m^{6}A in bone homeostasis and related diseases

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ABSTRACT

N^6-methyladenosine RNA methylation (m^{6}A) is one of the most common and widespread RNA modifications in eukaryotic cells. m^{6}A plays a crucial role in the regulation of pathophysiological processes of eukaryotes. Three types of m^{6}A regulators, including methyltransferases, demethylases and m^{6}A-binding proteins, are involved in the reversible epigenetic modification of m^{6}A. Bone is a vital organ with irreplaceable functions of movement, haematopoiesis, and protection of other organs. Its physiological homeostasis is mainly determined by the synergy of corresponding cells such as bone marrow derived stem cells, osteoblasts, and osteoclasts. Once the physiological equilibrium is broken, the bones will transform into a pathological state, resulting with diseases such as osteoporosis, osteoarthritis, rheumatoid arthritis, and osteosarcoma. Here, we review the composition of m^{6}A and its regulation mechanism in bone physiology and pathology.

Keywords:
Bone; m^{6}A; Osteoarthritis; Osteogenesis; Osteoporosis

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1. Introduction

Epigenetic regulation refers to the changes in gene expression and function caused by various modification methods without changing DNA sequence of the gene.[1] The most recent research suggests that the essence of epigenetics involves a range of covalent modifications on histones and nucleic acids, as well as the regulation of chromatin and chromosome three-dimensional structure. These mechanisms work together to control gene expression.[2] The primary approach for treating diseases through epigenetic methods lies in influencing the differential expression of mRNA.[3] So far, 17 different kinds of DNA modifications and more than 160 different kinds of RNA modifications have been identified to participate in many physiological and pathological activities.[4] Research of RNA modifications has lasted for more than half a century with many have been discovered, including N^1-methyladenosinea (m^{1}A), N^6-methyladenosine (m^{6}A) and 5-methylcytosine (m^{5}C) (**Figure 1**).[5]

m^{6}A, initially identified in 1974, has emerged as the most prevalent and widespread internal RNA modification across a majority of eukaryotic organisms, including mammals. m^{6}A serves a pivotal regulatory function in diverse RNA types, such as messenger RNA (mRNA), transfer RNA, ribosomal RNA, and numerous non-coding RNAs, therefore plays an extensive role in modulating various aspects of gene expression.[6-10] m^{6}A modification specifically refers to the process of adding or removing a methyl group at the N^6 position of an adenosine residue in mRNA, which subsequently trigger a series of biological effects. m^{6}A represents the most common dynamic and reversible modification mode of eukaryotic mRNA, accounting for 80% of RNA methylation modifications. m^{6}A is involved in nearly whole phases of the RNA lifecycle, including the regulation of transcription, maturation, translation, degradation, and stabilisation of mRNA.[11] m^{6}A modification system includes methyltransferases, demethylases, and m^{6}A-binding proteins.[12] Methyltransferases add a methyl group to adenosine in mRNA. Whereas Demethylases remove methyl groups from adenosine residues. m^{6}A-binding proteins recognise m^{6}A modifications, further affecting mRNA stability, translation efficiency, and degradation. Together, they collaboratively regulate a series of metabolic processes, including splicing, transport, and translation of mRNA.[13-15]

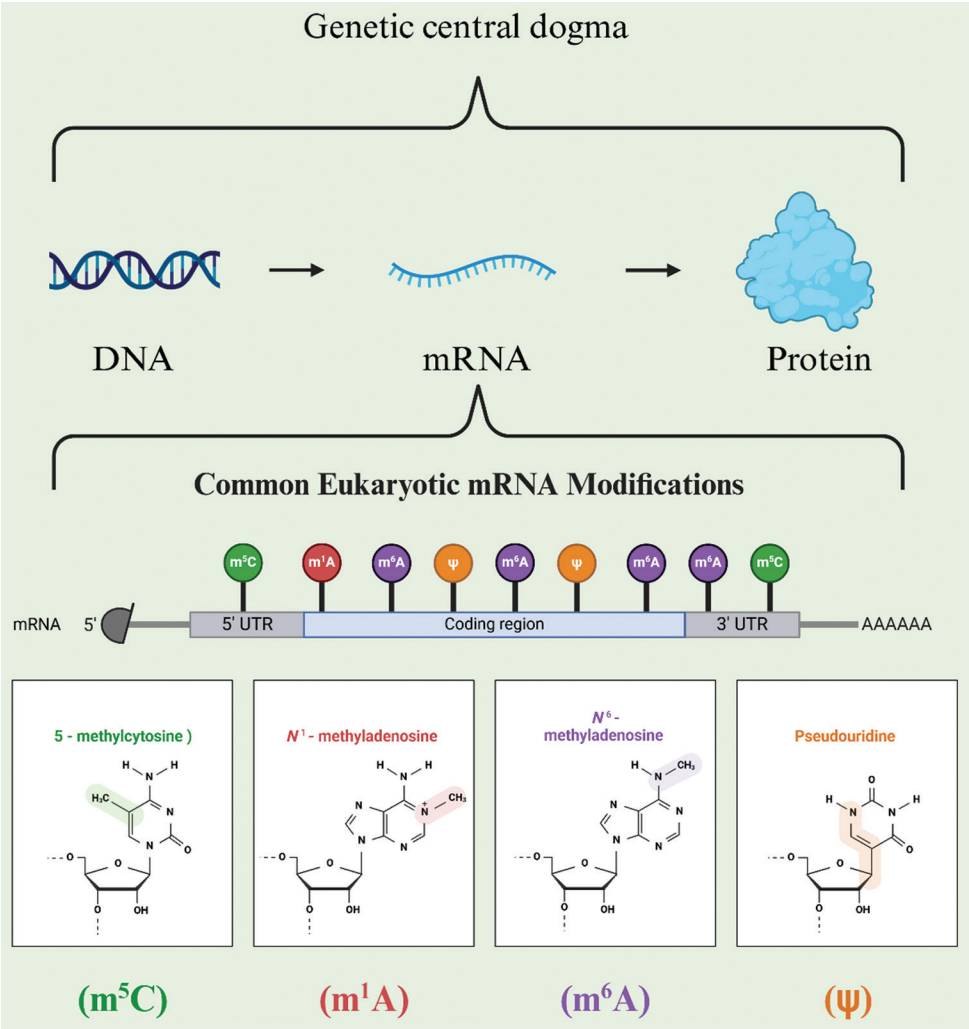

Figure 1. Common eukaryotic RNA modifications. Created with BioRender.com.
Abbreviations: m¹A: N¹-methyladenosine; m⁵C: 5-methylcytosine; m⁶A: N⁶-methyladenosine; UTR: Untranslated region; ψ: Pseudouridine.

Bone is a vital organ in mammals which serves many irreplaceable functions.[16,17] For example, the mechanical properties of bone provide support for movement and protection for internal organs. Bone contains multipotent stem cells and provides a favourable environment for their multi-directional differentiation. In addition, bone serves as an important storage reservoir for calcium and phosphate ions in the body.[18] The maintenance of bone homeostasis requires the coordinated effect of multiple factors, including normal mechanical loading, adequate nutrition and suitable hormone levels, etc.[19] At the cellular level, the physiological function of bone is sustained through the coordinated activity of various cell types, including bone marrow derived stem cells (BMSCs), osteoblasts and osteoclasts.[20]

Research on the bone homeostasis regulation from m⁶A has become a hot topic, with many results indicating that m⁶A is involved in many physiological activities of bone development as well as maintains a significant impact on the occurrence of related diseases such as osteoporosis and bone tumours.[19,21] Here, we reviewed the basic composition of m⁶A and its regulation on the physiological and pathological activities of the bone. To ensure a comprehensive review, we employed a systematic retrieval strategy. Literature was searched using the following databases: PubMed, Google Scholar, and Web of Science. The following terms were searched: "m⁶A", "N⁶-methyladenosine", "RNA modification", "osteogenesis", "osteoclastogenesis", "bone homeostasis", "bone diseases", "osteoporosis", "osteoarthritis", "rheumatoid arthritis", and "osteosarcoma". Boolean operators such as AND, OR, and NOT were applied to refine the search. We included studies published in English that focused on the role of m⁶A modification in bone-related physiological processes or diseases with experimental evidence or mechanistic insights providing. The related articles of our group were then screened to identify potential review writing for consideration.

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2. Composition of N^6-methyladenosine

The RNA methylation modification process of m^{6}A involves three main regulators: methyltransferases, demethylases and m^{6}A-binding proteins[22,23] (**Table 1** and **Figure 2**).

2.1. Methyltransferase

RNA methyltransferases catalyse the N6-methylation of adenine in RNA. This methylation process involves a methyltransferase complex (MTC) mainly composed of three core proteins, methyltransferase-like 3 (METTL3), methyltransferase-like 14 (METTL14), and Wilms tumour 1 associated protein (WTAP), as well as auxiliary factors such as RNA binding motif protein 15 (RBM15), viriliser like m^{6}A methyltransferase associated (VIRMA) and zinc finger CCCH-type containing 13 (ZC3H13).[24,25] Among which METTL3 is the most important component of the MTC. The METTL3-METTL14 complex exhibits higher catalytic activity than METTL3 or METTL14 alone, as they enhance each other's binding activity to RNA.[26] WTAP does not have catalytic function but can interact with METTL3 and METTL14, facilitating their localisation to nuclear speckles enriched in pre-mRNA processing factors for further m^{6}A catalysis.[27] ZC3H13 interacts with WTAP via its low complexity domain zinc finger structure, after which the MTC is retained in nuclear speckles, thereby enhancing its catalytic function.[28] Knockdown of ZC3H13 can influence the localisation and function of the MTC, thereby further influencing the level of RNA methylation.[29] RBM15 also lacks catalytic function but can bind to METTL3 and WTAP which direct them to specific RNA sites for m^{6}A modification.[30,31] VIRMA can recruit METTL3/METTL14/WTAP, the core components of the MTC, to achieve selective methylation in regions primarily located in the 3'untranslated region and nearby the stop codon.[32]

2.2. Demethylase

RNA demethylases catalyse the removal of the N^6-methyl group from adenine in RNA. The demethylases Fat mass and obesity-associated protein (FTO) and human Alk B homolog 5 (ALKBH5) were discovered in 2011 and 2013 respectively.[33,34] FTO is an α-ketoglutarate-dependent m^{6}A demethylase which demonstrates punctate expression pattern in the nucleoplasm and partial co-localisation with nuclear speckles. The crystal structure of FTO contains an active site domain similar to the ALKB family,[35] and it also has a unique C-terminal domain that may be involved in additional protein-RNA/protein-protein interactions.[36] ALKBH5 mainly affects mRNA synthesis and splicing. The immunoprecipitation experiments of ALKBH5 have identified its binding sites to RNA substrate, and it has been shown to be part of mRNA-binding proteins, indicating close interactions with mRNA and other RNA substrates.[34,37]

2.3. m^{6}A-binding protein

The discoveries of the above methyltransferases and demethylases demonstrate that m^{6}A modification is a reversible process. In order for the m^{6}A group to exert its biological function, methylated RNA must be recognised and bound by m^{6}A-binding proteins which further regulate processes such as RNA transport, translation as well as affect RNA stability. The well-known binding-proteins mainly include the YTH N6-methyladenosine RNA binding protein (YTH) family, insulin like growth factor 2 mRNA binding protein (IGF2BP) family, and heterogeneous nuclear ribonucleoprotein

Table 1. The major regulators of m^{6}A and their functions

Classification	Regulator	Function
Methyltransferase	METTL3	Catalyse m^{6}A modification
	METTL14	Recognise RNA sequences and stabilise the structure of MTC
	WTAP	Recruit METTL3 and METTL14 into nuclear speckles
	RBM15	Transfer of METTL3-14 heterodimers to specific RNA sites
	VIRMA	Recruit MTC and interact with polyadenylation cleavage factor
Demethylase	FTO	Remove m^{6}A methylation modification
	ALKBH5	Remove m^{6}A methylation modification
m^{6}A-binding protein	YTHDC1	Promote RNA cleavage and nuclear export
	YTHDC2	Improve the translation efficiency of target gene mRNA while reduce its intracellular content
	YTHDF1	Promote mRNA translation
	YTHDF2	Assiste mRNA degradation
	YTHDF3	Interact with YTHDF1 to promote mRNA translation and interact with YTHDF2 to promote mRNA degradation
	IGF2BP1	Promote the translation of mRNA and maintain its stability
	IGF2BP2	Promote the translation of mRNA and maintain its stability
	IGF2BP3	Promote the translation of mRNA and maintain its stability
	HNRNPA2B1	Assist primary miRNA processing and modification
	HNRNPC/G	Regulate mRNA splicing and content

Abbreviations: ALKBH5: AlkB homolog 5; FTO: Fat mass and obesity-associated protein; HNRNP: Heterogeneous nuclear ribonucleoprotein; IGF2BP: Insulin like growth factor 2 mRNA binding protein; m^{6}A: N^6-methyladenosine; METTL3: Methyltransferase-like 3; METTL14: Methyltransferase-like 14; mRNA: Messenger RNA; MTC: Methyltransferase complex; RBM15: RNA binding motif protein 15; VIRMA: Viriliser like m^{6}A methyltransferase associated; WTAP: Wilms tumour 1-associated protein; YTH: YTH domain.

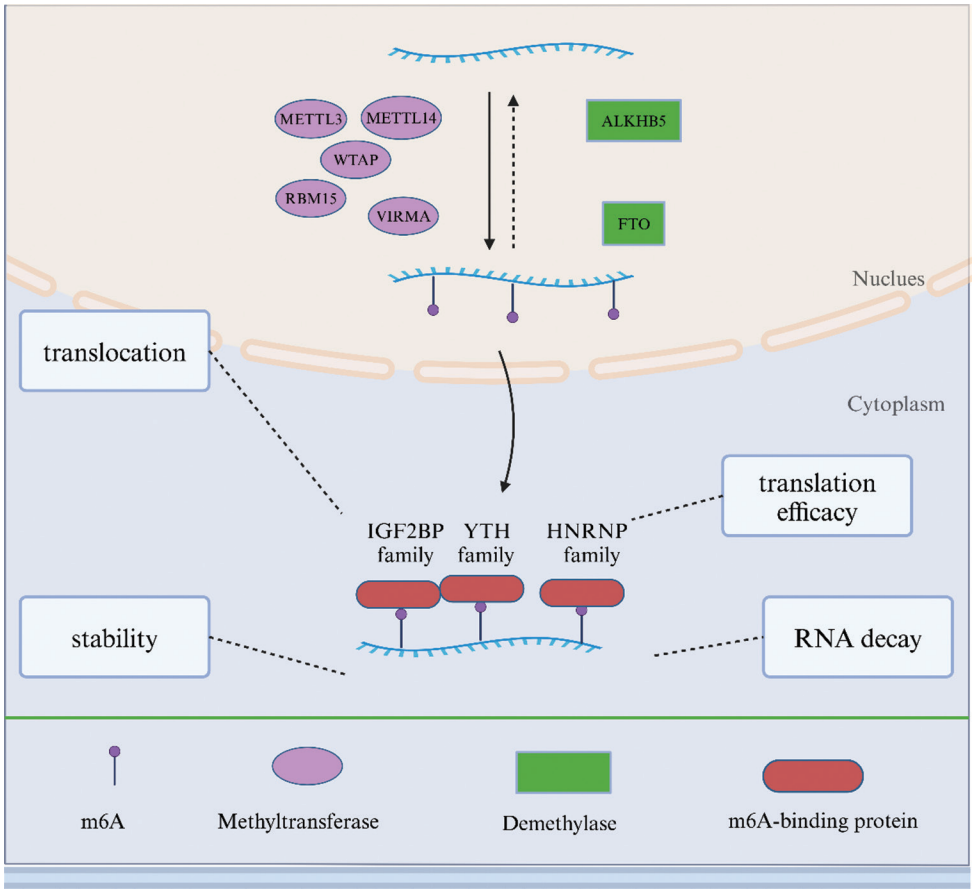

Figure 2. Common mediators of m⁶A. Created with BioRender.com.
Abbreviations: ALKBH5: AlkB homolog 5; HNRNP: Heterogeneous nuclear ribonucleoprotein; FTO: Fat mass and obesity-associated protein; IGF2BP: Insulin like growth factor 2 mRNA binding protein; m⁶A: N⁶-methyladenosine; METTL3: Methyltransferase-like 3; METTL14: Methyltransferase-like 14; RBM15: RNA binding motif protein 15; VIRMA: viriliser like m⁶A methyltransferase associated; WTAP: Wilms tumour 1-associated protein; YTH: YTH domain.

(HNRNP) family. YTHDF2 was the first discovered m⁶A reader protein. The interaction between its N-terminal region and the SH domain of the CNOT1 subunit can directly recruit the CCR4-NOT complex, leading to the degradation of m⁶A-containing RNA.[38] YTHDF1 enhances mRNA translation and protein synthesis through interacting with the mRNA start codon.[39] YTHDF3 is primarily found to have a synergistic effect which enhances RNA translation through interaction with YTHDF1 and promotes RNA degradation by binding to YTHDF2.[40,41] Therefore, we speculate that these three YTHDF proteins may function in a synergistic manner, affecting the corresponding m⁶A RNA metabolic processes. YTHDC1 can regulate the process of mRNA transport and selective splicing.[42] YTHDC2 is an RNA-induced ATPase with 3′→5′ RNA helicase activity which accelerates RNA degradation after recognising and binding to the m⁶A site. However, some studies have also shown that it can enhance mRNA translation efficiency and play a crucial role in processes such as spermatogenesis.[43,44] Recent research has found that the IGF2BP family promotes RNA stability in an m⁶A-dependent manner, therefore plays important roles in post-transcriptional gene regulation and cancer biology.[45] The HNRNP family can bind to m⁶A site on mRNA and further affect its processing, splicing, and cellular content.[46]

3. N⁶-methyladenosine is involved in formation and physiological homeostasis of bone

3.1. m⁶A regulates bone marrow derived stem cell osteogenic differentiation

The osteogenic differentiation of BMSCs is important for osteogenic differentiation, and many studies have shown that METTL3 is a key regulatory factor in this process (**Figure 3**). Wu *et al.*[47] found that conditional knockout of METTL3 in BMSCs in mice can induce osteoporosis, while constructing an ovariectomised mouse model with overexpression of METTL3 in BMSCs can protect mice from estrogen deficiency-induced osteoporosis. Further studies have revealed that METTL3 regulates the downstream parathyroid hormone (PTH)/parathyroid hormone receptor-1 (PTH1r) signalling axis. Knocking out METTL3 reduces the translation efficiency of PTH1r in BMSCs, disrupting PTH-induced osteogenesis and adipogenesis *in vivo*. Another study showed that METTL3 can mediate m⁶A modification of long intergenic non-protein coding RNA 657 (LINC00657) mRNA, therefore promote the osteogenic differentiation of BMSCs through the LINC00657/microRNA-144-3p/bone morphogenetic protein receptor type 1B axis.[48] However, some research

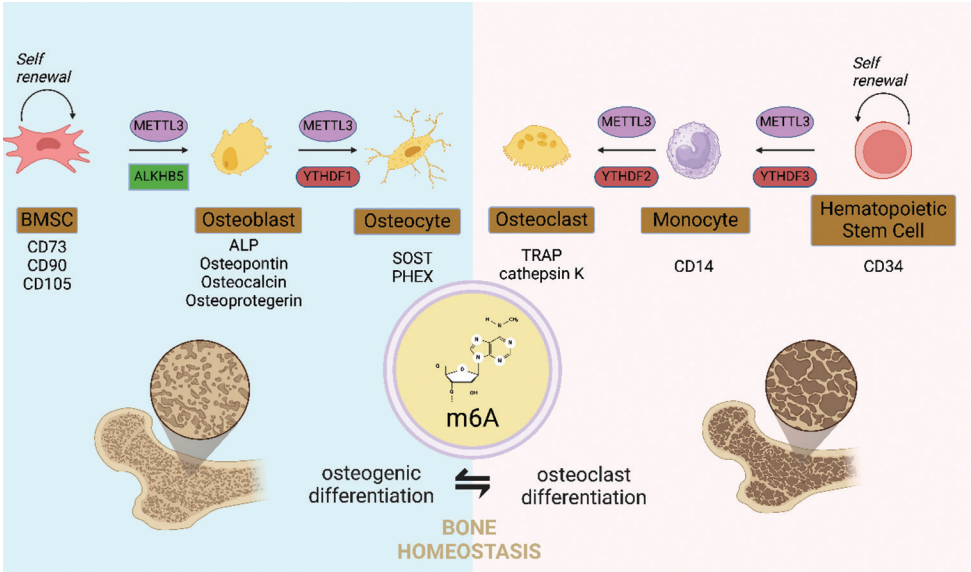

Figure 3. m^{6}A mediates bone homeostasis via regulating both osteogenic differentiation and osteoclast differentiation. Created with BioRender.com.

Abbreviations: ALKBH5: AlkB homolog 5; ALP: Alkaline phosphatase; BMSC: Bone marrow derived stem cell; FTO: Fat mass and obesity-associated protein; HNRNP: Heterogeneous nuclear ribonucleoprotein; IGF2BP: Insulin like growth factor 2 mRNA binding protein; m^{6}A: N^6-Methyladenosine; METTL3: Methyltransferase-like 3; METTL14: Methyltransferase-like 14; PHEX: Phosphate regulating endopeptidase; RBM15: RNA binding motif protein 15; SOST: Sclerostin; VIRMA: Viriliser like m^{6}A methyltransferase associated; WTAP: Wilms tumour 1-associated protein; YTH: YTH domain.

indicate that loss of METTL3 increases the synthesis and deposition of calcium nodules as well as enhances alkaline phosphatase activity of BMSCs,[49] which may reveal a potential inhibitory role of METTL3 in the osteogenic differentiation of BMSCs. The underlying molecular mechanism may be that METTL3 positively regulates the expression of myeloid differentiation primary response gene 88 through promoting its RNA methylation modification, and myeloid differentiation primary response gene 88 is a key upstream regulatory factor for activation of nuclear factor kappa-B (NF-κB), a widely considered osteogenic inhibitor. This negative osteogenic effect of METTL3 can also be reversed by the demethylase ALKBH5. Huang *et al.*[50] explored the potential regulatory role of the m^{6}A reader protein YTHDF1 in the osteogenic process of human BMSCs. They first constructed YTHDF1-knockout mice, which resulted in reduced bone mass in mice. Moreover, respectively lowering the content of YTHDF1 in human and mouse BMSCs *in vitro* reduced the osteogenic differentiation of both types of BMSCs. Further research found that the zinc finger protein 839 (Zfp839) is target site of YTHDF1. Moreover, Zfp839 enhances osteogenesis in mouse BMSCs by enhancing the transcriptional activity of Runt-related transcription factor 2 (Runx2). This study provides a new direction for research on m^{6}A regulation in mammalian osteogenesis.

3.2. m^{6}A regulates the function of osteoblasts

Osteoblasts are important for maintaining bone integrity with vital functions including the synthesis, secretion, and mineralisation of the bone matrix. Zhang *et al.*[51] found METTL3 was upregulated during the differentiation of mouse embryonic osteoblasts and downregulated after lipopolysaccharide (LPS) stimulation. Then they knocked out the *METTL3* gene in mouse embryonic osteoblasts and observed a decrease in the expression of Runx2 and Osterix as well as a decrease in the formation of mineralised nodules, cell ALP activity, and the phosphorylation level of Smad1/5/9. Knocking down METTL3 is similar as silencing YTHDF2 to elevate the mRNA expression and stability of Smad7, and Smurf1, which are the negative regulators of Smad signalling transduction. Furthermore, reducing METTL3 expression increased the expression of pro-inflammatory cytokines and enhanced the phosphorylation level of p38, Jun N-terminal kinase (JNK), extracellular regulated protein kinases (ERK), and p65 in the mitogen-activated protein kinase (MAPK) and NF-κB signalling pathways. In summary, knocking down METTL3 increases the mRNA levels of Smad7 and Smurf, which are degraded by YTHDF2, thereby inhibiting Smad1/5/9-dependent signalling which leads to attenuated differentiation of embryonic osteoblasts. In the meantime, it also leads to the activation of the LPS-interleukin 6 (IL-6)/interleukin 12 (IL-12)/tumour necrosis factor-alpha (TNF-α) induced inflammation through MAPK signalling pathway. Qian *et al.*[52] knocked out the FTO gene in mouse osteoblasts which makes the mRNA of heat shock protein family a member 1a and other genes in the DNA repair pathway more susceptible to damage from substances such as ultraviolet light and hydrogen peroxide, leading to a higher rate of apoptosis in osteoblasts. This research confirms that FTO protects osteoblasts from toxic damage through the heat shock protein family a member 1a/NF-κB signalling pathway.

3.3. m^{6}A regulates the function of osteoclasts

The differentiation of osteoclasts and their bone resorption function are also crucial for maintaining bone homeostasis and

integrity.[53] Wang *et al.*[54] found that METTL3 can methylate the 1956 bp site of circRNA-0008542 (circ-0008542), promoting its competitive binding with microRNA-185-5p (miRNA-185-5p), which result in increased expression of the target gene *RANK* and enhanced bone resorption function of osteoclasts. On the other hand, ALKBH5 inhibits the binding of circ-0008542 with miRNA-185-5p, thereby correcting excessive bone resorption. Therefore, METTL3/ALKBH5/circ-0008542/miRNA-185-5p/RNAK may be an important pathway for osteoclast activity regulation. Fang *et al.*[55] found that YTHDF2 can effectively reduce the LPS-induced osteoclast differentiation and alleviate inflammatory response caused by the enhanced expression of Nfact1, c-Fos, IL-1β, and TNF-α from NF-κB and MAPK signalling pathways which helps to maintain relative stability of the bone. Li *et al.*[56] found that the expression of METTL3 increases during osteoclast differentiation. Knocking down METTL3 in osteoclasts leads to increased cell size and decreased bone resorption function. It also suppresses the expression of osteoclast-specific genes (*Nfatc1, c-Fos, Ctsk, Acp5,* and *Dcstamp*), while the expression of the cell fusion gene Atp6v0d2 is upregulated. Additionally, knocking down YTHDF2 also increases the content of Atp6v0d2 in osteoclasts. Another result of knocking down METTL3 is that it disrupts the transport of TNF receptor-associated factor 6 (*Traf6*) mRNA from nucleus to cytoplasm, leading to a decrease protein content of *Traf6* in osteoclasts. Therefore, METTL3 can regulate differentiation and function of osteoclast through YTHDF2/Atp6v0d2 degradation pathway and affecting the transport of *Traf6* mRNA.

In conclusion, m⁶A modification affects the proliferation, differentiation and apoptosis of bone marrow mesenchymal stem cells, osteoblasts and osteoclasts through regulating the expression of related genes and cellular pathways such as phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) and Notch, thereby regulating bone metabolism. Therefore, a clear understanding of the regulatory mechanisms of m⁶A modification on bone metabolism may contribute to the improvement of treatment regimens for related bone metabolic diseases, such as osteoporosis and osteoarthritis.

4. N⁶-methyladenosine is involved in the occurrence and development of bone related diseases

4.1. m⁶A in osteoporosis

Osteoporosis is a metabolic disease characterised by the destruction of bone structure and decrease in bone mass, ultimately leading to fragility of bone and increased likelihood of fractures (**Figure 4**).[57] Yan *et al.*[58] found that METTL3 is significantly downregulated in patients with osteoporosis. They then constructed an ovariectomy-induced osteoporosis mouse model and similarly observed decreased expression of METTL3 in the mice. Subsequently, transfecting interfering fragment si-METTL3 into BMSCs reduced the expression of METTL3, leading to a decrease in the overall m⁶A level and osteogenic differentiation of BMSCs, as well as decreased calcium nodule deposition. Knockout of the METTL3 gene in mice also showed a decrease in bone mass. Conversely, adenovirus-mediated overexpression of METTL3 in BMSCs promoted osteogenic differentiation and overall calcium nodule deposition. Further research revealed that the effect of METTL3 on osteogenesis is mainly through increasing the methylation of the gene Runx2 and its precursor microRNA-320. Overexpression of microRNA-320 can alleviate the reduction of osteogenic differentiation of BMSCs caused by METTL3 deficiency. Therefore, increasing the expression of METTL3 in cells may become a new method for treating osteoporosis (**Figure 5**). However, Lin *et al.*[59] suggest that METTL3 may have the opposite effect. They found that high glucose and high fat induce an over 80-fold increase in the m⁶A level of apoptosis signal-regulating kinase 1 (ASK1), along with an increase in the expression of mitogen-activated protein kinase p38 in pre-osteoblastic MC3T3-E cells. Knocking out of METTL3 eliminated the activation of the high glucose and high fat-induced ASK1-p38 signalling pathway, indicating that the METTL3-ASK1-p38 axis may be a regulatory pathway for high glucose and high fat-induced osteoblast apoptosis. Wang *et al.*[60] discovered a decreased overall m⁶A methylation level with a continuous increase in FTO expression in BMSCs of osteoporosis patients. Through lentivirus-mediated overexpression of FTO in normal BMSCs, they verified that FTO overexpression inhibits the osteogenic potential of BMSCs. Further analysis showed that FTO reduces the overall m⁶A methylation level and the content of Runx2 mRNA in BMSCs. In contrast, inhibiting FTO expression can effectively increase osteogenic differentiation in osteoporosis mice. Shen *et al.*[61] found that during aging or osteoporosis in humans and mice, FTO is upregulated in the bone marrow through a growth differentiation factor 11 C-terminal fragment/estrogen-related receptor beta-dependent mechanism. In addition, the level of FTO increases during BMSCs differentiation into adipocytes, while FTO decreases during BMSCs differentiation into osteoblasts. Functional cell experiments demonstrated that FTO induces adipogenic differentiation of BMSCs and inhibits their osteogenic differentiation. Further mechanistic studies revealed that FTO demethylates the mRNA of peroxisome proliferator-activated receptor gamma (PPARG), a biomarker of osteoporosis, leading to an increase in PPARG mRNA expression. This indicates that FTO promotes the differentiation of BMSCs into adipocytes and inhibits their differentiation into osteoblasts through the growth differentiation factor 11/FTO/PPARG pathway. In contrast, Chen *et al.*[62] found that FTO expression is significantly downregulated in patients with osteoporosis and osteonecrosis. In vitro experiments showed that FTO is significantly upregulated during osteogenic differentiation of human BMSCs, and reducing FTO levels or using FTO inhibitors weakens the osteogenic differentiation of BMSCs. Animal experiments with FTO knockout mice also confirmed impaired osteogenic differentiation and reduced bone mass in these mice. Further research revealed that through YTHDF1, FTO reduces the stability of PPARG mRNA, thereby promoting the expression of ALP and osteopontin (OPN) in BMSCs, which enhances osteogenesis. Therefore, the FTO/YTHDF1/PPARG axis may become a new therapeutic pathway for treating osteoporosis in the future. In summary,

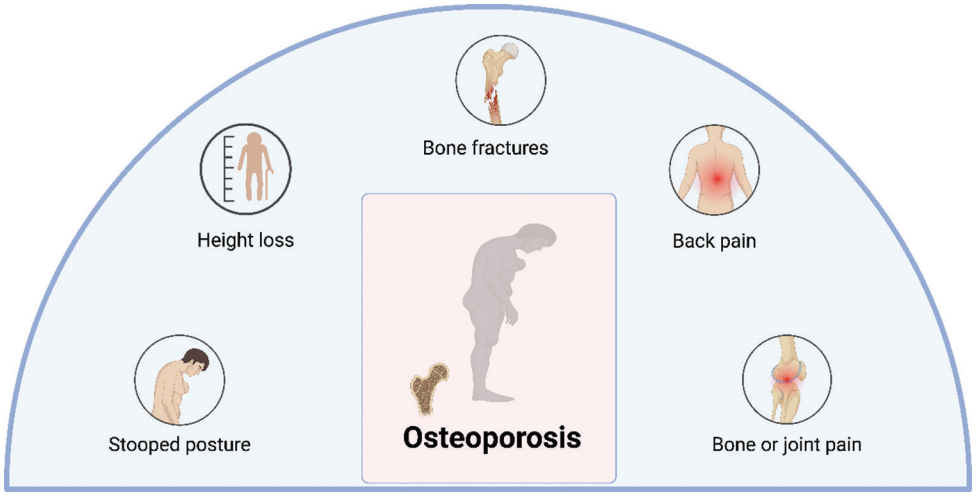

Figure 4. The main symptoms of osteoporosis. Created with BioRender.com.

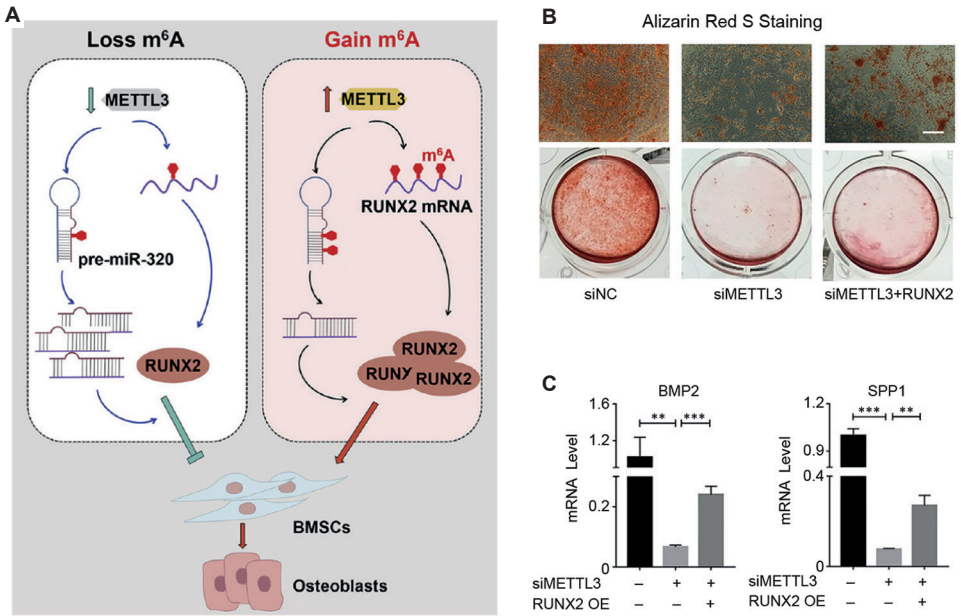

Figure 5. m^{6}A methylation of Runx2 by METTL3 controls BMSC osteogenic differentiation. (A) Graphical abstract. (B) Alizarin Red S staining results. Scale bar: 150 μm. (C) Runx2 overexpression exhibited a reversion effect on mRNA levels of BMP2 and SPP1. $^{**}P < 0.01$, $^{***}P < 0.001$. Reprinted from Yan *et al.*[58]

Abbreviations: BMP2: Bone morphogenetic protein 2; BMSC: Bone marrow derived stem cell; m^{6}A: N^6-methyladenosine; METTL3: Methyltransferase-like 3; mRNA: Messenger RNA; OE: Overexpression; Runx2: Runt-related transcription factor 2; SPP1: Secreted phosphoprotein 1.

m^{6}A modification regulates balance between osteogenic differentiation and osteoclast differentiation therefore regulating osteoporosis. Methyltransferases such as METTL3 and FTO may play an important role in this process.

4.2. m^{6}A in osteoarthritis

Osteoarthritis is the most common degenerative joint disease, characterised by progressive degenerative changes in the articular cartilage, subchondral bone sclerosis, joint pain and stiffness, synovitis, and osteophyte formation (**Figure 6**).[47,63] Chen *et al.*[64] found that in both human and mouse models of osteoarthritis, senescent fibroblast-like synoviocytes (FLS) significantly increase as osteoarthritis progresses. The primary mechanism involves reduced autophagy and upregulated senescence-associated secretory phenotype in senescent FLS. Reconstructing autophagy, accomplished by inhibiting the senescence regulator, transcription factor GATA-binding protein 4 (GATA4), helps counter this process. Specifically, METTL3-mediated m^{6}A modification negatively regulates FLS autophagy via decreasing the expression of the autophagy gene autophagy-related 7 (ATG7) which subsequently leads to overexpression of GATA4. Silencing METTL3 can enhance autophagy in FLS, and intra-articular injection of synovium-targeted METTL3-siRNA in mice can inhibit cellular senescence in the joints. Therefore, the METTL3/ATG7/GATA4 pathway may provide a new strategy for the treatment of osteoarthritis. Liu *et al.*[65] cultured the chondroprogenitor cell line ATDC5 in inflammatory

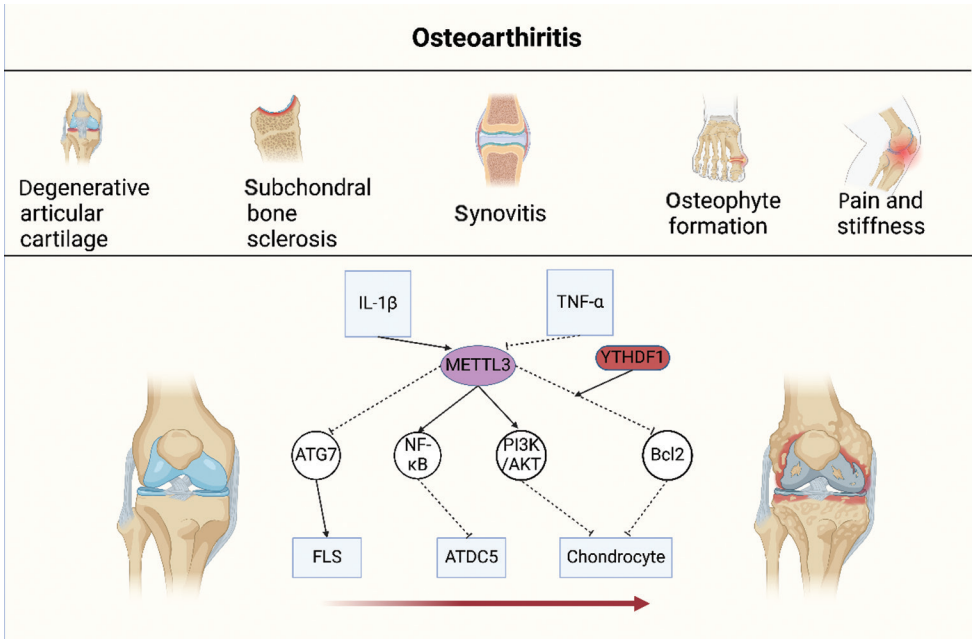

Figure 6. m⁶A regulates osteoarthritis. Created with Biorender.com.
Abbreviations: AKT: Protein kinase B; ATG7: Autophagy-related 7; FLS: Fibroblast-like synoviocyte; IL: Interleukin; m⁶A: N⁶-methyladenosine; METTL3: Methyltransferase-like 3; NF- κB: Nuclear factor kappa-B; PI3K: Phosphoinositide 3-kinase; YTH: YTH domain.

conditions via cytokine IL-1β *in vitro*. The results showed an increasing trend in both the expression level of METTL3 and the percentage of total m⁶A modifications level. In the meantime, the NF-κB signalling pathway in chondrocytes was activated. Conversely, silencing METTL3 reduced the levels of matrix metalloproteinase-13 and collagen X, while promoting the expression of Aggrecan and collagen II, thereby enhancing the degradation of the extracellular matrix. Overall, METTL3 can regulate the NF-κB signalling pathway and the extracellular matrix synthesis in chondrocytes, thus plays a role in the progression of osteoarthritis. This provides another perspective on the role of METTL3 in the development of osteoarthritis. Xiao *et al.*[66] discovered significant differences of METTL3 expression in patients with varying degree of degeneration of endplate cartilage, indicating that METTL3-mediated m⁶A methylation is closely related to endplate cartilage degeneration process. Subsequent cell functional experiments revealed that IL-1β can induce miR-126-5p to inhibit the PI3K/Akt signalling pathway by targeting the phosphoinositide 3-kinase regulatory subunit 2 (PIK3R2) gene, leading to disrupted cell viability and metabolic functions of endplate cartilage cells. Further experiments demonstrated that METTL3 can bind to the key protein DGCR8, involved in the processing of pri-miR-126-5p, thereby regulating the maturation of miR-126-5p. Based on the above, further research on the METTL3/DGCR8/miR-126-5p/PIK3R2/PI3K/Akt signalling pathway is expected to provide new therapeutic strategies for the degeneration disease of human endplate cartilage. In the study on the role of m⁶A in temporomandibular joint osteoarthritis, He *et al.*[67] found that the expression of METTL3 decreased both in temporomandibular joint osteoarthritis mice as well as chondrocytes under inflammatory stimulation *in vitro*. METTL3 was shown to inhibit TNF-α-induced apoptosis and autophagy in chondrocytes. Further bioinformatics analysis and RNA immunoprecipitation experiments confirmed that Bcl2 is the downstream gene modified by METTL3. The reader protein YTHDF1 binds to Bcl2 mRNA after METTL3 modification, and subsequently interacts with TNF-α-stimulated Beclin1 protein in chondrocytes. This study indicates that the METTL3/YTHDF1/Bcl2 signalling axis can inhibit apoptosis and autophagy of chondrocytes in temporomandibular joint osteoarthritis inflammation. In summary, while m⁶A regulates osteoporosis through mediating bone metabolism, it regulates osteoarthritis primarily functions by mediating the phenotype and inflammatory responses of chondrocytes. This process is strongly-related to downstream of NF-κB and Wnt/β-catenin signalling pathways, as well as exacerbating chondrocyte degeneration and inflammatory responses.

4.3. m⁶A in osteosarcoma

Osteosarcoma is the most prevalent primary malignant bone tumour, typically arising in the metaphysis of long bones and predominantly affecting children and adolescents. Unfortunately, the clinical prognosis for osteosarcoma patients is often relatively poor due to the highly aggressive nature of the disease, with high risk of recurrence and metastasis, making the treatment of osteosarcoma extremely challenging.[68] Over several decades, continuous exploration and improvement of chemotherapy drugs such as doxorubicin and methotrexate have successfully increased the 5-year survival rate of osteosarcoma patients to over 50%. However, when osteosarcoma patients become resistant to these chemotherapy drugs, their prognosis significantly worsens.[69,70] Zhou *et al.*[71] found that in osteosarcoma tissues, the tripartite motif containing 7 (TRIM7) protein promotes tumour cell migration and invasion as well as reduces sensitivity to chemotherapy drugs through the ubiquitination of breast cancer metastasis suppressor 1 (BRMS1). The high expression

of TRIM7 is caused by decreased degradation of m^{6}A-modified TRIM7 mRNA, which is regulated by METTL3 and YTHDF2. Therefore, targeting the METTL3/YTHDF2/TRIM7/BRMS1 pathway to downregulate TRIM7 expression may help inhibit osteosarcoma metastasis, enhance tumour sensitivity to chemotherapy, and ultimately improve patient survival rates (**Figure 7A**). Wei *et al.*[72] discovered that the expression level of YTHDF1 might be closely associated with poor prognosis in osteosarcoma patients. The expression of CCR4-NOT transcription complex subunit 7 (CNOT7) was significantly upregulated in osteosarcoma tissues and may be a potential downstream target of YTHDF1. METTL3 can promote the m^{6}A modification of CNOT7 mRNA. Therefore, the METTL3/YTHDF1/CNOT7 regulatory axis might be a new potential target for analysing osteosarcoma prognosis and further treatments. Wang *et al.*[73] sampled osteosarcoma tissues from patients in different Enneking stages of metastatic osteosarcoma and non-metastatic osteosarcoma to measure the expression differences of METTL3. The results showed that METTL3 expression was elevated in metastatic osteosarcoma. Artificial downregulation of METTL3 significantly diminished the migration and invasion capabilities of osteosarcoma cells. Furthermore, a positive correlation was observed between METTL3 and TRAF6 in metastatic osteosarcoma. This indicates that increased METTL3 expression in osteosarcoma enhances TRAF6 expression through m^{6}A modification, thereby promoting the migration and invasion of osteosarcoma. Yuan *et al.*[74] found that ALKBH5 expression was significantly decreased in osteosarcoma cells compared to normal osteoblasts or bone tissue, accompanied by an overall increase in m^{6}A methylation in osteosarcoma. On the other hand, overexpression of ALKBH5 significantly inhibited the growth, migration, and invasion of osteosarcoma as well as induced cell apoptosis. Further experiments showed that decreased ALKBH5 expression enhanced the m^{6}A methylation of pre-miR-181b-1 and Yes-associated protein (YAP) mRNA. Research on YAP and pre-miR-181b-1 revealed that increasing YAP expression or decreasing miR-181b-5p expression significantly weakened the antitumour activity induced by ALKBH5. This effect was due to YTHDF1 produced different effects after recognising their methylated mRNA. YTHDF1 binding to methylated pre-miR-181b-1 mRNA mediated further degradation, while methylated YAP mRNA recognition by YTHDF1 enhanced its translation. Therefore, the ALKBH5/YTHDF1/pre-miR-181b-1/YAP pathway may represent a novel therapeutic target for osteosarcoma treatment in the future (**Figure 7B**). Lv *et al.*[75] discovered that FTO can promote the metastasis and growth of osteosarcoma. RNA sequencing and methylated RNA immunoprecipitation sequencing identified Dishevelled-binding antagonist of β-catenin 1 (DACT1) was a downstream target gene of FTO. Further cell experiments showed that FTO can reduce the expression of DACT1 through m^{6}A modification, thereby activating the Wnt signalling pathway. Additionally, the m^{6}A reader protein IGF2BP1 was validated to be involved in the FTO-DACT1 interaction process. Notably, the study found

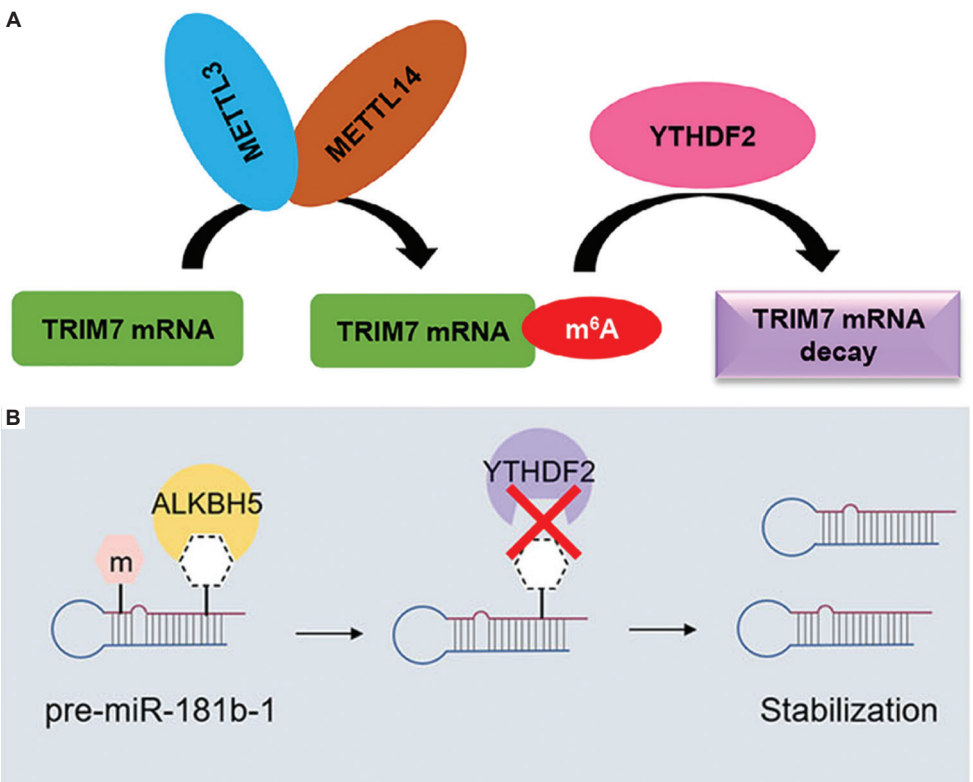

Figure 7. m^{6}A regulates osteosarcoma. (A) TRIM7-BRMS1 pathway positively regulates tumourigenesis and chemoresistance in osteosarcoma through m^{6}A modification. Reprinted from Zhou *et al.*[71] (B) ALKBH5 suppresses osteosarcoma via pre-miR-181b-1/YAP signalling axis. Reprinted from Yuan *et al.*[74]

Abbreviations: ALKBH5: AlkB homolog 5; BRMS1: Breast cancer metastasis suppressor 1; m^{6}A: N^6-methyladenosine; TRIM7: Tripartite motif containing 7; YAP: Yes-associated protein; YTH: YTH domain.

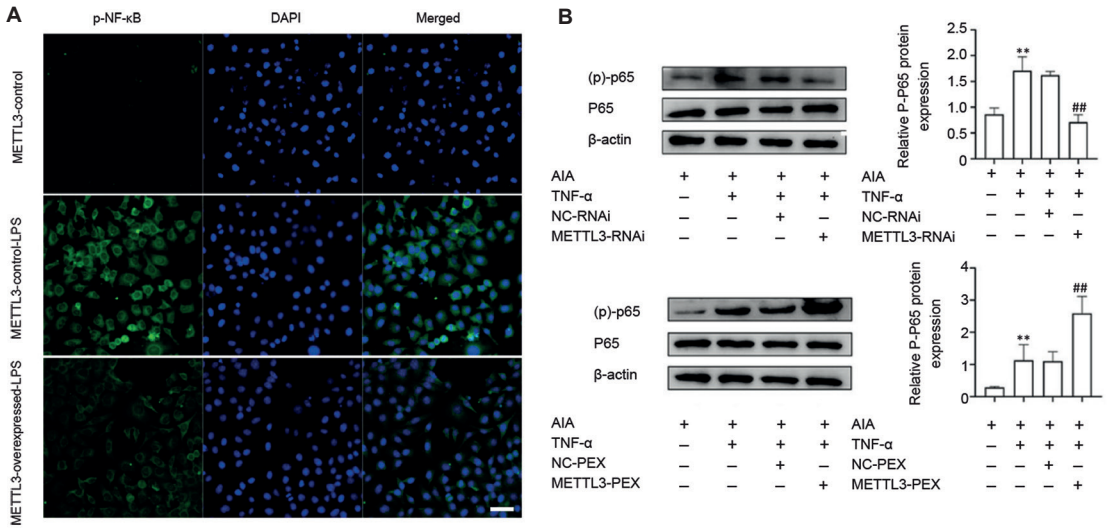

Figure 8. m⁶A regulates rheumatoid arthritis. (A) METTL3 attenuates LPS-induced inflammation in macrophages via NF-κB signalling pathway. Reprinted from Wang *et al.*[78] Scale bar: 50 μm. (B) METTL3 regulates (p)-p65 protein level in TNF-α-incubated RA-fibroblast-like FLS. $^{**}P < 0.01$, *vs.* control group; $^{\#\#}P < 0.01$, *vs.* NC-RNAi/NC-PEX group. Reprinted from Si *et al.*[79]
Abbreviations: (p)-p65: Phosphorylated p65; AIA: Adjuvant-induced arthritis; FLS: Fibroblast-like synoviocyte; LPS: Lipopolysaccharide; m⁶A: N⁶-methyladenosine; METTL3: Methyltransferase-like 3; NF-κB: Nuclear factor kappa-B; p-NK-κB: Phosphorylated NF-κB; PEX: Plasmid expression vector; RNAi: RNA interference; RA: Rheumatoid arthritis; TNF-α: Tumour necrosis factor-alpha.

that entacapone, a drug commonly used to treat Parkinson's disease, was effective in inhibiting the growth of osteosarcoma mediated by the FTO/IGF2BP1/DACT1/Wnt signalling axis. This study provides a new therapeutic approach for the disease.

4.4. m⁶A in rheumatoid arthritis

Rheumatoid arthritis is a chronic autoimmune disease characterised by synovial hyperplasia accompanied by inflammatory infiltration and progressive symmetrical joint destruction. It can cause cartilage and bone damage, and disability in severe cases.[76] Although some current medications are somewhat effective in relieving patient symptoms, many patients still do not respond well to various antirheumatic drugs. Prolonged pain and worsening joint deformities can severely impact the quality of life. Moreover, in most patients with rheumatoid arthritis, inflammation often persists even if the disease is well controlled.[77] Wang *et al.*[78] found that the expression of METTL3 in monocyte-macrophages in the peripheral blood of rheumatoid arthritis patients is significantly increased. LPS can increase the total m⁶A content in macrophages by upregulating METTL3. In vitro experiments showed that METTL3 can inhibit the proliferation of macrophages as well as its secretion of inflammation-related cytokines such as IL-6 and TNF-α. Furthermore, the negative impact of METTL3 in LPS-induced macrophage inflammation is dependent on the NF-κB signalling pathway. This research highlighting the key role of METTL3 in rheumatoid arthritis and providing a new therapeutic approach for treating rheumatoid arthritis (**Figure 8A**). However, another study found completely opposite functions of METTL3 in rheumatoid arthritis. Shi *et al.*[79] discovered that the expression of METTL3 is significantly upregulated in the synovial tissues of rheumatoid arthritis patients and animal models. However, knockdown of METTL3 inhibits the levels of IL-6, matrix metalloproteinase-3, and matrix metalloproteinase-9 in FLS cells of both patients and rat models. Further research demonstrated METTL3 may promote FLS cell activation and inflammatory responses through the NF-κB signalling pathway (**Figure 8B**).

5. Limitation

In this review, we aimed to provide a comprehensive analysis of the role of m⁶A RNA methylation in bone homeostasis and related diseases. However, we acknowledge several limitations in our review design. First, our work is based on currently available literature, which may cause publication bias, as studies with positive findings are more likely to be reported and included. Second, although we have discussed various cellular and molecular mechanisms, the complexity and heterogeneity of m⁶A regulation across different models and contexts are not fully addressed, which may limit the generalizability of some conclusions. Third, we recognise the presence of contradictory findings, such as the differing roles of METTL3 and FTO in various pathological processes, which reflects the ongoing need for further experimental validation. Lastly, while we have summarised key findings in this rapidly evolving field, emerging technologies like single-cell and spatial transcriptomics, which could provide more nuanced insights into m⁶A dynamics in bone biology, were not extensively covered. We hope that acknowledging these limitations will inspire future research to address these gaps and build a more comprehensive understanding of this field.

6. Conclusions

Epigenetic regulation is increasingly becoming a hotspot in current medical science research.[80] As the predominant internal RNA modification in eukaryotic cells, m⁶A is composed of methyltransferases, demethylases, and m⁶A-binding proteins.

Methyltransferases catalyse the N^6-methylation in mRNA, demethylases remove the N6-methyl group from adenine, and m^6A-binding proteins recognise and bind to m^6A sites to further exert their functions. Together, they collaboratively complete the reversible process of m^6A, which regulates many physiological activities. m^6A plays a crucial role in the differentiation of BMSCs, as well as the homeostasis and functional regulation of osteoblasts and osteoclasts in bones. Additionally, research on various bone-related diseases such as osteoporosis, osteoarthritis, osteosarcoma, and rheumatoid arthritis have revealed many mechanisms by which m^6A is involved.

Looking forward, future research in this field should address several key areas to deepen our understanding and enhance translational applications. First, further studies are needed to resolve contradictory findings regarding the roles of specific m^6A regulators, such as METTL3 and FTO, under different pathological conditions. Second, expanding the use of advanced technologies, such as single-cell RNA sequencing and spatial transcriptomics, could provide a higher-resolution understanding of m^6A-mediated regulation at the cellular and tissue levels. Additionally, exploring the interplay between m^6A and other epigenetic modifications, such as histone methylation and DNA methylation, could uncover novel regulatory networks involved in bone physiology and pathology.

From the application perspective, targeted modulation of m^6A regulators holds great potential for therapeutic development. For example, precise delivery of m^6A-related inhibitors or activators using nanotechnology or tissue-specific vectors could offer innovative treatments for bone-related diseases. Furthermore, leveraging m^6A as a biomarker for disease diagnosis or prognosis could improve early detection and personalised treatment strategies.

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