Molecular Therapy
Nucleic Acids
Review

Advances in mRNA 5-methylcytosine modifications: Detection, effectors, biological functions, and clinical relevance

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5-methylcytosine (m^5C) post-transcriptional modifications affect the maturation, stability, and translation of the mRNA molecule. These modifications play an important role in many physiological and pathological processes, including stress response, tumorigenesis, tumor cell migration, embryogenesis, and viral replication. Recently, there has been a better understanding of the biological implications of m^5C modification owing to the rapid development and optimization of detection technologies, including liquid chromatography-tandem mass spectrometry (LC-MS/MS) and RNA-BisSeq. Further, predictive models (such as PEA-m^5C, m^5C-PseDNC, and DeepMRMP) for the identification of potential m^5C modification sites have also emerged. In this review, we summarize the current experimental detection methods and predictive models for mRNA m^5C modifications, focusing on their advantages and limitations. We systematically surveyed the latest research on the effectors related to mRNA m^5C modifications and their biological functions in multiple species. Finally, we discuss the physiological effects and pathological significance of m^5C modifications in multiple diseases, as well as their therapeutic potential, thereby providing new perspectives for disease treatment and prognosis.

INTRODUCTION

Since the discovery of the first post-transcriptional RNA modification, pseudouridine (ψ), in the 1950s,[1] researchers have explored the mechanisms underlying gene regulation at the RNA level. This emerging field has been designated as "RNA epigenetics" or "epitranscriptomics."[2–4] Compared with the chemical modifications of DNA, RNA modifications are more abundant, with a total of 170 identified to date,[5] including N6-methyladenosine (m^6A), 5-methylcytosine (m^5C), N4-acetylcytidine (ac^4C), N1-methyladenosine (m^1A), ψ, and 7-methylguanylate (m^7G). These modifications constitute a relatively unexplored gene-expression regulatory layer, highlighting the complexity and diversity of RNA.[4] The structural diversity of modified nucleosides plays a key role in maintaining RNA stability,[6] translation,[7] and splicing.[8] Therefore, these modifications have critical roles in regulating the development and pathophysiology of several diseases.[9–11]

In m^5C modifications, a methyl group attached to the fifth carbon of the cytosine ring in DNA and RNA molecules. This modification was first identified on DNA[12] and later on RNA in the 1970s.[13] It is an important post-transcriptional modification (PTCM) that has significant roles in many biological processes.[14] Hence, m^5C modifications have recently garnered increased attention and have been reported in many species, including yeast, adenovirus, *Phaseolus vulgaris*, starfish, *Tetrahymena thermophila*, rat, *Drosophila melanogaster*, and wheat germ.[15–24]

Studies on m^5C modifications were initially focused on tRNA and rRNA.[25] In tRNA, m^5C has been shown to participate in optimizing codon–anticodon pairing, maintaining homeostasis, regulating stress response, and controlling translation efficiency and accuracy.[26–31] Meanwhile, m^5C modifications in rRNA play an important role in conferring bacterial drug resistance,[32,33] glioma sensitivity to bioactive substrates of the stress-related enzyme NQO1,[34] thermal

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adaptability (e.g., m^5C modifications mediated by ribosomal RNA small subunit methyltransferase F (RsmF) in *Thermus thermophilus* reduce its thermal adaptability),[35] and structural stability of the tertiary rRNA–tRNA–mRNA complex under stress (i.e., promotion of adaptive translation).[34] However, although there is a large amount of data on m^5C in tRNA and rRNA, little is known about its role in mRNA owing to the low abundance of mRNA and the lack of effective separation and purification technologies.[36] However, with recent advances in detection methods, studies on mRNA m^5C modifications have gradually increased.

In mRNA, m^5C modifications, along with multiple effector enzymes, such as NOP2/Sun RNA methyltransferase 2 (NSUN2),[37] NSUN6,[38] tRNA aspartic acid methyltransferase 1 (TRDMT1),[39] and Aly/REF export factor (ALYREF),[40] perform numerous functions, including the promotion of mRNA nuclear–cytoplasmic trafficking;[40] viral protein expression;[41] DNA damage repair;[42] mRNA stability;[43] cell tolerance,[44] proliferation and migration;[39] development, differentiation, and reprogramming of stem cells;[45,46] and regulation of mRNA splicing.[47] In addition, the distribution of m^5C varies among cell types.[24] m^5C modifications at specific positions of mRNAs exhibit different regulatory activities:[48] they can either promote or inhibit translation.[49,50] Therefore, abnormal mRNA m^5C modifications have been associated with the development and progression of multiple diseases, including cancer,[51] autoimmune diseases,[52] and arteriosclerosis.[53]

To date, no systematic review exists on the related effectors, specific biological functions in different species, or the clinical relevance of mRNA m^5C modifications. Therefore, in this review, we comprehensively summarize the current detection methods, predictive models, related effectors, and roles of mRNA m^5C modifications in normal physiology and diseased states, which may serve as a guide for researchers as well as new insights into the development of novel diagnostic methods, treatments, and prognostic biomarkers.

DETECTION METHODS

The identification of modified target genes is essential to investigating the biological functions of mRNA m^5C modifications. With recent advances in sequencing technology and experimental methods, methylation modifications of mRNA can be qualitatively and quantitatively analyzed at the single-nucleoside level, providing novel insights into its biological functions. Currently, the primary detection methods for m^5C modifications include: (1) physicochemical methods, such as chromatography, mass spectrometry (MS), high-performance liquid chromatography (HPLC; Figure 1), and liquid chromatography-tandem MS (LC-MS/MS; Figure 1); (2) chemical conversion, which combines next-generation sequencing (NGS) techniques, such as RNA bisulfite sequencing (RNA-BisSeq), with ten-eleven translocation (Tet)-assisted peroxotungstate oxidation sequencing (TAWO-seq; Figure 2); (3) immunoprecipitation combined with NGS techniques, such as aza-immunoprecipitation (5-Aza-seq; Figure 2) with m^5C individual-nucleotide-resolution cross-linking and immunoprecipitation (miCLIP; Figure 2); (4) third-generation sequencing (TGS), based on the differences in electrical signals (Nanopore-seq; Figure 2); (5) predictive models, such as a machine learning-based m^5C predictor trained with features extracted from the flanking sequence of m^5C modifications (PEA-m^5C), 5-methylcytosine sites via pseudo nucleotide compositions (m^5C-PseDNC), Pm^{5}CS-Comp-minimum redundancy maximum relevance (mRMR), identifying RNA 5-methylcytosine sites via pseudo nucleotide compositions (iRNA-m^5C-PseDNC), and m^5C-heuristic nucleotide physical-chemical property reduction (m^5C-HPCR). These methods are commonly used in the detection of m^5C modifications in target genes.

PHYSICOCHEMICAL METHODS

In early studies, the detection of modified nucleosides was based on techniques, such as thin-layer chromatography (TLC) and capillary electrophoresis (CE).[54,55] Although these methods are primarily used for the detection of m^5C in DNA, they are also suitable for RNA.[56,57] The advantages of these techniques include that they are well established, are easy to perform, and can be applied quantitatively.[58] Radioactive labeling has also been used to improve the low sensitivity associated with these early methods;[59] they remain unable to achieve single-base resolution, making quantitative assessment of methylation patterns in the natural sequence background difficult.

HPLC

The sensitivity of HPLC detection can reach the nanogram level; moreover, radioactive auxiliary compounds are not required for chromatographic separation, thus avoiding the effects of radioactive labeling on the nucleoside structure.[60] However, it remains necessary to develop HPLC for the simultaneous detection of multiple RNA modifications and to determine the appropriate HPLC conditions for tandem use with MS.

MS

MS is a physicochemical method that is used for detecting RNA modifications and has been used for more than 40 years.[61] It does not require the conversion of RNA into complementary DNA (cDNA) by polymerase chain reaction (PCR); rather, RNA sequences are directly treated with ribonucleases to catalyze their degradation into nucleosides, which are separated and identified using MS.[62,63] Modified nucleosides are identified based on the increases in mass and comparison with the mass-to-charge ratio of normal nucleosides.[64] Thus, the stripping of all edited bases and epigenetic information from molecules[65] and the occasional introduction of substantial artifacts[66] for reverse transcription can be avoided. MS can also be used for direct RNA sequencing (RNA-seq) and transcriptomic analysis (modification type/level). In theory, MS is suitable for all modification types, including m^5C and m^6A.[67,68] However, it has certain limitations: (1) it requires relatively high-target RNA purity and concentration; (2) its sensitivity must be improved compared with other amplification-based detection methods;[68] (3) considering that MS is primarily used in a serial mode (one RNA sequence at a time), it is unable to meet the required upper limit of the RNA sequence number for analysis, even with multi-channel technology.[69] In spite of these

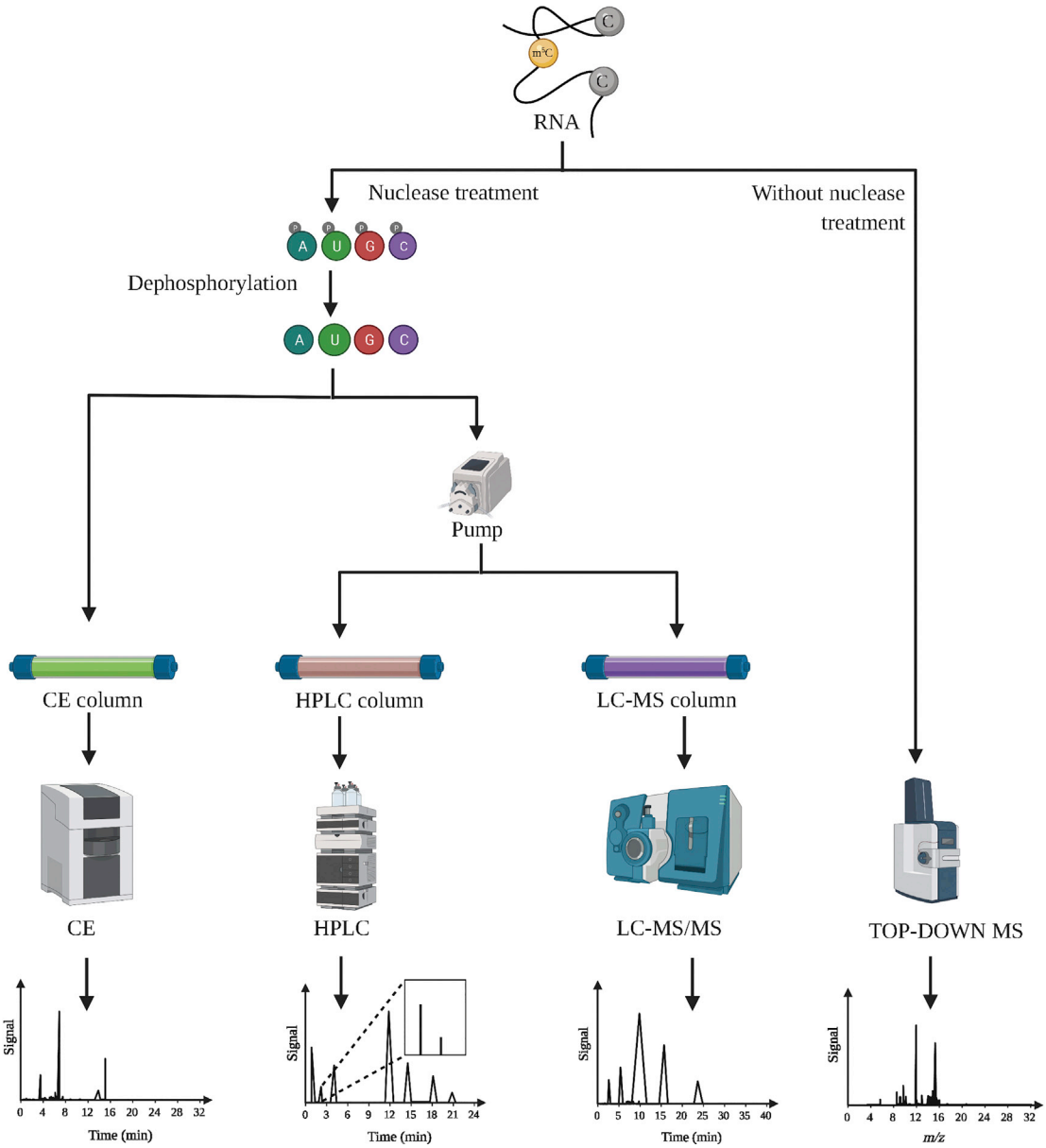

Figure 1. 5-methylcytosine (m^5C) detection methods based on chromatography and mass spectrometry (MS)

Nuclease is used to digest the RNA molecule into its constituting nucleotides, which are dephosphorylated. These nucleosides are prepared as input for capillary electrophoresis (CE), high-performance liquid chromatography (HPLC), and liquid chromatography tandem MS (LC-MS/MS). CE: in high-voltage electric field drive, nucleoside samples are separated using a quartz capillary column as a separation channel. LC-MS/MS: the LC system is connected to the mass spectrometer. TOP-DOWN MS: without nuclease treatment, using a high-resolution mass spectrometer and various fragmentation patterns of ribonucleotides, RNA can be directly analyzed using MS to obtain information, including the identification of post-transcriptional modifications, relative quantification, and positional information.

limitations, MS remains a powerful tool for studying RNA modifications.

LC-MS/MS

Owing to its superior qualitative ability, MS is often used in tandem with LC, e.g., LC-MS/MS. In fact, Cui et al.[70] used this method to identify dynamic m^5C mRNA modification in *Arabidopsis thaliana*. In LC-MS/MS, RNA is digested into oligonucleosides of different lengths by RNases, which are subsequently isolated by LC. Methylation modifications are identified according to the quality difference compared to standard nucleosides, and then modified residues are assigned to sequence sites in oligonucleosides according to gene sequence data.[71,72] Moreover, its sensitivity is at the femtomole level,[73] making it suitable for the detection of low-abundance

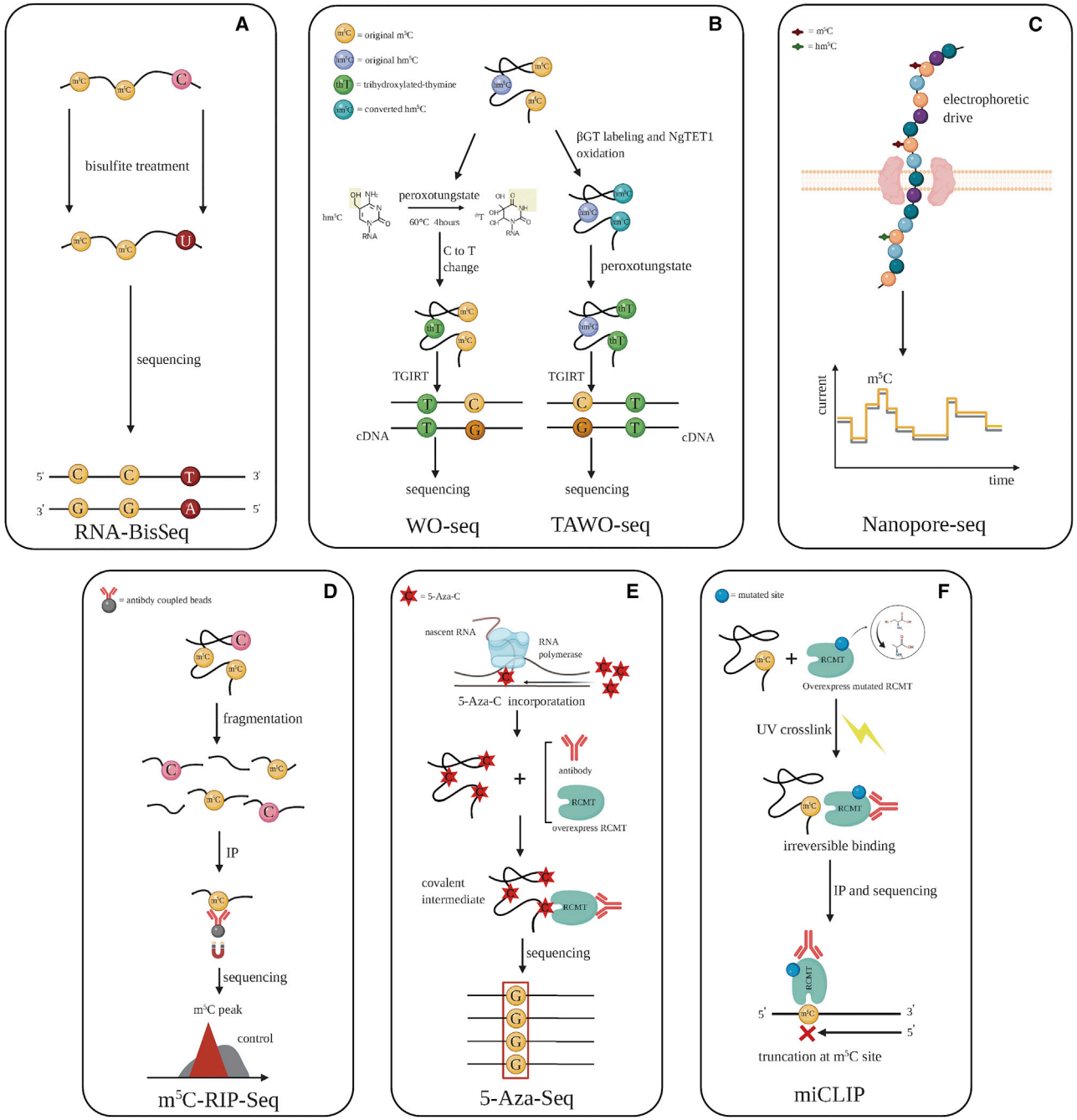

Figure 2. Transcriptome sequencing methods after m^{5}C modification

(A) RNA-BisSeq: unmethylated cytosines of RNA fragments could be transformed into uridines when treated with bisulfite, while bisulfite does not affect m^{5}C. (B) Peroxotungstate oxidation sequencing (WO-seq) and Tet-assisted WO-seq (TAWO-seq): for WO-seq, hm^{5}C can be transformed into trihydroxylated thymine (thT) by peroxotungstate, after which, the C-to-T mutation site was identified by sequencing. For TAWO-seq, m^{5}C was converted to hm^{5}C with NgTET1 oxidation, and hm^{5}C was converted to thT according to the principle of WO-seq. However, the original hm^{5}C of RNA was protected from being altered to thT by labeling with β-glucosyltransferase (βGT). The mutation of C to T was detected and identified as the original m^{5}C but not the original hm^{5}C. TGIRT, thermostable group II intron reverse transcriptase. (C) Nanopore-seq: a unique, scalable technology that enables direct, real-time analysis of long RNA fragments. The electrical current was monitored and recorded when nucleic acids passed through a protein nanopore. The modification signals were then decoded and identified along RNA fragments. (D) m^{5}C-RIP-seq: RNA fragments containing m^{5}C could be pulled down specifically by the anti-m^{5}C antibody and then used to construct a sequencing library. The m^{5}C peaks were identified against the input as background. (E) 5-Aza-seq: 5-azacytidine (5-Aza-C), a cytidine analog, is incorporated into the RNA molecule by RNA polymerase. RNA (cytosine-5)-methyltransferases

(legend continued on next page)

modifications (e.g., mRNA m^5C modifications). Besides screening for known modified nucleotides, it can also detect those that are unknown, such as the 3-(3-amino-3-carboxypropyl)-5,6-dihydrouridine in $tRNA^{Lys}$ (tRNA lysine, UUU) from *Trypanosoma brucei*.[74,75] In principle, LC-MS/MS requires at least tens to hundreds of nanograms of purified molecules, which is not feasible for heterogeneous and low-abundance mRNA. However, as this method involves processing RNA into nucleosides using enzymes, RNA samples cannot exist in their full-length form, which can mask the detection of modified sites, cause loss of base sequence context information, and increase the signal/noise in low-quality areas.[76]

Top-down MS

Unlike LC-MS/MS, top-down MS avoids the complications of enzymatic digestion and the need for oligoribonucleotide separation.[58] Thus, it provides the additional application in the detection of unknown modifications of RNAs. This method can also reveal site cytosine with altered mass and its modification type without labeling.[77] Although it uses a denaturing solution, reports have shown little effect on methylated bases.[78] Top-down MS has a unique potential for the detection, localization, and relative quantification, even though it relies heavily on specialized high-end mass equipment.

Nucleic acid isotope labeling-coupled MS (NAIL-MS)

Notably, the extent of m^5C modifications differs during the course of development; thus, the fate of methylated RNA during growth should be tracked to provide data on dynamically changing modified nucleosides. NAIL-MS was developed by Matthias Heiss et al.[79] and can be used to assess the influence of the external environment on the apparent transcriptome. However, due to the difficulty associated with adding internal isotope standards to cultured human cells, its utility is limited for these applications.

Nanoscale liquid chromatography coupled to tandem mass spectrometry (Nano-LC-MS/MS/MS)

With the exception of early TLC and CE, these methods are suitable for the detection of single RNA modification types; however, they cannot simultaneously detect multiple RNA modifications. In 2015, Fu et al.[80] established a nano-flow LC-MS/MS coupled with a stable isotope-dilution method capable of simultaneously detecting four modified nucleosides (m^5C, 2′-O-methylcytidine, m^6A, and 2′-O-methyladenosine). Compared with LC-MS/MS, the new method has a clear chemical specificity as well as superior sensitivity for the identification of analytes.[80] Moreover, the addition of stable isotope markers in the nucleoside mixture produces more stable and accurate results; thus, it is applicable to studies on RNA modification-related effectors (writer, reader, and eraser).[80]

2-dimensional mass-retention time hydrophobic end-labeling strategy into MS-based sequencing (2D-HELS MS Seq)

Another method for detecting multiple modification types is the 2D-HELS MS Seq. This method introduces 2D HEL into traditional MS, thereby improving the use efficiency of samples, while also broadening the application scope of RNA direct sequencing.[76,81]

CHEMICAL CONVERSION METHODS

RNA-BisSeq

As the gold standard for the detection of m^5C modifications,[82] RNA-BisSeq can recognize cytosine methylation sites at single-base resolution.[83,84] Bisulfite treatment chemically deaminates unmethylated cytosine in single-stranded RNA into uracil; however, methylated cytosine remains unchanged. The m^5C methylation sites on RNA can then be identified by constructing a library and sequencing[85,86] (Figure 2A). Currently, this method is widely used in the study of mRNA m^5C modification in the tissues and cells of multiple animals and plants, including humans, mice, *A. thaliana*, and *Oryza sativa*.[40,44,87]

Although RNA-BisSeq is a robust method for detecting m^5C modification sites in high-abundance tRNA and rRNA, it is associated with high rates of errors for low-abundance mRNAs.[25] One cause of this is the permanent loss of base-modification information, as the modified bases are less abundant than unmodified bases. Additionally, RNA-BisSeq converts bisulfite-treated RNA into cDNA, requiring NGS after PCR amplification.[65,88,89] Moreover, the common generation of artifacts can cause the data to become biased.[90] RNA-BisSeq also cannot sufficiently distinguish m^5C modifications from other cytosine modifications, such as hm^5C (5-hydroxymethylcytidine), which interferes with accuracy and validation.[70] Further, experimental pH conditions damage RNA and even lead to its degradation, thus affecting subsequent experiments and limiting the enrichment of RNA containing m^5C modifications.[91] Lastly, incomplete deamination occurs in unmodified cytidine treated with bisulfite, which is often present in the RNA stem and double-stranded RNA regions, resulting in false positives.[87] Therefore, the application of RNA-BisSeq to detect m^5C modification sites in mRNA requires further improvement.

TAWO-seq

WO-seq is a method for hm^5C detection.[92] Yuan et al.[86] used Tet enzymes to oxidize m^5C to hm^5C to establish a new detection method for m^5C, termed TAWO-seq (Figure 2B). Here, m^5C is first converted to hm^5C by peroxotungstate oxidation with Naeglaria Tet-like oxygenase (NgTET1) oxidation, while the original hm^5C is protected from oxidation by β-glucosyltransferase.[86] TAWO-seq causes less

(RCMT) can form a temporary intermediate with potential m^5C residues; however, 5-Aza-C can inhibit the complex separation. The RCMT-RNA complex was pulled down with a specific anti-RCMT antibody, and the pulled-down RNA was used for library construction and sequencing to identify the m^5C sites as C-to-G transversion (red underlined frame). (F) m^5C individual-nucleotide-resolution cross-linking and immunoprecipitation (miCLIP)-seq: mutating cysteine (C271) into alanine (C271A) for RCMT inhibited the separation of the enzyme-RNA complex with strong covalent bonding, which was achieved by UV cross-linking, resulting in stop position during PCR. The RCMT-RNA complex was pulled down with anti-RCMT antibody, the RNA was washed to construct the library, and the significant truncation site was considered as the m^5C site.

damage to RNA than does RNA-BisSeq. Furthermore, TAWO-seq does not alter unmodified cytosine but rather directly detects the transformation of modified cytosine, avoiding false-positive results caused by the incomplete transformation of unmodified cytosines in RNA-BisSeq. However, as it relies on the transformation of m^5C, its incomplete transformation presents a new limitation. Therefore, its transformation efficiency requires further optimization to improve its applicability for mRNA.[86]

IMMUNOPRECIPITATION

To address the issue of RNA damage associated with chemical transformation methods, immunoprecipitation, primarily using anti-m^5C antibodies that bind to modified RNA and form noncovalent complexes to locate RNA with m^5C modifications, has been employed.[93]

m^5C RNA immunoprecipitation sequencing (m^5C-RIP-seq)

Combining chromatin immunoprecipitation sequencing (ChIP-seq) and RNA-seq, this method can enrich for and identify m^5C modifications in the transcriptome without altering RNA sequence information or requiring RNA-modifying enzymes (Figure 2D). It also avoids the harsh chemical and temperature conditions required for other methods (e.g., the temperature for PCR amplification and acid-base conditions for RNA-BisSeq).[94] Therefore, m^5C-RIP-seq is theoretically suitable for detecting the distribution of multiple RNA modifications in the transcriptome[95] and has been applied to the analysis of bacterial, archaeal, yeast, and plant transcriptomes.[70] The limitations of m^5C-RIP-seq include its high dependence on specific antibodies and the risk of nonspecific binding to RNA.[93] To address these issues, Weichmann et al.[96] developed an experimental toolbox capable of verifying antibody performance, while improving the accuracy and credibility of m^5C-RIP-seq data. However, m^5C-RIP-seq cannot accurately identify the location of single nucleoside sites, as the length of the sequence reads it produces is generally 100–150 nt and is hindered by the RNA secondary structure.[70,94]

5-Aza-seq and miCLIP

These new derivatization methods (Figures 2E and 2F) have been developed based on immunoprecipitation. Compared with m^5C-RIP-seq, 5-Aza-seq can recognize specific targets of enzymes and specific catalytic sites of RNA (cytosine-5)-methyltransferases (RCMTs) at single-base pair resolution. Specifically, 5-azacytidine (5-Aza-C) is incorporated into RNA to prevent the decomposition of the methyltransferase-RNA complex, transforming the target site of methyltransferase from C to G. The m^5C modification site can then be identified.[36,37,97,98] This method can identify specific enzyme targets and the specific catalytic sites of RCMTs at the resolution of single bases, thus addressing the issue of nonspecific binding with MeRIP-seq, as well as the inability to accurately locate single nucleosides. Accordingly, Schaefer et al.[28] used 5-Aza-seq to characterize DNA methyltransferase (DNMT)2 function in the methylation of tRNA. However, 5-Aza-C destroys RNA integrity;[99] miCLIP forms irreversible covalent cross-linkages between the enzyme and RNA substrate by mutating the cysteine (Cys) in methyltransferase to alanine. Consequently, reverse transcription during amplification terminates at −1 of the methylation site, and the m^5C site is mapped at the +1 of the read sequence data.[100–102] Recently, Selmi et al.[103] mapped the m^5C modifications in the human transcriptome using miCLIP.

Both 5-Aza-seq and miCLIP depend on the formation of covalent bonds between RNA methylase and its substrate; however, the processes for the formation of this bond differ. Unlike 5-Aza-seq, miCLIP does not affect the methyltransferase step or the structure or stability of RNA.[37] However, miCLIP is time consuming and costly and depends on the mutation rate of methyltransferase, with a high rate altering the methylation pattern.[104] Nevertheless, both 5-Aza-seq and miCLIP can be used to identify targets of m^5C methylase. For example, in 2013, Khoddami and Cairns[97] discovered the target of methylase NSUN2 in noncoding RNAs, such as tRNA, using 5-Aza-seq. In the same year, Hussain et al.[102] analyzed and confirmed using miCLIP that NSUN2 can act on non-coding RNAs. Thus, both of these techniques can locate targets of the m^5C methylase NSUN2, thereby expanding the current knowledge regarding its scope of action and function.[105] Although these immunoprecipitation-based methods do not require PCR amplification, their application in trace-volume samples is limited. In addition, their reproducibility is poor due to batch-to-batch differences in antibodies and changes in their affinity.[106] Hence, it is necessary to develop more effective antibody-independent detection techniques in the future.

TGS

Although NGS can accurately localize modified nucleosides within the transcriptome,[107] sequence-by-synthesis technologies can only sequence short RNA reads,[65] limiting their ability to analyze long, repetitive sequences[108] and the subsequent plotting of genome-wide modification maps. Recently, third-generation, single-molecule long reads (TGS) generating long-read information have been developed, including Nanopore-seq technology as well as single-molecule real-time (SMRT) sequencing technology. These methods do not require RNA recombination or qPCR amplification when sequencing transcriptomes,[109] addressing the limitations of NGS and effectively improving continuity and integrity.[110]

Nanopore-seq technology offers unique advantages in the analysis of splice variants,[111] which provide more abundant biological information for RNA research. This technique is based on the principle that when RNA passes through nanopores, the bases cause characteristic changes in the current, which is unique to each normal and modified base. The corresponding bases can then be inferred from the signal-spatial data[112] (Figure 2C). In addition, SMRT technology uses reverse transcription to locate m^6A modification sites on RNA[113] and has been proposed to be capable of detecting other types of modifications in natural RNA.[114] However, TGS faces certain challenges. For example, RNA molecules readily degrade and fold, causing inaccurate results when sequencing longer RNA molecules. Although TGS has a high error rate, it can obtain complete genome sequences and transcriptional landscapes,[115] while addressing the issues associated with crosstalk and dependence among multiple RNA modifications in the same RNA molecule.[65] These advantages provide a solid

foundation for further studies on biological etiologies and disease pathologies.

PREDICTIVE MODELS

Although the above-mentioned experimental detection methods can obtain relatively accurate information on mRNA modifications, they are difficult to perform, time consuming, and costly; they are, therefore, often inappropriate for the in-depth analysis of m^5C-related mechanisms and functions.[116] In the post-genome era, computational tools for predicting m^5C sites have become both important and feasible. Studies on the optimization of these tools primarily focus on the construction of improved datasets by optimizing mixed datasets and determining the gold standard for negative groups to eliminate the influence of false negatives (unmodified samples). However, studies also focus on determining optimal composite feature extraction methods and classifiers.[14]

Currently, 12 models have been developed to predict mRNA m^5C modifications: PEA-m^5C,[117] a hybrid model for quickly and accurately identifying m^5C sites from non- m^5C sites in Homo sapiens RNA (Pm^{5}CS-Comp-mRMR), [118] m^5C-PseDNC, [119] iRNA-m^5C-PseDNC,[120] m^5C-HPCR,[116] prediction of RNA 5-methylcytosine sites based on three different kinds of nucleotide composition (RNA-m^5C-Pred),[121] identifying the occurrence sites of different RNA modifications by incorporating collective effects of nucleotides into PseKNC (iRNA-PseColl),[122] RNA-m^5C-finder,[123] iRNA-m^5C,[119] a novel method for predicting m^5C sites of RNA (m^5C-Pred-SVM),[124] a new predictor for multiple types of RNA modification sites using deep learning (DeepMRMP),[125] and a platform for simultaneously identifying multiple kinds of RNA modifications (iMRM) (Table S1).[126] With the exception of PEA-m^5C, which specifically predicts m^5C methylation sites in *A. thaliana*,[104,117] all of these models can predict human m^5C modifications. Compared with the m^5C-PseDNC model, Pm^{5}CS-Comp-mRMR is not affected by large or unbalanced datasets and adopts a mRMR algorithm with higher sensitivity;[118,119] however, these two models are not available on web servers.

In the early datasets used to construct predictive models, the numbers of positive and negative sequences were unbalanced, with the former far outnumbering the latter. In addition, these datasets contained minimal information on modifications and faced issues related to data redundancy. For example, iRNA-m^5C-PseDNC, created in 2017, retains highly similar sequences, leading to higher predictive results, whereas data redundancy in training datasets leads to a decline in generalizability.[120] In 2018, Zhang et al.[116] established a new calculation model based on Met1900 and Met1320. Their m^5C-HPCR server employs a HPCR algorithm to optimize encoding;[116] moreover, its area under the receiver operating characteristic curve, as well as the associated Matthews correlation coefficient, is superior to that of iRNA-m^5C-PseDNC.[116] In 2019, Fang et al.[121] selected three different features, K-nucleotide frequencies (Kmer) + K-spaced nucleotide pair frequencies (KSNPF) + pseudo-dinucleotide composition (PseDNC), and established a new model, RNA-m^5C-Pred, based on the Met935 database, with higher specificity and sensitivity than the models developed before 2019.

A total of seven site-prediction models have been developed to date: iRNA-m^5C-PseDNC, iRNA-PseColl, RNA-m^5C-finder, iRNA-m^5C, m^5C-Pred-SVM, DeepMRMP, and iMRM. Of the models capable of predicting m^5C modifications in humans and mice, iRNA-m^5C-PseDNC has the highest overall accuracy,[120] particularly when users want to detect more m^5C sites in human samples; it is preferred for its high sensitivity. However, this method also has poor specificity.[36] For instance, although iRNA-PseColl can identify the methylation sites of three different RNA modifications (m^1A, m^6A, and m^5C), it is accompanied by a high false-positive detection rate.[122]

Unlike iRNA-PseColl, the RNA-m^5C-finder model can recognize m^5C modification sites in eight tissues and cell types (mouse_embryonic stem cell, mouse_heart, mouse_kidney, mouse_liver, mouse_muscle, mouse_small intestine, mouse_brain, and human_HeLa);[123] however, it cannot predict methylation in fungi.[119] Meanwhile, the iRNA-m^5C model can detect m^5C modification sites in four different species (*Homo sapiens*, *Mus musculus*, *Saccharomyces cerevisiae*, and *A. thaliana*) and has a higher predictive capacity than RNA-m^5C-finder in terms of precision and accuracy.[119] The m^5C-Pred-SVM model can detect m^5C modification sites in *H. sapiens*, *M. musculus*, and *A. thaliana* by introducing position-specific propensity-related features; its performance, including sensitivity, specificity, overall accuracy, and Matthews correlation coefficient, is superior to that of other existing methods[124]. Similarly, DeepMRMP can predict m^1A, φ, and m^5C modification sites in *H. sapiens*, *M. musculus*, and *S. cerevisiae* RNA,[125] while integrating multiple modification types in different species, making it both time and cost effective. In addition, iMRM, a recently reported prediction tool based on eXtreme gradient boosting (XGboost), can predict m^1A, m^6A, m^5C, ψ, and A to I in the RNAs of *H. sapiens*, *M. musculus*, and *S. cerevisiae*.[126]

Predictive models for a single-modification type or species cannot provide information on the deep features or semantic information of such modification sites. Although these seven predictive models have effectively solved this problem, there remains much room for improvement in the use of these models in the prediction of the types of modification sites that may be involved. There is also a need to improve the predictive abilities of modification sites across different species. Therefore, additional experimental data are needed to improve the quality of datasets that can be used to establish more accurate prediction models.[36] However, this does not negate the importance of species-specific prediction models. Considering the high species specificity of eukaryotic m^5C modifications,[119] it is also necessary to optimize and develop more tailored models with higher performance for specific species.[119] Currently, the predictive technology of traditional computational models is highly dependent on the effectiveness of relevant feature extraction, whereas it remains challenging to determine the most relevant species-specific feature combinations.[125] Therefore, it is necessary to compare and verify

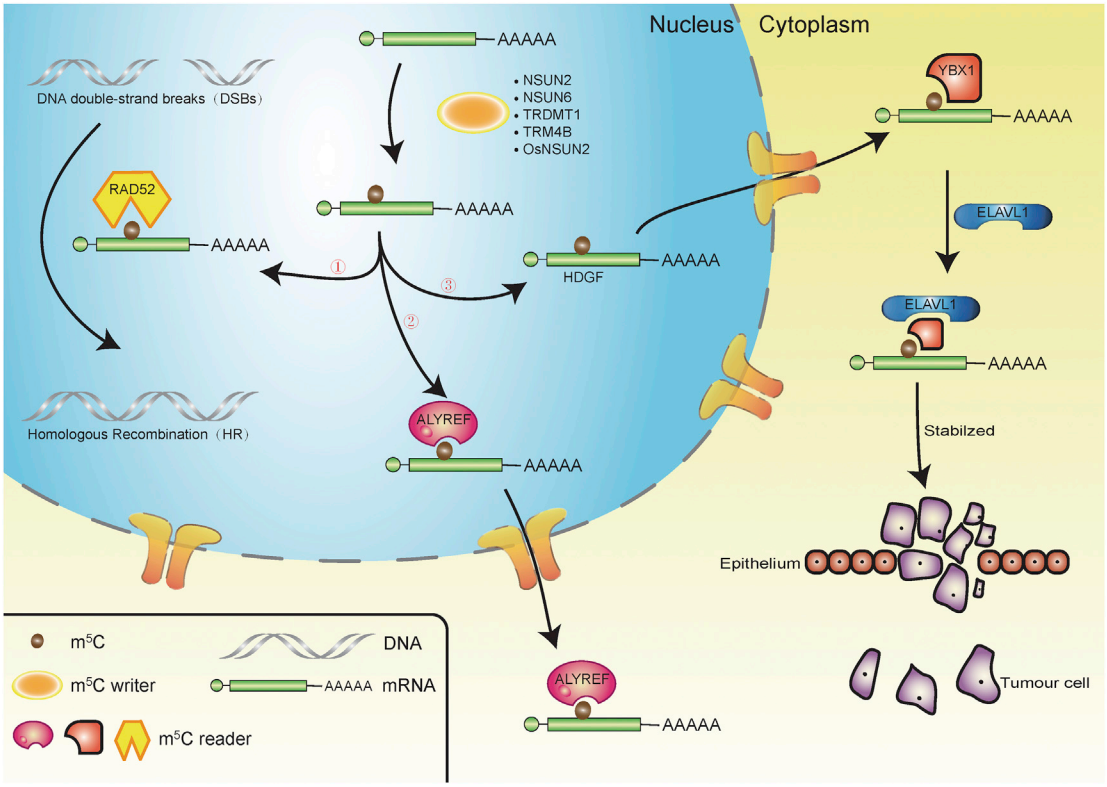

Figure 3. Overview of the effectors (writers, readers, and erasers) related to m^{5}C modifications of mRNA

NSUN2, NSUN6, TRDMT1, TRM4B, and OsNSUN2 are RNA methyltransferases responsible for m^{5}C modification. (1) m^{5}C reader RAD52 recognizes m^{5}C-methylated RNA to promote reactive oxygen species (ROS)-induced atypical HR repair of DSBs through the TRDMT1-m^{5}C-RAD52-RAD51 axis. (2) m^{5}C reader ALYREF recognizes m^{5}C-methylated mRNA and transfers mRNA from the nucleus to the cytoplasm. (3) In bladder cancer, m^{5}C reader YBX1 recognizes methylated heparin-binding growth factor (*HDGF*) mRNA and recruits ELAV-like protein 1 (ELAVL1) to stabilize *HDGF*, finally promoting the proliferation and metastasis of bladder cancer cells.

the predictive performance of models that integrate different feature extraction methods and avoid the impact of noise features on performance.

In summary, the establishment of computational models for predicting potential modification sites provides an excellent supplement to the experimental methods for identifying m^{5}C sites. The continued development and optimization of bioinformatic tools will help researchers to better understand experimental data. In the future, predictive and analytical tools will be effectively applied to increasingly complex samples and experimental conditions.

WRITERS, READERS, AND ERASERS RELATED TO m^{5}C MODIFICATIONS OF mRNA

The PTCM of RNA primarily involves three types of effectors:[65,127,128] (1) writers that "write" specific chemical groups into mRNA, which subsequently mediates mRNA modifications; (2) readers that "read" the information contained in these mRNA modifications to maintain mRNA stability and participate in RNA translation and splicing; and (3) erasers that "erase" mRNA modification signals, mediate mRNA modifications, and convert them back into unmodified nucleosides. Currently, identified writers of mRNA m^{5}C include NSUN2,[37] NSUN6,[38] TRDMT1,[39] the tRNA-specific methyltransferase 4B (TRM4B),[70] and *Osnsun2*,[44] and readers include ALYREF,[40] Y box binding protein 1 (YBX1),[43] and DNA repair protein RAD52 homolog (RAD52)[42] (Figure 3). To date, there have been no reported erasers.

Writers

Writing of m^{5}C into RNA is primarily accomplished by methyltransferases from two families, namely the DNMT family, i.e., TRDMT1, and the nucleolar protein 1 (NOL1)/NSUN protein family, which has seven members in humans (NSUN1 to NSUN7).[129,130]

NSUN2 is an enzyme with a wide target spectrum and was first discovered in association with a tRNA modification[131,132] and mRNA methylation.[133,134] It can catalyze the attachment of methyl to the 5th carbon atom of cytosine in mRNA.[89] The RNA-recognition motif of NSUN2, as well as the Rossmann-fold catalytic core containing S-adenosylmethionine (SAM) cofactor, plays major roles in catalytic modification. They use the covalent connection between two Cys and the cytosine in mRNA to activate the electron-deficient

pyrimidine heterocycle. The carbon 5 then nucleophilically attacks the methyl group of SAM, completing methylation.[135,136] One of the main biological functions of NSUN2-mediated mRNA m^5C modification is its influence on protein translation.[137] Modifications at different mRNA sites can promote or inhibit translation. For instance, Xing et al.[134] reported that NSUN2 methylates the 3′ UTR (C1733) of cyclin-dependent kinase 1 (CDK1) mRNA, enhancing its translation by increasing the assembly of ribosomes on CDK1 mRNA. Similarly, Wang et al.[138] found that NSUN2 mediates the methylation of cytosine at site 466 of interleukin 17A (IL-17A) mRNA to promote its translation in T lymphocytes, mediating homocysteine-induced upregulation of IL-17A expression. Additionally, correlations between increased expression of src homology 2 domain containing (SHC) proteins (p66SHC, p52SHC, and p46SHC), p21, and ICAM-1 (intercellular adhesion molecule 1) and mRNA m^5C modifications mediated by NSUN2 have been reported.[50,53,139] Tang et al.[140] reported that NSUN2 inhibits p27 translation by modifying C64 methylation (m^5C) in the 5′ UTR of p27 mRNA. Similarly, Mei et al.[141] found that m^5C modifications in mRNA 3′ UTR mediated by NSUN2 inhibit the expression of $p57^{Kip2}$. Schumann et al.[137] showed that the abundance of m^5C modifications in the coding sequence (CDS) region negatively correlates, whereas methylation enhancement in the 3′ UTR positively correlates, with mRNA translation efficiency. Therefore, the function of NSUN2 in m^5C modifications may be closely related to the position of the methylation or the specific modified target gene. Fang et al.[38] discovered NSUN6, a new mRNA m^5C methyltransferase, using CRISPR integrated gRNA and reporter sequencing (CIGAR-seq), a CRISPR-Cas9-based method for the unbiased screening of novel mRNA modification effectors. Using mRNA bisulfite sequencing in NSUN6- and/or NSUN2-knockout a near-haploid human leukemia cell line (HAP1) cells, they reported that the mRNA m^5C sites targeted by the two methylases do not overlap and that these enzymes are responsible for nearly all of the m^5C modifications in the mRNA.[38]

TRDMT1, another type of RCMT that regulates mRNA methylation and inhibits the proliferation and migration of HEK293 cells,[39] can catalyze m^5C modifications in tRNA.[28,130] This indicates that TRDMT1 has a wide target spectrum, similar to NSUN2, and that m^5C methylase may modify many types of RNAs. Therefore, further investigation is required to determine which methylase mediates m^5C methylation in different spatiotemporal states and RNA species. Unlike NSUN2, methylation by TRDMT1 only uses Cys at a single site.[136] Chen et al.[42] found that TRDMT1 is also a writer of RNA m^5C at a DNA damage site; its loss hampers homologous recombination (HR) and increases sensitivity to DNA double-strand breaks (DSBs). In addition, TRM4B in *A. thaliana* has mRNA m^5C methyltransferase activity.[70] Another study identified *Osnsun2* as an m^5C methyltransferase targeting mRNA in *O. sativa*.[44]

As m^6A is the most common modification type in eukaryotic mRNA,[127,142] many methylases have been reported to catalyze its installation.[143–150] Huang et al.[23] found that certain m^5C sites maintain high methylation levels in NSUN2 knockdown or knockout cell lines. Furthermore, these m^5C sites have different sequences and structural characteristics from those dependent on NSUN2, suggesting the involvement of unknown methyltransferases in the catalysis of m^5C sites, independent of NSUN2 in human mRNA.[23] Collectively, the diversity of m^6A writers suggests that numerous m^5C methylases remain to be discovered.

Interestingly, some studies have linked m^5C to m^6A. For example, m^5C mediated by NSUN2 and m^6A by METTL3/METTL14 have been shown to reciprocally promote each other and enhance p21 expression synergistically at the translation level during oxidative stress-induced cell aging.[50] Zhang et al.[133] found that NSUN2 acts on the 988th adenine of p16 mRNA to produce m^6A modification, suggesting that NSUN2 is an m^6A methyltransferase of mRNA. In addition, crosstalk between m^6A and m^5C regulators in human cancers has been proposed.[151] However, their precise interactions that are associated with the regulation of mRNA function remain unknown. It is also important to determine whether other types of modifications, apart from m^6A and m^5C, also interact. We speculate that these topics may become future research hotspots.

Readers

Reader proteins for m^6A modification in mRNA have been extensively studied and found to play roles in mRNA splicing, output, stabilization, and translation.[152–154] Relative to studies on mRNA m^6A readers, those on mRNA m^5C readers are in the early stages. For instance, ALYREF plays a role in the nucleoplasmic export of mRNA;[155] as a reader, it binds specifically to m^5C-modified mRNA via K171 (lysine at the 171st site) to regulate mRNA export.[40] Meanwhile, YBX1 participates in maintaining the stability of m^5C-modified mRNA.[43,51] In addition, RAD52 has a higher affinity for hybrid strands containing m^5C-modified RNA and DNA (complementary pair of RNA), indicating that it is an m^5C reader of DNA damage sites. Finally, RAD52 promotes the atypical HR repair of DSBs induced by reactive oxygen species through the TRDMT1-m^5C-RAD52-RAD51 axis.[42]

Erasers

The reversibility of mRNA m^6A modifications has been confirmed, with the mediation of RNA demethylases, such as fat mass and obesity-associated protein (FTO)[156] and AlkB homolog 5 (ALKBH5).[157] Early studies identified a demethylation pathway associated with m^5C modifications of DNA; that is, during DNA modification, m^5C is oxidized to 5-carboxycystine (5caC) by Tet and then cleaved by thymine-DNA glycosylase to complete the process.[158] The m^5C modifications can be hydroxylated to form hm^5C by the modification of mitochondrial (mt) tRNA methionine, with hm^5C further oxidized to 5-formylcytosine (F^5C).[159,160] However, the erasers of mRNA m^5C remain unknown. Shen et al.[161] reported that Tet2 mediates the oxidation of mRNA m^5C. However, in mouse cells, the level of mRNA hm^5C is lower than that of m^5C, and the presence of Tet2 protein does not increase the mRNA hm^5C content. Therefore, unknown proteins may reconvert hm^5C to m^5C,[161] suggesting that m^5C modifications may have a reversible erasure

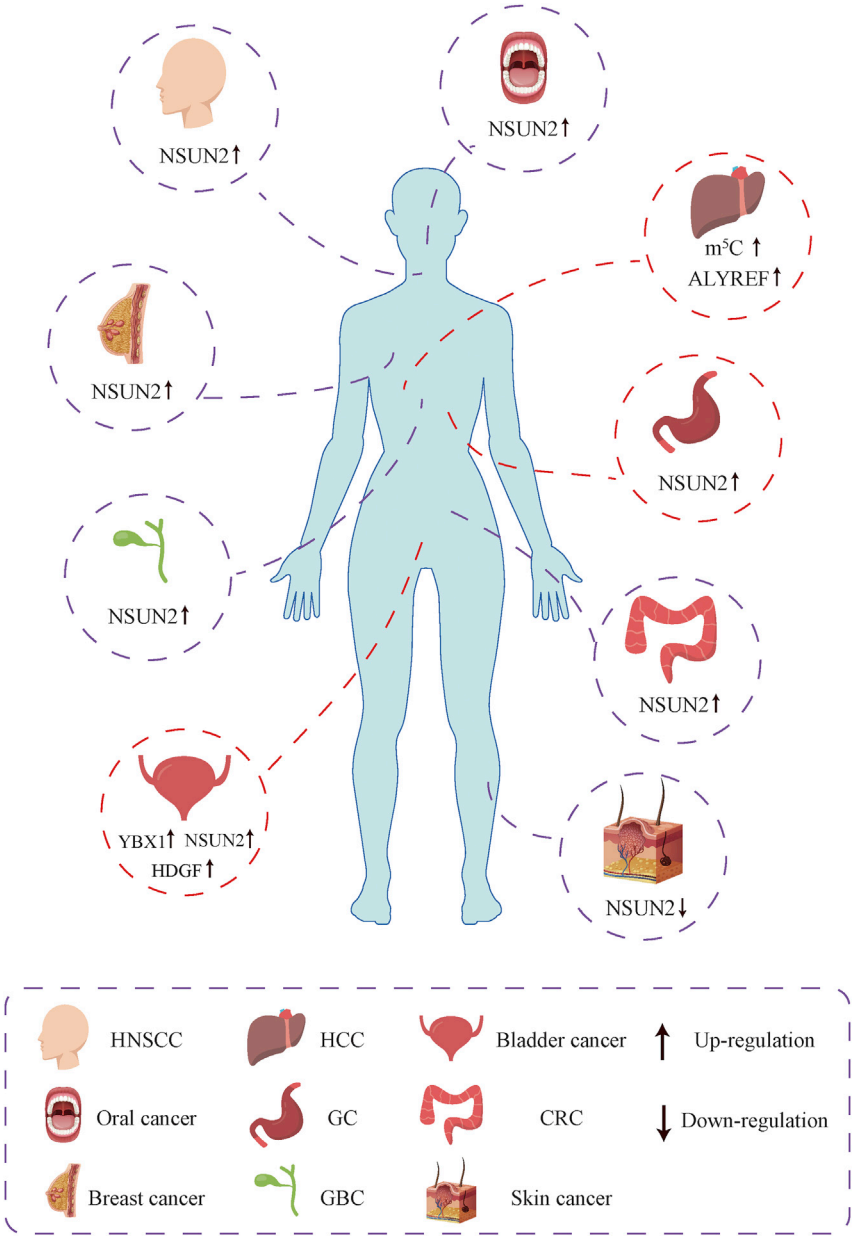

Figure 4. The mRNA m^{5}C modification associations with tumorigenesis and metastasis in multiple cancers

The red dotted line indicates that tumorigenesis and metastasis in multiple cancers are related to m^{5}C modifications, whereas the purple dotted line indicates that the disease correlation with m^{5}C modifications is uncertain.

pathway. Further research is needed to determine whether mRNA m^{5}C modifications are reversible.

mRNA m^{5}C MODIFICATION IN DISEASE

Cancer

Multiple mRNA m^{5}C effectors, which regulate m^{5}C modifications, have been identified as participants in the development and progression of cancer (Figure 4). Specifically, m^{5}C plays an important role in cancer cell proliferation and metastasis, as well as cancer stem cell development, by regulating mRNA stability, expression, and translation (Table 1).[51,141,166,167] Chen et al.[51] showed that YBX1 recognizes m^{5}C-modified mRNA via the W65 indole ring in the cold shock domain (CSD) and recruits ELAVL1 (ELAV-like protein 1) to stabilize HDGF (heparin-binding growth factor) mRNA, ultimately promoting the proliferation and metastasis of bladder cancer cells. Zhang et al.[168] found that the number of mRNA m^{5}C methylation peaks in liver cancer tissues was significantly higher than that in adjacent normal tissues, with significant differences in the distribution of genes in different positions. These results suggest that the development and progression of liver cancer correlate with mRNA m^{5}C modifications. High expression of m^{5}C-related effectors NSUN4 and ALYREF is correlated with poor prognosis of patients with hepatocellular carcinoma (HCC).[167] Although the specific underlying mechanisms remain unclear, these results suggest that m^{5}C modifications are closely related to the development, progression, and prognosis of HCC. Moreover, NSUN2 is reportedly significantly upregulated in gastric cancer tissues compared with levels in adjacent normal tissues and may promote the proliferation of gastric cancer cells in an m^{5}C-dependent manner by inhibiting the expression of $p57^{Kip2}$.[141] Collectively, these studies indicate that mRNA m^{5}C modifications play a role in certain cancers; however, whether m^{5}C is involved in the development and progression of other cancers needs to be investigated.

It should be noted that only a few studies have reported associations between mRNA m^{5}C modifications and cancer, most of which are focused on NSUN2, whereas no evidence has been presented to implicate NSUN2 in cancer development or progression by regulating m^{5}C modification (Figure 4). Gao et al.[162] found that a high expression of NSUN2 can promote the proliferation and tumorigenesis of gallbladder carcinoma cells both *in vitro* and *in vivo* by closely cooperating with ribosomal protein L6. Using immunohistochemistry, Yi et al.[163] reported that the expression of NSUN2 correlates with the clinical stage, tumor classification, and pathological differentiation of breast cancer. Furthermore, the hypomethylation of *NSUN2* led to its overexpression, thus promoting the proliferation, migration, and invasion of breast cancer cells. The upregulation of *NSUN2* has also been correlated with poor prognosis in head and neck squamous

Table 1. Clinical relevance of aberrant m^{5}C modifications

Disease	Effectors	Function	Regulation	Mechanism	Refs.
Bladder cancer	NSUN2	writer	upregulated	NSUN2 targets HDGF m^{5}C modification	51
Gastric cancer	NSUN2	writer	upregulated	NSUN2 promotes cell proliferation by repressing *p57^{Kip2}* expression in an m^{5}C-dependent manner	141
Gallbladder carcinoma	NSUN2	–	upregulated	NSUN2 promotes the proliferation and tumorigenesis cells by close cooperation with ribosomal protein L6 (RPL6)	[162]
Breast cancer	NSUN2	–	upregulated	hypomethylation of *NSUN2* leads to its overexpression	163
Head and neck squamous carcinoma	NSUN2	–	upregulated	–	164
Oral cancer and colorectal cancer	NSUN2	–	upregulated	–	165
Skin cancer	NSUN2	–	downregulation	–	166
Systemic lupus erythematosus	NSUN2	–	downregulation	–	52
Bladder cancer	YBX1	reader	upregulated	YBX1 recognizes m^{5}C-modified mRNA and recruits ELAVL1 to stabilize HDGF mRNA, finally promoting the proliferation and metastasis of bladder cancer cells	51
HCC	ALYREF	–	upregulated	high levels of ALYREF expression are associated with cell-cycle regulation and mitosis	167
Vascular endothelial inflammation	NSUN2	writer	–	NSUN2 promotes the expression of ICAM-1 by upregulating the methylation of ICAM-1 mRNA, thus promoting the development of vascular endothelial inflammation	53

–, unknown function.

carcinoma, oral cancer, and colorectal cancer.[164,165] In contrast, other studies have reported that its deletion in mice enhances the self-renewal potential of tumor-initiating cells, thus promoting the occurrence of skin cancer. Furthermore, the overexpression of NSUN2 in human skin cancer negatively correlates with disease malignancy.[166] Hence, certain questions remain and require further investigation. It is important to determine whether NSNU2 promotes or inhibits cancer development and progression by regulating specific cancer-causing or cancer-inhibiting mRNAs in different cancers. Additionally, validating whether NSUN2 plays a role in cancer development and progression via the regulation of m^{5}C modifications is necessary.

The m^{5}C modification and its effectors, such as NSUN2 and YBX1, are involved in the development and progression of multiple cancers, suggesting new targets for disease treatment. For example, inhibition of sphingosine kinase (SPHK) in breast cancer can reduce the expression of NSUN2, suggesting that SPHK1 is a potential breast cancer marker.[169,170] The YBX1 phosphorylation inhibitors TAS0612 (multi-kinase inhibitor) and everolimus (rapamycin complex 1 inhibitor), as readers, can overcome the resistance to estrogen in progressive breast cancer cells.[171] However, whether the role of YBX1 inhibitors in the treatment of breast cancer is related to the regulation of m^{5}C modifications remains to be elucidated. In particular, whether mRNA m^{5}C-related effectors can be used as markers to predict cancer prognosis and whether their inhibitors, combined with conventional chemotherapy drugs, represent effective anti-cancer strategies, remain to be confirmed.[65,166] Meanwhile, DNA modifications play an important role in regulating the characteristics of cancer stem cells. For instance, inhibitors of regulatory enzymes, such as those of histone deacetylase, e.g., vorinostat, and those of DNMT, e.g., azacitidine, decitabine, and SGI-110, are undergoing drug clinical trials for the treatment of multiple cancers.[172] Therefore, increasing our understanding of mRNA m^{5}C modifications may lead to improving the diagnosis, treatment, and prognosis of cancer.

Autoimmune diseases

Previously, we found that the 5-methylcytosine/cytosine (m^{5}C/C) ratio of CD4$^+$ T cells in patients with systemic lupus erythematosus (SLE) was lower than that in healthy controls. The distribution of m^{5}C modifications in the mRNA of SLE patients and healthy individuals is highly conservative and primarily concentrated near the mRNA translation initiation site. Hypermethylated and upregulated genes in SLE function in the immune system, including cytokine and interferon signaling pathways.[52] In addition, we observed the downregulation of NSUN2 and m^{5}C modifications in SLE patients; however, many genes were hypermethylated in SLE patients, suggesting the presence of other m^{5}C methylases.[52] For instance, IL-17A is an important mediator in many autoimmune diseases.[173] In rat T lymphocytes, NSUN2 promotes the translation of IL-17A by methylating its C466 site;[138] however, it is unclear whether m^{5}C modifications of IL-17A mRNA are involved in immune diseases. The specific mechanisms underlying mRNA m^{5}C modifications in the remission and recurrence of SLE are also not well understood. Further research is needed to address these questions.

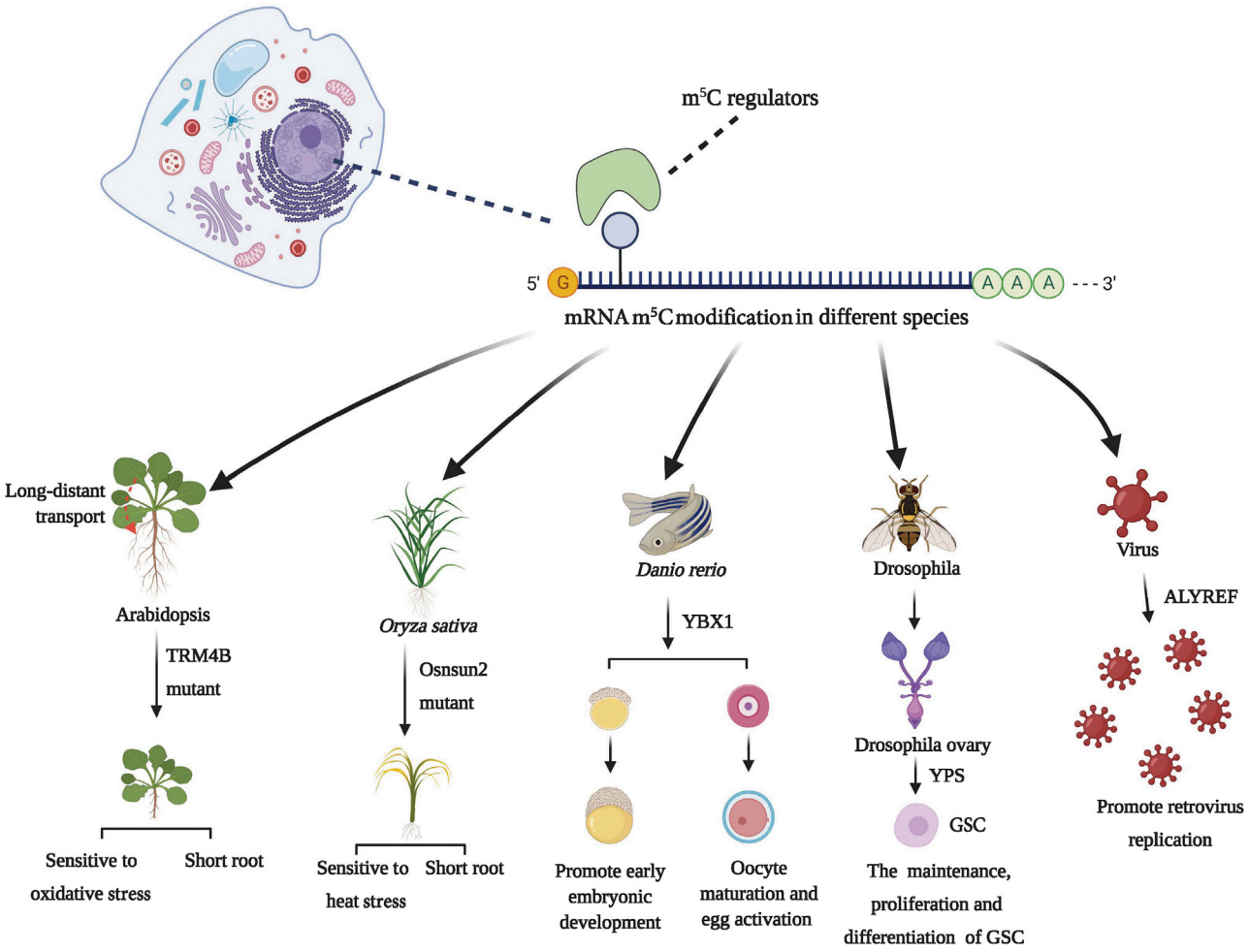

Figure 5. Physiological effects of m^{5}C modifications in different species

Vascular endothelial inflammation

Luo et al.[53] found that NSUN2 promotes the expression of ICAM-1 by upregulating the methylation of ICAM-1 mRNA, thus improving the adhesion between leukocytes and endothelial cells and promoting vascular endothelial inflammation, a key factor in the pathogenesis of multiple vascular diseases, including atherosclerosis, hypertension, restenosis, and ischemia/reperfusion damage.[53,174] Moreover, the lack of donor NSUN2 hindered the formation of allograft arteriosclerosis in a rat model of aortic allograft,[53] suggesting that the NSUN2-ICAM-1 regulatory axis is involved in vascular endothelial inflammation. Besides ICAM-1 mRNA, NSUN2 can also catalyze the methylation of other mRNAs and non-coding RNAs.[102,133] Therefore, the mechanism by which NSUN2 regulates the development and progression of vascular inflammation and atherosclerosis requires further investigation.

Clinical relevance and future directions

The clinical relevance of RNA methylation may potentially become a research hotspot in the coming years. However, current studies on the clinical effects of mRNA m^{5}C modification tend to focus on neoplastic diseases, with few focusing on non-neoplastic diseases (Table 1).[52] Hence, broadening the types of diseases in future studies will advance our understanding of the clinical consequences of m^{5}C modifications. Additionally, mRNA methylation may be a potential biomarker in the progression of specific diseases. Thus, it is essential to develop novel techniques for the rapid identification of modifications that give rise to diseased states and the quantification of the protein levels associated with these modifications.[175] Further exploration of the role of m^{5}C modifications in immune responses will provide a broader prospect for tumor immunotherapy[176] and tumor drug resistance.[177] There have been several companies targeting RNA apparent modifications.[178] The investigation of small-molecule inhibitors that are capable of targeting m^{5}C modification-related effector proteins in the context of disease treatment may be a promising research direction.[179] As the preclinical efficacy of these targeted drugs is revealed, the development of RNA epigenetic drugs will enter a new era.

mRNA m^{5}C MODIFICATIONS IN OTHER SPECIES

Plants

There is evidence for widespread mRNA modifications in plants.[70] Although the PTCMs of mRNA have been reported in *A. thaliana*, *O. sativa*, *Helianthus annuus*, *Triticum aestivum*, *Glycine max*, and *Solanum tuberosum*,[180] most studies on mRNA m^{5}C modifications have been conducted on *A. thaliana*[87] and *O. sativa*[44] (Figure 5).

A. thaliana

In *A. thaliana,* mRNA m^{5}C modification sites differ between tissue types, including seeding shoots, roots, siliques, and rosette leaves.[87,181] The low overlap between these sites indicates that mRNA performs tissue-specific functions through specific gene methylation patterns. Notably, the homologous protein TRM4B of human NSUN2 in *A. thaliana* and the homologous protein *Osnsun2* in *O. sativa* exhibit high sequence conservation. These homologous proteins exist widely in green algae and flowering plants and contain a conserved catalytic domain.[40,44] In addition, a TRM4B mutant has been reported to suppress division in root tip meristem cells, resulting in the short root phenotype of *A. thaliana*.[87] The TRM4B mutation is speculated to affect mRNA stability; i.e., decreased levels of m^{5}C modifications lead to decreased half-lives of *SHY2* and *IAA16*, which are critical for root development.[70] Furthermore, m^{5}C regulates the transport of TCTP1 (translationally controlled tumor protein 1) mRNA between the rootstock and scion in grafted seedlings, thus regulating root development.[181] Not only does m^{5}C influence plant development, but it also plays an important role in regulating environmental adaptation. In fact, its biological function is similar to that of m^{5}C in mammals.[40,166] Compared with wild-type *A. thaliana*, the TRM4B mutant is more sensitive to oxidative stress,[87] the mechanism of which may be related to the loss of tRNA methylation, reducing its stability. However, considering that TRM4B can act on multiple RNAs, whether methylation modifications of mRNA and other types of RNA participate in the oxidative stress response of *A. thaliana*, needs to be confirmed.

O. sativa

Tang et al.[44] reported that the conservation of m^{5}C sites between *O. sativa* and *A. thaliana* is relatively low, indicating that m^{5}C modifications in plants may not be as conserved as those in mammals. However, the distributions of m^{5}C in mRNAs of *A. thaliana* and *O. sativa* are similar, both primarily enriched in the coding sequence CDS located near the initiation and termination codons.[44,70,87] The mutant *Osnsun2* presents with short roots;[44] however, the underlying mechanism remains unclear. *Osnsun2* can also exhibit a heat-sensitive phenotype, as *Osnsun2* primarily plays a role in *O. sativa* resistance to heat damage by selectively methylating mRNAs involved in photosynthesis and detoxification-related processes.[44] Although heat-adaptation mechanisms of *Solanum lycopersicum*,[182] *Citrullus Schrad*,[182] and *Daucus carota*[183] have been reported, it remains to be determined whether mRNA m^{5}C modifications participate in them. Investigating the role of m^{5}C modifications in the environmental tolerance of crops can help to increase yields.

Danio rerio

Recently, Yang et al.[43] reported that the reader YBX1 recognizes m^{5}C-modified mRNA via a π-π interaction between the key residue Trp45 of the CSD and m^{5}C. YBX1 then recruits the poly(A)-binding protein Pabpc1a to maintain the stability of maternal mRNA, thus ensuring maternal-to-zygotic transition and promoting early embryonic development. However, the deletion of YBX1 or Pabpc1a leads to stagnation in the blastula and gastrula stages[43] (Figure 5). Another study in *D. rerio* revealed that YBX1 plays a role in oocyte maturation and egg activation by binding to individual mRNAs to inhibit the translation of related proteins[184] (Figure 5), suggesting that the biological function of YBX1 is far more complicated than simply recognizing and stabilizing m^{5}C-modified mRNA. Moreover, these roles of m^{5}C modifications in the embryonic development of *D. rerio* suggest that they may also be involved in coordinating embryonic development in other species.

D. melanogaster

Ypsilon Schachtel (YPS), a human YBX1 homolog, reportedly promotes the maintenance, proliferation, and differentiation of *D. melanogaster* ovarian germline stem cells (GSCs) by preferentially binding to m^{5}C-modified mRNA (Figure 5), whereas human YBX1 can functionally replace YPS to promote GSC development in the *D. melanogaster* ovary.[45] Considering that the function of YPS/YBX family proteins is highly conserved in *D. melanogaster* and humans, the *D. melanogaster* ovary is an attractive model to analyze the function of these proteins and the role of m^{5}C modifications in germ-cell development.

Viruses

As a post-transcriptional regulator of HIV-1 mRNA splicing and functions, m^{5}C has a direct impact on gene expression.[41] Meanwhile, m^{5}C modifications of murine leukemia virus (MLV) transcripts promote the replication and expression of viral genes.[185] m^{5}C is more common in the genomic RNA of MLV than in the mRNA of uninfected cells, promoting virus replication in an ALYREF reader-dependent manner[186] (Figure 5). Therefore, since m^{5}C regulates viral gene expression, viruses can activate the writers and readers of infected cells (hosts) to increase the expression and replication of their own genes. However, whether epitranscriptomic modifications have a beneficial role in viral life cycles remains controversial, as some studies have demonstrated that the methylation m^{6}A inhibits the replication of HIV-1[187] and Zika virus.[188] Therefore, in-depth investigations are needed to reveal the roles of these RNA modification types. The regulation of m^{5}C modifications during virus replication provides new insights into viral pathology and suggests the potential of m^{5}C methylase inhibitors as antiviral drugs.[189]

FUTURE PROSPECTS AND CONCLUSION

In conclusion, m^{5}C modifications have been detected in various organisms and exert unique biological functions in humans and plants,[44,87,89] with certain common features.[190] It is most abundant in tRNA, rRNA, and mRNA where it affects the RNA stability,[28] translational fidelity,[191] cell differentiation,[192] nervous system regulation, reproductive system development,[193,194] and viral viability.[195]

Therefore, m^5C modifications are important to understand a variety of physiological and pathological processes.

It is necessary to further improve and develop techniques for m^5C detection to clarify whether m^5C modifications actually exist in eukaryotic mRNA or are merely the result of experimental artifacts, as has been suggested by some studies.[196] We expect that TGS methods will improve the direct detection of RNA methylation. It is necessary to determine whether RNA m^5C modifications can be converted into hm^5C and F^5C,[159,160] whether m^5C is only a transient intermediate, and whether the dynamic balance between m^5C and its oxidation products have any functional relevance. Lastly, it is necessary to determine whether additional m^5C effector proteins exist and to investigate their potential mechanisms in disease to advance the current understanding. In summary, research related to the many mRNA m^5C modification-related enzymes and their biological functions is in its infancy, with countless significant discoveries left to be made.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.08.020.

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AUTHOR CONTRIBUTIONS

G.G., X.X., and H.Z. conceived the manuscript. G.G., K.P., S.F., and L.Y. drafted the manuscript. G.G., K.P., S.F., L.Y., X.T., Z.W., H.Z., and X.X. participated in the literature search, discussions, and manuscript revisions. K.P. and S.F. created figures. All authors read and approved the final manuscript for publication.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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