The microRNAs miR-204 and miR-211 maintain joint homeostasis and protect against osteoarthritis progression

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Osteoarthritis (OA) is a common, painful disease. Currently OA is incurable, and its etiology largely unknown, partly due to limited understanding of OA as a whole-joint disease. Here we report that two homologous microRNAs, miR-204 and miR-211, maintain joint homeostasis to suppress OA pathogenesis. Specific knockout of miR-204/-211 in mesenchymal progenitor cells (MPCs) results in Runx2 accumulation in multi-type joint cells, causing whole-joint degeneration. Specifically, miR-204/-211 loss-of-function induces matrix-degrading proteases in articular chondrocytes and synoviocytes, stimulating articular cartilage destruction. Moreover, miR-204/-211 ablation enhances NGF expression in a Runx2-dependent manner, and thus hyper-activates Akt signaling and MPC proliferation, underlying multiplex non-cartilaginous OA conditions including synovial hyperplasia, osteophyte outgrowth and subchondral sclerosis. Importantly, miR-204/-211-deficiency-induced OA is largely rescued by Runx2 insufficiency, confirming the miR-204/-211-Runx2 axis. Further, intraarticular administration of miR-204-expressing adeno-associated virus significantly decelerates OA progression. Collectively, miR-204/-211 are essential in maintaining healthy homeostasis of mesenchymal joint cells to counteract OA pathogenesis.
Osteoarthritis (OA) is a painful joint disease affecting more than 10% of the adult population\(^1,2\). Pathological changes of OA embody multiple manifestations such as articular cartilage (AC) destruction, synovial hyperplasia, osteophyte formation, and subchondral bone sclerosis. In addition, OA patients often show varied symptoms and respond differently to clinical interventions, suggesting that OA is a heterogeneous disease and its management demands therapeutics that offer greater precision\(^3,4\). Currently available studies ascribe the pathogenic causes of OA to dysregulation of anabolic and catabolic pathways that affect cartilage matrix maintenance and bone remodeling. However, clinical trials targeting related factors such as matrix metalloproteinases (MMPs), inflammatory cytokines, or growth factors, showed mixed results regarding the efficacy or safety of the therapeutics\(^5,6\). Thus, development of efficacious OA therapies calls for more in-depth understanding of OA and should benefit from identification of new molecular mechanisms that underlie the complex and multifaceted phenotypes of OA.

Multiple joint tissues undergo structural and functional failure during OA pathogenesis, including AC, meniscus, and synovium. These tissues are mesenchymal or contain significant portions of mesenchymal cells. Moreover, mesenchymal stem cells (MSCs) and mesenchymal progenitor cells (MPCs) are thought to exist in these joint tissues and play important roles in joint homeostasis and maintenance\(^7\). Importantly, the formation of osteocytes, a prominent radiographic feature of OA and a significant source of pain and joint dysfunction, requires MSCs derived from adjacent areas including periosteum and synovium to recapitulate endochondral ossification\(^8\). As miRNAs emerge as important regulators in skeletal pathophysiology\(^9,10\), their roles in MPC-derived skeletal tissues remain elusive. Through in vitro work we identified two homologous microRNAs, miR-204 and miR-211, which negatively regulate Runx2 in MPCs\(^11\). However, the in vivo functions of miR-204/-211 are largely unknown, especially regarding how they regulate MPCs and the pathophysiology of mesenchyme-derived skeletal tissues like synovial joints.

Here we identify a mechanism by which miR-204/-211 regulate the proliferation and differentiation of MPCs in the entire joint and maintain healthy metabolism of joint tissues, thus helping the joint to counteract OA pathogenesis in a well-rounded manner. Our study takes advantage of in vivo analysis of genetically modified mice with miR-204/-211 deficiency in MPCs and their descendant cells and we reveal that miR-204/-211 Runx2 axis is pivotal in maintaining joint tissue homeostasis. Disruption of this signaling pathway leads to multifactorial pathogenesis of OA, particularly with respect to noncartilaginous pathological changes such as synovial hyperplasia and osteophyte formation. In addition, our results demonstrate that intraarticular reintroduction of miR-204 alleviates OA progression in a surgically induced OA model.

**Results**

**Global deficiency of miR-204/-211 induces OA.** Our in vitro data showed that two homologous miRNAs, miR-204 and miR-211, suppress Runx2 expression in MPCs\(^11\). However, little is known about their in vivo functions. Thus, we have generated mouse models with conditional knockout (cKO) alleles for miR-204 and miR-211. A diagram of the targeting vector for miR-204 or miR-211 is shown in Fig. 1a. To remove the lacZ and Neo cassettes, we crossed the targeted mice with flipasse-expressing transgenic mice, and thus generated floxed alleles for the miRNAs (Fig. 1a, Supplementary Fig. 1a). Since miR-204 or miR-211 was reported to be expressed in a variety of tissues and play diverse pathophysiological roles\(^12,16\), we were interested to investigate if genetically modified animals with global deletions of both miRNAs, which were not yet available but are essential to elucidation of the miR-204/-211 functions, showed any aberrant phenotypes. Thus, we crossed CMV-Cre transgenic mice\(^17\) with miR-204\(^{lox/lox}\) and miR-211\(^{lox/lox}\) mice to generate global knockout (KO) of both miRNAs (Supplementary Fig. 1b, c), namely the mice genotyped as miR-204\(^{−/−}\); miR-211\(^{−/−}\) mice. These miR-204/-211-deficient mice did not show very apparent abnormality postnatally and postpubertally. However, our preliminary analysis of older global miR-204/-211 KO mice through the Mouse Metabolic Phenotyping Center at University of California, Davis revealed a spectrum of pathological changes such as hypermature cartilars with rupture, uveal melanosis and melanosis, retinal dysplasia, follicular dysplasia, valvular endocardiosis, etc. (Supplementary Table 1).

We also looked at the knee joint phenotype of the global KO mice. At the age of 9 weeks, the miR-204\(^{−/−}\); miR-211\(^{−/−}\) mice did not differ from the wild type in joint histology, particularly in terms of the tibial growth plate length and AC thickness (Supplementary Fig. 1d, f). At the age of 15 weeks, the miR-204\(^{−/−}\); miR-211\(^{−/−}\) mice still did not show difference in growth plate length, but started to possess increased AC abrasion (Supplementary Fig. 1e, h). Strikingly, examination of the aged miR-204\(^{−/−}\); miR-211\(^{−/−}\) mice showed swollen knee joints, which was demonstrated by μCT analysis to contain ectopically grown bone spurs, resembling osteophyte outgrowth in human OA patients (Fig. 1b). The structural deterioration in the knee joints of the mutant mice involved multiple types of tissues inside the joint, as displayed by highly abrasive AC, subchondral bone thickening, severe synovial hyperplasia, excessive osteophyte formation, and meniscus calcification (Fig. 1c–f). As the pathologic condition of human OA disease and a well-established OA model\(^18\) (Supplementary Fig. 1i) embodies all of these histological changes, we concluded that the miR-204/-211-deficient mice had undergone OA onset and progression.

Deletion of miR-204/-211 in MPCs promotes OA pathogenesis. The tissues affected by OA pathogenesis in the miR-204/-211 KO mice include AC, meniscus, subchondral bone, synovium and joint capsules, of which all are mesenchymal tissues. This suggested that the phenotype observed in the miR-204/-211 KO mice could be caused by the loss of miR-204/-211 in the mesenchymal stem/progenitor cells, which give rise to these joint tissues. Thus, we decided to specifically delete miR-204/-211 in MPCs by crossing the miR-204\(^{lox/lox}\)/miR-211\(^{lox/lox}\) mice with Prx1-Cre transgenic mice, in which the Prx1 promoter was reported to drive Cre expression in the early limb bud mesenchyme and in the MPCs\(^19,20\).

Single KO mice for miR-204 (miR-204\(^{lox/lox}\), Prx1-Cre) or miR-211 (miR-211\(^{lox/lox}\), Prx1-Cre) did not show obvious joint phenotype (Supplementary Fig. 2a, b). Thus, we focused on breeding and analyzing double KO mice (miR-204\(^{lox/lox}\)/miR-211\(^{lox/lox}\); Prx1-Cre, hereafter dKO). Interestingly, we had observed a significantly lower birth ratio of the dKO mice than that of the littersmates without Prx1-Cre. This prompted us to perform a whole-genome sequencing, which revealed that the Prx1-Cre transgene is located on Chromosome 7 (Supplementary Fig. 2c) where miR-211 also resides. Thus, the lower ratio of the dKO mice was largely contributed by the genetic linkage between miR-211 and transgenic Prx1-Cre. Meanwhile, we have not observed significant gross abnormality of the dKO pups, which survived as well as their Prx1-Cre− littersmates, suggesting that the ablation of miR-204/-211 has no apparent impacts on skeletal development and growth in the young mice.

We examined the dKO mice to identify if the OA phenotype caused by a global deficiency of miR-204/-211 could be
recapitulated by the loss of the miRNAs in MPCs. The μCT scanning of the knee joints of 42-week-old mice revealed striking osteophyte growth (Fig. 2a). Further histology analysis of the dKO mice confirmed strong OA-like phenotypes, including synovial hyperplasia, cartilage degradation, subchondral sclerosis, osteophyte formation, and narrowed knee joint space (Fig. 2b). Particularly, the OA phenotypes developed gradually with age, as exemplified by 42-week-old mice showing more severe synovial hyperplasia and cartilage degradation than 33-week-old mice (Fig. 2b). It is notable that osteophytes were accompanied by more ectopic cartilage formation (blue staining) in 33-week-old mice than in 42-week-old mice, suggesting that osteophyte growth was preluded by excess cartilage formation (Fig. 2b). In other words, osteophyte growth in the dKO knee joints resulted from de novo endochondral ossification that requires cartilaginous templates derived from MPCs. Osteoarthritis Research Society International (OARSI) scoring also demonstrated more severe cartilage destruction in the dKO mice and a progressive OA development (Supplementary Fig. 2d). As pain is the most common individual burden for OA patients and the most prominent symptom of OA, we also performed behavioral pain tests and found that the dKO mice had increased pain sensitivity compared with Prx1-Cre-negative littermates (Fig. 2c). Thus, our results suggested that the deficiency of miR-204/211 in MPCs and mesenchymal joint tissues contributed to the genesis of OA pain, possibly through the structural deterioration of the joint.

Next, we performed in situ hybridization (ISH) assays with highly specific locked nucleic acid (LNA)-enhanced miRNA probes to determine the expression of miR-204 and miR-211 in the control and dKO mice. Our results demonstrated that both miR-204 and miR-211 are expressed in knee joint tissues, such as AC, meniscus and synovium, which all have mesenchymal origin (Fig. 2d, e, Supplementary Fig. 2e, f). As well, immunohistochemistry (IHC) assays showed that the PrxI promoter drives Cre expression in AC, meniscus and synovium, suggesting a comprehensive deletion of miR-204/211 in joint tissues by
**Fig. 2** Deficiency of miR-204/-211 in mesenchymal progenitor cells recapitulates OA pathogenesis. a Representative µCT images of 42-week-old miR-204/-211 floxed (control) and conditional double KO (dKO, with Prx1-Cre) mice. Red arrowheads, osteophytes. n = 5. Scale bar, 1 mm. b Representative histology images of knee joints in 42- (top) or 33-week-old (bottom) control and dKO mice stained by Alcian blue/Orange G. Yellow arrowheads, loss of articular cartilage; red arrowheads, osteochondrophytes; green arrowheads, synovial hyperplasia; black arrowheads, subchondral sclerosis. c Results of von Frey test on 33-week-old control and dKO mice. **P < 0.01, unpaired Student’s t test. d, e In situ hybridization using highly specific LNA-enhanced miR-204 (d) or miR-211 (e) probe (green) on knee joint sections of 7-month-old mice. The bottom panels are the enlargements of the upper images as indicated. DAPI staining marks nuclei (blue). Red arrowheads, miR-204- or miR-211-positive cells. Scale bar, 100 μm. f, g qRT-PCR analysis of miR-204 and miR-211 expression in CD45^- BMSCs from control or dKO mice. **P < 0.01, ****P < 0.0001, unpaired Student’s t test. n = 3. Data are shown as the mean ± s.d.

Prx1-Cre (Supplementary Fig. 2g). Significantly diminished ISH signals of miR-204 (or miR-211) were displayed in AC, synovium and meniscus of dKO mice (Fig. 2d, e), suggesting that miR-204 and miR-211 had been effectively and specifically deleted in multiple types of mesenchymal tissues in the joint. Moreover, quantitative RT-PCR (qRT-PCR) assays demonstrated that both miR-204 and miR-211 significantly decreased in RNAs extracted from AC or synovium (Supplementary Fig. 2h, i), while the gene expression of the miRNA host genes (Trpm3 and Trpm1) had no significant change (Supplementary Fig. 2j, k). To further confirm the ablation of miR-204/-211 in other mesenchymal tissues particularly bone marrow MPCs, we also isolated and cultured CD45^- bone marrow stromal cells (BMSCs), of which the major fraction was shown to be MPCs22 (Supplementary Fig. 3). Subsequent qRT-PCR experiments showed a 90% reduction of miR-204/-211 in CD45^- BMSCs isolated from the dKO mice (Fig. 2f, g). Together, our results confirmed that miR-204/-211 are effectively deleted in mesenchymal tissues, including AC, meniscus and synovium of the knee joints.

Deficiency of miR-204/-211 elevates Runx2 and OA markers. The CD45^- BMSCs deficient of miR-204/-211 may be used for the confirmation of the interacting machinery between miR-204/-211 and Runx2 mRNA at the molecular level. Thus, we applied the cross-linking and immunoprecipitation (CLIP) technique23 to purify the complex comprising Argonaute (AGO) proteins, Ago-bound miRNAs and miRNA-targeting mRNAs. Our results demonstrated that miR-204/-211 bind to Runx2 mRNA at two binding sites (Supplementary Fig. 4a). Furthermore, ablation of miR-204/-211 in CD45^- BMSCs significantly decreased the ligated chimera between miR-204/-211 and Runx2 mRNA (Supplementary Fig. 4b), suggesting that miR-204/-211 deletion impaired the formation of the AGO-miR-204/-211-Runx2 mRNA tertiary complex and therefore relieved Runx2 suppression by miR-204/-211.

In addition, our IHC results showed that protein levels of Runx2 were significantly elevated in AC, meniscus and synovium, especially in thickened synovium and osteophyte tissues (Fig. 3a, Supplementary Fig. 4c). Besides mesenchymal tissues in knee...
joints, CD45− BMSCs of the dKO mice also showed higher expression of Runx2 proteins (Fig. 3b). These results collectively suggest that miR-204/-211 deletion in MPCs caused significant accumulation of Runx2 in a variety of MPC-derived mesenchymal tissues. Runx2 has been reported to induce matrix-degrading enzymes such as MMP13, Adamts5 and Adamts4 [25–28], which are upregulated during OA development and therefore deemed as OA marker genes. Thus, we performed IHC to determine if the OA phenotype in the dKO mice is associated with upregulation of cartilage-degrading enzymes. Our results showed that protein levels of MMP13, Adamts5 (Fig. 3c, Supplementary Fig. 4d, e) and Adamts4 (Supplementary Fig. 4f, g) were significantly elevated in joint tissues, such as AC, meniscus and synovium, of the dKO mice. In addition, we extracted total RNA from AC of knee joint and found that mRNA levels of Mmp13 and Adamts5 increased in the dKO mice, suggesting that Mmp13 and Adamts5 were upregulated at transcriptional level (Fig. 3d), possibly due to elevated Runx2 levels. Together, our results suggest that the ablation of miR-204 and miR-211 in mesenchymal tissues causes Runx2 accumulation and subsequent induction of proteases, including MMP13, Adamts5 and Adamts4, which mediate catabolic activities in AC degradation and contribute to OA pathogenesis.

OA is a complex degenerative joint disorder involving not only cartilage catabolism but also anabolic effects including osteophyte formation and subchondral bone sclerosis, two remarkable

Fig. 3 Deficiency of miR-204/-211 upregulates Runx2, cartilage-catabolic enzymes and osteogenic markers. a IHC results show enhanced Runx2 expression in the synovium, articular cartilage (AC), and meniscus of knee joints of dKO mice. Red arrowheads, Runx2-positive cells. Scale bar, 50 μm. b Western blot analysis shows Runx2 accumulation in CD45− BMSCs isolated from dKO mice. The numbers below the blot indicate the relative protein levels of Runx2 (normalized to β-actin) in each group compared to the control. n = 3. c IHC results of MMP13 and Adamts5 in control and dKO mice. Arrowheads, IHC-positive cells. Scale bar, 100 μm. d qRT-PCR analysis of Mmp13, Adamts5, nerve growth factor (NGF), and Runx2 expression in articular chondrocytes from control or dKO mice. Unpaired Student’s t test. n = 3. Data are shown as the mean ± s.d. e–g Alkaline phosphatase (ALP) staining (e), Alizarin red staining (f), and von Kossa staining (g) of BMSCs isolated from control or dKO mice. n = 3. h qRT-PCR analysis of Runx2, Osterix (Osx), Alp and Osteocalcin (OC) expression in CD45− BMSCs from control or dKO mice. Unpaired Student’s t test. n = 3. Data are shown as the mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Uncropped western blot scans are shown in Supplementary Data 1.
exhibitions of aberrantly enhanced osteogenesis. As Runx2 is a master regulator in bone formation, we asked if skeletal stem/progenitor cells from the dKO mice, which showed higher Runx2 levels than the cells from the control mice (Fig. 3b), possessed augmented osteogenic activity. We isolated and cultured BMSCs for alkaline phosphatase (ALP), Alizarin red and von Kossa staining, and found that osteoblast differentiation in the cells with miR-204/-211 deletion accelerated (Fig. 3e–g). We also purified CD45−BMSCs from the dKO mice and found that the mRNA levels of osteoblast markers such as Osterix, Alp and osteocalcin increased markedly in the dKO cells (Fig. 3h), suggesting that loss-of-function of miR-204/-211 in skeletal progenitor cells promotes osteoblast differentiation. Combined with the IHC data that demonstrated Runx2 accumulation in joint tissues, our results suggest that increased subchondral bone formation and osteophyte outgrowth could result from enhanced Runx2 levels and elevated osteogenic activity, which are mediated by miR-204/-211 deletion in mesenchymal tissues.

### Induced MPC proliferation/synovial hyperplasia in dKO joints.

We also observed synovial hyperplasia in the dKO mice, which represents an important pathological manifestation of OA. As fibroblast-like synovial cells are of mesenchymal origin and display MSC properties such as self-renewal and multipotentiality, we investigated if the deficiency of miR-204/-211 in MPCs caused aberrant proliferation of mesenchymal cells in synovium, which could underlie hypertrophy of nonhematopoietic tissues in synovium. Firstly, we isolated synovial cells from the dKO mice for flow cytometry to assess the frequency of MPCs in synovial cells. Four cell surface markers were used: CD45 is a hematopoietic cell marker while CD29, CD105 and Sca-1 are markers for MSCs. The frequencies of CD29+/CD105+/Sca-1+ MSCs in CD45− nonhematopoietic synoviocytes were significantly higher in the dKO mice than those in their Cre− littermates (Fig. 4a), suggesting that miR-204/-211-deficient mesenchymal cells in synovium underwent increased proliferation. To assess cell proliferation in the joint tissues, we performed IHC to examine the level of proliferating cell nuclear antigen (PCNA) or Ki-67, and found that positive staining for PCNA and Ki-67 was significantly elevated in the dKO joint tissues, particularly in hypertrophied synovium, indicating excessive proliferation of synovial cells (Fig. 4c, Supplementary Fig. 5a, b). Also, we injected BrdU into the dKO mice and performed flow cytometry to evaluate the percentage of BrdU-positive mesenchymal cells in bone marrow. Our results showed that the frequencies of BrdU+/CD45− cells, which represent proliferating nonhematopoietic cells, were significantly higher in the dKO mice than in the control mice (Fig. 4d, e), suggesting that mesenchymal cells with the miR-204/-211 deficiency retains an enhanced proliferative ability. As well, we observed that the frequencies of CD45−/CD29+/CD105+Sca-1+ cells in bone marrow cells were also higher in the dKO mice (Supplementary Fig. 5c, d). Together, our results suggest that miR-204/-211 ablation results in a greater proliferation rate of MPCs, which provides excessive mesenchymal cells and therefore contributes significantly to synovial hyperplasia in the dKO mice.
While our results demonstrated indispensable roles of miR-204/-211 in modulating mesenchymal cell differentiation and catabolism to ameliorate cartilage degradation and ectopic bone formation, our data also revealed an unanticipated facet of miR-204 regulation that could be related to the proliferation of MPCs. To further investigate the mechanism altering the proliferation of the dKO cells, we analyzed mRNA expression of cyclin-dependent kinase (CDK) inhibitors, such as p16, p21, p27, and p57, in CD45− BMSCs derived from the dKO mice. Consistent with increased proliferation of mesenchymal cells as shown by BrdU labeling experiments, the mRNA levels of CDK inhibitors decreased substantially (Fig. 4f), suggesting that miR-204/-211 loss-of-function has negative effects on CDK inhibitors to promote cell cycle progression in MPCs.

Elevated Akt signaling and NGF expression in dKO joints. Because Akt signaling is reported to be pivotal for cell cycle regulation through blocking transcription of CDK inhibitors, we performed western blot assays to determine if activation of Akt signaling is associated with enhanced proliferation of CD45− BMSCs from miR-204−/− BMSCs derived from the dKO mice. We found that miR-204−/− ablation in CD45− BMSCs dramatically increased the number of total Akt proteins and NGF expression in dKO mice, especially in hyperplasia and osteophyte outgrowth observed in the dKO mice. This is consistent with the increased proliferation of CD45− BMSCs from miR-204−/− BMSCs derived from the dKO mice. Therefore, our data suggest that miR-204 and -211 may play a critical role in modulating mesenchymal cell differentiation and catabolism to ameliorate cartilage degradation and ectopic bone formation.
Activation of Akt signaling cascade can occur with stimulation of receptor tyrosine kinases (RTK) by their ligands. We found that miRNAs coding for nerve growth factor (NGF) were substantially upregulated in CD45+ BMSCs derived from dKO mice (Fig. 5c), which have been demonstrated to be comprised of mainly MPCs (Supplementary Fig. 3). The mRNA expression of Ngf is abundant in CD45+ BMSCs (ACT ≈ 5 compared to β-actin), suggesting that NGF is an endogenous player in mesenchymal cells. We also performed IHC on the dKO knee joint sections and found that NGF protein levels increased significantly in periarticular tissues, especially in synovium and newly formed ectopic cartilage/bone (Fig. 5d, Supplementary Fig. 6b), suggesting that an aberrant increase of NGF, a major mediator of OA pain, is also associated with synovial hyperplasia and osteophyte formation in the dKO mice. To further confirm that NGF expression is increased by miR-204-211 loss-of-function and Runx2 accumulation, we introduced synthetic miR-204 mimics or Runx2 siRNAs into CD45+ BMSCs and found that both miR-204 and Runx2 siRNAs suppressed Ngf mRNA expression in CD45+ BMSCs (Fig. 5e), which may be mediated by the decrease of Runx2 levels by miR-204 or by Runx2 siRNAs (Supplementary Fig. 6c). These results indicate that miR-204 and miR-211 modulates NGF expression, Akt activation and cell proliferation through its regulation of Runx2. Retroviral overexpression of two NGF isoforms, i.e. NGF-A and NGF-B, both activated Akt signaling, as the results of western blots showed elevated levels of p-P13K, p-Akt and cyclin D1 in NGF-transduced CD45+ progenitor cells (Fig. 5f). As well, NGF overexpression increased CD45+ cell proliferation (Fig. 5g). Importantly, the overexpression of NGF in CD45+ BMSCs significantly inhibited the mRNA expression of CDK inhibitors, such as p16, p21, p27 and p57 (Fig. 5h). Accordingly, the knockdown of Ngf by its siRNA transfection significantly reduced p-Akt protein levels and cell proliferation rate in CD45+ BMSCs (Fig. 5i, Supplementary Fig. 6d). Collectively, these results suggested that Runx2 accumulation induced by miR-204-211 depletion upregulates Ngf expression to activate Akt signaling and then to promote MPC proliferation, which may constitute a major source of excessive mesenchymal cells contributing to deleterious anabolic events in OA, including synovial hyperplasia, osteophyte outgrowth and subchondral sclerosis.

Administration of miR-204 alleviates surgery-induced OA. The dKO mice displayed severe, comprehensive OA phenotype, including AC degradation, subchondral sclerosis, osteophyte formation and synovial hyperplasia, suggesting that endogenous miR-204-211 play a key role in maintaining joint tissue homeostasis and preventing OA development. Thus, we ask if OA could be treated by administrating miR-204, as miR-204 and miR-211 share redundant functions and overexpression of one miRNA rescues the double knockout of two homologous miRNAs. We performed partial meniscectomy to induce the onset of OA, and then used intraarticular injection of aden-associated virus (AAV) serotype 5 to express miR-204 in OA-inflicted knee joints. Three months after injection, we could still detect AAV expression in a variety of joint tissues including AC, meniscus and synovium (Supplementary Fig. 7), suggesting that AAV5 administered by intraarticular injection can drive potent and long-lasting gene expression in knee joints.

Next, we performed phenotypic analysis to investigate how miR-204 administration affects OA pathogenesis in the surgical OA model. Notably, μCT analysis showed that injection of AAV5-miR-204 diminished osteophyte growth significantly, compared with that of AAV5-control (Fig. 6a). Further, histological examination confirmed a substantial decrease of osteophyte formation and revealed marked mitigation regarding synovial hyperplasia and cartilage degradation in miR-204-injected joints (Fig. 6b, c). In pain-related behavioral tests, mice receiving miR-204 injection showed higher pain thresholds than those receiving control AAV5 injection (Fig. 6d), suggesting that miR-204 reintroduction in OA knee joints not only ameliorated histological features, including AC degradation and osteophyte formation, but also reduced pain, a prominent symptom affecting OA patients. We also used IHC to examine the protein levels of Runx2 and several OA markers, and found that miR-204 administration alleviated the upregulation of Runx2, Adamts5 and MMP13, which was induced by the surgery (Fig. 6e-h).

Together, our exploration of miR-204 administration in vivo suggest that miR-204 could act as an effective therapeutic agent to treat OA in a surgically induced OA mouse model, through inhibition of Runx2 expression in multiple joint tissues.

The Runx2-miR-204-211 axis controls OA pathogenesis. Accumulating evidence generated in this study points to miR-204-211-regulated Runx2 that acts as a central player in OA onset and progression. To alleviate Runx2 accumulation caused by the ablation of miR-204-211 in skeletal cells, we introduced Runx2 haploinsufficiency into the dKO mice by generating the miR-204-211+/−/+; miR-211+/−/+; miR-211−/−/+; Prx1-Cre mice, hereafter tKO. Comparison of tKO and dKO mice revealed that the OA phenotype observed in the dKO mice has been largely ameliorated in the tKO mice. Specifically, the tKO mice possessed less degradation of AC in their knee joints than the dKO littermates, as demonstrated by histological analyses. Moreover, we have observed an effective diminish of synovial hyperplasia and osteophyte outgrowth in the tKO than in the dKO mice (Fig. 7a, Supplementary Fig. 8). These results suggest that Runx2 insufficiency can significantly mitigate the severe OA phenotype caused by miR-204-211 ablation in the skeletal cells.

To investigate if reduction of Runx2 protein levels was a causal factor in OA phenotype alleviation, we performed IHC assays to examine Runx2 protein levels in the tKO mice. Our results showed that Runx2 insufficiency markedly attenuated the Runx2 accumulating effects resulting from miR-204-211 deficiency, in AC, meniscus and synovium (Fig. 7b). As Runx2 level returned to normal as similar as that in the control mice, the tKO mice also showed a remarkable extenuation in cartilage-catabolic factors including Adamts5 and MMP13 compared to the dKO littermates (Fig. 7c, d). Thus, our results suggest that heterozygous deficiency of Runx2 rescued the OA phenotype resulting from miR-204-211 deletion and readjusted Runx2 proteins to a normal physiologic level, and therefore significantly mitigated the severe OA phenotype observed in the dKO mice. Further, we conclude that miR-204-211 control Runx2 expression and the Runx2-miR-204-211 axis governs OA pathogenesis.

Discussion

Currently, many mechanistic OA studies focus on cartilaginous tissues, since cartilage is a key component of synovial joints and cartilaginous tissues are exposed to mechanical stress that was thought to be a major risk factor for OA. Therefore, cartilage destruction is deemed to be a landmark event during OA onset and progression. In this study, we have generated a new mouse model in which miR-204-211 are deleted in multiple joint tissues and a comprehensive OA-like phenotype is observed in the entire joint but not restricted to cartilage only. For example, we found that the dKO mice develop severe synovial hyperplasia, which may have instrumental effects on other manifestations of OA, such as induced matrix-degrading enzymes on cartilage breakdown and elevated NGF on increased pain sensitivity and MPC...
proliferation. Osteophyte formation is also a prominent feature displayed in the dKO mice, and we found that osteophyte outgrowth is significantly contributed by excessive proliferation of MPCs, which implicates a mechanistic similarity between osteophyte formation in OA pathogenesis and endochondral ossification in skeletal development. Thus, our dKO mice could represent a comprehensive OA model, of which the analysis provides multifaceted insights into the mechanism of OA initiation and progression as well as the metabolic maintenance of the joint.

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proliferation. Osteophyte formation is also a prominent feature displayed in the dKO mice, and we found that osteophyte outgrowth is significantly contributed by excessive proliferation of MPCs, which implicates a mechanistic similarity between osteophyte formation in OA pathogenesis and endochondral ossification in skeletal development. Thus, our dKO mice could represent a comprehensive OA model, of which the analysis provides multifaceted insights into the mechanism of OA initiation and progression as well as the metabolic maintenance of the joint.

When we were generating the dKO mice, we had observed a much lower ratio of the dKO pups than that of the miR-204fl/fl;Prx1-Cre and generated comprehensive pathological
changes not restricted to cartilage degradation. While the use of arguably more cartilage-specific promoters such as those of Aggrecan and Col2a1 would provide cartilage-focused information for OA studies, miRNA ablation in MPCs offers a unique and resourceful perspective which help to advance our understanding of pathological changes of the entire joint.

OA can be regarded as a mesenchymal disease as it affects the entire synovial joint, which contains multiple connective tissues derived from MPCs, or in a broader context, MSCs. In addition, MSCs are reported to be available in most joint tissues, including synovium, meniscus and cartilage, which act as reservoirs of MSCs are reported to be available in most joint tissues, including synovium, meniscus and cartilage, which act as reservoirs of MSCs. In addition, MSCs are reported to be available in most joint tissues, including synovium, meniscus and cartilage, which act as reservoirs of MSCs. In addition, MSCs are reported to be available in most joint tissues, including synovium, meniscus and cartilage, which act as reservoirs of MSCs. In addition, MSCs are reported to be available in most joint tissues, including synovium, meniscus and cartilage, which act as reservoirs of MSCs. In addition, MSCs are reported to be available in most joint tissues, including synovium, meniscus and cartilage, which act as reservoirs of MSCs. In addition, MSCs are reported to be available in most joint tissues, including synovium, meniscus and cartilage, which act as reservoirs of MSCs. In addition, MSCs are reported to be available in most joint tissues, including synovium, meniscus and cartilage, which act as reservoirs of MSCs. In addition, MSCs are reported to be available in most joint tissues, including synovium, meniscus and cartilage, which act as reservoirs of MSCs. In addition, MSCs are reported to be available in most joint tissues, including synovium, meniscus and cartilage, which act as reservoirs of MSCs. In addition, MSCs are reported to be available in most joint tissues, including synovium, meniscus and cartilage, which act as reservoirs of MSCs. In addition, MSCs are reported to be available in most joint tissues, including synovium, meniscus and cartilage, which act as reservoirs of MSCs. In addition, MSCs are reported to be available in most joint tissues, including synovium, meniscus and cartilage, which act as reservoirs of MSCs. In addition, MSCs are reported to be available in most joint tissues, including synovium, meniscus and cartilage, which act as reservoirs of MSCs. In addition, MSCs are reported to be available in most joint tissues, including synovium, meniscus and cartilage, which act as reservoirs of MSCs. In addition, MSCs are reported to be available in most joint tissues, including synovium, meniscus and cartilage, which act as reservoirs of MSCs. In addition, MSCs are reported to be available in most joint tissues, including synovium, meniscus and cartilage, which act as reservoirs of MSCs. In addition, MSCs are reported to be available in most joint tissues, including synovium, meniscus and cartilage, which act as reservoirs of MSCs. In addition, MSCs are reported to be available in most joint tissues, including synovium, meniscus and cartilage, which act as reservoirs of MSCs. In addition, MSCs are reported to be available in most joint tissues, including synovium, meniscus and cartilage, which act as reservoirs of

Fig. 7 Runx2 insufficiency mitigates OA phenotype in dKO mice. a Representative histology images of 9-month-old TKO (miR-204/fox; miR-211/fox; Runx2+/-fox; Prx1-Cre) mice compared with dKO (miR-204/fox; miR-211/fox; Prx1-Cre) and control (miR-204/fox; miR-211/fox) mice. Yellow arrowheads, articular cartilage degradation; green arrowhead, synovial hyperplasia; black arrowhead, subchondral sclerosis. b-d IHC results of Runx2 (b), Adamts5 (c), and MMP13 (d) expression in the control, dKO and tKO mice. Red arrowheads, Runx2-, Adamts5-, or MMP13-positive cells. Scale bar, 50 μm.
targeting of miRNAs are minimal (146 nt at 5′ and 110 nt at 3′ for both floxed alleles) and did not affect the expression or intron splicing of host genes, Trpm1 alleles) and did not affect the expression or intron splicing of host genes, which will be used as a marker for detection of the miR-204 expression introduced by AAV. We performed the surgery of partial meniscectomy on the right knee of wild type (WT) C57BL/6 mice to induce OA 10 days before they turned 3-month-old. Ten days later, we performed intraarticular injection. Specifically, a longitudinal skin incision was made to visualize the patellar ligament; the patella then was resected by a small needle into the area underneath the patella of the right leg.

**AAV intraarticular injection and surgically induced OA model.** We chose intraarticular injection of recombinant AAV for in vivo overexpression of miR-204. Both AAV5-EF1a-miR-204-GFP and AAV5-EF1a-control-GFP were purchased from Vector Biolabs (Malvern, PA, USA). The pri-miR-204 sequence is located in the intron with a poly(A) tail that drives GFP transcription, which will be used as a marker for detection of the miR-204 expression introduced by AAV. We performed the surgery of partial meniscectomy on the right knee of wild type (WT) C57BL/6 mice to induce OA 10 days before they turned 3-month-old. Ten days later, we performed intraarticular injection. Specifically, a longitudinal skin incision was made to visualize the patellar ligament; the patella then was resected by a small needle into the area underneath the patella of the right leg.

**Micro-Ct, histology, ISH, IHC and histomorphometry.** We used a Scanco µCT35 scanner (Scanco Medical, Bruttisellen, Switzerland) with 55 kVp source and 145 μAmp current for formalin-fixed mouse legs with a resolution of 10 μm. The scanned images from each group were evaluated at the same thresholds to allow three-dimensional structural rendering of each sample. For histology, ISH and IHC, tissues were fixed in 4% paraformaldehyde, decalcified, paraffin embedded through three levels; one section was mounted on a glass slide, the sec

**Flow cytometry.** After 2-week culture in α-MEM plus 10% FBS, mouse BMSCs were trypsinized, pelleted and then resuspended in PBS containing 2% FBS to a concentration of 2 × 10^5 cells/ml. Freshly isolated bone marrow cells were used for cell isolation and were seeded at 1 × 10^4 cells/ml in 3% FBS. Stem cells were incubated with anti-mouse CD45 PE-Cy7 (Biolegend, San Diego, CA, USA), 1/200 NF antibody (ab199, Abcam, Cambridge, MA, USA), 1/1000 CD45 antibody (ab185722, Abcam, Cambridge, MA, USA) antibody overnight at 4°C and incubated with 1/400 secondary biotinylated goat anti-rabbit or anti-mouse antibody (Vector Laboratories, Burlingame, CA, USA) antibody overnight at 4°C and then incubated with secondary antibody conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) for 30 min. Images of histology, ISH and IHC were captured using CellSens Imaging Software (Olympus) on an Olympus BX43 microscope, or a Zeiss LSM700 confocal microscope. Histomorphometric measurements were performed using OsteoMeasure software (Osteometrics, Inc., Atlanta, GA, USA).
12-1051-81), and anti-mouse Ly-6A/E (Sca-1) APC (BioLegend, San Diego, CA, USA). For BrdU Incorporation, the mice were intraperitoneally injected twice (a 16-h interval) with 1 mg/mouse/injection BrdU and euthanized 2 h after the second injection. Freshly harvested bone marrow was treated with 1× RBC Lysis Buffer (BioLegend, San Diego, CA, USA). The remaining cells were stained with anti-mouse CD45 PerCP-Cy5.5, anti-mouse/rat CD29 PE-Cy7, anti-mouse CD105 (Endoglin) PE, and anti-mouse Ly-6A/E (Sca-1) Alexa Fluor 700 (BioLegend, 56-5981-81) before permeabilization, DNase treatment and staining with anti-BrdU APC according to the instruction of APC BrdU Flow Kits (BD Pharmagen, Franklin Lakes, NJ, USA). The percentage of BrdU-positive fractions was assessed using FlowJo analysis software (Tree Star, Ashland, OR, USA).

Animal behavioral tests. Testing for mechanical allodynia (vom Frey sensitivity) was performed using a calibrated set of vom Frey filaments (Stoelting, Wood Dale, IL)39. Before the vom Frey test, we allowed animals to adapt to the environment, including an elevated mesh platform for 15 min. The set of vom Frey filaments was used to poke from below to the hind paw to calculate the 50% force withdrawal threshold using an iterative approach. The tests were performed in a blind manner that the investigator is not aware of the identification of animals as well as the study groups.

Experimental design and statistical analyses. The sample size for each experiment was determined based on our previous experiences. All the data were expressed as mean ± s.d., as indicated in the figure legends. Statistical analyses were completed with Prism GraphPad. Unpaired Student’s t test (for two groups), one-way ANOVA (for multiple groups) and two-way ANOVA (for multiple groups and time points) were used followed by the Tukey–Kramer test. P < 0.05 was considered statistically significant.

Data availability

The data set that supports the findings of this study are available within the article and its Supplementary Information files or from the corresponding author upon reasonable request.

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Author contributions

J.H. generated miR-204 and miR-211 floxed mice, designed the research and performed experiments, analyzed data and wrote the manuscript. L.Z. designed and performed experiments, analyzed data and revised the manuscript. Y.F. and L.L. performed mouse surgery and intraarticular injection. G.X. and P.X.M. provided expertise and feedback. D.C. designed the project, analyzed the results and revised the manuscript.

Additional information

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