The function of microRNAs in cartilage and osteoarthritis

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ABSTRACT

MicroRNAs are small double-stranded RNAs, which negatively regulate gene expression and have been shown to have key roles in both chondrocyte development and cartilage homeostasis with age.

Deletion of all microRNAs in chondrocytes leads to skeletal growth defects in mice, whilst deletion of specific microRNAs, e.g. miR-140, leads to premature articular cartilage degradation and increased susceptibility to post-traumatic osteoarthritis.

Studies comparing microRNA expression in normal human articular cartilage compared to osteoarthritic cartilage show differential expression, but varying sample groups make interpretation difficult. MicroRNAs have been proposed as circulating biomarkers of osteoarthritis, but again, this differs amongst patient cohorts. Many microRNAs have been shown to have roles in chondrocyte phenotype via signalling pathways, apoptosis, autophagy and senescence.

Modulating microRNAs in the joint has been shown to reduce osteoarthritis in animal models and translating this to man as a novel therapeutic strategy will be key.

Introduction

Osteoarthritis (OA) is a degenerative joint disease characterised by degradation of articular cartilage, thickening of the subchondral bone, synovial inflammation and formation of osteophytes at the joint margin. It is a leading cause of disability. The aetiology of OA is complex with genetic, developmental, biochemical and biomechanical factors contributing to the disease process. The pattern of gene expression and the transcription factors that control the development of cartilage (chondrogenesis) are known in detail, though mechanisms leading to altered gene expression in OA are less well understood.

There is a large body of research that demonstrates that microRNAs, negative regulators of gene expression, are crucial in the development of the skeleton and in maintaining the homeostasis of cartilage across age. Further, microRNAs are dysregulated in cartilage during OA and have a functional effect on disease progression. This means that modulating specific microRNAs in cartilage may be a novel therapy in osteoarthritis. MicroRNAs may also act as circulating biomarkers in disease. In the following sections, we briefly review each of these areas.

The biology of microRNAs

The first microRNA, lin-4, was discovered in 1981 in *Caenorhabditis elegans* (1). Ambros and Ruvkun found that lin-4 downregulated lin-14 (a protein coding gene), controlling a specific step in development (1-3). The lin-14 3'UTR harboured multiple sites of imperfect complementarity to lin-4 and it was proposed that lin-4 bound to these sites and blocked the translation of lin-14. A second microRNA, let-7 was discovered in *C.elegans* and since this had homologues in higher species, this model moved across species. The term 'microRNA' was then coined for this class of non-coding gene regulators in 2001 (4-6).

MicroRNAs are evolutionarily conserved, short (~22nt long), double-stranded RNA molecules that negatively regulated gene expression at a post-transcriptional level by binding to specific sequences within target mRNAs (7).

To date, 1917 microRNAs have been identified in human cells (miRBase v22; http://mirbase.org/) and each is predicted to regulate several target genes (8). Many known microRNAs are either intergenic or located in introns of protein coding genes; a lower percentage of

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microRNAs originate from exons (9). A significant number of microRNA are found in polycistronic units encoding more than one microRNA and these are often functionally related (4, 5).

The majority of microRNAs are transcribed by RNA polymerase II and subsequently capped and mainly polyadenylated (10, 11). Transcription results in a primary microRNA transcript (pri-microRNA) harbouring a hairpin structure (12) (Fig. 1). Within the nucleus, the RNAse II-type molecule Drosha and its cofactor DGCR8 process the pri-microRNAs into 70- to 100-nt-long pre-microRNA structures (13), which in turn are exported to the cytoplasm through the nuclear pores by Exportin-5 (14). Subsequently, the RNAse III-type protein Dicer generates a double-stranded short RNA in the cytoplasm (15). This duplex microRNA is unwound by a helicase into single-stranded short RNA in the cytoplasm. One strand of the microRNA duplex is selected as the guide microRNA and remains stably associated with the microRNA-induced silencing complex (mRISC); the other strand, known as the passenger strand, can be rapidly degraded (16). The strand with less-stable base pairing at its 5' end is usually destined to become the active strand (17), however, some microRNA 'passenger' strands are themselves active and can negatively regulate gene expression. The mature microRNA guides the mRISCs mainly to the 3'UTR of its target microRNA (18). The seed sequence, comprising nucleotides 2-8 at the 5'-end of the mature microRNA, is the most important for binding of the microRNA to its target site in the mRNA (18), though other sequences such as the supplementary region (nucleotides 13-16) are increasingly seen as vital for effective target repression (19). In an alternative pathway for microRNA biogenesis, short hairpin introns termed mirtrons are spliced and debranched to generate pre-microRNA hairpin mimics (20). These are then cleaved by Dicer in the cytoplasm and incorporated into typical microRNA silencing complexes. The presence of mirtrons may be an evolutionary strategy to diversify mi-RNA-based gene silencing (21).

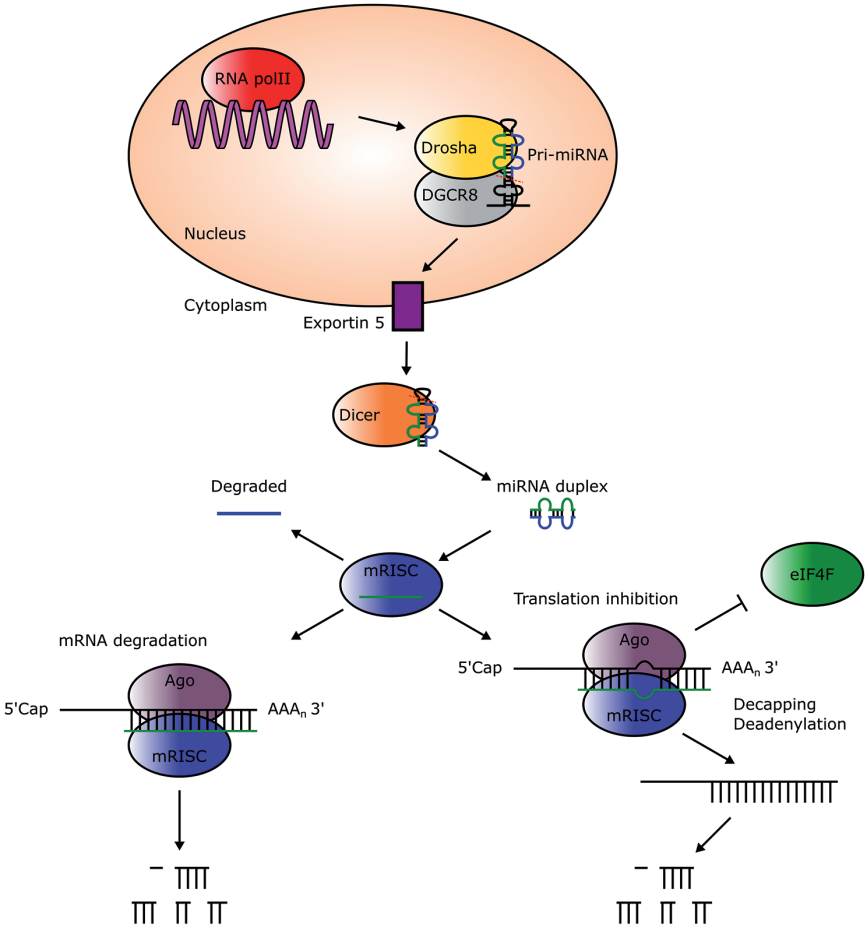

Fig. 1. Biogenesis of microRNAs. MicroRNAs are transcribed as primary transcripts (pri-microRNAs) and processed by Drosha/DGCR8 to hairpin precursors (pre-microRNAs). These are exported to the cytoplasm and further processed by Dicer into the mature double-stranded microRNA. The duplex is unwound and one strand (guide) incorporated into RISC whilst the other strand (passenger) is degraded. The microRNA-RISC complex acts either to degrade its mRNA target where homology is high, or to inhibit translation, leading to mRNA degradation, where homology is lower.

The degree of base pairing between the microRNA and its target in the mRISC determines the fate of the transcript. If there is perfect complementarity between the microRNA and target, the mRNA target is cleaved by Ago2 at the annealing site, with subsequent degradation of the mRNA. In contrast, where the microRNA is only partially complementary to its corresponding 3'UTR (almost all cases in mammals), inhibition of target mRNA translation occurs via Ago1 (22).

The skeletal impact of deleting microRNAs

The mutation or deletion of Dicer, prevents the biogenesis of the majority of microRNAs, demonstrating a role for the class in skeletal development. Conditional knockout of Dicer in limb mesenchyme at the early stages of embryonic development leads to the formation of a smaller limb (23). Dicer null growth plates show lack of chondrocyte proliferation, but also enhanced hypertrophy (23). Conditional knockout of Dicer in chondrocytes results in skeletal growth defects and premature death (24) with a similar phenotype in conditional Drosha knockouts (25). The deletion of specific microRNAs is discussed below.

Differential expression of microRNAs in cartilage

There have been many reports of microRNAs being differentially expressed in cartilage from osteoarthritis patients compared to normal cartilage (*e.g.* (26-29)). For microarray or RNA-Seq screens of expression, these do not give

consistent differentially expressed microRNAs, likely due to sample differences. However, the identity of specific microRNAs has been the starting point for several studies.

An analysis of the published literature on the expression of microRNAs and OA was performed by Cong *et al*. This identified 46 differentially expressed microRNAs involved in a number of processes including apoptosis and autophagy, differentiation, metabolism, ECM degradation and inflammation in chondrocytes (30).

The 'functional' microRNAs within chondrocytes have been assessed using AGO2 immunoprecipitation to identify microRNAs in the RISC complex. In cells taken from the cartilage of either OA patients or normal controls, miR-27b-3p was most abundant, with miR-140-3p (see below) eighth most abundant (31).

A powerful technique was utilised by Coutinho de Almeida *et al*. who performed both mRNA-Seq and microRNA-Seq from OA lesion and macroscopically normal cartilage from the same patient (32). This enabled the construction of a so-called interactome of differentially expressed mRNAs and microRNAs. Amongst much data, miR-99a-3p was downregulated in OA lesions and targets 36 differentially expressed mRNAs, three of them showing strong correlation (FZD1, ITGB5, GSF6), whilst miR-143-5p is increased in the OA lesions and targets 16 differentially expressed mRNAs including the strongly correlated DCAKD, AMIGO1 and SMAD3 genes.

Some information on the role of microRNAs in cartilage development, homeostasis and osteoarthritis is given below.

MicroRNAs and chondrogenesis

Many microRNAs have been identified which target transcription factors or signalling molecules involved in chondrogenesis and which therefore regulate the process. MicroRNAs may therefore be useful in cartilage repair or engineering (*e.g*. (33, 34)).

Amongst myriad examples, Sox9 induces expression of many microRNAs, with miR-140 particularly responsive (35). The microRNA-140 null mouse has a clear skeletal phenotype, as below, with a decrease in proliferating chondrocytes in the growth plate (36, 37). This is at least in part via its ability to target Sp1 and alter cell cycle (38). A number of other targets for miR-140-5p have been identified during hMSC chondrogenesis *in vitro*, including RALA and FZD6 (39, 40).

MiR455 is co-regulated with miR-140 in both ATDC5 and hMSC models of chondrogenesis (39, 41) and is also highly Sox9 inducible (39). miR-455-3p has also been shown to act in early chondrogenic differentiation via direct targeting of Runx2 (42) and potentially HDAC2 and HDAC8 (43). It may also impact on DNA methylation during chondrogenesis via DNMT3A (44).

MicroRNA and signalling

MicroRNAs have been shown to regulate and be regulated by many signalling pathways implicated in OA and it is beyond the scope of this review to explore them in any detail.

To give one example, TGFβ both regulates the expression of a number of microRNAs and TGFβ/Smad signalling is regulated by both the same and different microRNAs. For example, TGFβ reduces the expression of miR-140 (45) whilst Smad3 was identified as a direct target of miR-140-5p (46). Similarly, TGFβ reduces expression of miR-29 in many cell types including chondrocytes (47), and this is Smad3 dependent, at least in renal fibrosis (48). However, miR-29 can suppress TGFβ signalling (47). Conversely, TGFβ induces expression of miR-455 in chondrocytes, whilst miR-455-3p suppresses TGFβ signalling via directly targeting Smad2 (41). Hence, both feedforward and feedback loops exist between TGFβ signalling and microRNAs.

MicroRNAs have been shown in chondrocytes to regulate Smad2 and 3 (above) but also Smad4. MicroRNA-483 was identified as significantly decreased in expression in chondrocyte hypertrophy (49). Overexpression of miR-483 showed that it downregulated Smad4 to suppress chondrogenesis and reduce extracellular matrix production. Interestingly, levels of Smad4 mRNA were not altered by miR-483 overexpression, but protein levels were and this is the same for miR-140-5p and Smad3 above (46).

MicroRNAs have been shown to impact on the majority, if not all, of the signalling pathways pertinent to OA, though the role this has in pathogenesis is unclear.

MicroRNAs and chondrocyte apoptosis / senescence / autophagy

A number of different microRNAs were initially shown to inhibit apoptosis, but sometimes also to enhance apoptosis via a number of targets and mechanisms. This research has broadened to implicate microRNAs in the regulation of autophagy and senescence too, all potentially key components of OA pathogenesis. Some examples are given below.

MicroRNA-34a was the first microRNA to be linked to chondrocyte apoptosis, with an inhibitor of miR-34a reducing IL-1-induced apoptosis in rat chondrocytes (50). MicroRNA-34a is increased in expression in human OA cartilage compared to control, whilst SIRT1, shown to be a direct target, was decreased (51). This led to less acetylation of p53, increase in the pro-apoptotic Bax, decrease in the anti-apoptotic Bcl-2 and an increase in chondrocyte apoptosis. Markers of autophagy were also decreased in chondrocytes by miR-34a over expression (52). Zhang *et al*. 2018 reported that a miR-34a mimic increased both cell death and senescence in chondrocytes via targeting the Notch pathway (53). Both Zhang *et al*. and Yan *et al*. showed that intraarticular injection of a miR-34a inhibitor in a model of OA abrogates cartilage destruction (51, 53).

MicroRNA-24 regulates the cell cycle inhibitor P16INK4a, a marker of senescence. P16INK4a and other markers of the senescence-associated secretory phenotype (SASP) increase in OA and in terminal chondrogenesis, whilst miR-24 is decreased (54).

MicroRNA-495 is also elevated in human OA cartilage (55). MicroRNA-495 overexpression increases chondrocyte apoptosis and also increases markers of senescence (SA-β-gal and p16), via direct targeting of AKT1 and the S6-

mTOR system. Intra-articular injection of a miR-495 inhibitor in a rat model of OA induced by anterior cruciate ligament transection (ACLT) decreased OA and decreased chondrocyte apoptosis. Yang *et al*. 2019 also show that inhibition of miR-495 suppresses apoptosis through activation of the NFκB pathway and CCL4, the latter a potential direct target for miR-495 (56).

When cells are under stress, autophagy can be activated to prevent apoptosis and a number of microRNAs have been reported to do this. Recent examples include: expression of miR-335-5p was lower in OA than normal chondrocytes, with overexpression increasing autophagy whilst reducing inflammation (57); Zhao *et al*. 2019 showed that expression of miR-107 was lower in OA chondrocytes than control and again, overexpression inhibited apoptosis and increased autophagy (58).

Interestingly, extracellular vesicles (EVs) from senescent cells taken from chondrocytes from human OA cartilage can transfer senescence to non-senescent chondrocytes and inhibit cartilage formation by these cells (59). These EVs contained less miR-140-3p and more miR-34a that EVs derived from non-senescent chondrocytes. Clearance of senescent cells (SnC) resulted in decreased miR-34a in synovial fluid derived EVs from young mouse joints with post-traumatic OA (ACLT), though no differences were seen in older animals. A complete analysis of microRNAs in synovial EVs from mice with PTOA showed that a number of microRNAs alter, though only miR-223 decreases in both young and old mice with PTOA after senolytic treatment (59).

Deletion of specific microRNAs

Whilst the initial studies showed that microRNAs, as a class, are functionally important in skeletogenesis (see above), the role of individual microRNAs in development or in osteoarthritis *in vivo*, is less well explored.

By far the most studied microRNA in cartilage is microRNA-140. Originally described as expressed in a skeletally restricted pattern in the developing zebrafish (60), this was then confirmed in the mouse (61). MicroRNA-140-5p has been shown to directly mediate expression of *e.g*. IGFBP-5, MMP13, Hdac4, Cxcl12, Bmp2 and Smad3 (45, 46, 62), all of which are implicated in chondrocyte development and/or cartilage homeostasis. However, it appears that miR-140-3p is the most abundantly expressed in cartilage and it may be that miR-140-5p is most important in development (39, 40), whilst miR-140-3p potentially has a greater role in tissue homeostasis.

The expression of miR-140 in knee cartilage from osteoarthritis patients was decreased compared to normal cartilage (though these were not age-matched groups) (63). The complete knockout of miR-140 in mice leads to mild dwarfism, with impaired chondrocyte differentiation / proliferation (36, 37). Deletion of miR-140 also predisposed mice to the development of spontaneous age-related cartilage breakdown (36, 37) and increased cartilage destruction in surgically-induced OA (36). Key targets identified include *ADAMTS5* (36) and the aspartyl aminopeptidase Dnpep (37), but many more targets exist which likely contribute to the phenotype. The deletion of miR-140 also interacts with inhibition of let-7 microRNAs in the mouse to give a more severe skeletal phenotype than either single mutation (64).

The relevance of miR-140 to human disease was recently proven when a rare autosomal dominant skeletal dysplasia, found in two families, was tracked to a mutation in the seed sequence for miR-140-5p (65). This single base substitution was reiterated in knock-in mice, demonstrating a neomorphic phenotype and not simply loss of function.

Another well-researched microRNA is miR-146a, whose expression is stimulated by many inflammatory mediators and with roles in the immune system and inflammation (66). MicroRNA-146a is highly expressed in cartilage from early stage human osteoarthritis patients (67) and may modulate pain in disease (68). It is reported to have effects on inflammation, chondrocyte autophagy and apoptosis and the expression of extracellular matrix genes (*e.g*. (69-72))

Recently, a miR-146a null mouse showed decreased cartilage destruction both spontaneously and in three different models of induced osteoarthritis (destabilisation of the medial meniscus DMM, partial medial meniscectomy PMM and anterior cruciate ligament transection ACLT) (73). Intraarticular delivery of a miR-146a inhibitor decreased cartilage destruction in surgically induced OA, with target genes, calcium/calmodulin-dependent protein kinase II delta (Camk2d) and protein phosphatase 4, regulatory subunit B, beta isoform (Ppp3r2) identified as essential in regulating cartilage homeostasis. In contrast to this, Guan *et al*. (74) showed that miR-146a expression is decreased in lesions compared to non-lesions in human OA cartilage. They went on to characterise miR-146a null mice as developing early onset spontaneous OA, whilst conversely, a mouse overexpressing miR-146a in a chondrocyte-specific manner was protected from such disease. Furthermore, miR-146a null mice were more susceptible to instability induced OA and the conditional overexpressing mice were again protected. This appears to be via the ability of miR-146a to target Notch1, with intraarticular delivery of a Notch1 inhibitors rescuing joint destruction in the miR-146a null mice. Clearly these conflicting data require resolution.

MicroRNA-21-5p was increased in expression in human cartilage from osteoarthritis patients compared to normal controls. Cartilage specific deletion of miR-21 in mice decreased spontaneous cartilage lesions and protected from induced osteoarthritis (75).

Finally, miR-128a is increased in expression in human cartilage from osteoarthritis patients compared to normal controls and targets Atg12 to repress chondrocyte autophagy (76). A recent meeting report suggests that a cartilage-specific miR-128a null mouse had decreased osteoarthritis in both DMM and collagenase-induced OA models (77)

As described above for miR-140 and miR-146a above, the overexpression of a microRNA *in vivo* may also allow the dissection of its function. In order to explore data suggesting that intraar-

ticular delivery of miR-483-5p increased severity and progression of OA in the DMM model, Wang *et al*. used an inducible transgenic mouse overexpressing miR-483. This mouse again showed an increase in spontaneous and induced OA via targeting of matrilin 3 and Timp2 (78).

MicroRNAs as biomarkers of OA

Since microRNAs are found in the circulation and exhibit good stability there, the possibility that their plasma levels act as a biomarker of disease status is clearly attractive.

Beyer *et al*. 2014 used a Taqman microarray screen to measure the levels of 374 microRNAs in the pooled plasma of a sub-group of patients in a longitudinal cohort who had or had not progressed to joint replacement. Identified microRNAs were then assayed in the entire cohort to show that let-7e, miR-454 and miR-885-5p were potentially predictive of OA progression. Let-7e was decreased in the plasma of osteoarthritis patients compared to control with robust statistical analyses (79).

A similar Taqman microarray screen was performed by Borgonia Cuadra *et al*. 2014 which identified 12 microRNAs with statistically different levels in OA plasma compared to controls in two cohorts (80).

In 2017 Kong *et al*. used a microarray to measure 2578 mature microRNAs in OA compared to normal plasma identifying 70 differentially expressed microRNAs. Eight of these were validated by qPCR to identify miR-19b-3p, miR-122-5p and miR-486-5p as independent factors for the risk of knee OA, with miR-19b-3p and miR-486-5p positively correlating with disease severity (81).

Ntoumou *et al*. 2017 also used a further microarray to measure 2549 microRNAs in OA *versus* control plasma, identifying 279 differentially expressed microRNAs. QPCR validation identified miR-140-3p, miR-33b-3p and miR671-3p as potential biomarkers (82).

Across these studies, there were no validated microRNAs which were identified in all four as significant. A number of microRNAs (miR-122, miR-885, miR-140, miR-93 and miR-663a) were identified in two of the studies. This likely reflects differences in patient groups and numbers but also differences in the extent and methodology of measurement.

Several other studies have measured specific microRNAs as potential circulating biomarkers of osteoarthritis too (83-86).

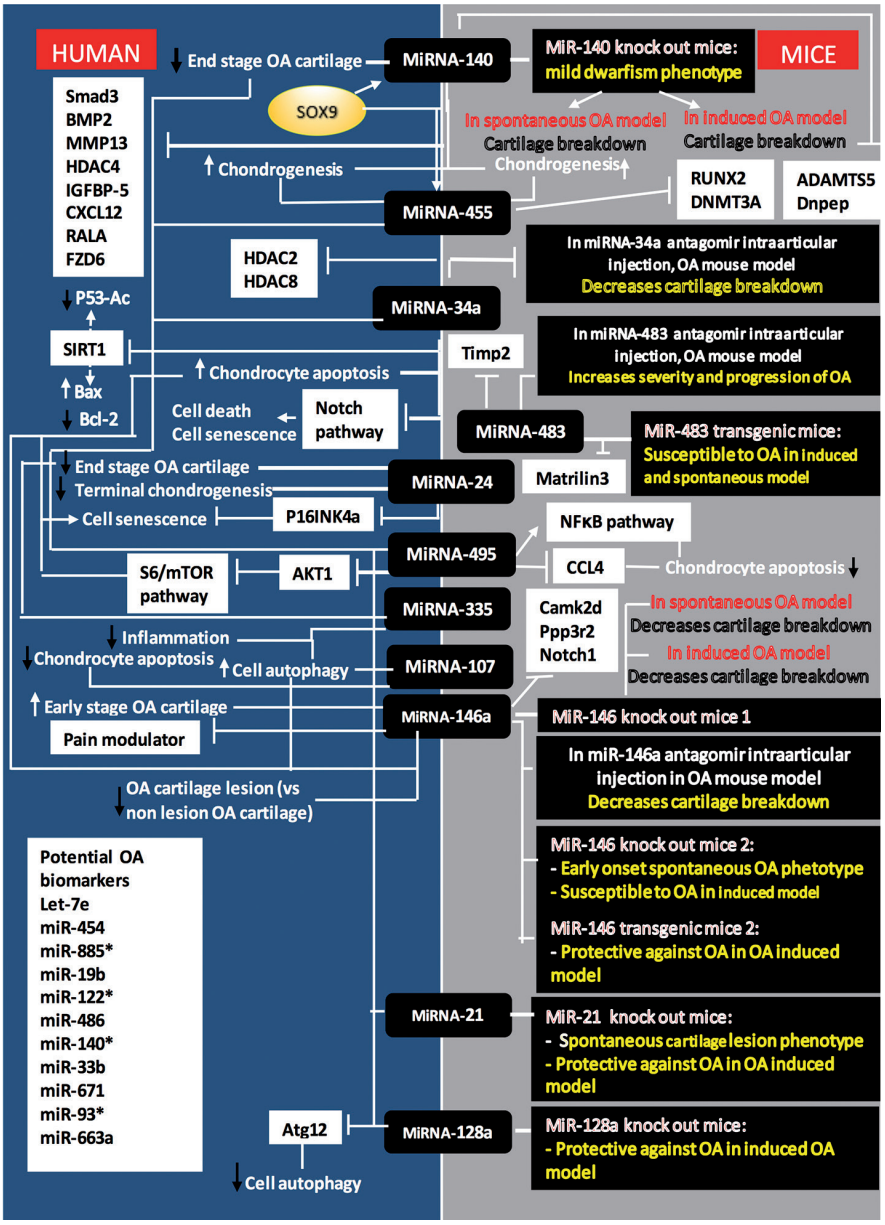

Fig. 2. Examples of the roles of microRNAs in osteoarthritis.
A number of microRNAs have been shown to be dysregulated in osteoarthritic cartilage and to have functions in chondrogenesis, chondrocyte autophagy, apoptosis, senescence, intracellular signalling and inflammation. Data from human cartilage, cells or plasma is shown on the left hand side with complementary data from mouse models on the right hand side.

The therapeutic use of microRNAs in OA

In order to be used therapeutically, microRNA mimics or their inhibitors must be successfully introduced to the joint and taken up into joint tissues, *e.g*. cartilage. As proof-of-concept of this, the majority of studies have used intraarticular injection of microRNA- or inhibitor-expressing lentivirus in mouse and rat models of OA. Examination of cartilage integrity shows efficacy, but does not demonstrate uptake into cartilage itself, though measurement of known microRNA targets in cartilage tissue act as a surrogate for this. However, Lian *et al*. used lentivirus to

deliver miR-128a or its inhibitor to the joint. They then demonstrated uptake into cartilage and chondrocytes using in situ hybridisation (ISH) as well as functional outcome where microRNA-128a mimic increased OA score and miR-128a inhibitor decreased OA score (76). Similarly, Wang *et al*. delivered miR-483 intraarticularly using lentivirus and showed uptake into cartilage using ISH and also via co-expressed GFP expression in mid and deep zone chondrocytes. Dai *et al*. use an adenoviral delivery of miR101 or an inhibitor to the joint in a rat MIA model and again use a GFP tag to demonstrate full depth penetration of the virus and expression (87).

Viral delivery to the mouse knee is simply proof-of-concept and is associated with several risks that make it unlikely to be translated to man. Non-viral delivery systems are also being developed. Interestingly, Wang *et al*. also delivered an inhibitor of miR-483 directly to the joint (*i.e*. not virally) and show functional outcome (78). This was also achieved by Nakamura *et al*., who inject a short (16 mer) antisense LNA oligonucleotide intraarticularly in the DMM model of OA and show both histological improvement of OA and a concurrent decrease in OA markers (88).

MicroRNAs can be packaged into exosomes, nanometre scale particles produced by the majority of cells. Indeed, microRNAs can circulate through the bloodstream in this way and exosomes can be taken up by distant cells (89). This strongly suggests that exosomes could be used for therapeutic delivery of microRNAs. Mao *et al*. used exosomes produced by human mesenchymal stem cells which were transfected with miR-92a-3p or an inhibitor and introduced these by intraarticular injection into a collagenase-induced OA model in the mouse. They used *in situ* hybridisation to show expression of miR-92a-3p by cartilage chondrocytes and demonstrated a functional effect (90). Similarly, Tao *et al*. use exosomes from miR-140-5p transfected synovial MSC injected intraarticularly in a rat ACLT model to show decreased OA (91)

All of these studies are in rodent models and trials of microRNA delivery to the joint in man have yet to be conducted.

Crossing over with the use of microRNAs as biomarkers in OA, Kohle *et al*. measured exosomal microRNAs in the synovial fluid of patients with OA compared to normal controls. This study demonstrated differential levels of several microRNAs in OA, with gender-specific differences in the pattern of microRNAs identified. This may both help understand the pathogenesis of disease and microRNAs which may be therapeutic in OA (92).

Conclusion

As in many fields of biology, a huge number of microRNAs have been implicated in cartilage physiology and pathology. Detailed studies on the function of single microRNAs *in vitro* and ultimately *in vivo* in knockout or transgenic mice will delineate the roles of specific microRNAs and intraarticular delivery of these microRNAs or their inhibitors may be therapeutic in OA. However, given that a single microRNA can target many mRNAs and that each mRNA can be the target for several microRNAs, it is clear that really understanding the system will require a mathematical modelling approach. Combining data from the research described above and further research, at all scales, will enable us to build better networks of microRNA (and mRNA) function in osteoarthritis, identifying key microRNAs as ideal targets for therapeutic intervention. Whilst this review has focused on cartilage, a similar story exists in other relevant tissues of the joint and again, understanding the interplay between these tissues at the level of microRNAs will be crucial.

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