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Post-transcriptional methylation of transfer and ribosomal RNA in stress response pathways, cell differentiation and cancer

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Abstract

Purpose of the review—Significant advances have been made in understanding the functional roles of evolutionary conserved chemical modifications in RNA. By focusing on cytosine-5 methylation, we will highlight the latest insight into the mechanisms how post-transcriptional methylation contributes cell fate decisions, with implications for cancer development.

Recent findings—Several mutations in RNA-modifying enzymes have been identified to cause complex human diseases, and linked post-transcriptional modifications to fundamental cellular processes. Distinct post-transcriptional modifications are implicated in the regulation of stem cell maintenance and cellular differentiation. The dynamic deposition of a methyl mark into non-coding RNAs modulates the adaptive cellular responses to stress and alterations of methylation levels may lead to cancer.

Keywords

Cytosine-5 methylation (m⁵C); NSun proteins; cellular stress response; non-coding RNA; stem cells; cancer

Introduction

Homeostasis in most mammalian tissues is achieved by stem cells that continuously maintain their population (self-renewal) while generating numerous differentiated cells. During self-renewal stem cells have to avoid cell cycle exit and differentiation; whereas during differentiation stem cells must evade uncontrolled proliferation. The unique properties of stem cells are controlled by a dynamic interplay between extrinsic and intrinsic regulatory mechanisms. While extrinsic mechanisms include signalling from the neighbouring niche cells, intrinsic mechanism include epigenetic, transcriptional and translational pathways. The transcriptional regulation of stem cells is now increasingly understood in both embryonic and adult tissues, yet the precise roles of post-transcriptional mechanisms in regulating stem cell maintenance and differentiation remain unclear.

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Conflicts of interest

There are no conflicts of interest.

Best-characterized is the role of the microRNA pathways in regulating stem cells self-renewal and differentiation, and the importance of specific microRNAs in repressing selected mRNAs in stem and differentiated daughter cells has been shown in embryonic stem cells, germline stem cells and somatic tissue stem cells [1]. The overall function of the microRNA pathways has been studied by using for instance DGCR8 and Dicer knockout mouse embryonic stem (ES) cells. Both proteins are essential for the production mature microRNAs. Interestingly, in both cases the knockout ES cells are viable but exhibit severe growth and differentiation defects [2, 3]. However, all cellular RNAs are subject to processing, quality control and surveillance pathways and at each step RNA is dynamically associated with RNA-binding proteins. Post-transcriptional modifications have emerged as important determinants for the dynamic and temporal accurate binding of proteins to their targeted RNA molecules.

Post-transcriptional methylation in multicellular organisms

More than one hundred distinct chemical modifications have been found in eukaryotic RNAs [4]. However, in multicellular organisms the enzymes responsible for each of the modification as well as the biological roles of these modifications remain unclear. Methylation is one of the most common enzyme-catalyzed modifications [4, 5]. The majority of the methyl-based modifications are conserved from bacteria to mammals and plants, and their functions include structural and metabolic stabilization as well as functional roles in regulating protein translation [6-9]. The existence of methylated nucleosides in RNA including 5-methylcytidine (m^5C) and N^6 -methyladenosine (m^6A) in non-coding and coding RNA has been described decades ago, but their precise location, abundance and cellular functions have until recently remained controversial [10-15].

New approaches to capture m^5C and m^6A modifications transcriptome-wide confirmed dynamic and conserved methylation throughout the mammalian transcriptome [16-22]. In only a couple of years it has become evident that post-transcriptional methylation of both nucleosides regulate a wide range of fundamental cellular processes and that loss-of-function mutations in methylating and demethylating enzymes are cause of severe human diseases, including neurological disorders [23-25]. The m^6A modification is most prevalent in messenger RNAs (mRNA) and has been extensively reviewed elsewhere [24, 26-29]. Therefore, we will focus on the functional and biological roles of m^5C which is mainly found in non-coding RNAs [15].

Cytosine-5 methylation enzymes

Post-transcriptional cytosine-5 methylation is a widespread mark in the transcriptome of multicellular organisms [19]. All RNA: m^5C methyltransferases contain a catalytic domain with a common structural core and the S-Adenosyl methionine (AdoMet) binding site. Two conserved cysteines, both located within a sequence with similarity to a methyltransferase active site are required for the transfer of the methyl group [30]. To initiate methylation, the catalytic cysteine residue forms a covalent bond with the cytosine pyrimidine ring [31]. The second conserved cysteine residue is then required to break the covalent adduct and releases the methylated RNA and the enzyme [30, 32].

The first confirmed RNA:m⁵C methyltransferases in multicellular organisms were the DNA methyltransferase homolog Dnmt2 and the NOP2/Sun domain RNA methyltransferase family member NSun2, both of which were found to methylate transfer RNA (tRNA) [33, 34]. In the last couple of years three more NSun family members, namely NSun4, 5 and 6 have been described to methylate mitochondrial and cytoplasmic ribosomal RNA (rRNA) and tRNA respectively (Fig. 1A) [9, 35-38]. m⁵C is predicted to be deposited by at least three more RNA:m⁵C methyltransferases (NSun 1, 3 and 7), yet their substrate specificity remains to be confirmed in mammals.

One common methylation target of Dnmt2, NSun2 and NSun6 is nuclear encoded tRNA. However, the deposition of the methyl mark by these three enzymes takes place in a strict site-specific manner. While Dnmt2 targets the anti-codon loop (C38) of aspartate, valine and glycine tRNAs [33, 39], NSun2 methylates cytosines located to the variable loop (C48-50) of most transcribed tRNAs [34, 39, 40]. NSun6 targets the 3' end of the tRNA acceptor stem (C72) of cysteine and threonine tRNAs [35]. In contrast to Dnmt2 and NSun6, NSun2 additionally methylates other non-coding RNAs such as vault RNAs [15, 21]. The only confirmed RNA target of NSun4 is the mitochondrially encoded ribosomal RNA 12S [38]. NSun1 and 5 have also been linked to rRNA. NSun1 (Nop2/p120) orthologs in yeast and *Arabidopsis* have been suggested to methylate 25S rRNA [9, 41-43]. Worm, plant, fly and yeast homologs of NSun5 methylate 28S/25S rRNA in a region located in close proximity to the peptidyltransferase centre [9, 36, 41, 42]. Human NSun5 co-precipitates with ribosomes suggesting that 28S RNA may be a conserved substrate of NSun5 in mammals [44]. The substrate specificities of NSun3 and 7 are unknown. In contrast to m⁵C on DNA or N⁶-methyladenosine in mRNA, an enzyme that removes 5-methylcytosine from RNA has yet to be identified.

Functional roles of 5-methylcytidine

The functional consequences for Dnmt2- and NSun2-mediated methylation of tRNAs are now well-understood. Deposition of m⁵C by both enzymes protects tRNAs from endonucleolytic cleavage [39, 40, 45]. Lack of m⁵C at the tRNA variable loop in the absence of NSun2 increases the affinity of the endonuclease angiogenin (Fig. 1B). Angiogenin cleaves the tRNA into half and the small non-coding RNAs derived from 5' end of the tRNAs accumulate, whereas the 3' tRNA fragments degrade in the cells [40]. The molecular function of 5' tRNA fragments is to repress cap-dependent translation by displacing translation initiation and elongation factors from mRNAs or by interfering with efficient transpeptidation [46-49]. Accordingly, global protein synthesis is reduced in the absence of NSun2 *in vitro* and *in vivo* [40]. In contrast, loss of Dnmt2-mediated methylation of tRNA Asp^{GTC}, Gly^{GCC}, and Val^{AAC} at C38 causes tRNA-specific fragmentation patterns which leads to specific codon mistranslation [50]. Cytosine-5 methylation of vault non-coding RNAs by NSun2 alters their processing into Argonaute-associated small RNA fragments that can function as microRNAs and regulate expression of targeted mRNAs [18].

Cytosine-5 methylation of rRNA is fundamental for ribosome biogenesis and therefore, influences protein synthesis. Loss of NSun4-mediated 12S rRNA methylation affects ribosomal subunits assembly and leads to a strong inhibition of mitochondrial translation

[38, 51, 52]. The molecular role NSun1 and NSun5 mediated methylation in multicellular eukaryotes is unknown, but work in yeasts showed a key role in ribosome assembly and translational regulation. Loss of NSun1 yeast homolog Nop2 causes defects in pre-rRNA processing, 60S biogenesis and a significant reduction in polysome assembly [41, 43, 53, 54]. In contrast, loss of NSun5 yeast homolog Rcm1 does not affect polysome assembly, but it relaxes the 25S rRNA structure, which alters the translation fidelity only under certain stress conditions [36, 41].

The correct deposition of m⁵C into both tRNA and rRNA is essential for normal development. Consistent with NSun4's essential mitochondrial function, its deletion in mice is embryonic lethal [38]. NSun2 is highest expressed in brain, skin and testis, and deletion of NSun2 impairs the development of all three tissues. In brain, loss-of-function of NSun2 causes neuro-developmental deficits (i.e. microcephaly) in mouse and human [40, 55-58]. Morphologically brain, skin and testis show significant delays in cell differentiation during development [39, 59, 60]. In skin, NSun2 expression is restricted to a distinct progenitor population in the hair follicle, and loss of NSun2 causes delays the activation of stem cells causing a delay in differentiation of hair lineages [59].

Morpholino-mediated loss of Dnmt2 in zebrafish reduced the size of the morphants by half and specifically affected liver, retina and brain development due to a failure to conduct late differentiation [61]. Deletion of Dnmt2 in mice is not lethal but endochondral ossification is delayed in newborn Dnmt2-deficient mice, which is accompanied by a reduction of the haematopoietic stem and progenitor cell population and a cell-autonomous defect in their differentiation [50].

Together, cytosine-5 RNA methylation pathways play a diverse role during development, yet they all converge in their function to ensure efficient and correct protein translation, a function that seems to be dispensable for tissue stem cell self-renewal but required for accurate lineage commitment and differentiation.

Cytosine-5 methylation pathways and the cellular stress response

Post-transcriptional control is not only important to direct the proper steps during RNA metabolism pathways, but also plays a prominent role in modulating rapid cellular responses to environmental changes [62, 63]. Deletion of m⁵C methyltransferases in yeast, flies, worms and mice is usually not lethal [33, 36, 41, 54, 59, 64, 65]. However, in virtually all cases, the level of m⁵C modification plays a key role in regulating the cellular response to stress-stimuli, including drugs, DNA damage, oxidative stress or environmental cues [40, 45, 64, 66].

Angiogenin-mediated tRNA cleavage is a conserved response to oxidative stress in eukaryotes [67-69]. Several lines of evidence suggest that angiogenin-mediated stress response is directly controlled by tRNA modifications. Levels of m⁵C in tRNAs change in response to oxidative stress in yeast [70]. Loss of m⁵C through deletion of Dnmt2 or NSun2 increases stress-induced cleavage of tRNAs and sensitizes flies, mice and human cells to oxidative stress *in vivo* [40, 45]. Stress-induced cleaved 5' tRNA fragments inhibit protein synthesis by displacing translation initiation factors from mRNAs [46, 71]. The reduction of

global protein synthesis is an integral part of stress responses to allow cells to alleviate cellular injury or alternatively induce apoptosis [72]. Interestingly, 5' tRNA fragments are sufficient and required to trigger cellular stress responses, and induce the apoptotic pathway in the absence of NSun2 [40]. Thus, cellular survival during the oxidative stress response may directly require the “*re-methylation*” of tRNAs to ensure translational fidelity and relieve stress-induced translational bias [50, 73, 74].

Stress-induced reprogramming of the translational machinery might be a conserved mechanism. For instance changes in m⁵C levels in Leu^{CAA} tRNA or 25S rRNA cause selective translation towards specific transcripts during stress responses in yeast [36, 74, 75]. While loss of m⁵C in tRNA sensitizes yeast, flies, mice and human cells to stress, loss of NSun5-mediated rRNA methylation increases resistance to stress stimuli and the lifespan in yeast, worms and flies [36, 40]. Ribosomes are able to modulate their translation capacity in response to various environmental stimuli by differential expression of ribosomal building blocks but one can also hypothesize that post-transcriptional modifications of rRNAs such as NSun5-mediated methylation is required to shape the ribosome in rapid responses to stress stimuli [36]. For instance ribosomes that lack pseudouridine modifications show a direct impairment in binding to internal ribosome entry (IRES) elements [73]. IRES is an RNA structured element positioned at the 5' untranslated region (UTR) of specific mRNAs that can recruit the ribosome directly to the initiation region of mRNAs with a reduced requirement for canonical initiation factors [53], and more than 10% of cellular mRNAs rely on cap-independent mechanisms which are used in response to stress stimuli [54]. Ribosomal profiling has recently uncovered enriched ribosomal occupancy in many novel upstream open reading frames (uORFs), a shift in the translation machinery that appears in stress responses [20, 52, 76]. How this shift of ribosomal occupancy is regulated is still poorly understood. Manipulating the levels of post-transcriptional modifications in rRNA may allow cells to quickly adapt and “specialize” their ribosomes, a paradigm that is emerging for heterogeneity in ribosomes as a mechanism for stress adaptation in bacteria [77].

Implications for cytosine-5 methylation pathways in cancer

The rate of tRNA synthesis is regulated by both oncogenes and tumours suppressors and tRNA levels can drive changes in mRNA translation and cell growth [78]. Besides their canonical function as decoders of the genomic code, tRNAs emerged as regulators of cell signaling through tRNA-derived small non-coding RNAs [79]. Differential abundance of small non-coding RNAs derived from cleaved tRNAs has been described for human breast and prostate cancer [80-82]. Elevated protein synthesis and perturbations in several components of the translational apparatus have been linked to increased cancer susceptibility [83].

The precise role of cytosine-5 methylation and their respective RNA methylases in cancer development, progression and metastasis is currently unclear. Dnmt2 is upregulated in multiple tumour samples [84]. Some of the 61 somatic mutations found in Dnmt2 in tumours originating from different tissues strongly modulated the enzymatic activity of Dnmt2, indicating a potential role in cancer [85]. Inhibition of Dnmt2 decreased the metabolic

activity of human cancer cell lines [86]. NSun1 (also called NOP2 or p120) regulates cell cycle progression in tumorigenesis [53, 87-91]. Whether and how the enzymatic activity of NSun1 is required to mediate these cellular processes remains unclear.

NSun2 was originally identified as a transcriptional target gene for the oncogene c-Myc [92]. Genomic gain of 5p15 that includes the *NSUN2* gene can result in high expression NSun2 in a range of epithelial tumours [93]. However, genomic gain of *NSUN2* is not indicative of tumour malignancy and although growth of human squamous-cell-carcinoma xenografts into host mice is decreased when NSun2 is inhibited by RNAi, deletion of NSun2 in cancer cell lines does not affect proliferation or cell cycle progression [92, 93]. These opposing effects may rather reflect a role for NSun2 in stress response pathways rather than proliferation, and expression of NSun2 may increase survival of tumour cells. In fact recent evidence showed that combined knockdown of NSun2 and the tRNA methyltransferase Mett1l in HeLa cells potentiated the sensitivity of cells to 5-Fluorouracil [66]. Whether NSun2's role in tumorigenesis is entirely mediated through its methylating activity is currently unknown. Considering that NSun2 methylates about 80% of all expressed tRNAs and that lack of NSun2-mediated methylation causes tRNA cleavage resulting in a strong reduction of global protein synthesis [40], the main role of NSun2 downstream of c-Myc may be the coordination of protein synthesis. Myc plays an important role in regulating protein translation through transcriptional regulation of RNA and protein components of ribosomes and translation initiation factors [94, 95].

Conclusions

The role of post-transcriptional RNA methylation is far from being fully understood, but a consensus on a role in fine-tuning of protein synthesis to regulate stem cell function and differentiation is emerging. Since the vast majority of cellular non-coding RNA is involved in translation, it is not surprising that post-transcriptional modifications of RNA provide means for fast regulation of cellular protein content when necessary, for example in response to a changing microenvironment.

RNA methylation has been extensively studied in yeast, however many orthologs with a known function in yeast have not been identified in mammalian cells yet. Since many mechanisms, for instance translation initiation are considerably more complex in mammals than in yeast, novel genetically modified cells and mouse models are needed to investigate the function of RNA methyltransferases whose targets have not yet been established. Furthermore, the level of protein redundancy in RNA methylation pathways is unknown and depletion of a single RNA methylase may not be sufficient to comprehensively understand their roles in translation processes.

Recent studies revealed that modulation of RNA methylation alters cellular function and deregulation of these pathways can lead to complex diseases. However, more investigation is required to establish which molecular mechanisms are involved in mediating RNA methylation and what are the direct consequences of impaired RNA methylation pathways for instance in response to stress *in vitro* and *in vivo*. Understanding the precise molecular pathways of RNA methylation and the pathological consequences of impaired RNA

methylation will allow us to develop novel therapeutic strategies for human diseases and cancer. One notable success is the development of the Mammostrat® test that uses the tRNA methyltransferase 2 homolog A (Trmt2A) as a biomarker to categorize breast cancer patient into distinct risk groups [96].

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Summary

Post-transcriptional modifications such as cytosine-5 methylation are dynamically regulated and may influence tumour development, maintenance and progression.

Key Points

- The functional roles of post-transcriptional methylation in regulating RNA functions are complex but converge in modulating protein synthesis in response to stress stimuli.
- Distinct post-transcriptional modifications are dispensable for stem cell self-renewal but implicated in the accurate temporal and spatial activation of cellular differentiation programs.
- Aberrant deposition of a methyl mark into non-coding RNAs impairs the adaptive cellular response and differentiation pathways and may contribute to cancer development, growth and resistance.

Figure 1.

(A) Cytosine-5 methylation is placed on tRNAs by the site-specific RNA methyltransferases Dnmt2, Nsun2 and Nsun6; on vault RNA by Nsun2; on mrRNA by Nsun4 and on rRNA by Nsun1 and Nsun5. (B) NSun2 and Dnmt2 tRNA methylation prevents from cleavage by angiogenin. In the absence of Nsun2- and Dnmt2-mediated tRNA methylation, tRNAs are cleaved and 5' fragments accumulate, which results in repression of protein synthesis.

tRNA: transfer RNA; mrRNA: mitochondrially transcribed ribosomal RNA; rRNA: nuclear transcribed RNA. Arrows indicate RNAs shuffling in and out of the nucleus.