Cofactor of BRCA1 promotes epithelial-mesenchymal transition and cell proliferation in intermediate stage hepatocellular carcinoma cell line

Mennatallah Ghouraba

Follow this and additional works at: https://fount.aucegypt.edu/etds

APA Citation

MLA Citation
Ghouraba, Mennatallah. Cofactor of BRCA1 promotes epithelial-mesenchymal transition and cell proliferation in intermediate stage hepatocellular carcinoma cell line. 2018. American University in Cairo, Master's thesis. AUC Knowledge Fountain. https://fount.aucegypt.edu/etds/446

This Thesis is brought to you for free and open access by AUC Knowledge Fountain. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of AUC Knowledge Fountain. For more information, please contact mark.muehlhaeusler@aucegypt.edu.
School of Sciences and Engineering

COFACTOR OF BRCA1 PROMOTES EPITHELIAL-MESENCHYMAL-TRANSITION AND CELL PROLIFERATION IN INTERMEDIATE STAGE HEPATOCELLMULAR CARCINOMA CELL LINE

A Thesis Submitted to the
Biotechnology Master’s Program

In partial fulfillment of the requirements for the
Degree of Master of Science

By
Mennatallah Hani Ghouraba
Bachelor of Pharmaceutical Sciences

Under the supervision of
Dr. Asma Amleh
Associate Professor, Department of Biology
The American University in Cairo
May 2018
COFACTOR OF BRCA1 PROMOTES EPITHELIAL-MESENCHYMAL-TRANSITION AND CELL PROLIFERATION IN INTERMEDIATE STAGE HEPATOCELLULAR CARCINOMA CELL LINE

A Thesis Submitted by

Mennatallah Hani Ghouraba

Submitted to the Biotechnology Master’s Program

May 2018

In partial fulfillment of the requirements for the degree of

Master of Science in Biotechnology

Has been approved by

Thesis Committee Supervisor/Chair

Affiliation

Thesis Committee Reader/Examiner

Affiliation

Thesis Committee Reader/Examiner

Affiliation

Thesis Committee Reader/External Examiner

Affiliation

Dept. Chair/Director Date Dean Date
DEDICATION

To my parents, who believe in me endlessly and love me unconditionally. To my husband, whose support and care are immeasurable. And to my son, the apple of my eyes.
ACKNOWLEDGMENTS

I would like to thank my advisor and mentor Dr. Asma Amleh for all the effort and time she has invested in teaching me. I am very proud and thankful to grow as a scientist in her lab. I am very grateful to all the AUC professors who taught me. I would like to thank all my team mates who taught me, assisted me, and gave me emotional support. At the end, I would like to thank the AUC for providing us with research funds and fellowship opportunities.
COFACTOR OF BRCA1 PROMOTES EPITHELIAL-MESENCHYMAL-TRANSITION AND CELL PROLIFERATION IN INTERMEDIATE STAGE HEPATOCELLULAR CARCINOMA CELL LINE

ABSTRACT

Hepatocellular carcinoma ranks as one of the most lethal types of cancer worldwide and in Egypt. Being complex and heterogenous clinically and molecularly, HCC has a very poor prognosis, and it lacks adequate diagnostic and prognostic molecular markers. Cofactor of BRCA1 (COBRA1); also known as NELF-B, is one of the NELF complex components that stalls RNA polymerase II during transcription elongation and regulates several processes in the cell. Previous studies have shown the upregulation of COBRA1 in Hepatocellular carcinoma (HCC) compared to healthy liver tissue. It was also shown to support cell proliferation and migration in early stages of HCC. In our study, we investigated the role of COBRA1 in the epithelial-mesenchymal transition (EMT)- a critical process in cancer metastasis, and progression of HCC. Functional analysis was done by silencing the expression of COBRA1 in an intermediate stage HCC cell line SNU449, followed by examining the effect of the knockdown on some cancer hallmarks. COBRA1 knockdown cells showed decreased cell proliferation, induction of apoptosis, accompanied by a downregulation of Ki67, Survivin that mark for proliferation and anti-apoptosis respectively. Moreover, COBRA1 Knockdown cells showed a decrease in cell migration and invasion, accompanied by decreased expression of TWIST1- one of the key transcription factors that mediate EMT. Our results demonstrated that COBRA1 supports cell proliferation, migration and invasion, and inhibits apoptosis, which implicates it has a critical role in the progression of HCC.
TABLE OF CONTENTS

DEDICATION .................................................................................................................. iii
ACKNOWLEDGMENTS ................................................................................................. iv
ABSTRACT ................................................................................................................... v
TABLE OF CONTENTS ............................................................................................... vi
LIST OF ABBREVIATIONS ......................................................................................... vii
LIST OF TABLES .......................................................................................................... viii
LIST OF FIGURES ....................................................................................................... ix
1. Introduction .............................................................................................................. xi
  1.1. Hepatocellular Carcinoma .................................................................................. 1
    1.1.1. Incidence and epidemiology ....................................................................... 1
    1.1.2. Etiology and risk factors ........................................................................... 1
    1.1.3. HCC molecular pathogenesis ................................................................... 3
    1.1.4. Surveillance and diagnosis ....................................................................... 3
    1.1.5. HCC molecular markers .......................................................................... 4
    1.1.6. Treatment ................................................................................................. 4
  1.2. Cofactor of BRCA1 ........................................................................................... 6
    1.2.1. Interactions and roles .............................................................................. 6
    1.2.2. Role of COBRA1 in Cancer ..................................................................... 9
HYPOTHESIS AND SPECIFIC OBJECTIVES .............................................................. 11
2. MATERIALS AND METHODS .............................................................................. 12
  2.1. Cell Culture ..................................................................................................... 12
  2.2. Trypan Blue Exclusion Test for Cell Viability .................................................. 12
  2.3. Gene Silencing ................................................................................................ 12
  2.4. siRNA Transfection ........................................................................................ 13
  2.5. RNA Extraction ............................................................................................... 14
  2.6. Reverse Transcription Polymerase Chain reaction (RT-PCR) ....................... 14
  2.7. Western Blot Analysis ....................................................................................... 15
  2.8. Wound Healing Assay ..................................................................................... 17
  2.9. Transwell Invasion Assay ................................................................................ 17
  2.10. Data Analysis .................................................................................................. 18
3. RESULTS .................................................................................................................. 19
3.1. Optimization of COBRA1 knockdown in SNU449 .......................................................... 19
  3.1.1. Forward transfection: .............................................................................................. 19
  3.1.2. Reverse Transfection ............................................................................................... 20
3.2. Estimation of COBRA1 knockdown efficiency on mRNA level, and its effect on cell morphology .. 20
3.3. COBRA1 knockdown significantly downregulates the expression of NELF-A, and does not affect NELF-C/D or NELF-E expression ................................................................................................................................. 24
3.4. COBRA1 Knockdown significantly decreases cell count and expression of proliferation marker Ki67 .......................................................................................................................................................................................... 24
3.5. COBRA1 knockdown suppresses the mRNA expression of Survivin and induces apoptosis ........ 27
3.6. COBRA1 knockdown decreases cell migration .................................................................. 28
3.7. COBRA1 knockdown decreases cell invasion .................................................................... 28
3.8. COBRA1 knockdown suppresses the expression of transcription factor TWIST1 ................. 30
4.DISCUSSION.......................................................................................................................... 32
  4.1. Co-regulation among NELF subunits ............................................................................ 32
  4.2. COBRA1 supports cell proliferation in SNU449 ........................................................... 33
  4.3. COBRA1 inhibits apoptosis and promote HCC cell survival ......................................... 33
  4.4. COBRA1 promotes Epithelial-Mesenchymal Transition ................................................ 35
  4.5. TWIST1 mediates EMT in SNU449 ............................................................................... 36
  4.6. Insights about role of COBRA1 and its mode of action ............................................... 37
CONCLUSION ......................................................................................................................... 38
FUTURE RECOMMENDATIONS ........................................................................................... 39
REFERENCES ......................................................................................................................... 40
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP</td>
<td>Alpha-fetoprotein</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator Protein-1</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>BCLC</td>
<td>Barcelona clinic liver cancer</td>
</tr>
<tr>
<td>BHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>Bps</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer type 1 susceptibility protein</td>
</tr>
<tr>
<td>CDK4</td>
<td>Cyclin-dependent kinase 4</td>
</tr>
<tr>
<td>COBRA1</td>
<td>Cofactor of BRCA1</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography scan</td>
</tr>
<tr>
<td>DSIF</td>
<td>DRB sensitivity-inducing factor</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ERα</td>
<td>Estrogen receptor-alpha</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HBx</td>
<td>hepatitis B viral protein</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HepG2</td>
<td>Well differentiated human hepatocellular carcinoma cell line</td>
</tr>
<tr>
<td>Hr</td>
<td>Hour</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>mins</td>
<td>Minutes</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NELF</td>
<td>Negative elongation factor</td>
</tr>
<tr>
<td>nM</td>
<td>Nano moles</td>
</tr>
<tr>
<td>nm</td>
<td>Nano meter</td>
</tr>
<tr>
<td>p53</td>
<td>Tumor suppressor protein p53</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEI</td>
<td>Percutaneous ethanol injection</td>
</tr>
<tr>
<td>PRB</td>
<td>Progesterone receptor B</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>RFA</td>
<td>Radiofrequency ablation</td>
</tr>
<tr>
<td>RNAPII</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>secs</td>
<td>Seconds</td>
</tr>
<tr>
<td>siCOBRA1</td>
<td>COBRA1 siRNA</td>
</tr>
<tr>
<td>siNTC</td>
<td>Non-targeting siRNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
</tr>
<tr>
<td>SNU449</td>
<td>Intermediate stage HCC cell line</td>
</tr>
<tr>
<td>TACE</td>
<td>Transcatheter arterial chemoembolization</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>0.01% Tween-20 in 1X TBS</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TFF1</td>
<td>Trefoil factor 1</td>
</tr>
<tr>
<td>UGCs</td>
<td>Upper gastrointestinal cancers</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>ZEB</td>
<td>zinc-finger E-box-binding</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1. COBRA1 siRNAs, their target sequences, and their corresponding exons: .... 13

Table 2. RT-PCR Primers sequences, annealing temperature, number of cycles, and amplicon sizes (F: forward primer, R: reverse primer, bp: base pair): ......................... 15
LIST OF FIGURES

Figure 1 Optimization of COBRA1 knockdown transfection conditions in SNU449. ................................................................. 21
Figure 2. COBRA1 siRNA transfection significantly knocks down COBRA1 on protein and mRNA levels............................................................... 22
Figure 3. No Change in morphology was observed upon transfection and knockdown of COBRA1, but cells were less in density........................................ 23
Figure 4. Significant downregulation of NELF-A expression upon COBRA1 knockdown, and no effect on NELF-C/D, NELF-E expression levels.............. 25
Figure 5. COBRA1 significantly reduces cell proliferation and downregulated the expression of Ki67 ......................................................... 26
Figure 6. COBRA1 knockdown significantly downregulates the expression of Survivin .......................................................................................... 27
Figure 7. COBRA1 knockdown significantly decreases cell migration rate. ...... 28
Figure 8. COBRA1 knockdown decreases cell invasion......................................... 29
Figure 9. Detection of fragmented nuclei in COBRA1 knockdown cells marking for cell apoptosis................................................................. 30
Figure 10. COBRA1 knockdown significantly downregulates the expression of TWIST1 ............................................................................. 31
1. Introduction

1.1. Hepatocellular Carcinoma

1.1.1. Incidence and epidemiology

Liver cancer ranks second among the leading causes of cancer deaths, accounting for 788,000 death cases in 2015, with around half to one million newly diagnosed cases every year (1,2). Less developed regions have an incidence of almost 95% of worldwide incidence, and 96% of all mortality (3). In Egypt, liver cancer is the most common type of cancer among men, and the second common among women, and the first in both, accounting to 23% of all diagnosed cancer cases (4). Liver cancer is a male predominant cancer, with an incidence ratio of almost 4:1 between men and women (5). There are several types of primary liver cancers, among which hepatocellular carcinoma (HCC) represents 85-90% of the cases (6). The occurrence of HCC is the highest at the age of 70 years and above, with rare occurrence at ages younger than 40 (7).

1.1.2. Etiology and risk factors

Several risk factors predispose to the occurrence of HCC, major ones include; Hepatitis B virus (HBV) and Hepatitis C virus (HCV) infections, aflatoxins consumption, and alcoholism. Other factors that also increase the risk of HCC include obesity, diabetes and non-alcoholic fatty liver (8). The chronic active exposure to the different HCC risk factors cause insult to the liver cells, which is usually accompanied by chronic inflammation, hepatocytes necrosis, formation of fibrotic tissue, and liver cirrhosis. Cirrhotic liver is closely related to HCC, where around 80% of HCC arise in livers that are either cirrhotic or chronically inflamed (9).

HBV and HCV infections alone associate with 75% of the diagnosed cases worldwide, and HBV has double the contribution of HCV (10). In Egypt where HCV prevalence is the highest worldwide affecting 14% of the population (11,12), HCV is estimated to
predispose to around 40-50% of HCC cases, with a decline in HBV associated HCC to 25% of the cases (13,14). That decline in HBV-associated HCC could be attributed to the introduction of obligatory HBV vaccination, and the higher incidence of HCV nationally (15).

The mechanisms by which the risk factor mediates hepatocarcinogenesis are variable. HBV infection is viewed by many authors as the most potent factor among them (16). HBV is a DNA virus, it mediates carcinogenesis through several mechanisms that include and not limited to; 1- integration of its genomic DNA into the host genome, causing genomic instability. 2- Causing DNA insertional mutations that disrupt genes involved in processes like cell proliferation, cell cycle, and differentiation. 3- Production of a proto-oncogene HBx , that transforms in hepatocytes, to bind to p53 inhibiting DNA repair and cell apoptosis. HBx has also been reported to activate cell signals that induce cell proliferation (17,18,19).

HCV is an RNA virus, it doesn't integrate into the host genome, however it produces proteins that directly or indirectly induce mechanisms like liver fibrogenic and survival pathways, affect immunity interactions and metabolism (20). It also induces mutations by causing double-stranded DNA breaks (21).

Aflatoxin consumption as well is considered one of the major risk factors to HCC, especially in countries with humid and warm climates, where environment is suitable for fungal growth. Aflatoxin is a mycotoxin which is produced by fungus such as Aspergillus flavus, that grow on foods like peanuts, rice and corn, especially when improperly stored. (22). Aflatoxins produce very potent mutant metabolites inside the body, that cause DNA damage and accumulation of mutations. It was found to cause mutations in p53 gene, tumor suppressor regulating DNA repair and cell apoptosis, and that mutation has been reported in around 30-60% of HCC cases in regions known to be aflatoxin-endemic (23,24). There is growing evidence that aflatoxin consumption in Egypt is becoming one of the major risk factors (15). Evidences of interaction between HBV-Aflatoxin have been reported, which augments their risk of HCC development (25,26,27). Chronic alcohol consumptions attributes to liver damage through causing an oxidative stress, and endotoxin induced inflammation (28).
1.1.3. HCC molecular pathogenesis

Advancement in the next generation sequencing technologies resulted in huge progress into understanding the genetic and epigenetic alterations associated to the process of hepatocarcinogenesis. Molecular alterations include somatic mutations of genes that play key roles in carcinogenesis such as telomerase promoter and p53. As discussed earlier, different etiologies can contribute to the occurrence of certain mutations. Dysregulation of several critical cellular pathways was also found to be frequently altered in HCC, which include pathways regulating signaling of growth factors such as insulin like growth factor (IGF) and hepatocyte growth factor, others involved in cell differentiation such as Notch and Wnt pathways, and pathways related to angiogenesis as well. Molecular alterations in HCC were not limited to genetic ones only, but also epigenetic modifications were discovered that include changes in DNA methylation, histone modifications, chromatin remodeling, and changes in the regulating non-coding RNAs; microRNAs and long non-coding RNAs (29).

1.1.4. Surveillance and diagnosis

HCC is asymptomatic, and mostly diagnosed at late stages. Due to increased awareness, and implementing active surveillance, diagnosis at earlier stages has increased recently. Screening every 6 months is recommended for high risk patients, especially those who have cirrhosis (30). Screening tools include ultrasound and measuring Alpha-fetoprotein (AFP) levels serum levels (31). AFP has been shown not to be very useful lately, as it doesn’t necessarily correlate to tumor growth (32). Ultrasound detects 85-90% of tumors ranging between 3-5 cm in size, and that ability decreases to 60-80% for tumors less than 1 cm in size (33,34). Other imaging techniques like magnetic resonance imaging (MRI) and computed tomography (CT) scan can be employed to further investigate smaller undetectable lesions. Uncommon or unclear features detected by imaging may require the use of an invasive image guided biopsy, which can give many false-negative results (35).
1.1.5. HCC molecular markers

The lack of ideal molecular biomarkers caused the dependence of the current surveillance tools on the morphological features, and serological AFP levels that lack sensitivity and selectivity, which makes detection of HCC at very early stages not always possible. Identification of sensitive and specific molecular markers for diagnosis, prognosis and monitoring of therapy is inadequate, and can change the face of HCC management. Extensive research is currently done, aiming for setting molecular classification and profiling of HCC different stages, and even etiologies, in attempts to identify novel biomarkers that can be used in diagnosis and prediction of prognosis (36,37).

1.1.6. Treatment

There are several treatment options available for HCC, and the choice of regimen depends on stage at which patient is diagnosed. Several staging systems exist, among which the Barcelona Clinic Liver Cancer (BCLC) classification is the most used and most efficient tumor staging system, as it takes into account the stage of the tumor, the patient performance status, and the liver function reserve, which are factors that impact the appropriate choice of treatment (5,38). Curative therapies include liver transplantation, and tumor resection which are applicable at early stages, also locoregional treatment options like radiofrequency ablation (RFA) and percutaneous ethanol injection (PEI) can be used at early stage. For intermediate stages; transarterial chemoembolization (TACE) and radioembolization are used. Recently, systemic molecular targeted agents started emerging for treatment of advanced HCC. Sorafenib is currently available in the market, and others are still under investigation (39,40,41). Even with the availability of various treatment options, HCC poor prognosis persists, with less than 5% 5-year survival rate, as most patients are diagnosed at stages at which they are no longer eligible for curative therapy (42).

Liver transplantation is considered as the best treatment option for patients with cirrhotic liver, as it doesn’t only eliminate the tumor, but the underlying disease as well. Eligibility
for transplantation is determined by a set of criteria referred to as Milan criteria; liver has one lesion 5 cm or smaller or has a maximum of 3 lesions that are 3 cm in size or smaller. Following Milan criteria results in a 75% 5-year overall survival rate, and less than 15% recurrence rates (43-44). To extend the use of liver transplantation as it is the most effective treatment approach, some centers either expand Milan criteria, or use some local treatments like RFA and TACE to downsize the tumor size prior transplantation (41). Despite the effectiveness of liver transplantation, the procedure is limited by the availability of donor organs, as patients wait for long periods to receive the transplant, through which their disease deteriorates (45).

Tumor surgical resection is recommended for patients that have single nodules, with properly functional liver that is not cirrhotic (46). However, it has a very high recurrence rate that reaches 50-80% within the first 5 years, that can be attributed to the emergence of a new tumor in the diseased liver or due to dissemination from the resected tumor (5, 42).

For early stage tumors that are not suitable for surgery, RFA is the standard ablative therapy, replacing percutaneous ethanol ablation as it has shown better survival rates and local control. RFA can create necrotic areas of size up to 3 cm (41).

For management of intermediate stage of HCC where tumors are unresectable, TACE is considered as the standard of care, at which chemotherapy is injected into the segmental branches feeding the tumor and was shown to increase survival and delay tumor progression (47). Radioembolization is another emerging therapy suggested for intermediate stage HCC, that is under development and optimization (48,49,50).

Despite the thorough and advanced studies on HCC, we are still far from having a clear understanding of its molecular pathogenesis. This difficulty is attributed to the high complexity and heterogeneity of the molecular mechanisms. More studies are needed to characterize pathways involve in the initiation and progression of HCC, which will facilitate the development of targeted therapies and biomarkers for diagnosis and prognosis.
1.2. Cofactor of BRCA1

Cofactor of BRCA1 (COBRA1) also known as NELF-B, is a leucin enriched 580 amino acid protein, that has three LXXLL motifs. It was first isolated and identified in 2001 as a novel protein that interacts with BRCA1 via yeast two hybrid screen (51). Not before long, COBRA1 was found to be identical to the NELF-B, a subunit of the negative elongation factor (NELF) complex, a four-subunit complex that interacts with other factors to pause the transcriptional activity of RNA polymerase II (RNAPII), and regulates several important processes in the cell (52). COBRA1 lacks DNA binding domain in its structure, so it always mediates its gene expression regulatory roles through interacting with other factors, that are not limited to BRCA1 or NELF. COBRA1 was found to interact with various transcription factors such as Activator protein-1 (AP-1), and nuclear receptor such as Estrogen receptor-alpha (ERα), progesterone receptor B (PRB), Glucocorticoid receptor (GR), and androgen receptor (AR). The interaction of COBRA1 with several transcription complexes postulates its importance in regulating vital cellular processes. Roles and interactions of COBRA1, in addition to its effect in cancer will be thoroughly discussed below.

1.2.1. Interactions and roles

1.2.1.1. COBRA1, as cofactor of BRCA1

Breast cancer susceptibility gene 1 (BRCA1) is a tumor suppressor gene, its mutations/dysregulation are highly associated to the ovarian and breast cancer predisposition.it has been shown to regulate multiple processes such as DNA repair and gene transcription (53,54,55). A study that was performed to elucidate the mechanism of BRCA1 action, and to identify the co-factors it recruits to initiate large scale chromatin unfolding. This study employed yeast two-hybrid screen, through which a novel protein was found to interact with BRCA1, that was then named cofactor of BRCA1 (COBRA1). COBRA1 was found to be recruited by the first BRCT repeat of BRCA1 to the chromosome site, and it also showed having independent ability of chromosome unfolding (51).
1.2.1.2. The NELF complex:

Couple of years after the identification of COBRA1, a study of the NELF complex subunits revealed that COBRA1 is the NELF-B subunit of NELF (52). The NELF complex recruitment takes place along with DRB sensitivity-inducing factor (DSIF), to mediate pausing of transcriptional elongation via binding to RNA polymerase II (56, 57). NELF regulates a substantial number of genes that are involved in metabolism, cellular stress response, proliferation and cell cycle control (58). In vivo studies demonstrated that NELF-B is essential in early embryogenesis, as its knockout was embryo lethal (59).

NELF complex is composed of 4 subunits; NELF-A, NELF-B, NELF C/D and NELF-E that are essential for the formation of a functional complex. NELF-C and NELF-D have a similar structure and are thought to represent translation variants of the same mRNA, and only either of them would exist in the formation of a NELF complex. NELF-B and NELF-C/D form the core of the complex that brings together the other two subunits. NELF-A binds directly to RNAPII through its RNAPII binding domain, and NELF-E contains an RNA binding domain, through which it binds to the RNA (52).

In contrast to the initial view of the RNAPII pausing as a transcriptional repressor, several studies have shown that in many genes, NELF mediated RNAPII stalling has a positive role in transcription regulation (60, 61). The mechanism was first demonstrated in a study at which NELF was depleted in Drosophila cells, and microarray analysis showed the downregulation of most of the NELF regulated genes. Further investigation showed that this positive role was facilitated through maintaining a permissive chromatin structure by NELF, to enhance gene expression (60).

1.2.1.3. COBRA1 as corepressor of Steroid hormone receptors

Steroid hormone receptors are a group of transcription factors that bind to DNA and activate gene expression in a legend-dependent or independent manner, and the potency of their activity is mostly determined by the availability of the cognate hormones and a set of transcription co-regulators (62, 63, 64). COBRA1 displayed transcriptional co-repressor
role, by binding to ERα and repressing the expression of its downstream targets in breast cancer cell line (65). Further studies were done to elucidate the effect of COBRA1 on other steroid hormone receptors. COBRA1 was found to have a very high affinity to androgen receptor (AR), and less affinity to glucocorticoid receptor (GR), while the least affinity detected was to progesterone receptor B (PRB). Knockdown of COBRA1 showed a remarkable enhancement of expression of AR transcriptional activity, and a moderate effect on GR and PR. This study did not only denote the negative effect of COBRA1 in regulating steroid hormone receptor-dependent transcription, but has also showed COBRA1 dual role as it is also involved in alternative splicing of the regulated genes (66). The interaction between COBRA1 and the steroid hormone receptors goes in line with the presence of 3 LXXLL motifs identified in its structure, as that motif is frequently found in transcription coregulators of steroid hormone receptors (51).

1.2.1.4. Interaction with AP-1 complex

In addition to the Interaction of COBRA1 with NELF subunits and BRCA1, it was also found to interact with a transcription factor; AP-1 and regulating its transcriptional activity. Ectopic expression of COBRA1 was found to inhibit AP-1 transcriptional activity, while COBRA1 knockdown enhanced AP1 activity (67). AP-1 complex was reported to promote cell survival, and in other context it was found to induce apoptosis. It also regulates genes involved in differentiation, proliferation, and neoplasm formation (68).
1.2.2. Role of COBRA1 in Cancer

COBRA1 involvement in several major transcription regulation machinery has been demonstrated, regulating many critical cellular pathways. That suggested the potential role COBRA1 could play in a disease like cancer. However, COBRA1’s role in carcinogenesis is still unclear, and has only been studied in breast, gastrointestinal, and liver cancers.

1.2.2.1. COBRA1’s role in breast cancer

COBRA1’s potential role in cancer was first studied in breast cancer. That was first demonstrated by Aiyar et al in 2004, where COBRA1 was found to bind to ER-α repressing its transcriptional activity. That was elucidated by knockdown of NELF proteins, that resulted in enhancement of cell proliferation mediated by ER-α transcription. This study showed that this regulation was mediated through RNAPII stalling (65). COBRA1 transcriptional regulatory role in breast cancer occurs in both estrogen-dependent and estrogen-independent manner. A huge overlap between the genes regulated by BRCA1 and COBRA1 was identified, of which many are involved in breast cancer progression (69,70). All these evidences of the potential role of COBRA1 as a tumor suppressor in breast cancer from in vitro studies were further confirmed by examining its levels in breast carcinoma tissues. Low levels of COBRA1 detected on mRNA levels, were highly correlated to breast cancer metastases and recurrence, and predicted poor prognosis (71).

1.2.2.2. COBRA1’s role in upper gastrointestinal cancer

Contradictory to what was shown in breast cancer, COBRA1 was found to be upregulated in the majority of upper gastrointestinal adenocarcinomas (UGC). COBRA1 overexpression was highly correlated to downregulation of TFF1, a protein that is normally expressed in upper gastrointestinal system, and its loss is associated to gastric
tumorigenesis. The study demonstrated that COBRA1 negatively regulates AP-1 complex activation of TFF1 promoter, causing the downregulation of TFF1. It also showed that regulation of AP-1 complex by COBRA1 in gastric cancer cells is estrogen independent, unlike breast cancer. This study demonstrated the oncogenic potential of COBRA1 in UGCs for the first time and highlighted that COBRA1’s role in cancer is cancer-type dependent (72).

1.2.2.3. COBRA1 in hepatocellular carcinoma

Owing to the potential role of COBRA1 in cancer demonstrated in breast and UGC, and our interest in understanding molecular pathogenesis of HCC, we studied COBRA1 for the first time in HCC at our lab. Levels of COBRA1 were found to be upregulated in HCC tissue samples compared to their adjacent non-neoplastic liver samples. The same pattern of COBRA1 overexpression was obtained through in silico analysis of HCC microarray database. In silico analysis has also demonstrated an overexpression in the other NELF subunits, with NELF-E showing the highest differential expression. This pattern of NELF subunits simultaneous deregulation is consistent with their interdependent nature reported previously (73).

The levels of COBRA1 was also evaluated among five cell lines representing different stages of HCC and a control. Results have shown highest levels of expression at early HCC stages, with a gradual decrease as the stages advanced, with significant downregulation in high grade HCC cell line (74). To investigate the role of COBRA1 in HCC, loss of function analysis was performed using HepG2 Cell line that represents an early stage of HCC. Knockdown of COBRA1 was achieved using short interfering RNA (siRNA), and cells showed a significant decrease in proliferation and migration. That was also accompanied by down regulation in Ki-67 the standard proliferation marker, and Survivin, a key anti-apoptotic protein that is overexpressed in most cancers. (73)
COBRA1 is involved in transcriptional regulation of numerous genes, being an important component of key transcription complexes. An overexpression pattern of COBRA1 was found in hepatocellular carcinoma, and it was shown to support cell proliferation and migration in early HCC stages. Therefore, we hypothesize it has a key role in the progression of hepatocellular carcinoma. To evaluate this hypothesis, we had the following as our main objectives:

1-To optimize and achieve an efficient COBRA1 knockdown using short interfering RNA in intermediate stage hepatocellular carcinoma cell line SNU449.

2-To analyze the effect of COBRA1 knockdown on major cancer hallmarks; cell proliferation, apoptosis, migration and invasion.

3-To examine the differential gene expression of some key players in cancer on mRNA level upon COBRA1 knockdown.
2. MATERIALS AND METHODS

2.1. Cell Culture

SNU449 cell line was used in all experiments. The cell line was a generous gift from Dr. Mehmet Ozturk at the Department of Molecular Biology and Genetics, Bilkent University, Turkey. SNU449 represents an intermediate stage of HCC, grade II-III/IV. The cell line was derived from a 52 years old, Asian male. Hepatitis B viral DNA was detected in the cell line (75). Cells were used at passage numbers 15-25 in all experiments, in their logarithmic growth phase. Cells were cultured in RPMI 1640 (Lonza) media, supplemented with 10% FBS (Invitrogen), 100 units/ml penicillin and 100mg/ml streptomycin (Invitrogen). Cells were incubated at 37°C and supplied with 5% CO₂ in a humidified incubator. Inverted microscope (Olympus IX70, USA) was used to observe cells.

2.2. Trypan Blue Exclusion Test for Cell Viability

Trypan blue method was used to determine viable cell count. 20µl of Cell suspension was mixed with equal volume of 0.4% trypan blue in phosphate buffer Saline (PBS). Viable cells were counted using hemocytometer (Hausser Scientific). Ten microliters of cell suspension were loaded in each chamber, and cells in the four outer squares of each chamber were counted. Cell count per ml was determined using the following equation:

\[
\text{Cells number/ml} = \frac{\text{Total number of viable cells}}{\text{total number of squares}} \times \text{dilution factor} \times 10,000.
\]

2.3. Gene Silencing

For COBRA1 knockdown, we utilized a pool of 4 siRNAs that target different exons of COBRA1 mRNA (siGENOME SMARTPool; M-015839-02, Dharmacon). Table 1 showing
exon numbers and target sequences of COBRA1 siRNAs (siCOBRA1). Allstars negative control siRNA (SI03650318, Qiagen) was used as a control to the siRNA Transfection procedure effect. Negative siRNA (siNTC) in non-targeting has no homology to mammalian genes (76). All siRNAs were reconstituted using RNase-Free water, to a concentration of 20µM, according to manufacturer instructions.

<table>
<thead>
<tr>
<th>SiGENOME SMARTPool (M-015839-00)</th>
<th>siRNA Target Sequence (5'-3')</th>
<th>Target exon</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CCGAAAGCUUCACUAAGUU</td>
<td>9&amp;10</td>
</tr>
<tr>
<td>2</td>
<td>GCGACUUGGCCUUUGCGA</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>GAGCCUGGGACAUGAUCGA</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>CGUCUAAGCUGGAGGCGU</td>
<td>12</td>
</tr>
</tbody>
</table>

**Table 1.** COBRA1 siRNAs, their target sequences, and their corresponding exons:

**2.4. siRNA Transfection**

COBRA1 knockdown was performed by reverse transfection of COBRA1 siRNA into SNU449 cells using Lipofectamine3000 (Life technologies), that facilitates the delivery of siRNA into the cells through the cell membrane by forming liposomes that carry the siRNA. Optimized Transfection conditions were done in 6 well plates. Controls used; Untreated cells cultured in 1.5 ml RPMI 1640 supplemented with FBS and 0.5 ml of OPTI-MEM (Gibco) media, Mock cultured in the same media in addition to lipofectamine, and Negative siRNA control. For transfection of COBRA1 and negative SiRNA, 500 microliters of OPTI-MEM media were transferred to a 1.5 ml Eppendorf tube, 40nM (4 microliters of the 20µM stock solution) siRNA were added to the Opti-mem media and mixed with 3.75 microliters of lipofectamine 3000 by pipetting. siRNA and lipofectamine3000 were incubated for 15-20 at room temperature minutes to promote complex formation. siRNA-Lipofectamine mix was then transferred to the corresponding wells, at which 1.5 ml of
antibiotic-free media (RPMI+10% FBS) containing $1.5 \times 10^5$ cells approximately. Growth media were changed 24 hours post-transfection. Cells were harvested 96hrs post-transfection for RNA and Protein analysis. All Transfections were performed in an RNase free environment. Evaluation of knockdown efficiency was performed by analyzing the proteins levels of COBRA1 using western blot analysis.

2.5. RNA Extraction

RNA extraction was performed using Trizol reagent (Invitrogen, USA) according to the manufacturer’s recommendations. RNA was dissolved in nuclease-free water, and RNase free conditions were maintained during the procedure. RNA was quantified using SPECTROstar Nano by measuring absorbance at 260 nm, purity was estimated by obtaining 260/280 ratio, and RNA quality was assessed using RNA integrity gels.

2.6. Reverse Transcription Polymerase Chain reaction(RT-PCR)

Semi-Quantitative RT-PCR was used to analyze the differential gene expression. 0.5 µg of the total RNA was reverse transcribed using random primers in a total volume of 20µl, using RevertAid First Strand cDNA synthesis kit (Thermo Scientific, USA), according to the manufacturer’s protocol.

PCR was performed using MyTaq DNA Polymerase kit (Bioline). One µl of cDNA was used per PCR reaction. GAPDH was used as an internal control. PCR conditions were similar among all analyzed genes except for the cycle numbers and annealing temperatures listed in Table (2); 94°C for 3 minutes, followed by cycles of (94°C for 30 secs, annealing temperature for 30 secs, and 72°C for 45 secs), followed by 7 minutes at 72°C for final extension. PCR products were run on a 2.5% agarose gel and visualized by Gel Doc EZ System (Bio-Rad, USA).
2.7. Western Blot Analysis

Protein was extracted from cells for Western blotting using ice-cold 1xLaemmli Lysis buffer (50 mM Tris pH6.8, 2% sodium dodecyl sulfate, and 10% glycerol), supplemented by 1x Halt protease inhibitor cocktail (ThermoScientific, USA) (10µl of the protease inhibitor for 1ml Laemmli Lysis buffer). For 6 well plate, cells from each well were extracted using 80-100 µl of the extraction buffer, by pipetting several times, followed by an incubation period of 30-40 minutes at 4°C with shaking.

Table 2. RT-PCR Primers sequences, annealing temperature, number of cycles, and amplicon sizes (F: forward primer, R: reverse primer, bp: base pair):

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer sequence (5’-3’)</th>
<th>Annealing Temperature</th>
<th>Cycle Number</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>F: CCACCCATGGCAAATTCCATGGCT</td>
<td>60.5 °C</td>
<td>26 cycles</td>
<td>598</td>
</tr>
<tr>
<td></td>
<td>R: TCTAGACGCCAGGTCAGGTCCACC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COBRA1</td>
<td>F: ACATCACCAAGCAGAGGAA</td>
<td>59.5°C</td>
<td>32 cycles</td>
<td>366</td>
</tr>
<tr>
<td></td>
<td>R: GATCCAGCTGTTCCAGCTTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki67</td>
<td>F: CTTTGGGTGCAGACTTGACG</td>
<td>60°C</td>
<td>27 cycles</td>
<td>199</td>
</tr>
<tr>
<td></td>
<td>R: GTCGACCCCGCTCCTTTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivin (BIRC5)</td>
<td>F: TTGAATCGCGGGACCCCGGTGG</td>
<td>61°C</td>
<td>28 cycles</td>
<td>Isoform 1: 477</td>
</tr>
<tr>
<td></td>
<td>R: CAGAGGCCCTCAATCCATGGCA</td>
<td></td>
<td></td>
<td>Isoform 2: 359</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Isoform 3: 546</td>
</tr>
<tr>
<td>NELF-A</td>
<td>F: GTCGGCAGTGGAAGCTCAAGT</td>
<td>60°C</td>
<td>32 cycles</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>R: TTCACACTCACCCACCTTTTCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NELF-C/D</td>
<td>F: GAAGAAGGAGAGACCCAGC</td>
<td>56°C</td>
<td>28 cycles</td>
<td>443</td>
</tr>
<tr>
<td></td>
<td>R: GTGCACAAGGCTAGTGTGAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NELF-E</td>
<td>F: TGGTGAAGTCCAGGCACATCAG</td>
<td>63°C</td>
<td>28 cycles</td>
<td>565</td>
</tr>
<tr>
<td></td>
<td>R: CGCCGTTCAGGGAATGAATC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TWIST1</td>
<td>F: AGCTACGCCCTTCTCCGTCTG</td>
<td>60°C</td>
<td>37 cycles</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>R: CTCTTTCTCTGGAAAATAATGACA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
To eliminate viscosity through denaturing, a heating step for 5 minutes at 90 °C was done, if viscosity persists, more buffer was added. Samples were centrifuged at 12,000 rpm for 20 minutes at 4°C, and the supernatant was collected in a fresh vial. Protein was quantified using BCA Protein Assay kit (Pierce Biotechnology, USA) according to the manufacturer’s protocol.

20-30 µg of whole cell lysate proteins were mixed with 4x-SDS-Laemmli with loading dye to give a final volume of 28 µl and boiled for 5 minutes at 99°C. Samples were loaded on to 10% SDS-Polyacrylamide gel. After protein separation on SDS-Polyacrylamide gel, proteins were blotted on to a nitrocellulose membrane. To block the membrane, membranes were soaked in 5% non-fat dry milk in 1x TBST (0.01% Tween in TBS buffer) for 1 hour at room temperate. Afterward, membranes were incubated with primary antibodies overnight at 4°C. Membranes were then washed 3 times (5minutes each) using 1X TBST, then incubated for 1 hour with the secondary antibody at room temperature. Membranes were subsequently washed 3 times (5 minutes each) using 1XTBST, followed by addition of 2 ml of BCIP/NBT Phosphatase colorimetric substrate (KPL) for 1-5 minutes (depending on the bands color development). Upon the development of bands, membranes were washed using distilled water to stop the color development. Membranes were visualized using Gel Doc EZ system (Bio-Rad, USA).

Primary antibodies used; anti-GAPDH (Abcam, ab8245) (1:10,000 in 5% non-fat dry milk), anti-COBRA1 (Abcam, ab167401) (1:1000 in 3% non-fat dry milk). Secondary antibodies used are anti-mouse (KPL, 4751-1806) (1:10,000 in 5% non-fate dry milk), and anti-rabbit (KPL, 4751-1516) (1:10,000 in 5% non-fat dry milk). All non-fat dry milk used was dissolved in 1XTBST.
2.8. Wound Healing Assay

Wound Healing Assay was performed to assess the effect of knockdown on cell migration rate. Transfection process was performed in 24-well plate as described before in section 2.4., after downscaling all quantities of reagents used to 1/5 of the original amounts to suit the well size. At 96 hours post-transfection, media was aspirated, and two perpendicular scratches were generated in the confluent (~90% confluent) cells monolayer using the sterile 20 µl yellow tip. Cells were then washed gently twice with PBS to remove floating cells and cell debris, and fresh media were added. Pictures at different fields of the wound were taken at two timepoints from wound generation (0 and 24 hours from scratch generation) using an inverted microscope. Wound area was measured using ImageJ Software. Percentage of wound closure was calculated using the following equation:

Percentage wound closure = [(wound area 0 hr - wound area 24 hr)/ wound area 0hr] x 100

2.9. Transwell Invasion Assay

To test the effect of COBRA1 knockdown on cell invasion, we used the Transwell invasion assay. Cells were collected 96-hours post-transfection, and 2x10^5 cells of COBRA1 knockdown and negative control cells were suspended in 100 µl RPMI1640 media supplemented with 1% FBS. Cells were applied to the top chamber of 24-well cell culture insert (GBO) with pore size 8µm. Cell culture inserts were coated with 40µg of Collagen I (SERVA, 47254), left to dry in the incubator overnight and were rehydrated with media 30 minutes before applying the cells. The lower chamber of the 24-well plate contained 600 µl RPMI1640 media supplemented with 10% FBS. Cells were incubated in the chambers for 22 hours, cells in the upper side of the chamber were removed, then cells that migrated to the lower side of the chamber were fixed with 4% formaldehyde and Stained with DAPI (KPL,71-03-01) (1:1000 in PBS). Cells were visualized using fluorescent microscopy at 200X magnification. Cells in 5 random fields were counted for each condition, and average numbers of invading cells were compared.
2.10. Data Analysis

For PCR and Western Blot Differential gene expression analysis, ImageJ software was used to quantify the bands’ intensities, and bands were normalized to loading control GAPDH. Relative gene expression was presented as fold change to either Untreated control, or the Negative siRNA Control.

Data were presented as the mean value ± Standard deviation (SD) from two independent experiments, unless specified otherwise. Unpaired Student’s t-test (two-tailed) was used to determine statistical significance for pairwise comparison. GraphPad Prism 5.0 software was used to perform multiple experimental groups comparisons by One-way ANOVA, followed by a Bonferroni post-test. p-value less than 0.05 was considered significant (*p-value ≤ 0.05, ** p-value ≤ 0.01, *** p-value ≤ 0.001).
3.RESULTS

3.1. Optimization of COBRA1 knockdown in SNU449

Different conditions and methods of knockdown were tested in the process of knockdown optimization, to achieve efficient knockdown that would promote the aimed functional analysis. Two methods of siRNA transfection: Forward and Reverse, different siRNA concentrations and different incubation periods were tested. The efficiency of knockdown was estimated on protein level by Western blot analysis.

siRNA is known to mediate transient silencing of the targeted gene by promoting the degradation of its mRNA hence preventing translation. Upon the degradation of siRNA, the target gene starts going back to its expression levels. Incubation period required for optimal knockdown vary according to each cell line and gene, and usually range from 48-96 hours.

The concentration of the siRNA employed requires optimization, to obtain an efficient knockdown, with the minimal amount of siRNA. Excessive amounts of siRNA can cause an off-target activity, at which mRNAs other than the targeted are being degraded.

3.1.1. Forward transfection:

For forward transfection, cells were seeded one day prior to the addition of the siRNA-lipid transfection complex.

3.1.1.a) Duration of incubation:

To test the efficiency of transfection at different incubation time points, forwards transfections were done using 25nM of siRNA, and harvested at 48, 72, and 96 hours. Knockdown was maximal at 72 hours (Figure 1.A). For subsequent optimization trials, cells were incubated for 72 hours.
3.1.1.b) siRNA concentration:

Three different concentrations were tested; 25nM, 40nM and 60nM. Cells were incubated for 72 hours post-transfection, then harvested for protein analysis. Lowest protein levels were obtained by the 25nM, while higher concentrations didn’t show enhanced knockdown efficiency (Figure 1.B.). However, maximum knockdown levels obtained by forward transfection weren’t very high, and further optimization was carried on to test whether higher knockdown levels could be obtained by employing reverse transfection.

3.1.2. Reverse Transfection

To further optimize COBRA1, reverse transfection was performed, at which cells are seeded during transfection and mixed with the siRNA-lipid complex as a cell suspension before adhering to the cell culture plate. Two different concentrations; 25nM, and 40nM were tested, at two different incubation periods; 72, and 96 hours (Figure 1.C.). Optimum knockdown was obtained by using 40nM siCOBRA at 96 hours incubation period with only 4% COBRA1 residual protein expression. The optimum knockdown conditions were performed on all the subsequent experiments used for further analysis.

3.2. Estimation of COBRA1 knockdown efficiency on mRNA level, and its effect on cell morphology

Optimum transfection conditions resulted in a significant protein knockdown (p-value ≤ 0.001) with an average of 96% decrease in protein expression (Figure 2.a.). mRNA levels of COBRA1 were also analyzed to estimate the transfection efficiency on mRNA level, and a significant decrease in expression was obtained (p-value ≤ 0.01), with an average of 93.5% decrease (Figure 2.b.). Images of transfected, (mock, negative siRNA- siNTC, and COBRA1 siRNA- siCOBRA1) and untransfected cells were compared to observe whether the knockdown effects on cell morphology (Figure 3). No significant change in morphology was noticed, however, COBRA1 knockdown cells were less dense than the other three controls, which could be due to decreased cell proliferation or cell survival upon knockdown (Figure 3).
Figure 1 Optimization of COBRA1 knockdown transfection conditions in SNU449. Western blot assay employed for determining COBRA1 protein expression levels among different optimization conditions. A) Forward transfection, different incubation periods: using 25nM siRNA concentration, testing different incubation periods (48, 72, and 96 hours). Silencing was highest at 72 hours. B) Forward transfection, different siRNA concentrations: 25nM, 40nM, 60nM, incubated for 72 hours. Maximum knockdown was achieved by 25nM concentration; higher concentrations did not achieve enhanced silencing efficiency. C) Reverse Transfection: testing two different concentrations (25nM, and 40nM), and two different incubation periods (72 and 96 hours). Maximal knockdown was obtained by 40nM siRNA concentration, and 96 hours incubation. All bands were quantified by ImageJ and normalized to GAPDH as an internal control, presented as fold change to untreated control samples.
Figure 2. COBRA1 siRNA transfection significantly knocks down COBRA1 on protein and mRNA levels. A) Effect of COBRA1 siRNA knockdown on protein level compared to the negative siRNA control. All bands were quantified by ImageJ and normalized to GAPDH as an internal control, presented as fold change to siNTC. Statistically significant at *** p<0.001 (Student t-test, two-tailed). B) Effect of COBRA1 siRNA knockdown on mRNA level. All bands’ intensities were analyzed using ImageJ software and normalized to GAPDH the internal control, presented as fold change to negative siRNA (siNTC) control. Presented data represent mean ±SD of two independent experiments (n=2). Statistically significant at ** p<0.01 (one-way ANOVA, Bonferonni’s post-test).
Figure 3. No Change in morphology was observed upon transfection and knockdown of COBRA1, but cells were less in density. Images of untreated, mock, siNTC and siCOBRA1 were obtained at 200X magnification. Morphology seemed comparable between different controls and COBRA1 knockdown cells; however, COBRA1 knockdown cells seemed less dense than the controls used.
3.3. COBRA1 knockdown significantly downregulates the expression of NELF-A, and does not affect NELF-C/D or NELF-E expression

The effect of COBRA1 knockdown on the expression levels of the other NELF subunits was measured by semiquantitative RT-PCR (Figure 4). No significant difference was noticed in the expression levels of NELF-C/D or NELF-E. NELF-A, on the other hand, was significantly downregulated (p-value ≤ 0.001) with an average of 30% decrease (Figure 4).

3.4. COBRA1 Knockdown significantly decreases cell count and expression of proliferation marker Ki67

The number of cells 96-hours post-transfection was calculated using the Trypan blue exclusion assay, to determine the effect of COBRA1 knockdown on cell proliferation. A significant decrease (p-value ≤ 0.05) in cell count due to COBRA1 knockdown was estimated, by an average of 55% decrease in cell count compared to siNTC negative control (Figure 5A). No significant change in cell count was shown among the controls (Untreated, mock and siNTC).

For additional examination of the effect of COBRA1 on cell proliferation, the levels of Ki67 gene expression were estimated using RT-PCR. Ki67 was significantly decreased (p-value ≤ 0.05) by an average of 51% compared to siNTC (Figure 5B).
Figure 4. Significant downregulation of NELF-A expression upon COBRA1 knockdown, and no effect on NELF-C/D, NELF-E expression levels. The effect of COBRA1 knockdown on NELF subunits expression on mRNA levels was assessed using RT-PCR. No significant effect was noticed in NELF C/D, or NELF-E. NELF-A, on the other hand, was significantly downregulated. All bands were quantified using ImageJ, normalized to GAPDH as an internal control, and represented as fold change to Negative (siNTC) control. Data represent the mean of two independent experiments (n=2). Statistically significant at *** p ≤0.001 (one-way ANOVA, Bonferonni's post-test).
Figure 5. COBRA1 significantly reduces cell proliferation and downregulated the expression of Ki67

A) Cell proliferation was determined by counting the cells after 96 hours of the transfection using the Trypan blue exclusion assay. Untreated, Mock and siNTC cells showed comparable cell counts, while siCOBRA1 showed a significantly lower cell count relative to the siNTC control. Data presented as the mean ±SD (n=2) of the cell count relative to the siNTC, statistically significant at * p≤0.05 (one-way ANOVA, Bonferonni’s post-test).

B) The Effect of COBRA1 knockdown on the Ki67 expression on mRNA levels was assessed using RT-PCR. Ki67 was significantly downregulated compared to siNTC. All bands were quantified using ImageJ, normalized to GAPDH as an internal control, and represented as fold change to Negative siNTC control. Data represent the mean of two independent experiments (n=2). Statistically significant at * p ≤0.05 (Student t-test, two-tailed).
3.5. COBRA1 knockdown suppresses the mRNA expression of Survivin and induces apoptosis

Survivin is a member of the Inhibitor of apoptosis proteins and is highly overexpressed in most types of cancers, inhibiting apoptosis and supporting cell cycle progression (77). Survivin has been previously shown to be affected by the COBRA1 knockdown in the early stage HCC cell line the HepG2 (73). We wanted to test if this correlation is consistent with more advanced HCC stages. Our results show a significant downregulation (p-value ≤ 0.001) in Survivin mRNA levels by an average of 53% compared to the siNTC (Figure 6). Significant downregulation was observed only in the wild type variant of Survivin (477 bp), while a decrease in Survivin-deltaex3 (369 bp) was found to be non-significant. The third variant that could be detected by the primers we used; Survivin-2B (546 bp) was not expressed in our cells.

Upon staining the Cells with DAPI stain to visualize the nuclei, fragmented nuclei were observed in COBRA1 knockdown cells and not the siNTC control, which marks for cell apoptosis (Figure 9).

Figure 6. COBRA1 knockdown significantly downregulates the expression of Survivin The Effect of COBRA1 knockdown on Survivin expression on mRNA levels was assessed using RT-PCR. Survivin was significantly downregulated compared to siNTC. All bands were quantified using ImageJ, normalized to GAPDH as an internal control, and represented as fold change to Negative siNTC control. Data represent the mean of two independent experiments (n=2). Statistically significant at *** p ≤0.001 (Student t-test, two-tailed).
3.6. COBRA1 knockdown decreases cell migration

Classical wound healing assay was performed to test the effect of COBRA1 knockdown on cell migration. The wound was created at 96 hours post-transfection, and pictures were taken for the wound at 0 hours of its creation, and at 24 hours. Percentage of wound closure was calculated by measuring the area of the wound at the two mentioned time points. The cell migration rate significantly ($p$-value $\leq 0.01$) decreased in COBRA1 knockdown cells, showing around 45% decrease in migration rate compared to the siNTC negative control (Figure 7).

![Image of wound healing assay](image)

**Figure 7. COBRA1 knockdown significantly decreases cell migration rate.** Wound healing assay was used to assess the effect of COBRA1 knockdown on cell migration. The wound was created at 96-hours post transfection and monitored over 24 hours. Changes in wound area were analyzed by ImageJ software and percentage of wound healing was calculated in both siCOBRA1 and siNTC. Data presented as the mean ±SD (n=2) of the change in migration rate relative to the siNTC, statistically significant at ** $p<0.01$ (Student t-test, two-tailed).

3.7. COBRA1 knockdown decreases cell invasion

Transwell invasion assay was used to assess the effect of COBRA1 knockdown on cell invasion through extracellular matrix (ECM). 96 hours post-transfection, cells were moved
to cell culture insert covered by a layer of collagen and incubated for 22 hours. The number of cells that could invade through collagen decreased by around 43% upon COBRA1 knockdown compared to siNTC (Figure 8).

![Image](85x457 to 532x650)
![Image](205x284 to 407x442)

**Figure 8. COBRA1 knockdown decreases cell invasion.** Transwell assay was used to assess the cell invasion ability through collagen. Invasive cells were fixed, stained by DAPI, and visualized by fluorescent microscopy at 20X magnification. COBRA1 knockdown cells showed around 43% decrease in the number of invasive cells compared to siNTC.
3.8. COBRA1 knockdown suppresses the expression of transcription factor TWIST1

Cell invasion and migration that occur during cancer progression are mediated through epithelial-mesenchymal transition (EMT) process. Many transcription factors mediate this process, TWIST1 is one of the main transcription factors that mediate EMT in HCC. We tested the expression levels of TWIST1 using RT-PCR, and a significant downregulation ($p$-value $\leq 0.05$) by an average of 42% was detected upon COBRA1 knockdown compared to siNTC (Figure 10).
Figure 10. COBRA1 knockdown significantly downregulates the expression of TWIST1 The Effect of COBRA1 knockdown on the TWIST1 expression on mRNA levels was assessed using RT-PCR. TWIST1 was significantly downregulated compared to siNTC. All bands were quantified using ImageJ, normalized to GAPDH as an internal control, and represented as fold change to Negative siNTC control. Data represent the mean of two independent experiments (n=2). Statistically significant at * p ≤0.05 (Student t-test, two-tailed).
4. DISCUSSION

Hepatocellular carcinoma is a disease of very aggressive course and poor prognosis. Being asymptomatic at its early stage, and lack of appropriate diagnostic markers, contribute to its late diagnosis, at stages where surgical resection/transplantation are mostly non-applicable or non-efficient. There is also lack of effective prognostic and molecular biomarkers for monitoring treatment efficiency, which makes recurrence rates one of highest.

HCC molecular heterogeneity and complexity make it difficult to characterize the molecular machinery attributing to its development and progression. The thorough study of the underlying molecular mechanisms, and discovery of key player biomarkers will not only provide better diagnostic and prognostic markers but will also promote the development of effective targeted therapies.

In our lab, a potential biomarker was studied for the first time in HCC; COBRA1, also known as NELF-B. It is a part of transcription regulatory machinery that attributes to the regulation of several important processes in the cell. The role of COBRA1 was previously studied in breast and UGC cancers, and showed opposite roles, signifying its tissue/context-dependent function.

In HCC, we found it to be upregulated compared to their paired non-neoplastic tissue, and it was also found to support proliferation and migration at early stages of HCC (73). As an extension to our previous work, we aimed to determine its role in maintenance and progression of HCC at more advanced HCC stages. To achieve that, we performed a loss of function analysis by transient silencing of COBRA1 in an intermediate stage HCC cell line; SNU449, followed by an analysis of the effect of that on some of the main cancer hallmarks, and expression of some of the well-characterized biomarkers in cancer.

4.1. Co-regulation among NELF subunits

NELF consists of four subunits; NELF-A, NELF-B, NELF-C/D, NELF-E, that are all essential for the assembly of the functional complex. They are characterized by being interdependent, as the knockdown of any of them results in the downregulation of the
other subunits via post-transcriptional modifications, exclusively (61,58,71). Upon examining the mRNA levels of NELF subunits post-COBRA1 knockdown, we detected significant downregulation of NELF-A, which was not reported on the transcriptional level before, to the best of our knowledge.

Pathologically, the simultaneous dysregulation of more than one NELF subunit was observed more than once. In breast cancer, COBRA1 and NELF-C/D were downregulated, and showed an inverse relationship with tumor aggressiveness (71,78). On the other hand, COBRA1 and NELF-E are upregulated in HCC, with the later described as a risk factor for recurrence and supporting cell proliferation (78). However, the overlap of modes of action between those subunits is to be further elucidated.

4.2. COBRA1 supports cell proliferation in SNU449

Sustaining cell proliferation is considered the most fundamental cancer hallmark as described by Hanahan & Weinberg, where cancer cells can evade the tight regulation of growth signals. COBRA1 was shown to support cell proliferation at initial stages of HCC (73). We aimed to examine whether that effect was only required during tumor initiation only, or for maintenance as well at more advanced HCC stages. Our study revealed a significant effect of COBRA1 in supporting cell proliferation, and positive correlation with Ki-67 expression, the most commonly used proliferation marker. Ki-67 is essentially expressed throughout the different stages of cell cycle, promoting its progression and maintaining cell proliferation (80). On the contrary, in breast cancer, COBRA1 is inversely related to cell proliferation (65) which highlights the cancer-type dependent role of COBRA1.

4.3. COBRA1 inhibits apoptosis and promote HCC cell survival

Apoptosis is programmed cell death, which promotes the maintenance of healthy cells and tissues, by eliminating old, unhealthy, or unnecessary cells (81,82,83). Apoptosis
pathways are highly dysregulated in cancer, which promotes the survival of defective cancer cells. Several stimuli trigger the apoptosis process via activation of caspases, leading to cell death (82). Survivin, also known as BIRC5, is one of eight members in the inhibitor of apoptosis proteins family (IAPs), that blocks the apoptosis process via direct binding to caspases (83). Overexpression of IAPs has been demonstrated in different cancer types, with survivin being the most overexpressed family member in majority of tumors, and the most potent antiapoptotic protein studied to date (77). It is undetectable in most differentiated, adult normal tissues, and expressed at low levels in few (84,85).

The role of survivin in cancer is not limited to its anti-apoptotic activity, but it also promotes cell proliferation. It was found to supports the progression of the cell cycle at early mitosis, via binding to the microtubules of the mitotic spindles, protecting them from hydrolysis (86,87). Its overexpression in early hepatoma cells showed its interaction with cyclin dependent kinase 4 (CDK4), leading to G1 arrest counteracting, and phosphorylation of the tumor suppressor retinoblastoma protein (Rb) (88). It was also found to induce angiogenesis in tumor stroma, by facilitating the endothelial cells proliferation and migration, and expression of VEGF (Vascular endothelial growth factor) (89,90).

In our study, a direct correlation was shown between COBRA1 and survivin expression, as survivin expression was significantly downregulated upon COBRA1 knockdown. The effect of survivin downregulation as demonstrated in our study, that significantly decreased in cell number upon knockdown of COBRA1, infers a reduction in cell proliferation, and increased cell apoptosis, which was confirmed by the detection of nuclear fragments (91).

Regulation of survivin by COBRA1 has been consistent in early-stage HCC cell line HepG2, in the intermediate stage HCC cell line (SNU449), and also in the breast cancer cell line (73, 65). This conserved correlation over different types and stages of cancer makes the investigation of the exact mechanism by which COBRA1 regulates survivin very important.
4.4. COBRA1 promotes Epithelial-Mesenchymal Transition

Epithelial mesenchymal transition (EMT) is the process by which epithelial cells lose their epithelial phenotype, and acquire mesenchymal phenotype via events of biochemical changes, to become more invasive, migratory and resistant to apoptosis. This process normally occurs and is tightly regulated during development, wound healing, and was found to be also occurring during cancer progression promoting metastasis (92).

The initiation of EMT process and its completion include a series of molecular processes, where the activation of certain transcription factors suppresses the expression of epithelial markers and induce the expression of mesenchymal markers. These markers include expression of specific cell-surface proteins, cytoskeleton proteins reorganization, and the secretion of the extracellular matrix (ECM) degrading enzyme (93). The changes that occur during the EMT enable those cells to enter the cascade of invasion-metastasis through which primary tumors spread to other organs. Metastases cascade comprises; local invasion, intravasation into blood and lymph vessels, transport and survival through circulation, extravasation, micrometastases formation, and eventually macroscopic metastases colonization (94,95,96).

As cancer metastases is a very critical hallmark of cancer progression, the estimation of the effect of COBRA1 silencing on cell migratory and invasive ability was essential. For examining cell migration, we used the classical wound healing assay, the standard *in vitro* technique for cell migration assessment (97). Our results demonstrated a significant decrease in cell migration upon COBRA1 silencing, going in line with what was previously reported for early-stage HCC cell line HepG2 (73), which suggests a similar role throughout the early and intermediate HCC stages. We also demonstrated the effect of COBRA1 on cell invasion *in vitro* as COBRA1 silencing decreased the cells' ability to invade through an ECM equivalent. Our results show for the first time in HCC that COBRA1 supports the invasion and not only migration. These results are contradictory to what has been previously reported in breast cancer, where COBRA1 inversely correlates to metastases (71).
Several transcription factors contribute to the initiation of EMT, among which zinc-finger transcriptional repressors (SNAIL and SLUG), TWIST and zinc-finger E-box-binding (ZEB) are considered the main regulators of the process, activated at its early stage (93). The contribution and co-operation between these transcription factors vary among different tissues, and they cooperate to repress the epithelial genes, and thus promote mesenchymal ones (98,99).

4.5. TWIST1 mediates EMT in SNU449

In our study we examined the expression levels of *TWIST1*, to investigate the possible mediators of COBRA1 effect on EMT. TWIST1 is a member of the evolutionary conserved basic loop-helix-loop (BLHL) transcription factors family (100). TWIST1 has an essential role in development (101), and its role in promoting metastases via EMT is well established by studies done in various cancer types (102, 103). TWIST1, as well as other EMT transcription factors (SNAIL and ZEB superfamilies) have an antiapoptotic effect that is exerted through inhibition of p53, retinoblastoma protein (RB), in addition to other mechanisms, which benefits the survival of invasive cells through the process of metastases (104). Apart from its role in metastases, two recent studies on breast and skin cancer have shown that TWIST1 mediates the development of cancer stem cell properties, by promoting proliferation and inhibiting apoptosis during tumor initiation. They showed that TWIST1 was required at low levels to promote tumor initiation, and higher levels promote metastases as the tumor progresses, and the tumor initiation property is mediated via mechanisms independent from EMT, through different downstream targets. (105,106)

TWIST1 is overexpressed in several types of cancers such as HCC, prostate, gastric, and breast. It is overexpressed in 43% of primary HCC tumors and correlates to poor prognosis. A study on EMT regulators in HCC demonstrated that TWIST and SNAIL, not SLUG were the Key regulators of EMT in HCC. Both were proven to be critical for HCC EMT/metastases, independently regulated, and have an additive effect. (107)
Our study shows downregulation of TWIST1 upon COBRA1 silencing, which goes in line with the decreased rates of migration and invasion, indicating the role of COBRA1 in promoting epithelial-mesenchymal transition via TWIST1. However, the effect of COBRA1 on other EMT transcription factors is yet to be examined to elucidate whether COBRA1 mediates its effect through TWIST1 only, or through other factors as well.

4.6. Insights about role of COBRA1 and its mode of action

Youssef et al. have previously shown the differential expression of COBRA1 among HCC cell lines of different stages, with the highest expression levels detected in early stages, while a gradual decrease of COBRA1 levels was measured as the HCC stages advance (74). That result could suggest that COBRA1 role is important for HCC initiation, and not maintenance or progression. To test that hypothesis, we examined the SNU449 cell line derived from a more advanced HCC tumor, stage II-III/IV. The loss of function analysis we employed refuted that hypothesis, as COBRA1 was shown to support the progression of HCC at that stage by mediating several critical cancer hallmarks. However, that needs further investigation for several reasons; 1- HCC is very heterogeneous in terms of molecular profile, and that mechanism could be variable among different HCC tumors. 2- SNU449 originates from an HBV positive patient, other etiologies of HCC could contribute differently to the progression of the disease. 3- COBRA1 role in more advanced HCC stages hasn’t been studied yet, at which it could behave differently.

Through the examination of expression levels of several cancer biomarkers, we found out that COBRA1 affects the regulation of three important markers of cancer in SNU449 cell line; TWIST1 reported for the first time to be affected by COBRA1, Survivin and Ki-67, previously reported in early HCC stage HepG2 cell line. It is worth noting that the three were downregulated upon COBRA1 knockdown, despite COBRA1 acting predominantly through the NELF complex that stalls RNA polymerase II at early elongation stages. Several mechanisms could attribute to these genes’ regulation; they could be indirectly regulated via transcriptional repressors stalled by the NELF complex, be directly activated by RNA polymerase stalling, or other unidentified mechanisms. Activation of gene
transcription via RNA polymerase stalling was first reported by Gilchrist et al. in 2008, in a study done on *Drosophila* cells where NELF was silenced and microarray analysis was performed. Unexpectedly, only one-thirds of the NELF target genes were upregulated, and the other two-thirds were downregulated. The authors further examined the mechanism through which NELF mediated its regulation of the downregulated genes and revealed that polymerase stalling at these genes activates the transcription by sustaining chromatin accessibility near the promoter-proximal region, while abolishing Polymerase II contributed to increased nucleosome occupancy (60). The same observation was found in a study on mammalian breast cancer cells, and the same mechanism was proposed to be conserved in the mammalian cell (58).

**CONCLUSION**

In conclusion, our study reveals that COBRA1 plays a vital role in the progression of HCC in SNU449, supporting critical cancer hallmarks; cell proliferation, inhibition of apoptosis, migration and invasion. We also showed that COBRA1 positively regulates key players in these processes; *Ki67, Survivin* and *TWIST1*, that is reported to be affected by COBRA1 for the first time in HCC. These results need further investigations to fully understand the underlying mechanisms of COBRA1 and need to be extended to more HCC cell lines of different properties; nonetheless, COBRA1 shows great potential as a possible therapeutic target and prognostic factor of HCC.
FUTURE RECOMMENDATIONS

COBRA1 plays key role in HCC initiation and progression. However, that role needs to be examined thoroughly and extended. Since HCC has different etiologies that affect its molecular profiles, and due to its heterogeneity in general as a disease, we suggest the examination of COBRA1 roles in more cell lines of different etiologies, stages, and genetic backgrounds. Employing techniques such as microarray and RNA seq analysis after gene silencing will promote a much broader discovery of the genes regulated by COBRA1 and can dramatically enhance the process of functional analysis. Another tool that is highly important is *in vivo* testing of COBRA1 silencing effect using an animal model, as *in vitro* testing alone cannot simulate all the physiological factors that interplay with cancer progression.

To allow a better understanding of the COBRA1 role in promoting EMT, it is worth examining the levels of transcription factors other than *TWIST1* that could promote metastases in HCC, and more EMT markers such as; E-Cadherin, N-Cadherin, B-Catenin localization, and matrix metalloproteinases. Also, further investigation of other apoptotic markers and genes could be valuable to understand how COBRA1 inhibits apoptosis.

We also think that examining the transcriptional regulation mechanisms of the genes affected by COBRA1, and whether that regulation is direct or indirect is important to give more insight about the COBRA1 role.
REFERENCES


