5. Conclusions and future directions

The major roadblocks in treatment for GBM are resistance to therapy and recurrence of GBM cells. Regardless of various treatment strategies, the average survival of GBM patients is poor and incidence of recurrence remains high. The presence of GSCs, a dynamic cellular system within GBM contributes to chemo/radio resistance and recurrence. The plasticity of GSCs is supported by reversible biological processes including DNA methylation, histone modifications and RNA modifications. m^6A is a reversible mRNA methylation which regulates various steps of RNA processing. In this thesis, we attempted to elucidate the METTL3- mediated m^6A as a molecular mechanism behind the dynamic nature of GSCs. It comprises of two parts:

Part 1- The landscape of METTL3-dependent m^6A-epitranscriptome in GSCs and the functional orchestration of m^6A targets

Part 2- Essential role of METTL3 mediated m^6A modification in glioma stem-like cells maintenance and radioresistance

In first part, we demonstrate levels of m^6A modified RNAs and METTL3 are maintained high in GSCs and they are attenuated during serum induced differentiation. Other members involved in m^6A modification are either down regulated or unregulated in GSCs which confirms the dependency of GSCs in METTL3 for creating m^6A marks. In addition, previous reports provide support that solely METTL3 carry catalytic domain for SAM-binding. Based on these facts, we elucidate the METTL3-dependent global m^6A modification in GSCs and the impact of METTL3 targets in coordinating various functions. By comprehensive analysis of m^6A-RIP seq and whole transcriptome post METTL3 silencing in GSCs, we identified the direct and indirect targets for METTL3-mediated m^6A modification. The genes which preserve stem cell properties and aid in tumorigenesis were predominantly inhibited by METTL3 silencing in GSCs suggesting a universal oncogenic role of METTL3 in GBM. Large subset of genes was down regulated after METTL3 silencing suggesting a global destabilization of transcripts. The enrichment of m^6A peaks near stop codon and 3'UTR indicates functional importance of METTL3 in RNA stabilization and translation termination. In addition to protein coding genes, METTL3 fine-tunes the expression of non-

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coding RNAs which includes lncRNAs, anti-sense RNAs etc. Interestingly, we identified m^6A peaks which encompass miRNA target sites and we hypothesize that m^6A modification may scrutinize the binding affinity of miRNAs. We further determined the inter-play between chromatin remodeling with m^6A epitranscriptome. Genes which carry active chromatin marks and transcription factor binding are further stabilized by METLT3-dependent m^6A modification. The altered secondary/tertiary structure induced by m^6A may act as loading site for various RBPs and achieve various RNA processing functions. Our analysis deduced RBPs HuR and QKI preferentially bind to m^6A marked RNAs and helps in enhancing the expression of m^6A modified targets. Together, this study provides a panoramic view on global m^6A modification mediated by METTL3 in GSCs.

In second part, we examined the crucial role of METTL3 in glioma stem cell physiology. Inhibition of METTL3 hinder the neurosphere formation and stem cell properties of GSCs. Anti-METTL3 RIP studies combined with m^6A RIP-seq results identified SOX2 as a key m^6A mediator of METTL3 and the m^6A marks created by METTL3 sustains SOX2 transcript stability. The exogenous over expression of 3'UTR-less SOX2 significantly alleviated the inhibition of neurosphere formation observed in METTL3 silenced GSCs. METTL3 interaction and m^6A modification *in vivo* required intact three METTL3/m^6A sites present in the SOX2- 3'UTR. Further, we found that HuR recruitment to m^6A modified RNA is essential for SOX2 mRNA stabilization by METTL3 and at global level HuR-RNA interaction prefers the m^6A modified transcripts. METTL3 silenced GSCs showed enhanced sensitivity to γ-irradiation due to reduced DNA repair. Exogenous overexpression of 3'UTR-less SOX2 in METTL3 silenced GSCs rescues efficiency of DNA repair and specifically homologous recombination repair. It also resulted in the significant rescue of neurosphere formation from METTL3 silencing induced radiosensitivity. GBM tumors have elevated METTL3 transcripts and silencing METTL3 in GSCs inhibited tumor growth and prolonged mice survival. METTL3 transcript levels predicted poor survival in GBMs which are enriched for GSC-specific signature. Thus our study reports the importance of m^6A modification in GSCs and uncovers METTL3 as a potential molecular target in GBM therapy.

Future perspective

The combined m^6A-RIP and transcriptome analysis in GSCs adds exciting new dimensions for future stem cell biology. The mechanistic view on METTL3-mediated regulation of specific direct targets and the functional impact needs further study. We identified miRNA seed sites present within m^6A peaks at 3'UTR of target genes which were stabilized by METTL3. It warrants further studies in order to decipher the coordination between miRNA binding and m^6A modification. The m^6A modification alters the RNA duplex stability and conformation due to addition of extra methyl moiety and it may act as recruitment site for various RBPs. We identified RBPs- HuR, QKI and IGF2BP3 exhibit preferential binding to m^6A modified targets. The functional outcome of the coordination between m^6A modification and RBPs needs further investigation. We observed a positive correlation of genes by METTL3-mediated m^6A stabilization with activated by H3K4me3 or by key stem cell transcription factors MYC, SOX2, OCT4, Nanog via. It suggests a collaborative regulation between METTL3 and epigenetic modulation. Additional studies are required to substantiate our observations. It will be interesting to investigate the specific clustering of m^6A peaks near splice sites since it can recruit RNA processing factors and may determine choice of exons during alternative splicing. Our study shows METTL3-dependent m^6A modification in several lncRNAs including XIST, MALAT1 and H19. The regulation of lncRNAs by m^6A modification is obscure and it requires further studies to examine how modified lncRNAs differ in performing the functions from unmodified ones. In second part, we showed METTL3 being regulated by gamma irradiation. Previous report on phosphoproteome profiling of irradiated cells shows METTL3 as an ATM kinase candidate which get phosphorylated post irradiation (Matsuoka et al, 2007). Since ATM gets activated upon radiation, it will be interesting to identify phosphorylation induced changes in METTL3. To conclude, our investigation instigates interesting questions which need further studies to expand the knowledge in the field of m^6A modification.