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NEWS AND COMMENTARY

Tackling chondrocyte hypertrophy with multifunctional nanoparticles

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Osteoarthritis (OA) is the most common form of arthritis and leads to irreversible changes in all articular tissues and associated skeletal muscle.[1,2] OA is a collection of different phenotypic subtypes involving different relative contributions of the stressors and pathogenic pathways that trigger and drive, respectively, disease development (Figure 1). Recent investigations have shown that OA is driven by interplay between local joint inflammation (synovitis) and chondrocyte impaired bioenergy and protein homeostasis (see Liu-Bryan and Terkeltaub[2] for an excellent review). Another important pathway being studied in the OA field is that chondrocytes acquire a phenotype similar to terminal differentiating chondrocytes found at the growth plate and express markers of hypertrophic chondrocytes including type X collagen, matrix metalloproteinase-13 (MMP13) and vascular endothelial growth factor (VEGF).[3] These findings suggest that the loss of phenotypic stability of chondrocytes in OA reflects an early futile process to repair stressed cartilage that ultimately leads to pathologic cartilage calcification.

Chondrocyte hypertrophy is regulated by the activation of a constellation of transcription factors and other signalling molecules. Studies with knockout and transgenic mice have shown that deletion of many of these regulators results in resistance to OA development, thus suggesting that blocking chondrocyte hypertrophy by targeting these regulators in chondrocytes would be a valid therapeutic approach for OA. The direct exposure of the cartilage to the joint cavity makes chondrocytes amenable to targeting via intra-articular (IA) injection. This route offers the advantages of high joint bioavailability while reducing the risk of off-target effects. Nevertheless, the delivery of IA-injected small molecules and macromolecules into chondrocytes has been limited by the fast clearance out of the synovial cavity and poor diffusion through the tight cartilage matrix.[4] This problem has been recently tackled through the use of a new class of drugs based upon multifunctional nanoparticles designed to selectively and safely deliver therapeutic agents to a diseased site.[5] In an OA prevention study published in a recent issue of *Gene Therapy*, Pi *et al.*[6] described the use of polyetheleneimine (PEI) to fabricate chondrocyte-targeting nanoparticles. PEI was first conjugated to a chondrocyte-affinity peptide (CAP; DWRVIIPPRPSAC) and, next, used to condense an siRNA against hypoxia-inducible factor-2α (Hif-2α) in nanoparticles. The nanoparticles were injected weekly into the knees of 8-week-old male Chinese Kun Ming mice at the onset of OA, that is, 3 days after surgical destabilization of the knee joints by dissecting the anterior cruciate ligament (ACL), medial collateral ligament (MCL) and anterior horn of the medial meniscus. After seven weeks, mice treated with CAP-coated nanoparticles showed a significant reduction in cartilage breakdown and synovitis compared with sham-treated mice or OA mice treated with nanoparticles coated with non-targeting peptides. By providing proof-of-principle that chondrocytes can be therapeutically targeted in a surgical OA mouse model through the use of nanoparticles, this investigation provides a key step in the development of novel OA approaches aimed at blocking chondrocyte hypertrophy. Nevertheless, many questions remain to be addressed before such approaches can be translated into the clinic, as we capsulize below.

A pragmatic approach to tackle chondrocyte hypertrophy through multifunctional nanoparticles involves the identification of a molecular target involved in chondrocyte hypertrophy along with the assembly of a suitable combination of components in order to maximize the accumulation of drugs inside, or in proximity to, chondrocytes, while avoiding off-targeting.[5] Many transcription factors, including runt-related transcription factor-2 (RUNX2),[7] HIF-2α,[8,9] CCAAT/enhancer-binding protein-β (C/EBPβ)[10] and small mothers against decapentaplegic 1/5/8 (Smad1/5/8),[11,12] to name a few, have been identified as targets and their inhibition has been regarded as an ideal therapeutic strategy for OA. However, the activation of many of these transcription factors is not constant during OA development—for example, HIF-2α is overexpressed in early and progressive stages of OA but is downregulated in the late stages of OA[9]—which makes the time window for the therapeutic use of gene inhibitors potentially narrow. Moreover, the therapeutic index of gene inhibitors against some of these transcription factors may be limited given their homeostatic role in tissues other than cartilage, for example, HIF-2α is required for maintenance of cardiorespiratory homeostasis and carotid body function and SMAD1/5/8 are key regulators of neural development.[13,14] However, these limitations could potentially be offset through local IA use of chondrocyte-targeted nanoparticles, which could boost the accumulation of drugs inside chondrocytes and abate off-targeting effects. Pi *et al.*[6] were able to boost the accumulation of anti-Hif-2α siRNA inside chondrocytes using PEI nanoparticles coated with targeting agents against receptors expressed on anabolic chondrocytes. To this end, chondrocyte-targeted therapeutic approaches for OA would benefit from using peptides against receptors overexpressed on hypertrophic, rather than homeostatic, chondrocytes.

Recent studies have discovered that 5′ AMP-activated protein kinase (AMPK) activity is potentiated by a positive feedback loop among AMPK, sirtuin-1 (SIRT1) and liver protein kinase B1 (LKB1).

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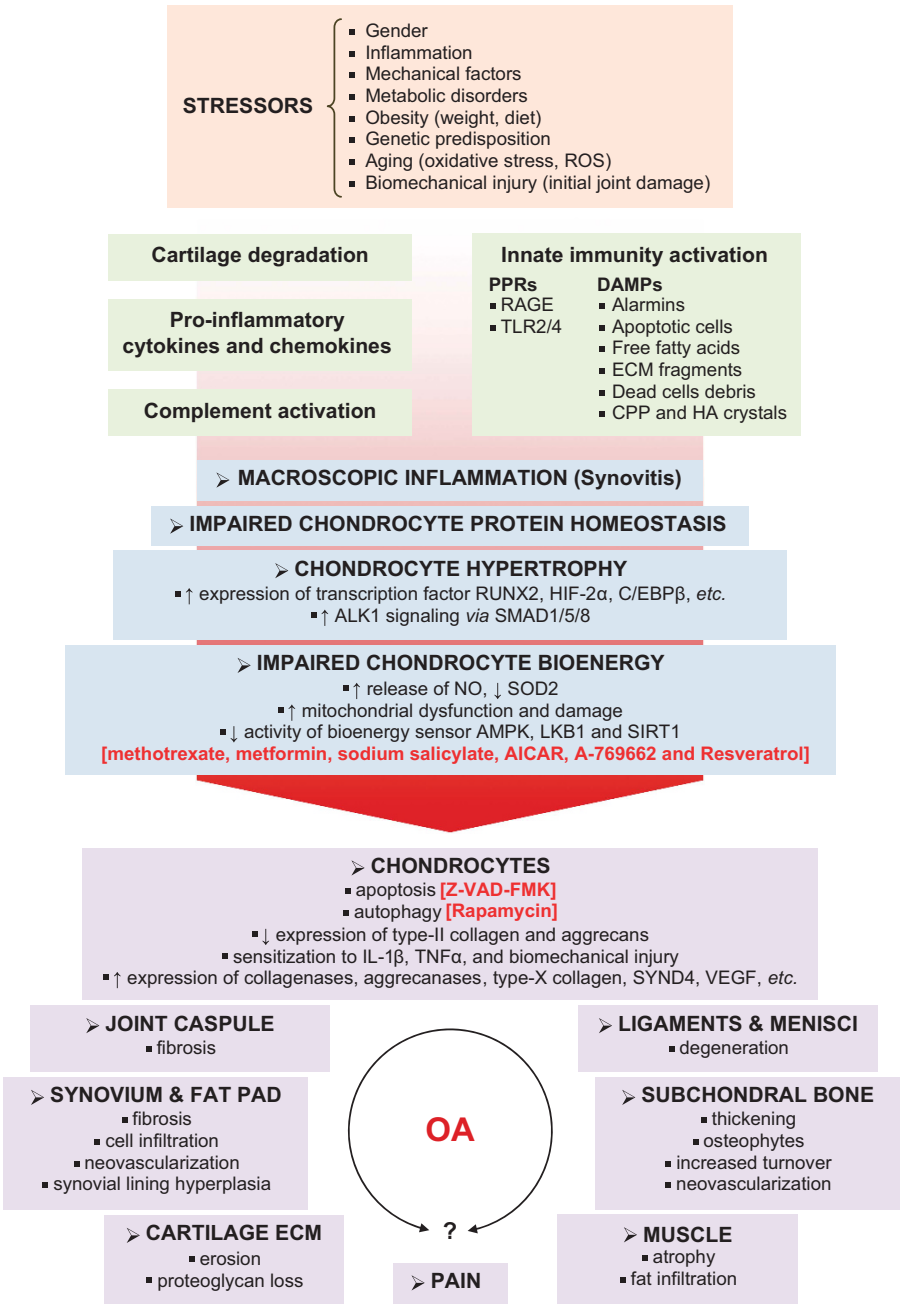

Figure 1. The role of chondrocytes in OA development. Stressors (light orange box), including genetic predisposition, obesity, ageing and biomechanical injury, to name a few, trigger initial meniscal and hyaline cartilage degradation and activate a complex network of inflammatory meditators, including PRRs (expressed in both synovium and cartilage) and their DAMP ligands, pro-inflammatory cytokines, chemokines and proteins of the complement system (light green boxes). This network promotes macroscopic inflammation, including synovitis, impaired chondrocyte bioenergy and protein homeostasis, and chondrocyte hypertrophy (light blue box), and trigger a self-perpetuating loop of changes in all articular tissues and associated skeletal muscle (light purple box). The mechanisms of cross-talk and feedback among the tissues relevant to OA development are not completely understood (question mark). Although more than one road leads to OA development, blocking chondrocyte hypertrophy has been envisaged as an ideal therapeutic approach for OA. Some of the drugs used to activate cell bioenergy regulators involved in OA pathophysiology or inhibit apoptosis and negative regulators of autophagy are reported in red. These drugs might be successfully translated into the clinic for the treatment of OA in the near future. ALKs, activin-like kinases; AMPK, 5′ AMP-activated protein kinase; C/EBPβ, CCAAT/enhancer-binding protein-β; CPP, calcium pyrophosphate dehydrate; ECM, extra-cellular matrix; ER, endoplasmic reticulum; DAMPs, danger-associated molecular patterns; DDR2, discoidin domain receptor 2; HA, hydroxyapatite; HIF-2α, hypoxia-inducible factor 2α; IL-1β, interleukin 1-β; LKB1, liver protein kinase B1; MMPs, matrix metalloproteinases; NGF, nerve growth factor; NO, nitric oxide; OA, osteoarthritis; PRRs, pattern recognition receptors; RAGE, receptor for advanced glycation end-products; ROS, reactive oxygen species; RUNX2, runt-related transcription factor-2; SIRT1, sirtuin-1; SMAD1/5/8, small mothers against decapentaplegic 1/5/8; SOD2, superoxide dismutase 2; SYND4, syndecan 4; TLR2/4, toll-like receptor 2/4; TNF-α, tumor necrosis factor-α; UPR, unfolded protein response; VEGF, vascular endothelial growth factor.

AMPK activation is induced via phosphorylation of AMPKα subunit and inhibits chondrocyte pro-catabolic responses to interleukin-1β (IL-1β) and tumour necrosis factor-α (TNFα) by inhibiting the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB).[15,16] AMPK activation also alleviates endoplasmic reticulum stress and promotes autophagy, a cellular homeostatic program for the destruction of damaged cell components in conditions of oxidative stress, which is defective in ageing and OA chondrocytes.[16,17] Drugs already in clinical use for arthritis and other diseases, including methotrexate, metformin, sodium salicylate, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and A-769662 (Figure 1),[18] activate AMPK and might be successfully translated into the clinic also for the treatment of OA. The clinical benefits in OA of these drugs would be improved by using degradable nanoparticles targeted to hypertrophic chondrocytes. Nanoparticles targeted to hypertrophic chondrocytes could also be used to improve the clinical translatability of resveratrol (a SIRT1 activator), rapamycin (a suppressor of mammalian target for rapamycin (mTOR), a negative regulator of autophagy) and Z-VAD-FMK (a pan-caspase inhibitor), in OA.[19–21]

As an alternative to inhibiting transcription factors or activating bioenergy sensors in chondrocytes, blocking upstream signalling through receptors that have a part in chondrocyte hypertrophy, for example, activin-like kinase 1 (ALK1),[11,12] discoidin domain receptor-2 (DDR2),[22] syndecan-4 (SYND4),[23] and toll-like receptor 2 and 4 (TLR2/4),[24] by means of specific receptor antagonists can interfere with OA progression. Novel OA therapies aimed at interfering with the signalling through these receptors would benefit from using degradable nanoparticles targeted to damaged cartilage components. This will improve nanoparticle retention within cartilage damaged zones and drug release in proximity to hypertrophic chondrocytes.

The efficacy of halting chondrocyte hypertrophy with multi-functional nanoparticle-based OA therapies depends also on the type of nanoparticle. The physico-chemical properties of nanoparticles, including size, geometry and flexibility, can affect their ability to navigate through the cartilage extra cellular matrix (ECM). The latter is a three-dimensional bio-material composed of a collagen network with pores of ~60–200 nm in size and filled by large and highly negatively charged aggrecan proteoglycans and many other macromolecules, including small proteoglycans, glycoproteins, lipids and hyaluronic acid. Early studies have shown that proteoglycans form the main impediment to the transport of molecules with a molecular weight greater than 70 kDa (for example, dextran) through the cartilage ECM.[25] These results suggest that the pore size of cartilage ECM is much smaller than the spacing among collagen fibrils, that is, from few to few tens of nanometres, and strongly limits the maximum size of nanoparticles able to break through the cartilage matrix. A recent study showed that spherical nanoparticles with an average size of 38 nm penetrated into mouse cartilage, while nanoparticles with an average size of 96 nm did not.[26] However, the nanoparticles had a broad size distribution, thus, it can be hypothesized that only the smaller particles of the batch with a 38 nm-centred size distribution penetrated the cartilage. Further investigations are necessary to assess the size limit for spherical nanoparticles able to penetrate the cartilage.

Geometry also governs the mobility of nanoparticles through the cartilage. Our group has described the trafficking profile of carbon nanotubes (NTs) modified with polyethylene glycol (PEG) chains (PEGylated NTs or PNTs) in the cartilage of both healthy and OA 4-month-old mice.[27] OA was induced by destabilization of the medial meniscus (DMM).[28] PNTs are biocompatible and biodegradable nanoparticles with a needle-like shape and a diameter of few nanometres and an average length of ~100 nm.[29,30] We found that PNTs could penetrate the dense cartilage ECM barrier and accumulate into chondrocytes of both healthy and OA mice 3 days after IA injection even though their length was greater than the mesh size of the cartilage matrix. This phenomenon can be explained by considering that PNT nanoscale diameter and one-dimensional structure, along with dynamic compression during ambulation, could have facilitated nanoparticle penetration in the dense cartilage ECM. This phenomenon can also be explained by considering a general property of nanoparticles in biologic milieu. As soon as they enter a biologic environment, nanoparticles are coated by a corona of proteins, lipids and biomacromolecules (also named bio-corona), which mediates the effects of nanoparticle physico-chemical properties on their biologic performance, that is, trafficking, clearance, biocompatibility and degradation.[31,32] In a recent study, we have identified the major plasma protein constituents of PNT 'plasma bio-corona' and shown that an increase in surface PEG density affected the competitive adsorption of the major constituents of PNT bio-corona.[33] In particular, an increase in surface PEG density led to a greater relative abundance of β-2-glycoprotein (Apo H) in PNT bio-corona, which changed the pharmacokinetic profile of systemically administered PNTs, that is, promoted shorter blood circulation half-life, faster excretion and greater relative spleen vs liver accumulation. Among the constituents of PNT bio-corona, inter-α-trypsin inhibitor (ITI) family proteins and fibronectin were identified. ITI family proteins bind HA, whereas fibronectin is a ligand of $\alpha_5\beta_1$ integrins on chondrocyte surface.[34–36] As these proteins were found in both healthy and OA synovial fluids,[37] it can be hypothesized that the 'synovial bio-corona' adsorbed onto PNTs as soon as they enter the synovial cavity consists of proteins that may engage specific cartilage ECM constituents and/or chondrocyte receptors. Further investigations will be useful to shed the light on the relationship between nanoparticle physico-chemical properties, synovial bio-corona and biologic performance in OA mice.

Recent investigations have suggested that the *in vitro* and *in vivo* formed bio-corona could reduce the targeted uptake of nanoparticles by cells,[38] while other studies have provided evidence that nanoparticles could be designed in such a way that the bio-corona of adsorbed proteins could be exploited for targeted delivery of nanoparticles and their cargo.[39] The crucial role of the bio-corona on nanoparticle ability to target a desired location has stimulated the search for 'natural' drug nanocarriers that exploit their intrinsic bio-corona to target specific tissues/cell sub-populations, while avoiding the adsorption of biomolecules from the surrounding milieu.[31] Headland *et al.*[40] have recently used synovial neutrophil-derived microvesicles (MVs) to activate anabolic gene expression in chondrocytes and lessen cartilage breakdown caused by rheumatoid arthritis (RA). Following IA injection, MVs entered the cartilage and bound formyl peptide receptor 2 (FPR2)/ALK receptor on chondrocytes through the anti-inflammatory protein annexin A1 (AnxA1) overexpressed on their surface, inducing the production of transforming growth factor-β1 (TGF- β1) and deposition of cartilage ECM. Microscopy revealed that a proportion of MVs localized inside chondrocytes. The ability to target chondrocytes was most likely in response to chemokine gradient; however, the authors were not able to identify the chemoattractant. It is worth noting that MVs entered cartilage despite their wide size distribution, from few nanometres to ~500 nm and with a median value of 143 nm, while synthetic microcapsule of comparable size did not. Although the mechanism of MV migration was not described, it can be hypothesized that a combination of active targeting via their natural bio-corona and flexibility allowed the MVs to enter cartilage. The use of MVs may also significantly impact OA therapy. Investigations are warranted to assess the use of MVs not only native as anti-inflammatory drugs *per se* but also engineered to encapsulate and release inside hypertrophic chondrocytes therapeutic agents.

The choice of a nanoparticle is also driven by the amount and type of therapeutic agents one wishes to deliver inside or in proximity to hypertrophic chondrocytes. Pi *et al.*[6] performed seven

weekly injections of ~50 nm-in-diameter PEI-based nanoparticles in the knee of 8-week-old mice 3 days after surgery to transport siRNA inside chondrocytes. However, the authors did not describe the amount of siRNA accumulated inside chondrocytes. As we have mentioned before, the dense cartilage matrix could have enabled only smaller nanoparticles to reach chondrocytes, thus limiting the maximum intracellular siRNA concentration. It is worth noting that, as age is one of the strongest risk factors for OA, experiments should be performed on surgical OA models based on older animals. It has been reported that DMM on 1-year-old mice led to more severe OA than on younger mice, thus using surgical OA models based on older animals may improve the navigation of bigger nanoparticles through the damaged cartilage.[41] Experiments are also warranted to assess the accumulation inside or in proximity to chondrocytes of drugs administered at different doses and/or dosing intervals. To this end, several types of nanoparticles can be used, including polymeric nanoparticles, liposomes, dendrimers and PEGylated carbon nanotubes. Polymeric nanoparticles with sizes ranging from few tens of nanometres to several microns can be fabricated by cross-linking polymer monomers via both covalent and non-covalent procedures. Polymeric nanoparticles can entrap a wide variety of small molecules and biomacromolecules, and release the cargo in a controlled way via diffusion and/or matrix degradation due to the presence of specific enzymes or changes in pH in the surrounding environment. Liposomes are approved drug delivery systems composed of a single or multiple nested lipid bilayers and have a core filled by an aqueous solution. They can entrap both hydrophobic and hydrophilic molecules within the lipid bylayers and inside the core, respectively. Dendrimers are tree-like structures with sizes ranging from few to several tens of nanometres and are characterized by the ability to adsorb large amounts of drugs within their branches via electrostatic attraction. Drug release from dendrimers could be due to diffusion and/or matrix degradation. As previously described, PNTs are needle-like particles with a diameter of few nanometres and an average length of ~100 nm. We have recently employed PNTs to deliver antisense oligomers (ASOs) against a green fluorescent protein (GFP) inside chondrocytes of healthy and OA GFP-transgenic 4-month-old mice and inhibit GFP expression.[27] ASOs were adsorbed onto PNTs via π-stacking and released within chondrocyte due to internal stimuli, that is, decrease in affinity for nanotube sidewalls due to the proximity to ASO targets. On-going studies have shown that a single IA injection of ~5 μg of PNTs, which is equivalent to ~1 μM of PNTs in the synovial fluid, in healthy mice led to 100–500 pM of PNTs inside chondrocytes 3 days after administration (unpublished data). Since 50–100 ASO molecules adsorbed onto each nanotube, we calculated that a maximum ASO concentration of ~50 nM was reached inside chondrocytes. It has been described that polyaromatic small molecules (for example, Paclitaxel, Doxorubicin) adsorbed via π-stacking in similar amounts onto PNTs and are released at the site of interest through either external (for example, near-infrared (NIR) radiation) or internal (for example, decrease in affinity for nanotube sidewalls) stimuli.[42,43] Thus, we posit polyaromatic small molecules can be accumulated within chondrocytes in concentrations similar to those calculated for ASOs by employing PNTs as delivery systems.[29] Similar calculations about the amount of drugs released by other types of nanoparticles inside or in proximity to chondrocytes have not yet been performed.

Finally, the translatability of approaches aimed at tackling chondrocyte-hypertrophy depends on the choice of the animal model. A detailed discussion about animal models of OA is beyond the scope of this commentary (see Malfait and Little[44] for an excellent review); however, some basic points will be discussed below. Surgical mouse models, such as the DMM or the one used by Pi *et al.* based on dissecting the ACL, MCL and anterior horn of the medial meniscus, have been the most commonly used animal OA models to assess nanoparticle ability to penetrate joint tissues and therapeutic efficacy. However, as OA is an age-related disease —OA resulted from injury accounts for about ~12% of total OA cases—the use of spontaneous OA mouse models due to ageing would be preferable to surgical OA mouse models. In addition, it is worth noting that the translation of the biologic performance of multifunctional nanoparticles in the cartilage from a mouse model to human is difficult. Mouse knee cartilage is ~50 μm thick, whereas that of human ranges from 1 to 2 mm. Thus, particle permeability of extremely thin mouse cartilage is not a good predictor of the biophysics of transport within the cartilage of OA patients and evaluation in bigger animals is necessary to exhaustively predict nanoparticle clinical translatability. Last, as OA is a highly heterogeneous disease, comprising overlapping yet distinct sub-types, the ability of multifunctional nanoparticles to deliver drugs into chondrocytes and/or other joint tissues should be carefully assessed in different animal models of OA at different stages of disease.[41,44]

Few multifunctional nanoparticles have been approved by regulatory agencies, and those approved are mostly focused on cancer therapy.[45,46] The benefits of multifunctional nanoparticles to cancer patients and new insights on the molecular mechanisms driving OA have recently driven academic research towards the development of nanotechnology-derived therapeutic approaches for OA aimed at tackling chondrocyte-hypertrophy. However, these approaches are still in their infancy, and their therapeutic efficacy has not been established in OA animal models as of yet. It can be anticipated that, as chondrocyte hypertrophy is caused by a shift in the balance between catabolic and anabolic cartilage processes, the use of multifunctional nanoparticles to simultaneously block degradative pathways and activate anabolic pathways (for example, by activating HIF-1α)[47] into chondrocytes would likely restore chondrocyte homeostasis more efficiently than using nanoparticles targeting only catabolic pathways. In addition, the use of multifunctional nanoparticles may be useful to unravel the mechanisms of cross-talk and feedback among the pathways within tissues that are relevant to OA progression. These mechanisms are not yet fully understood. For instance, nanoparticles can be used to target a pathway in one tissue and observe if changes in that pathway occur in another tissue. Last, further investigations are warranted to better understand the biologic performance of nanoparticles entering the synovial cavity and targeting specific cartilage sub-compartments. To this end, studies about the synovial bio-corona adsorbed onto nanoparticle surfaces will help to unravel the molecular mechanisms driving the biologic performance of nanoparticles in the joints and shed light on the potential use of nanoparticles in novel strategies for OA therapy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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