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Knockdown of MDM2 in HepG2 Cell Line: A Step Towards Understanding the Relation Between miRNA590-3p and its Downstream Target Gene, MDM2, in Hepatocellular Carcinoma

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Knockdown of MDM2 in HepG2 cell line: A step towards understanding the relation between miRNA590-3p and its downstream target gene, MDM2, in Hepatocellular carcinoma

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By

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Declaration of Authorship

I, [Jihad Mahmoud Khaled], declare that this thesis titled, "[**Knockdown of MDM2 in HepG2 cell line: A step towards understanding the relation between miRNA590-3p and its downstream target gene, MDM2, in Hepatocellular carcinoma.**]" and the work presented in it are my own. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University.
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- Where I have consulted the published work of others, this is always clearly attributed.
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work.
- I have acknowledged all main sources of help.
- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself.

Signed:

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Date:

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Dedication

To

My beloved parents, my lovely sisters (Asmaa and Neama), my awesome brothers (Khaled and Ahmed), and my beautiful aunts (Amel and Fatma) without whom I could not have undertaken this journey

Abstract

Background: miRNA 590-3p is a small non-coding RNA that has previously been associated with the occurrence and progression of several cancer types. Its expression pattern and biological role in Hepatocellular carcinoma (HCC), however, remain controversial. Interestingly, a previous study in our lab revealed a tumor suppressing activity of miR-590-3p in HCC and identified the *MDM2* gene as the miR-590-3p target gene.

Aim: The current study aimed to knock down the expression of MDM2 in HepG2 cells to understand how the inhibition of MDM2, as a validated downstream target of miR-590-3p, would affect different functional pathways in HCC. In addition, we aimed to identify further downstream targets in the "mir-590-3p-MDM2" pathway and understand their role in the development of HCC.

Methods: RNAi-mediated knockdown was used to inhibit mRNA and protein levels of MDM2. Clonogenic cell survival assay was utilized to assess HepG2 cell proliferation, while transwell assay was carried out to evaluate the migratory behavior of these cells. miR-590-3p (mimics and NC) transfected HepG2 cells were used to assess the effect of miR-590-3p overexpression on target genes. In silico analysis was employed to identify further downstream targets in the "mir-590-3p-MDM2" pathway. Potential target genes predicted by bioinformatics tools were subjected to RT-qPCR analysis.

Results: The transient knockdown of MDM2 in the HepG2 cells had a silencing effect up to 80% at mRNA level and almost 70% at protein level. In clonogenic cell survival assay and transwell assays, MDM2 gene silencing was shown to inhibit cell proliferative and migratory behavior of HepG2 cells. Gene expression analysis revealed that this miRNA functions, at least in part, by influencing the expression of genes that regulate EMT progression, which was also downregulated following MDM2 knockdown in HepG2 cells. Additionally, FOXO3 was identified as a novel target in the miR-590-3p/MDM2 pathway.

Conclusion: Our results not only reveal a crucial role for MDM2 in the regulatory mechanism of EMT in HCC but also demonstrate novel targets for miR-590-3p in HCC. Moreover, these results shed light on the important role of the miR-590-3p/MDM2 pathway in HCC.

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List of abbreviation

3’ UTR	**3’ Untranslated region**
Anti-OncomiR	Anti-oncomiRNA
BCA	Bicinchoninic acid
CSmiRTar database	Condition-Specific miRNA Targets
DMEM	Dulbecco's Modified Eagle's Medium
EMT	Epithelial-Mesenchymal Transition
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FOXO	Forkhead box protein O
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GENT2	Gene Expression database of Normal and Tumor tissues 2
HBV	Hepatitis B Virus
HCC	Hepatocellular carcinoma
LDLT	living-donor liver transplantation
LT	Liver Transplantation
MC	Milan Criteria
MDM2	Mouse double minute 2 homolog
MMPs	Matrix metalloproteinase
N-cadherin	Neural Cadherin
P-value	Probability value
p53	Tumor antigen p53 gene

PBS	phosphate buffered saline
Pen-Strip	Penicillin-Streptomycin
pRb	Retinoblastoma protein
Pre-miRNA	microRNA Precursor
Pri-miRNA	Primary microRNA
RISC	RNA induced silencing complex
RISC complex	RNA inducing silencing complex
RNAi	RNA interference
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
siNTC	Non-targeting siRNA
siRNA	Small interfering RNA
Snail	Snail family zinc finger 1
TBST	0.01% Tween-20 in 1X TBS
ZEB	Zinc finger E-box-binding homeobox

Chapter 1 : Background

1.1 Hepatocellular carcinoma

1.1.1 Incidence and Epidemiology

With an estimated almost 905,000 newly diagnosed cases and 830,000 deaths over the world, liver malignancy constitutes the 6th most frequent cancer and the 3rd leading cause of cancer death (Sung et al., 2021). Liver cancer is classified as primary or secondary (metastatic). HCC accounts for roughly 80% of primary hepatic malignancy, thereby representing the most common type of liver malignancy (Singal et al., 2020). Regionally, incidence rates are highest in Northern and Western Africa and Eastern Asia, followed by mid-levels in Southern European countries, such as Italy, Spain, and Greece, whereas Northern Europe and South Central Asia have the lowest rates (Mittal & El-Serag, 2013; Suresh et al., 2020). Among men, HCC is more prevalent, with an incidence ratio of almost 4:1 between males and females (Llovet et al., 2021). The highest incidence of HCC is among adults aged 45–65 years old, rarely occurring during the first 4 decades of life (El-Serag, 2012; Suresh et al., 2020).

From a worldwide perspective, HCC is a major global health burden, with epidemiological data varying by location (Sung et al., 2021). According to health authorities in Egypt, HCC is the most challenging health issue, ranking as the 2nd most commonly diagnosed malignant tumor in males and the 6th most common malignancy in females (Omar et al., 2013; Rashed et al., 2020). It has been estimated that the frequency of all liver-related malignancies in Egypt increased by 2-fold from 4.0% in 1993 to 7.2% in 2003 (Rashed et al., 2020). The reason for increased incidence is primarily due to the rising prevalence of viral hepatitis and its related

complications, which are a significant factor contributing to the occurrence of HCC worldwide (Omar et al., 2013).

1.1.2 Etiology and Risk factors

In almost 80- 90% of the cases, HCC develops against a background of cirrhosis, suggesting that cirrhosis is a crucial prerequisite for liver carcinogenesis (El-Serag, 2012). Prominent causes of cirrhosis are therefore considered risk factors for HCC development, including infection with blood-borne viruses, aflatoxin-contaminated food, and heavy alcohol intake (Dhanasekaran et al., 2012). Other risk factors, such as metabolic syndrome (diabetes or obesity), nonalcoholic fatty liver disease, and tobacco smoke inhalation, have also been proposed to lead to HCC, albeit to a limited extent. Of note, HCC screening, early detection, prevention, and management could all be improved with a deeper understanding of the risk factors that affect the burden of HCC.

In developing countries, most cases of HCC (approximately 80%) are linked to chronic HBV or HCV infections (Alqahtani & Colombo, 2020). HBV infection is a type of viral infection caused by a small DNA virus that belongs to the "Hepadnaviridae" family (Yuen et al., 2018). Although HBV infection is the strongest epidemiologic factor associated with HCC worldwide, its influence on the etiology of HCC in Egypt has declined in the last two decades. The reason for reduction could be attributed to the successful nationwide vaccination strategy and the high prevalence of the hepatitis C virus. Despite the decline, chronic HBV infection continues to be a dominant cause of HCC because national infant immunization programs were not implemented until the 1980s, and therefore most individuals aged 20 years or older have not been vaccinated (Omar et al., 2013).

Unlike HBV infection, HCV is a type of viral infection caused by a single-stranded virus that belongs to the family Flaviviridae (Dhanasekaran et al., 2012). Many studies conducted in Egypt have indicated that HCV infection is emerging as a predominant cause of chronic hepatic disease that progresses to HCC, accounting for 40-50% of cases, which is considered the biggest contribution to the incidence of HCC (Omar et al., 2013).

Another contributable risk factor to developing HCC is exposure to Aspergillus-derived aflatoxin (Chidambaranathan-Reghupaty et al., 2021). Aflatoxins are toxic and carcinogenic fungal metabolites produced by certain molds, particularly *Aspergillus* species (Kimanya et al., 2021). In Africa and several countries in Asia, the presence of Afla toxins is very common due to hot and humid climates that promote fungi growth. Recent investigations in Egypt argue that aflatoxin contributes to HCC development, and this is becoming an increasingly common cause of HCC (Omar et al., 2013).

1.1.3 Treatment

The current treatment modalities available for HCC are classified based on the stage of HCC. For example, curative treatments, including hepatic resection, liver transplant, and local tumor ablation, are recommended for the HCC at the early stage (Bruix,.2014). In the advanced stage, standard treatments, which FDA has approved, such as sorafenib and regorafenib, are being used. It is obvious that treatment choices in the advanced stage are limited (Kim et al., 2017). Unfortunately, a significant proportion of HCC cases are diagnosed in the advanced stage. Consequently, only 15% of cases are eligible for these curative therapies. Even after using curative treatment such as surgical resection, about 70% of patients may suffer from tumor recurrence (Fujiwara et al., 2018). Yet, it is noted that these treatments and management approaches exist with various pros and cons.

1.1.3.1 Surgical Rection

Surgical resection is the best therapeutic treatment for non-cirrhotic patients with excellent liver function. In surgical resection, the entire tumor or as much as of the tumor is removed (Llovet et al., 2021). Although extensive resections can be conducted with minimal morbidity, strict selection criteria are required to check for the existence of cirrhosis because post-operative complications, such as liver failure, are more common (Schlachterman et al., 2015). Yet, despite the rigorous criteria used in selecting and screening out eligible candidates, HCC continues to have a high recurrence rate, affecting almost 70% of the cases at five years post-resection (Schlachterman et al., 2015; Ferrante et al., 2020). Unfortunately, when tumor recurrence occurs, the tumor tends to be more aggressive and challenging to cure (Schlachterman et al., 2015). In this case, evaluation for repeat resection should be considered. Regardless of this, hepatic resection remains the best therapeutic option for HCC cases with no underlying cirrhosis and early HCC due to its availability and improved survival. Finally, for HCC cases with a high risk of recurrence, pre-emptive liver transplantation has been suggested.

1.1.3.2 Liver Transplantation (LT)

To date, LT is regarded as an ideal therapeutic approach for treating HCC as it offers the potential for curing both the tumor and the underlying cirrhosis (Santopaolo et al., 2019). A well-established criterion to select and screen the LT candidates is the Milan criteria-defined as having one solitary tumor < 5 cm in diameter or up to three tumor lesions each < 3 cm, without extra-hepatic spread (Mazzaferro et al., 1996; Attwa & El-Etreby, 2015). Many reports indicate that patients who fulfill the Milan criteria before LT have a 5-year survival of 68% and a recurrence rate of 10–15% (Kumari et al., 2018). The critical hurdle in LT is that the shortage of donor organs leads to long waiting times on the transplant list. During this period, failure to treat HCC will invariably result in extra-hepatic masses and a higher degree of invasiveness that disqualifies patients for LT (Kumari et al., 2018). Current data indicate that

the dropout rate due to progression while on the waitlist is estimated at 25% at one year (Schlachterman et al., 2015).

It is essential to point out that living-donor liver transplantation (LDLT) could shorten the waiting period and reduce the exclusion rate for HCC patients requiring transplant and has comparable results to cadaveric donor transplantation (Brown, 2008). Specifically, when waiting period exceeds 7 months, a decision analysis of LDLT based on cost, dropout, and donor mortality proves beneficial (Bhardwaj et al., 2016). Not surprisingly, surgeons generally prefer LDLT as an alternative to deceased donor LT and a possible option for transplantation for HCC cases that do not fulfill the criteria (Kaido & Uemoto, 2010; Schlachterman et al., 2015).

1.1.3.3 Other Treatments

For patient at an earlier stage of HCC and outside the relam of curative therapies, such as LT and liver resection, ablative treatments are the best therapeutic option (Tejeda-Maldonado et al., 2015; Kumari et al., 2018). In these treatments, HCC tumor mass is destroyed either through chemical injection (such as ethanol, acetic acid, or boiling saline) or through the application of different energy sources (heat and cold) (Dhanasekaran et al., 2012; Ferrante et al., 2020). It is significant to note that these treatments can be utilized not only as an effective therapy but also as a bridge therapy to keep patients on the liver LT list (Gish et al., 2013). Despite these advances in HCC treatment, the prognosis for HCC cases remains poor owing to the high recurrence rate (Khemlina et al., 2017). Indeed, a comprehensive understanding of HCC oncogenic processes and the signaling pathways would assist in identifying more effective diagnostic and curative solutions for HCC at earlier stages.

1.1.4 Molecular and Cellular pathogenesis of HCC

The regulatory mechanisms underlying HCC tumorigenesis are still quite unclear. Advanced studies in hepatic carcinogenesis identified multiple dysregulated signaling pathways in liver carcinogenesis, such as WNT-β-catenin, P13/PTEN/AKT, RAS/MAPK, IGF, HGF/MET, VEGF, EGFR, and PDGF (M. Wu et al., 2019; Dimri & Satyanarayana, 2020). Genomic instability, including telomerase activation, chromosome translocation, inactivation of DNA-damage-response components, and others, have also been described in HCC. More recently, a comprehensive analysis of miRNA expression patterns in HCC revealed dysregulation of several miRNAs (Bruix et al., 2014; Farazi & DePinho, 2006). Consequently, all these events may lead to cells that sustain proliferation, evade growth suppressors, and undergo epithelial to mesenchymal transition (Farzaneh et al., 2021).

As for the relationship between miRNA and HCC, although it is now obvious that miRNAs serve a crucial role in tumorigenesis, the exact mechanisms by which miRNAs exert their roles in oncogenesis still need further investigation (Morishita & Masaki, 2015). Therefore, it is urgent to elucidate the underlying mechanisms of HCC tumorigenesis. In addition, exploring new potential biomarkers is urgent to allow for early diagnosis and may be the treatment of HCC. In current investigation, we investigated the role of the miRNA 590-3p/MDM2 axis in HCC.

1.2 miRNAs

1.2.1 Introduction and Mechanism of Action

miRNAs are defined as endogenous short non-coding RNAs with a length < 25 nucleotides and without peptide-coding functions (Reddy, 2015). Several elegant studies have revealed that miRNAs regulate several physiological and pathologic processes. In the human genome, more

than 2,588 miRNAs have been discovered, and these miRNAs can control almost 60% of all human genes (Friedman et al., 2009; Shu et al., 2017). Recently, several miRNAs have been found to be abnormally expressed and involved in regulating many processes associated with malignant progressions, such as EMT, proliferation, survival, and metastasis.

To exert their regulatory functions, miRNAs bind to the 3'-UTR of their target mRNA, resulting in translational inhibition (Morishita & Masaki, 2015; Peng & Croce, 2016). In studies examining the regulatory mechanisms of miRNA, researchers have revealed that miRNAs mainly cause translational repression, but not a direct cleavage, due to partial complementarity between the miRNA and its target mRNA (Bartel, 2009; Oliveto et al., 2017). Additionally, owing to the short base pairing between miRNA and its target mRNA, one miRNA may regulate hundreds of RNA targets.

1.2.2 Biogenesis of miRNAs

Figure (1) represents miRNA biogenesis in humans. The biogenesis of miRNA begins with the transcription of a gene into a long double-stranded primary miRNA (pri-miRNA), which is subsequently cleaved by the RNase III Drosha in the nucleus, resulting in the generation of a sequence consisting of almost 70 nucleotides, referred to as precursor miRNA (pre-miRNA). Once pre-miRNAs are generated, nuclear transporter named Exportin 5 translocates pre-miRNAs outside the nucleus. In the cytoplasm, the pre-miRNA is cut by RNAase III Dicer enzyme into ~22 nt double-stranded mature miRNA. Finally, the antisense miRNA is incorporated into RNA inducing silencing complex (RISC complex) and serves as a guide for the RISC complex that binds, and sequence-specifically inhabits or degrades complementary target mRNAs (Acunzo et al., 2015; Rupaimoole et al., 2016; O'Brien et al., 2018).

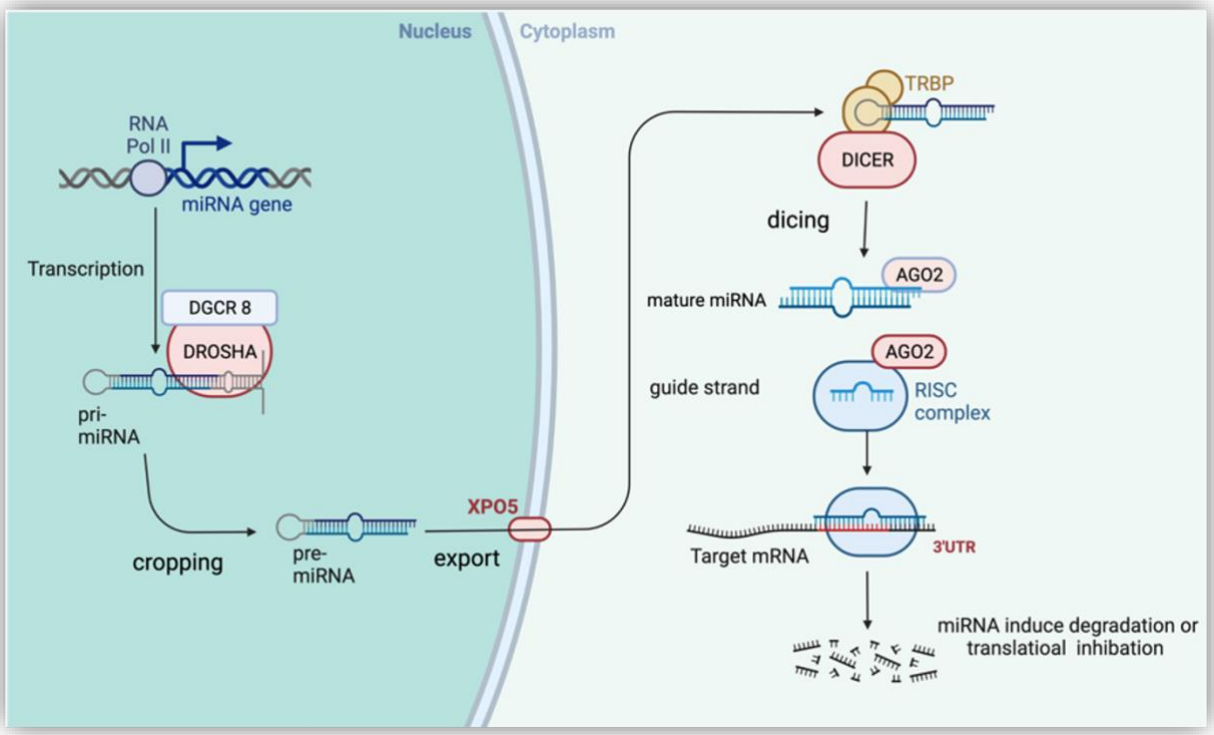

Figure (1) Biogenesis of miRNA inside the cell. Created with Biorender.com

1.2.3 Biological Role of MiRNA590-3p in HCC

miR-590-3p, located at chromosome 7q11.23, is a small non-coding RNA previously shown to exert significant roles in cancer (Dong & Qiu, 2017). It has been suggested that miR-590-3p acts as oncogenic miRNA or antioncomiR in several human cancers. Several reports indicated that miR-590-3p is underexpressed and exerts anti-oncogenic roles in diverse malignancies, such as breast cancer, glioblastoma, bladder cancer, and bone cancer (Abdolvahabi et al., 2019; Pang et al., 2015; Mo et al., 2013; W.-T. Wang et al., 2018). On the other hand, it is overexpressed and acts as oncomiR in ovarian cancer, T cell acute lymphoblastic leukemia, and colorectal cancer (Salem et al., 2018; Miao et al., 2016; Sun et al., 2017).

However, there is a disagreement regarding its expression pattern and biological function in HCC (H. Yang et al., 2013; Ge & Gong, 2017a). For instance, a recent investigation reported

that miR-590-3p plays oncogenic roles in HCC by targeting tumor suppressors, such as *PDCD4* and *PTEN*, to promote HCC tumorigenesis (H. Yang et al., 2013). On the contrary, evidence that miR-590-3p exerts a tumor suppressive role in HCC via regulating *TEAD1* oncogene was also demonstrated (Ge & Gong, 2017a; He et al., 2017). Similarly, a previous study in our lab revealed a tumor suppressing activity of miR-590-3p in HCC and identified the *MDM2* gene as the miR-590-3p target gene (Youssef, 2020).

1.3 MDM2

1.3.1 MDM2 Discovery

The *Mdm2* (murine double minute) gene was initially discovered as one of three genes (*Mdm1, Mdm2, and Mdm3*) in the 3T3DM cell line, a tumorigenic mouse cell line. In 3T3DM cells, the *Mdm2* gene was amplified more than 50 times on extrachromosomal amplified DNAs, called double minutes (Cahilly-Snyder et al., 1987; Fakharzadeh et al., 1991). Molecular analysis revealed that the three genes have distinct sequences, and *MDM2* was the only amplified gene detected in human malignancies (Mendoza et al., 2014). Early studies in cell culture later reported that the MDM2 gene product is responsible for the immortalization of rodent primary fibroblasts as well as the induction of transformation in cultured cells (Freedman et al., 1999; Senturk & Manfredi, 2012). A turning point in understanding the function of MDM2 was the discovery that MDM2 binds and efficiently inactivates the tumor suppressor gene *p53* (Momand et al., n.d.; Xu et al., 2013). During the same period, several reports demonstrated that increased expression of MDM2 occurs in over one-third of p53-wild type soft tissue sarcomas (Oliner et al., 1992). These stunning results established the validity of the hypothesis that MDM2 overexpression is one of the molecular mechanisms by which the cell might deactivate p53 during the development of tumors (Iwakuma & Lozano, 2003; X. Zhou et al., 2017).

1.3.2 Gene Structure and Protein Function

The murine double minute 2 (*MDM2*), located on chromosome 12q13-14, is about 34 kilobases (kb) in size. Similar to the murine *Mdm2* gene present in mouse, the human *MDM2*(*HDM2*) consists of 12 exons capable of generating distinct proteins. While up to 70 diverse MDM2 isoforms have been detected in different cancer and normal tissues, the MDM2 isoforms (MDM2-A, B, and C) are the most frequently detected isoforms in various human cancer (Volk et al., 2009; Y. Zhao et al., 2014; Saadatzadeh et al., 2017). The majority of the variants result from the use of alternative internal splice sites. Many of these do not contain sequences encoding the p53 binding site, implying that they exert a function independent of p53 (Rosso et al., 2014). Notably, some of these splice variants may not serve a significant function and may result from the loss of splicing fidelity during carcinogenesis. In addition, MDM2 consists of two promotors, P1 and P2. It has been proposed that basal transcription of MDM2 is initiated by the p1 promotor, whereas wildtype p53-mediated MDM2 transcription is driven by the P2 promote.

Structurally, MDM2 protein has several conserved functional domains (Figure 2). At the amino terminus is the p53 binding domain. This domain exerts a crucial role in blocking the transactivation function of p53 (J. Chen et al., 1993; J. Chen et al., 1995). This region is also implicated in the binding process to other proteins. Downstream to the p53 binding domain is the nuclear localization sequence (NLS) and the nuclear export signal (NES) that contribute to the nucleo-cytopasmic shuttling of MDM2 protein (Roth et al., 1998; Lohrum et al., 2000). The following region is the acidic domain, which mediates its interaction with ribosomal protein L5 and its associated 5S ribosomal rRNA (Marechal et al., 1994). Other domains are the zinc finger domain of an unknown function. Further toward the carboxyl-terminus are two additional zinc fingers in a RING finger confirmation that bind specifically to RNA sequences

(Elenbaas et al., 1996). The RING motif of MDM2 also possesses intrinsic E3 ubiquitin ligase activity. Due to its E3 ligase activity, MDM2 protein has several substrates, of which the *p53* gene is the most common (Honda et al., 1997a).

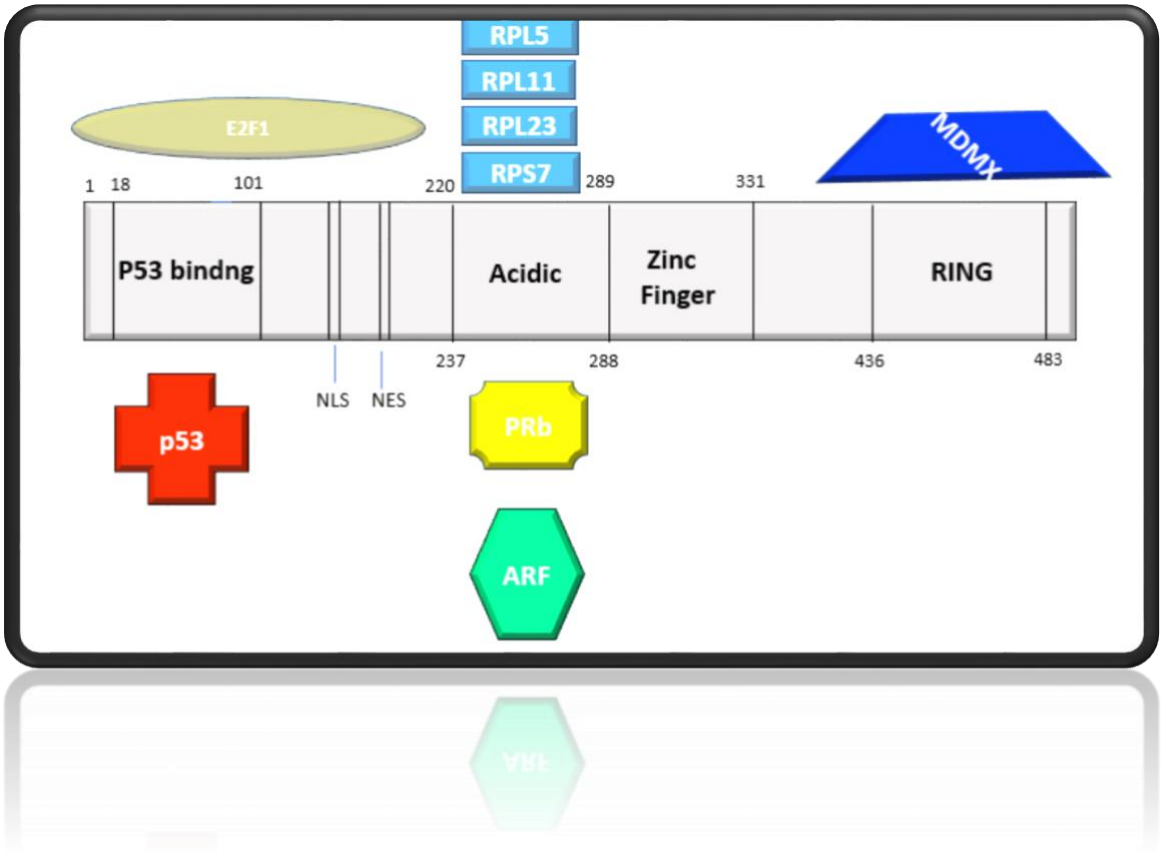

Figure (2) MDM2 proteins domains. The figure was created using PowerPoint.

1.3.3 The Biological Function of MDM2

1.3.3.1 The relationship between MDM2 and p53

p53 is a transcription factor that maintains genomic stability and prevents tumor development by regulating a set of p53 target genes (Leenders & Tuszynski, 2013; Liu et al., 2019). Depending on the magnitude of the DNA damage, p53 initiates cell cycle arrest or apoptosis. Since high expression of the antiproliferative and proapoptotic p53 can be lethal to normal cell development, the level of p53 is closely regulated in unstressed cells (Hafner et al., 2019). A

well-known negative regulator of p53 is the proto-oncogene MDM2, which mediates p53 ubiquitination and degradation (Haupt et al., 1997). Under conditions of cellular homeostasis, MDM2 can inhibit p53 function through multiple mechanisms (Carr & Jones, 2016). As an E3 ubiquitination ligase, MDM2 recruits a ubiquitin conjugating E2 enzyme to facilitate p53 proteasomal degradation (Honda et al., 1997b). MDM2 can also inhibit p53 by blocking its ability to transactivate gene expression. Using its NES sequence, MDM2 can promote the transport of p53 from the nucleus to the cytoplasm, resulting in the degradation of the p53 protein (Roth et al., 1998). However, under conditions of stress, p53 levels rise rapidly in response to stress signals. This increase in p53 protein levels induces the expression of MDM2, forming an autoregulatory feedback loop that maintains normal p53 activity in control. As such, amplification of MDM2 contributes significantly to tumor formation by suppressing p53 function (Jones et al., 1998; Momand et al., 1998; Shaikh et al., 2016).

1.3.4 MDM2 as an Oncogene: The evidence

Since its discovery three decades ago, MDM2 has emerged as a key oncoprotein. It has been reported that MDM2 expression is highly increased in numerous cancers, including colorectal cancer, Burkitt's lymphoma, lung cancer, breast cancer, and sarcoma (Wade et al., 2013). MDM2 is shown to be upregulated in several cancers due to one of three mechanisms: gene amplification, increased transcription, or enhanced translation (Freedman., 1999). A study of 3889 samples from 28 different cancer types detected approximately 7% overall increased frequency of MDM2 amplification. Soft tissue sarcoma exhibited the highest frequency (20%), followed by osteosarcoma (16%). In addition, almost 5.9 % of breast cancer patients demonstrated a 17-fold increase in the *MDM2* gene. Other examples include brain cancers (6.7%) and lung tumors (5.7%) (Shaikh et al., 2016).

Given MDM2 well-researched role in negatively regulating the expression of p53 through multiple mechanisms, it stands to reason that its oncogenic activity is due to p53 (Honda et al., 1997a). Recent data, however, indicated that MDM2 exerts p53-independent oncogenic effects on cells when overexpressed (Brekman et al., 2011; Nag et al., 2013). Approximately 10% of human malignancies exhibit both overexpression of MDM2 and mutations in p53 (Bohlman & Manfredi, 2014). It has been revealed that patients who possess the two alterations have a significantly worse prognosis than those patients with either alteration alone (Onel & Cordon-Cardo, 2004). If MDM2 acts as an oncogene only by inhibiting p53, having both abnormalities in the tumor would be a redundant mechanism to suppress p53 function. These observations indicate that MDM2 possesses additional p53 independent roles, which also appear to contribute to tumorigenesis. Regardless of p53 status, MDM2 transgenic mouse found to develop a higher percentage of sarcoma as compared with mice with p53 deletion (Senturk & Manfredi, 2012). The second line of evidence comes from mice with targeted overexpression of MDM2 to the mammary epithelial cells that elucidate the fascinating phenotype of increased ploidy, which is a marker for genomic instability regardless of p53 status (Lundgren et al., 1997; Marine & Lozano, 2010).

1.3.5 MDM2 Role in Cell Migration, Invasion, and Metastasis in Different Cancer Types

Most notably, MDM2 overexpression was clinically showed to be closely linked to migration, EMT progression, invasion, and metastasis in numerous cancers.

In breast cancer, assembling evidence highlighted *MDM2* as an oncogene with critical roles in cell EMT and metastasis via p53-independent mechanisms. In a study by Yung et al. (2006), MDM2 was found to facilitate the ubiquitination and degradation of E-cadherin, a key cellular adhesion molecule (J.-Y. Yang et al., 2006). In a later investigation by X. Chen et al. (2013), high expression levels of MDM2 in invasive ductal breast carcinoma tissue were associated with poorer disease-free survival outcomes and enhanced expression of *MMP9* gene. By performing in *vitro* functional assays, the authors of that study indicated that overexpression of MDM2 promotes cell motility by enhancing MM9 expression (X. Chen et al., 2013). A further investigation by Lu et al. proposed that MDM2 affects other processes of breast cancer metastasis. Importantly, overexpression of MDM2 was showed to induce EMT by upregulating the expression of the EMT-TF Snail, whereas inhibition of Snail was shown to abolish MDM2 induced-EMT (X. Lu et al., 2016). These data imply that MDM2 contributes to cell EMT, migration and invasion, thus promoting breast cancer metastasis.

In ovarian cancer, high expression levels of MDM2 were detected in 94% of the patients, and MDM2 overexpression was correlated with the stage and metastasis of ovarian cancer cells. Notably, MDM2 was found to facilitate the activation of the TGF-ß-Smad pathway, resulting in inhibition of surface markers, such as E-cadherin, and the elevation of mesenchymal markers, most notably, Vimentin and N-cadherin. In addition, MDM2 was found to induce the transcription of EMT-TFs, such as Snail and Slug, indicating that MDM2 contributes to ovarian

cancer metastasis by serving as a critical factor in driving EMT and metastasis of ovarian cancer cells (Y. Chen et al., 2017). In this respect, another interesting study demonstrated that Nutlin3, a selective MDM2 inhibitor, suppressed TGF-ß-induced EMT in ovarian cancer (Y. Wu et al., 2014). Collectively, these findings implicate an oncogenic role of MDM2 in ovarian cancer by facilitating ovarian cancer cell migration, invasion, and metastasis.

Similarly, in lung cancer, a study exploring the association between MDM2 and the Smad2/3 signaling pathway suggested that MDM2 promotes lung cancer metastasis by activating the Smad 2 and Smad 3 signaling pathway. In that study, the overexpression of MDM2 was found to upregulate the expression of EMT-associate factors, such as Slug, Snail, Vimentin, and N-cadherin and inhibit the expression of E-cadherin, whereas, the inhibition of MDM2 by shRNA showed the opposite trend in *vitro* and in *vivo* (Tang et al., 2019). It should be noted that negative regulation of the EMT-TF Slug by MDM2 in lung cancer has also been observed via MDM2-mediated degradation of Slug protein. In particular, Lin and Hsu found that the recombinant Ling Zhi-8 (rLZ-8), a protein analogous to an effective medicinal ingredient in the mushroom *Ganoderma lucidum*, enhanced the interaction between MDM2 and Slug protein, leading to Slug degradation in CL1–5 cells (Lin & Hsu, 2016). In another study, the negative regulation of Slug protein by MDM2 was reported to be p53 dependent, as p53 was found to modulate Slug protein levels by binding it with MDM2 to generate the MDM2-p53-Slug complex, resulting in MDM2-mediated degradation of Slug protein (S.-P. Wang et al., 2009a). Collectively, MDM2 exerts an important role in lung cancer metastasis.

Recent investigations exploring the impact of MDM2 overexpression on different animal tumor models, including glioma, lung cancer, and breast cancer cells, indicated that MDM2 over-expression can promote the expression of EMT-related genes, such as *Snail*, *Slug*, and

ZEB1 via the B-Raf signaling pathway (Ou et al., 2021). Khor et al. emphasized that MDM2 expression is upregulated in prostate cancer patients, and its expression is correlated with distant metastasis in prostate cancer (Tang et al., 2019). In renal cell carcinoma, one interesting finding demonstrated that MDM2 could promote cell motility and invasiveness independent of its ubiquitin ligase function (Polański et al., 2010).

In hepatocellular carcinoma, the upregulation of MDM2 expression and its association with cell invasiveness, EMT, and metastasis have been reported in many contexts. However, the underlying mechanisms still remain unclear (Ranjan et al., 2016). One study has highlighted the negative regulation of Snail protein by MDM2 by forming the p53-Snail-Slug complex, resulting in MDM2-mediated Snail degradation (Lim et al., 2010). Whether MDM2 is involved in the regulation of *Snail* at mRNA levels is yet to be investigated. This study focuses on the mRNA downstream targets of MDM2.

Interestingly, the present study revealed various functional pathways by which MDM2 can promote HCC development and progression. We performed functional assays to assess the effect of MDM2 depletion on cell proliferative and migratory behavior of HepG2 cells. Moreover, we measured the effect of MDM2 knockdown on EMT-TFs, such as Snail, Slug, ZEB1, and ZEB2 in HepG2 cells. Last but not least, we uncovered the relationship between the *FOXO* gene family and MDM2 at the mRNA level.

1.4 Objective and scope of the study

The expression pattern and biological role of miR-590-3p in HCC are still controversial (H. Yang et al., 2013; Ge & Gong, 2017a). One study uncovered that miR-590-3p serves as oncogenic miRNA in HCC by targeting tumor suppressors, such as *PDCD4* and *PTEN*, to promote HCC carcinogenesis (H. Yang et al., 2013). On the contrary, another evidence indicated that miR-590-3p has a tumor-suppressing effect in HCC partly by targeting *TEAD1* oncogene (Ge & Gong, 2017a). Similarly, our previous work revealed that miR-590-3p exhibits antiproliferative and anti-migratory activities in HCC, and the *MDM2* gene had been identified as its downstream target using bioinformatics and RT-qPCR analysis (Youssef, 2020). In the current investigation, we aimed to expand on our previous findings and further investigate the function of the miR-590-3p/MDM2 pathway in HCC. We hypothesized that the anti-oncogenic roles of miR-590-3p in HCC are achieved through MDM2, and inhibition of MDM2 can mimic the effect of miRNA overexpression in HCC.

- Specific Aim 1

Establish siRNA-mediated MDM2 knockdown in HepG2 cancer cell line

- Specific Aim 2

Test and analyze the effect of MDM2 silencing on the tumor suppressor gene *p53*

- Specific Aim 3

Test and analyze the effect of MDM2 silencing on HCC tumorigenicity (cell proliferation, migration, and EMT markers)

- Specific Aim 4

Conduct in silico analysis to identify further downstream targets in the “mir-590-3p -MDM2” pathway.

- Specific Aim 5

Test and analyze the effect of miR-590-3p overexpression on genes that were predicted by in silico analysis using qPCR.

Chapter 2 Methods and Materials

2.1 Cell Culture

The HCC-derived cell line, HepG2, was obtained from NAWAH scientific Inc. The cells were cultured in DMEM (Lonza, USA) with supplementation of 10% fetal bovine serum (FBS) (GIBCO, USA) and 5% pen-strep antibiotic (GIBCO, USA) at 37°C with 5% CO2. In all experiments described below, cells were frequently subcultured upon reaching 70-90% confluency (passage numbers 7-25) and were examined under an inverted microscope (Olympus IX70, USA).

2.2 Trypan Blue Exclusion Assay

Trypan blue exclusion assay was carried out to determine the viability of both the siMDM2 and NC treated cells. Briefly, 20 microliters of the cell suspension were mixed with 20 microliters of trypan blue in 1.5 mi Eppendorf. Ten microliters of the mixture were then loaded into one of the chambers of the hemocytometer (Hausser Scientific, USA). The four squares in the chamber containing viable cells were counted, and the average number of cells was obtained by dividing the total by 4. The number of cells per 1 ml was obtained through this equation:

Number of viable cells /ml = Average number of viable cells x dilution factor x 10,000.

2.3 miR-590-3p Overexpression

cDNA samples from our previous work were utilized to analyze the effect of miR-590-3p on its target genes.

2.4 RNA Interference

The knockdown of the *MDM2* gene(NCBI Reference Sequence: NG_016708.1) was achieved using ON-TARGETplus SMARTpool siRNA (siMDM2 SMART Pool; L-003279-00-0020) (Dharmacon). Target sequences of the MDM2 siRNAs are presented in Table 1. AllStars Negative Control siRNA (SI027280) (Qiagen) is a validated siRNA that lacks homology to human genome and is used to distinguish sequence-specific silencing from non-specific effects. All siRNA pellets were dissolved in RNase-free water to a final concentration of 100 μM, in accordance with the supplier's protocol.

2.5 SMARTpool siRNA Transfection

The MDM2 SMARTpool siRNA was delivered by reverse transfection into HepG2 cells using Lipofectamine 3000 (Life Technologies) as a transfection reagent. For each well of the 12-well plate to be transfected, approximately $70*10^3$ cells were reverse transfected with 60 nM MDM2 siRNA using 2 μl of the transfection reagent, according to the supplier's instructions in a final volume of 500ul serum free media. In a 1.5 ml Eppendorf tube, 3 μl siRNA solution (10 μM) was diluted in 25 μl of Opti-MEM (GIBCO) without FBS, and 2 μl of Lipofectamine was diluted in 25 ul Opti-MEM. The mixture was mixed gently and incubated for 5 min at RT. Following incubation, dilutions of siRNA and Lipofectamine were combined for a total of 50 ul. The mixed solution was incubated for 15-20 mins to allow liposome formation. siRNA-lipid complex (liposome) was then added dropwise to one of the wells of a 12-well plate, after which 100 μL of the diluted cell suspension were added. At 4h post-transfection, another 500ul

media supplemented with 20% FBS was added to get a final concentration of 10%FBS in the well. At 24 h after transfection, growth media was replaced with fresh complete growth media to reduce cell toxicity. Two days post-transfection, the cells were collected for RNA extraction. MDM2 silencing efficiency was evaluated by RT-qPCR.

Table 1 ON-TARGETplus SMARTpool siRNA sequences (5'-3')

siRNA1	**GCCAGUAUAUUAUGACUAA**
siRNA 2	GAACAAGAGACCCUGGUUA
siRNA 3	GAAUUUAGACAACCUGAAA
siRNA 4	GAUGAGAAGCAACAACAUA

2.6 RNA Isolation

Total RNA from the cell pellet was extracted with the help of triazol reagent. (Invitrogen, USA), under the guidance of the manufacturer's instructions. As recommended, an RNAse-free environment was kept during the isolation. Isolated RNA was resuspended in RNase-free water. The quality and quantity of extracted RNA were assessed using the UV spectrophotometer (Shimadzu, Japan) by measuring OD260 and OD260/280 ratios, respectively. RNA integrity was determined by agarose gel electrophoresis.

2.7 RT-qPCR

To detect mRNA expression, 2ug total RNA was first reverse transcribed by random primers in a final volume of 10-15 μl in accordance with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA).

The PCR amplification was conducted using PowerUp SYBR Green kit (Applied Biosystems) in a final volume of 10ul, consisting of 5ng/reaction cDNA along with 250-300 nM forward or reverse primers and 5ul of 10x syber green master mix. The PCR reaction conditions were as follows: UDG activation at 50°C for 2 min, pre-denaturation at 95°C for 2 min, after which denaturation at 95°C for 15 s and annealing extension at 60°C for 1 min, with a total of 40 cycles. Melt curve analysis was conducted at 60-95°C to confirm the specificity of the PCR products.. The standard $2^{-\Delta\Delta Ct}$ method was utilized to calculate the gene expression levels relative to the GAPDH expression level. Primers were purchased from byEurofins Genomics

2.8 Western Blotting

Transfected cells were rinsed with 1 ml PBS and rocked gently. PBS was then discarded, and cells dislodged in another freshly added PBS using a scraper. The mixture was transferred to 2ml microcentrifuge tube and centrifuged at 2000 rpm for 5 min. The cell pellet was resuspended and lysed using 1X ice-cold CelLytic™ M Cell lysis Reagent (Sigma- C2978) mixed with a freshly added 1X Halt Protease Inhibitor Cocktail. BCA Protein Assay Kit (Pierce Biotechnology, USA) was used to quantify proteins under the guidance of the manufacturer's protocol.

Equal amounts (15 μg) of proteins were mixed with 4X Laemmli loading dye (240mM Tris/HCl pH 6.8, 8% SDS, 40% glycerol, 0.04% bromophenol blue, and 5% freshly added β-mercaptoethanol) and loaded on 10% SDS-Polyacrylamide gel and separated by electrophoresis at 150V for an hour. SDS gels were blotted to nitrocellulose membranes (GE

Healthcare). Membranes were then left for overnight blocking at 4 C in 5% non-fat dry milk diluted in 1X Tis buffer saline with 0.01% Tween 20 (TBST). Following blocking, membranes were incubated with primary antibodies. Primary antibodies mentioned here are Anti-GAPDH (Invitrogen, MA5-15738) (1:10,000), MDM2 (Invitrogen, MA1-113) (1:200).

Table 2 qPCR primer sequences

Gene Name	Primer Sequence (5'-3')
GAPDH	F: AAGGTCATCCCTGAGCTGAAC R: ACGCCTGCTTCACCACCTTCT
MDM2	F: CCCAAGACAAAGAAGAGAGTGTGG R: CTGGGCAGGGCTTATTCCTTTTCT
P53	F: TCAACAAGATGTTTT R: ATGTGCTGTGACTGC
Snail	F: ACTATGCCGCGCTCTTTCCT R: GCTGCTGGAAGGTAAACTCTGG
Slug	F: CAAGGCGTTTTCCAGACCCTG R: AAGAAAAAGGCTTCTCCCCCGT
ZEB1	F: TGCTGGGAGGATGACACAGG R: CTGCTTCATCTGCCTGAGCTT
ZEB2	F: TTCCTGGGCTACGACCATACC R: CAAGCAATTCTCCCTGAAATCC
N-cadherin	F: GCGTCTGTAGAGGCTTCTGGT R: TCTGCAGGCTCACTGCTCTC

Vimentin	F: CTCAATCGGCGGGACAGCAG R: GACACGGACCTGGTGGACAT
FOXO3	F: TGGGCAAAGCAGACCCTCAA R: GGCGTGGGATTCACAAAGGTG

2.9 Colony Formation Assay

The cell colony forming ability of HepG2 cells after MDM2 knockdown were assessed using colony formation assay. Forty-eight hours post-transfection, viable HepG2 transfectants (siMDM2 and NC) were harvested by trypsinization (Gibco) and reseeded into a six-well plate as 500 cell/well. The cells were maintained in an incubator at 37C in complete media for ten days. On day 10, colonies were rinsed twice with phosphate-buffered saline (PBS), fixed with 100% ice-cold ethanol for 5 mins, and stained with crystal violet(brand) for 30 min. Excess staining was then removed by washing with tap water, and colonies of more than 50 cells were counted manually. The experiment was repeated three times.

Clone formation rate was calculated as the following formula: Clone formation rate = number of formed colony / number of seeded cells × 100%(X.-Y. Li et al., 2014)

2.10 Transwell Assay

The migration ability of HepG2 cells after MDM2 knockdown was evaluated using Transwell Assay. 48 hours post-transfection, the transfected cells(siMDM2&siNTC) were trypsinized with 0.05% Trypsin-EDTA (Gibco), centrifuged, and resuspended in serum-free media. A cell suspension containing $150*10^3$cells/well was placed in the upper chamber of 8μm cell culture translucent inserts (GBO) placed in a 24-well plate. In each well of the 24-well plate, 6ooul culture medium containing 15% (v/v) FBS was added to serve as a chemoattractant to allow

for cell migration through the microporous filter. After 10 h incubation at 37C, cells that didn't migrate were removed carefully with cotton swab, while cells migrated to the lower chamber were fixed with 3.7% (v/v) formaldehyde for 15 min at room temperature. Inserts were then rinsed twice with PBS and stained with DAPI (KPL,71-03-01) (1:1000 in PBS) for 15 min. The migratory cells were visualized under a fluorescent microscope at 20X magnification, and five independent fields of view per insert were photographed. Cells were counted using Image J software. Experiment was repeated a minimum of three times.

2.11 Bioinformatics Analysis

FOXO3 mRNA expression in HCC tissues vs their normal-adjacent tissues was ascertained by the GENT2 database. Potential downstream targets of miR-590- 3p were predicted using Condition-Specific miRNA Targets (CSmiRTar) database (http://cosbi4.ee.ncku.edu.tw/CSmiRTar/). To feature the sequence alignment between the miR-590-3p seed region and the 3'-UTR of its target genes, the TargetScan platform (www.targetscan.org) was used.

2.12 Statistical Analysis

The relative expression of target genes in HepG2 cells following MDM2 knockdown or miR-590-3p overexpression were calculated using the comparative ΔΔCT method. Fiji ImageJ software(https://imagej.net/Fiji) was used for the analysis of Transwell assay results, while Image Lab Software was used for the densitometric analysis. All experiments in vitro were carried out in triplicate unless specified. GraphPad Prism 8.02 statistical packages was used to perform all statistical analyses(www.graphpad.com). Differences between two experimental groups (treated and negative control), were analyzed using unpaired Students' t-test. P-value less than 0.05 was considered to indicate a statistically significant difference.

Chapter 3 : Results

3.1 RNAi-mediated knockdown of MDM2 in HepG2 cells

Considering the data from our previous work indicating that MDM2 expression is highly increased in HCC tissues and cell lines, we set out to analyze the impact of siRNA-mediated silencing of MDM2 on HepG2 cells (Cheng et al., 2011; Shan, 2010). To efficiently silence MDM2, the different transfection conditions (seeding density, siRNA concentration, transfection reagent, and incubation duration) needed to be optimized following the manufacturer's instructions to fulfill the highest knockdown efficiency.

3.1.1 Seeding density

To optimize the transfection conditions, it is recommended to perform transfection with at least two different levels of confluency (Sakurai et al., 2010; C. Yang et al., 2011). Herein, we tested two different cell densities (60-70% and 90%). As presented in Fig. 3A, the 60-70% cell density showed higher transfection efficiency than 90% seeding density. Therefore, cell density was maintained at 60-70% confluency for all subsequent experiments.

3.1.2 siRNA concentration

The optimal concentration of siRNA is a crucial transfection parameter that may vary depending on the cell lines used and the gene target itself (Shan, 2010). In this study, we tested two different concentrations (30 nM and 60 nM) of siRNA to optimize transfection in HepG2 cells. Knockdown efficiency was evaluated at 48 hr following transfection by RT-qPCR. As presented in Fig. 3B, transfection efficiencies of dsRNA in HepG2 cells were remarkably higher at the concentrations of 60 nM than at the concentrations of 30 nM. It should be noted

that both concentrations did not show any toxicity. Since the 60 nM resulted in higher efficient mRNA knockdown, subsequent experiments were conducted using this concentration.

3.1.3 Incubation duration

siRNA-mediated knockdown is known to be a transient knockdown, especially when dealing with rapidly growing cells where the intracellular siRNA is diluted with each cell division (Haiyong, 2018). To achieve maximum mRNA silencing while leaving enough time for phenotypic alterations to appear, silencing efficiency was assessed at 36h and 48h after transfection. As illustrated in Fig. 3C, knockdown efficiency after 48h was higher than that of the 36h. Therefore, all remaining assays were carried out 48 hrs following transfection.

3.1.4 Transfection reagent volume

The transfection reagent is another important transfection parameter that needs careful optimization. Transfection reagent volumes that are too low can limit transfection, whereas volumes that are too high will result in cytotoxicity. To identify the optimal siRNA-lipid volume that results in efficient silencing of the target gene without toxicity, two volumes of cationic lipids were tested. We found that low volume showed high transfection efficiency, whereas the high volume resulted in very high mortality preventing the estimation of knockdown efficiency (data not shown).

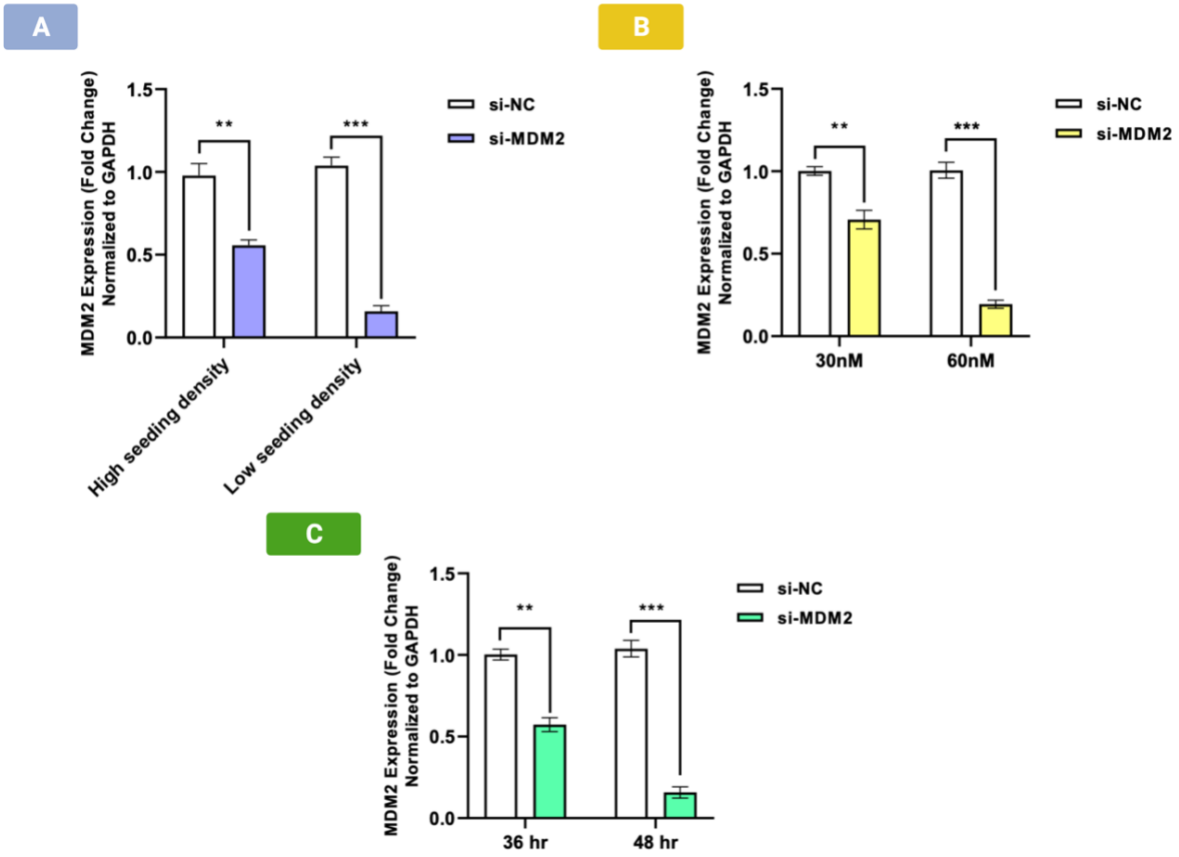

Figure (3) Small interfering RNA (siRNA) transfection optimization using RT-qPCR. HepG2 cells were transfected with siRNA targeting MDM2 in 12-well plates. Optimization of (A) seeding density. Two different cell densities (60-70% and 90%) were tested. Volume of transfection reagent (2 µl), incubation time (48 hr) and concentration (60nM) were kept constant (B) siRNA concentration. Two different concentrations (30nM and 60 nM) were tested. Volume of transfection reagent (2 µl), incubation time (48 hr) and low cell confluency (60-70%) were kept constant for all tested concentrations. (C) Incubation duration. Silencing efficiency was assessed at 36 h and 48 h after transfection. Volume of transfection reagent (2 µl), siRNA concentration (60 nM), and low cell confluency (60-70%) were kept constant. The transfection conditions (60-70% cell density, 48h incubation period, and 60 nM of siRNA) showed higher transfection efficiency. ***P<0.001, **P<0.01

3.2 SMARTPool siRNA effectively inhibits MDM2 protein expression in HepG2

The on target smart pool MDM2 siRNA was successful in knocking down *MDM2* expression at mRNA level, achieving an average of more than 80% knockdown, compared to NC-transfected cells. To investigate whether the smart pool siRNA also induced protein

knockdown, MDM2 protein levels were determined using western blot of cell lysate prepared from transfected cells. The results indicated that the siRNA pool significantly silenced MDM2 at the protein level (almost 70% reduction compared to the control group) (Figure 4), which is comparable to the knockdown detected at the mRNA level.

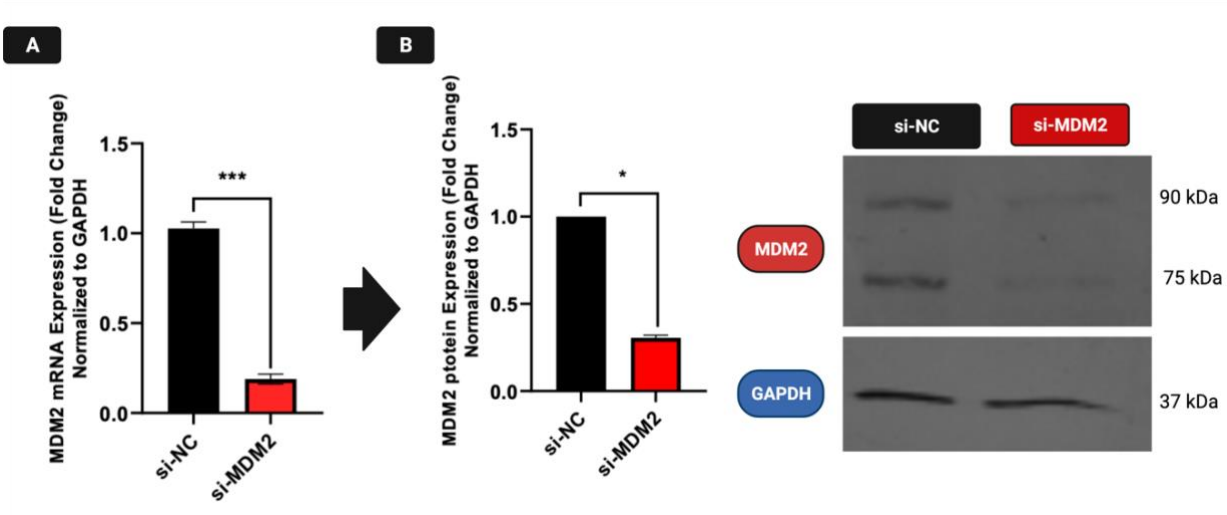

Figure (4) MDM2 siRNA effectively silences MDM2 expression at both the transcript and protein levels. (A) RT-qPCR showing the expression of *MDM2* after transfecting HepG2 cells with MDM2 siRNA or control siRNA. (B) Western blot showing the successful depletion of MDM2 protein expression. Data values are expressed as the mean ± SEM (N=3) for qPCR, (N=2) for western blotting. *P<0.05, ***P<0.001

3.3 Knockdown of MDM2 activates p53 in HepG2 cells

Given MDM2 well-studied role in targeting the p53 tumor suppressor function, we sought to determine if this inhibition of MDM2 could exert an effect on the downstream target gene *p53*. As presented in Fig. 5, MDM2 silencing significantly increased the amount of *p53* mRNA, suggesting that the MDM2 silencing process effectively affects the downstream target genes of MDM2.

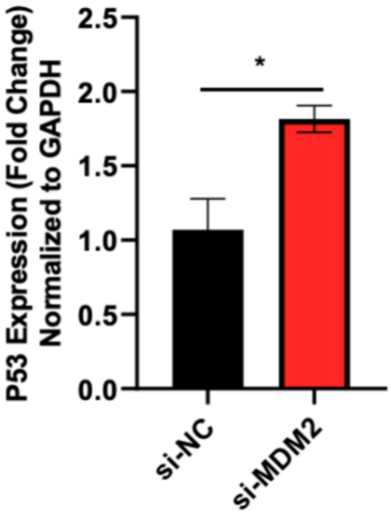

Figure (5) MDM2 knockdown upregulates p53 expression. At 48h post-transfection of si-MDM2 or si-NC in HepG2 cells, *p53* transcript levels were analyzed by RT-qPCR. The transcript levels of *p53* gene showed a significant increase following MDM2 depletion in HepG2 cells. Data are presented as the mean ± SEM (N= 2). *P<0.05; Student t- test, siMDM2-MDM2 siRNA, siNTC- negative siRNA.

3.4 Knockdown of MDM2 suppresses clonogenicity in HepG2 cells

To characterize the role of MDM2 in HepG2 cells, we investigated the effect of MDM2 silencing on the HepG2 cells with regard to colony forming. The self-renewing capacity was evaluated by the plate colony forming assay. As shown in Fig. 6, the colony formation rates were markedly reduced for si-MDM2 transfected HepG2 cells in comparison with the control cells (P=0.0099).

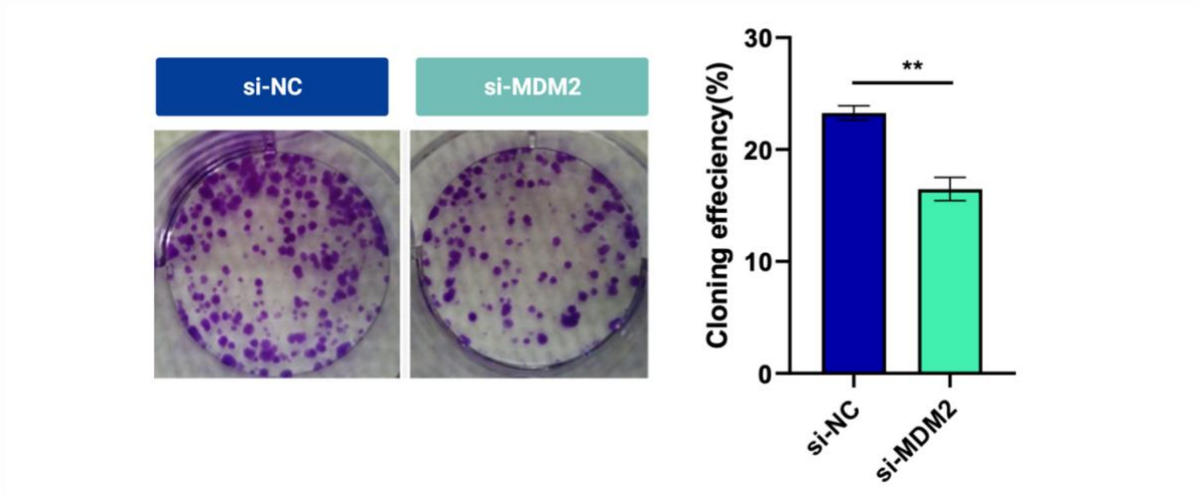

Figure (6) MDM2 silencing impairs colony forming ability of HepG2. Colony formation assay was conducted to evaluate the capability of HepG2 cells to form colonies following MDM2 knockdown. Two days post-transfection, transfected cells (si-MDM2 and si-NC) were plated onto six-well plate. On day ten, cells were fixed, stained with crystal violet, and counted manually. The colony formation rate was lower in HepG2 transfected with MDM2 siRNA than that in NC treated group. The data values are expressed as the mean ± SEM (N = 3). **P < 0.01 indicates statistical significance; Student t-test. siMDM2-MDM2 siRNA, siNTC-negative control siRNA.

3.5 Silencing of MDM2 attenuates the migration of HepG2 cells

To examine whether MDM2 functionally contributed to migratory capacities of HepG2 cells, transwell assay was carried out. As presented in Fig. 7, MDM2 silencing potentially decreased the number of cells that migrated through the membrane (38% reduction compared to the negative control, P=0.005). Therefore, we concluded a functional role for MDM2 in mediating cell migratory activity of HepG2 cells.

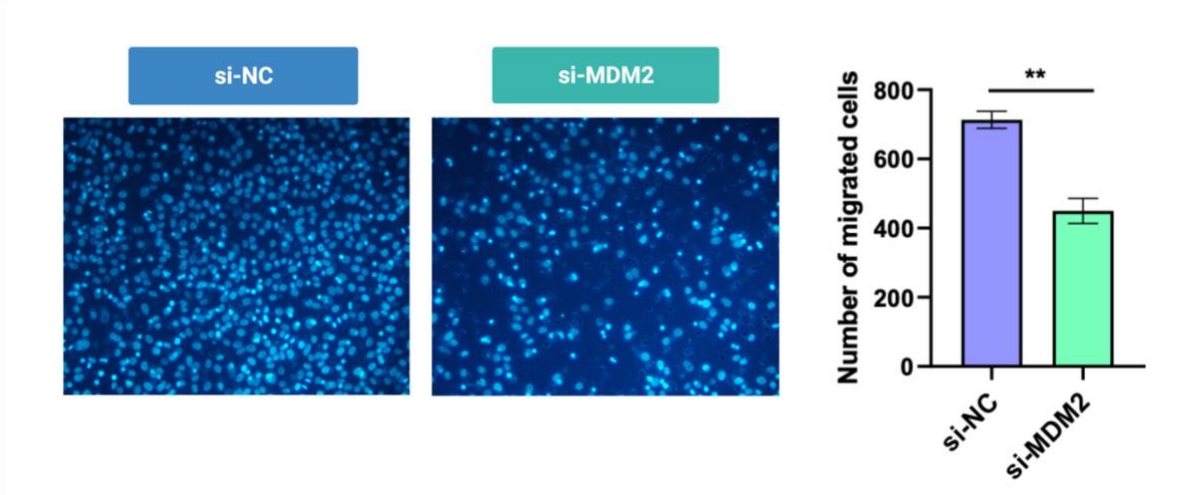

Figure (7) Effect of MDM2 silencing (si_MDM2) on migration of HepG2 cells, as determined by Transwell chamber assays. Two days post-transfection, transfected cells (siMDM2&siNTC) were seeded onto the upper chamber of 8 µm transwell inserts. Cells penetrating the membrane were fixed, Dapi stained after 10 h as described in experimental procedures. MDM2 knockdown showed a 38 % decrease in the number of migrated cells, relative to si-NTC in the HepG2 cell line. Data are expressed as the mean ± SEM (N = 3). **P < 0.01 by Student t-test. si-MDM2-MDM2 siRNA, si-NC- negative siRNA

3.6 Silencing of MDM2 suppresses EMT-inducing transcription factors in HepG2 cells

Having observed that MDM2 knockdown exerted a critical role in suppressing HepG2 cell motility, we investigated its effect on markers of EMT using RT-qPCR. First, we examined the transcript levels of EMT-TFs, such as *Snail1*, *Slug*, *ZEB1*, and *ZEB2* in MDM2 knocked down HepG2 cells. As shown in Fig. 8, the transcript levels of *Snail1*, *Slug*, *ZEB1*, and *ZEB2* dramatically decreased in MDM2-silenced cells compared with the control group.

It is well documented that the activation of EMT-TFs leads to elevation in the expression of mesenchymal markers and downregulation in the expression epithelial markers (Ribatti et al., 2020). Vimentin and N-cadherin are well-known mesenchymal markers that play significant roles in EMT. To determine the regulatory impact of MDM2 on the mesenchymal associate genes, we examined the transcript levels of *Vimentin* and *N-cadherin* using RT-qPCR. Quantitative data disclosed that mRNA levels of both genes were markedly downregulated in

si-MDM2 transfected HepG2 cells relative to the negative control (Fig. 9). These findings reveal that MDM2 regulates EMT progression in HepG2 cells.

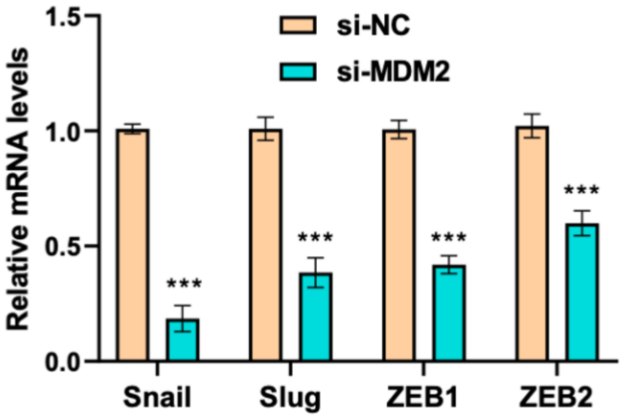

Figure (8) MDM2 depletion suppresses EMT-inducing transcription factors in HepG2 cells. 48h after transfection of MDM2 or negative siRNA in HepG2 cells, *Snail*, *Slug*, *ZEB1* and *ZEB2* expression were analyzed by RT-qPCR and was normalized to the expression of *GAPDH*. The expression of all 4 EMT-inducing transcription factors was significantly reduced following MDM2 knockdown. Data are presented as the mean ± SEM (N= 3). ***P < 0.001; Student t- test, siMDM2-MDM2 siRNA, siNTC- negative siRNA.

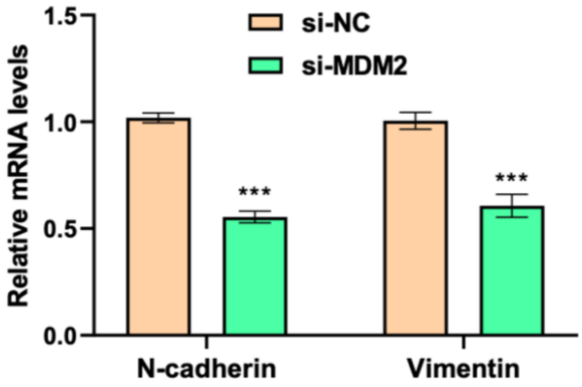

Figure (9) MDM2 knockdown inhibits mesenchymal genes (Vimentin and N-cadherin) expression. The transcript levels of *Vimentin* and *N-cadherin* were analyzed using RT-qPCR. The mRNA levels of both mesenchymal genes were significantly decreased following MDM2 knockdown. Data are depicted as the mean ± SEM; ***P < 0.001 by Student t- test.

3.7 MiR-590-3p plays tumor suppressive roles by inhibiting EMT-inducing transcription factors in HepG2 cells

The aforementioned study done in our lab demonstrated that miR-590-3p exerts anti-oncogenic roles in HCC partly by targeting *MDM2* oncogene. In addition, they demonstrated that miR-590-3p targets *N-cadherin* (Youssef, 2020). Subsequently, in the present work, we aimed at analyzing whether miR-590-3p overexpression suppresses further EMT-related genes, specifically those affected by MDM2 inhibition. To test this possibility, we searched the Condition-Specific miRNA Targets (CSmiRTar) database for direct targets of miR-590-3p and focused on EMT-TFs, such as Sanil, Slug, ZEB1, and ZEB2, as potential candidates (Fig. 10A). CSmiRTar collects data from the four most commonly used miRNA target prediction algorisms: miRDB, TargetScan, microRNA.org, and DIANA-microT. The sequence alignment between the seed region of miR-590-3p and its target genes was located using TargetScan (Fig. 10B). The data demonstrate that miR-590-3p contains a sequence complementary to the 3′-UTR of *Slug*, *ZEB1*, and *ZEB2*, but surprisingly not *Snail*.

Following the proof of complementarity between miR-590-3p and *Slug*, *ZEB1*, and *ZEB2* 3-UTR, the expression of *Slug*, *ZEB1*, and *ZEB2* genes was measured by RT-qPCR. As MDM2 knockdown caused a reduction in *Snail* expression, and *MDM2* is the miR-590-3p downstream target gene, we hypothesized that miR-590-3p is indirectly involved in *Snail* regulation. To test this hypothesis, *Snail* expression was also subjected to PCR analysis (Fig. 10C). As anticipated, the PCR data clearly indicate that miR-590-3p overexpression markedly reduced the transcript levels of *Snail*, *Slug*, *ZEB1*, and *ZEB2*. Based on these findings, we conclude a potential role for miR-590-3p in negatively regulating EMT.

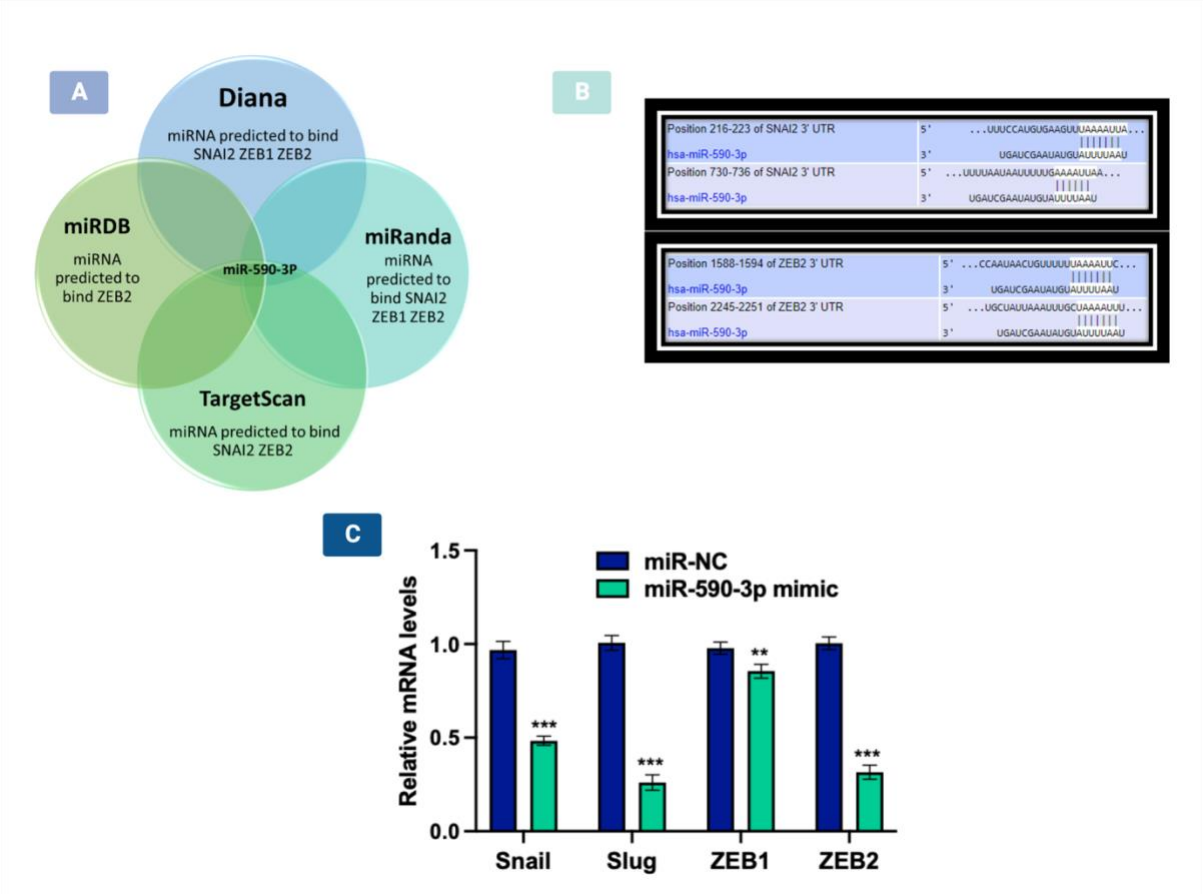

Figure (10) miR-590-3p overexpression inhibits EMT-inducing transcription factors (A) CSmiRTar database analysis showing *Slug*, *ZEB1* and *ZEB2* as miR-590-3p potential target genes. (B) TargetScan analysis showing the sequence alignment between the seed region of miR-50-3p and its downstream target genes (C) RT-qPCR showing the expression of EMT-TFs after transfecting HepG2 cells with miR-NC or miR-590-3p mimics, with GAPDH as an internal control. Data values are expressed as the mean ± SEM (N=3 for *Slug* and *ZEB2*, N=2 for *ZEB1* and *Snail1*), Statistically significant at ** $P < 0.01$, *** $P < 0.001$(Student t-test, two-tailed).

3.8 Knockdown of MDM2 inhibits FOXO3 mRNA expression levels in HepG2 cells

Although the distinct deregulation of FOXO3 expression has been extensively discussed in HCC, the cellular regulators implicated in this deregulation are not fully explored (Fondevila et al., 2021). To investigate the function of *FOXO3* gene in HCC, we analyzed *FOXO3* expression patterns in cancer and normal samples using GENT2 database. The results indicated that *FOXO3* expression is slightly upregulated and activated in HCC tissues (Log 2-fold change; 0.233, P value; 0.001, Figure 11A).

It is known that MDM2 mediates proteasomal degradation of FOXO3 protein under increased cellular oxidative stress. Nonetheless, assembling evidences indicated that MDM2 binds to and increases the mRNA stability of certain genes. Whether MDM2 influences the mRNA of *FOXO* is yet to be established. Toward this, the transcript level of *FOXO3* was analyzed using RT-qPCR. We found that *FOXO3* expression was decreased in si-MDM2 transfected HepG2 cells in comparison with the control group (Fig. 11B). These data indicate that MDM2 positively regulates *FOXO3* at mRNA level.

3.9 miR-590-3p directly targets FOXO3 in HepG2 cells

Next, we continued to ask whether miR-590-3p directly regulates *FOXO3* expression. To do this purpose, we searched the CS-miTar database and found that *FOXO3* is a potential target of miR-590-3p (Fig. 12A). Afterward, we used TargetScan to locate miR-590-3p target sequences in the *FOXO3* 3′-UTR (Fig. 12B). To validate this potential direct interaction, we measured *FOXO3* expression in response to miR-590-3p mimic using RT-qPCR. The PCR data clearly revealed that miR-590-3p overexpression markedly suppressed the transcript levels of *FOXO3* (Fig. 12C). Overall, the results are indicative of post-transcriptional silencing of *FOXO3* gene by miR-590-3p in HepG2 cells.

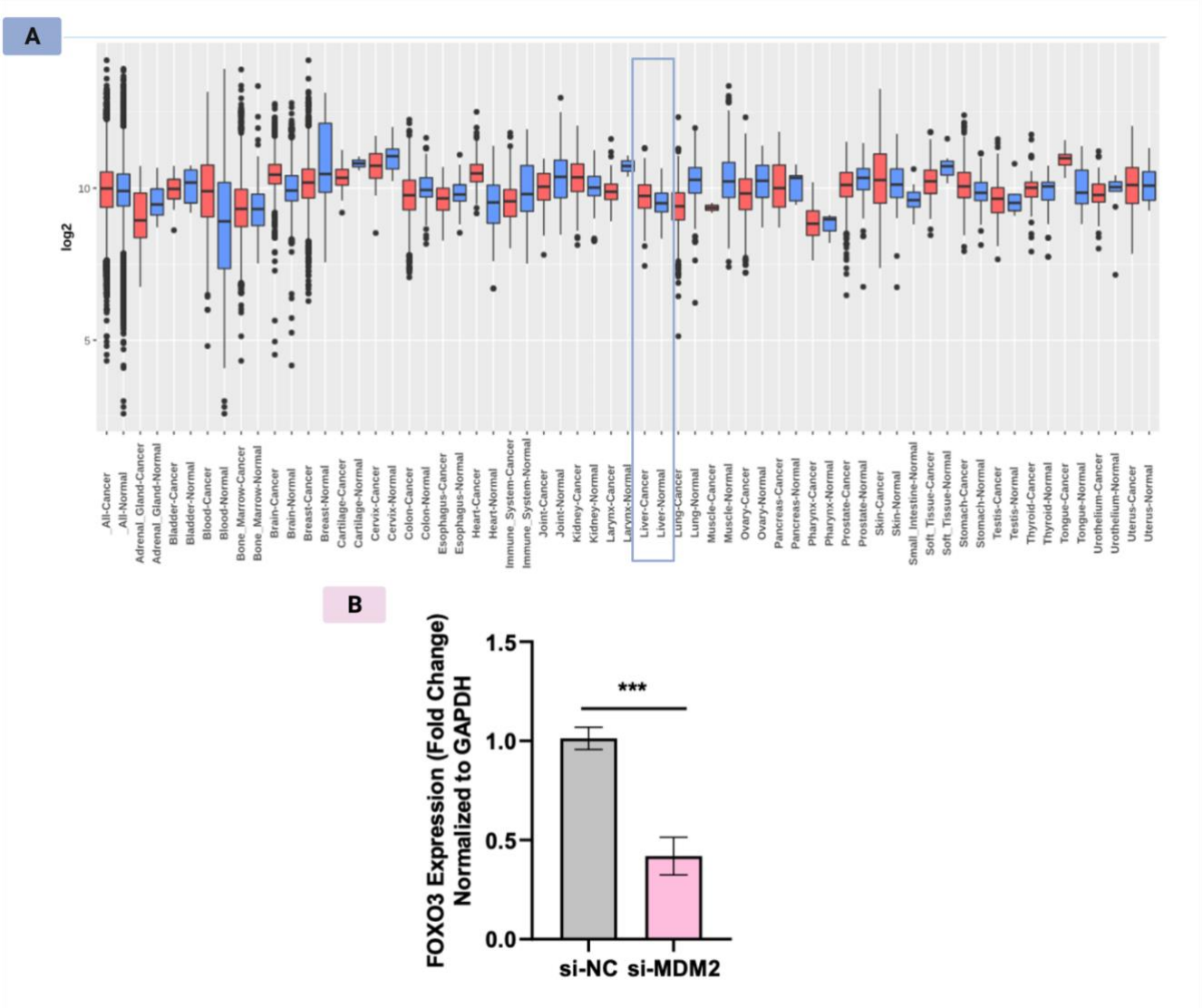

Figure (11) MDM2 depletion represses FOXO3 expression ((A) GENT2 database showing the expression pattern of *FOXO3* across different tissues (B) RT-qPCR showing the expression of *FOXO3* after transfecting HepG2 cells with MDM2 siRNA or control siRNA. Data values are expressed as the mean ± SEM (N=3). *** $P < 0.001$ by Student t-test.

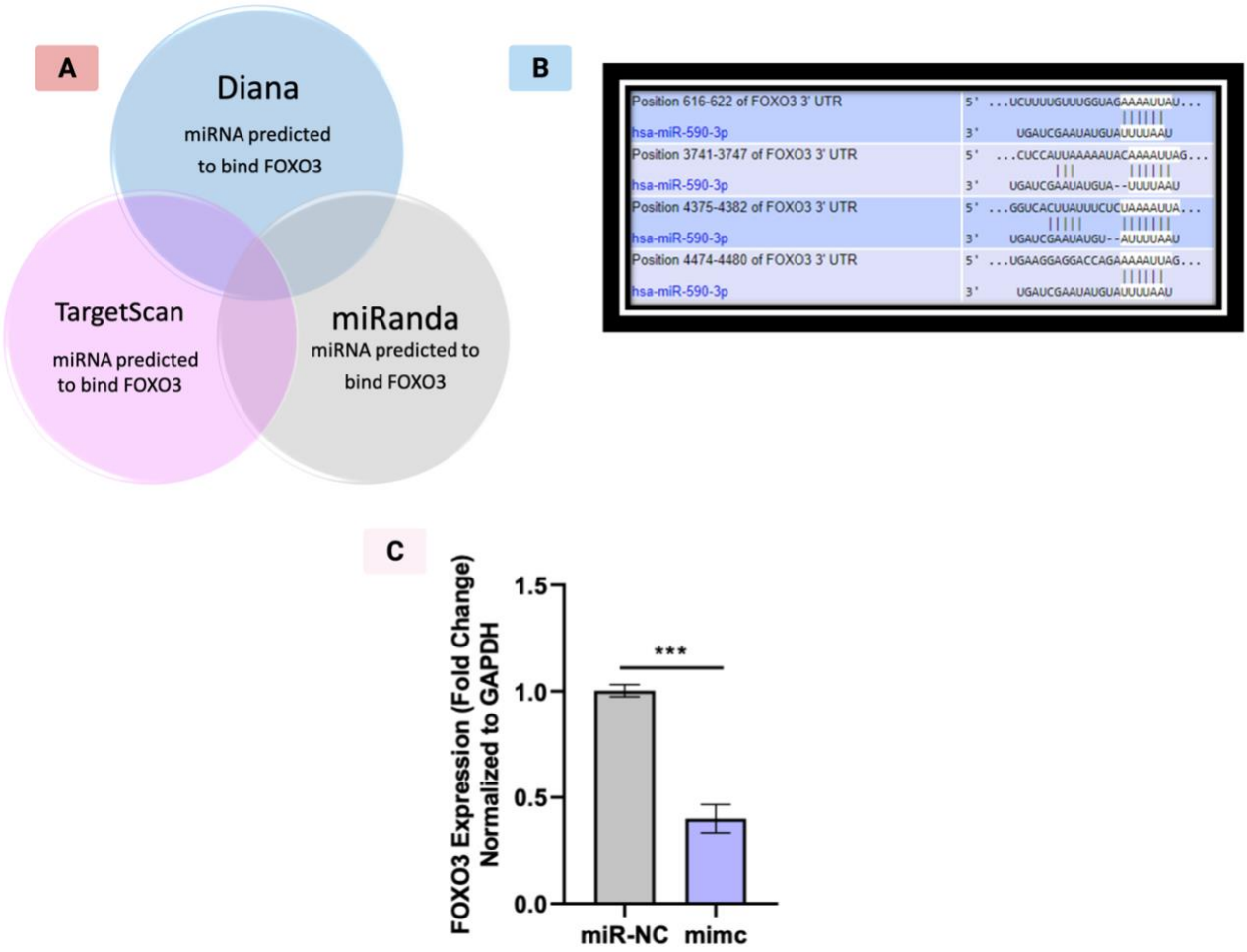

Figure (12) miR-590-3p directly targets FOXO3 in HepG2 cells. (A) CSmiRTar database analysis showing *FOXO3* as the miR-590-3p potential target gene. (B) TargetScan analysis showing the sequence alignment between the seed region of miR-590-3p and its downstream target gene (C) RT-qPCR showing the expression of *FOXO3* in miR-NC and miR-590-3p mimics transfected HepG2 cells. Data are from 3 independent experiments (N= 3) and are presented as the mean ± SEM. *** P < 0.001; Student t-tests. miR-590-3p- miRNA 590-3p, miR-NC – miRNA negative control.

Chapter 4 : Discussion

HCC incidence is increasing rapidly and remains one of the largest health burdens globally (Sung et al., 2021). The major causes of fatality in HCC patients include recurrence and metastasis, with 68% of HCC patients developing this metastatic disease (Bruix et al., 2014; Schütte et al., 2020; Firkins et al., 2021). Therefore, it is urgent to elucidate and understand the underlying mechanisms of HCC development and progression and identify new biomarkers that can allow early diagnosis.

miRNAs are defined as endogenous short non-coding RNAs that mediate gene expression at the post-transcriptional level (Bartel, 2009; Acunzo et al., 2015). Cumulative evidence indicates that miRNA deregulation can serve as a biomarker for the early diagnosis of HCC (X. Li et al., 2014; Mizuguchi et al., 2016; Jin et al., 2019). Interestingly, previous work in our lab revealed that miR-590-3p has antiproliferative, and anti-migratory effects in HCC and identified the *MDM2* gene as the miR-590-3p downstream-target gene using bioinformatics and PCR analysis (Youssef, 2020).

MDM2, a proto-oncoprotein that has roles both dependent and independent of p53, is overexpressed in several tumors, including HCC (Shaikh et al., 2016). Recent reports indicated that MDM2 expression in HCC is correlated with increased malignancy, EMT progression, a higher degree of invasiveness, and greater metastatic potential (Ranjan et al., 2016; W. Wang et al., 2019; Cao et al., 2020). However, the key roles and detailed regulatory mechanisms of MDM2 in HCC are still far from clear.

Therefore, this study aimed to expand upon our prior studies by characterizing and investigating the molecular role of the miR-590-3p/MDM2 axis in HCC. We used RNAi-mediated knockdown to inhibit MDM2 expression, to evaluate its effect on cell proliferation and migration, and to uncover the regulatory mechanisms of MDM2 implicated in the EMT progression of HepG2 cells. In silico analysis was conducted to identify further downstream targets in the "mir-590-3p-MDM2" pathway. Potential target genes predicted by bioinformatics tools were subjected to RT-qPCR analysis. The findings of these assays could characterize the role of the miR-590-3p/MDM2 in HCC.

4.1 ON-TARGETplus Human MDM2 siRNA effectively knocked down MDM2 in HepG2 cells

In light of our lab's prior findings that MDM2 expression is upregulated in HCC tissues and cell lines, the present investigation sought to determine the effect of RNAi-mediated silencing of MDM2 on HepG2 cells. RNAi-mediated gene knockdown is a cellular mechanism by which siRNAs trigger transcriptional gene silencing in a sequence-specific manner (Mocellin & Provenzano, 2004). In the RNAi pathway, the antisense or the guide strand of siRNA is incorporated into RNA Induced Silencing Complex (RISC Complex) and guides the RISC complex to the homologous mRNA that is subsequently degraded. Due to its simplicity and superb efficiency, siRNA is considered a method of choice for researchers conducting loss of function studies (Svoboda, 2020).

Despite being an indispensable tool in functional genomics and drug development, siRNA can result in the silencing of unspecific genes, named off-target effects (Jackson & Linsley, 2010). Due to the concerns over off-target effect frequently associated with siRNAs, two main techniques have been developed to minimize siRNA off-target activity (Neumeier & Meister, 2020). Chemical and structural modification to the sense and antisense strands of the siRNA is

a very common approach that minimizes the off-target effect (Jackson et al., 2006). The second common approach that helps to mitigate the off-target effect is delivering a pool of multiple individual siRNAs targeting the same gene (Jackson & Linsley, 2010; Kittler et al., 2007; Neumeier & Meister, 2020). Due to competition between the siRNAs in the pool, the off-target signature of the pool as a whole is less than that of any individual siRNA. Nevertheless, it is worth emphasizing that off-target activity cannot be ruled out, and the available research regarding how it might be reduced is largely contradictory (Jackson & Linsley, 2010).

In this study, the transfection of the MDM2 smart pool (4 chemically modified individual siRNAs combined) was shown to transiently inhibit the mRNA and protein levels of MDM2 in HepG2 cells. Our results indicate that the on-target smart pool siRNAs can induce remarkably strong and detectable knockdown (more than 80% at mRNA level and almost 70% at protein level). This efficient inhibition was crucial for our research because it had previously been documented that a silencing threshold must be crossed before a discernible loss of function (LOF) phenotype can be observed. In this regard, it has also been claimed that experiments conducted using siRNA pools are more likely to generate LOF phenotypes than individual siRNAs duplexes (Parsons et al., 2009). Finally, since our experimental silencing of MDM2 had met our objective, we proceeded with our subsequent assays.

4.2 Knockdown of MDM2 enhances p53 expression

An essential hallmark of cancer cells is to evade tumor suppressors, most notably p53 (Hanahan & Weinberg, 2011). p53 is a transcription factor that regulates a group of p53 target genes to maintain genomic stability and tumor prevention (Aubrey et al., 2018). Various studies revealed that inhibition of p53 transcriptional activity is a key characteristic of elevated MDM2 expression in cancer (Zhu et al., 2003; Meng et al., 2014). In this respect, we tested the effect

of MDM2 knockdown on the transcript levels of *p53* and observed that the amount of mRNA was increased in MDM2 knocked down cells compared to the control. The HepG2 cells harbor wild-type p53. In accordance with our observations, several reports indicated that inhibiting the interaction between MDM2 and p53 may stabilize the mRNA and protein levels of p53 and induce p53-mediated cell cycle arrest or apoptosis in HCC and several other human cancers (Rayburn et al., 2009a; Meng et al., 2014; W. Wang et al., 2020). It is important to emphasize, however, that the oncogenic activity of MDM2 is not only via p53. Assembling evidence indicated that MDM2 interacts with certain proteins that play key roles in cell proliferation, EMT progression, and metastasis, independent of p53 (Ranjan et al., 2016).

4.3 Knockdown of MDM2 suppresses the clonogenic ability of HepG2 cells

A well-recognized feature of tumor cells is their capacity to sustain proliferation. Clonogenic cell survival assay is an in *vitro* assay that tests the capability of a single cell to self-renew and undergo ‘‘unlimited’’ division in response to treatment (Rafehi et al., 2011; Franken et al., 2006). In the current investigation, we performed clonogenic cell survival assay to test the effect of MDM2 silencing on clone formation abilities of HepG2 cells and showed that depletion of MDM2 could reduce colonies number to more than a 30%-fold decrease. A recent report has shown that inhibition of MDM2 by SP-141, a specific MDM2 inhibitor, represses the colony formation capacity of HepG2 cells (W. Wang et al., 2019). Additionally, earlier studies have revealed that MDM2 possesses a proliferation promoting activity in HCC and several other human cancers, independent of p53 (Martin et al., 1995; Wunderlich & Berberich, 2002; Z. Zhang et al., 2005; Guan et al., 2020). Notably, MDM2 was found to increase the expression of E2F1, a transcription factor implicated in cell cycle progression, either directly or through pRb degradation, and promotes cell proliferation and tumorigenesis (Gnanasundram

et al., 2020a; S. Zhang et al., 2022). To sum up, the present work confirmed these prior findings by demonstrating that knockdown of MDM2 attenuated cell proliferation in HCC.

4.4 Knockdown of MDM2 attenuates the migration of HepG2 cells

Since cell motility is a key cellular determinant of metastatic potential of carcinomas, the present work investigated the regulatory effects of MDM2 on cell motility using transwell assay and showed that knockdown of MDM2 could impair HepG2 cell migratory behavior. In support of this observation, assembling evidence have indicated a significant stimulatory effect of MDM2 on cell motility of numerous cancers, including ovarian cancer, breast cancer, lung cancer and renal cancer carcinoma (Rayburn et al., 2009b; Polański et al., 2010; Y. Chen et al., 2017; Tang et al., 2019; Gao et al., 2019; Guan et al., 2020). The detailed mechanism of how MDM2 promoted cell migration and invasion was also reported in other cancer types. For instance, in ductal breast cancer cells, MDM2 was reported to induce the production and activation of MMP9, a key protease enzyme involved in the digestion of the extracellular matrix proteins (X. Chen et al., 2013; Shi et al., 2014; Zhang et al., 2014). In another investigation, MDM2 was shown to enhance cell motility and invasiveness of breast cancer cells by targeting E-cadherin for proteasomal degradation (J.-Y. Yang et al., 2006). The current work provided evidence that MDM2 silencing attenuated HepG2 cell migration. The possibility that the MMPs and E-cadherin proteins are implicated in the regulative effect of MDM2 on HepG2 cell migration cannot be excluded.

4.5 Silencing of MDM2 Suppresses EMT

Considering that MDM2 knockdown inhibited HepG2 cell migration, we investigated its effect on markers of EMT using RT-qPCR. EMT is a highly conserved trans-differentiation program whereby cells shed their epithelial properties and adopt a motile mesenchymal phenotype

(Kalluri & Weinberg, 2009a; Babaei et al., 2021). During EMT, the expression of several genes critical to cancer metastasis is altered (Ribatti et al., 2020). However, owing to its complexity and the lack of a consensus definition, evaluating the EMT status of cells is often confounding. It is well-accepted that the assessment of EMT status is based on cellular characteristics, surface markers, and EMT-related transcription factors (EMT-TFs) (J. Yang et al., 2020). SNAIL, ZEB, and TWIST are well-known EMT-TFs, which can both inhibit epithelial genes associated with epithelial cell phenotype and upregulate genes involved in the mesenchymal cell phenotype (Ansieau et al., 2014).

4.5.1 Regulation of Snail family by MDM2

The first family of EMT-TFs is the Snail family, including Snail1 (Snail) and Snail2 (Slug) (Škovierová et al., 2018). The two transcription factors are overexpressed in many malignancies, including HCC, and their upregulation is related to the invasion and metastasis of HCC (Sugimachi et al., 2003; X. Zhao et al., 2014; Ranjan et al., 2016). Snail and Slug act as transcriptional repressors via binding to the E-box of the *E-cadherin* promoter to decrease its expression (Cano et al., 2000; Alves et al., 2009). Indeed, cellular factors modulating Snail and Slug expression may also serve as a major determinant of cellular invasiveness. MDM2 is one of these cellular factors that regulates the Snail family at both transcriptional and protein levels, of which the latter mechanism appears to be well-studied (Jung et al., 2013; S.-P. Wang et al., 2009a; Lim et al., 2010).

In the current work, we observed that knocking down MDM2 suppressed the expression of *Snail* and *Slug* mRNAs, suggesting that MDM2 positively regulates *Snail* and *Slug* at the mRNA level. These results contradict the MDM2 well-studied role of promoting Slug and Snail protein degradation. Notably, p53 modulates Snail and Slug protein levels, binding them

together with MDM2 to generate the MDM2-p53-Slug complex or MDM2-p53-Snail complex, which promotes MDM2-mediated ubiquitination and degradation of Snail or Slug proteins (S.-P. Wang et al., 2009b; Lim et al., 2010). Although this is consistent with the anti-oncogenic function p53, the negative regulation of Snail or Slug proteins by MDM2 does not relate to the oncogenic role of MDM2 in human cancers. According to previous studies, MDM2 expression is elevated in HCC tissues, and this increased expression is more frequent in the advanced stage than in early-stage ones (M.-F. Zhang et al., 2009; Meng et al., 2014; Ranjan et al., 2016). Additionally, MDM2 was consistently shown to promote invasion, metastasis, and the EMT process (Rayburn et al., 2009a; Hauck et al., 2017; Y. Chen et al., 2017). To be said, an extensive body of studies has focused on the protein targets of MDM2, whereas its mRNA targets have received less attention. In fact, MDM2 is also capable of binding to and stabilizing the mRNA of some genes (S. Zhou et al., 2011; Gnanasundram et al., 2020b; Faruq et al., 2022).

Recently, one interesting study focusing on the mRNA targets of MDM2 demonstrated that MDM2 increased *Slug* transcript levels by binding to and stabilizing the *Slug* mRNA in both p53-null and p53-expressing cancer cells (Jung et al., 2013). Surpassingly, this effect of MDM2 was found to elevate the protein levels of Slug in p53-null but not p53-expressing cancer cells. These data argue that the MDM2 stimulation of Slug protein degradation is p53 dependent. In other words, Slug protein appears to be determined by a balance between MDM2-mediated accumulation of *Slug* mRNA and MDM2-mediated degradation of Slug protein, with p53 to be required for the letter function. In support of this assumption, our data imply that MDM2 serves as a positive regulator of *Slug* at the mRNA level. It is important to emphasize that the present work is the first work testing the effect of MDM2 knockdown on the Snail family at

mRNA level in HCC. Indeed, more studies focusing on the mRNA targets of MDM2 that are involved in HCC progression might aid the comprehension of HCC metastasis.

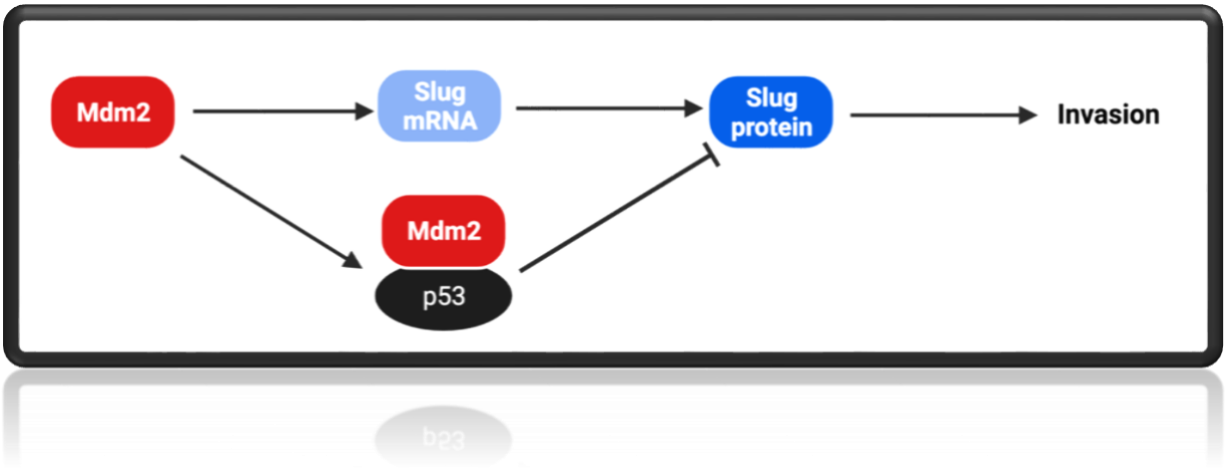

Figure (13) A schematic representation for the MDM2-mediated regulation of cellular Slug levels and invasiveness. MDM2 positively regulates the mRNA level of *Slug* independent of its E3 ligase activity. In the presence of p53, MDM2 bind to Slug protein and form MDM2/Slug/p53 complex to promote Slug protein degradation using its E3 ligase activity. Adopted from (Jung et al., 2013).

4.5.2 Regulation of ZEB family by MDM2

The second family of EMT-TFs is the Zinc finger E-box-binding homeobox, including ZEB1 and ZEB2 transcription factors. Like other EMT-TFs, the ZEB factors can suppress E-cadherin expression and trigger EMT (Škovierová et al., 2018). Herein, we measured the effect of MDM2 silencing on the transcript levels of *ZEB1* and *ZEB2* and found that the transcript levels of both transcription factors were dramatically decreased in MDM2-silenced cells in comparison with the control group. In line with this, recent investigations exploring the effect of MDM2 overexpression on different animal tumor models, including glioma, lung cancer, and breast cancer cells, indicated that MDM2 over-expression can promote the expression of EMT-related genes, such as *Snail*, *Slug*, and *ZEB1* via the B-Raf signaling pathway (Ou et al., 2021). Further evidence has been provided by two independent studies with novel MDM2 inhibitors, such as SP141 and AMG232, wherein the depletion of MDM2 caused a significant

decrease in the expression level of ZEB1 in HCC and glioblastoma cells (Her et al., 2018; W. Wang et al., 2019). In the case of ZEB2, despite no direct correlation observed between MDM2 levels and ZEB2 in the published literature and STRING database (supplemental data, Fig. 15), our study showed that MDM2 knockdown affected *ZEB2* expression. Recent evidence highlighted that Smad2 and Smad3, central mediators of TGF-β signaling pathways, are well correlated with the ZEB family (*ZEB1/2*) to inhibit E-cadherin expression (Singh & Settleman, 2010). In another study, MDM2 was shown to activate Smad2 and Smad3 proteins, resulting in EMT progression (Tang et al., 2019). Therefore, it is possible that MDM2 may indirectly regulate ZEB2 expression by regulating the activity or the expression of Smad2 and Smad3. In short, these findings indicate that MDM2 is involved in the regulation of the ZEB factors in HCC.

4.5.3 Regulation of EMT effectors by MDM2

Indeed, activation of EMT-TFs can lead to molecular alterations, including the inhibition of surface markers, most notably E-cadherin, and the elevation of mesenchymal markers, like Vimentin and N-cadherin, etc (Chen et al., 2017). Herein, we observed a downregulation in the *Vimentin* and *N-cadherin* transcript levels in HepG2 cells where MDM2 had been knocked down. Consistent with these data, siRNA-mediated suppression of MDM2 in breast cancer cells caused a marked decrease in the expression of N-cadherin and Vimentin and enhanced the expression of E-cadherin in *vitro* (X. Lu et al., 2016). On the contrary, MDM2 overexpression was shown to upregulate the expression of EMT-TF, which further caused a reduction in the expression of E-cadherin along with upregulation in the expression of N-cadherin and Vimentin at both transcript and protein levels in glioblastoma and lung cancer (Ou et al., 2021; Tang et al., 2019). Collectively, these data imply that MDM2 displays

oncogenic activity that promotes EMT progression of human cancers. Nonetheless, whether miRNAs are involved in the regulative effect of MDM2 on EMT is yet to be determined.

4.6 MiR-590-3p overexpression inhibits EMT-inducing transcription factors in HCC

It has been documented that alterations in the expression of specific microRNAs are essential for initiating and completing an EMT (Kalluri & Weinberg, 2009b). As MDM2 caused a reduction in the transcripts level of EMT-markers and MDM2 is the miR-590-3p downstream target gene, we sought to determine whether miR-590-3p overexpression would have the same effect of MDM2 on EMT. Toward this, using bioinformatics tools, transcription factors (Slug, ZEB1, and ZEB2) were predicted as possible candidates. Theoretically, *Snail* gene was not a direct target of miR590-3p as predicted by the CS-miRTar database. Yet, we hypothesized that miR-590-3p could affect the expression of *Snail* gene by targeting *MDM2*. To test this hypothesis, we measured the transcripts level of *Snail* in response to miR590-3p mimic using RT-qPCR analysis. We observed that miR-590-3p overexpression was sufficient to suppress EMT-TF, such as *Snail*, *Slug*, *ZEB1*, and *ZEB2*. The data demonstrate that miR-590-3p suppresses *Slug*, *ZEB1*, and *ZEB2* through direct binding to its markers. However, miR-590-3p may target *Snail* through other players that are primarily regulated by miR-590-3p, such as *MDM2*. These results are consistent with two previous reports wherein miR-590-3p was shown to suppress EMT in glioblastoma and intrahepatic cholangiocarcinoma (Pang et al., 2015; Zu et al., 2017). The current findings also confirm the anti-oncogenic role of miR-590-3p in HCC in agreement with previous reports (He et al., 2017; Ge & Gong, 2017b). However, our data disagree with what Yang et al. reported, wherein miR-590-3p was found to promote HCC tumorigenesis (H. Yang et al., 2013). To the best of our knowledge, this is the first investigation revealing that miR-590-3p is an EMT suppressive miRNA in HCC.

4.7 MDM2 positively regulates FOXO3 at the mRNA level

Last but not least, we were interested in identifying further possible targets in the miR-590-3p/MDM2 pathway that may potentially be implicated in HCC carcinogenesis. Among various genes found in published literature and predicted by in silico analysis, FOXO3, a transcription factor belonging to the FOXO family, which has previously been linked to HCC progression, has been selected as a potential target (S. Yang et al., 2021).

FOXO, an essential subfamily belonging to the evolutionary conserved FOX family, represents a group of transcription factors (FOXO1, FOXO3, FOXO4, and FOXO6) that plays pivotal roles in cellular proliferation, transformation, differentiation, longevity, cell cycle arrest, oxidative stress and apoptosis (Carbajo-Pescador et al., 2014). These transcription factors share a high degree of evolutionary conservation. Like other FOXO proteins, FOXO3 consists of four different core functional domains, including the winged-helix DNA binding domain, the nuclear localization sequence, the nuclear export sequence, and the transactivation domain (Schmitt-Ney, 2020). Through this unique structural feature, FOXO3 regulates various target genes implicated in tumour suppression, such as *Bim* and *FasL*, resulting in apoptosis induction (Y. Wu et al., 2020; S. Yang et al., 2021).

Although FOXO3 has been extensively investigated and found to serve a pivotal role in key cellular processes, there is still disagreement about its expression pattern and mechanistic role in oncogenesis, finding that an increased level of FOXO3 may serve either as a tumor-suppressor or an oncogene, depending on the cancer type, cellular context, specific circumstances or genomic profile (M. Lu et al., 2019a). As such, identifying the cellular factors that regulate FOXO levels is a must for better understanding of its role in HCC.

Herein, the GENT2 database was used to analyze *FOXO3* expression in HCC. We found that *FOXO3* mRNA is slightly upregulated in HCC samples compared to normal ones. In support of this, Lu et al. revealed that FOXO3 expression is elevated in HCC samples, and its overexpression is related to extensive liver damage and increased expression of HCC-associate genes, indicating that high levels of FOXO3 are implicated in tumor progression (M. Lu et al., 2019b). Song et al. emphasized that FOXO3 is overexpressed in HCC patients, and FOXO3 expression is associated with Edmondson grade, TNM stage, presence of metastases, and increased AFP level (Song et al., 2020). A systematic review and meta-analysis suggested a significant association between increased FOXO3 expression and HCC development, shorter overall survival, and risk of invasion (Fondevila et al., 2021). Ahn et al. provided evidence that FOXO3 expression is upregulated in HCC samples and its expression is related to aggressive phenotypes of HCC (Ahn et al., 2018). In that study, silencing of FOXO3 impaired cell proliferative and migratory activity of HepG2 cells, indicating a tumor-promoting function of FOXO3 in HCC.

Contrary to all the above-mentioned results, Chen et al. evidenced that FOXO3 expression is downregulated in HCC specimens, and decreased FOXO3 expression is associated with advanced TNM stage and vein invasion (Y. Chen et al., 2017). Similarly, Lu et al. highlighted that FOXO3 is underexpressed in HCC tissues, and its decreased expression is significantly associated with histological grade, cirrhosis, and tumor size (M. Lu et al., 2009). Taking together, HCC reports concerning the expression pattern of FOXO3 in HCC show contradictory results, being necessary to determine the exact function of FOXO3.

In this study, we further demonstrated that MDM2 positively regulates *FOXO3* at the mRNA level. As already mentioned, most of the studies in the literature focused on the protein targets

of MDM2. Previously, Yang et al. provided evidence that MDM2 is implicated in the ubiquitination of FOXO3a protein and suggested that FOXO3a phosphorylation by ERK through an unknown mechanism promotes MDM2 binding to FOXO3a protein (J.-Y. Yang et al., 2008). The article by Fu et al. described that MDM2 serves as an E3 ubiquitin ligase for FOXO3 protein, downstream of p53, to induce its proteasomal degradation (Fu et al., 2009). Interestingly, in that study, MDM2 was found to interact with FOXO3 in the nucleus and had an effect on FOXO family that is separable from degradation. Using transient transfection reporter assays, the author of that study observed a positive effect of MDM2 on the transcriptional activity of FOXO1. These findings demonstrate that MDM2 is involved in different layers of regulation of the FOXO family, which is likely to depend on cellular status, environment stimulus, and p53 status. However, whether miR-590-3p is also implicated in the regulation of FOXO3 remains to be solved.

4.8 MiR-590-3p overexpression inhibits FOXO3 in HCC

It has been documented that miRNAs rely on their downstream genes to carry out their biological roles (Bartel, 2009). In the current study, our bioinformatics analysis predicted *FOXO3* as the miR-590-3p target gene. We measured the effect of miR-590-3p overexpression on *FOXO3* mRNA and found that miR-590-3p suppressed *FOXO3* expression. In this respect, we believe that FOXO3 contributes to the tumor suppressive activity of miR-590-3p in HCC. Our findings certainly support the data reported by the majority of studies and defend the tumor-promoting function of FOXO3 in HCC (Fondevila et al., 2021; L. Yang et al., 2021). However, additional large-scale studies are needed in HCC to prove such promising findings.

4.9 Regulation of HCC cell proliferation, migration, and EMT by miR-590-3p/ MDM2 axis

Figure (14) represents the role of miR-590-3p/MDM2 pathway in HCC. Our previous work revealed that overexpression of miR-590-3p suppressed cell proliferative and migratory behavior of HepG2 cells (Youssef, 2020). Interestingly, similar to overexpression of miR-590-3p in HCC, silencing of MDM2 exerted tumor-suppressive effects in the present work. We further identified the target genes of miR-590-3p and MDM2 through in silico analysis and published literature. We observed that overexpression of miR-590-3p or silencing of MDM2 caused a marked decrease in the expression of EMT-TFs, such as *Snail*, *Slug*, *ZEB1*, and *ZEB2*. Although *Snail* was not predicted as a direct target of miR-590-3p, we observed a marked reduction in the transcript levels of *Snail*, suggesting that miR-590-3p may target *Snail* through other players primarily regulated by miR-590-3p, such as *MDM2*. The present work also revealed that MDM2 knockdown suppressed the expression of *N-cadherin* and *Vimentin*. Surprisingly, our previous work indicated that miR590-3p targets *N-cadherin* but not *Vimentin*, suggesting that the effect of MDM2 on *Vimentin* mRNA is due to another pathway.

Furthermore, we attempted to find more downstream targets of the miR-590-3p/MDM2 pathway, and *FOXO3* was one of the genes that captured our attention. Herein we uncovered that MDM2 positively regulates *FOXO3* at the mRNA level, and *FOXO3* is a novel target of miR590-3p. Our finding may be considered to contribute to the understanding of the miR-590-3p/MDM2 molecular role in HCC.

Chapter 5 : Conclusion

In conclusion, we report the following findings: (*i*) *MDM2* is a proto-oncogene associated with cell proliferation, migration, and EMT progression in HCC (*ii*) Depletion of MDM2 showed a suppressive effect similar to miR-590-3p overexpression in HepG2 cells, confirming that *MDM2* is a functional target of miR-590-3p (*iii*) miR-590-3p is an EMT tumor suppressive miRNA. (*iv*) *Slug*, *ZEB1*, and *ZEB2* are direct target genes of miR-590-3p. (*v*) miR-590-3p regulates *Snail* expression through other players, such as *MDM2*. (*vi*) *FOXO3* is a novel target in the miR-590-3p/MDM2 pathway. Our findings indicate a critical function of the miR-590-3p/MDM2 pathway in HCC that could serve as a promising candidate for the treatment of HCC.

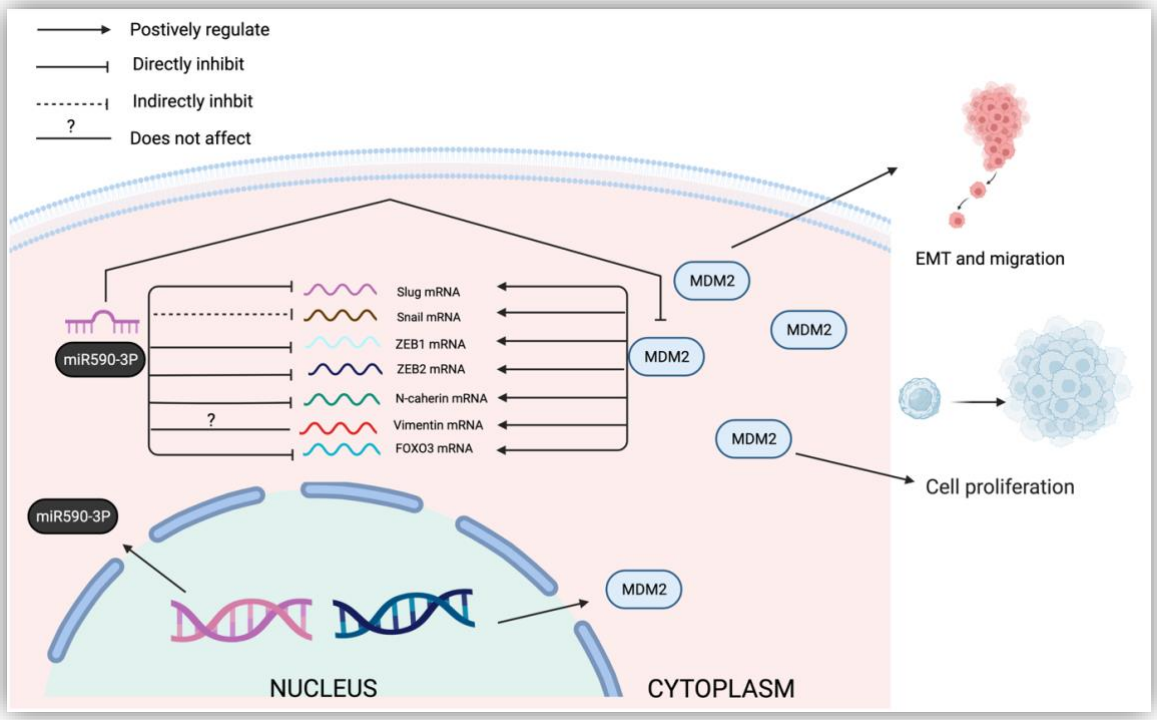

Figure (14) Hypothetical model for the role of miR-590-3p/MDM2 axis in HCC. *MDM2* acts as an oncogene in HCC. miR-590-3p exerts tumor suppressive roles in HCC by directly targets *MDM2*. The figure was created with Biorender.com

****Note: The effect of miR0590-3p on N-cadherin and Vimentin were tested in our past study*

Chapter 6 : Limitation of the study and future respective

Our study has some limitations. As already mentioned above, the siRNA off-target activity is frequent in the RNAi experiments. Assembling evidence suggests that using 2-3 individual siRNAs targeting the same gene and comparing their siRNA phenotypic output can increase confidence that an observed phenotype results from the function of a particular gene (Jackson & Linsley, 2010). In this regard, we recommend using 2-3 siRNAs against the *MDM2* gene to increase confidence in the results. In addition, overexpressing MDM2 in HepG2 cells can further validate the specificity of our results.

In this current research, we did not evaluate the impact of MDM2 knockdown on its downstream target genes at the protein level. Indeed, measuring the effect of MDM2 on the previously reported targets at the protein levels will improve our understanding of the precise mechanism of HCC metastasis. Since p53 was activated following MDM2 knockdown, annexin V flow cytometry is recommended to detect cell cycle arrest or apoptosis. As MDM2 was shown to positively regulate *Snail* family at mRNA level, it would be interesting to confirm these findings using pull down assay and mass spectrometry. The association between MDM2, MMPs, and E-cadherin was reported in several cancers. However, whether MMPs are implicated in the regulative effect of MDM2 on the migration of HCC cells is yet to be determined. Last but not least, loss and gain of function assays to uncover the exact mechanism by which *FOXO3* acts as an oncogene in HCC are highly needed to get more knowledge of its oncogenic roles in HCC.

Supplemental data

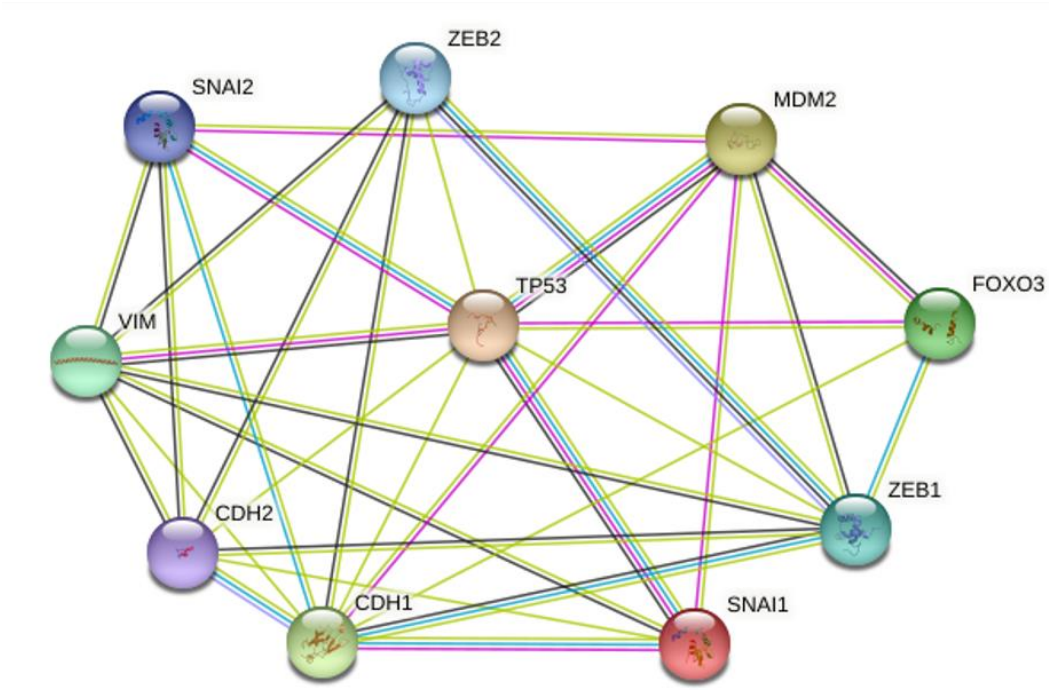

Figure (15) Protein-protein interaction (PPI) network of MDM2 based on STRING database. ZEB2 protein does not interact with MDM2.

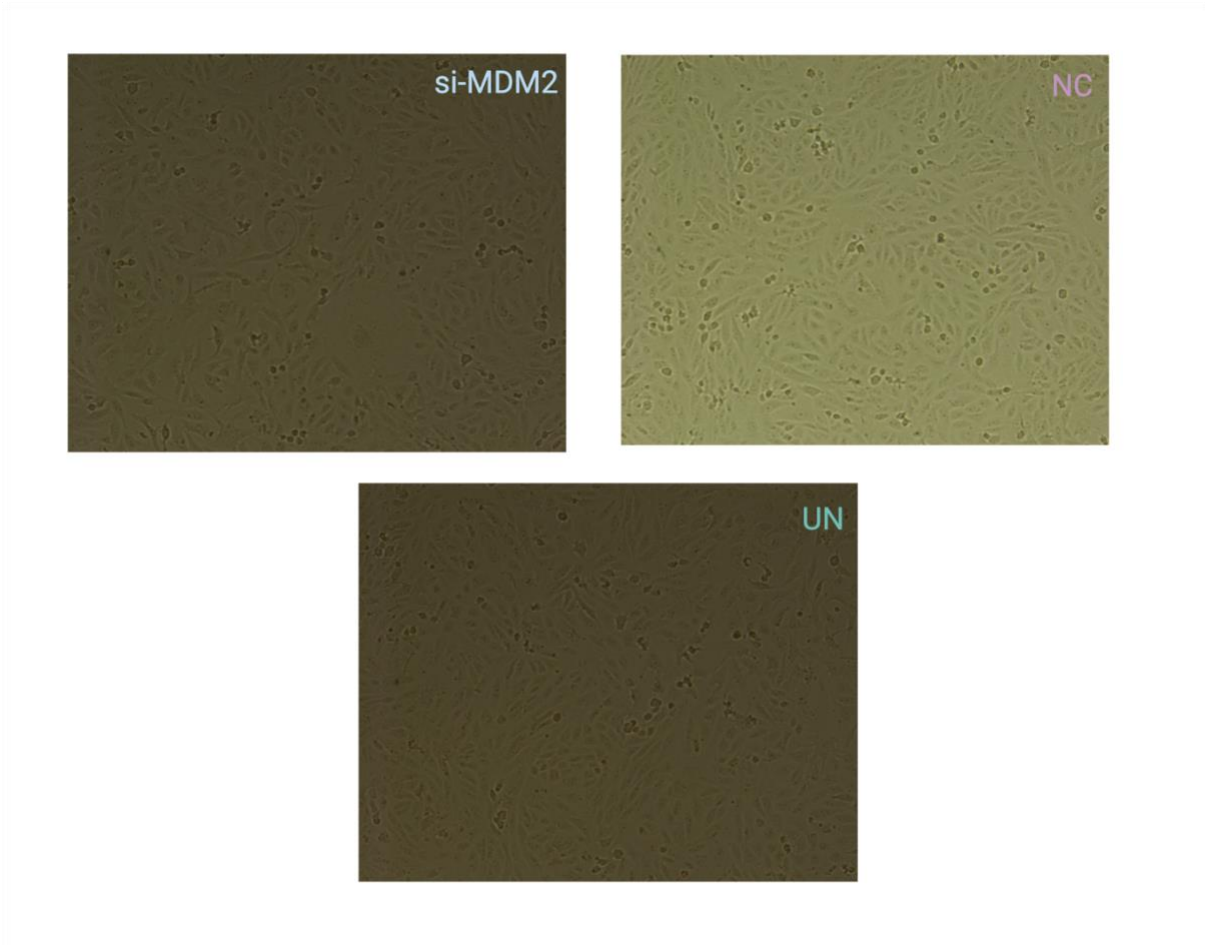

Figure (16) No toxicity was observed following MDM2 or negative siRNA transfection in HepG2 cells. The pictures were taken 48h following transfection. siMDM2-MDM2 siRNA, siNTC- negative siRNA, UN-untreated

Table 3: Datasets used for the FOXO3 expression validation via GENT2 web server

Cancer type	Datasets	Source
Liver Cancer	GSE14323 GSE60502 GSE60502 GSE5364 GSE14323 GSE12630 GSE12630 E-TABM-36 E-TABM-292 GSE14323 GSE60502 GSE5364 GSE14323 GSE12630	Affymetrix UI33A and UI33 Plus2 microarray platform

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