.

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