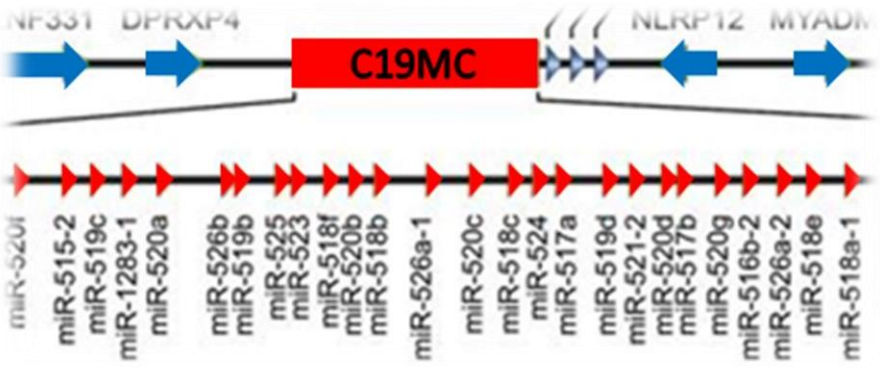
University of East Anglia

Understanding the oncogenic effects of C19MC on hepatocellular carcinoma through epigenetic manipulations.



Master's by Research Thesis

Emine Efendi (100171126)

Supervisor: Dr. David Monk

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Contents

1. Abstract.

Aberrant epigenetic alterations, such as DNA methylation, histone modification and miRNA-mediated processes, are associated with several types of cancer including hepatocellular carcinoma (HCC). HCC is the third leading cause of cancer-related fatalities worldwide. Despite the improvements in surgical and medical treatment HCC associated deaths are still showing an increase. Methylation defects at the chromosome 19 miRNA cluster (C19MC) have been shown to be a molecular alteration specific to liver cancers and is an attractive candidate for novel HCC therapies. Several C19MC miRNAs have been reported to be over-expressed in HCC and C19MC hypomethylation may account for this cancer-associated expression. This present study assesses the oncogenic effects of C19MC cluster in HCC using epigenetic manipulations. Using pharmaceutical and novel targeted epigenome editing tools demethylation was induced in HCC cell lines showing a normal hypermethylated state. Demethylation was shown to be sufficient to re-activate C19MC miRNAs throughout the cluster. Following overexpressing *miR-512-3p* through miRNA mimics, we showed that upregulation of *miR-512-3p* significantly promotes cell invasion. Since abnormal miRNA expression has been associated with metastatic spread of tumors, studying changes in miRNA expression could help to improve diagnosis and prognosis and provide molecular targets for new therapeutic strategies against HCC. Our study suggested that *miR-512-3p* can be a robust marker for HCC prognosis and diagnosis.

2. Introduction.

2.1. Cancer and basic classifications.

Cancer is clinically defined as a vast number of complex diseases which have distinct behaviours. The behaviours depend on the cell types which they originate from and the genetic modifications that occur within each cancer type. The two main properties observed in all cancer cells are abnormal cell growth and division and their ability to

spread and colonize other parts of the body, known as metastasis. Cancer cells become dangerous when cells divide uncontrollably in combination with uncontrolled metastatic spread (Klug *et al.*, 2016). Therefore, cancer is the most common cause of human fatalities worldwide and its incidence and mortality have shown a rapid increase in recent years (Kelly *et al*., 2017).

There are two main cancer types: benign and malignant. Benign tumor results from loss of genetic control over cell growth and is a noncancerous growth (King., 2019). Benign tumors neither invade neighbouring tissues nor metastasize to other parts of the body. On the other hand, malignant tumors are capable of entering the blood stream or lymphatic system, invading other tissues and metastasize. Therefore, malignant tumours can become life threatening whereas benign tumors are more common and mostly harmless (Klug *et al*., 2016; King, 2019). Although malignant tumors can spread and invade other tissues, all cancer cells in the primary and secondary tumors are clonal. In other words, these cells originate from common ancestral cells that accumulate certain mutations that cause cancer (Klug *et al*., 2016). For instance, breast-derived bone metastases are tumors formed from cancer cells released from the breast and growing in bone tissue. Therefore, these bone metastases are not bone cancer but could be considered as ectopic cancerous breast cells that have started growing inside the bone. Treatment of these secondary cancers within the bone are difficult, however with the correct treatment the size of metastases can be reduced, slow their growth, lessen the symptoms, and increase the patients' life span (Case-Lo, 2016).

2.2. Common molecular mechanisms to Oncogenesis.

The major changes that characterize cancer are represented by altered oncogenes and tumor-suppressor genes (Botezatu *et al*., 2016). Oncogenes are mutant genes derived from proto-oncogenes and are potential carcinogenetic factors (Bagci and Kurtgoz, 2015; Klug *et al*., 2016; Nelson,2019). Somatic genetic alterations including gene mutations, arrangements, and amplifications (Croce, 2008) result in oncogene activation. Mutations causing activation of one allele of an oncogene can interfere with normal cell growth and differentiation (Roland, 2009; Klug *et al*., 2016), leading to cancer initiation

and progression (Bagci and Kurtgoz, 2015; Nelson, 2019). On the other hand, tumor-suppressor genes (TSGs) normally function to suppress cell division (Klug *et al*., 2016). Thus, loss of function of tumor-suppressor genes due to mutations or deletions result in activation of cell division and tumor formation (Mendelsohn *et al*., 2014).

Since cancer is a heterogenous disease, uncontrolled growth of malignant cells occurs due to combined genome aberrations, methylation changes, as well as altered miRNA expression (Capper *et al*., 2018; Martincorena *et al*., 2017). Studies have been focused on the origin of tumor cells for over 50 years. Previously it was suggested that mutations, amplifications, deletions, and rearrangements were responsible from tumorigenesis (Vogelstein and Kinzler, 2004). Our understanding of cancer as a genetic disease has evolved since cancer cells gain their characteristics at distinct times in different microenvironments during cancer progression, via wide range of mechanisms (Hanahan *et al*. ,2011; Vogelstein *et al*., 2013). Genome instability is defined as enhanced tendency of the genome to acquire mutations. Genome instability and increased mutation frequency can result from defects in DNA repair, DNA replication, chromosome segregation or cell cycle control. For instance, defects in DNA mismatch repair (MMR), primarily alterations of the *MLH1*, *MSH2* and *PMS2* genes result in deletions or random insertion and expansion of repetitive DNA sequences (microsatellites) and are a characteristic feature of several cancers, including ovarian, lung and colorectal cancer (Kim *et al*., 2000; Pikor *et al*., 2013). Thus, genome instability and faulty repair mechanisms cause mutation accumulation which increases over time, playing a role in carcinogenesis (Deman *et al*,. 2001; Langie *et al*., 2015). Moreover, alterations in the epigenome may indirectly cause genome instability. For example, epigenetic modifications can influence DNA repair efficiency and fidelity through altering the expression of DNA repair genes (Langie *et al*., 2013; Langie *et al*., 2014) or genome architecture through widespread hypomethylation. As will be discussed later, epigenetic changes in DNA was shown to be associated with cancer progression and DNA methylation was the first epigenetic event studied in tumor cells (Ehrlich, 2009; Shen and Laird, 2013).

2.3 Hepatocellular carcinoma; causes and sub-types.

Hepatocellular carcinoma (HCC) is one of the most common tumors and is the third cause of cancer-related deaths across the globe. Early stage HCC patients are initially treated with surgical resection in combination with adjuvant therapy to improve survival (Bruix *et al.*, 2016). However, frequent tumor recurrence has a negative impact on HCC patients' prognosis (Augello *et al*., 2018). Most of HCCs are related to hepatitis C virus (HCV) or hepatitis B virus (HCB) infections, yet dysplastic liver nodules are often linked with different molecular aberrations and gene expression signatures (Augello *et al*., 2012). Although genetic mutations and copy-number alterations are well described in HCC, Deng *et al*. (2009 and 2010) characterized infection mediated liver carcinogenesis and demonstrated that abnormal promoter methylation may also play an important role. Furthermore, several studies have established that epigenetic alterations are one of the hallmarks of tumorigenesis (Jones and Baylin, 2002; Shen and Laird, 2013).

2.4 Epigenetic regulation in health and disease.

Epigenetics is the complex mechanisms that control gene expression by DNA or chromatin modifications, without altering the underlying DNA sequence (Pujadas and Feinberg, 2012; Klug *et al*., 2016). Epigenetic processes involve DNA methylation, histone modifications and small RNAs. These processes are known to regulate cellular mechanisms independent of each other. However, joint activities of different epigenetic events were shown to have common consequences, indicating cross talk and interdependence between these epigenetic processes (Murr, 2010).

Much of our understanding of gene expression has been obtained through determination of gene regulatory elements (Matharu and Ahituv, 2015). Gene expression is not only controlled by the promoters but also by distal regulatory elements such as repressors which inhibit transcription from promoters, enhancers which target gene promoters resulting in gene expression, insulators which disrupt enhancer-promoter cross talk when present between them and barriers which prevent heterochromatin spreading through

maintaining borders between euchromatin and heterochromatin regions (Lin *et al*,. 2011). Looping factors, gene regulatory elements and non-coding RNAs play a crucial role in enhancer-promoter interactions which have been shown to be associated with transcriptional regulation. Hence, abnormal enhancer-promoter interactions can result in several diseases, including cancer (Matharu and Ahituv, 2015). Moreover, epigenetic modifications have impacts on cellular processes such as transcription regulation, gene expression and DNA repair, therefore altered epigenetic regulation is a known cause of human diseases (Nojima *et al*., 2016).

As mentioned above, the roles of epigenetic alterations in health and disease are becoming increasingly noticeable. Deregulation of epigenetic processes is one of the significant causes of cancer, hereditary and neurogenerative diseases (Langie *et al*., 2015; Holtzman and Gersbach, 2018). Epigenetic aberrations can be categorized into four main groups (Fig.1). First category is the loss of function mutation of epigenetic modifiers (Holtzman and Gersbach, 2018). DNA methylation modifiers involve DNA methyltransferases (DNMT enzymes) and DNA demethylation modifiers involve ten-eleven translocation (TET) methylcytosine dioxygenases. Loss of TET protein function results in aberrant DNMT activity and DNA hypermethylation. Accumulation of methylation due to loss of TET activity and enhanced DNMT activity has been associated with malignant transformation. Furthermore, it is known that all three *TET* genes are mutated and have decreased expression, and the proteins have disrupted activity in various cancer types (Rasmussen and Helin, 2016). For example, somatic alterations of *TET2* were observed in both myeloid and lymphoid malignancies as well as acute myelogenous leukaemia (AML) (Scourzic *et al*., 2015). Therefore, precise regulation of DNA methylation patterns, which involves TET enzymes, is essential for normal development and provides a vital protection against cellular transformation (Rasmussen and Helin, 2016). The second category states that a disease can also stem from a mutation in gene regulatory regions which alters downstream gene expression levels. The third category is the inheritance or *de novo* introduction of epigenetic abnormalities. A well-known example is hypomethylation of the *H19/IGF2* locus which may cause transformation, proliferation and hence cancer. The fourth category is stochastic stress

that result from various factors including aging, metabolism, and environmental factors (Fig. 1) (Holtzman and Gersbach, 2018).

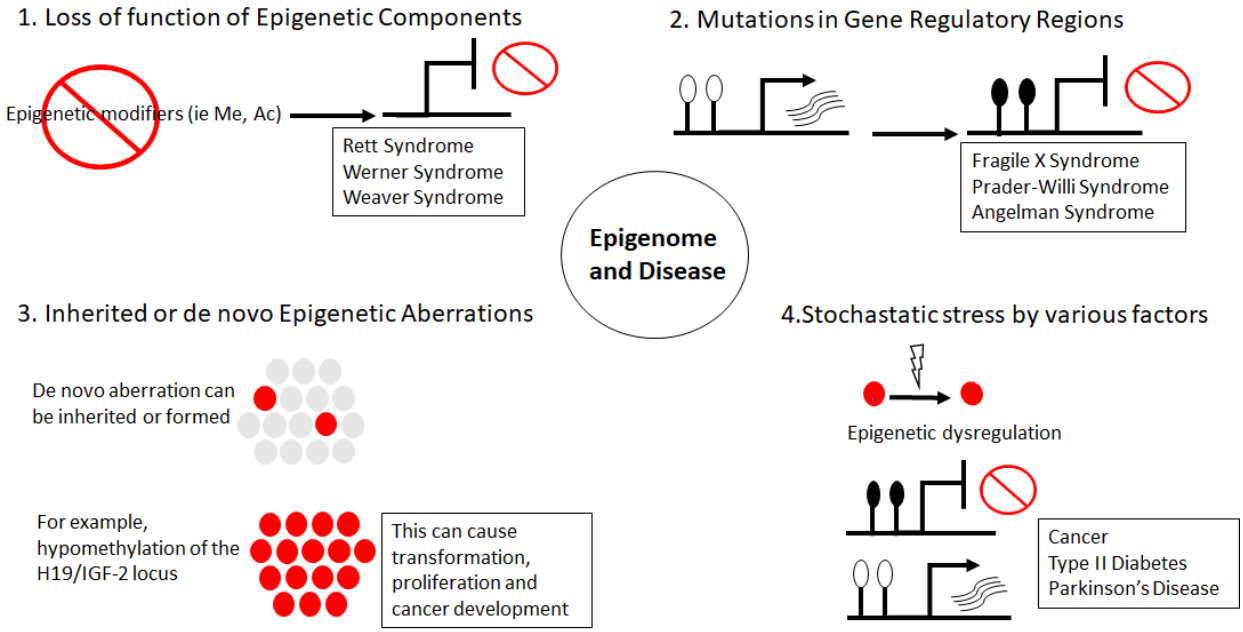


Figure 1 Epigenome and Disease. (Figure adapted from Holtzman and Gersbach, 2018) Several cellular processes including transcription, DNA repair and gene expression are regulated by the epigenetic processes. Therefore, aberrant epigenetic modifications result in human diseases. The loss of function mutation of epigenetic modifiers can cause Rett syndrome (neurological disorder) and Werner syndrome (autosomal recessive disorder). A mutation in gene regulatory regions which alters downstream gene expression levels can also result in diseases. Epigenetic abnormalities can be inherited, or *de novo* introduced. An example is hypomethylation at the imprinted *H19/IGF2* locus which may cause tumor development. Moreover, some other factors such as aging, metabolism or environmental factors may impact cellular functions leading to epigenetic abnormalities and disease including type 2 diabetes, cancer, etc.

2.5 DNA methylation.

DNA methylation is the major epigenetic phenomenon involved in the regulation of numerous cellular processes such as imprinting, X-chromosome inactivation, chromatin organization, genome stability and gene expression. DNA methylation is classically the

covalent addition of a methyl group (-CH3) to the 5' cytosine that precedes guanine in the DNA sequence; the CpG nucleotide, forming 5-methylcytosine (5mC) (Sablok and Tatarinova., 2013). DNMTs are responsible from catalyzing the transfer of cytosine to 5-methylcytosine (Fig.2). Three DNMTS have been identified in mammals; DNMT1, DNMT3A and DNMT3B. DNMT1 recognizes hemimethylated CpG nucleotides in the parent DNA strand during replication and produces symmetrically methylated sites by catalyzing the transfer of the methyl group to the cytosine residues in the unmethylated daughter strand. This ensures that propagation of DNA methylation with cell division takes place accurately (Newell-Price *et al*. ,2000; Klug *et a*l., 2016; Gowher and Jeltsch, 2019). On the other hand, DNMT3A and DNMT3B can methylate previously unmethylated DNA, and are known as *de novo* methylation, responsible for establishing new methylation patterns during development (Bestor *et al*., 1988; Okano *et al*., 1998; Okano *et al.*, 1999; Holtzman and Gersbach, 2018; Gowher and Jeltsch, 2019).

DNA demethylation is the reverse of methylation; the removal of a methyl group from 5' cytosine of the from CpG dinucleotides (Fig 2). The TET enzyme family (Tet1, Tet2, Tet3) play crucial roles in demethylation. TET enzymes catalytically convert 5-mC into 5-hydroxymethylcyotsine (5-hmC) in presence of 2-oxoglutarate and iron (II) reversing the DNMT actions (Tahiliani et al., 2009; Wu and Zhang, 2011; Guo *et al*., 2011; Rasmussen and Helin., 2013). In addition, all three TET enzymes have been reported to further oxidize 5-hmc to 5-formylcytosince(5-fC) and 5-carboxylcytosine (5-caC) (He *et al*., 2011; Zhang *et al*., 2012). The modified cytosines frequently undergo deamination, glycosylase dependent excision or replaced with unmodified cytosines through DNA repair mechanisms (Bhutani et al., 2011; Branco et al., 2012). Together, this indicates that TETs are actively involved in DNA demethylation (Bhutani *et al*., 2011; An *et al*., 2017).

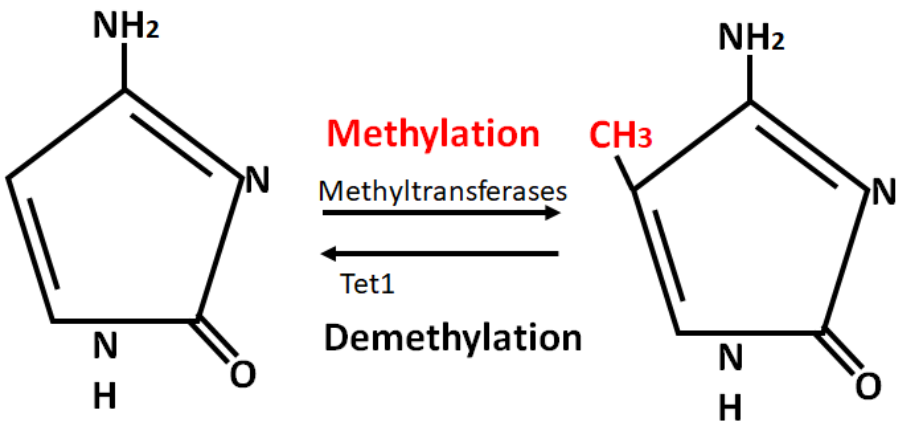


Figure 2 DNA methylation. In DNA methylation, de novo methyltransferases DNMT3A and DNMT3B add a methyl group (CH3) to 5' cytosine of the CpG dinucleotides, forming 5-methylcytosine. After methylation is established by *de novo* methyltransferases, maintenance methyltransferases such as DNMT1 and DNMT3 maintain the methylation marks. Demethylation is reverse of methylation in which the methyl group is removed actively by TET enzymes.

2.6 Interaction between DNA methylation and histone modifications.

Methylation of CpG nucleotides can have effects on gene expressions (Fig.3). Cytosine methylation is one of the main epigenetic mechanisms responsible for gene silencing as it is highly correlated with the inhibition of gene transcription. There are two different mechanisms by which DNA methylation suppresses gene expression. The first mechanism involves inhibition of binding of specific transcription factors through methylation of recognition sequences (Comb and Goodman, 1990; Lewondowska and Bartoszek, 2011). The second mechanism involves the recruitment of methylcytosine binding proteins (MBPs) and corepressors to methylated DNA. This induces a closed chromatin conformation within the area of MBP binding, limiting the access to transcription factors, leading to gene silencing (Zardo *et al*., 2005; Klose and Bird, 2006; Lewandowska and Bartoszek, 2011).

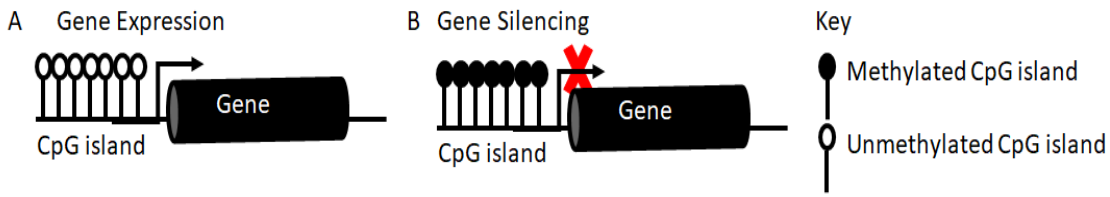


Figure 3 Methylation of CpG islands causes gene silencing. A) When a gene's promoter is unmethylated, the gene can be transcribed. B) Methylation of a promoter through the addition of a methyl group to 5' cytosine of the CpG islands inhibits transcription hence the gene is silenced.

In addition to DNA methylation, other histone modifications such as histone methylation, deacetylation and chromatin binding proteins influence local chromatin structure and hence regulate transcription (Baylin, 2005). Some of histone post-translational modifications can form extended domains of modified histones by spreading along the genome independently of the DNA sequence, influencing genome stability and gene function (Zhou *et al.*, 2011). For instance, di/trimethylation of histone 3 at lysine 9 (H3K9me2/3) and lysine 27 (H3K27me2/3) are enriched in heterochromatin and result in gene silencing (Talbert and Henikoff, 2006; Grewal and Jia, 2007; Simon and Kingston, 2009). H3K9me2/3 can spread around specific response elements within the genome (Jermann *et al*., 2014), resulting in repression of genes within the methylated domains (Fig.4) (Baur *et* al., 2001; Akhtar *et al*., 2013). Therefore, aberrations in histone modifications and the enzymes involved can cause cancer (Geutjes *et al*., 2012; Plass *et al*., 2013, Holtzman and Gersbach, 2018).

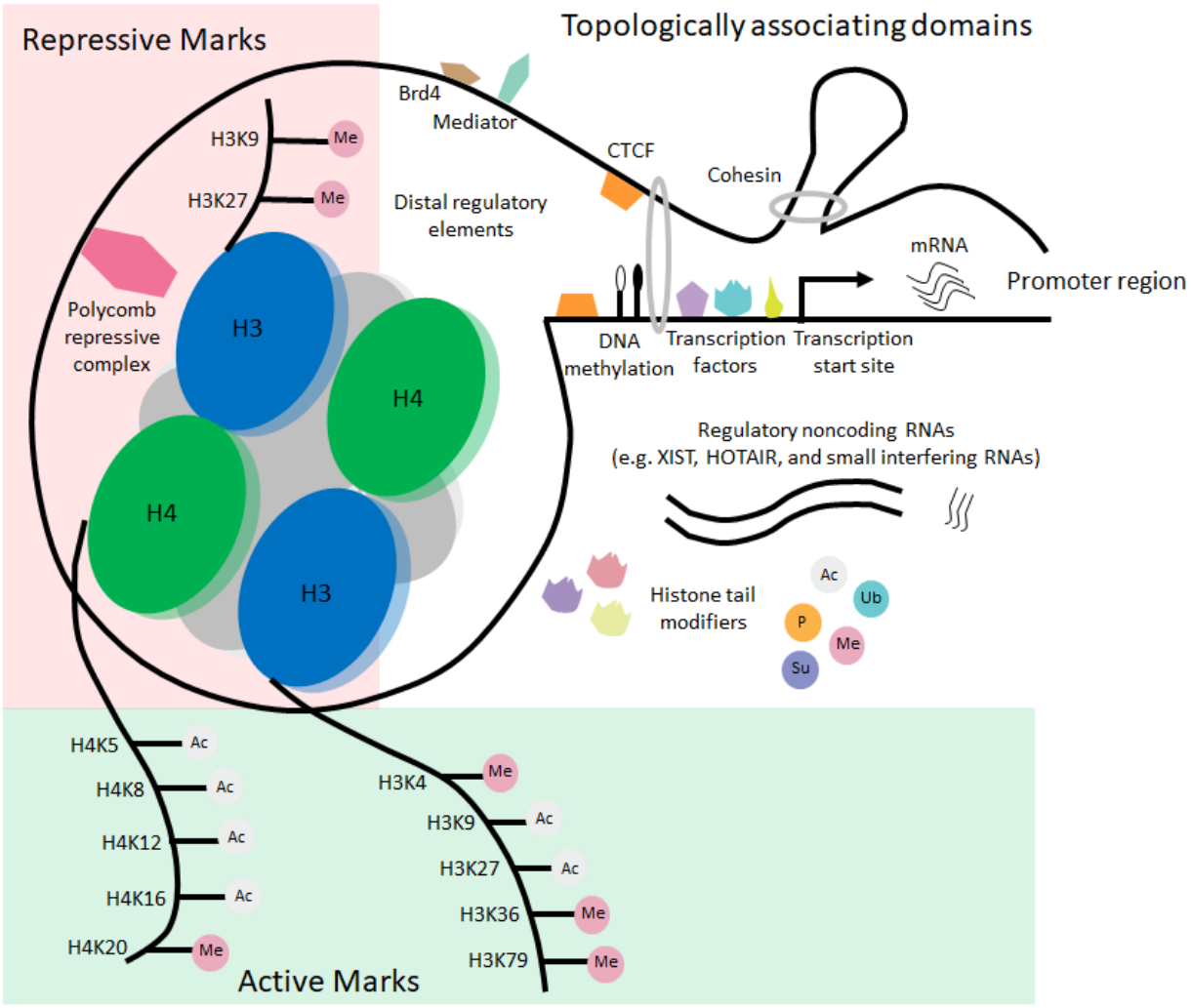


Figure 4 Epigenome is complex and diverse. (Figure adapted from Holtzman and Gersbach, 2018) Epigenetic events involve histone tail modifications, DNA methylation, chromatin looping. Several interacting elements facilitate the deposition and removal of these epigenetic features. Epigenetic marks and gene regulatory elements can affect gene expression. Significant histone modifications associate with active or repressed chromatin (green and pink background respectively). Histone modifiers catalyse the accumulation and removal of methylation (Me) or acetylation (Ac). Moreover, histone modifications may facilitate other processes, for instance phosphorylation (P), SUMOylation (Su) and ubiquitination (Ub).

2.7 Common epigenetic signatures associated with cancer

Aberrant epigenetic regulation is known to facilitate tumor development. For instance, differences in DNA methylation patterns is significant between cancer and non-cancer

tissues and cancer-specific epigenetic properties have been identified for every cancer type (Nojima *et al*., 2007; Nojima *et al*., 2009; Nojima *et al*., 2016). Although, only global DNA hypomethylation was initially shown to be related to the carcinogenic process, linked to genome instability and reactivation of retroviral sequences, it is now known that alterations in DNA methylation in neoplastic cells are characterized by localized hypomethylation and hypermethylation of specific genes (Feinberg and Tycko,2004; Ehrlich *et al*., 2016; Ehrlich, 2019). To be more significant, epigenetic modifications in oncogenes and TSGs also cause tumor development. Hypomethylation of oncogene promoters can activate oncogenes. For example, activating mutations in *RAS* oncogenes are one of the most common processes in numerous cancers including thyroid and colorectal cancers and this gene is frequently hypomethylated in cancer (Botezatu *et al*., 2016). On the other hand, hypermethylation of the core promoter in CpG islands has been shown to lead to the loss of transcription of classical tumor-suppressor genes including *p16* and *MLH1* in several cancers (Esteller, 2008; Wu *et al*., 2012). Numerous TSGs in an extensive range of cancers have been reported to be inactivated due to promoter hypermethylation which frequently occurs in the context if the promoter is embedded within a CpG island (Onay *et al.*, 2009; Sebova *et al*., 2011; Carmona *et al.*, 2012). Thus, the methylation status of oncogenes and TSGs not only drive cancer but can serve as a potential marker for diagnosis and prognosis prediction (Morris *et al*., 2010; Ricketts *et al*., 2012).

As mentioned above, the two main differences between the DNA methylomes of cancer cells and normal cells is that cancer cells exhibit a global reduction in DNA methylation and abnormal hypermethylation of some sequences, primarily CpG islands (Enrilch, 2009). Aberrant hypermethylation inactivates TSGs and hence is often associated with cancer progression (Boyes and Bird, 1992; Feinberg and Tycko, 2004; Sebova *et al*., 2012; Carmona *et al*., 2012). However, analysis of cancer methylomes have shown that aberrant CpG hypermethylation takes place predominantly at genes that are already silent and is hence not associated with transcriptional silencing of TSGs (Sproul and Meehan, 2013). In order for abnormal hypermethylation to directly cause cancer via gene silencing, the affected genes must be expressed prior to hypermethylation (Antequera *et*

al., 1990; Sproul *et al*., 2012). Transcriptionally silenced genes were shown to be the primary target of cancer-associated aberrant hypermethylation through the analysis of cancer methylomes and gene expression data (Sproul *et al*., 2012). However, a study in colon cancer (Hinoue *et al*., 2012) demonstrated that 93% of the hypermethylated genes in tumors had unaltered expression when compared to normal tissue. The results of this suggested that the genes were already repressed, often by bivalent chromatin and H3K27me3, in the normal tissue (Hinoue *et al*., 2012).

2.8 Hypermethylation may affect CFCT sites and later enhancer-promoter looping.

In vertebrates, insulator protein cohesin and CCTC-binding factor (CTCF) facilitate enhancer-promoter interactions by enabling chromatin looping (Krivega and Dean, 2012; Matharu and Ahituv, 2015). The role of CTCF proteins in establishing enhancer-promoter has been recognised in several previous studies. For instance, Murrell *et al.* (2004) examined DNaseI hypersensitive sites (DHS) in the *Igf2-H19* locus. These regions have different methylation status on maternal and paternal alleles and the methylation of these sites were found to impact the binding of CTCF proteins. This was shown to enable allele-specific looping of DHs to *Igf2* promoter, enhancing the loops and thus resulting in stable transcription (Murrel *et al*., 2004).

Genomic alterations removing CTCF-associated boundaries cause abnormalities in enhancer-gene interactions and modify gene expression (Lupianez *et al*., 2015) due to CTCF binding being methylation sensitive (Flavahan *et al*.,2016). Therefore, cancer-associated hypermethylation is not only involved in gene suppression by promoter hypermethylation, but can alter CTCF localization (Hark *et al*., 2000; Bell and Felsenfeld, 2004), resulting in loss of insulation between domains causing aberrant gene expression (Flavahan *et al*., 2016). It is crucial to note that altered CTCF loops can cause both transcriptional activation and repression, showing the importance of CTFC in genomic organisation and gene expression regulation (Pentland and Parish, 2015).

2.9 Cancer associated hypomethylation.

Global DNA hypomethylation occurs frequently in neoplastic tissues. Loss of maintenance methyltransferases DNMT1 or TET-mediated oxidation of methyl cytosine can cause passive and active DNA hypomethylation respectively (Cortellino *et al*., 2011; Smith and Meissner, 2013). Although promoter CpG islands are often hypermethylated in tumors, DNA hypomethylation is found in neighbouring CpG island shores and repetitive elements (Luo *et al*., 2014). Hypomethylation of intronic and intergenic regions occur early in the transition from normal to neoplastic, indicating the role of DNA hypomethylation in genome instability and cancer initiation (Sheaffer *et al*., 2016). Not only DNA hypomethylation but also promoter hypomethylation can lead to cancer. For instance, hypomethylation of an intergenic CpG island on chromosome 19 is associated with the reactivation of a large microRNA cluster (C19MC) specifically in HCC (Rui *et al*., 2020).

2.10 MiRNAs.

MicroRNAs (miRNAs) are small non-coding RNAs derived from 70-100 nucleotide long precursors molecules consisting of a double-stranded stem loop as well as single-stranded regions (Klug *et al*., 2016; Takahashi *et al*., 2019). These double-stranded stem loop structures are recognized and cleaved by nuclease enzymes in the nucleus during RNA-induced gene silencing (RNAi), revealing the involvement of miRNAs in RNAi gene silencing (Fig.5) (Klug *et al*., 2016). It has been shown that the sequence specific binding of miRNAs to 3’ untranslated region (UTR) of their target messenger RNA (mRNA) can induce transcript degradation or inhibition of protein translation (Takahashi *et al*., 2019). In addition, miRNA can block the translation or facilitate the cleavage of their target mRNA via binding to their coding sequences (Hausser *et al*., 2013; Forman *et al*., 2014; Ito *et al*., 2017). Hence, miRNAs negatively regulate their target genes, silencing gene expression (Takahashi *et al*., 2019). miRNAs are expressed by many eukaryotic plants and animals (Carthew and Sontheimer, 2009). Transcription of miRNAs is carried out by RNA polymerase II (Rossi, 2009). Occasionally several miRNAs can be produced from a single transcript, whereas other miRNAs are individually produced from distinct transcription units (Bartel, 2004). Thus, a transcript can encode either clusters of different

miRNAs or a miRNA and a protein-coding mRNA. Studies of the latter type of locus reveals that the miRNA sequence is often located within an intron of the mRNA (Lu *et al*., 2008; Carthew and Sontheimer, 2009).

The processing of miRNAs can be either from the sense or antisense strand of the gene encoding them and takes place in the nucleus and in the cytoplasm (Rossi *et al.*, 2009). In the nucleus, miRNA genes are transcribed by RNA polymerase II as primary miRNA (pri-miRNA) (Rossi *et al*., 2009; Takashai *et al*., 2019). These primary transcripts are further processed in the nucleus into precursor miRNA (pre-miRNA) by a protein complex that involves a double-stranded RNA (dsRNA) specific ribonuclease Drosha (Banaudha and Verma., 2012). These pre-miRNAs are then exported into the cytoplasm by the nuclear export protein exportin-5 where they are further cleaved into short, linear double stranded miRNAs by the Dicer complex (Klug *et al.*, 2016; Takashai *et al*., 2019). Either the RNA-induced silencing complex (RISC) or the RNA-induced transcription silencing complex (RITS) recognizes the short double stranded RNA molecules and degrades one of the strands. The RISC complex is guided by the anti-sense single-stranded RNA in the RNAi pathway and marks the target mRNA substrates for degradation or inhibiting translation. The mature miRNA binds to complementary sequences in the 3’ UTR or coding regions of its target mRNA, causing mRNA degradation or transcriptional repression (Takashai *et al*., 2019). On the other hand, in the transcription silencing pathway, the RITS complex recognizes the genomic DNA which is complementary to the single strand of the miRNAs. The RITS complex recruits enzymes that alter chromatin structure and suppress transcription (Fig.5). Having impacts on chromatin-mediated gene silencing proposes the involvement of miRNA molecules in epigenetic events including gene imprinting and X chromosome inactivation (Klug *et al*., 2016).

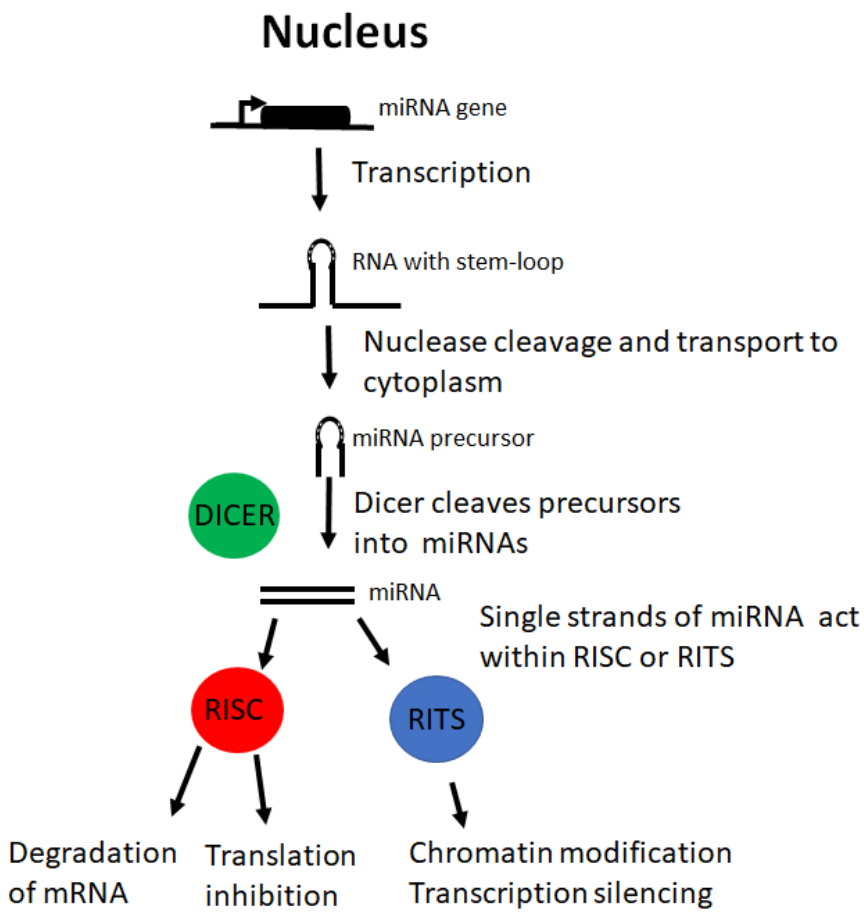


Figure 5 Gene regulation by RNA-induced gene silencing mechanism. (Figure adapted from Klug *et al*., 2016). Stem loop structures of miRNAs are cleaved by nuclease within the nucleus and are transported to cytoplasm. In the cytoplasm, the Dicer complex processes the miRNA precursors into short double stranded RNA molecules. These double stranded RNA molecules are recognized by either the RISC or the RITS complex, resulting in the degradation of one strand. In RNAi pathway, RISC complex guided by antisense single-strand RNA recognizes target mRNA substrates and marks them for degradation or translation inhibition. The RITS complex acts in the nucleus in the transcription silencing pathway and is responsible for recruiting enzymes that alter chromatin and suppress transcription. Degradation of mRNAs, inhibition of translation and chromatin modification result in silencing of gene expression.

2.11 miRNAs and their involvement in cancer.

miRNAs can function as oncogenes or tumor suppressor genes (Gailhouste *et al*., 2013; Takahashi *et al*., 2014; Takahashi *et al.*, 2015; Nezu *et al*., 2016). This is because in addition to gene silencing, miRNAs have crucial roles in several biological processes including cell proliferation (Hwang and Mendell,2006), development (Karp and Ambros, 2005), differentiation (Chen *et al*., 2004; Shivdasani, 2006), metabolism (Wienholds and Plasterk,2005), genome instability (Caffarelli *et al*., 2011) and DNA repair (Chowdhury *et al*., 2013). Therefore, dysfunctional miRNAs cause abnormal cell behaviors and shown to be associated with the development and progression of many human diseases, notably cancer (Lages *et al.*, 2012; Lan *et al*., 2015).

Oncogenic miRNAs and oncogenic epigenetic changes have been reported in HCC (Shen *et al*., 2012; Xia *et al*., 2013). For instance, *miR-216a* and *b* were shown to have upregulated expression and oncogenic behavior in HCC (Xia *et al.*, 2013). *miR-182* was shown to keep *HIF1α* pathway continuously active by targeting *PHD2* and *FIH1* which could facilitate tumor cell adaption to hypoxic stress during prostate tumor progression (Giraldez *et al.,* 2018). However, certain miRNAs have oncogenic activities in one scenario but can be tumor suppressive in another (Svornos *et al*., 2016). For example, *mir-125b* functions as an oncogenic miRNA in several hematological malignancies but as a tumor suppressor in many solid tumors (Shaham *et al*., 2012; Sun *et al*., 2013). The location of cancer cells may determine whether a miRNA has a net oncogenic or net tumor suppressive effect (Svornos *et al.*, 2016).

Aberrant miRNA methylation has been linked with cancer, underlying their biological significance in general tumorigenic processes (Ramasson *et al*., 2018). Initially, 155 out of 332 human miRNAs were found to be regulated by DNA methylation (Weber *et al*. ,2007). In another previous study, following stable depletion of DNMT1 and DNMT3B in a colorectal cell line, *miR-124a*, *miR-373* and *miR-517c* were proposed to be transcriptionally inhibited by methylation (Lujambo *et al*., 2007). Moreover, *miR-9*, *miR-34b/c*, and *miR-418a* were demonstrated to be silenced due to hypermethylation in metastatic cell lines from colon, melanoma, head, neck, primary colon, head, breast, lung

cancers (Lujambo *et al*., 2008). In HCC, several miRNAs including *miR-1*, *miR-9*, *miR-34b*, *miR-124*, *miR-148a* and, *miR-200b* were confirmed to be aberrantly methylated (Furtura *et al*., 2010; Xie *et al*., 2014; Zhang *et al.*, 2015; He *et al.*, 2015). Therefore, miRNAs are regulated by epigenetic modifications including DNA methylation. However, miRNAs can regulate epigenetic machinery at the post-transcriptional level through establishing epigenetic loops. For example, DNMT1 is aberrantly upregulated in breast cancer which results in hypermethylation of *miR-148a* and *miR-152* promoters, forming an epigenetic loop (Braconi *et al*., 2010; Zhang *et al*., 2011; Xu *et al*., 2013).

2.12 Cancer-associated hypomethylation and miRNAs.

miRNA expression can be controlled by DNA methylation although the correlation is not always positive or negative and is region dependent (Manodoro *et al.*, 2014). Even though DNA methylation modifications in CpG islands within promoters have been extensively studied, the dynamical and functional importance of DNA methylation in other regions, for instance non CpG island sites, including gene bodies is not fully understood (Yang *et al*., 2014; Nojima *et al*., 2016). Tumors often have reduced levels of mature miRNAs (Lu *et al*., 2005) due to genetic loss, epigenetic silencing, errors in the biogenesis pathway or widespread transcriptional repression (Jansson and Lund, 2012). Hence, cancer can result from upregulation of potentially oncogenic miRNAs due to cancer-specific miRNA cluster hypomethylation (Brueckner *et al*., 2007; Iorio *et al*., 2007; Nojima *et al*., 2016; Lu *et al.*, 2018). Recently, Lu *et al*. (2018) showed upregulation of *miR-10b-3p* due to promoter hypomethylation, which subsequently resulted in the up-regulation of *FOXO3* in esophageal squamous cell carcinoma. In some cases, hypomethylation and the reactivation of miRNAs is cancer tissue-specific, such as the C19MC in HCC (Nojima *et al.*, 2016).

2.13 C19MC.

A cluster of miRNAs located on human chromosome 19, known as the chromosome 19 miRNA cluster (C19MC) is primate-specific and exclusively expressed in the placenta (Dumont *et al*., 2017). C19MC consists of 46 highly homologous miRNA genes within a 100 kb genomic region. Since it comprises approximately 8% of all known human miRNA

genes, C19MC is regarded as the largest miRNA gene cluster in the human genome (Fig.6) (Bortatin-Cavaille *et al*., 2009; Donker *et al*., 2012). C19MC cluster of mature miRNAs is believed to originate from tandem duplication of one member of the neighbouring *miR-371-3* cluster (Zhang *et al*,. 2008; Fronori and Bullerdiek, 2012; Rui *et al*., 2020).

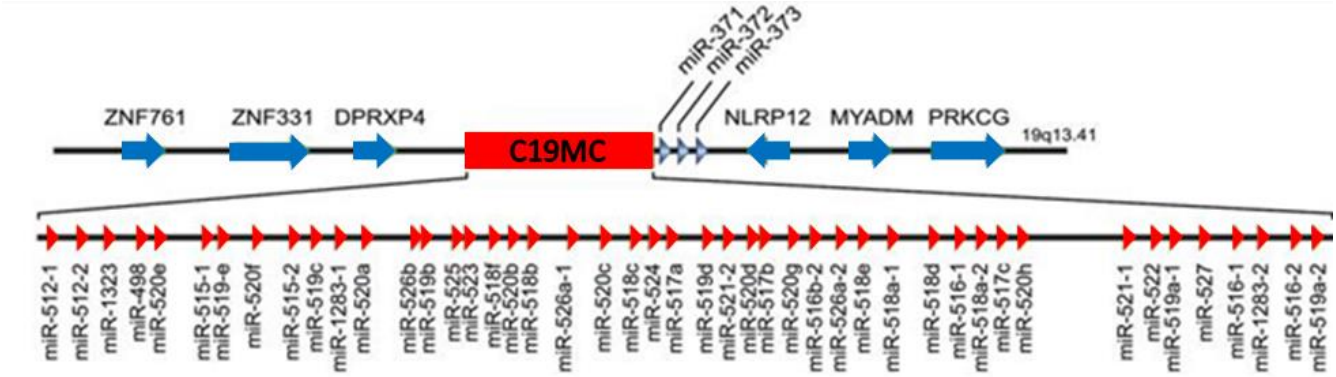


Figure 6 Diagram of C19MC cluster. (Diagram adapted from Donker *et al.*, 2012) C19MC cluster is 100kb long and consists of 46 miRNAs. These miRNAs are exclusively expressed in placenta and regulate gene expression in maternal tissue.

C19MC miRNAs have crucial roles not only in reproduction but also development and differentiation in primates (Lin *et al.*, 2010). Being restrictively expressed in reproductive tissues and not in other adult tissues shows the developmental importance of C19MC (Liang *et al*., 2007; Razak *et al*., 2013). Additionally, C19MC miRNAs can regulate gene expression in maternal tissue and influence maternal physiology when secreted within the exosomes from the trophoblast layer into maternal circulation (Dumont *et al.*, 2017).
In the human placenta, C19MC is expressed *en bloc* from the paternal allele which is controlled by a major promoter located 17.6 kb upstream of the first miRNA in the cluster (Noguer-dance *et al*., 2010). Bortolin-Cavaille and collaegues (2009) suggested that the transcription of the C19MC cluster is controlled by specific methylation dynamics of this upstream CpG-rich promoter region to generate a primary transcript containing the entire cluster. Hypermethylation of both paternal and maternal alleles (Noguer-dance *et al*., 2010), is responsible for the restricted expression profile and silencing in normal somatic tissues (Liang *et al*., 2007; Lin *et al*., 2010). On the other hand, the maternal allele was

found to be unmethylated in the placenta resulting in paternal-allele expression of the pri-C19MC transcript (Tsai *et al*., 2009; Noguer-dance *et al*., 2010). This can subsequently be splicing to produce the individual precursor miRNA species which are later processed by the DGRC-8 Drosha microprocessor complex to generate the mature miRNAs (Bortolin-Cavaille *et al*., 2009). Uniquely, the C19MC miRNAs are related to each other and located within 400-700 bp repeated sequences bounded by Alu repeats (Alus) (Fig.7) (Borchert *et al*., 2006; Nouger-Dance *et al*., 2010).

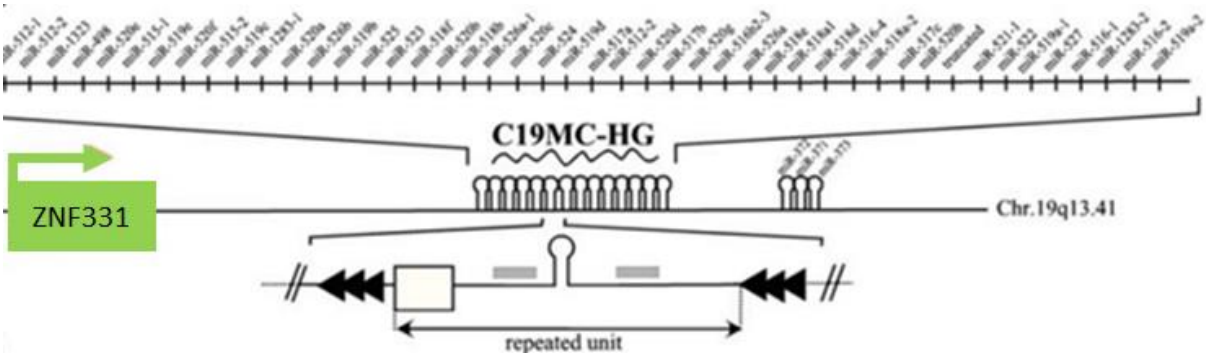


Figure 7 Schematic diagram of C19MC cluster. (Diagram adapted from Nouger-Dance *et al*., 2010). In this diagram, black triangles represent Alu repeats and stem-loop structures represent pre-miRNA genes. Most pre-miRNA genes are localized within repeated introns flanked by Alu repeats.

Borchert *et al.* (2006) suggested that the upstream Alus have Pol-III promoters which drive the expression of the downstream C19MC pri-miRNA genes. However, re-assessment of the organization and expression of C19MC miRNAs suggests it is unlikely that Pol-III-dependent transcription occurs and instead the miRNAs are processed from the large non-coding placenta specific transcripts generated by Pol-II (Bortolon-Cavaille *et al.*, 2009).

2.14 Oncogenic effects of C19MC.

Results of a recent study (Nyguen *et al*., 2017), showed that C19MC miRNAs were selectively activated in cancer cells, suggesting the functional role in promoting cancer development. The re-expression of the *miR-515-3p*, *miR-518a-3p*, *miR-520f* and *miR-525-3p* have all been shown to be selectively over-expressed in HCCs (Augello *et al*.,

2012). Preliminary data from the Monk laboratory demonstrated that C19MC promoter hypomethylation is specific to HCC and occurs in 30-40% of cases and may account for this cancer-associated expression. This is supported by the fact that a comparison of normal and cirrhotic liver tissue revealed that over-expression of *miR-519d* occurs only in the HCC samples with hypomethylation (Fornari *et al*., 2012).

Re-expression often impacts almost all C19MC miRNA members resulting in dynamic interactions between miRNAs and their target genes. Some C19MC miRNAs are associated with oncogenic functions whereas other have tumor-suppressive effects (Flor and Bullerdiek, 2012). Interestingly, several C19MC miRNAs are known as context-dependent miRNAs having both oncogenic and tumor-suppressive properties (Kasinksi and Slack, 2011). C19MC *miR-519a-3p*, *miR-519b-3p* and *miR-519c-3p* were classified as potential tumor-suppressors as they were found to induce cellular senescence by suppressing *HuR* (Marasa *et al.*, 2010; Abdelmhosen *et al*., 2010). However, *miR-519d* was shown to directly target *CDKN1A, PKTEN, AKT3* and *TIMP2*, resulting in promotion of cell proliferation and invasion, and inhibition of apoptosis (Fornari *et al*., 2012).

2.15 The significance of miRNAs in cancer diagnosis and prognosis.

Endogenous circulating miRNAs drew distinctive attention since they have potential applications in the diagnosis, prognosis, and metastasis of cancer (Lu *et al*., 2018). Tumor-derived miRNAs were first discussed in plasma by Mitchell *et al.* (2008) and it has been indicated that investigation of plasma miRNAs is highly promising for clinical uses (Lu *et al*., 2018). Although extensive resources are available for studying the influence of miRNAs on human diseases, there are undiscovered associations between miRNAs and diseases. Thus, it is essential to improve understanding towards the involvement of miRNAs in human diseases (Chen *et al*., 2019).

Despite improvements in tumor diagnosis and therapy, survival rate is approximately 5 years and still remains low (Wu *et al*., 2018). Hence, it is vital to explore underlying molecular mechanisms in order to facilitate the early diagnosis and therapy for cancer. The biological functions and expression of C19MC members in cancer cells have not

been investigated in a cluster-wide manner (Nguyen *et al*., 2017). Since pathogenesis, growth and metastatic spread of tumors have been associated with abnormal miRNAs expression, miRNAs have been suggested to be novel potential, diagnostic or predictive biomarkers in HCC (Augello *et al*., 2012; Lin *et a*l., 2012; Vaira *et al*., 2015). Therefore, studying changes in miRNA expression could help not only to improve diagnosis and prognosis but also provide molecular targets for new therapeutic strategies against HCC (Augello *et al.*, 2018).

2.16 Introducing epigenetic manipulation.

Less than 3% of the human genome is made up of gene coding regions (Encode proj. Consort, 2012) and intergenic or intronic regions consist of approximately 90% of the single-nucleotide polymorphisms (SNPs) which are associated with human diseases (Hindorff *et al*., 2009). Targeted deletions and mutations in intergenic regions, for instance enhancer, have significant impacts on gene expression, suggesting that there is a regulatory network between coding and non-coding regions of the genome (Korkmaz *et al*., 2016).

Epigenetic components including DNA methylation, histone modifications, chromatin accessibility and DNA architecture are significantly associated with cellular processes, hence their dysregulation alters gene expression and cause disease (Ernst *et al*., 2011 ;Rao *et al*., 2014;.Kundaje *et al*., 2015; Merkenschlager and Nora, 2016; Yin *et al*., 2017). Selective modification of epigenome not only improves our understanding of the function of epigenetic modifications but also allows manipulation of cell phenotype for research or therapeutic purposes (Holtzman and Gersbach, 2018). The improvements in genome-editing tools that can target specific DNA sequences with increased precision and efficiency has led to development of targeting platforms comprising mainly zinc fingers (ZFs), transcription activator-like effectors (TALENs) and the CRISPR/dCas9 system (Gaj *et al*., 2013). These platforms are different from each other in terms of ease of use, implantation, and flexibility (Thakore *et al*., 2016). The Monk laboratory has an interest in epigenome editing tools encompassing the CRISPR/dCas9 system since it is relatively easy to use and a flexible platform (Holtzman and Gersbach, 2018).

2.17 Epigenetic manipulations by non-specific small molecules.

Genetic manipulation techniques involve use of non-targeting small molecule inhibitors. Small molecule libraries are collections of chemical compounds which have biological activity. Small molecule libraries have been extensively used in various high-throughput screens with the aim of identifying targets associated with certain effects. The use of small molecules, primarily in the pharmaceutical industry, has led to the discovery of wide range of drugs including mevastatin and cyclosporin A. Some of these drugs target enzymes which deposit epigenetic marks and are predominantly used for research and anticancer treatment purposes. DNMT1 and DNMT3 inhibitors azacitidine (5-azacitidine, 5-aza) and decitabine (5-aza-deoxyctidine, 5-aza-DC) as well as histone deacetylase (HDAC) inhibitors suberoylanilide hydroxamic acid (SAHA) and romidepsin (depsipeptide ir FK228) are some of the commonly used small molecules in the clinic (Holtzman and Gersbach, 2018). Since these compounds lack specificity, they may cause unknown effects at other loci and tissues. Additionally, lack of specificity limits the dose range which they can be effectively used, limiting the potency of inhibition (Holtzman and Gersbach, 2018).

2.18 Locus-specific examples, ZNF- fusions.

To overcome the non-specific nature of small molecular remodelling of DNA methylation, DNA-binding zinc finger proteins (ZNF) were utilized in targeted editing, initiating a new era not only in genomic but also in epigenomic manipulation (Urnov *et al*., 2011; Adli, 2018). These DNA binding proteins consisting of protein motifs or fingers recognize and bind to three DNA nucleotides (Urbano *et al*., 2019). DNA-binding domains (DBD) are engineered in ZNFs, hence they can recognize specific target nucleotide sequences (Urnov *et al*., 2011). Combination of different ZNF modules are used based on their respective affinities for a specific three base sequence so that specific genomic regions can be targeted (Urbano *et al*., 2019). Therefore, DBDs of ZNFs are mainly fused with a nuclease or other effector protein to mediate a site-specific genetic or epigenetic response (Urnov *et al*., 2011; Grimmer *et al*., 2014; Chatterjee and Eccles, 2015; Adli, 2018). However, since each ZNF-fusion requires a unique set of ZNF modules to

recognise DNA, each region to be targeted needs separate ZNF-fusion which requires a huge cloning effort.

2.19 Epigenetic manipulations, TALEN-fusions.

Transcription activator-like effectors (TALENs) are also DNA-binding proteins whose DBDs are designed to target specific nucleotide sequences (Christian *et al*., 2012). TALEs were isolated from the *Xanthomonas* bacteria and were next developed for targeted editing (Adli, 2018). TALEs are dimeric transcription factors or nucleases made from arrays of amino acid modules. Like ZFNs, fusion of Fok I DNA cleavage domain to a combination of TALE modules produce an effective nuclease called TALENs (Li *et al,*. 2011). Like ZNF proteins, TALES enable sequence-specific DNA binding, yet TALES can bind individual bases at a target locus. Also similar to ZNFs, TALES can induce a certain effector response at a targeted locus when fused with specific effector proteins (Joung *et al.,* 2013; Adli, 2018), but suffer from the same disadvantages.

2.20 Epigenetic manipulations, dCas9-fusions.

Although ZNF and TALE-based technologies enable genomic and epigenomic editing at a single locus, these techniques are difficult and laborious as each targeting site requires a full re-design and re-engineering of a new set of proteins. On the other hand, CRISPR-based technologies have simpler and easily targetable systems and provide a better level of editing efficacy (Urbano *et al*., 2019).

The first CRISPRs were detected 33 years ago during the analysis of the gene responsible for isozyme conversion of alkaline phosphates in *Escherichia coli* (E. *coli*) (Ishino *et al*., 1987). The CRISPR/Cas9 system was first explored as an adaptive immune response mechanism of bacteria against invading viruses (Mojica *et al*., 2005). The CRISPR loci consists of a clustered set of *Cas* (CRISPR associated) genes surrounded by identical repeat nucleotide sequences with spacer in between (Jansen *et al*., 2002). The nucleotide spacers were obtained by Cas enzymes from exogenous protospacers following the invasion of viruses. If the same virus invades, the Cas9 cleaves the same specific genetic element as the spacers recognize and target the same foreign agent.

Consequently, double-stranded cleavage is induced in the foreign DNA as an adaptive immune response (Doudna *et al*., 2014).

The CRISPR- based tool modulating DNA methylation at a target locus is represented by the fusion of the effector protein component to the CRISPR-deactivated Cas9 (dCas9) targeting protein (Adli, 2018). For instance, DNMT3A enzyme has been fused to dCas9 for targeted methylation editing (Fig.8A). Moreover, the fusion of TET dioxygenase enzyme with dCas9 allows selective demethylation of the epigenome (Fig.8B) (Urbano *et al*., 2019). Apart from selective methylation and demethylation, CRISPR-dCas9 system is also used to manipulate distal regulatory elements. Hilton *et al*. (2015) showed that CRISPR-dCas9 based acetyltransferase results in the activation of genes from promoter and enhancers manipulations, suggesting dCas9-fusions have a diverse range of potential uses (Hilton *et al*., 2015).

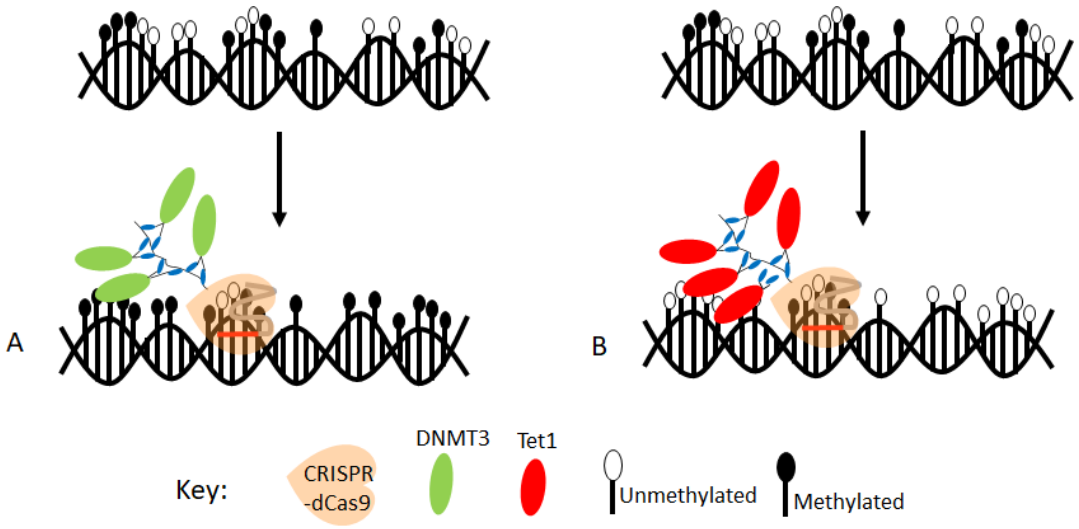


Figure 8 CRISPR/Cas9 for selective methylation and demethylation. (Figure adapted from Urbano *et al.*, 2019) The light orange image represents the CRISPR-dCas9 protein complex which is required along with a unique guide RNA sequence fused to the single-guide RNA plasmid construct for targeted editing. A) For locus-specific methylation, the epieffector DNMT3A (green) catalyzes the addition of methyl groups. B) For locus-specific demethylation, the epieffector used is Tet1 (red) enzyme which removes the methyl marks (Urbano *et al.*, 2019).

2.21 The principles of dCas9 epigenetic targeting.

The best characterized system for genome and epigenome manipulations, as described above is the type II CRISPR system used by *Streptococcus pyogenes.* This system involves the Cas9 nuclease, a CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA). The crRNA hybridizes with the tracrRNA recruiting Cas9 and binds to foreign protospacer elements (Ran *et al*., 2013). The two RNAs can be joined forming a chimeric, single-guide RNA (sgRNA) (Jinek *et al*., 2012). Modification of this guide RNA (gRNA) molecule through alteration of the 20 bp guide sequence in the spacer can direct Cas9 to almost any target (Urbano *et al*., 2019). In the CRISPR-Cas9 system derived from S.*pyogenes*, the target sequence is required to immediately follow a 5'-NGG protospacer-adjacent motif (PAM). PAM recognition is essential for ATP-independent strand separation. In addition, PAM recognition is required for gRNA complexing with target genetic elements (Jinek *et al*., 2012).

For epigenome manipulation, the underlying DNA sequence does not need to be cleaved. Thus, the Cas9 nuclease is deactivated for removal of the catalytic activity. Single-amino acid substitutions of Asp10 to Ala10 and His840 to Ala840 create nuclease-deficient dCas9 (Doudna, 2014). Ongoing researches aim to optimize and improve the use of CRISPR-deactivated Cas9 (dCas9) for targeted editing (Urbano *et al.*, 2019). The fundamental requirement for CRISPR dCas9 fusion for epigenome editing comprises of three vital parts: a DNA-binding targeting protein, an effector protein, and a unique gRNA sequence (Fig 9). The CRISPR-dCas9 system is an optimal targeting protein complex since it can be targeted by gRNAs to multiple sites and is insensitive to CpG methylation (Perez-Pinera *et al*., 2013; Hilton *et al*., 2015). I took the advantage of the fact the Monk laboratory has cloned several dCas9-fusion constructs, including dCas9-DNMT3-CD and dCas9-TET-CD that contain the minimal catalytic domains of methyltransferase and demethylases respectively, for my studies.

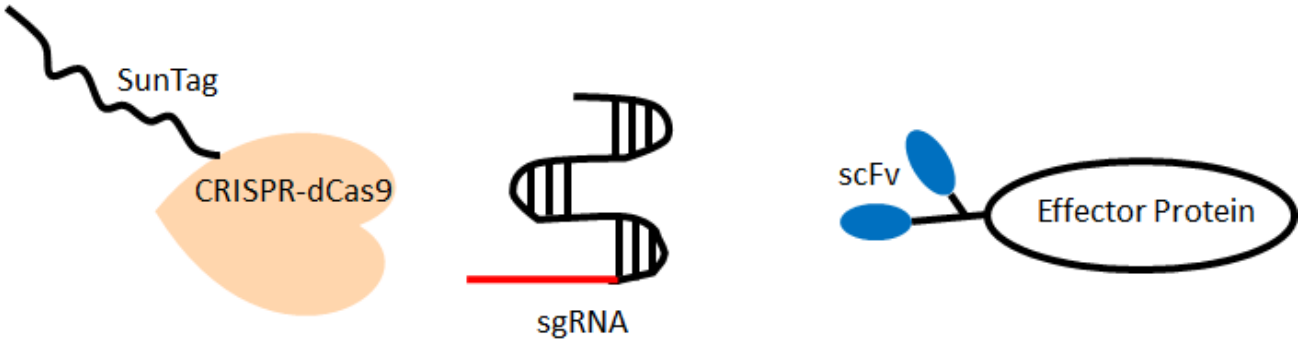


Figure 9 CRISPR/dCas9 components. (Figure adapted from Urbano *et al.*, 2019) The CRISPR-dcas9 protein complex, a unique gRNA sequence fused to the sgRNA and the effector domain are required for selective methylation or demethylation. dCas9 serves as a DNA binding domain (Enriquez,2016). The effector domains are DNMT3A or TET for methylation and demethylation, respectively. dCas9 protein can be fused to repetitive peptide epitopes (SunTag) to recruit multiple copies of antibody-fused effector domains (Huang *et al*.,2017).

3. The aim of this project.

The goal of this study was to examine the oncogenic effects of the C19MC cluster in hepatocellular carcinomas through epigenetic manipulation. Taking the advantage from previous studies in the Monk laboratory, I investigated the links between aberrant DNA hypomethylation in HCC cell lines that drive the re-expression of oncogenic miRNAs. I focused my experiments in HCC that have retained hypermethylation of the C19MC promoter, including HepG2 and HLF. Specifically, I tried to:

(1) Profile C19MC methylation and determine miRNA expression in HCC cell lines.

(2) Re-express C19MC associated miRNAs using small molecular inhibitiors 5-aza-deoyctidine (5-aza-DC) and Trichostatin A (TSA) which results in global demethylation and histone acetylation, respectively (Mossman *et al*., 2010).

(3) Generate stably expressing HLF cell containing the dCas9-Tet1 construct to demethylate the C19MC promoter in a targeted fashion through the use of multiple gRNAs.

(4) Determine the effect of specific C19MC miRNA re-expression using miRNA mimic.

(5) For the cells generated in aims 3 and 4, assessed cellular behavior, migratory potential and invasiveness by colony forming assays, scratch tests and trans-well chamber assays, respectively.

4. Materials and Methods.

4.1 Cell Culture Protocols.

4.1.1. Growing cell lines.

Human hepatocellular carcinoma cell line HLF was purchased from American Type Culture Collection and HepG2 was a gift from Prof Manel Esteller, IDIBELL, Barcelona. The cells were cultured in Dulbecca's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and 1% penicillin- streptomycin (P/S) at 37°C and 5% CO_2. Cryopreserved cells were rapidly defrosted to room temperature, resuspended in 5ml of growth media and centrifuged at x1100g for 5 min. Following centrifugation, the media was removed, and the cell pellets were resuspended in an appropriate volume of growth medium and dispensed into sterile flasks. During passaging of cells, 3 ml per 25 cm^2 of trypsin-EDTA was added to the culture flasks once the culture media had been removed and the flasks were incubated for 5 min. Once the cells were detached, 3 ml of DMEM media containing FBS was added to inhibit the reaction and recentrifuged to collect the cells (x1100g for 3 min). The media was subsequently aspired, the cell pellets resuspended in appropriate volume of growth medium and seeded in sterile flasks.

4.1.2. 5-aza-DC treatment, optimization of concentration and duration.

To facilitate global demethylation, HLF cells were incubated with 5-aza-DC (Sigma-Aldrich). The stock solutions were stored at –80°C and the various working concentrations of 5-aza-DC were prepared fresh daily by diluting in PBS. To determine the optimal concentration for my experiments, I performed MTT to identify the IC50 and cell counts. Five different concentrations of 5-aza-DC were compared to a control (0 μM); 0.5 μM, 1 μM, 2.5 μM, 5 μM and 10 μM for either 24h, 48h, 72h and 96h. HLF cells were seeded in 6-well plates and when at ~40% confluency the 5-aza-DC treatments began. Cell counts were performed using 10 μl of trypsinized cells, the remainder stored at -80°C for subsequent DNA extraction.

MTT assay was carried out to assess cell viability. HLF cells were seeded in 96-well plates with each well containing 10,000 cells in 100 μl of media. Thiazolyl blue tetrazolium bromide (Sigma-Aldrich) of 0.1 g was dissolved in 20 ml of PBS 24h before the MTT

assay. The media in 96-well plate was removed and 100 µl of DMEM media and 100 µl MT2 solution was added to each well. After 3h of incubation, the media was once again removed and 250 µl of DMSO was added. The plate was put on a rocker for 30 min to allow the crystal to dissolve. A plate reader was used to determine the absorbance at wavelength of 560 nm as the amount of absorbance is proportional to the cell number.

4.1.3 Combined 5-aza-DC and TSA experiment.

When the laboratories reopened following the COVID-19 lockdown, I regrew and stabilized my HLF cells. The cells were then treated with 5-aza-DC alone, in combination with TSA and TSA alone (Sigma, Aldrich) to induce global demethylation and histone deacetylation, respectively. HLF cells were seeded when at 60% confluency and 24h prior to the treatment. The cells were added with 10 µM of 5-aza-DC and incubated for 3 days, 7 days and 10 days. Previous studies showed that 100-400 nM TSA in combination with 5-aza-DC (for the last 24h of the culture) results in high expression (Mossman et al., 2010; Zych et al., 2013). Hence, we added 100 nM of TSA at the last 24h before harvesting cells for DNA and RNA extraction. The culture media was replaced every 24h with fresh media containing 5-aza-DC.

4.1.4. Transfections with Lipofectamine, PEI and JetPrime.

Initially, common transfection reagent Lipofectamine (Invitrogen) (10 µl for 5 µg of DNA in 250 µl DMEM) was used to deliver the dCas9-TET-CD construct into HCC cell lines. Green fluorescent protein (GFP) was used as a marker for detecting transgenic expression as our initial construct contained T2A-GFP. Transfection controls included the pMax-GFP plasmid. The transfected cells were incubated for 48h and analysed under a fluorescent microscope. In addition, dCas9-TET-CD fusion was delivered into the cells with Polyethylenimine (PEI) solution (120 µl PEI solution for 10 µg dCas9 plasmid in 500 µl DMEM). In addition to the dCas9-TET-CD-T2A-GFP plasmids, we also used a construct in which GFP had been replaced with the puromycin-N-acetyltransferase gene. Following 72h of incubation with this second plasmid, the transfected cells were seeded into new plates and exposed to media containing puromycin (ThermoFisher Scientific) (DMEM+ 10% FBS + 1% FBS + 400 µl puromycin) and cells were analyzed 72h later.

Lastly, JetPrime (Polyplus transfection agent) was used for dCas9-TET-CD delivery (200 µl JetPrime buffer and 4 µl of JetPrime reagent for 2 µg of DNA) to try and find the best delivery agent for these large constructs.

4.1.5 Infections of dCas9-TET lentivirus.

Lentiviral transfer of pLV hUbC-dCas9-TET-T2A-GFP and Fuw-dCas9-TET-CD (Addgene 84475-LVC, 1.8x10^8 TU/ml), containing 5'LTR sequences flanking the transgenes, into the HCC cell lines was utilized. HLF cells were seeded into 6-well plate in 2 ml media 24h prior to lentiviral infection. Lentivirus of 15 µl was added to 100 mg/ml polybrene containing culture media and each well was added with 500 µl Lentivirus/Polybrene mixture. The virus treated HLF cells were cultured for 48h and were seeded into a 6-well dish and 10 cm plates. The cells in 6-well dish were then infected with Fuw-dCas9-TET-CD lentivirus for the second time. Media was replaced with complete DMEM 48h after infections. The cells were grown for 2 weeks to prevent transiently expressing cells masking the stable cell lines. Colonies were split so that cultures would be maintained while DNA was extracted for dCas9 PCR.

4.1.6 miRNA mimics.

For miRNA over-expression experiments we used chemically synthesized miRCURY LNA miRNA mimic (Qiagen)(Fig.10). The *miR-512-3p* mimic of 5 nmol was initially resuspended in 75 µl of H_2O to yield a concentration of 66.67 µM. Stock solutions of 50 nM were stored at -20°C. JetOptimus DNA transfection reagent (Polyplus transfection) was used to transfer the mimic into HLF cells. The cells were seeded into 6-well plates 24h prior the transfection. To each well we added with 200 µl of JetOptimus buffer, 3 µl of JetOptimus transfecting reagent and 1.5 µl of the mimic. We used Cel-mir-39 control mimic in addition to control HLF cells that were exposed to JetOptimus with cargo DNA. The cells were incubated for 48h for scratch assay and RNA extraction.

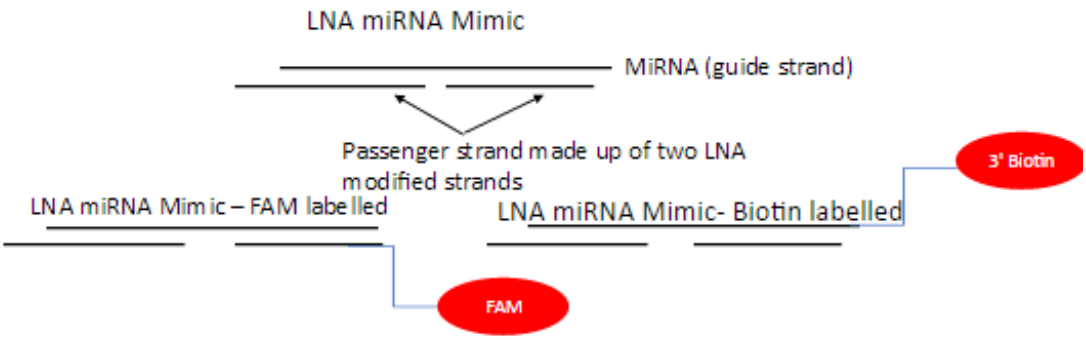


Figure 10 miRCURY LNA miRNA mimic. Generally, the mature miRNA mimics involve unique triple RNA design; the guide strand and a passenger strand made up of two LNA modified strands. The miRNA strand sequence is complementary to miRBase annotation. Mimics with the fluorescent label (FAM) help to assess transfection efficiency and mimics with biotin label enable to isolate targets by RNA pull down. In our study, the mimic did not contain either the FAM or the biotin labelling. To summarize, the use of tree RNA strands makes sure that only the miRNA strand is incorporated into the RISC complex with no miRNA activity form the two complementary strands.

4.1.7 Scratch Test.

Scratch assay was performed to study cell migration. HLF cells were seeded into 6-well dishes in 2 ml complete media and incubated for 24h to create a 70-80% confluent monolayer. The cell monolayer was scratched with a p20 pipette tip across the center of the wells. This was followed by washing the cells with media twice to remove debris. The cells were added with 2 ml complete media with 1% FBS. This was done to limit proliferation and growth for additional 48h. The cells were then washed twice with PBS and fixed with 3.7% paraformaldehyde for 30 min. The fixed cells were stained with 1% crystal violet in 2% ethanol for 30 min. The gap distance was observed with a phase-light microscope.

4.2 Bioinformatics.

Human primers were designed for amplification of target regions in cDNA, genomic DNA or bisulphite converted DNA (Table 1). USCS genome browser (https://genome.ucsc.edu/) was used to identify specific sequences.

4.2.1. Primer Design- RT-PCR primers

RT-PCR primers (22-24 bp in length) (Table 1) were designed to span introns or exon-exon junctions and encompass an amplicon of ~150 bp in regions without SNPs. Following designing, the primers sequences were run through BLAT (https://genome.ucsc.edu/cgi-bin/hgBlat) for sequence alignment.

4.2.2 Primer Design- Bisfulphite PCR primers

To design primers for bisulphite converted sequence, the genomic sequence of interest was changed to the predicted sequence after conversion, using find/replace function in word. Specifically,

CG > XG (e.g. AAAGGGCGCGCCCCGGA > AAGGGXGXGCCCCGGA)

C > T (e.g. AAAGGGCGCGCCCCGGA > AAAGGGTGTGTTTTGGA)

XG > CG (e.g. AAAGGGXGXGCCCCGGA > AAAGGGCGCGCCCCGGA)

Since no online tool is available for designed bisulphite PCR primers, multiple primer pairs were designed to each loci ensuring that as much complexity was included in the primer sequences (as there would be significantly less C present) and avoid long stretches of mononucleotide Ts (Table 1).

Table 1 Primer sequences. Table shows sequence of the primers we used during the project including those for bisulphite PCR, RT-PCR and dCas9 selection.

Loci/PCR name	Forward Primer Sequence	Reverse Primer Sequence
C19MC outer	AATGTTAGGTTTATTTATTTTTTGT	TTTTTTTTTGAGGGATTAGAATTTG
C19MC inner	GTTATTTGGAATTAATATTTTGG	GTTATTTGGAATTAATATTTTGG
C19MC-2 outer	TGGGGAAAAAAAGGGTAGTTT	GTGTTTGTTTGTTTGTTGAGATTTTTGTG
C19MC-2 inner	GTAATTTTAGTATTGGAGGAG	GGGTAATATAGTGAGATTTTT

C19MC pugi	TGTTTGGAACGGGGTTGTTTATGTA	CCCTCAAAAAAAAACCAAAATATTAATTC
Pri-C19MC RT	TGCCTTGCTACTTCAAGCAGT	CGAAAGTTGCATCTGTACGACTGG
pGEMT insert	GATGGTGCTGCAAGGCGATTAAGTTG	ATGTTGTGTGGAATTGTGTAGCGGA
RPL19 RT	GCGGAAGGGTACAGCCAAT	GCAGCCGGCGCAAA
RNU6B RT	CTCGCTTCGGCAGCACA	AACGCTTCAGAATTTGCGT
JMJDC1	TTCCTTAAATGGATACAGAGTGAGAG	GGATCGACATTCTGTGGTTCT
LATS1	TGGACACACGATTCTAAGTAC	CAACCAAAGAATGTGCTAGAC
KATA6	GCCAGAGGAACTCATCTCCTC	TCTCGACAGGAGCTGCATGTT
dCas9 plasmid	GTCTAATTTCGACCTGGCAGAG	TGGTGGTGCTCATGATACCGCT

4.2.3 Statistical Analysis

IBM SPSS Statistics Version 23 (IBM Corp,2015) was used for all statistical analyses. One tailed and two tailed t-test was performed where appropriate. One tailed t-test was used to look at differences in groups in a specific direction whereas two tailed t-test was used to study the possibilty of both positive and negative differences.

4.3 Experimental Protocols.

4.3.1. Extracting RNA.

Total RNA was isolated from all cell lines using the Invitrogen TRIzol Reagent (ThermoFisher) according to the manufacturer's instructions with a few modifications. The cell pellet was resuspended in 1 ml of Trizol and left at room temperature for 20 min before the addition of 500 µl of chloroform. Following centrifugation, 400 µl of the aqueous phase was transferred to a new Eppendorf tube and 320 µl of isopropanol was added. The tube was centrifuged for 1h at 11,000 g/4°C and the RNA pellet washed in 70% ethanol. A repeated spin step ensured the RNA was washed and the pellet was dried and finally

resuspended in DEPC-H_20 or TE. RNA concentration was determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and the RNA was stored at -80°C until use.

4.3.2. Extracting DNA.

Genomic DNA was isolated by the standard phenol/chloroform extraction. Cell pellets were washed with PBS and centrifuged at 1000 rpm for 5 min. The pellets were resuspended in 1 ml of Lysis buffer to which proteinase K and SDS was added. This was incubated at 37°C overnight on a heat block. The following day, an equal volume of phenol/chloroform was added to the cell lysates into phase-lock gel tubes (Prime5) and mixed. To separate organic and aqueous phases, centrifugation was performed at 1000 rpm for 5 min. The phenol/chloroform extraction was repeated 3 times until a clean aqueous phase was obtained. Three subsequent phase-lock spins with chloroform only were performed. Genomic DNA was precipitated by adding 1 in 10 of the volume of 3 M AcNa and 2.5 volumes of 100% EtOH. Genomic DNA pellets were washed once with 70% EtOH and air-dried. Dried pellets were resuspended in TE or H_2O. The quantity and purity of the DNA was determined by measuring absorbance at 260 nm (A260) and 280 nm (A280) using a NanoDrop 2000 Spectrophotometer (Thermo Scientific). An A260/280 ratio of 1.8-2.0, indicates DNA free of contaminating phenol or protein. All genomic DNA was stored at -20°C until use.

4.3.3. Making cDNA using random primers for mRNA expression.

cDNA for gene expression utilized 1 µg of total RNA in a 20 µl reaction volume. RNA was first treated with DNase I, Amplification Grade (ThermoFisher) according to the manufacturer's instructions. Following the inactivation of DNase I by 25 mM EDTA, the RNA in a volume of 11 µl was heated for 5 min at 70°C. Subsequent random primer RT was then carried out using M-MLV Reverse Transcriptase (Promega) in a Veriti 96-Well Thermal Cycler (Applied Biosystems). An RT-negative control of cDNA synthesis (omission of the MMLV RT) was performed to detect possible contamination with genomic DNA. Before use in qPCR, the integrity and efficiency of RT conversion was assessed by standard RT-PCR using *RPL19* house-keeping gene.

4.3.4. miRNA specific cDNA synthesis and qRT-PCR

The TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems) was used to generate miRNA compatible cDNA for the miRNA amplification in the HCC cell lines. The cDNA synthesis was performed according to the manufacturers' protocol and included four different reaction steps: the poly(A) tailing reaction, adapter ligation, reverse transcription (RT) reaction and the miR pre-Amp reaction (Fig.11). Since the Advanced assays do not allow for normalization to *RNU6B*, the reverse primer along with *RNU6B* and *RPL19* were "spiked-in" the RT step. Relative expression levels of *RPL19 and RNU6B were used to check the effciency of the RT reaction.* Afterwards, expression levels of *miR512-3p*, *miR-518f-5p*, *miR-520d-5p*, *miR-525-5p*, *miR-517-3p* and *miR-122-5p* were quantified using TaqMan Advanced miRNA Assays (Applied Biosystems) according to the manufacturer's instructions. The qPCR reactions were carried out in quadruplicate for each miRNA and endogenous controls on a 96-well plate and amplified in a 7500 Fast Real-Time PCR system.

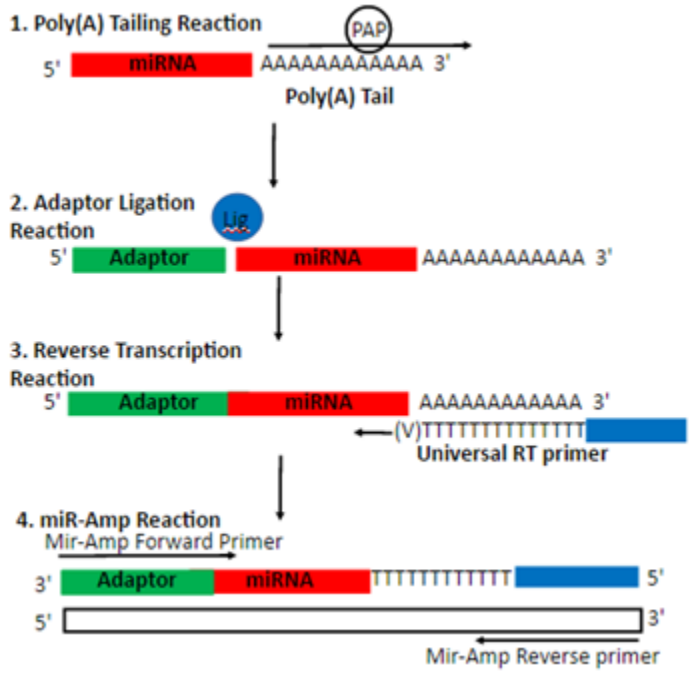


Figure 11 cDNA template preparation. The first step of cDNA synthesis is the addition of 3'-adenosine tail to the miRNA by Poly(A) polymerase. Poly A tail added miRNA undergoes adaptor ligation at the 5' end. The adaptor serves as the forward-primer binding site for the miR-Amp reaction. The third step involves binding of a universal RT primer to the 3' Poly (A) tail, reverse transcribing the miRNA. CDNA is created through reverse transcription. The last step is miR-Amp

reaction. This step uses universal forward and reverse primers to increase the number of cDNA molecules.

4.3.5. RT-PCR Optimization.

With the aim of optimizing RT-PCR, reactions were performed in a MicroAmp Fast Optical 96-Well Reaction Plate using either SYBR green (for mRNAs and pri-miRNA) or advanced taqman probes (miRNAs). A template dilution series of 5 µl of 1:10, 1:100 and 1:1000 was set up so that correlation coefficient slopes and melting curve could be generated to ensure reactions worked efficiently.

4.3.6. qRT-PCR deltaCT.

The delta-delta Ct method (devised by Livak and Schmittgen, 2001) was used to obtain the relative fold miRNA/gene expression of the control and HCC samples when performing qPCR. Ct stands for the cycle threshold and represented the cycle number where the fluorescence produced by the PCR product can be distinguished from the background noise. Delta Ct is the difference in Ct values for the gene of interest and the housekeeping gene. In this study, the average of *RLP19* and *ACTB* genes was used to normalize pri-C19MC and target mRNA gene expression levels. Moreover, we initially normalized mature miRNAs to *RNU6B*. However, *RNU6B* has been reported to be unsuitable for normalization as it is highly variable in HCC and liver samples (Lamba *et al*., 2014; Lou *et al*., 2015). Therefore, we selected another two control miRNAs as normalizers which were less variable. Labma *et al*. (2014) recommend normalizing miRNA against *miR-152* and *miR-23b* in HCC samples, thus we normalized our miRNAs not only to *RNU6B* but also *miR-152* and *miR-23b*. An in-house excel template with all the required calculations was used to obtain the Ct values which was in turn used to quantify pri-C19MC and mature miRNAs in the HCC cancer cell lines and control liver samples.

4.3.7. Bisulphite conversion.

DNA Methylation Kits from ZYMO Research were used for bisulphite conversion. In general, we used 1 µg of DNA as a template and following manufacturers' protocol. To

20 µl of 1 µg DNA, 130 µl of CT conversion kit was added and the reaction was incubated in a thermocycler. Binding Buffer was added to a Zymo-Spin IC Column with the corresponding collection tubes. Samples were loaded into the columns and mixed by inverting the column several times. After centrifuging at 10,000 g for 30 sec and discarding the flow-through, washes were performed with 100 µl of M-Wash Buffer and same centrifuge conditions. To finish the conversions in which all non-methylated cytosines are deaminated to uracil (Fig.12), we added 200 µl of M-Desulphonation buffer to each column and incubate at room temperature for 20 min. After the incubation, we centrifuged at 10,000 g for 30 sec and repeated the washing step twice, this time, with 200 µl of M-Wash Buffer. Finally, the columns were placed into a 1.5ml microcentrifuge tube and performed a double elution with 10 µl of M-Elution Buffer by centrifuging 11000 g for 30 sec. BS-converted DNA was stored at -20ºC until use.

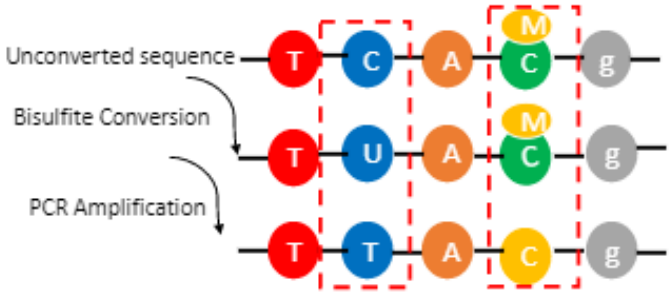


Figure 12 Bisulfite Conversion. This technique is widely used to determine pattern of methylation in which unmethylated cytosine (C) is deaminated to uracil whereas methylated cytosine remains intact. Thymines (T) and guanines (G) are not affected throughout the process. During PCR amplification, urcails (U) are recognized as thymines (T) while methylated cytosines are recognized as cytosines (as indicated by red squares). This therefore enables to distinguish methylated residues from unmethylated residues.

4.3.8. PCR Reactions.

For standard PCR, we used around 50 ng of genomic DNA or the cDNA equivalent of 50-100 ng RNA (generally 1 µl of cDNA) as template in a 13 µl reaction using Biotaq Taq-polymerase (Bioline). For all amplifications using Bisulphite-converted DNA or other difficult templates, we utilized hot-start Immolase Taq-polymerase (Bioline) in 13-25 µl reactions. All PCRs performed included a non-template negative control for amplification

from contamination sources. For Immolase enzyme-based reactions an initial denaturation step of 96°C for 10 min was required for enzyme activation and if the PCR was performed for methylation analysis, 45 cycles were required to obtain suitable amplification for downstream applications such as sequencing or cloning (Fig.13).

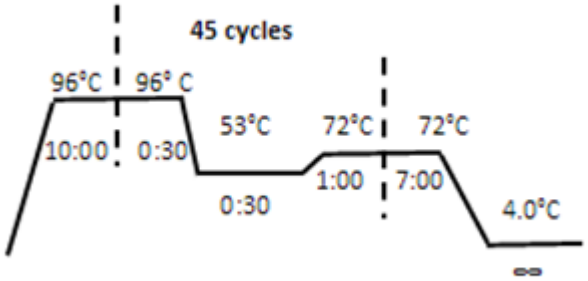


Figure 13 PCR conditions. The PCR conditions with Immolase Taq Polymerase were: one cycle of 96°C for 10 min, 45 cycles of 96°C for 30 sec, 53°C for 30 sec and 72°C for 1 min, followed by one cycle of 72°C for 7 min.

4.3.9 Cloning of bisulphite PCR products for strand-specific, base-pair resolution methylation.

PCR sub-cloning was performed in T-Vector Systems since some thermostable DNA polymerases add a single A nucleotide to the 3' end of blunt DNA, producing a "sticky-end" PCR product ideal for 3' T vector cloning. The A-tailed PCR products were directly ligated to the pGEM-T easy vector (Promega) (Fig.14) using ~3.5 µl of PCR product + H20, 1 µl (50 ng) of vector, the appropriate amount of ligation buffer and 1 µl of T4 DNA ligase. The ligation reaction was left at 4ºC overnight and used later for bacterial transformation or stored at 4°C until use.

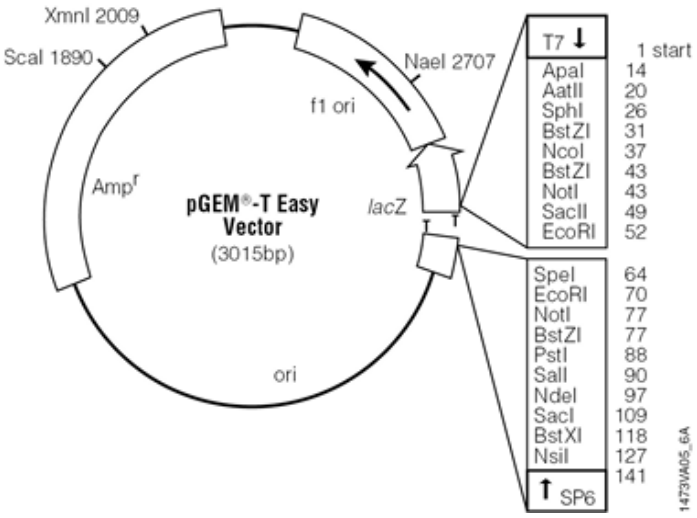


Figure 14 pGEMT-easy vector Map. Taq Immunolase amplified PCR products were inserted in pGEMT-easy vector which contains T7 and SP6 RNA polymerase at a multiple cloning region within alpha-peptide coding region of beta-galactosidase. 3'-T overhangs at the insertion sites allows efficient ligation of a PCR product into the plasmid as the overhangs provide a matching overhang for PCR products.

The ligations were transformed into E.*coli* competent cells (either JM109s of DH5α) by heat shock (30 min in ice, 45 sec at 42°C and 2 min in ice) followed by a growth in LB without antibiotic for 30 min-1h shaking at 37°C. The transformed cultures were then spread on LB-agar plate with Ampicilin, X-Galactose (Promega) and IPTG (Sigma Aldrich) and grown at 37°C overnight. The vector allows for blue/white selection based on hydrolysis of β-galactosides. Positive white clones were picked and grown in 50 µl of LB-media without ampicillin for an hour. A selection PCR with primers designed to flank the T-cloning/multiple cloning sites was performed with 1 µl of the culture acting as the template. All appropriately sized amplicons were sequenced by Sanger sequencing using M13F or T7 primer located immediately internal to the PCR oligonucleotides.

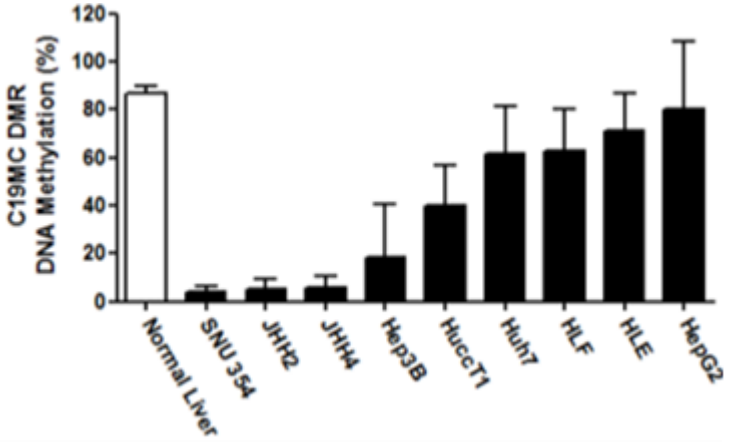
5. Results.

5.1 Initial methylation and expression observations in HCC cell lines.

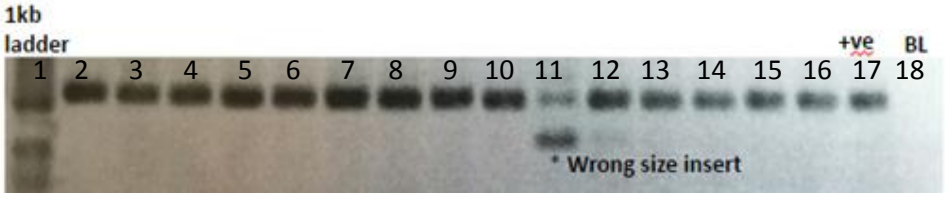
5.1.1 DNA methylation profiling.

I initially characterized the C19MC promoter methylation in nine HCC cell lines and normal liver samples through bisulfite PCR. We used a placenta control for a partial

methylated control since one-allele is methylated. These were dependent on either pyrosequencing, direct Sanger sequencing or cloning of individual DNA strands. C19MC promoter is silenced in normal liver, hence we used normal liver biopsies as controls. This revealed that SNU354, JHH2 and JHH4 were aberrantly unmethylated (<10%) whereas Huh7, HLE, HLF and HepG2 were highly methylated similar to normal liver (>70%). Statistical analysis showed that Huh7, HLE, HLF and HepG2 were significantly methylated like normal liver (p value 2.39×10^{-5} , <0.05) when compared to SNU354, JHH2 and JHH4. On the other hand, partial methylation was observed with HepB3 and HuccT1 (25-40%) (Fig.15A). Pyrosequencing gives the average methylation within an amplicon, but we also wanted to look at methylation status of individual DNA strands in HepG2 and HLF cells. Using strand-specific cloning approach I confirmed that both the HepG2 and HLF promoter is fully methylated on all strands (Fig.15B,C&D). Looking at methylation of individual strands in these two cell lines, strands were more methylated in HepG2 than HLF (Fig.15C&D), consistent with the C19MC methylation percentage data obtained using pyrosequencing (Fig.15A). Statistical analysis, using one tailed t-test, also revealed that HepG2 cell line is significantly more methylated than HLF cell line as the p value (0.045) was less than the significance level of 0.05.



A



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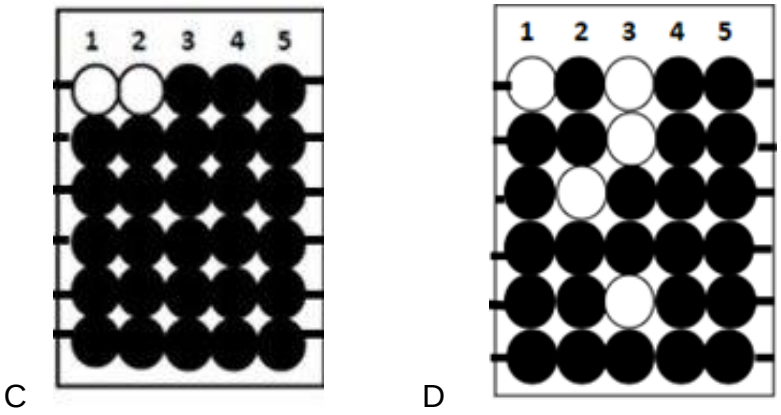


Figure 15 DNA methylation profiling. A) SNU354, JHH2 and JHH4 were aberrantly unmethylated, which is cancer related hypomethylation, whereas Huh7, HLE, HLF and HepG2 were significantly highly methylated like normal liver, with p value being 2.39×10^{-5} (less than the significance level of 0.05). Also, Hep3B and HuccT1 were shown to be partially methylated. This figure was produced with a biological replicate of three, thus the standard error bars indicate how the data is spread around the mean value. B*)* pGEMT PCR was run for the HepG2 cells cloned with 350bp insert. *Lane 1, 1kb ladder; Lane 2-9 HepG2 PCR product with the correct insert; Lane 10, Hepg2 PCR product with the wrong insert: Lane 11-15, HepG2 with the correct insert; Lane 16 positive control; Lane 17 Blank control.* The size of PCR products was compared with the positive control which was approximately 600 bp. All appropriately sized amplicons were sequenced by Sanger sequencing. C) Sequencing data for all 5 CpGs within the C19MC-2 amplicon showed that six DNA strands were cloned and HepG2 promoter was methylated on all strands, indicated by the black circles. D) Similarly, all CpGs were sequenced within the same amplicon for HLF revealing that the interval was largely methylated. However, HLF had more unmethylated positions than HepG2.

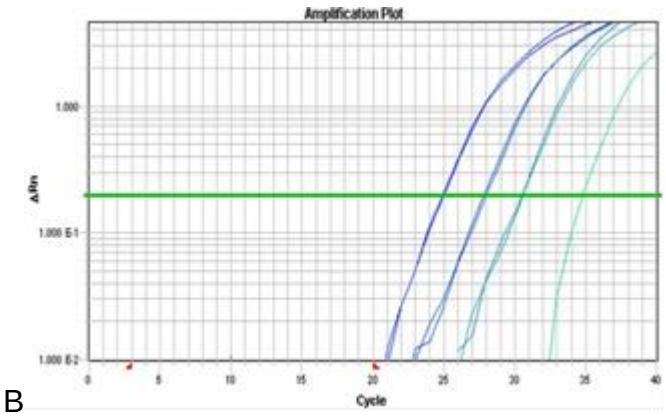
5.1.2 miRNA expression optimizing.

Next, we wanted to determine miRNA expression in HCC cell lines. Previous studies in the Monk laboratory observed reactivation of *miR-517*, *miR-525* and *miR-520h* in JHH2 and SNU345 cells. This is consistent with the hypomethylation observed. Standard microRNA real-time PCR assays normalized to *RNU6B* were used to obtain these results. I initially needed to optimize amplification, thus using Taqman “Advanced” miRNA assays I quantified more C19MC-derived miRNAs in the same sample cDNA. The miRNA

expressions were normalized to *miR-152* and *miR-23b* as well as *RNU6B* spike-in, due to the reasons stated previously (Lamba *et al*., 2014; Lou *et al*., 2015).

In addition to amplifying mature miRNAs, I have designed qPCRs to the pri-C19MC with the aim of measuring the unprocessed transcript. To achieve this, I designed primer sets in single copy regions of the C19MC, near miRNA sequences (*miR-518* and *miR-520d*). This was difficult since the structure of the locus is made up of SINE-miRNA blocks. Primers of 22-24 bp in length were designed to amplify ~150 bp without underlying SNPs. The sequences were subjected to BLAST and BLASTn to make sure they were single copy. Using standard PCR, and visualizing on an agarose gel, we confirmed the efficiency of PCR amplification. For qPCRs, reactions were carried out in triplicate in a MicroAmp Fast Optical 96-Well Reaction Plate using SBYR Green. A dilution series of template (5 µl of 1:10; 1:100 and 1:1000) was amplified to ensure the reactions worked efficiently as indicated by the correlation coefficient slope of –3.08 (a specific and highly efficient qPCR has a ratio of –3.3) for *pri-miR-518* and –3.4 for *pri-miR-520d* (Fig.16A,B&C).

A



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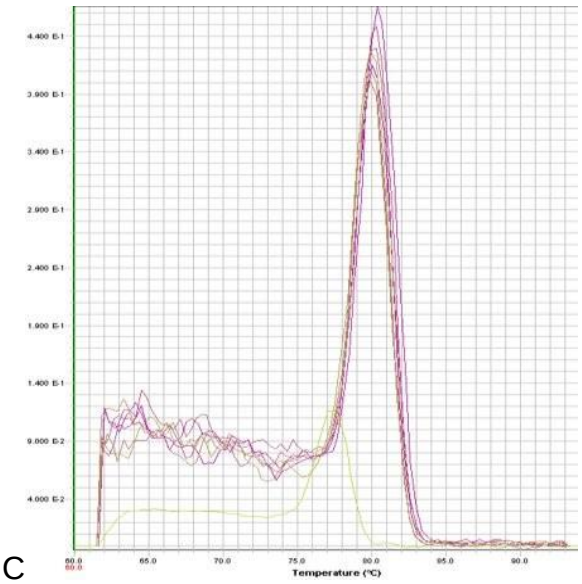


C

Figure 16 Optimizing qPCR. A) The standard curve was created by setting a serial dilution of template and amplifying with qPCR. The correlation coefficient slopes obtained were –3.08 and -3.4 for *pri-mir-518* and *pri-mir-520d*, respectively. B) The blue amplification plots appeared to be clear indicating that difference in normalized fluorescence is large hence the density range is appropriate. C) The melt curves measure the amount of fluorescence and are normally produced after the amplification cycles are completed.

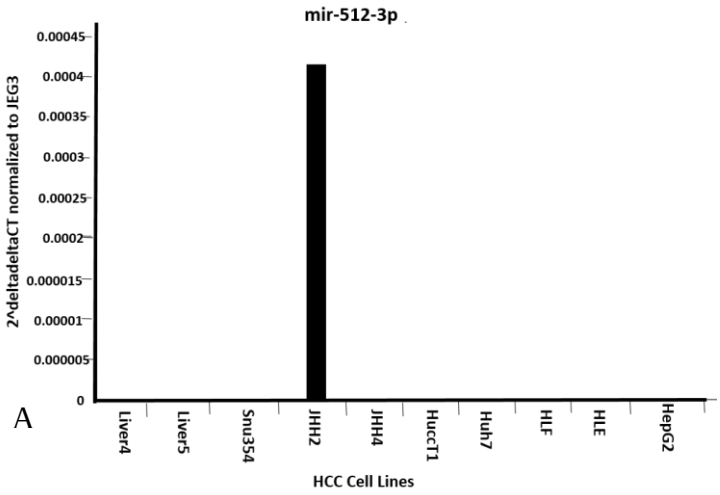
5.1.3 miRNA expression profiling.

Taking the advantage from the previous studies in the Monk lab, in this study we assessed expression of *miR-512-3p, miR-518f-5p*, *miR-525-5p*, *miR-520d-3p* and *miR-517c-5p* in nine HCC cell lines. It was essential to investigate miRNA expression with the aim of assessing the links between aberrant DNA hypomethylation in HCC cell lines and re-activation of oncogenic miRNAs. C19MC miRNAs are not expressed in normal liver. To discriminate between a sample with poor quality RNA and tissue-specific absence of expression I confirmed miRNA expression using a previously described liver specific miRNA. I chose *miR-122-5p* as the control miRNA because this non C19MC miRNA is not only highly expressed in liver but also is downregulated in cancer (Jopling, 2012). Based on the *miR-122-5p* expression profile Liv5 was used as the control cell line. On the other hand we used the placenta trophoblast JEG3 cell line as the control for C19MC, as these cells highly express the miRNAs from this cluster. miRNA expressions were

normalized to the average of *miR-152* and *miR-23b* as well as *RNU6B* for reasons stated previously.

miR-512-3p was only detected in JHH2 following both control miRNA and *RNU6B* normalization (Fig.17A&B). On the other hand, *miR-518f-5p* was detected in all HCC cell lines with highest in SNU354 (Fig.17C). When normalized to *RNU6B*, *miR-518f-5p* expression was less than when normalized to two control miRNAs and was not detectable in JHH2 and HepG2 (Fig.17D). Expression of *miR-525-5p* was observed in JHH4, SNU354 and HLF, for both control miRNA and *RNU6B* normalization (Fig.17E&F). *MiR-520d* was expressed in JHH4, SNU354 and HLF when normalized to miRNAs (Fig.17G) however, it was detected in JHH2 when normalized to *RNU6B* (Fig.17H). *miR-517c-3p* activity was observed in SNU354 and HLF with a modest expression in JHH4 (Fig.17I&J). The control *miR-122-5p* was shown to be detected in most HCC cell lines with highest levels in the normal liver control, as expected. m*iR-122-5p* activity was not detectable in JHH4 (Fig.17K&L).

Overall, we detected similar miRNA activity when normalized to both two control miRNAs or *RNU6B* (note, the scale of the y-axis changes due to the levels of endogenous controls). We expected to detect miRNA expression in the unmethylated cell lines (JHH2, JHH4 and SNU354) since unmethylated promoters facilitate expressions. However, in some cases such as *miR-512-3p*, we did not detect miRNA expression in all the unmethylated cell lines. It is also interesting to note that some miRNAs were expressed highly in HLF although this cell line has a partially methylated C19MC promoter. Finally, despite detecting varying amounts of these miRNA in the HCC lines, their abundance was several magnitudes less the the endogenous levels of the positive control, the placent cell line JEG3.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0.000005
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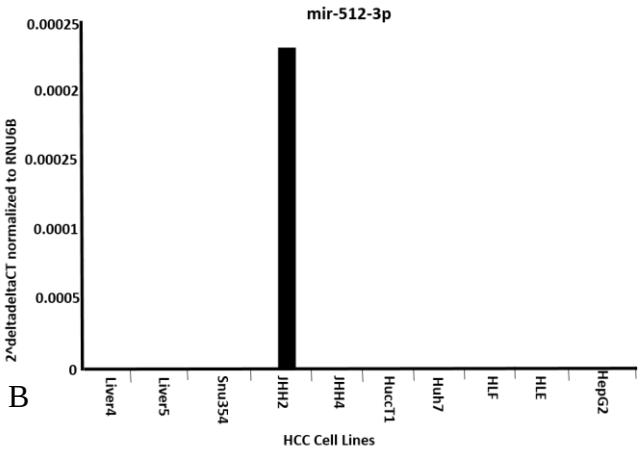
Snu354
JHH2
JHH4
HuccT1
Huh7
HLF
HLE
HepG2
HCC Cell Lines

A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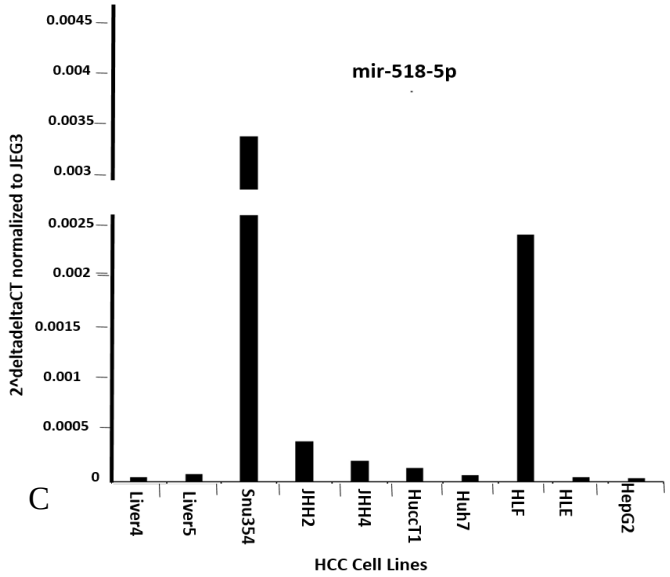
HepG2
HCC Cell Lines

B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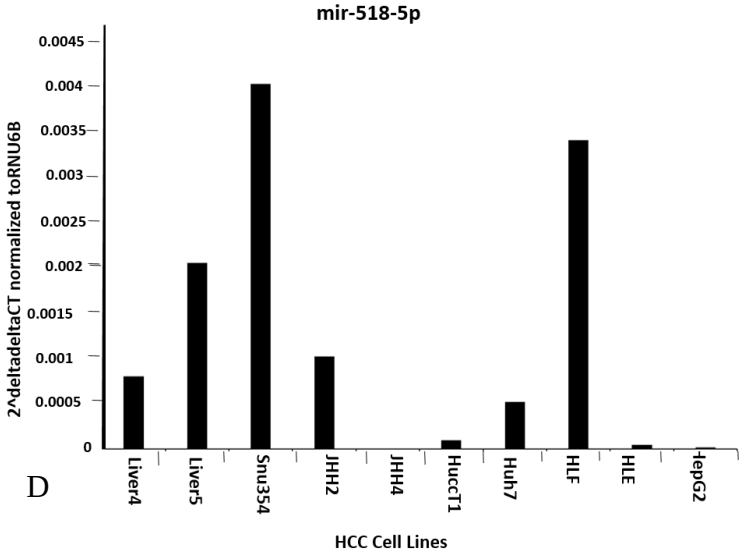
JHH4
HuccT1
Huh7
HLF
HLE
HepG2
HCC Cell Lines

C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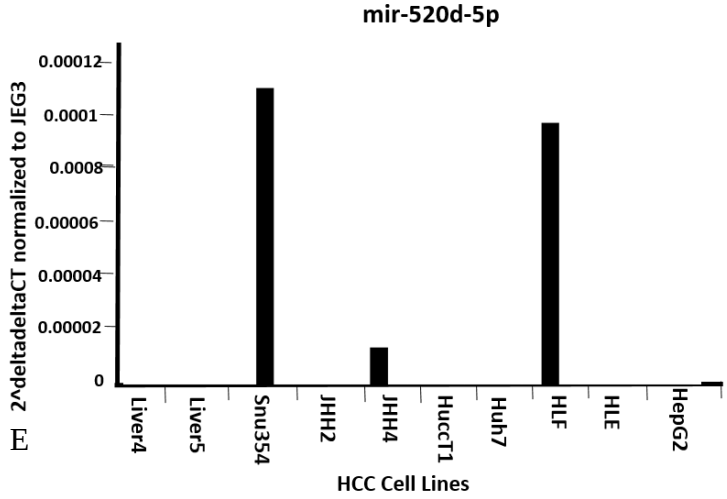
HCC Cell Lines

D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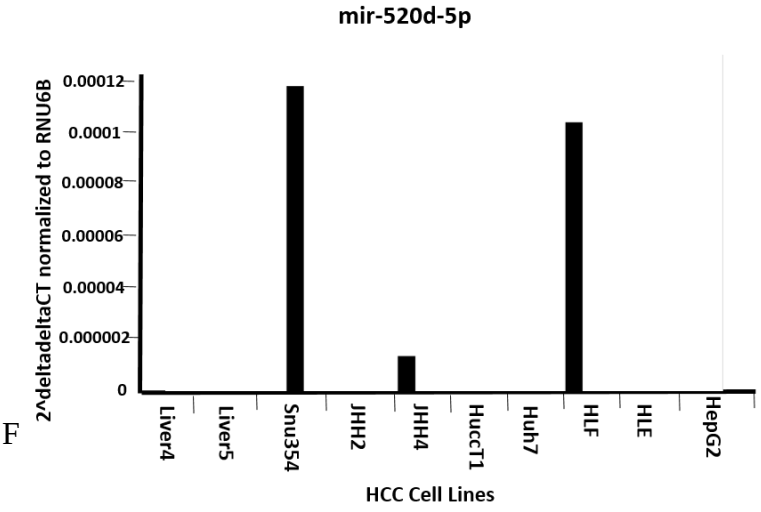
HepG2
HCC Cell Lines

E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Huh7
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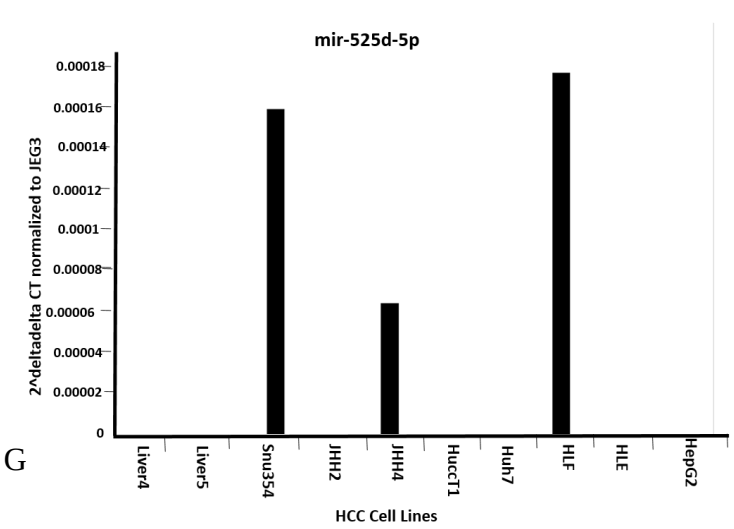
HLE
HepG2
HCC Cell Lines

F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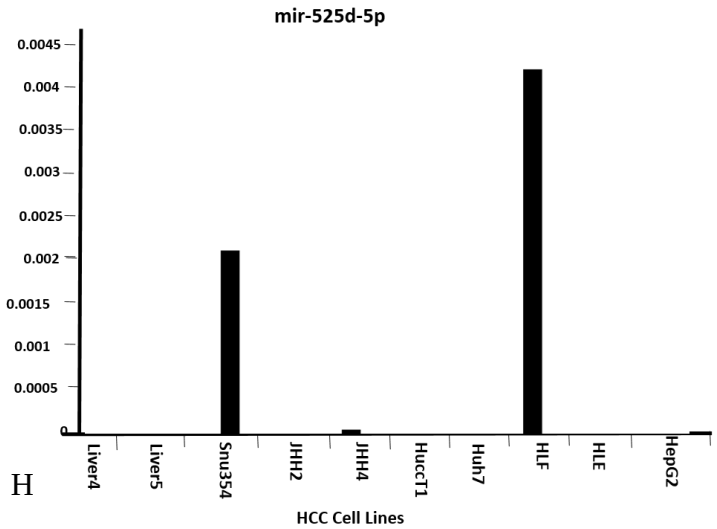
Snu354
JHH2
JHH4
HuccT1
Huh7
HLF
HLE
HepG2
HCC Cell Lines

G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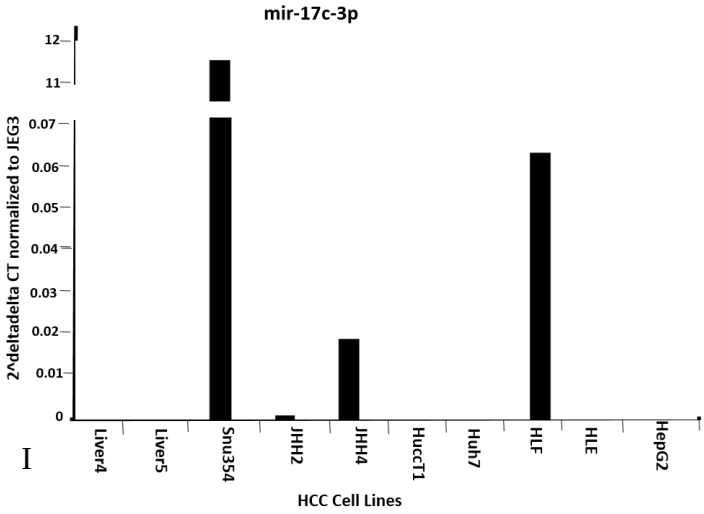
JHH4
HuccT1
Huh7
HLF
HLE
HepG2
HCC Cell Lines

H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0.01
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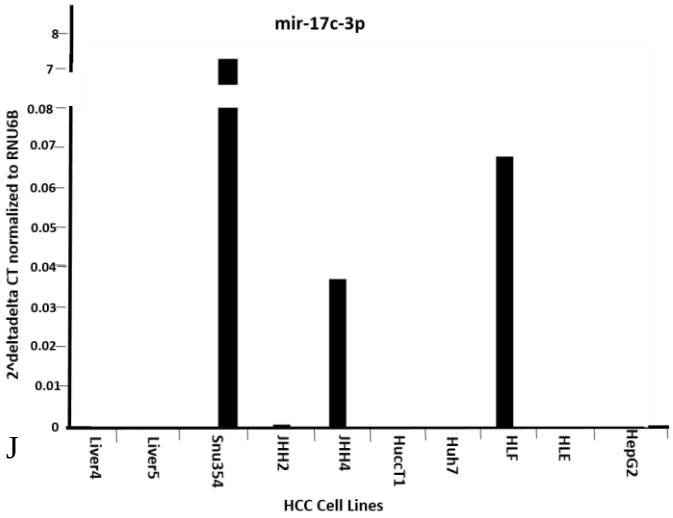
JHH4
HuccT1
Huh7
HLF
HLE
HepG2
HCC Cell Lines

I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7
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0.07
0.06
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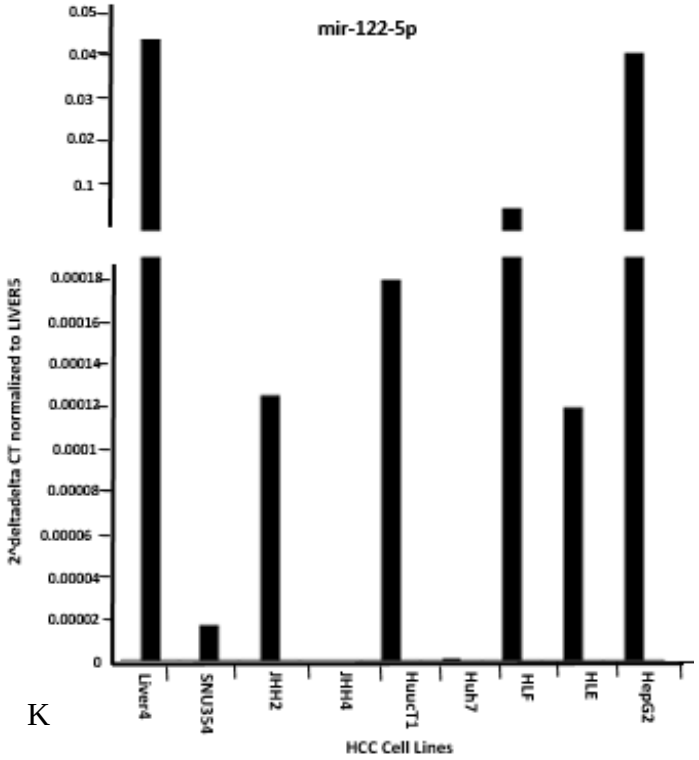
HCC Cell Lines

J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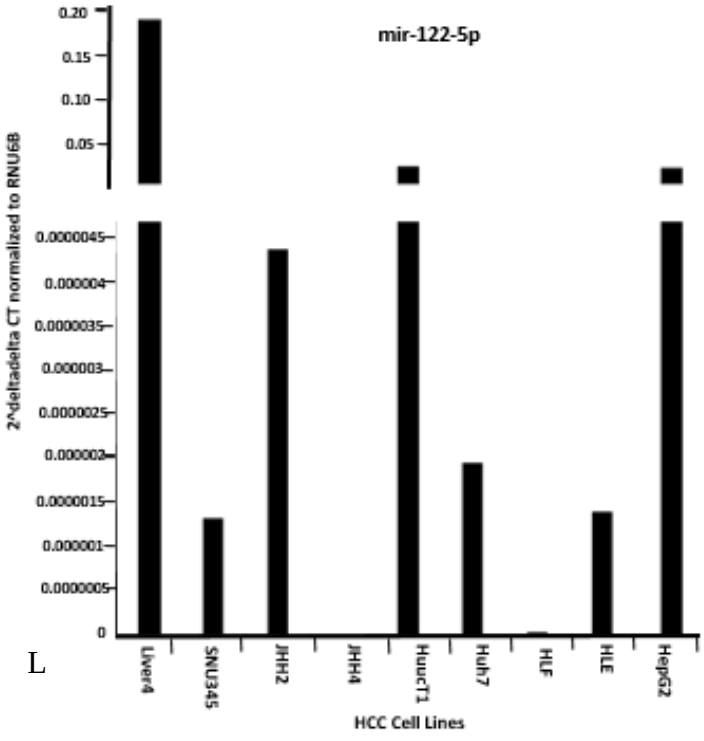
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JHH2
JHH4
HuucT1
Huh7
HLF
HLE
HepG2
HCC Cell Lines

L

Figure 17 miRNA expression profiling. Standard miRNA real-time PCR assays normalized to control small RNAs; the average of *miR-152* and *miR-23b* or *RNU6B,* respectively. These graphs were produced with a technical replicate of three and a biological replicate of one, therefore standard error bars could not be added.

5.2 Epigenetic manipulations with small molecule inhibitors.

5.2.1 Optimizing 5-aza-DC treatments.

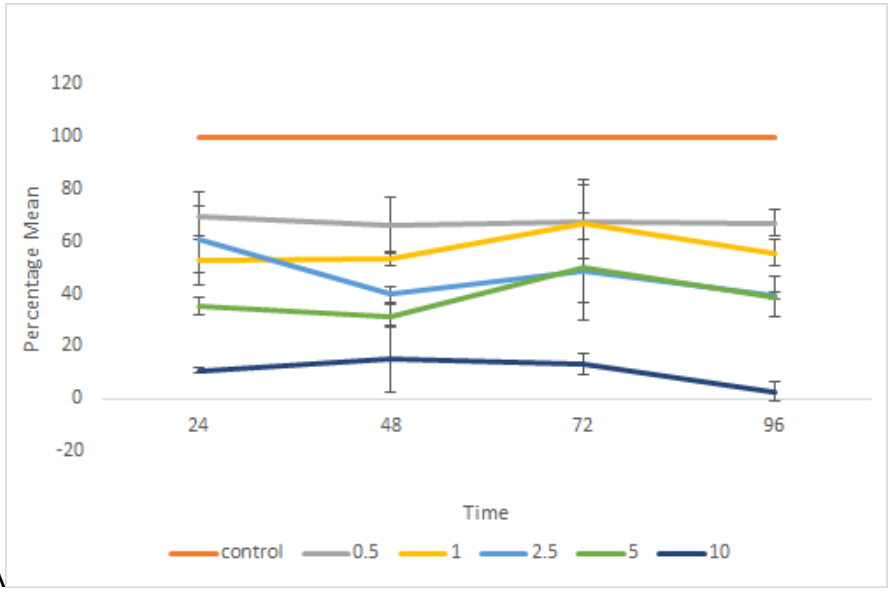
Small molecule inhibitors, 5-aza-DC and TSA were used in this study to demethylate the methylated HCC cell lines. Before we could assess the inhibitory effect of small molecule drugs on DNA methylation in our HCC cell lines, I needed to determine the general toxicity and consequence on cell replication for 5-aza-DC treatment. For this I determined proliferation by counting cells every day for a 5-day time course during which the cells were exposed to different concentrations of 5-aza-DC. Following literature searches, several publications suggested that the working concentration, to result in global demethylation, was between 1-10 μM (Pall et al., 2008; Zhang et al., 2016). For total cell counts I determined the percentage mean number of cells for each concentration. When compared to controls, the percentage mean for the lowest concentration, 0.5 μM, remained stable at approximately 80% of controls, suggesting that there may have been an initial low-level toxicity with a decrease of 20%. As the concentration increased, there was a proportional effect on cell counts at 24h, after which the cells maintained at this number. In general, the number of cells obtained from 1 μM, 2.5 μM and 5 μM incubations after 72h-96h were approximately 50% less of the untreated controls. The most severe effect was for the highest concentration 10 μM, for which there were 85% less cells compared to the control (Fig.18A).

Rather than relying on cell count alone, I further assessed the effect of 5-aza-DC treatment using MTT assays, a colorimetric test to determine metabolic activity. Using the same drug dilutions and similar exposure time, I observed that increased concentration of 5-aza-DC caused an increase in cell death, with the largest effect recorded for 10 μM. This was consistent with the cell count data. Despite the sharp decrease in viability observed at 10 μM, and the relatively large standard deviation (indicating the variance for

each replicate), the IC50 value, that is, the concentration of compound which exhibits 50% cell viability, was determined to be 10 μM (Fig.18B).

Based on classical toxicology, 10 μM for a minimum of 3 days should be selected, however our final experimental read out is the total decrease in C19MC promoter methylation. To address this, I extracted DNA from the cells exposed to various concentrations of 5-aza-DC and used them for bisulphite conversion for C19MC PCR. The sequencing results revealed a reduction in global methylation and showed that the C19MC promoter region was ~30% demethylated compared to untreated control cells. I also performed statistical analysis which showed that cell death occured the most at 10 μM (p values <0.05). Based on these observations, I finally selected the 5-aza-DC concentration of 10 μM for all experiments.

A



B

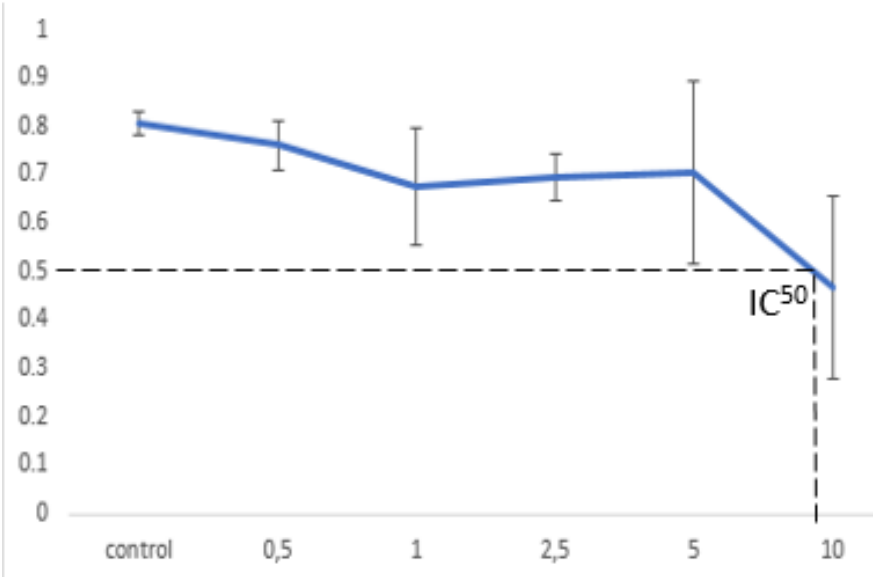


Figure 18 Optimizing the concentration and duration for 5-aza-DC. These figures were generated with a biological replicate of three and standard error bars represent the variance for each replicate A) The percentage mean number of cells was calculated for each concentration and each time period. Comparison of percentage mean number of cells revealed higher concentration of 5-aza-DC resulted in less cells, with 10 μM causing the highest number of cell death compare to untreated controls. This was statistically shown to be significant as the p value obtained from one tailed t-test was 0.040 (<0.05) B) MTT assay is a colorimetric assay uses reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) to measure cellular metabolic activity as a proxy for cell viability. The absorbance value is proportional to cell number. After 72h, the IC50 was reached using 5-aza-DC at 10 μM which was again shown to significantly result in the highest cell death with a p value of 0.033 (<0.05).

5.2.2 Methylation profiling following combined 5-aza-DC and TSA experiments.

We treated HLF cells with 5-aza-DC to inhibit methylation, TSA to inhibit histone deacetylation and with both to obtain maximum transcription from the C19MC promoter. We could detect residual expression for some C19MC-derived miRNAs in HLF. However, the expression was not to the levels of unmethylated SNU354 cells or endogenously expressing JEG3 control cells, suggesting that all auxilary factors were present yet expression was limited by the promoter hypermethylation. As a result, we selected HLF cell line for the drug experiment and all future experiments. To address whether

demethylation was induced upon drug treatment, I extracted DNA from the cells exposed to the drugs for 3 days, 7 days and 10 days. I also extracted DNA from the untreated control cells which enabled us to assess the impacts of the drugs on methylation. The extracted DNA was used for bisulfite conversion for C19MC PCR. Sequencing results showed that the control cells were fully methylated whereas 10-20% of demethylation was achieved in the cells treated with 5-aza-DC and 5-aza-DC plus TSA for 3 days. The cells exposed to the drugs for 7 days were 15-20% demethylated. On the other hand, 20-30% demethylation was observed from the cells exposed to 5-aza-DC and 5-aza-DC plus TSA for 10 days. We did not observe demethylation in cells treated with TSA only, suggesting that histone acetylation did not affect DNA methylation. To summarize, our results suggested that demethylation was achieved in our HLF cells treated with 5-aza-DC as well as combined 5-aza-DC and TSA. Treating the cells with the drugs for 10 days significantly resulted in more demethylation than 3 days and 7 days (p value<0.05) (Fig.19).

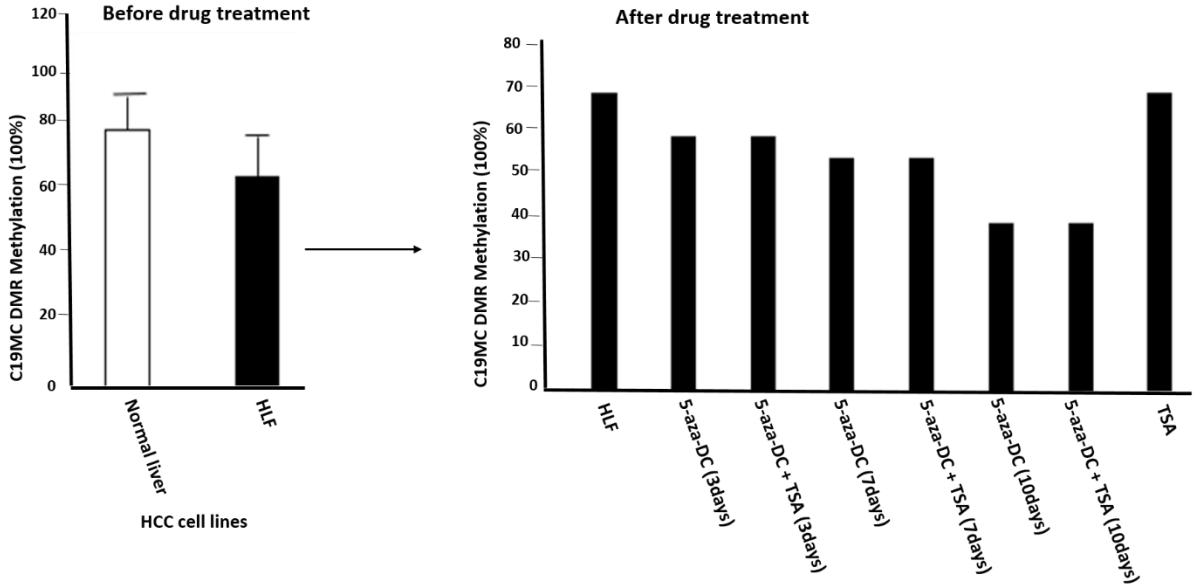


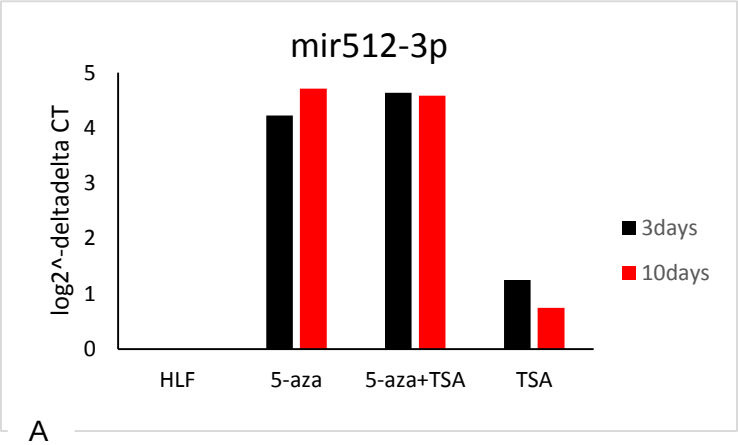
Figure 19 Methylation status after the drug treatment. Left side, before the treatment, we showed that HLF cell line is methylated similar to normal liver. HLF cells were then exposed to 5-aza-DC, 5-aza-DC plus TSA for 3 days, 7 days and 10 days. TSA was added 24h before harvesting the pellets. Following, DNA extraction, bisulfite conversion and C19MC2 PCR, the samples were sequenced to assess the C/T ratio. Right side, the results showed demethylation

following treatment with 5-aza-DC and 5-aza-DC plus TSA. Treating the cells with the drugs for 10 days significantly resulted in more demethylation since the p value was found to be 0.001, with a two-tailed t-test. TSA alone did not affect methylation suggesting that methylation was not affected by histone acetylation. Standard error bars were not applicable since this experiment was biologically repeated once.

5.2.3 miRNA reactivation in 5-aza-DC and 5-aza-DC plus TSA treated cells .

Once we confirmed that 5-aza-DC and TSA treatment resulted in demethylation of the C19MC promoter in HLF, we next wanted to determine if demethylation was sufficient to massively reactivate our miRNAs. Following RNA extraction from the drug treated HLF cells, cDNA was made and C19MC-derived miRNAs in the cDNA were quantified by using Taqman “Advanced” miRNA assays. We also assessed the expression of pri-C19MC transcript in control and treated cells. For miRNA qPCRs, reactions were carried out in triplicate in a MicroAmp Fast Optical 96-Well Reaction Plate and normalized to *miR-152* and *miR-23b* as well as *RNU6B*. The quantification of of pri-C19MC was normalized to *RPL19* and *ACTB*.

The qPCR results revealed that the miRNAs were lowly expressed in the untreated control cells but were readily reactivated in 5-aza-DC and 5-aza-DC plus TSA treated HLF cells, initially detectable following 3 days treatment, and maximal at 10 days. In addition, we showed that pri-C19MC long-coding RNA was also re-expressed upon treatment. Logarithmic scales were empolyed to visualize the resulting data as induction of C19MC miRNA expression was massive comapred to the untreated controls. Following treatment for 10 days, *miR-512-3p* significantly had the highest increase in fold among all miRNAs when normalized to both control miRNAs or *RNU6B* (p value<0.05)(Fig.20A&B). In addition, h igh levels of *miR-518f-5p*, *miR-525-5p*, *miR-520d-5p* and *miR-517c-3p* expression were observed and there was no expression in TSA-only treated cells (Fig.20C-J). In general, miRNAs significantly had higher expression in 5-aza-DC plus TSA treated cells when compared to 5-aza-DC only, which was also greater for the 10 days treated cells when compared to only 3 days (p values <0.05). Cells exposed to TSA only had only marginal miRNA reactivation which was independent of methylation.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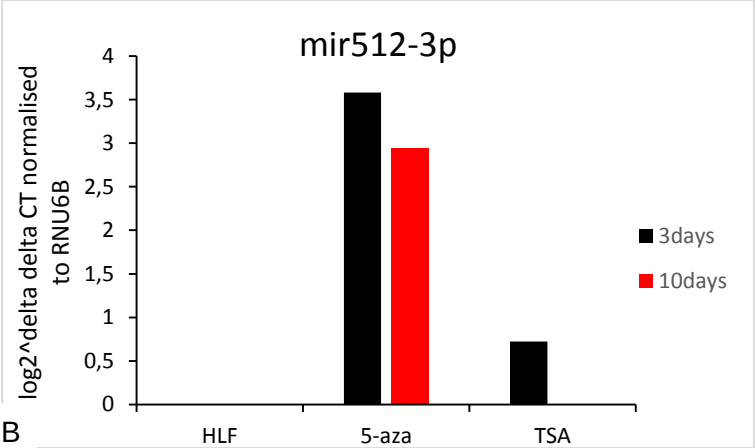
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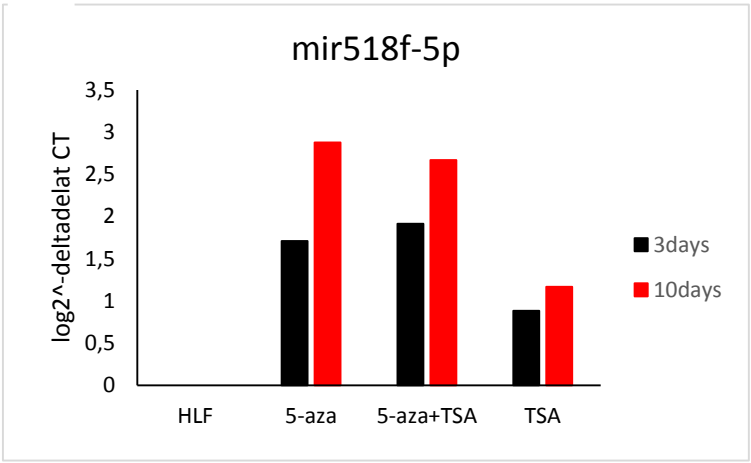
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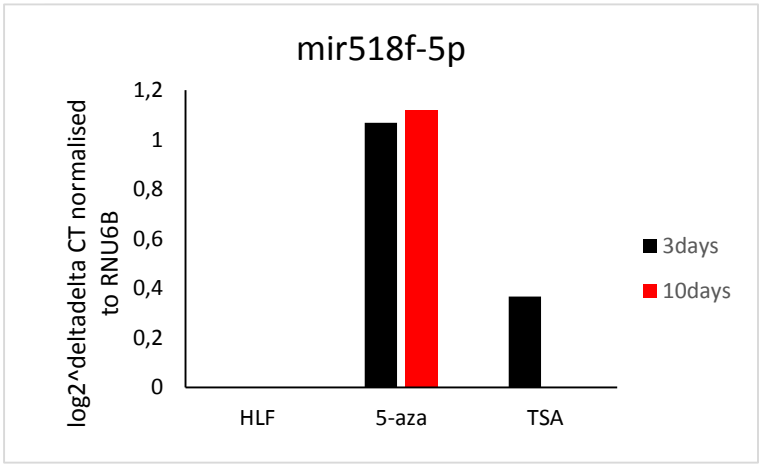
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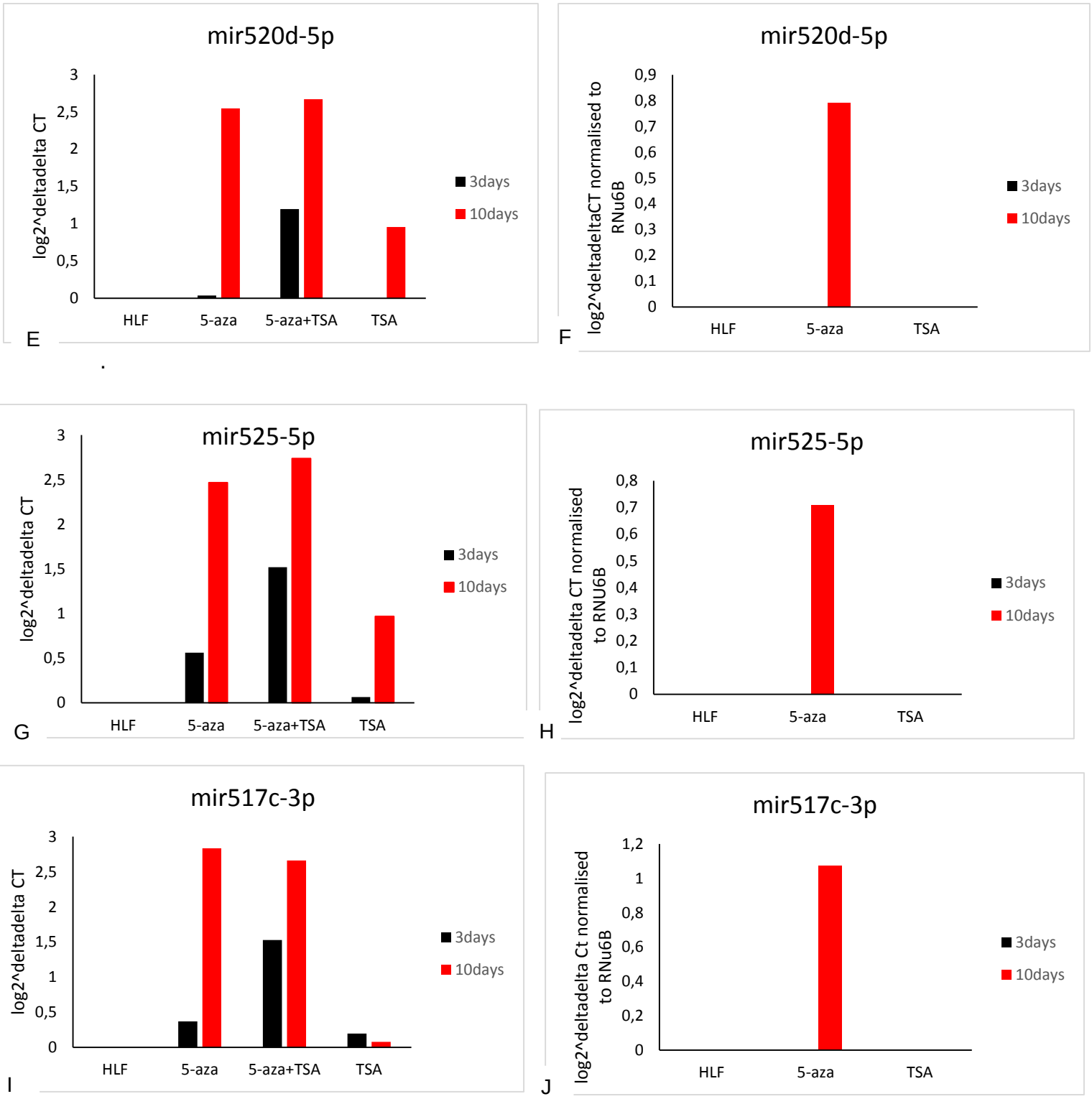

Figure 20 miRNA reactivation normalized to two control miRNAs and RNU6B. Standard microRNA real-time assays normalized to control small RNAs; the average of *miR-152* and *miR-*

23b or *RNU6B,* respectively, was used to obtain these results. All 5 miRNAs were not expressed in control HLF cells and were reactivated in drug treated cells. One-tailed t-test results revealed that *mir-512-3p* significantly had the highest increase in fold among all miRNAs with the p value of $2.99x10^{-6}$. Overall, miRNA expression was significantly higher in the combined 5-aza-DC and TSA treated samples (p value 0.03) for 10 day exposure (p value $2x10^{-4}$). Because these graphs were produced with a technical replicate of one but biological replicate of one, standard error bars could not be included to show the distrubition of the data around the mean value.

5.3 Epigenetic manipulations with dCas9-TET strategy.

5.3.1 Designing crRNA for dCas9-TET experiments.

Treatment with small molecule inhibitors presumably affected the whole genome, including C19MC promoter. In order to study C19MC specifically, we wished to use the dCas9-TET strategy. Before transfecting HCC cells with the pLV hUbC-dCas9-TET1-T2A-GFP construct I designed the crRNAs to the C19MC promoter interval. The DNA sequence of the C19MC promoter CpG islands was obtained from the UCSC genome browser and mapped all CpG dinucleotides. Using Cas9-target function of UCSC browser I identified 22 different gRNA/crRNA sequences which were high scoring using 3 algorithms (MIT guide specificity, efficiency defined by Doench *et al*. 2016 and Moreno-Mateos *in vitro* score) located throughout the promoter interval. I then selected the nine best distributed gRNAs throughout the interval mapping to both DNA strands, all possessing the NGG PAM sequence (Table 2). Since the promoter consists of five imperfect ~50 bp repeats, I also designed a crRNA for this as it may allow for multiple interactions using a single crRNA sequence. Importantly, the bisulphite PCRs that would allow for methylation to be quantified are located in the center of the CpG island, overlapping 3 crRNAs.

Table 2 gRNA sequences. The table shows the sequences of nine gRNAs which possess the NGG PAM sequence as well as the repeat sequence

gRNAs	Sequence
1. gRNA	GTGTTGATTCTTGCGGAACA
2. gRNA	CCCAAGCGGGTACATTTGCC
3. gRNA	TGGGCGTGGATCTCCTCACCTGCAGCGCT

4. gRNA	AGGTGTGCTCCCAGGGTCTCCACATCCCTAA
5. gRNA	CCGCAAGGCTGGCCTCTTTA
6. gRNA	CTGTTTCcgCTGCcgGcgTC
7. gRNA	TGGACcgAGGTCTCTAGAGCTGC
8. gRNA	TGCGACAATCTTCCGGTGCC
9. gRNA	GCTGGGCACGGTAGTTCGCAT
Repeat Sequence	CCATCAGGGCGCCCATTAAG

5.3.2 Comparing methodologies for introducing dCas9-TET-CD constructs into cells

For the initial delivery of the dCas9-TET-CD-T2A-GFP constructs I used lipofectamine. This resulted in ~10-20% GFP positive cells. However, the GFP positive cells did not survive for the FACs selection and all cells subsequently died in culture. The control pMax-GFP plasmid had a higher efficiency with 50% green suggesting that the size of the dCas9-TET plasmid which is >14 kb might be the limiting factor (Fig.21A). Next, we transfected the cells with using PEI (Fig.21B). Unfortunately, cells failed to survive again.

We also used JetPRIME transfection reagent but despite many attempts using these reagents we failed to efficiently deliver the plasmid into the cells. Therefore, we could not obtain sufficient positive cells for FACs. The same plasmid is available in the Monk laboratory with the GFP gene replaced with a puromycin gene to allow for antibiotic selection. Again, following Lipofectamine transfection, 2 rounds of puromycin selection for 4 weeks failed to result in stable colonies (and additional replicated became infected and died). Finally, we changed strategy and used the lentiviral pLV hUbC-dCas9-Tet-T2A-GFP and Fuw-dCas9-TET-CD plasmids. These plasmids are smaller from Addgene

and lack the T2A-GFP and both contain 5'LTR sequences flanking the transgenes (Fig.21C).

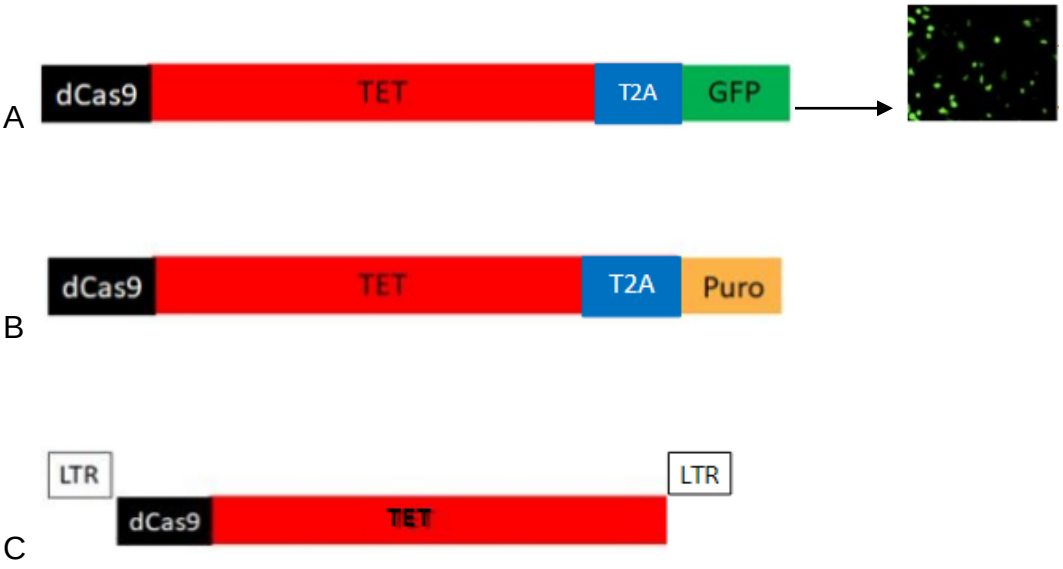

Figure 21 dCas9-TET Experiments. We used Lipofectamine, PEI and JETPRIME transfecting reagents to deliver the dCas9-TET-CD construct into HLF cells. A) We did not observe green cells with GFP containing construct. However, treatment with a smaller GFP-pMax vector resulted in high number of positive cells. This suggested that our construct might be too big for an efficient delivery. B) We then added puro to our construct and put our cells in puromycin media following transfection. Unfortunately, our cells did not survive. C) We changed strategy and used a lentiviral transfection. This construct lacked the T2A-selection gene, making it significantly smaller and included LTR repeat sequences. The virus utilizes these LTR repeats to stably intregrate the DNA into the cell genome.

5.3.3 TET Lentiviral introduction of dCas9-TET constructs into cells.

Since our transfection approach has failed after numerous attempts we switched to using dcas9-TET lentivirus infections. Following 48h of incubation with the virus, cells were trypinised and divided into two aliquotes, one for DNA/RNA extraction to ensure the cells were positive for dCas9 plasmid, the other to continue growing. Amplifying single transfected cells with dCas9 primers resulted in bright bands. PCR positive cells were expanded and divided into two further aliquotes, one to generate stable cell lines and the

other reinfected with the lentivus one more. Following PCR confirmation that the cells contained the dCas9 construct, both single and double infected cells were expanded to generate monoclonal stable cells. The double infected cells were subjected to FACs to ensure single cells were dispensed into each well of 96 well plates for subsequent expansion (Fig.22). Of the 380 indiviudal cells plated, 44 gave rise to colonies. Duplicate plates were generated to allow for both PCR selection and continued culture. All tested colonies were PCR positive and selected for continous expansion (Fig.23A&B). In addition to confirming the presence of the dCas9-TET-CD construct by PCR, the function and copy-number of the plasmid was needed to ensure functional protein was being produced in adequate amounts. However, due to having limited time I could not perform these experiments.

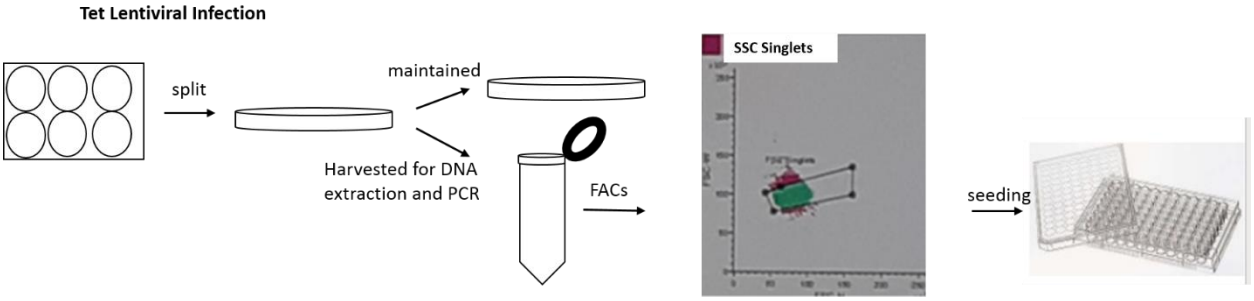

Figure 22 Schematic diagram of dCas9-TET lentiviral infection strategy. The work-flow represents the tissue culture procedure carried out for infecting HLF cells with dCas9-TET lentiviral. Initially, the cells were seeded in 6-well plates and infected with lentiviral. Following incubation, 50% of these cells were seeded in 10 cm dishes and half were harvested for dCas9 PCR. After confirming dCas9 was present in the cells, we infected the cells for the second time. Again, some of these cells were maintained in culture and some harvested for a second dCas9 PCR. One dish of double infected cells was subjected to FACs analysis. Using appropriate FAC gating, single cells were added to each well of a 96-well plates with “A1” containing 100 cells as a bulk control. Following three weeks of culture, colonies were obtained from some of the single cells. These stable cells were used for a final dCas9 PCR to ensure they were positive cells and expanded to generate monoclonal stable cells.

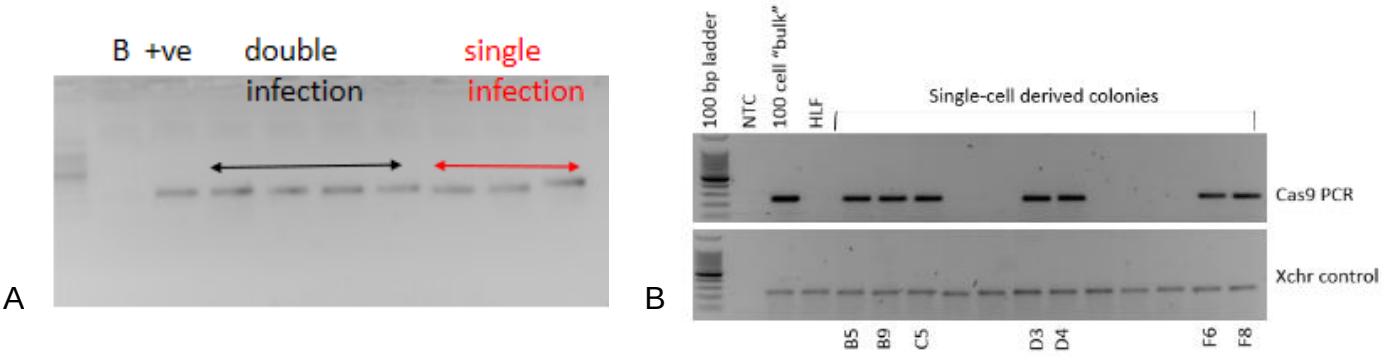

Figure 23 Representative gel Image of dCas9 PCR. A) Both double and single infected HLF cells were subjected to PCR with dCas9 primers to validate dCas9 integration. PCR amplicons were obtained from HLF cells with both first and second rounds of infections suggesting that the cells were positive for dCas9. B) The second gel image is an example showing the bands obtained from dCas9 PCR on the expanded monoclonal cultures. Along with the dCas9 PCR, Xchr primers were used to show the presence of genomic DNA.

5.4 miRNA mimic experiments.

5.4.1 Validating *miR-512-3p* over-expression.

The *miR-512-3p* was consistently re-expressed in our 5-aza-DC experiments and I therefore selected this miRNA to over-express using miRNA mimic technology. HLF cells were transfected with 50 nM and 500 nM of the mimics for 48h. Subsequently, extracted RNA was used for miRNA Advanced cDNA synthesis. To ensure over-expression was specific for *miR-512-3p*, qRT-PCR was performed not only for *miR-512-3p* but also for fellow C19MC-derived *miR-518f-3p* and normalized to the average of *miR-152* and *miR-23b* in control cells, cells transfected with a C. Elegans mimic (as a scrambled control) and cells transfected with *miR-512-3p* mimic. We did not detect *miR-512-3p* expression in either control cells as expected. The cells transfected with *miR-512-3p* mimic had higher expression using 500 nm than with 50 nM. The over-expression levels obtained using 500 nM were less than the endogenous amounts observed in the control JEG3 cells, suggesting that we had not saturated the HLF cells with mimic and that functional studies could be performed (Fig.24).

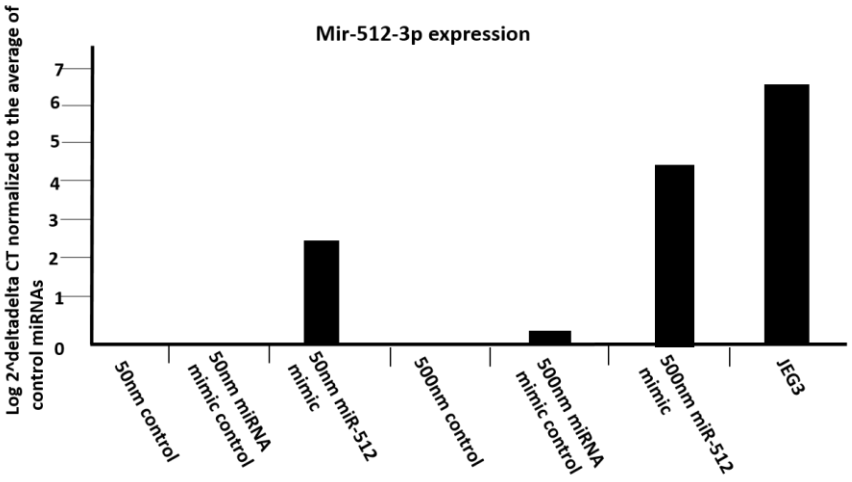

Figure 24 *mir-512-3p* over-expression through mimic transfection. HLF cells were transfected with 50 nM and 500 nM of miRNA C. Elgans or *mir-512-3p* mimic. Two days post-transfection RNA was extracted and used for miRNA cDNA synthesis. Expression of *mir-512-3p* was compared to the average of control miRNAs and compared with endogenous levels in the placenta-derived JEG3 cells. Two-tailed t-test revelaed that significantly higher mir-512-3p expression was detected with the cells treated with mir-512-3p mimic when compared to untreated control cells and cells treated with C.Elegans control mimic (p value 0.001). This graph was generated with a technical repeat of three and biological repeat of one therefore standard error bars were not applicable.

5.4.2 Impacts of *miR-512-3p* over-expression on cell migration.

To determine whether *miR-512-3p* over-expression had any potiential oncogenic effects, we performed scracth tests to monitor the cell invasion capacity. In cells 2 days post-transfection with 500 nM mimics, the resulting confluent cell monolayer was scratched and the cells were put in only 1% FBS containing media to ensure they could no longer divide (thus separating the effects of proliferation from migration). After 5 days in culture, cells were visualised under a light-phase microscope and 10 field of view images recorded. Subsequently 3 measurements per field of view were recorded at predefined locations (Fig.25A,B&C). In total, 30 measurements were taken per replicate and the experiment was performed three times in total.

In general, the average distance from three plate condtitions was 9.14, 9.22 and 5.53 (arbitrary units, AU) for non-transfected control, C. Elegan miRNA and *miR-512-3p* mimics respectively. We performed a T-test with paired two sample for means in SPSS and compared the significance of cell invasion (for the mean of replicates) between control and *miR-512-3p* mimic as well as miRNA control mimic and *miR-512-3p* mimic. Our findings suggested that *miR-512-3p* mimic significantly promoted cell invasion when compared to the control and miRNA control mimic, with p values being less than the significance level 0.05 ($6.78x10^{-20}$ and $1.19x10^{-22}$)(Table.3).

Table 3 Statistical Analysis. The table represents the results obtained from statistical analysis, including mean, variance and p values. T-test was calculated for paired two sample for means in order to compare data obtained from *miR-512-3p* with control and C. Elegan control respectively. p value (red) was checked to assess significance (p value less than 0.05). The mean of replicates was compared to obtain the p value.

t-Test: Paired Two Sample for Means	Control (AU)	*miR-512-3p* (AU)	Control mimic (AU)
Mean	9.14	5.53	9.23
Variance	5.47	4.31	4.49
Observations	90	90	90
Pearson Correlation	0.12		0.18
Hypothesized Mean Difference	0		0
df	89		89
t Stat	11.65		13.04
P(T<=t) one-tail	6.78E-20		1.19E-22
t Critical one-tail	1.66		1.66
P(T<=t) two-tail	1.36E-19		2.37E-22
t Critical two-tail	1.99		1.99

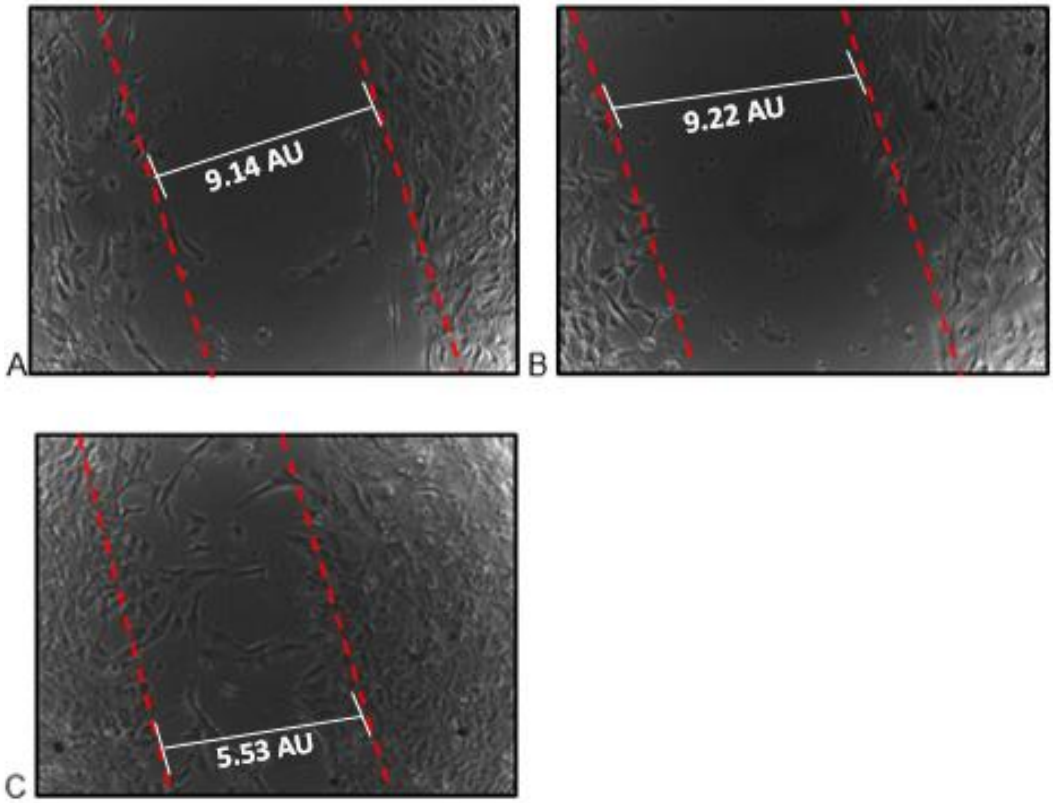

Figure 25 Cell Invasion Assay. Scratch test was performed to assess cell invasion in (A) untransfcted control cells, (B) C. Elegans miRNA control and (C) *mir-512-3p* mimics. The red stippled lines represent gap borders and the size bars represent the average gap distance obtained for each three plate conditions. The gap remained open with the untransfected control cells and cells transfected with C.Elegans miRNA control mimic (9.14 and 9.22 AU respectively). On the other hand the gap was almost closed with cells treated with the *mir-512-3p* mimic (5.53 AU).

Thus, the results showed that *mir-512-3p* over-expression significantly promoted cell invasion when compared to control cells and miRNA mimic control (p value<0.05) (Fig.25A, B&C, Table 3). As cell invasion has a crucial role in metastasis it is one of the hallmarks of cancer. Therefore, our results indicated the oncogenic properties of *mir-512-3p* upregulation in HCC.

6. Discussion.

6.1 miRNA expression profiling.

C19MC miRNA cluster is the largest miRNA cluster that has been detected in human genome and consists of approximately 46 mature miRNAs (Flor and Bullerdiek, 2012) which have been reported to have roles in tumorigenesis (Veronese *et al*., 2010; Ward *et al*., 2014; Rui *et al*., 2020). Preliminary studies in the Monk lab observed reactivation of *miR-517*, *miR-525* and *miR- 520h* in JHH2 and SNU345 cells which is consistent with the C19MC promoter hypomethylation observed. Moreover, Pang *et al*. (2014) showed that *miR-525-3p* is often upregulated in HCC tissues and regulated tumor migration and invasion via downregulating the expression of *ZNF395*. This zinc finger gene is responsible for activating several cancer-associated genes, including *MACC1, PEG10*, *CALCOCO1*, and *MEF2C* (Jordanovski *et al*., 2013). Another publication reported that *miR-520h* had significantly altered expression in HepG2 cells when compared to normal liver tissues (Sun *et al.*, 2016). According to the results, *HDAC1* was negatively related to the expression of *miR-520h*, suggesting this interaction had important roles in prognosis of HCC. During the course of this study, Rui et al. (2020) showed that upregulation of *miR-512-3p* and *miR-519a-5p* was associated with poor survival, suggesting that these two C19MC miRNAs promote oncogenic pathways in HCC.

Here I have assessed the expression of *miR-512-3p*, *miR-518f-5p*, *miR-525-5p*, *miR-520d-5p* and *miR-517c-3p*. I initially quantified these C19MC derived miRNAs nine HCC cell lines to determine the association between their expression and promoter methylation status. It was also crucial to look at miRNA expression before 5-aza-DC and TSA treatments in terms of confirming that changes in miRNA expression observed were solely from the drug treatment. Our results suggest that the C19MC-derived miRNAs were predominatly expressed in the unmethylated HCC cell lines, with the exception of HLF which was detectable in varying degrees in the cell lines. However, it is important to note that not all unmethylated cells had miRNA expression. Thus, we hypothesized that more epigenetic signals, such as acetylation and chromatin modification, or transcription factors loading might be needed for expression of these miRNAs.

More interestingly, we showed that some miRNAs had detectable expression in HLF cells. Our methylation studies revealed that this cell line has a hypermethylated C19MC promoter (being ~70% methylated). In-depth evaluation of the strand specific methylation in HLF cells represented that some individual CpG positions were unmethylated. Therefore, methylation-sensitive transcription factors might be able to bind to this interval in a sub-population of cell, facilitating transcription (Fig.25). However, more complicated scenarios may explain the discrepancy between expression and observed promoter methylation. Recently translocation within the C19MC domain have been shown to result in fusions which link active promoters with the C19MC miRNA cluster. In this case the methylated C19MC promoter would no longer be distal to the miRNA cluster and would not exert any regulation of their expression (Fig.26). This is the case for 19q13.4 translocations that selectively activate C19MC miRNAs in thyroid adenomas (Nguyen *et al*., 2017). Additional studies have shown that fusions between the C19MC and the *TTHY1* promoter (an embryonic chloride channel protein) facilitate C19MC miRNA expression in embryonal tumors with multilayered rosettes (ETMRs) (Kleinman *et al*., 2014). Sin-Chan *et al*. (2019) suggested that C19MC may promote or maintain a primitive neural/embryonic epigenetic cell phenotype in ETMRs. Moreover, their study suggested that a C19MC-LIN28A-MYCN oncogenic circuit controlled by hijacked super-enhancer may be a therapeutic vulnerability in ETMRs (Sin-Chan *et al.*, 2019).

Another possibility is that transcription factors might bind to a different promoter which is hypomethylated (Fig.27). Normally, TFs regulate gene expression by binding to gene promoter regions or to distal regions, enhancers. Moreover, TFs may bind to DNA indirectly through interacting with another TF even though by definition TFs possess DNA binding domains (Dekker and Heard, 2015).

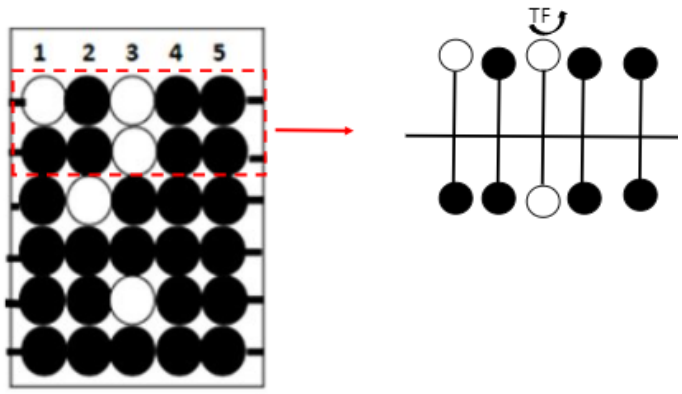

Figure 25 Expression with methylation. As mentioned before, methylation results in gene silencing. However, in one of our methylated HCC cell lines, HLF, we still detected high miRNA expression. Our strand-specific experiments showed that some DNA strands were unmethylated in HLF. This methylation might not be sufficient enough for silencing. Moreover, transcription factors might be binding to unmethylated and/or less methylated sites resulting in expression.

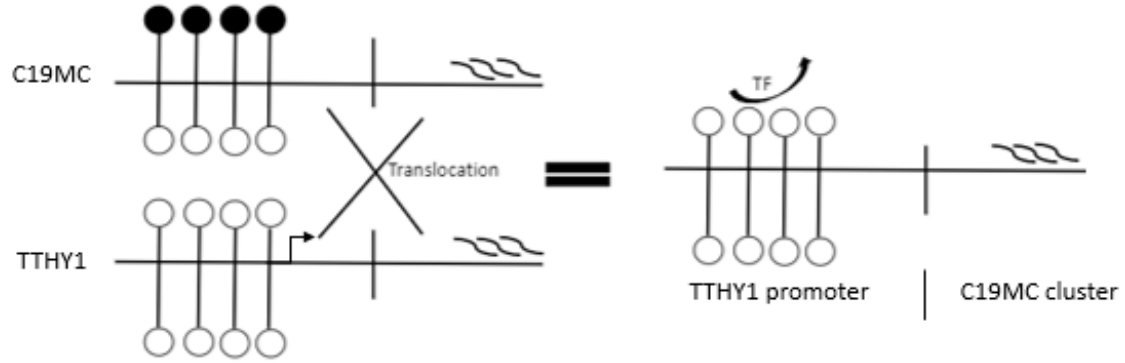

Figure 26 Translocations in cancer. Translocations are pathogenic events in cancer. In our case, translocation of a methylated region with an unmethylated region (for instance translocation of methylated C19MC with unmethylated TTHY1 promoter) could have facilitated transcription as transcription factors could bind and result in expression.

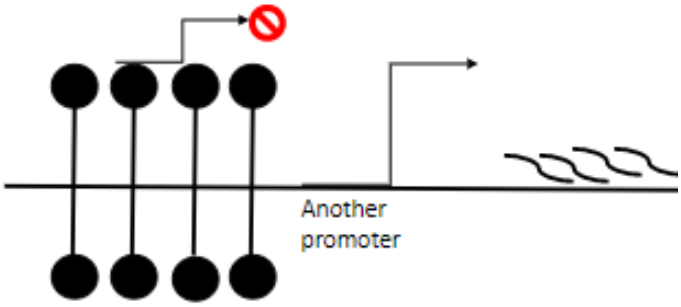

Figure 27 Alternate transcription start site. TFs could be binding to a different promoter that is revelaed if the intergenic C19MC interval is hypomethylated, allowing transcription initiation from a downstream position.

6.2 Epigenetic Manipulations with small molecule inhibitors

Epigenetic changes are known to disrupt gene function and in cancer epigenetic changes include hypermethylation of the CpG islands in the promoter regions, global DNA hypomethylation, chromatin modifications and loss of imprinting (Hatziapostolou and Iliopoulos, 2011; Mouthino and Esteller, 2017). The involvement of miRNA epigenetic regulation in cancer was first demonstrated by using an epigenetic drug, 5-aza-2'-deoxycytidine (5-aza-DC) (Saito *et al*., 2006). 5-aza-DC is a commonly used small-molecule that inhibits DNMT1 and DNMT3 (Fig.28). Epigenetic manipulations are widely used not only to improve our understanding of how these modifications regulate transcription and drive phenotypes (such as cancer), but also for their potential therapeutic use (Holtzman and Garsbach, 2018).

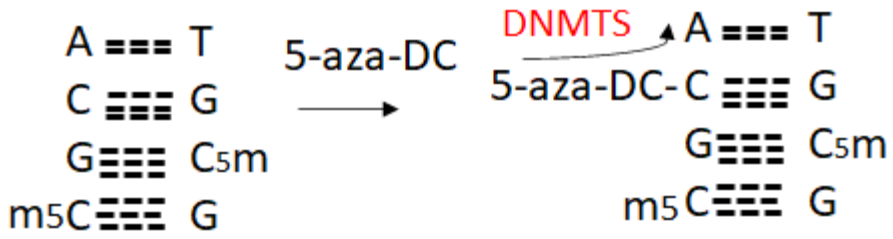

Figure 28 Mechanism of methylation inhibition by 5-aza-DC. This drug prevents maintenance methylation by trapping DNMTs to methylated C. This reduces cellular DNMT levels whic in turn results in reduced methylation and global hypomethylation

The characterization of DNA methylation revealed that the C19MC promoter in HLF cells is fully methylated and therefore was an ideal region to test the sensitiveness to demethylating agents, such as 5-aza-DC, to induce global demethylation. After determining the optimum concentration of 5-aza-DC for my HCC cells, which was 10 μM for a minimum of 72h, I exposed the cells to 5-aza-DC for 3-10 days. In some cases, treatment with 5-aza-DC was sufficient to result in transcription, but additional induction of expression was observed with the addition of TSA, a histone deacetylase inhibitor.

Treating cells with the drugs for 3 days and 10 days resulted in 10-20% and 20-30% C19MC promoter demethylation, respectively. These results suggest that, in addition to the reported global demethylatation induced by 5-aza-DC, the normally methylated C19MC promoter was also sensitive to this small molecule inhibitor.

Synthetized 40 years ago, the DNMT inhibitor 5-aza-DC is not only widely used in research but also in clinics for the treatment of malignant diseases. This agent has an effective anti-metabolic activity on tumor cells, primarily in the setting of acute myeloid leukemia (AML). It is unknown if the anti-cancer effects of 5-aza-DC are due to epigenetic remodeling or via its toxic effect at high dosage. There is evidence that 5-aza-DC inhibits DNA methylation and interferes with metabolic circuitries (Bezu *et al*., 2019). Moreover, *in vitro* studies in various solid tumor models have revealed that its main anti-tumoral impact is by increasing cancer cell lysis (Weber *et al*., 1994; Almstedt *et al*., 2010; Krishnadas *et al*., 2014). However demethylating effect of 5-aza-DC on genomic DNA might be restricted to specific regions with some regions not being affected (Tabolacci *et al*., 2016). It is possible that 5-aza-DC could be used to treat HCC through inhibiting DNA methylation and reactivation TSGs, however due to the lack of specificity, and possible side effects, other more tailored therapies are still preferable (Holtzman and Gersbach, 2018).

6.3 miRNA reactivation upon global demethylation.

After confirming demethylation was induced in HLF cells using 5-aza-DC and TSA, we looked at miRNA activity to see if this resulted in miRNA reactivation. In comparison to the initial miRNA expression profiling, all miRNAs were reactivated upon drug treatment. Combination of 5-aza-DC and TSA resulted in higher expression than 5-aza-DC on its own. In addition, we observed higher miRNA expression in cells following longer exposure to the drug, with 10 days treatments having consistently higher expression compared to 3 days. This is consistent with the observation that treating cell for 10 days resulted in more demethylation than for 3 days. Therefore, our results suggested a direct link between the extent of demethylation and the abundance of the re-expressed miRNAs.

As mentioned above, all tested miRNAs were reactivated following drug treatment. However, expression levels of each miRNA were different even though these miRNAs are all derived from the same long non-coding RNA molecule. miRNAs are initially transcribed as pri-miRNAs and are then processed into pre-miRNAs by Drosha. Pre-miRNAs are exported and processed in the cytoplasm by Dicer, giving rise to mature miRNAs. Drosha and Dicer are crucial for processing of intermediates to mature miRNAs. More importantly, these enzymes introduce variations in miRNAs abundance through preferential processing (Fig.29) (Tijsterman *et al*., 2004; Kim and Nam, 2006; Lee *et al*., 2006; Vaz *et al.,* 2013) so that differential expression may occur in which some have higher abundance than the others.

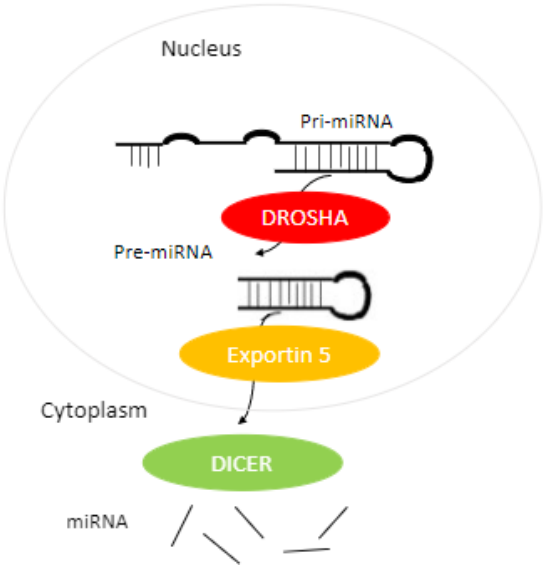

Figure 29 DROSHA/ DICER processing. miRNAs are initially processed as pri-miRNAs. Pri-miRNAs are then processed into pre-miRNAs by Drosha, in the nucleus. Exportin 5 exports pre-miRNAs into the cytoplasm where pre-miRNAs are processed into mature miRNAs by Dicer. Therefore, Drosha and Dicer are crucial for processing intermediates to mature miRNAs and introducing variations in miRNAs. This means that miRNAs that are processed from the same precursor might be differ in miRNA processing.

Additionally, each miRNA may have a different number of targets. A single miRNA can bind to several target mRNAs and several miRNAs can bind to a single target (Lewis *et al.*, 2003; Vaz *et al*., 2013), all of which affects free miRNA within the cell. miRNAs with

more targets may have a lower free miRNA levels than those with less targets (Yu *et al*., 2007). For instance, our MiRDB research showed that *miR-525-5p* and *miR-520d-5p* have 808 and 2404 predicted targets respectively whereas *miR-512-3p* has 521 predicted targets. In fitting with this theory, I observed higher expression of *miR-512-3p* than *miR-520d-5p* and *miR-525-5p*, although it is not true for all C19MC-derived miRNAs. Therefore, more experimental and computational approaches are essential to study the association between miRNA expression and number of targets, as well as the role of 'sponge' RNAs. Sponge transcripts are generally non-coding and also known as competing endogenous RNA in humans. Ebert and collogues (2007) revealed that miRNA function was lost due to the presence of miRNA sponges which also increased the levels of endogenous targets. The *H19* imprinted non-coding was one of the first molecular sponge reported. The *H19* trancript inhibits miRNA *let-7* and is associated with human genetic disorders and cancer (Gabory *et al*., 2010; Gao *et al*., 2014). Previous data suggest that miRNA sponges regulate miRNA activity in many eukaryotes, including plants (Franco-Zorrilla *et al.*, 2007) and mammals, as well as tumor biology (Poliseno *et al*., 2010; Cesana *et al*., 2011; Karreth *et al*., 2011; Tay *et al*., 2011; Sumazin *et al*., 2011). Poliseno *et al*. (2010) studied the functional association between the mRNAs produced by the *PTEN* tumor suppressor gene and its pseudogene *PTENP1*. They found that *PTENP1* regulates cellular levels of *PTEN*, playing a growth-suppressor role, which is often lost in human cancer. Overall, their findings showed that pseudogenes may play a role in tumorigenesis by fine tuning miRNA-mRNA interactions (Poliseno *et al*., 2010). In subsequent systematic screen (Sumazin *et al*., 2011), a post-transcriptional regulation network was found to have more than 248,000 miR-mediated interactions, 7000 of these acting as miR sponges and 148 having non-sponge interactions. This network was shown to regulate established oncogenic pathways in glioblastoma through mediating drivers of tumor initiation, such as *PTEN*, PDGFRA and *RB1* (Sumazin *et al*., 2011).

Therefore, it is possible that these C19MC miRNAs have different abudance levels and target activites because of the reasons stated above; different miRNA processing by Dicer and Drosha, different number of targets, and/or the sponge effect.

6.4 Epigenetic manipulations.

6.4.1 CRISPR-Cas9 Strategy.

Treatment with small molecule inhibitors presumably resulted in demethylation in the whole genome including C19MC. Therefore, in this study, I designed a CRISPR/dCas9 strategy to induce selective demethylation of the normally hypermethylated C19MC promoter in HLF cells.

Targeted editing enables for efficient genomic and epigenomic manipulations. New techniques, such as CRISPR interference (CRIPSRi) have valuable applications in research (Choudary *et al*., 2015) and substential potential in the clinic. CRISPRi utilizes dCas9 fused to effector domains to influence transcription and provides a complementary approach to standard RNAi. The difference between CRISPi and RNAi is that CRISPRi regulates gene expression primarily at the transcriptional level, while RNAi methods control expression at the mRNA level. It has been shown that CRIPSRi can knock down a large proportion of the human genome efficiently which can help to identify genetic sequences involved in hereditary diseases such as certain forms of cancer (Qi *et al*., 2013). In addition, the significance of increased gene expression in a disease can be studied by CRIPSRi through the attachment of transcription activators (Larson *et al*., 2013). Although this technique is promising in genome editing, it has some limitations associated with it. For instance, it can result in unexpected side effects by affecting nearby genes. Furthermore, the number of genes that can be targeted is limited due to the fact that CRIPSRi, like CRISPR/Cas9, relies on the use of PAM (Larson *et al*., 2013). Nevertheless, the major advantage of this technique is that it can result in efficient and significant manipulation in gene expression. Additionally, it is potentially easy to modify CRIPSRi, thus it can be used in *ex-vivo* cell therapy and sequence-targeted medicines (Qi *et al*., 2013).

I initially proposed to employ two different dCas9 constructs, one fused with the catalytic domain of DNMT3 to specifically methylate the C19MC promoter in JHH2 and JHH4 HCC cell lines that are aberrantly unmethylated and express some C19MC-associated miRNAs. However, upon resuscitation from liquid nitrogen, these cell lines failed to

proliferate at a rate suitable for transfection (i.e. they were hard to manipulate). Thus, for the purpose of this study, I focused on demethylating the C19MC promoter in HLF cells using a dCas9-TET fusion (Fig.30) as these cells divide at a much faster rate.

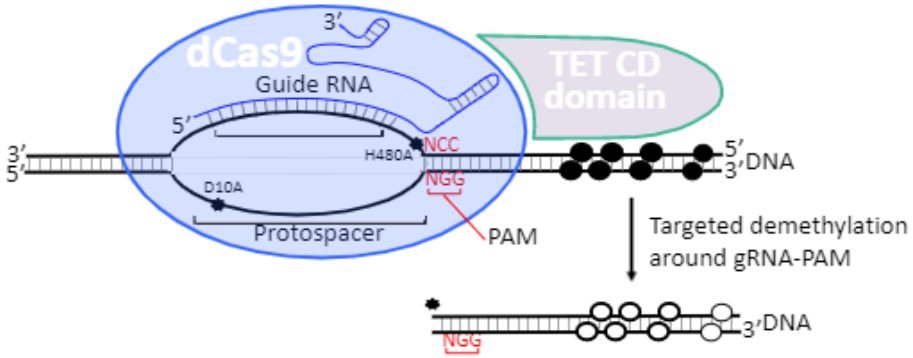

Figure 30 dCas9-Tet strategy. CRIPSR uses a cas9 nuclease, CRIPSR RNA (crRNA) and transcript-activating RNA. Fusion of these two RNAs generates the guide RNA which can be modified for selective targeting. In this strategy, the target must follow a 5' NGG PAM sequence. PAM motif is critical as it allows binding of guide RNA to the target. Fusion of the dCas9 with the catayltic domain of TET enzyme with the guide RNA enables targeted demethylation which occurs around gRNA-PAM.

6.4.2 dCas9-TET experiments.

The dCas9-TET-CD constructs were already established in the Monk laboratory and previous experiments using *in vitro* transcribed mRNA injected into mice embryo efficiently resulted in targeted demethylation. This confirms that the dCas9-TET-CD construct works when efficiently introduced into cells with gRNAs. Furthermore, several studies published since 2016 have used the dCas9-TET fusion system, reporting targeted demethylation with an associated induction of transcription and increased mRNA abundance of target genes (Liu *et al*., 2016; Xu *et al*., 2016). One study in particular used transient and lentiviral-based dCas9-Tet systems with four sgRNAs to selectively target the *BRCA1* promoter to induce gene expression which resulted in a 20% decrease in methylation at 3 CpGs and a significant increase in expression (highest upregulation of the gene was observed with TDE-I and sgRNA-2) (Choudhury *et al*., 2016).

I carefully designed multiple crRNAs to non-repetitive sequences in the C19MC promoter to ensure on-target recruitment of the construct. Furthermore, the C19MC CpG island contains a tandem repeat of approximately 50 bp to which I also designed a crRNA since it had multiple PAM sequences. It is possible that by targeting this interval with a single crRNA that the dCas9-TET would be recruited multiple times to each repeat unit. This approach of targeting repeat elements has been used to epigenetically manipulate LINE-1 sequences throughout the mouse genome and was also successfully when using a single TET-CD TALEN (Jachowicz *et al*., 2017). My initial dCas9-TET-CD construct delivery with lipofectamine resulted in 10-20% GFP positive cells which unfortunately did not survive for FACs selection. We hypothesized that the size of the dCas9-TET-CD plasmid (>14kb) might be the limiting factor as the control pMax-GFP plasmid resulted in 50% higher efficiency. Following transfection of a T2A-puro version of the plamsid using PEI, our cells failed to survive selection and no colonies resulted. Ultimately, our attempts to efficiently deliver the construct with lipofectamine, PEI and JetPRIME failed and we could not obtain sufficient positive integrated cells.

When the laboratories re-opened after the COVID-19 lockdown, I tried to generate stable expressing dCas9-TET-CD HepG2 and HLF cells using lentiviral transfer. Recombinant lentiviruses have previously been used to infect HCC cells, including HepG2, to enhance gene expression (e.g. for *CYP3A4*) suggesting that our cells lines are accepting of such methodology and therefore hold great potential for this work (Chiang *et al*., 2014). The construct generated by the Monk lab is based on the pLV hUbC-dCas9 backbone that contain 5'LTR sequences flanking the transgene. However, the sequences contained within the 5'LTR sequences, is very large, over 12 kb, which is beyond the recommend packaging capacity for most lentiviruses. In parallel, I also tried the Fuw-dCas9-TET-CD lentivirus from the Jaenisch laboratory from the Whitehead Institute/MIT as this has a smaller transgene because it lacks the T2A-GFP/T2A-puro downstream of dCas9-TET-CD. Unfortunately, HepG2 cells failed to proliferate and survive, thus we utilized lentiviral transfection into HLF cells only. Following infection, we expanded the cell cultures for screening through PCR with dCas9 primers. After confirming that our cell population was positive for dCas9, we continued culture to expand only cells with the integrated construct.

To produce monoclonal HLF cells with dCas9-TET-CD, I subjected the bulk cultures to FACs in order to isolate single cells to grow colonies (performed with the Earlham Institute). I obtained several colonies that were all shown to be dCas9 positive by PCR. However, being PCR positive does not give information about the plasmid integration site or copy number, as a single molecule could have entered a region of heterochromatin and be transcriptionally silent. To ensure the monoclonal HLF lines express functional dCas9-TET levels, western blotting would need to be performed before transfecting gRNA and quantifying the underlying DNA methylation. Unfortunately my time in the lab ended before I could finish these experiments and they will be completed by other lab members.

6.5 *miR-512-3p* over-expression.

Frequent upregulation of C19MC in HCC has been reported indicating the high correlation between co-upregulated C19MC expression and tumorigenesis (Augello *et al*., 2012; Nguyen *et al*., 2017), promoting the invasion and metastasis. Toffanin *et al*. (2011) and Fornari *et al*. (2012) showed the oncogenic and pro-invasive roles of four C19MC members, *miR-517a*, *miR-520c*, m*iR-519d* and *miR-519c-3p*. Upregulation of *miR-512-3p*, the first miRNA on the C19MC cluster, was shown to be associated not only with tumor burden, stage and grade but also with the poor survival of HCC (Rui *et al*., 2020). Rui and collogues (2020) subsequently used miRNA mimics to overexpress *miR-512-3p* and showed that upregulation of this miRNA inhibited the direct target genes, *MAP3K2* and *MAP2K4*.

In this study, we also used miRNA mimics to overexpress *miR-512-3p*. We initially proposed to assess the impacts of miRNA upregulation on the target genes. Using miRNA target prediction database (miRDB) we checked the targets of *miR-512-3p* and selected 8 targets based on their score, oncogenic properties and expression in liver. The targets selected were *CCDC6*, *SFMTB1*, *LATS1*, *DYRK2*, *FOXR2*, *PPARA*, *JMJDIC* and *KAT6A*, however due to having limited time we could not study the impacts of *miR-512-3p* upregulation on these target genes.

6.6 Impacts of miRNA over-expression on cellular behavior.

In this study, I performed scratch assays to study cell invasion upon *miR-512-3p* over-expression through mimics. Similarly, Rui *et al*. (2020) performed trans-well assay and *in vivo* studies to assess cell invasion and HCC metastasis in the xenograft models (in nude mice) respectively. Their trans-well assay results validated that over-expression of *mirR-512-3p* significantly promoted the invasiveness in the HCC cell lines when compared with the control. Additionally, *miR-512-3p* transfected mice had significantly larger tumor volume and weight when compared to the control group. Overall, findings of this study suggested that upregulation of *miR-512-3p* promoted HCC malignancy and early recurrence (Rui *et al*., 2020). Although we did not study cell invasion *in vivo*, our results also suggested that over-expression of *miR-512-3p* significantly promoted cell invasion in HLF cells when compared to the control cells and miRNA control mimic cells. The findings of both studies indicated that *miR-512-3p* can promote HCC, hence suggesting that this C19MC miRNA could be a marker for not only detection of HCC but also for prediction of therapy targets and outcomes.

6.7 Using miRNA Epigenetics for cancer treatments.

Despite the improvements in the approaches to prevent (Siegel *et al*., 2018), detect (Chang *et al*., 2016), diagnose (Liu *et al.,* 2016) and treat (Marrero *et al*., 2018) HCC remains as one of the major factors of tumor-related fatalities. Although the use of targeted agents as a part of pharmacological treatment has notably increased the overall survival (Llovet *et al*., 2008; Serper *et al*., 2017; Kudo *et al*., 2018), HCC-related deaths continue to rise (Yang *et al*.,2019). It is therefore essential to further study the underlying mechanisms of HCC and explore therapeutic targets (Rui *et al*., 2020). In clinic, use of hypermethylation as a prognostic marker could help to predict the effectiveness and efficiency of treatments. Moreover, it could be helpful for predicting the disease outcome (Mouthino and Esteller, 2017).

The C19MC hypomethylation we observed in this study is HCC specific. C19MC miRNA biomarkers can be used to detect HCC and predict worsening of the disease. C19MC miRNAs were studied as biomarkers in several cancers. It was reported (Strub *et al*.,

2016) that detection of C19MC miRNAs in the circulation of infants with infantile hemangioma (IH; the most common vascular tumor of infancy) is promising for IH diagnosis in a noninvasive means. *miR-519a/d* is highly expressed in IH and over-expression of *miR-519a* targets *RBL2*, activating DNMT3B as well as forming embryonal tumors (Kleinman *et al*., 2014). Targets of these miRNAs, including inhibitors of cell proliferation and angiogenesis, suggested that C19MC is important in IH pathogenesis (Wu *et al*., 2010; Fornari *et al*., 2012; Kameswaran *et al*., 2012; Haecker *et al*., 2012; Vlaschos *et al*., 2015). Therefore, C19MC miRNA detection may be helpful for identifying patients for appropriate therapy as well as monitoring treatment response (Strub *et al*., 2016). In addition, to early detection of cancer, detection of circulating C19MC miRNAs; *miR-516b*, *miR-517-5p*, *miR-520a-5p*, *miR-525-5p* and *miR-526a* has been suggested to be associated with preeclampsia (pregnancy related complication) (Hromadnikova *et al*., 2013), and the presence of high plasma levels of *miR-517-5p* might be a predictive of preeclampsia (Hromadnikova *et al*., 2017). Therefore, screening HCC patient plasma for free or exosome-derived C19MC miRNAs maybe useful for early disease detection.

Finally, our results showed that upregulation of *miR-512-3p* significantly promotes cell invasion, revelaing the oncogenic properties of this miRNA. Therefore*, miR-512-3p* could serve as a marker to detect and predict the worsening of HCC. In addition to HCC, *miR-512-3p* could be studied in other cancer types, such as colorectal and breast cancer, in which C19MC-derived miRNAs were reported to be reactivated (Kleinman *et al.,* 2014; Ma *et al*., 2016). Association between *miR-512-3p* upregulation and cell invasion could be translated in other relevant studies in order to validate potential roles of *miR-512-3p* as a diagnostic biomarker in various cancer types. However, using miRNAs as diagnostic markers can be challenging due to poor diagnostic specificity and reproducibility of some miRNAs. It is crucial to optimize the methods used for miRNA detection prior to use for diagnostic purposes to generate useful data (Wang *et al*., 2016). It is also challenging to discover specific miRNAs that can be used as biomarkers in a wide range of patients as well as to develop accurate, simple and cheap methods that involve pre- and post-analytical producers (Condrat *et al*., 2020).

7. Future Work.

I quantified five C19MC-derived miRNAs in a panel of HCC cell lines, showing that there is a direct relationship between their expression and the promoter methylation status. We showed that global methylation could be induced by small molecule inhibitors (5-aza-DC and TSA) and that C19MC hypomethylation was sufficient to reactivate miRNAs. We could not perform all of the experiments we proposed to do due to COVID-19 lockdown restrictions. This following section highlights some of the future work required to complete this study.

7.1 dCas9-TET Experiments – TET Expression.

We aimed to induce selective demethylation in the C19MC promoter through recruiting a dCas9-Tet fusion using multiple guide RNAs. Despite several attempts with different transfection reagents, we failed to deliver our construct into our cells. COVID-19 lockdown interrupted my study when I was about to use the lentiviral transfection. I did not have enough time to validate Tet activity in our stable lines. TET enzymes have been shown to have crucial roles in active DNA demethylation through oxidizing 5mC to 5hmC (Gong and Zhu, 2011; Nettersheim *et al*., 2013). Therefore, examining TET expression could validate that our cell lines stably express the construct and give more evidence for demethylation. Western blotting and immunostaining techniques could be used to test the nuclear-localisation of the dCas9-Tet fusion protein. In addition, Epigenase 5mc-Hydroxylase TET Activity/Inhibition Assay Kit (Colorimetric) could be used to measure Tet hydroxylase activity by detecting Tet-converted hydroxymethylated products (BioCat GmbH).

If we observed targeted demethylation of the C19MC promoter in HLF cells and the concomitant reactivation of the associated miRNAs, it would be benefical to study their exprssion using RNA-FISH. Our collaborators (Jérôme Cavaillé at CNRS Toulouse) have optimized an imaging technique specific for monitoring C19MC Alu-miRNA expression. They use FISH with fluorescent oligonucleotides (Augello *et al*., 2012), which could be

useful to not only to validate the qPCR miRNA results but also to determine the interaction with the miRNA processor complex.

7.2 dCas9-DNMT experiments

As mentioned above, we could not utilize dCas9-DNMT3 in our study as the hypomethylated cell lines JHH2 and JHH4 cells did not grow sufficiently. However, several studies have shown that dCas9-DNMT fusions can target DNA methylation to specific locations of the genome and hence reduced gene expression. For instance, Votja and colleagues (2016) utilized a direct fusion of dCas9 to the catalytic domain of DNMT3A (dCas9-DNMT3A-CD) to increase the CpG methylation by 60% at the *BACH2* loci in human HEK293T cells (human embryonic kidney cells) (Votja *et al*., 2016). While this is an impressive increase in methylation, one possible reason for the lack of complete hypermethylation is that DNMT3A generally requires a tetramer formation for efficient DNA methylation (Huang *et al*., 2017). To increase the percentage of methylation further, chimeric methyltransferase (MTase) fusion proteins were produced. Stepper *et al*. (2017) showed that DNMT3A-DNMT3L chimeric fusion protein induced higher levels of methylation than dCas9-DNMT3A-CD alone. Furthermore, a chimeric fusion of three dCas9 fused to DNMT3A, DNMT3L and Krupple-associated box (KRAB) protein respectively, were shown to result in a greater improvement in methylation efficacy (Amabile *et al*., 2016). This approach would not only target DNA methylation via the action of the MTase, but also H3K9 methylation, an epigenetic mark associated with hypermethylation due to the recruitment of the co-repressor complex by KRAB-domain. In fact, very recent work in the Monk lab has shown that dCas9-ZFP57[KRAB] constructs can maintain DNA methylation during embryonic reprogramming, suggesting that KRAB recruitment of the co-repressor complex also involved endogenous DNMT3 (personal communication Ana Monteagudo-Sánchez). In another study, dCas9-DNMT3A fusion protein were used to target the promoters of human *CDKN2A* and *ARF* and mouse *CDKN1a* (McDonald *et al*., 2016). The results of this study demonstrated that the dCas9-DNMT3A induced methylation and reducing the expression of all three gene, but importantly, only when multiple gRNAs were used. For this reason, I have designed multiple crRNAs to the C19MC promoter.

Overall, many dCas9-based techniques have resulted in a successful (epi)genomic editing *in vitro* in cell lines derived from numerous tissue types, including muscle, liver, and kidney. However, it remains limited to cell lines that are easily accept constructs and that replicate well in culture. Urbano *et al*. (2019) proposed that controlling gene regulation with epigenetic manipulations will become an increasingly remarkable tool with potential for therapeutic use.

7.3 Impacts of miRNA upregulation on target genes.

As mentioned before, *miR-512-3p* had the highest increase in fold thus we overexpressed *miR-512-3p* using a mimic. Firstly, miRNA target prediction database (mirDB) was used to determine the targets of this miRNA. Rank and score were checked for each of the targets. I undertook extensive literature research to determine the oncogenic targets and checked the expression of the candidate targets in normal liver and in HCC on human tissue atlas. Based on our findings, I selected *CCDC6*, *SFMTB1*, *LATS1*, *DYRK2*, *FOXR2*, *PPARA*, *JMJDIC* and *KAT6A* genes. Despite optimizing the qRT-PCR conditions for these transcripts I did not have sufficient time to look at their expression profile following miRNA over-expression. Futhermore, it would be ideal to identify differentially expressed genes in an unbiased manner to find novel *miR-512-3p* targets by RNA-seq and correlate the results with the presence of the miRNA binding motif. A direct regulation of the target mRNAs could be confirmed by using 3'UTR luciferase assays.

8. Conclusion.

In the present study, I investigated and quantified several C19MC-derived miRNAs in a panel of HCC cell lines (and possible primary cancer tissues) and showed that their expression is directly related to the promoter methylation status. Next, we showed that hypomethylation induced by 5-aza-DC and TSA resulted in reactivation of miRNAs. Last but not least, overexpressing *miR-512-3p* with mimics promoted cell invasion which suggested that this miRNA, with further experimental data, has the potential to be used as a predictive marker for HCC and well as a function target to limit HCC invasion.

To conclude, as mentioned above, abnormal miRNA expression has been linked with pathogenesis, growth and metastasis of tumors and can be used as a novel diagnostic or predictive biomarkers in HCC (Augello *et al*., 2012; Borel *et al*., 2012; Vaira *et al*., 2015). Therefore, studying changes in miRNA expression could help not only to improve diagnosis and prognosis but also provide molecular targets for new therapeutic strategies against HCC (Augello *et al.,* 2018).

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