ABSTRACT

Investigating miR-23a/b Regulation of KDM6A Protein Expression in Epithelial-Mesenchymal Transition Using Breast Cancer Cell Models

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Director: Joseph H. Taube, Ph.D.

Tumor progression of epithelial cancers is facilitated by epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET), reverse processes that enable invasive cellular behavior. A key regulator of EMT/MET is lysine (K)-specific demethylase 6A (KDM6A), an activator of epithelial gene expression that is abundant in epithelial cell phenotypes. During EMT progression, the level of KDM6A protein expression decreases but there is no change in KDM6A transcript. Understanding the post-transcriptional regulators of KDM6A may reveal new modulators of EMT and potential therapeutic targets. MicroRNAs are short non-coding RNAs that bind mRNA transcripts to repress protein translation. To identify miRNAs that likely target KDM6A, we utilized seven databases as well as in vitro and clinical data to identify miR-23a and miR-23b as putative regulators of KDM6A. We then overexpressed miR-23a/b in breast cancer cells and, unexpectedly, observed no impact on KDM6A protein expression. Further research may explore other means of post-transcriptional regulation of KDM6A, including additional miRNA screening and alternative splicing analysis.

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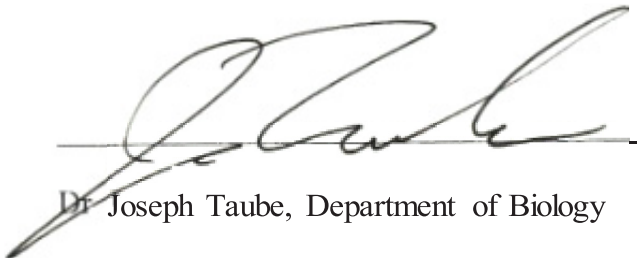

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INVESTIGATING MIR-23A/B REGULATION OF KDM6A PROTEIN EXPRESSION IN EPITHELIAL-MESENCHYMAL TRANSITION USING BREAST CANCER CELL MODELS

A Thesis Submitted to the Faculty of

Baylor University

In Partial Fulfillment of the Requirements for the

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By

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CHAPTER ONE

Introduction

Cancer cell metastasis, or the development of secondary tumors that are distant from the initial tumor location, is the leading cause of cancer patient mortality. It is estimated that breast cancer, the most common form of cancer in females, will prove fatal for 30-40% of patients through tumor metastases at distant sites (Jin and Mu, 2015). The metastatic cascade is characterized by local tumor invasion of cancer cells, intravasation and circulation in blood and lymphatic vessels, extravasation at a secondary site, and tumor survival and growth at this distant location (Talmadge and Fidler, 2010). Despite its detrimental effects on human health, the mechanisms of metastasis are poorly understood. Therefore, there is an urgent need to explore the fundamental molecular pathways and cellular properties that contribute to metastatic progression of cancer.

Epithelial-Mesenchymal Transition (EMT) in the Metastatic Cascade

Epithelial-mesenchymal transition (EMT) and its reverse process, mesenchymal epithelial transition (MET), are conserved biological processes that contribute to migratory cellular behavior. During EMT, polarized epithelial cells undergo a series of biochemical changes to acquire the spindle-shaped morphology and invasive properties of mesenchymal cells (Yang et al., 2020). These changes include loss of cell polarity and intercellular adhesion, increased motility, resistance to apoptosis, and restructuring of the extracellular matrix. Progression of EMT is characterized by loss of epithelial markers,

most notably the cell-adhesion molecule E-cadherin, and gain of mesenchymal markers such as N-cadherin and vimentin (Kalluri and Weinberg, 2009). EMT is accomplished through complex regulation and pleiotropic signaling of specific activating transcription factors (e.g. Snail, Slug, Twist, Zeb1/2), inhibitory miRNAs, and epigenetic and post-translational factors (Nieto et al., 2016). Several signaling pathways participate in the induction of EMT, including TGFβ, Wnt, NOTCH, and mitogenic growth factor signaling pathways (Dongre and Weinberg, 2018).

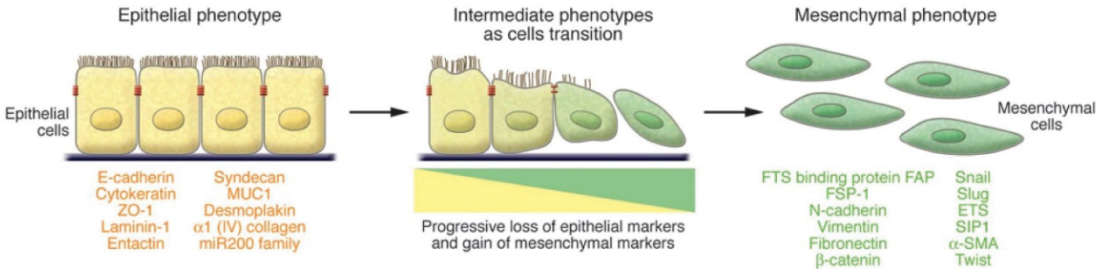

Figure 1.1: EMT Progression and Protein Markers (Kalluri and Weinburg, 2009)

EMT is implicated in embryonic development, differentiation, wound healing (tissue repair) and fibrosis, and pathological processes such as fibrosis and cancer migration/tumor invasiveness (Kalluri and Weinburg, 2009). Based on these functional roles regarding its biological relevance, EMT is classified into three types. Type 1 is involved in formation of motile mesenchymal cells during embryogenesis, type 2, in wound healing and tissue regeneration, and type 3, in cancer neoplasm (Blick et al., 2008; Trimboli et al., 2008). Within discussion of the EMT subtypes, the analogous capacities of embryogenesis and carcinogenesis for cellular migration led researchers to posit EMT as a significant contributor to epithelial tumor progression (Cano et al., 2000; Thiery, 2002). Therefore, it is feasible that cancer co-opts the natural role of EMT in beneficial

human functions for neoplastic malignancy and metastatic colonization (Hanahan and Weinberg, 2011).

However, EMT's role in cancer is not fully known and remains a topic of frequent debate, largely due to limited ability to assess EMT in clinical samples (Diepenbruck and Christofori, 2016). A study published in 2015 by Fischer et al. challenged the relevance of EMT in cancer metastasis by demonstrating a predominantly epithelial cell profile in breast-to-lung metastases, with only a small proportion of cells undergoing EMT. In response, researchers who support the role of EMT in cancer metastasis cite contributions of inter- and intra-tumor heterogeneity on the invasive tumor front, resulting in a gradient from full, partial, to no EMT cell states (Huang et al., 2013). Further, various states of EMT have been observed in heterogeneous circulating tumor cells (CTCs), a tumor cell sub-population with stem cell-like properties contributing to aggressive malignancies at distant metastases (Barriere et al., 2014). CTCs found in breast cancers are shown to have higher frequency of mesenchymal cells, with the triple-negative breast cancer subtype favoring the mesenchymal phenotype (Yu et al., 2013). A revised model for EMT emphasizes hybrid intermediate stages of the partial EMT program during the induction and metastasis of CTCs (Nieto et al., 2016). Overall, despite ongoing discussion regarding EMT relevance to cancer in vivo, its viable involvement in tumor intravasation and subsequent extravasation through MET contribute to its prominence in cancer metastasis research.

Epigenetic Plasticity and Modifications of the "Histone Code"

The dynamic and reversible nature of EMT suggests regulation through epigenetic modifications, or structural changes to chromatin excluding genetic alteration of the DNA

sequence (Tam and Weinberg, 2013). The chromatin landscape is composed of DNA and histones, as well as transient proteins that enhance and inhibit the transcription of genes through spatial and temporal molecular interactions. Alterations to this landscape can influence cell fate and differentiation through gene activation and silencing (Waddington, 1957; Flavahan et al., 2017).

Chromatin reorganization can be achieved through modulation of the "histone code," or the complex sequence of proteins subject to post-translational modifications, particularly on their tail domains (Strahl and Allis, 2000). Of these post-translational markers, trimethylation of histone H3 at lysine 27 (H3K27me3) enforces gene silencing (Cao et al., 2002; Ringrose et al., 2004). Its presence in concert with H3K4me3, a gene activator, establishes a bivalent domain that is characterized by both silencing and activating marks at the same gene promoter, maintaining a more flexible pre-differentiation state (Santos-Rosa et al., 2002; Voigt et al., 2013). Loss of one of these marks leads to lineage-specific commitment by the mark that is retained, leading either to gene silencing by H3K27me3 or activation by H3K4me3 (Bernstein, 2006).

Previous research identifies high presence of bivalent promoter domains in EMT-induced mesenchymal cells, with modulation of H3K27me3 as the predominant mediating presence between epithelial and mesenchymal cell states in gene expression (Malouf et al., 2013). Thus, removal of the H3K27me3 mark may be significant in MET and re-establishment and colonization of distant tumors.

KDM6A: An Enzyme That Regulates Chromatin Structure and EMT

Lysine-specific demethylase 6A, or KDM6A (also known as Ubiquitously Transcribed Tetratricopeptide Repeat Protein X-Linked, or UTX), is a ~154 kDa histone

demethylase enzyme that acts canonically as a component of the COMPASS-like complex to remove the H3K27me3 mark and activate target genes (Schulz et al., 2019). As a part of the COMPASS-like complex, KDM6A's secondary functions include methylation of lysine 4 on histone H3 (H3K4) and acetylation of H3K27 (Shilatifard, 2012; Ford and Dingwall, 2015). KDM6A performs its demethylase function through the JmjC catalytic domain, which is shared by KDM6A paralogs KDM6B/JMJD3 and KDM6C/UTY (Hong et al., 2007; Walport et al., 2014).

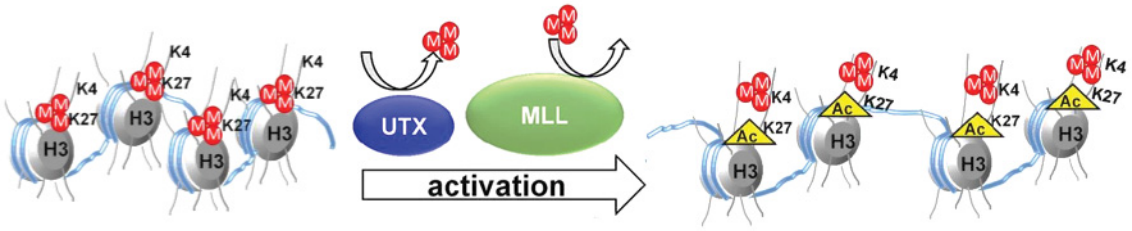

Figure 1.2: KDM6A/UTX in Gene Activation Through Chromatin Remodeling (Schulz et al., 2019)

Pathologically, hereditary mutations in KDM6A are known to cause Kabuki syndrome (Van Laarhoven et al., 2015). However, in cancer, KDM6A's role as a tumor suppressor or oncogene is less clear. Evidence suggests that KDM6A's cancer promoting or inhibitory properties are contingent on cancer type and interactions with transcription factors. In urothelial bladder cancers and certain T-cell leukemias, KDM6A loss supports tumor suppressor capabilities, with mutations leading to cancerous outcomes (Ntziachristos et al., 2014; Hurst et al., 2017; Kobatake et al., 2020). Conversely, the expression of KDM6A in breast, prostate, and cervical cancers confers pro-oncogenic activity (Kim et al., 2014; Toska et al., 2017; Morozov et al., 2017; Soto et al., 2017).

Interestingly, recent research implicates KDM6A in EMT and the metastatic pathway. KDM6A coordinates downregulation of the TGFβ pathway and expression of E-cadherin to favor the epithelial cell state (Li et al., 2020; Zha et al., 2016). Further, loss of KDM6A has been shown to induce metastatic pancreatic cancer in females, and re-expression of KDM6A may be an important factor in colonization of metastatic tumors through MET (Andricovich et al., 2018; Taube et al., 2017). Therefore, by understanding KDM6A expression and its regulation throughout EMT, we can clarify its role in the metastatic cascade and propose new targets for cancer medicine applications.

Regulation of mRNA Translation by MicroRNAs

MicroRNAs (miRNAs) are short ~22 nucleotide regulatory RNAs that bind to target mRNA sequences and block translation or signal mRNA degradation (Bartel, 2018). In the canonical pathway for miRNA biogenesis, the miRNA begins as a pri-miRNA precursor sequence transcribed by RNA polymerase II, which is then loaded into the Microprocessor complex (consisting of Drosha and DGCR8 proteins) and cleaved to form the pre-miRNA hairpin. (Denli et al., 2004). The pre-miRNA is exported from the nucleus to the cytoplasm by the Exportin 5/RAN-GTP complex, and the pre-miRNA is further cleaved near the terminal hairpin loop by the endonuclease Dicer to generate the miRNA duplex (Okada et al., 2009; Hutvágner, 2001). Finally, with the help of chaperone proteins, one strand of the miRNA duplex is loaded into an Argonaute (AGO) protein to form the RNA-induced silencing complex (RISC) while the other is degraded. Selection between the 5' and 3' miRNA strands of the duplex is based on binding affinity and complex stability. The RISC goes on to regulate gene translation, using the mature miRNA as a guide (Iwasaki et al., 2010; Kawamata and Tomari, 2010). The miRNA

"seed sequence," or nucleotide positions 2-7 of the miRNA 5' end, bind the 3' untranslated region (UTR) of the target mRNA sequence through Watson-Crick base-pairing (Lewis et al., 2003). In humans, miRNA and target mRNA pairing is imperfectly complementary, allowing one miRNA to bind to multiple mRNA targets (Bartel, 2018).

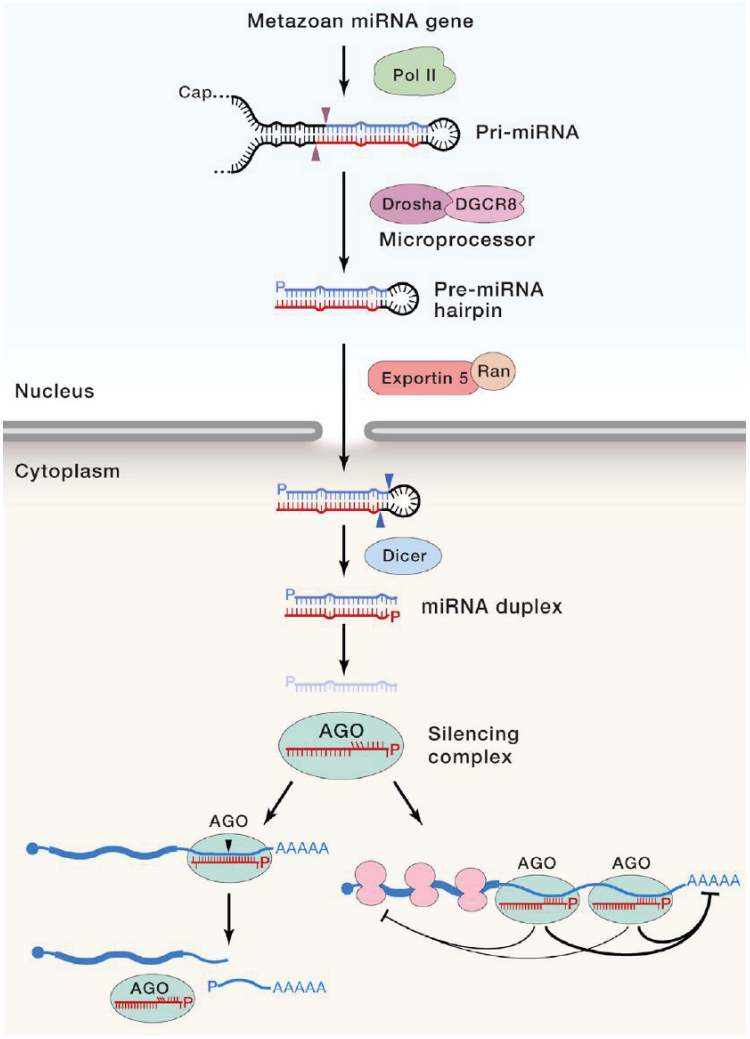

Figure 1.3: miRNA Biogenesis and Functions (Bartel, 2018)

Of the hundreds of miRNAs that have been identified in humans, many have conserved sequence homology with the miRNAs other animals. Additionally, human miRNA interactions with most mRNA transcripts are highly conserved. Thus, research

suggests that bioregulation by miRNAs in virtually all developmental processes, with miRNA dysregulation leading to developmental defects and conditions such as epilepsy, infertility, and immune disorders (Friedman et al., 2009; Bartel, 2018). Of particular interest, aberrant amplifications and deletions of certain miRNAs are associated with malignant neoplasms, including leukemia, lung, and breast cancer (Peng and Croce, 2016). Further, miRNAs play a crucial role in EMT progression. For example, miR-34 and the miR-200 family repress expression of EMT transcription factors such as ZEB1 and SNAI1, and loss of miR-203 is required for EMT and cancer stem cell properties (Lamouille et al., 2013; Taube et al., 2013). Given their widespread influence in biological processes and dynamic roles in translational regulation, miRNAs are ideal candidates of research for understanding molecular relationships and development of cancer therapies.

Rationale and Hypothesis

Despite its controversial nature, a rapidly growing body of EMT research supports the valuable insight EMT provides in clarifying the molecular mechanisms of primary tumor metastasis. One mechanism for EMT modulation is facilitated by post-transcriptional modifications of the histone code, including the marker H3K27me3, a histone modification that enforces gene silencing and accumulates in cells undergoing EMT. H3K27me3 is targeted by KDM6A, a histone demethylase enzyme that induces the epithelial cell phenotype. By understanding KDM6A expression and its regulation throughout EMT, we can clarify its role in the metastatic cascade.

Previous research in our lab has determined that KDM6A is not dynamically regulated at the transcription level. We now aim to investigate the post-transcriptional

mechanisms that modulate KDM6A expression patterns. We hypothesize that KDM6A mRNA transcripts are regulated by specific miRNAs that inhibit expression of KDM6A protein, influencing EMT and downstream cellular morphology.

CHAPTER TWO

Materials and Methods

miRNA Prediction Tools and Database Search

Seven databases (miRsearch, DIANA, MIRANDA, MIRBRIDGE, PICTAR, PITA, RNA22, and TARGETSCAN) were utilized through the miRSystem tool to extract miRNAs that putatively target *KDM6A* based on computational sequence analysis using available genomic data to predict putative miRNA binding at the *KDM6A* 3' UTR (Paraskevopoulou et al., 2013; Krek et al., 2005; Kertesz et al., 2007; Miranda et al., 2006; Agarwal et al., 2015; Lu et al., 2012).

To further narrow miRNA candidates, miRbase, miRmine, and UCSC Xena (uses data from The Cancer Genome Atlas, an NIH reference based on cancer patient data), repositories for experimental and clinical data, were used to make final preliminary miRNA selections (Kozomara, 2019; Panwar, 2017; Goldman, 2020).

Cell Culture

MCF10A cells (ATCC) were cultured in DME/F12 media (GE Healthcare Life Sciences) supplemented 5% horse serum (GE Healthcare Life Sciences), 1% penicillin/streptomycin (Lonza), 20 ng/mL EGF (Sigma), 10 ng/mL insulin (Sigma), 500 ng/mL hydrocortisone (Acros Organics), and 100 ng/mL cholera toxin (Enzo Life Sciences). Cells were plated at 10,000 cells/cm^2 and passaged every other day to maintain consistent densities. For TGFβ treatment, media was supplemented with 5 ng/mL recombinant human TGFβ-1 (R&D Systems; resuspended in 4 mM HCl, 0.1% BSA).

MCF7, MDA-MB-231, and Hs578t cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L glucose, L-glutamine, sodium pyruvate, 10% fetal bovine serum and penicillin/streptomycin. The cells were indefinitely incubated at 37° C and 5% CO2. The cells were passaged periodically when confluent, at 80% plate coverage, using 0.15% Trypsin.

For viral transfection of doxycycline-inducible miR-23a-3p shMIMIC and control cell lines, HEK-293T cells (ATCC) were cultured in DMEM (Corning) supplemented with 10% FBS (Equitech-Bio) and 1% penicillin/streptomycin (Lonza).

Transfection and Transduction

Bacterial stocks containing miR-23a-3p shMIMIC inducible lentiviral vector were purchased from Dharmacon (reference number: MIMAT0000078). To package the miR-23a plasmids into the lentivirus, HEK293T cells were plated and exposed to a mixture of Fugene 6 (Promega), lentiviral packaging plasmids (delta8.2 and pVSVG (Addgene)), and the plasmid of interest for 24 hours. Fugene 6 allowed the mix to enter the 293T's and the cells began to produce lentivirus. The supernatant was then removed and placed on MCF7 and MDA-MB-231 cells and incubated for 24 hours. Puromycin (10 μg/mL) was then administered to select for cells containing the plasmid of interest. Following gene-positive cell selection, overexpression of miR-23a was induced through doxycycline (doxy) administration in treatment groups. Cells were harvested 48 hours after doxycycline induction, and GFP images were acquired using an inverted Nikon Eclipse Ts2R fluorescent microscope.

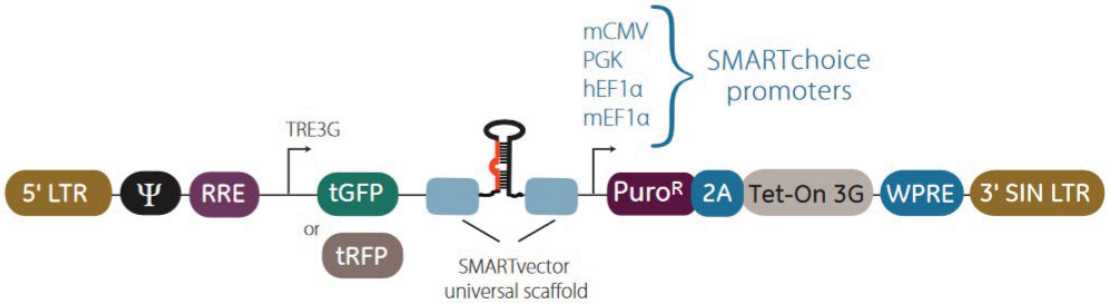

Figure 2.1: Elements of the shMIMIC Inducible Lentiviral miRNA Vector (Dharmacon, Horizon Discovery)

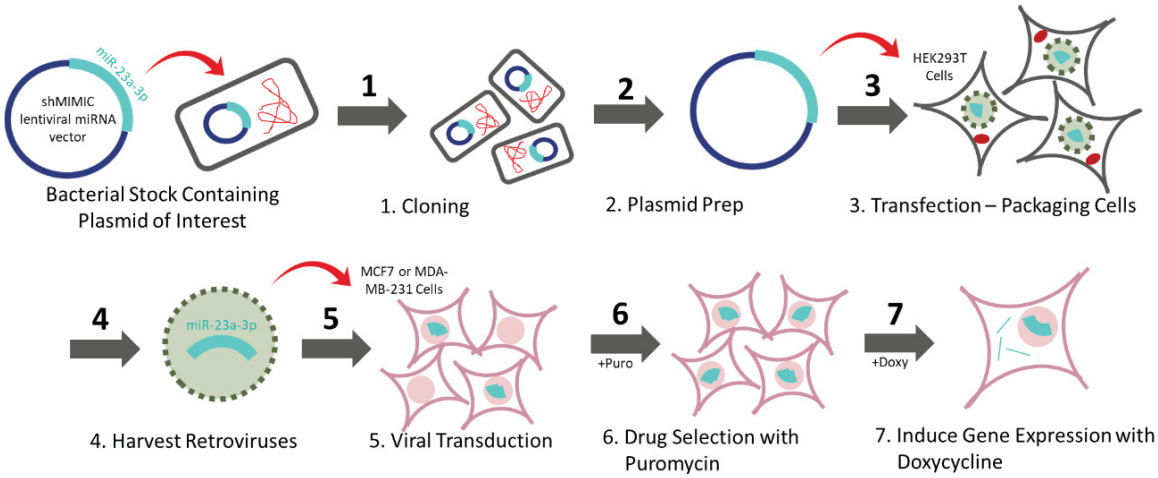

Figure 2.2: Inducing miR-23a/b Overexpression Through Lentiviral Transduction

Antibodies and Immunoblotting

Primary antibodies, used for immunoblotting and immunofluorescence, were raised against the following antigens: KDM6A (NBP1-80628; Novus Biologicals, Centennial, CO, USA) and beta-actin (612657; BD Transduction Laboratories, San Jose, CA, USA). For immunoblotting, proteins were extracted by lysing cells in ice-cold radioimmunoprecipitation (RIPA) buffer containing protease and phosphatase inhibitors (Alfa Aesar, Ward Hill, MA, USA). Protein was quantified using the BCA Assay (Thermo Scientific, Rockford, IL, USA). Cell lysates (20-30 µg) were resolved using

SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were probed with primary antibodies overnight at 4°C, followed by extensive washing with TBST, and incubation with secondary antibodies for 1 hour at room temperature. Chemiluminescent signals were detected with ECL™ prime (Cytiva, Marlborough, MA, USA) using the Biorad ChemiDoc system.

RNA Extraction and Quantitative Reverse-Transcription PCR

Cells were lysed in the presence of Trizol Reagent (Life Technologies) and total RNA extracted following manufacturer protocol recommendations. RNA was reverse transcribed to obtain cDNA, which was then amplified in a quantitative polymerase chain reaction (RT-qPCR). RT-qPCR of KDM6A mRNA levels was performed using the SYBR Green assay with GAPDH as the reference gene.

For RT-qPCR of miRNAs, two methods were utilized. Initial miRNA quantification of miR-23a, -23b, -199a, and -144 was performed using the poly-A tailing method, where a poly-adenine nucleotide "tail" is added to the 3' end of the miRNA before reverse transcription and subsequent amplification (Figure 2.3A). Using the poly-A tailing method, miRNAs were quantified using the SYBR Green assay with reference to SNORD44. After identification of miR-23a/b as miRNA of interest and further investigation, a miR-23a-specific stem-loop primer was used for reverse transcription (Figure 2.3B), and miR-23a/b relative levels were quantified using the TaqMan assay with reference to U6 (RNU6-1) snRNA (Applied Biosystems, Thermo Scientific).

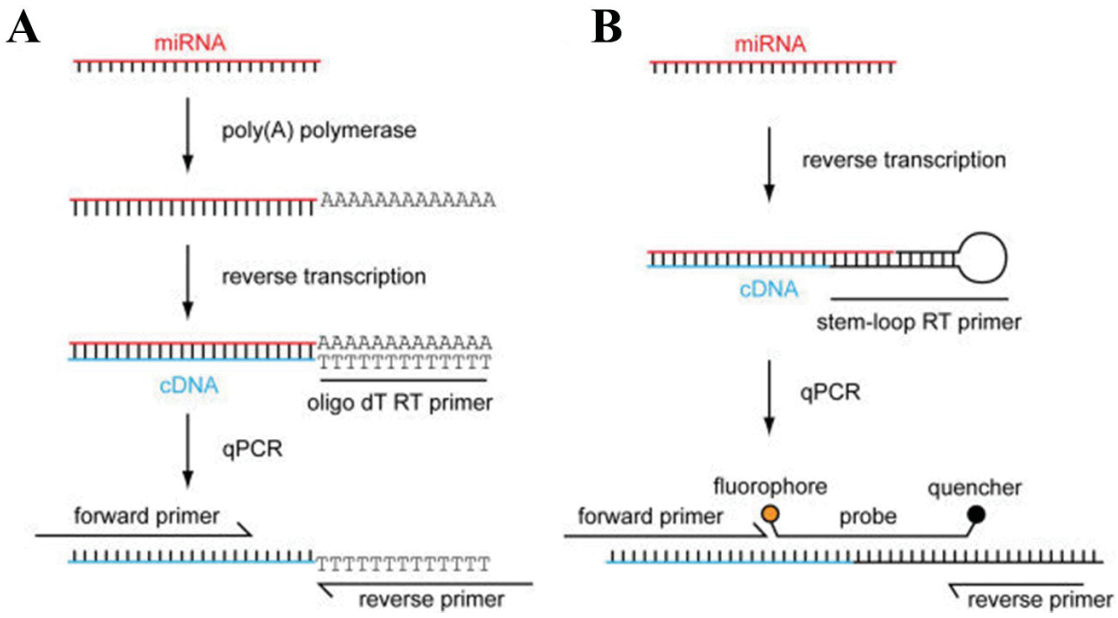

Figure 2.3: RT-qPCR Quantification Methods for miRNAs (Roberts et al., 2014). (A) Poly-A tailing method with SYBR Green detection assay. (B) Stem-loop primer method with TaqMan detection assay.

Preliminary experiment primers for KDM6A (F: TTCCTCGGAAGGTGCTATTCA, R: GAGGCTGGTTGCAGGATTCA), miR-23a-3p (ACATTGCCAGGGATTTCCAA), miR-23b-3p (TCACATTGCCAGGGATTACCA), miR-144-5p (CAGTTTTCCCAGGAATCCCTAA), and miR-199a-3p (CAGTAGTCTGCACATTGGTTAAAAA) were obtained from Eton Biosciences, and miR-23a-3p stem-loop primers were obtained from Applied Biosystems (sequence proprietary). Relative quantification was calculated from qPCR data using the comparative Ct method with the formula $2^{-\Delta\Delta Ct}$. All RT-qPCR experiments were run in quadruplicate and a mean value was used for the determination of mRNA levels.

CHAPTER THREE

Results

Disparity in KDM6A RNA and protein dynamics confirm unlikely KDM6A *regulation during transcription*

KDM6A is highly expressed in epithelial cells, decreases during EMT, and is re-expressed during MET. TGFβ is a cytokine that induces EMT when applied to epithelial cells and promotes MET during its withdrawal. During previous work in our lab, we

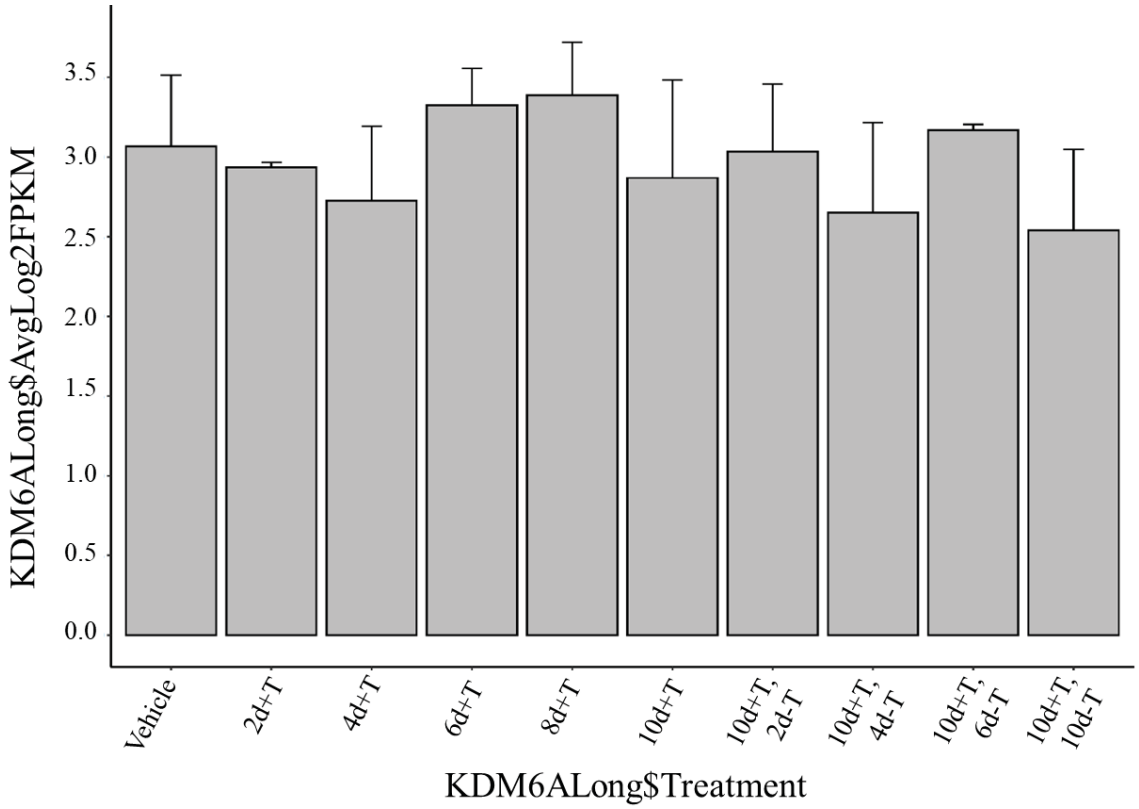

Figure 3.1: Preliminary RNA-Seq data showing KDM6A expression in TGFβ-induced EMT and MET. Treatment samples as shown on the x-axis corresponds to days of TGFβ treatment and withdrawal. For example, "10d+T, 2d-T" denotes 10 days of TGFβ treatment and 2 days of TGFβ withdrawal. (Credit: Kelsey Johnson)

induced EMT and MET in MCF10A, a non-cancerous epithelial mammary cell line, through administration and withdrawal of TGFβ. We then collected RNA samples at specific EMT and MET timepoints and measured expression of KDM6A. RNA-Seq analysis of our data showed inconsistent patterns of *KDM6A* RNA expression and no significant difference between EMT and MET timepoints (Figure 3.1).

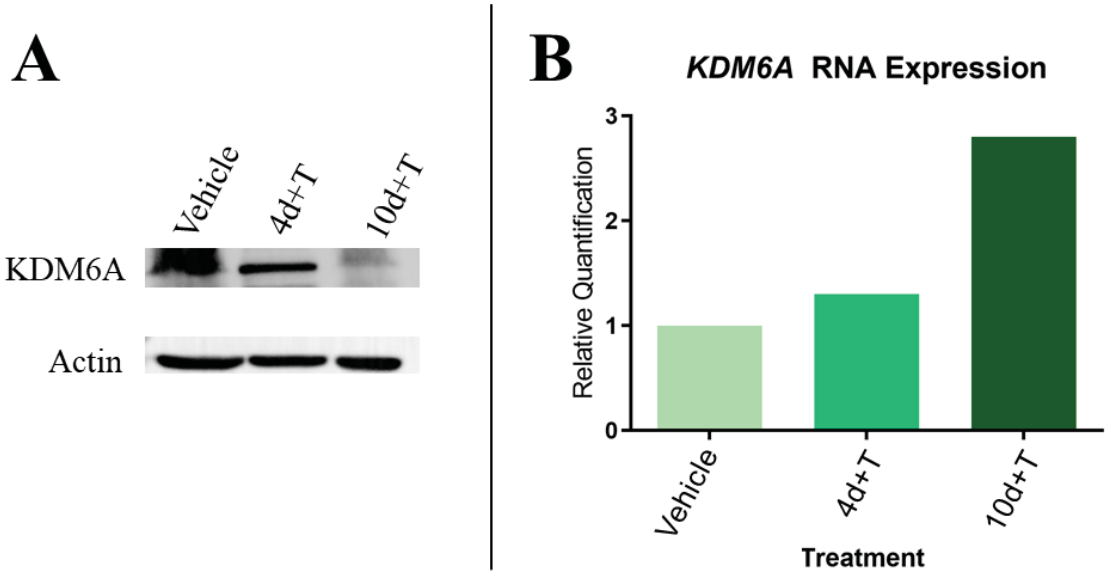

Figure 3.2: Validation of KDM6A expression during TGFβ-induced EMT. Experimental groups include: Vehicle (0d+T), 4d+T, and 10d+T. (A) Western blot detecting relative abundance of KDM6A protein. Western blots were prepared by running 20 µg of protein in 12% SDS-PAGE gels. (B) RT-qPCR showing KDM6A RNA relative to Vehicle control.

To further validate KDM6A RNA and protein expression, we performed RT-qPCR and Western blot experiments, respectively, on TGFβ-induced EMT samples. Results show increased RNA expression (Figure 3.2B) but decreased protein expression (Figure 3.2A) for KDM6A following EMT. Should KDM6A have been regulated during transcription, we would expect *KDM6A* RNA levels to decrease during EMT, with all downstream forms of KDM6A following similar expression patterns; however, according

to our data, this is not the case. Therefore, we conclude that KDM6A regulation does not occur during gene transcription but through post-transcriptional mechanisms.

miR-23a, -23b, -145, and -199a are predicted to target KDM6A

A list of ~160 miRNAs that may target *KDM6A* was compiled from seven databases (A)

Top miRNA candidates were selected by number of deep sequencing reads, observed amounts in breast cancer cell lines, and frequency in patient data (B)

A

Database	DIANA	MIRANDA	MIRBRIDGE	PICTAR	PITA	RNA22	TARGETSCAN
Hits	84	105	2	13	54	9	35

B

miRNA	Database Hits	Reads in miRbase	log 2 RPM 578t	log 2 RPM MCF7	Detection in TCGA
hsa-miR-23a-3p	5	898,000	9.55	10.72	Frequent
hsa-miR-23b-3p	6	915,000	10.30	11.56	Frequent
hsa-miR-145-5p	4	1,456,000	8.72	0.74	Frequent
hsa-miR-199a-3p	4	767,000	12.07	2.76	Frequent
hsa-miR-323b-5p	4	1	0	0	None

Figure 3.3: *In silico* database analyses to identify miRNAs that target KDM6A RNA.

To investigate post-transcriptional regulators of KDM6A, we aimed to identify gene expression modulators at the translational level. One mechanism of translational regulation occurs through miRNAs, which canonically inhibit translation by binding to target mRNA sequences. Given this reciprocal relationship, we hypothesized that miRNAs that target *KDM6A* would be present at inverse levels compared to *KDM6A* during EMT and present in high amounts in the mesenchymal cell phenotype. To identify candidate miRNA regulators of *KDM6A*, we searched for sequence homology to the *KDM6A* 3' UTR using seven miRNA target prediction algorithms through the miRSystem tool (Figure 3.3A). Of the resulting ~160 projected miRNAs, we further selected for top miRNA candidates by filtering results through the following biologically relevant parameters: number of deep sequencing reads (miRbase), observed amounts in

breast cancer cell lines (miRmine), and frequency in patient data (UCSC Xena) (Figure 3.3B). Final outcomes of database analysis determined miR-23a-3p, miR-23b-3p, miR-145-5p, and miR-199a-3p as putative regulators of KDM6A.

miR-23a and -23b are dynamically expressed throughout TGFβ treatment and withdrawal

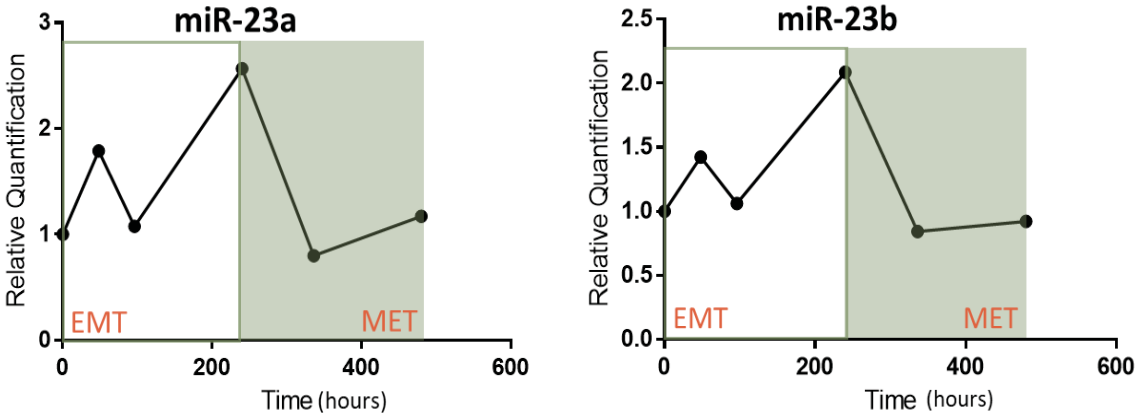

Figure 3.4: RT-qPCR data of miR-23a and -23b expression during TGFβ-induced EMT and MET, relative to Vehicle control. Experimental groups include: Vehicle (0 days); 2d+T; 4d+T; 10d+T; 10d+T, 4d-T; and 10d+T, 10d-T.

To experimentally validate putative miRNA regulators of *KDM6A* in biological samples, RNA was taken at various timepoints during TGFβ-induced EMT and MET in MCF10A cells. RT-qPCR was performed using miR-23a, -23b, -145, and -199a primers. Results were then analyzed by ΔΔCt analysis and relative miRNA expression was graphed (Figure 3.4). Based on the data presented, miR-23a and -23b generally increase during EMT and decrease during MET in vitro, corroborating computational miRNA prediction of miR-23a and -23b. According to RT-qPCR data, miR-145 and -199a were not detected at significant levels. Thus, of the four miRNA candidates predicted in silico,

miR-23a and -23b were identified as likely post-transcriptional regulators of KDM6A, as their relative quantification levels follow expected patterns throughout EMT/MET.

Figure 3.5: Putative binding regions between the 3' UTR of KDM6A and seed sequences of miR-23a-3p and miR-23b-3p.

The 3' strand predominates in the miR-23a and -23b miRNA duplex, as denoted by "-3p" in the miRNA naming convention. miR-23a and -23b sequences are shown in Figure 3.5, differing in only one nucleotide and possessing identical seed sequences. Due to sequence similarity and analogous expression profiles during preliminary experimentation (Figure 3.4), miR-23a and -23b is assumed to serve parallel biological functions and will herein be referred to as miR-23a/b.

miR-23a/b are well-studied miRNAs that are part of the miR-23-3p family alongside miR-23c and miR-130a-5p. miR-23a/b have been characterized as tumor suppressors, though conflicting evidence presents miR-23a/b as oncomiRs, indicating variable roles of miR-23a/b depending on cellular environment and cancer type (Gregorova et al., 2021; Hu et al., 2017). Recent studies have reported that high levels of miR-23a/b correspond to poor gastric cancer patient prognosis and that miR-23a promotes TGFβ-induced EMT by targeting E-cadherin in breast cancer cells (Hu et al., 2017; Ma et al., 2017). Further, miR-23b has been shown to promote metastatic breast cancer dormancy and enable metastasis in pancreatic tumors (Ono et al., 2014; Michael et al., 2019). Therefore, based on current literature and preliminary experimentation, we

conclude that miR-23a/b potentially inhibits the expression of KDM6A protein as cells undergo EMT and gain metastatic properties.

MCF7 and MDA-MB-231 breast cancer cells are suitable models to study the relationship between miR-23a/b and KDM6A expression

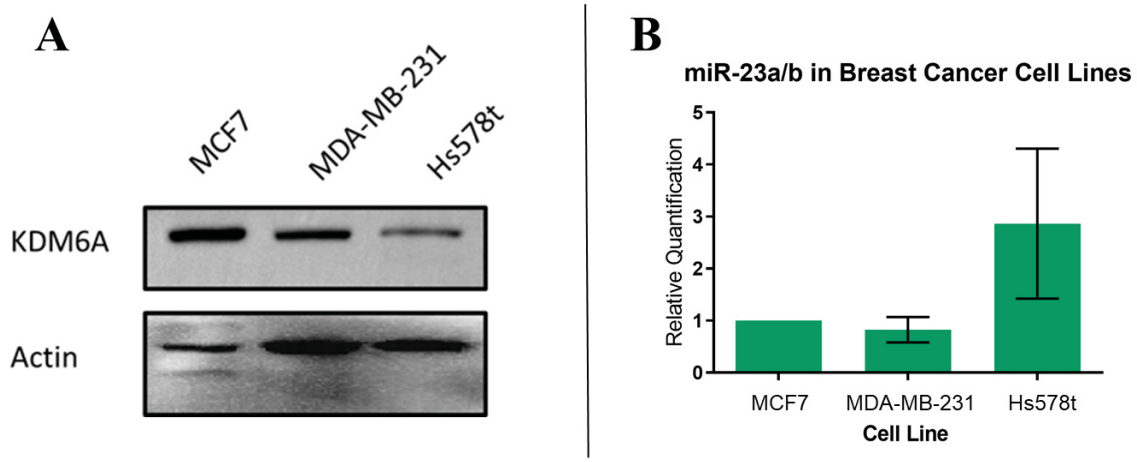

Figure 3.6: KDM6A and miR-23a/b expression in breast cancer cell lines. (A) Western blot detecting relative abundance of KDM6A protein. Western blots were prepared by running 30 μg of protein in 10% SDS-PAGE gels (Credit: Shuxuan Song). (B) RT-qPCR of endogenous miR-23a/b levels relative to MCF7 in breast cancer cell lines. Error bars represent variability between three technical replicates of one biological sample.

To determine whether experimental manipulation of miR-23a/b downregulates KDM6A protein expression, we aimed to overexpress miR-23a/b and observe changes in KDM6A protein levels. In order to induce viable miR-23a/b overexpression to observe appreciable KDM6A expression changes, we aimed to identify the cell lines with relatively high KDM6A protein expression and corresponding low miR-23a/b levels. We measured endogenous KDM6A and miR-23a/b levels in three common breast cancer models: MCF7, an epithelial cell line, and MDA-MB-231 and Hs578t, mesenchymal cell lines. We observed high relative quantities of miR-23a/b in Hs578t cells compared to MCF7 and MDA-MB-231 (Figure 3.6B), inversely corresponding to KDM6A protein

expression in Figure 3.6A. Thus, of the three breast cancer cell lines measured, MCF7 and MDA-MB-231 cells provide the best models to study miR-23a/b overexpression effects on KDM6A protein levels.

Inducible miR-23a/b overexpression is stable in MDA-MB-231 cells in the presence of doxycycline

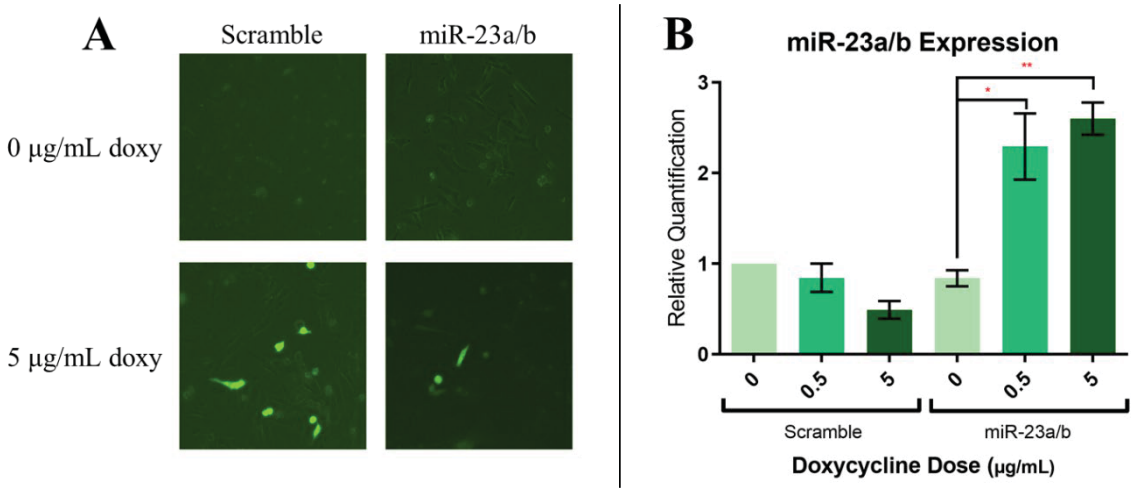

Figure 3.7: Validation of miR-23a/b overexpression in MDA-MB-231 cells. (A) GFP images of treatments and controls 48 hours after doxycycline (doxy) administration. (B) RT-qPCR of miR-23a/b expression levels relative to Scramble 0 doxy control. Experimental groups were treated with varying doxycycline dosages. Error bars represent variability between three biological replicate samples. Statistics of overexpression treatments were analyzed using unpaired, two-tailed t-tests (0.5 µg/mL treatment: $P < 0.05$; 5 µg/mL treatment: $P < 0.01$).

After determining relevant cell lines to perform miR-23a/b overexpression experiments, we overexpressed miR-23a/b (Figure 2.2) in MDA-MB-231 cells, a mesenchymal breast cancer cell line, for the purpose of performing KDM6A protein quantification experiments. The GFP gene element is co-localized on the shMIMIC lentiviral miRNA vector, and GFP visualization acts as an indicator of successful miR-23a/b overexpression and incorporation into the target cell genome (Figure 2.1). GFP

visualization confirms transduction of scramble and miR-23a/b lentiviral vectors into MDA-MB-231 cells (Figure 3.7A). There is a significant difference between relative quantities of miR-23a/b treatment groups compared to control groups in RT-qPCR data, confirming miR-23a/b overexpression and viable controls (Figure 3.7B).

miR-23a/b may not inhibit KDM6A protein expression in MDA-MB-231 cells

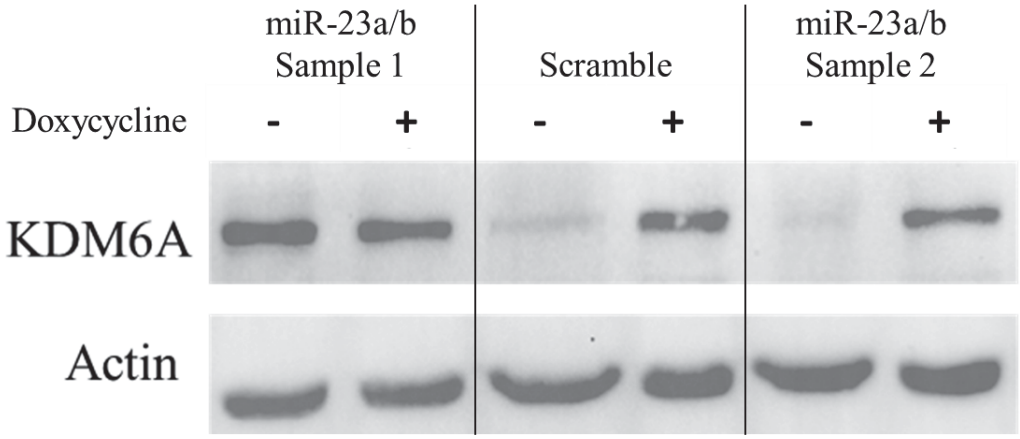

Figure 3.8: Western blot detecting relative abundance of KDM6A protein. Two biological sample replicates are shown. 600 ng/mL of doxycycline was administered in treatment groups (+) relative to 0 ng/mL doxy in controls (-). Western blots were prepared by running 30 µg of protein in 10% SDS-PAGE gels.

After establishing miR-23a/b overexpression, we quantified relative KDM6A protein abundance. As a likely target of miR-23a/b, KDM6A protein was expected to decrease following miR-23a/b induction. Contradictory to our hypothesis, results indicate that KDM6A does not decrease in cells overexpressing miR-23a/b, showing no change or increased expression in doxy-treated cells (Figure 3.8). However, doxy-treated controls also exhibited increased abundance of KDM6A protein, suggesting possible doxycycline or off-target interference in KDM6A expression. Still, it is necessary to revisit technical execution and experimental preparation to ensure biological validity of shown data.

CHAPTER FOUR

Discussion

In this present study, we explored the post-transcriptional regulation of KDM6A by identifying miR-23a/b as a putative regulator of KDM6A translation, inducing overexpression of miR-23a/b in breast cancer cells, and observing KDM6A expression changes in the presence of miR-23a/b upregulation. Contradictory to our initial hypothesis, results tentatively indicate that KDM6A is not translationally repressed by miR-23a/b. However, as shown in Figure 3.9, the control samples show questionable induction of KDM6A protein expression following doxy treatment, indicating that further experiments are necessary to draw firm conclusions on the relationship between KDM6A and miR-23a/b. Further, miR-23a/b overexpression experiments can be repeated in other cell lines, particularly MCF7, to clarify the effect of miR-23a/b on KDM6A protein expression. Additionally, more biological replicates in measuring endogenous miR-23a/b levels in various cell lines, as well as expanding the cell line panel to include additional EMT models would lend further evidence in miR-23a/b's role in EMT.

While miRNAs are best characterized for translational repression and mRNA degradation of their targets, current literature has described alternative biological functions of miRNAs. Surprisingly, a 2007 study by Vasudevan et al. has shown that under certain quiescent cell conditions, miRNA binding to the 3' UTR of target mRNAs activates translational upregulation. Quiescence is the reversible exiting of the cell cycle that can be induced by cellular stress. The conditions that Vasudevan et al. describe as

necessary for miRNA-mediated translational activation include quiescent cell state, high (A+U)-rich elements in the target mRNA 3'UTR, and recruitment of FXR1 to the RISC.

Quiescence is a hallmark of stem cells, including cancer stem cells (CSCs), a cancer cell subset that are resistant to chemotherapies (Chen et al., 2016). In relation to EMT, mesenchymal cells have been shown to contain higher proportions of CSCs, including MDA-MB-231 cells (Sheridan et al., 2006). In our data, the binding region between miR-23a/b and the 3' UTR of KDM6A mRNA show high (A+U) enrichment (Figure 3.3), and in literature, miR-23b has been shown to induce dormancy (Ono et al., 2014). Further, Figure 3.3B shows similar abundance of miR-23a/b in epithelial (MCF7) and mesenchymal (Hs578t) cell lines, and in Trial 2 of Figure 3.7A, KDM6A expression increased after induction of miR-23a/b. Therefore, it is possible that miR-23a/b may be implicated in both the activation and repression of KDM6A translation during EMT, dynamically mediating opposite regulatory functions as CSCs achieve quiescence and then re-establish aggressive tumor functions at distant metastatic sites.

Further research on miRNA regulation of KDM6A may utilize alternative means of miRNA prediction analysis. First, data from our bioinformatic analysis (Figure 3.3) may be revisited to propose other potential miRNAs for KDM6A regulation. Alternatively, new miRNA candidates may be identified by performing a synthetic miRNA screen. Such an experiment may be implemented by transfecting a reporter vector containing the KDM6A 3' UTR into a viable cell model, exposing the cells to multiple synthetic miRNAs of interest, and then measuring for downregulation of KDM6A expression using a luciferase assay.

Additional studies of KDM6A inhibition may explore different mechanisms of post-transcriptional regulation other than miRNA translational repression. One such program is alternative splicing, or the process by which a single gene can generate multiple protein products through mRNA transcript editing (e.g. intron excision, exon skipping) (Baralle and Giudice, 2017). According to the UCSC Genome Browser, there are nine distinct KDM6A splice isoforms. In KDM6A, the JmjC catalytic domain responsible for KDM6A's histone demethylase activity is contained on amino acid positions 1095-1258 on the KDM6A protein, and alternative splicing of KDM6A transcripts may attenuate KDM6A's catalytic functions (Kent et al., 2002). Differential processing of KDM6A exons in epithelial versus mesenchymal cells may contribute to translational repression of KDM6A during EMT. Within this alternative splicing framework, we can hypothesize that an inactive isoform of KDM6A, with the JmjC domain spliced out during mRNA transcript processing, is the predominant isoform present in mesenchymal cells as the cell undergoes EMT.

Overall, given our data, miR-23a/b is highly predicted to repress translation of KDM6A. It remains to be seen whether miR-23a/b truly does not inhibit KDM6A protein expression, and further validation and testing is necessary to clarify this relationship. Additionally, the biological role of miR-23a/b may be influenced by cellular proliferation and quiescence, highlighting the dynamic nature of KDM6A modulation. Future studies in post-transcriptional modifications of KDM6A can clarify KDM6A's role in metastatic activity and contribute new targets for research in clinical applications.

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