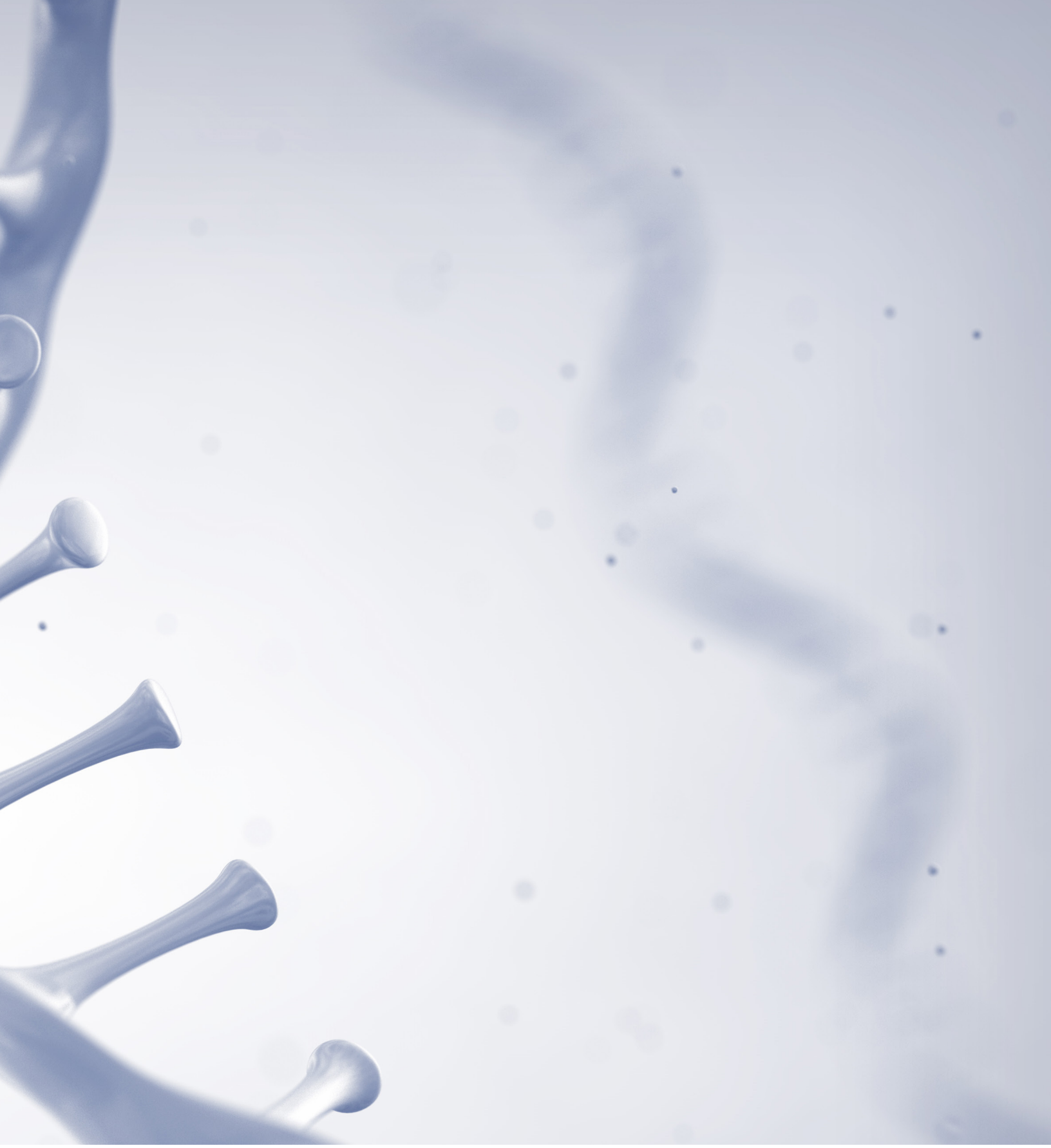


RNA and 5-Methylcytosine

Epitranscriptomics

GLEN RESEARCH

part of Maravai LifeSciences

Introduction

Glen Research provides RNA nucleoside phosphoramidites for the synthesis of RNA oligonucleotides containing rare RNA bases positioned in sequence context. Minor RNA phosphoramidites are available with 2'- TBDMS or 2'-TOM protecting groups. 5-methyl-U and 5-methyl-C monomers containing 2'-methoxy are also available. These relatively rare, or minor, RNA base modifications are increasingly found to be post-translational sequence- and context-specific modifications of RNA polynucleotides. Among these rare RNA modifications, over a hundred have been described, and, armed with rapid sequencing and improved analytical methods, researchers have begun to elucidate their biologic roles.

This upsurge in RNA research will be briefly introduced below. For example these advances have rendered the minor RNA nucleotide 5-methylcytosine (m5C) readily detectable using next-generation sequencing (NGS). This note explores RNA transformations affecting the presence of 5-methylcytidine (m5C) on RNA, shown as a methyl group on an RNA cytidine residue in Figure 1. This note also examines a recent publication where, using a glioblastoma cell line, cytosine methylation of miRNA abolishes the tumor suppressor function of miRNA-181a-5p, and the underlying mechanism of cytosine methylation in these cells is revealed. These results may impact glioblastoma multiforme (GBM) patients since the loss of the tumor suppressor function of miRNA-181a-5p is associated with a poor prognosis.

Background

The term epigenetics, at present, refers to changes in gene expression or cellular phenotype caused by mechanisms other than changes in the genomic DNA sequence. Most researchers are somewhat familiar with epigenetically heritable phenotypes and mechanisms controlling gene expression that involve DNA methylation and histone modification. However, less well-appreciated is the emerging research on various RNA polynucleotides that are also subject to epigenetic modification that can significantly affect gene expression. Like DNA epigenetics, we are learning that some RNA modifications are not random, and instead, are sequence specific, dynamically controlled, reversible, and impact gene expression; it remains an intriguing question the degree to which RNA epigenetics are heritable.

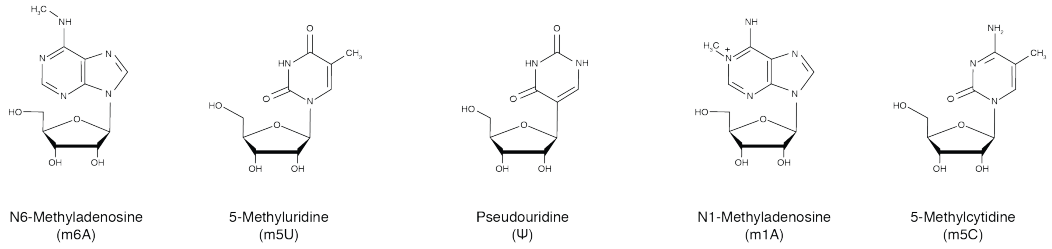


Figure 1. Important RNA epigenetic modifications

The idea that post-transcriptional modification of RNA could impose information on top of the sequence information contained in the RNA, analogous to epigenetic information for DNA and chromatin, prompted coining of the terms "RNA epigenetics"[1] and "epitranscriptomics".[2] Most of the 171 modified RNA bases described in the scientific literature are rare and of unknown functional importance.[3] It is now recognized that the presence of certain RNA modifications has profound effects on RNA stability, molecular recognition and regulation of gene expression. Several of the rare modifications with known functions include pseudouridine (ψ), N6-methyladenosine (m6A) and 5-methylcytidine (m5C) (Figure 1).

Pseudouridine is long-known to be present in both tRNA and rRNA and is highly conserved in the prokaryotic and eukaryotic kingdoms. In addition, it is irreversibly introduced into mammalian rRNA via conserved ribonucleoprotein complexes and, recently, it is now recognized as an important component involved in host immune response to pathogens, a property now being incorporated in vaccine and therapeutic mRNA development.[4]

N6-methyladenosine is the most abundant, reversible, internal modification occurring in eukaryotic mRNA. This methyl modification is now known to be managed in vivo by mammalian m6A writers and erasers that install and remove the methyl group, respectively, and reader proteins that bind to m6A, at defined sequence motifs.[5] In contrast, while the addition of a methyl group to uridine yielding 5mU is a known RNA modification, this transformation has been discovered only very recently in eukaryotic mRNA and the details of its maintenance and role remain to be revealed.[6] The N1-methyladenosine methyl group installs a positive charge to this base, significantly disrupting Watson-Crick base pairing with a corresponding nucleic acid strand.

Another ‘minor’ RNA modification is 2’-O-methylation on the ribose ring. The position of this O-methyl group within the mRNA sequence is key to the increased stability and translational efficiency of 5’-capped mRNA structures. However this modification has also recently been confirmed at single base resolution in other locations in mRNA and snRNA.[7] Details of this modification and its biologic implications are outside the scope of this note, except to note that Glen Research has available for researchers 2'-OMe-m5U Phosphoramidite and 2'-OMe-m5C Phosphoramidite.

The distribution of 5-methylcytidine differs among different organisms. For example, it has been common to find m5C methylation substrates in the tRNA and mRNA of eukaryotes and archaea, while no m5C methylation substrates have been detected in bacteria.[8] Detection of sequence specific RNA methylation to yield m5C is now revealing a role in transcriptional regulation. And in addition to rRNA, m5C has also been detected in long non-coding RNA (lncRNA) and smaller non-coding RNAs, for example enhancer associated RNA (eRNA), vault RNA (vtRNA) and microRNA (miRNA).

Methyl Group Maintenance in RNA

The RNA epitranscriptome is now recognized to contain a dynamic layer of information, controlled largely by the enzymatic activities of methyltransferases, demethylases and, for pseudo-U, the ψ synthases. The methyl group writers, erasers and reader protein systems modify m1A, m6A and m5C. The m6A methylations of mRNA affect mRNA identification by YTH reader proteins that are involved in stem cell differentiation or with distinct subsets of m6A sites with differing expression outcomes.[9] A majority of current knowledge about m5C in RNA comes from research on the abundant rRNAs and tRNAs, but this is changing as new methods of studying these rare modifications are developed.

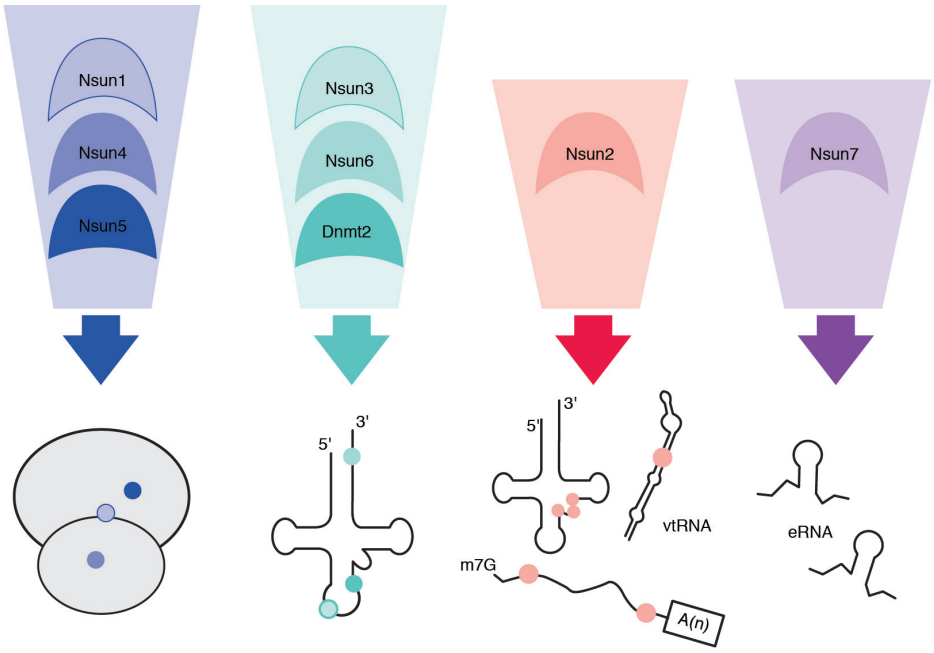


Figure 2. Summary of eukaryotic RMCT activities

The methylation of cytosines in RNA utilize the cofactor S-adenosyl-L-methionine (SAM) as methyl group donor and one of the RNA m5C methyltransferases (RCMT). RCMTs with confirmed in vivo activity belong to either the DNMT2 or the NOL1/NOP2/sun (Nsun family) subgroups of methyltransferases. In mammals, the Nsun family of enzymes comprises seven genes that include Nsun1 through Nsun7.

A great deal is known about the roles of these methyltransferases and their RNA substrates involvement in regulation of cellular processes (Figure 2). For example, the RCMT group (1) of NSUN1, 4 and 5 is implicated in methylations associated with ribosome assembly and translational fidelity. Group (2) of NSUN3, NSUN6 and DNMT2 catalyze methylations conserved in certain tRNA molecules. In contrast, NSUN2 modifies a different region of the tRNA molecule, and also the vault RNAs and key locations on mRNA. NSUN7 is involved in methylation of eRNAs, the recently discovered non-coding enhancer RNAs that appear to play a role in transcriptional regulation. A comprehensive discussion is outside the scope of this note. However it is noteworthy to our discussion below that little is known about methylation of small RNA and microRNA.

RNA demethylation through enzymatic oxidation of methyl groups uses molecular oxygen and alpha-ketoglutarate cofactor. Demethylations of m6A occur through fat-mass and obesity-associated protein (FTO) enzyme action[10] or ALKBH5 demethylase, depending on cellular context.[11] The ALKBH3 oxidizes the m1A methyl group and its activity responds to various types of cellular stress.[12] 5-methylcytosine and m5C in DNA and RNA, respectively, can be oxidized by Tet-family dioxygenase enzymes to 5-hydroxymethylcytosine (hm5C).[13] Below we discuss m5C detection and recent methodology that is applicable to high throughput NGS.

Research on proteins that “read” methyl marks on RNA is at a very early stage. The regulatory roles of m6A are mediated by YT521-B homology (YTH) domain-containing proteins. It was recently found that the activity of the nuclear export factor ALYREF/THOC4 is strongly affected by the methylation status in CG sequence context near the translational start codon of its target mRNAs. Along with several lines of evidence ALYREF appears to be a reader protein involved in mRNA export from the nucleus. For other newly-mapped mRNA modifications besides m6A, identification of their writer, eraser, and reader proteins would help us understand their biological functions. The discovery of m6A readers relied on the RNA pull-down assay using synthetic RNA oligonucleotides containing m6A and biotin, synthetic modifications available from Glen Research.[14] Such methods are likely to be useful to identify readers for m1A, m5C and others.

Advances in Detection of m5C

Until recently detection of the sequence context of methylcytosine in RNA involved using antibody or specific protein binding to fragmented RNA followed by immunoprecipitation (IP) or otherwise collection of the bound RNA complexes, then the RNA is released for sequencing. The most-used methods are methylated-RNA-immunoprecipitation (meRIP-seq) and aza-IP, where 5-azacytidine (5-azaC) is incorporated into RNA by feeding cells with the modified nucleoside; in this way an RCMT may be covalently trapped at an RCMT target site, allowing IP using antibody against the RCMT. Another IP method, methylation-individual nucleotide resolution crosslinking and immunoprecipitation

(miCLIP), is based on the use of a deletion mutant RCMT that lacks a cysteine essential for release of the covalently bound RNA substrate. Reverse transcription of the captured RNA terminates at the position of the bound enzyme-RNA complex allowing the m5C position to be identified.[15] These are cumbersome, highly technical methods with various limitations.

Bisulfite sequencing of DNA is widely used; the bisulfite anion $HSO3^-$ reacts exclusively with the cytosine carbon-carbon double bond at acidic pH resulting in deamination and formation of uracil-6-sulfonate adduct which converts to uracil at alkaline pH. The resulting C-to-U conversion is detected by sequencing the untreated and bisulfite treated samples, revealing which U positions in the treated sample were originally U or C.

In principle, the bisulfite reaction can also occur on m5C, however, in practice, the chemistry of the bisulfite reaction with m5C is nearly two orders of magnitude slower than with cytosine base, likely due to steric and electronic factors. The harsh reaction conditions used in the DNA bisulfite protocol include denaturation at 95°C and an alkaline pH, precluding use of standard bisulfite protocols with RNA.

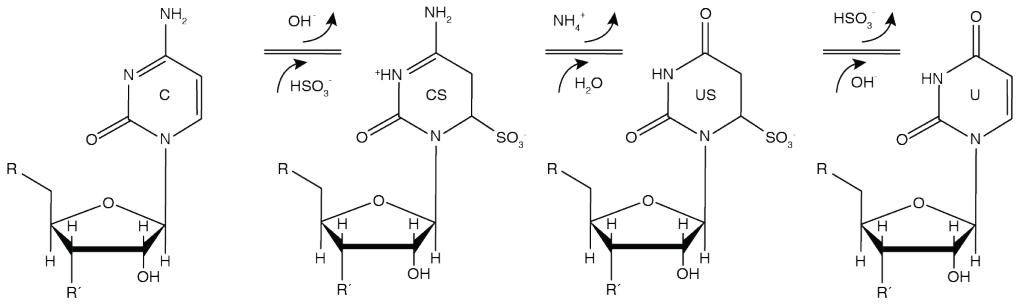


Figure 3. Bisulfite sequencing chemistry for RNA

Schaefer et al. reasoned that conditions optimized for efficient cytosine to uracil conversion and that were compatible with RNA would allow identification of m5C within the RNA sequence since m5C would be unmodified by bisulfite treatment (Figure 3).[16] Using a commercial EpiTect® Bisulfite Kit designed for use with DNA and containing a buffer to maintain pH control, the conditions of bisulfite treatment of RNA were investigated. By lowering denaturation temperature to 60°C and extending bisulfite incubation to 180 min, complete conversion of C to U occurs with negligible RNA degradation/ m5C deamination.

The Schaefer team developed a stem-loop RT primer-based method coupled with use of a reverse-primer that hybridizes to the deaminated RNA allowing generation of PCR amplicon. The method, and amplicon cloning and sequencing was applied to tRNAs and rRNAs to demonstrate that m5C is reproducibly and quantitatively detected. This methodology has proven to be a breakthrough for quantitative detection of m5C.

Cytosine Methylation of MicroRNA (miRNA)

Soon researchers began using the bisulfite methodology. Squires et al. identified widespread m5C in both coding and non-coding RNA.[17] Using HeLa cells, these researchers confirmed 21 of the 28 previously known m5C sites in human tRNAs and identified 234 novel tRNA candidate sites. However, most surprising was the discovery of 10,275 sites containing m5C in mRNAs and in non-coding RNAs. The mRNAs contained elevated m5C in untranslated regions and near Argonaute binding regions. By carrying out RNAi-mediated knockdown of the RNA methyltransferases TRDMT1 and NSUN2, five new sites were determined to be modified by NSUN2, these include another tRNA, a subunit of RNase P and two mRNAs. In their screen they searched for m5C site distribution within mRNAs; 83% of candidate sites identified were in mRNAs, and there was significant enrichment within the 5'- and 3'-untranslated regions and much less enrichment within coding regions. The authors concluded that results were "highly suggestive of an involvement of cytosine modification in post-transcriptional gene regulation."

RNA Methylation and Disease

The RNA subclass of short noncoding microRNAs (19-24nt) are important regulatory molecules and only recently were discovered to contain methylated RNA bases. Early reports of miRNA N6-adenosine methylation[18] have been followed by reports of guanosine methylation that promotes miRNA processing[19], and also that the specific miRNA-175p can be cytosine methylated and the methylated form may be useful as a biomarker for pancreatic cancer.[20]

The Cheray lab was interested in understanding the molecular mechanisms involved in cytosine methylation of miRNAs and chose to study cytosine methylation in miRNAs in glioblastoma multiforme (GBM) cells and tumor samples.[21] Synthetic miRNAs containing m5C and unmethylated control RNA oligos used for this work were synthesized by a commercial source.

Initially, the researchers sought to determine m5C's presence in the miRNAs within glioblastoma cell lines. Thus, U87 cells were cultured, the miRNA was extracted, and the m5C contents were analyzed using a combination of methods; 1) miRNA migration in 5% agarose gel electrophoresis, 2) HPLC of 5-methylcytosine (m5C) in miRNA 3) dot blot using m5C antibody, 4) quantification by an ELISA method[22], and 5) miRNA bisulfite sequencing and miRNA array analysis of anti-5-methylcytosine antibody-mediated miRNA immunoprecipitation 6) followed up by array analysis of methyl group enriched miRNA in three additional glioblastoma cell lines: T98G, U118 and LN229. The results of electrophoresis, HPLC, quantitation by dot blot and ELISA essentially confirmed the presence of m5C in the isolated U87 glioblastoma miRNA.

In order to identify the specific methylated miRNAs present, two independent sequencing approaches were compared. First, sequencing was carried out using the NGS bisulfite method and second, miRNA containing samples were immunoprecipitated using m5C antibody and subjected to miR array sequencing[23]. The result was the detection of 12 and 13 miRNAs, respectively (Figure 4).

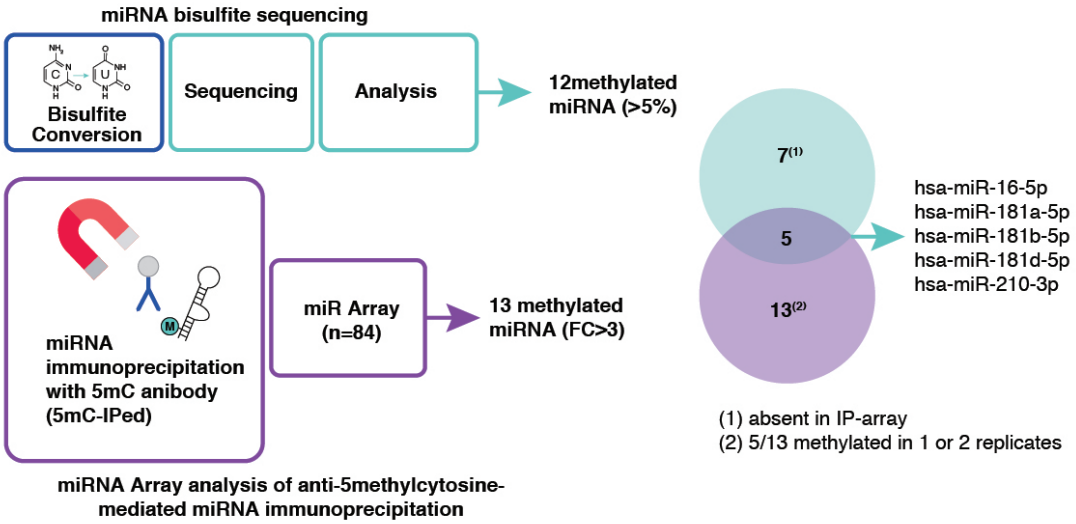


Figure 4 Sequencing via bisulfite and array methods

Considering these results further, five miRNA sequences containing m5C were found in common to both sequenced sets. These 5 highest methylated miRNAs were miRNA16-5p (24%), hsa-miR-29c-3p (11%), hsa-miR-210-3p (9%), hsamiRNA-181a-5p (9%), and hsa-miRNA-339-3p (9%).

In a follow-up experiment using 3 other glioblastoma cell lines, T98G, U118 and LN229, the researchers analyzed the m5C miRNA contents via the same m5C-immunoprecipitation method and miRNA array sequencing. The heat map results show some variation in expression of the less enriched miRNAs, however all three cell lines, like U87, showed the highest, 500 to 1,000-fold, enrichment of miR-181a-5p (Figure 5).

In a series of elegant experiments these researchers provided evidence that two proteins interact to form a DNMT3A/AG04 protein complex that promotes methylation of miR-181-5p. The interested reader can find the details in the original article.

In order to examine the impact of m5C in miRNA-181a-5p on gene expression the researchers analyzed the expression of BIM, a protein whose expression in GBM is associated with overall survival prognosis.[24] BIM, or Bcl-2-like protein 11, is a member of a

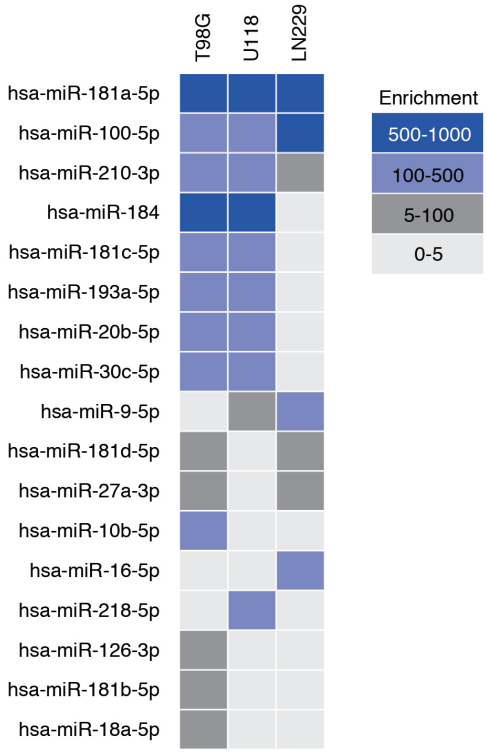


Figure 5. miRNA Array Heat Map of Glioblastoma Cell lines

protein family that shares Bcl-2 homology (BH) domains. The Bcl-2 family proteins are recognized for regulation of apoptosis at the mitochondrion surface. The 3'-UTR of BIM mRNA is one of the targets of miRNA-181a-5p and they sought to evaluate the impact of variations in the miR-181a-5p sequence and resulting effects on BIM expression in cells treated with these variant miR-181a sequences (Figure 6).

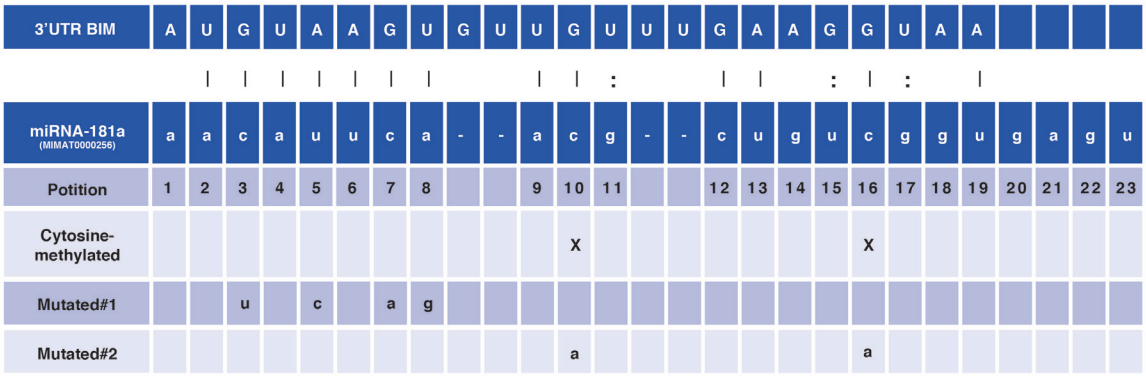


Figure 6. 3'-UTR of BIM and miRNA-181a oligo variants tested

Thus, U87 Cells were transfected and treated with the listed synthetic miRNA, either wild-type, mutated or cytosine methylated (m5C modified), and the resulting BIM expression was evaluated via detection of BIM protein by ELISA (Figure 7, left side) and confirmed by western blot (not shown). miRNA-451a was used as a negative control that does not target BIM. Authentic wild-type and synthetic non-5-methyl-modified miR-181a (miR181a-UnM) significantly block expression of BIM. In contrast, methyl-modified miR-181a-M and the two mutant sequences both lose the repressor function as indicated by high BIM expression.

In an alternative test of this control of expression function by miRNA-181a-5p, the BIM 3'-UTR was inserted into the 3'-UTR of a luciferase reporter gene (pmiR-BIM-3'-UTR). Cells were transiently transfected with the indicated mRNA containing the BIM 3'-UTR-reporter construct. Luciferase activity with the various miRNAs present was determined 48 h after transfection. The luciferase activity of pmiR-BIM-3'UTR was significantly reduced by wild type miR-181a and unmethylated synthetic miR-181a-UnM, but was highly expressed, or only weakly reduced by methylated or either mutated forms of miR-181a-5p (Figure 7, right side) similar to previous results using BIM expression.

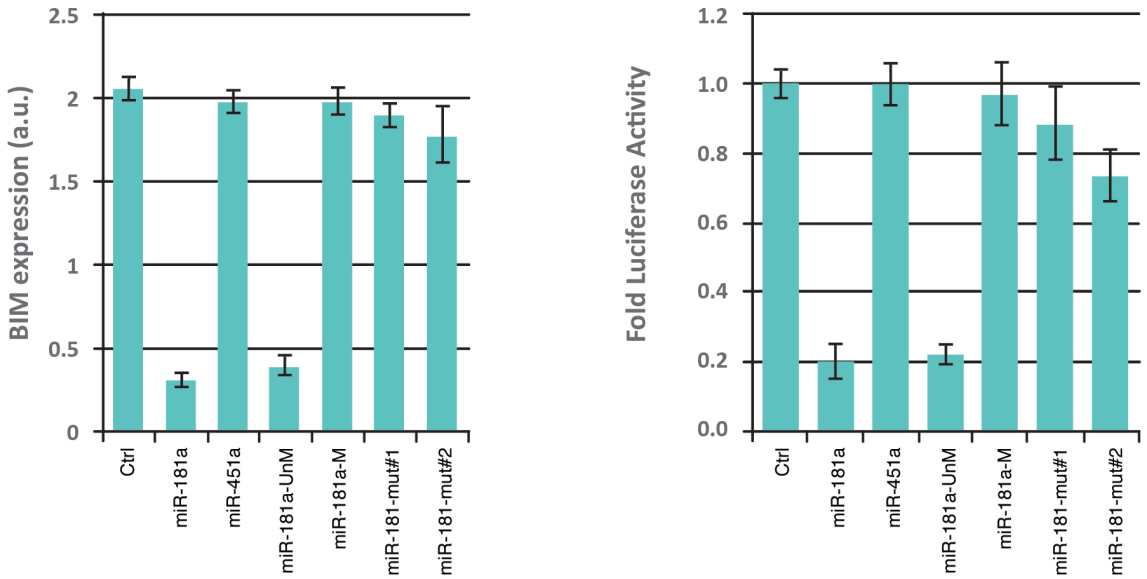


Figure 7. BIM expression and luciferase activity after transfection

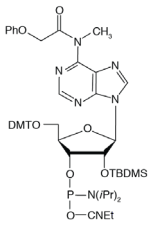
The data demonstrate that m5C on miRNA-181a-5p abolished its repressive function towards BIM in the GBM cell line U87. Interestingly, the mutation of cytosine to mismatched adenosine at positions 10 and 16 showed the same high expression effect as the presence of m5C on the function of miRNA-181a-5p towards BIM suggesting an important role that these two cytosines play in controlling the repressive function of miRNA-181a-5p. Further results implicating m5C modification on miR-181a-5p in GBH are described in the report, and the interested reader is directed to the original article.

Summary

Our understanding of RNA epigenomics is rapidly accelerating with the availability of new analytical methods for sequence context identification of the rare methyl marks on coding and noncoding RNA of all types. The facile synthetic access to site-specific modified RNA oligonucleotides containing minor methyl-modified bases is facilitating research promising to greatly improve our understanding of gene expression in normal and disease conditions.

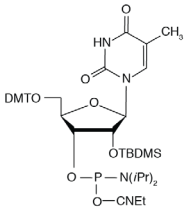
Glen Research provides a 5-methylcytidine phosphoramidite for use in preparing site specific m5C modifications to RNA oligonucleotides including the methylcytidine modified microRNAs used in the above study. We also provide additional rare RNA phosphoramidites for the synthesis of m6A, m5U, m1A and pseudouridine monomers and doubly methyl-modified monomers m5Um and m5Cm containing 2'-O-methyl ribose (Figure 8) that can be used for studies of epitranscriptomics, epigenetic enzymology or biochemistry research, or diagnostic test R&D.

N6-Methyl-A-CE Phosphoramidite



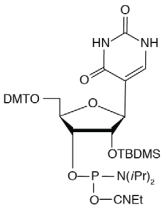
Glen Research offers the N6-Me-A RNA monomer with a phenoxyacetyl protecting group to minimize potential branching. The N6-Me-A-CE phosphoramidite has been shown to be compatible with all popular RNA synthesis and deprotection methods.

5-Me-U-CE Phosphoramidite



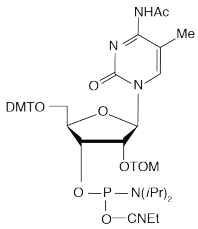
In addition to its utility in analyzing RNA structure and activity relationships, 5-Methyl-Uridine is a common highly-conserved modification in rRNA and tRNA, that has only recently been discovered in eukaryotic mRNA.

PseudoUridine-CE Phosphoramidite



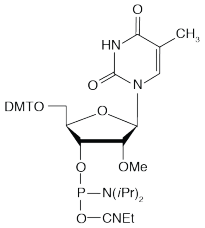
PseudoUridine is one of the most common modified nucleosides found in RNA. Research interest includes termination suppression in premature termination codons, potential alternative codon coding and the function of conditional pseudouridylation of mRNA.

5-Me-C-TOM-CE Phosphoramidite



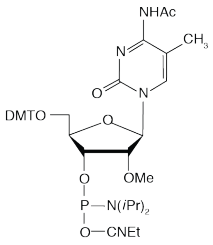
Glen Research offers 5-Methyl-Cytidine with a TOM protecting group. This monomer is useful for analyzing RNA structure and activity relationships, for example, in ribozyme studies.

2'-OMe-5-Me-U-CE Phosphoramidite



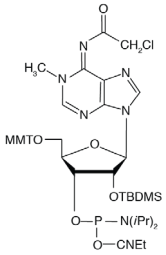
Glen Research offers several phosphoramidites that aid in the evaluation of structural analyses of complexes containing 2'-OMe-RNA sequences. 2'-OMe-5-Methyluridine is useful in triplex studies.

2'-OMe-5-Me-C-CE Phosphoramidite



Oligos containing 2'-OMe-5-Me-C would be of interest to researchers involved in triplex and antisense studies using 2'-OMe-RNA. This monomer increases duplex stability and nuclease resistance.

1-Me-A-CE Phosphoramidite



N-1 methylation excludes participation of the adenine base in canonical Watson–Crick base pairing and provides a positive charge to the nucleobase. This modification also alters hydrophobicity of the base, stacking properties, ordering of water molecules and chelation properties.

Table 1. Ordering Information

Item	Pack Size	Catalog No.
N6-Methyl-A-CE Phosphoramidite	0.25g	10-3005-02
	100µmol	10-3005-90
	50µmol	10-3005-95
5-Me-U-CE Phosphoramidite	0.25g	10-3050-02
	100µmol	10-3050-90
	50µmol	10-3050-95
PseudoUridine-CE Phosphoramidite	0.25g	10-3055-02
	100µmol	10-3055-90
	50µmol	10-3055-95
5-Me-C-TOM-CE Phosphoramidite	0.25g	10-3064-02
	100µmol	10-3064-90
	50µmol	10-3064-95
2'-OMe-5-Me-U-CE Phosphoramidite	0.25g	10-3131-02
	100µmol	10-3131-90
2'-OMe-5-Me-C-CE Phosphoramidite	0.25g	10-3160-02
	100µmol	10-3160-90
1-Me-A-CE Phosphoramidite	0.25g	10-3501-02
	100µmol	10-3501-90
	50µmol	10-3501-95

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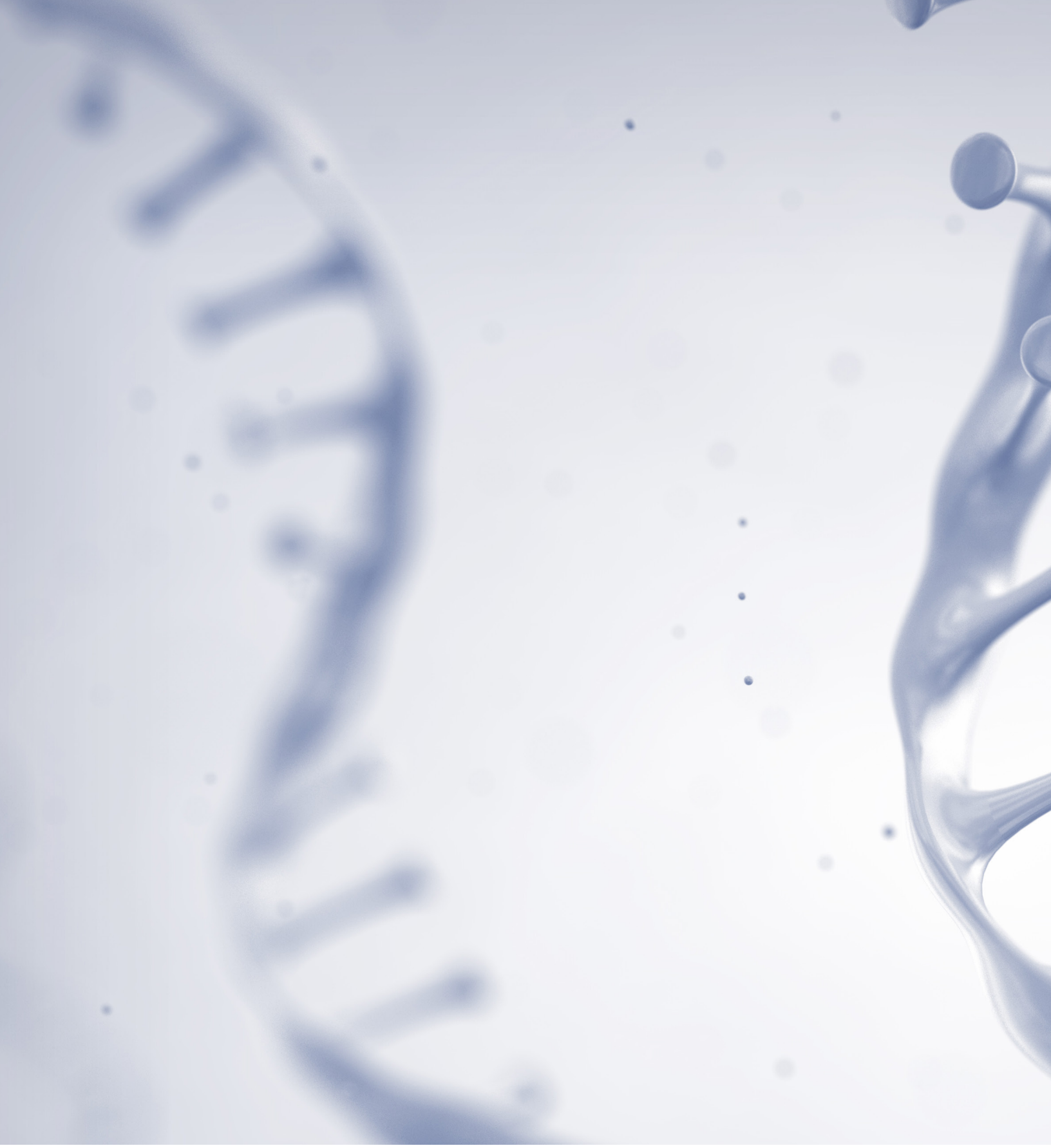

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