論文の内容の要旨

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論文題目

Studies on translational buffering upon splicing inhibition

（スプライシング阻害時に働く翻訳緩衝機構に関する研究）

Introduction

Eukaryotic cells possess many steps to produce mature mRNAs for proper translation. Errors during the processing of mRNA may lead to the production of aberrant proteins that are usually detrimental to the cell. Several layers of quality control systems in the nucleus and the cytoplasm prevent formation of these deleterious proteins. Splicing of primary transcripts (pre-mRNA) to remove introns between the exons in eukaryotes is a crucial step in the formation of mature mRNAs for the proper translation. Studying the molecular mechanism underlying regulation of splicing and the fates of improperly spliced transcripts presents a means to understanding a fundamental cellular process and its relation to disease. The cell possesses multiple mechanisms to prevent leakage of pre-mRNAs from the nucleus and their translation in the cytoplasm such as non-sense mediated decay (NMD). Even though premature termination codons inside intronic sequences should trigger degradation of the transcript via the NMD pathway, some pre-mRNAs escape NMD, resulting in the production of truncated proteins. This study focuses on the control mechanisms that govern how cells sense and cope with the production of potentially deleterious peptides from unspliced pre-mRNAs.

Using specific small molecule inhibitors to modulate the function of a biological system presents a potent technique to study the function of essential gene products, whose mutation would like result in non-viable cells. Here, I use spliceostatin A (SSA) as a tool compound to study the cellular effect of splicing inhibition (Kaida *et al.*, 2007). The removal of intron sequences is catalyzed by a multimeric ribonucleoprotein called the spliceosome. Five small nuclear ribonucleoprotein particles (snRNPs)- U1, U2, U4, U5 and U6, each containing a small nuclear RNA (snRNA) and associated proteins bind to their target sites on pre-mRNAs in a stepwise and specific manner. Two sequential transesterification reactions occur, thereby forming a phosphodiester bond between the 5' and 3' exons. SSA is the stable methyl ketal derivative of the natural product FR901464, originally isolated from *Pseudomonas* sp. It strongly binds the SF3B subcomplex of U2snRNP, thereby inhibiting splicing before the first esterification step. It has potential as a biochemical probe to dissect the splicing-related cellular machinery. Furthermore, SSA holds clinical promise as it showed potent antitumor activity *in vivo* and *in vitro*. However, the mechanism by which tumor cells are selectively affected by splicing inhibition remains elusive.

In this study, I discovered the presence of a novel mechanism that downregulates protein synthesis upon production of truncated proteins from unspliced transcripts by suppressing the mTOR signaling pathway. mTOR a serine/ threonine kinase is a key regulator of cellular homeostasis and protein synthesis. In addition to mTOR inhibition, translation of many gene transcripts highly expressed in tumors were downregulated by SSA, providing a basis for further exploration of the antitumor activity of splicing inhibitors such as SSA.

Chapter 1: Translation of transcripts with retained introns

Previous studies on transcription, splicing, translation or RNA quality control were limited by studying each process in isolation. With the advent of high-throughput sequencing-base methods, it has become possible to observe how perturbation in one system affects gene regulation on the whole. To monitor global changes in transcription and translation upon splicing inhibition by SSA, I set out to perform ribosome profiling and RNA sequencing of HeLa S3 after SSA treatment. While mRNA sequencing has become an established method for measuring gene expression, it actually only provides information on transcript sequence and transcript concentration. mRNA abundance by itself, however, is not a good indicator of gene expression as high transcript levels do not necessarily result in high protein output. Therefore, I combined my analysis with ribosome profiling to ascertain which messages actually reached the translation machinery. Ribosome profiling is a powerful method based on the deep sequencing of ribosome-protected mRNA fragments. This technique provides a high resolution map of actively translating ribosomes down to nucleotide level. It provides the best overview of proteome dynamics to date, encompassing gene-specific translation regulation rather than only monitoring transcript expression (Ingolia *et al.*, 2011). The combination of transcriptome sequencing and ribosome profiling furthermore allows determining translation efficiency (TE), defined as the ratio of ribosomal footprints per mRNA. TE analysis overcomes the limitations of RNA sequencing alone, as it accounts both for transcript abundance and the amount of resulting protein output.

Global analysis confirmed the translation of a subset of transcripts with retained introns. Exon skipping and intron retention were observed under SSA treatment, recapitulating the results of previous studies on SF3B inhibitors (Yoshimoto *et al.*, 2017). Significant retention of 5920 introns was observed. Among these a significant number of introns (1106) showed ribosome footprints, demonstrating that some messages do reach the translation machinery. Furthermore, ribosomal footprints were generally not detected 3' of premature stop codons, giving further evidence for the production of truncated proteins. It had been reported earlier that SSA induced expression of a C-terminally truncated version of the p27 cyclin-dependent kinase (CDK) inhibitor. The shortened protein, dubbed p27*is stable and biologically active in 3' inhibiting CDK 2 (Kaida *et al.*, 2007). However, p27* production alone cannot account for all cellular effects observed upon splicing inhibition. This suggests that further factors play a role in SSA's biological effect. Ribosome profiling enabled the discovery of further truncated polypeptides, which likely account for the observed changes in cellular signaling upon splicing inhibition.

Chapter 2: Differential expression analysis upon SSA reveals inhibition of translation

Under splicing inhibition mRNA production decreased by about half. However, protein production sank about four-fold, much more than one would expect from changes to mRNA levels alone. Metabolic labeling of nascent proteins with a puromycin derivative recapitulated the inhibition of protein synthesis observed in the ribosome profiling experiment. Increasing resolution to the single gene level, we determined the translation efficiency (TE) for all detected transcripts, looking for transcripts whose protein output especially decreased in the presence of SSA.

Since SSA has not been reported to affect mitochondrial translation, we used mitochondrial footprints as internal standards and quantified cytosolic transcripts and ribosome footprints. This provides a robust control to more accurately quantitate changes in gene expression. This normalized differential expression analysis identified significantly upregulated and downregulated genes upon splicing inhibition. Functional pathway analysis using Gene Ontology (GO) revealed that pathways associated with cancer, cell cycle, systemic lupus erythematosus, and chromatin assembly were significantly changed at both mRNA and footprint levels. Genes involved in the ribosome, tight junctions, the mTOR pathway and the cell cycle experienced a particularly significant TE decrease. Moreover, many of the oncogenes were downregulated by SSA at the translational level, which may at least in part account for the antitumor activity of splicing inhibitors.

Chapter 3: Splicing inhibition induces mTORC1 mediated translation repression

Following up on our gene ontology analysis, we detected significant inhibition of the mTOR pathway. As mTOR signaling affects translation initiation and ribosome biogenesis, its inhibition could also account for both the decrease in protein output as well as the TE decrease in genes associated with ribosome production. mTOR, a serine/threonine protein kinase plays a central role in cell proliferation. The mTOR kinase is the essential component of two distinct protein complexes: mTORC1 and mTORC2. mTORC1 is a key regulator of translation initiation via phosphorylation of eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4EBP1). Phosphorylation of 4EBP1 by mTORC1 results in dissociation from eIF4E, allowing eIF4F complex formation and 5' cap-dependent translation to occur. Therefore, mTOR inhibition leads to increased 4EBP1 binding to eIF4E and decreased translation initiation. mTORC1 furthermore regulates ribosomal protein S6 kinase via phosphorylation of p70S6 kinase 1 (S6K1). S6K in turn plays a critical role in ribosome biogenesis and translation activation (Laplante and Sabatini, 2012). To validate mTORC1 inhibition by SSA, we observed dephosphorylation of its key substrates 4EBP1 and S6K1 in a time dependent manner. The effect on mTOR, however, is indirect as SSA did not inhibit the kinase *in vitro*.

mTORC1 plays a fundamental role in controlling translation of the 5' terminal oligopyrimidine (5' TOP) motif containing mRNAs which are largely mRNAs encoding ribosomal proteins. The translation efficiency of TOP motif containing mRNAs was indeed strongly inhibited by SSA, giving further evidence for the relation between the SSA action and mTORC1 inhibition. Genes downregulated by SSA significantly overlapped with genes sensitive to the mTOR inhibitor pp242. I further tested whether a similar effect could be produced upon knockdown of SF3B1 or treatment with other splicing inhibitors. SF3B1 knockdown induced the dephosphorylation of the key mTOR substrates 4EBP1 and S6K1, indicating inhibition of mTORC1 activity. Pladienolide B (PlaB), another small molecule

SF3B inhibitor, also downregulated mTORC1 activity. Therefore, it is splicing inhibition in general that accounts for mTOR inhibition, not SSA in particular.

Chapter 4: Truncated proteins induced by SSA cause proteotoxic stress and feed back to repress translation via mTORC1

Finally, I tried to elucidate the mechanistic link between splicing inhibition and mTOR downregulation. It has been shown that proteotoxic stress caused by heat shock and stressor compounds is immediately sensed by c-Jun N-terminal kinase (JNK), thereby leading to the inhibition of mTORC1. Indeed, phosphorylation of JNK was significantly increased by SSA treatment. Therefore, it seemed likely that some of the truncated proteins observed earlier were responsible for JNK activation. The ribosome profiling data provided a number of plausible candidates. A selection of proteins was ectopically expressed in their predicted truncated form. Especially, the shortened form RAS oncogene family protein (RAB32*) could recapitulate SSA's effect in the absence of drug. In immunoblot assays on cell lysate RAB32* could not be detected in the soluble fraction, but only in the pellet, which suggests that the truncated protein aggregates and consequently induces cellular stress. The phosphorylation of JNK and simultaneous dephosphorylation of 4EBP1 and S6K1 were observed upon RAB32* expression. Furthermore, knockdown of JNK rescued the SSA-induced dephosphorylation of 4EBP1 and the translation inhibition of 5' TOP motif containing mRNA. These results suggest that some of truncated proteins with translated intron sequences activate the proteotoxic stress response via JNK, thereby leading to mTORC1 inhibition.

Conclusion

Maintenance of proper gene expression is monitored by several layers of control mechanisms. As exemplified by chemical splicing inhibition, a number of transcripts circumvent NMD and reach the translation stage despite containing intronic sequences. Here we analyzed the relationship between splicing inhibition and global translation through ribosome profiling, in combination with a specific splicing inhibitor. Our results demonstrate that translation of improperly spliced transcripts leads to downregulation of global protein synthesis via inhibition of translation initiation probably through the misfolded protein-mediated suppression of the mTOR pathway. Beyond many RNA quality control pathways in cell, our study infers the presence of a negative feedback mechanism to respond to the production of truncated proteins, possibly to prevent further damage and to survive till the source of stress has subsided.

References:

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