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THE AMERICAN UNIVERSITY IN CAIRO
الجامعة الأمريكية بالقاهرة

School of Sciences and Engineering

**COFACTOR OF BRCA1
AS A MODULATOR OF HEPATOCELLULAR
CARCINOMA GROWTH AND MIGRATION**

A Thesis Submitted to the
Biotechnology Master's Program

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

By

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Bachelor of Pharmaceutical Sciences

Under the supervision of

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September 2015

The American University in Cairo
School of Sciences and Engineering (SSE)

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In partial fulfillment of the requirements for the degree of
Master of Science in Biotechnology
Has been approved by

Thesis Committee Supervisor/Chair

Affiliation _____

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Dept. Chair/Director

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Dean

Date

DEDICATION

*To my beloved parents and brothers,
without whom none of my success would
have been possible*

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All Praise is to Allah for Everything

I would like to thank Dr. Asma Amleh for her mentorship and guidance during my graduate studies. Her discussions and insights were indispensable to the progress of the project and have helped me a lot. I am forever grateful to the time that she dedicated and invested on my training.

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COFACTOR OF BRCA1 AS A MODULATOR OF HEPATOCELLULAR CARCINOMA GROWTH AND MIGRATION

ABSTRACT

Cofactor of BRCA1 (COBRA1) is one of the four subunits that make up the Negative Elongation Factor Complex (NELF) which is involved in the stalling of RNA polymerase II early during transcription elongation. As such, COBRA1 is able to regulate a substantial number of genes involved in a number of pathways, including cell cycle control, metabolism, cell proliferation and DNA repair. In the field of cancer, the role of COBRA1 is not yet fully understood. The aim of our study was to investigate the functional role of COBRA1 in the tumorigenesis of hepatocellular carcinoma (HCC). We investigated the gene expression pattern of COBRA1 in HCC tumors using the publicly available Oncomine Cancer Microarray Database. Results from three different microarray datasets reveal the frequent overexpression of COBRA1 in HCC tumors versus their normal counterparts. To elucidate the biological significance for this overexpression in HCC, RNA interference was used to silence the expression of COBRA1 in the well differentiated HCC cell line, HepG2. The silencing efficiency was confirmed by both reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. Interestingly, knockdown of COBRA1 resulted in a significant decrease in cell proliferation, accompanied by a concomitant decrease in the expression of the proliferation marker, Ki-67. A scratch wound healing assay revealed a significant decrease in the migratory potential of the HepG2 cell line in culture upon COBRA1 knockdown. In addition, silencing of COBRA1 was associated with a significant decrease in the expression of survivin, suggesting that survivin might be one of the mechanisms by which COBRA1 mediates its role in the tumorigenicity of HCC. Collectively, data findings presented here highlight an oncogenic role for COBRA1 in hepatocellular carcinoma. To the best of our knowledge, our study provides evidence for the first time to support a positive role for COBRA1 in the growth and migration of HCC.

TABLE OF CONTENTS

Dedication.....	iii
Acknowledgements.....	iv
Abstract.....	v
List of Abbreviations	viii
List of Tables	x
List of Figures	xi
Chapter 1: Literature Review	
1.1. Hepatocellular Carcinoma.....	1
1.1.1. Statistics and Epidemiology.....	1
1.1.2. Etiology.....	1
1.1.3. Surveillance and Diagnosis.....	2
1.1.4. Prognosis and Available Treatments.....	2
1.1.5. Molecular Classification of HCC.....	4
1.1.6. Molecular Biology of HCC Progression.....	5
1.2. Cofactor of BRCA1.....	6
1.2.1. Breast Cancer Type 1 Susceptibility Protein	7
1.2.2. Negative Elongation Factor Complex.....	8
1.2.3. Nuclear Receptors	9
1.2.4. AP-1 Complex	10
1.3. COBRA1 in Cancer	10
1.4. COBRA1 in Hepatocellular Carcinoma	12
Hypothesis and Study Objectives.....	13
Chapter 2: Materials And Methods	
2.1. Cell lines and Culture.....	14
2.2. Viable Cell Count.....	14
2.3. RNA Interference.....	14
2.4. siRNA Transfection.....	15
2.5. RNA Extraction.....	15
2.6. Primer Design	15
2.7. Reverse Transcription-Polymerase Chain Reaction	16
2.8. Immunoblotting	16
2.9. Scratch Wound Healing Assay.....	17
2.10. Data Analysis.....	18
2.11. Data Mining.....	18

Chapter 3: Results

3.1. Expression of COBRA1 in HCC Tumors.....	20
3.2. Optimization of Knockdown	20
3.3. SMARTPool siRNA Efficiency.....	24
3.4. Cell Proliferation.....	26
3.5. Cell Migration.....	27
3.6. Survivin Expression	27

Chapter 4: Discussion

4.1. Expression of COBRA1 in HCC Tumors.....	31
4.2. SMARTPool siRNA Efficiency.....	32
4.3. Cell Proliferation.....	33
4.4. Cell Migration.....	34
4.5. Interdependent Nature of NELF Subunits	35
4.6. Survivin Expression	35
Conclusion.....	38
Future Recommendations.....	39
References	40

LIST OF ABBREVIATIONS

AASLD	American Association for the Study of Liver Diseases
AFP	Alpha-fetoprotein
ANOVA	Analysis of Variance
AP-1	Activator Protein-1
AR	Androgen receptor
Bps	Base pairs
BRCA1	Breast cancer type 1 susceptibility protein
CAP2	Cyclase-associated protein2
CLD	Chronic liver disease
COBRA1	Cofactor of BRCA1
DEPC	Diethylpyrocarbonate
DSIF	DRB sensitivity-inducing factor
ER α	Estrogen receptor-alpha
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GPC3	Glypican-3
GR	Glucocorticoid receptor
GS	Glutamine synthetase
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HepG2	Well differentiated human hepatocellular carcinoma cell line
HLE	Human hepatoma cell line
Hr	Hour
Hsp70	Heat shock protein 70
IAP	Inhibitor of apoptosis
LOF	Loss-of-function
LT	Liver transplantation

mins	Minutes
NELF	Negative elongation factor
NRs	Nuclear receptors
PBS	Phosphate-buffered saline
PBST	0.01% Tween-20 in 1X PBS
PCR	Polymerase chain reaction
PRB	Progesterone receptor B
RDBP	RD RNA binding protein
RNAi	RNA interference
RNAPII	RNA polymerase II
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulfate
secs	Seconds
siCOBRA1	COBRA1 siRNA
siNTC	Negative siRNA
siRNA	Short interfering RNA
T47D	Metastatic human breast carcinoma cell line
TFF1	Trefoil factor 1
UGCs	Upper gastrointestinal cancers
UTR	Untranslated region
WT	Wild type
µls	Microliters

LIST OF TABLES

Table 1. Target sequences and exons of COBRA1 siRNAs (NCBI Genebank: NM_015456) used in the study	15
Table 2. Semi-quantitative RT-PCR primer sequences, amplicon sizes and optimized PCR conditions (F: forward primer, R: reverse primer, bp: base pair)	16
Table 3. Oncomine microarray datasets used in this study	19

LIST OF FIGURES

Figure 1. Overexpression of COBRA1 mRNA in hepatocellular carcinoma versus normal liver tissue	21
Figure 2. Optimization of transfection conditions of COBRA1 siRNA in HepG2.....	23
Figure 3. COBRA1 siRNA effectively silences COBRA1 expression at both the RNA and protein level.	24
Figure 4. No significant morphology changes associated with COBRA1 knockdown observed in HepG2 cells (40X).	25
Figure 5. COBRA1 knockdown does not affect the expression of any of the remaining NELF subunits	26
Figure 6. Knockdown of COBRA1 inhibits the cell proliferation of HepG2 cells.....	28
Figure 7. Effect of COBRA1 knockdown on the expression of the cellular proliferation marker, Ki-67.....	28
Figure 8. Silencing of COBRA1 impairs HepG2 cell migration	29
Figure 9. Silencing of COBRA1 suppresses Survivin expression	30
Figure 10. Alternatively Spliced Survivin Transcripts	36

CHAPTER 1. LITERATURE REVIEW

1.1 Hepatocellular Carcinoma

1.1.1 Statistics and Epidemiology

Liver cancer is the second most frequent cause of cancer-related deaths worldwide, responsible for more than 0.5 million deaths globally per year and accounting for 0.5–1 million newly diagnosed cases each year (Ferlay *et al.*, 2013; Jemal *et al.*, 2011). Hepatocellular carcinoma (HCC) accounts for 70- 85% of all liver cancer cases; thereby representing by far the most common type of liver cancer (Jemal *et al.*, 2011). It is a male predominant cancer, affecting males 3 to 4 times more frequently than females (Guy & Peters, 2013). The incidence of the disease increases with age, occurring rarely before the age of 40 years and reaching its peak at about 70 years of age (El-Serag, 2011).

HCC is considered one of the most aggressive cancers worldwide affecting primarily developing countries, which harbor more than 80% of all presented cases worldwide (El-Serag, 2011). According to GLOBOCAN, Egypt has one of the highest incidences of liver cancer, ranking first in the most common cancer affecting men while second in those affecting women (Ferlay *et al.*, 2013). Incidence rates in Egypt were reported to have nearly doubled from 4.0% in 1993 to 7.2% in 2003 (el-Zayadi *et al.*, 2005). This is primarily attributed to the high hepatitis C virus (HCV) prevalence rates in the country, which is by far the highest worldwide reaching up to 20% and representing one of the major risk factors for the development of HCC (Lehman *et al.*, 2008; Omar, Abou-Alfa, Khairy & Omar, 2013).

1.1.2 Etiology

The majority of HCC cases (80 -90%) develop from cirrhotic livers; rarely does a HCC case develop from a patient that has no cirrhotic liver (El-Serag, 2011). Factors predisposing to liver cirrhosis are therefore considered risk factors for the development of HCC and include chronic hepatitis B virus (HBV) infection, chronic HCV infection, chronic alcohol consumption and dietary aflatoxin consumption (Montalto *et al.*, 2002). Other risk factors such as diabetes, obesity, metabolic syndrome, hemochromatosis and nonalcoholic fatty liver disease have been proposed to play a role in the development of HCC, however, to a much lesser extent (Farazi & DePinho, 2006).

Chronic HCV infection has currently the largest share in the incidence of HCC, accounting for about 40-50% of cases (Omar *et al.*, 2013). Prior to the introduction of the HBV vaccine,

HBV infection was the risk factor that had the largest contribution towards the development of HCC in Egypt. The influence of HBV on the etiology of HCC has however declined over the past 2 decades, owing to the introduction of routine mandatory HBV vaccinations and to the large increase in the prevalence of HCV (Lehman *et al.*, 2008; Omar *et al.*, 2013). Despite this decline, chronic HBV infection still remains an important risk factor for HCC as the vaccination program, although estimated to have a childhood coverage of 95-100%, was not initiated until the 1980s and therefore most adults over the age of 30 years are not vaccinated (Lehman *et al.*, 2008).

Another major contributor to the development of HCC worldwide is chronic aflatoxin consumption. Aflatoxins are carcinogenic fungal metabolites, produced mainly by fungal species *Aspergillus flavus* and *parasiticum* and contaminates many food products such as peanuts, maize and cottonseed. The presence of aflatoxins is more common in countries with hot and humid climates, which support the growth of the fungi (Waly Raphael, Yangde, & Yuxiang, 2012). Several lines of evidence are recently suggesting that aflatoxin is a growing risk factor for HCC in Egypt (Omar *et al.*, 2013).

1.1.3 Surveillance and Diagnosis

Early stage HCC is asymptomatic and therefore difficult to clinically diagnose. As a result, most HCC patients are usually diagnosed at an advanced stage, when treatment options are limited. Surveillance is thus recommended for patients at high risk of HCC in order to allow for the early detection of HCC (El-Serag, 2011; Forner, Llovet, & Bruix, 2012).

According to both the American Association for the Study of Liver Diseases (AASLD) and the ESMO Clinical Practice Guidelines, surveillance for HCC in liver cirrhosis patients should be done every 6 months using abdominal ultrasound (El-Serag, 2011; Verslype, Rosmorduc, & Rougier, 2012). Although alpha-fetoprotein (AFP) is the most widely used serological marker for the diagnosis and follow-up of HCC, it suffers from a limited sensitivity of 60-80% and thus guidelines do not recommend its use for surveillance (Forner *et al.*, 2012).

1.1.4 Prognosis and Available Treatments

Despite the different treatment regimens available, prognosis of HCC still remains very poor with an overall 5-year survival rate less than 5% (X. Huang, Zheng, & Yuan, 2013). More than 0.5 million lives are lost worldwide every year to hepatocellular carcinoma (Jemal *et al.*, 2011). This is attributed to the fact that potential curative therapies, which

includes either surgical resection or liver transplantation, are ideal for only a small subset of early HCC cases. Unfortunately, more than 50% of all HCCs are diagnosed at an advanced stage or at metastasis, making them ineligible to either treatment (X. Huang *et al.*, 2013). For patients with early HCC and well-compensated cirrhosis, the superiority of one treatment over the other remains controversial and management will depend from one patient to another (Kim & Hemming, 2009)

Liver transplantation (LT), theoretically, is considered an ideal treatment as it does not only remove the tumor, but cures the underlying disease as well (Forner *et al.*, 2012). It has been associated with better overall and disease-free survival in well-selected patients (Bismuth *et al.*, 1993). When used for small HCCs, survival rates have been reported similar to transplantations performed for non-HCC indications (Hemming *et al.*, 2001). The criteria used to define ideal candidates for LT are collectively referred to as the Milan criteria (Forner *et al.*, 2012; Morise *et al.*, 2014). Within the Milan criteria, overall and disease-free survival at 4 years have been reported to be 92% and 85%, respectively while recurrence rates have been demonstrated to be as low as 4-10% (Kim & Hemming, 2009). However, LT suffers from a major limitation, besides from being an option available only to a small subset of patients. As a result of a shortage in donor organs, the transplant list is usually associated with long waiting times that patients do not have. With the inevitable progression of the HCC tumor, patients do eventually drop out from the list without receiving a transplant as they become with time ineligible for the procedure (Fortune, Umman, Gilliland, & Emre, 2013).

Surgical resection suffers from a major drawback. Even with the thorough criteria put in the selection of surgical candidates, tumor recurrence is very common reaching up to 50-80% of the patients at 5 years following resection, with the majority occurring in the first 2 years (El-Serag, 2011; Kim & Hemming, 2009). It is believed that recurrence may either represent a dissemination from the primary tumor or a '*de novo*' tumor in the remnant liver. Controversy however exists about the contribution of each. Dissemination from the primary tumor is considered the result of either microscopic tumor foci that have been missed by common imaging techniques and thus have not been taken into consideration, or cancerous cells that have spread during the surgical procedure (X. Huang *et al.*, 2013). Despite the discussed drawbacks, liver resection remains an attractive option due to its availability and the fact that it is curative in 45% of eligible patients (Kim & Hemming, 2009).

Besides surgical options, other treatments do exist for the management of HCC and include ablative (radiofrequency ablation, transarterial chemoembolization), and medical treatments (chemotherapy), however, they are usually performed either as palliative

treatment for patients who are ineligible for surgical treatment or as 'bridging' therapy for patients awaiting liver transplantation (Jelic & Sotiropoulos, 2010)

1.1.5 Molecular Classification of HCC

The inadequate availability of molecular markers has led to significant reliability on morphological features for the diagnosis of HCC. This makes early stages of HCC difficult to diagnose. This is due to the fact that clinicopathological features of early stages deviate only slightly from chronic liver disease (CLD). Important diagnostic features typically associated with more advanced forms of the disease such as radiologic findings, increased vascularity, elevated serological markers and histological atypia are not typically present in the early stages. Consequently, over 50% of HCCs are detected at an advanced stage and thus are limited to more palliative forms of treatment (X. Huang *et al.*, 2013; Jelic & Sotiropoulos, 2010).

Research currently aims to identify expression profiles and signatures associated with early HCC (Mas *et al.*, 2009; Sakamoto, Effendi, & Masugi, 2010). From ~12,600 genes analyzed in a gene-expression profiling study performed on nodule-in-nodule type HCC, heat shock protein 70 (hsp70) was reported to be significantly upregulated in the early component of the cancer when compared to either its progressed counterpart or noncancerous liver (Chuma *et al.*, 2003). The fact that hsp70 expression was undetectable in other benign nodular lesions, hepatocellular adenoma and focal nodular hyperplasia, indicated the potential of such a marker in differentiating early HCC from other benign liver lesions (Sakamoto, 2009). Comparable approaches have suggested other potential markers of early HCC as well such as, cyclase-associated protein2 (CAP2) (Shibata *et al.*, 2006), glypican-3 (GPC3) (Hippo *et al.*, 2004) and glutamine synthetase (GS) (Osada *et al.*, 1999). Di Tommaso *et al.* (2007) demonstrated that a combination of the 3 markers, HSP70, GPC3 and GS can promote the accuracy of diagnosing early HCC, indicating thus the applicability of using a panel of markers instead of just one to allow for increased accuracy in diagnosis.

Similar attempts were carried out to subclassify HCC. Studies successfully identified gene expression profiles that can distinguish HBV- from HCV-associated HCC, early from late intrahepatic recurrence of HCC, and HCCs with different prognoses (Farazi & DePinho, 2006; Mas *et al.*, 2009; Roessler *et al.*, 2010; Wurmbach *et al.*, 2007).

Proper HCC management remains largely hindered by the lack of molecular biomarkers for the early diagnosis, prognosis & therapy monitoring of HCC. The hope of finding truly

sensitive and specific molecular markers to subclassify HCC and hence, to support patient management is still in its infancy and requires further extensive research.

1.1.6 Molecular Biology of HCC Progression

The exact mechanisms lying behind the development and progression of HCC remain very much a mystery. Several signaling pathways are known to be deregulated in HCC such as the IGF, Wnt/ β -catenin, TGF- β , Ras/MAPK, VEGF, EGFR, HGF/MET and P13/PTEN/AKT pathways (Cornellà, Alsinet, & Villanueva, 2011; Waly Raphael *et al.*, 2012). In addition to the disrupted signaling pathways, HCC is associated with a large number of activated oncogenes and suppressed tumor-suppressor genes such as TP53, ErbB receptor family members, E-cadherin and cyclooxygenase 2. Genomic instability has also been described in HCC in the form of defects in chromosomal segregation (Farazi & DePinho, 2006).

Despite significant progress in the understanding of the genetic and epigenetic changes associated with HCC, we are still a long way from a clear understanding of the molecular mechanisms that govern the multi-step progression or evolution of the disease. This is largely due to the heterogeneity and underlying complexity of the disease. Therefore, research in HCC is currently directed towards the characterization of these molecular mechanisms. A more comprehensive understanding of this progression will in effect allow the discovery of novel treatment strategies (Cornellà *et al.*, 2011; Wong & Ng, 2008).

Up until recently no systemic chemotherapy was available for HCC. However, in 2007, a recently discovered Raf kinase inhibitor (Sorafenib, NEXAVAR®) was approved by the US Food and Drug Administration (FDA) for use in HCC. By targeting the RAF/MEK/ERK pathway, Sorafenib shows antiproliferative, antiangiogenic, and proapoptotic activity (S. Wilhelm *et al.*, 2006). In a phase III randomized controlled trial (SHARP), it was able to increase overall survival in patients with advanced HCC by about 3 months (Llovet *et al.*, 2008). Shortly after its discovery, Sorafenib was shown capable of inhibiting a number of other receptor tyrosine kinases as well involved in tumor progression. Thus, why it is currently referred to as a multi-kinase inhibitor (S. M. Wilhelm *et al.*, 2008).

As discussed earlier, characterization of the unique genetic changes associated with HCC will not only allow a more comprehensive understanding of the genetic basis of the disease but will also allow the discovery of more promising targets for therapy. In this study, our interest lies in one of the genes recently implicated as a potential player in the development and progression of several cancers, known as COBRA1.

1.2 Cofactor of BRCA1

Cofactor of BRCA1 (COBRA1) was originally isolated from a human ovary cDNA library. It encodes a 580-amino acid leucine-enriched protein with 3 repeats of the LXXLL motif (L-leucine, X-any amino acid). This motif, commonly present in transcription coactivators, is responsible for mediating ligand-dependent interactions with nuclear receptors (NRs) (Ye *et al.*, 2001). The importance of COBRA1 was later established through *in vivo* studies, where a general knockout of the murine *Cobra1* gene was shown to be lethal in the embryo. In addition, no COBRA1^{-/-} embryos appeared to develop beyond embryonic day 5, indicating an important role for COBRA1 in early embryogenesis (Amleh *et al.*, 2009).

COBRA1 was initially identified as a novel BRCA1 interacting protein through a yeast two-hybrid screen (Ye *et al.*, 2001). It was not until later that Narita *et al.* (2003) identified COBRA1 as the same protein as NELF-B, one of the 4 subunits that make up the human negative elongation factor (NELF) complex. NELF is a well-characterized complex involved in the stalling of RNAPII during transcription elongation and hence, responsible for the regulation of a significant number of genes involved in cell cycle control, proliferation, metabolism as well as cellular responses to stimuli and stress (Sun & Li, 2010).

In eukaryotes, COBRA1's role in the regulation of gene expression is not limited by any means to its interaction with BRCA1 and NELF. With no DNA binding domain, COBRA1's ability to regulate genes occurs via its interaction with other site-specific transcription factors. In fact, it was shown to act as a cofactor for another complex known as Activator Protein-1 (AP-1), to regulate AP-1's transcriptional activity of downstream targets (Zhong *et al.*, 2004). Given the major role the AP-1 signaling pathway plays in determining cellular fate, it is postulated that COBRA1 will have a similar contribution to those cellular decisions.

In 2004, Aiyar *et al.* demonstrated a novel role for COBRA1 as a transcriptional corepressor in the repression of hormone-responsive transcription. It was shown to directly bind to estrogen receptor-alpha (ER α) and repress ER α -mediated gene transcription (Aiyar *et al.*, 2004). Given the positive role ER α -mediated signaling has on the development of breast cancer (Foster, Henley, Ahamed, & Wimalasena, 2001; Persson, 2000), COBRA1's physiological role in suppressing the estrogen-dependent growth of breast cancer cells was illustrated and established (Aiyar *et al.*, 2004). In agreement, lack of COBRA1 in breast cancer was later demonstrated a poor predictor of prognosis (Sun *et al.*, 2008).

In addition to ER α , COBRA1 was also reported to bind with various degrees of affinity to other nuclear receptors such as the progesterone receptor B (PRB) and the glucocorticoid

receptor (GR). In particular, COBRA1 was demonstrated to strongly bind to the androgen receptor (AR) & repress AR-mediated transcription (Sun, Blair, Aiyar, & Li, 2007). Similar to the ER α -signaling pathway, AR-mediated signaling is also well established in mediating the development and/or progression of different malignancies such as prostate cancer and HCC (Lonergan & Tindall, 2011; Ma, Lai, Yeh, Cai, & Chang, 2014). However, the role that COBRA1 or NELF-B plays in these different malignancies has not been yet studied and remains unknown.

Even though COBRA1 has been extensively studied over the years, in the field of cancer, the role of COBRA1 is not yet fully understood. In this study, our interest lies in unraveling the role of COBRA1 in HCC. A summary of current knowledge of COBRA1 however will be first reviewed below.

1.2.1 Breast Cancer Type 1 Susceptibility Protein (BRCA1)

BRCA1 is the first breast and ovarian cancer susceptibility gene to be identified. Germ line mutations in BRCA1 predispose patients to familial breast and ovarian cancers and are responsible for 50% of all familial early onset breast cancers, which account for ~2-5% of all breast cancers (Couch & Weber, 1996; Ye *et al.*, 2001). It represents one of the most recognized tumor suppressors and is currently widely used to diagnose the likelihood of patients to develop breast and ovarian cancer.

Being a predominantly nuclear protein, BRCA1 is normally involved in multiple nuclear functions, including the regulation of transcription, recombination, checkpoint control and DNA repair. These functions are mediated via its ability to induce large-scale chromatin unfolding (Ye *et al.*, 2001). In an attempt to identify cofactors recruited by BRCA1 to induce chromatin unfolding, Ye *et al.* (2001) employed a yeast two-hybrid screen and discovered a novel protein that was named cofactor of BRCA1 (COBRA1). Surprisingly, COBRA1 was reported sufficient for the induction of large-scale chromatin decondensation in the absence of BRCA1 (Ye *et al.*, 2001).

In a gene expression profiling study performed in breast cancer, a significant overlap was uncovered in the genes that are regulated by COBRA1 or BRCA1. Both have been shown to share common downstream target genes, many of which have been implicated in breast cancer progression (Aiyar, Cho, Lee, & Li, 2007). In agreement, similar to BRCA1's recognized role as a tumor suppressor in breast cancer, several lines of evidence have been reported that highlight a role for COBRA1 as a tumor suppressor in breast cancer as well (Sun *et al.*, 2008). However, this will be discussed later.

1.2.2 Negative Elongation Factor Complex (NELF)

Shortly after its initial discovery in 2001, COBRA1 was identified by Narita *et al.* (2003) as the same protein as NELF-B, one of the 4 subunits that make up the NELF complex. NELF is a complex that is activated with the assembly of its 4 subunits; NELF-A, NELF-B (or COBRA1), NELF-C/D and NELF-E. Upon assembly and activation, the complex is recruited together with DRB sensitivity-inducing factor (DSIF) to induce the stalling of RNA polymerase II (RNAPII) early during transcription elongation. It is believed that NELF recruitment occurs immediately after transcription initiation, stalling the elongation of the RNA transcript in a process referred to as promoter proximal pausing. This is characterized by the association of NELF with a paused Pol II complex 30–50 bps downstream of the transcription start site (Yamaguchi *et al.*, 1999).

NELF is a 4-subunit complex; NELF-A (66 kDa), NELF-B (COBRA1; 62 kDa), NELF-C (60 kDa) or NELF-D (59 kDa) and NELF-E (46 kDa). The NELF-C and NELF-D subunits represent translation variants from a common mRNA through the alternative use of translation initiation codons. However, either NELF-C or NELF-D will be present in a NELF complex at any one point. NELF-C/D together with COBRA1 (NELF-B) form the core of the NELF complex, bridging NELF-A and NELF-E. The NELF-A subunit contains the RNAPII binding domain, through which the NELF complex is able to bind to RNAPII. NELF-E is the smallest subunit and contains a conserved RNA binding domain, through which the NELF complex is able to bind to nascent RNA transcripts (Narita *et al.*, 2003).

It is important to mention that although NELF-A and NELF-E are the binding domains by which the NELF complex will bind to RNAPII and RNA, respectively, all of the four subunits including COBRA1 (NELF-B) and NELF-C/D are necessary for the assembly and function of the complex (Narita *et al.*, 2003). In fact this explains the well-established interdependent nature of the NELF subunits, whereby knockdown of any NELF subunit results in the simultaneous co-depletion of the remaining subunits (Narita *et al.*, 2007; Sun & Li, 2010; Sun *et al.*, 2008).

There are three major steps to transcription: initiation, elongation and termination, each of which is now known to be tightly controlled. Originally, the transcriptional regulation of eukaryotic genes was believed to occur almost exclusively at the initiation phase of transcription. Hence, it had been the focus of much research and is why it is considered to be the most understood phase of transcription. However, recent findings have revealed that the elongation step of transcription is also a very highly regulated step and can actually

be rate-limiting in the regulation of the expression of many genes in eukaryotic cells (J.Sims III, Belotserkovskaya, & Reinberg, 2004).

The elongation step is regulated both during the early steps of elongation as well as during the phase of productive elongation. During early elongation, just before the RNAPII transitions to productive elongation, RNAPII can pause at the promoter proximal region, 30–50 base pairs (bps) downstream of the transcription start site in a process referred to as promoter proximal pausing. Contrary to transcriptional termination or arrest, promoter proximal pausing is reversible and represents one of the important mechanisms by which transcription can be regulated at the elongation phase (Adelman & Lis, 2012; J.Sims III *et al.*, 2004; Jonkers & Lis, 2015).

Even though, the process of RNAPII stalling was well established, it was reported only at the promoters of very few genes such as Hsp70 (Wu *et al.*, 2003), estrogen-responsive genes in breast cancer (Aiyar *et al.*, 2004) and mammalian proto-oncogene *junB* (Aida *et al.*, 2006), leading many to view it as a rare phenomenon. It was not until later that genome-wide studies in *Drosophila* demonstrated the association of RNAPII with the promoters of thousands of genes with either low levels of transcription or none at all, in an indication that the process of RNAPII stalling is more widespread than originally anticipated (Muse *et al.*, 2007; Zeitlinger, Stark, Kellis, & Hong, 2007). Now, the regulation of RNAPII pausing during transcription elongation is believed to be cornerstone in the repression of a large number of genes in a variety of biological systems (Gilchrist *et al.*, 2008).

Surprisingly, stalled RNAPII was found associated in the promoter regions of ~50% of the most highly expressed genes in *Drosophila*, demonstrating the capability of promoter proximal pausing of enhancing gene expression rather than only repressing it (Gilchrist *et al.*, 2008; Lee *et al.*, 2008). Even though this was the first time the NELF-mediated RNAPII stalling event was shown to have a positive effect on activating gene expression, a previous microarray study reported the downregulation of a significant number of genes upon NELF-depletion (Narita *et al.*, 2007). However, it was not until later that this phenomenon was understood and linked directly to the NELF-mediated RNAPII stalling event. In fact, it is now even believed that the stalling process of RNAPII might enhance gene expression by maintaining a permissive chromatin architecture at the promoter-proximal region of those genes (Gilchrist *et al.*, 2008). This was again further demonstrated in yet another study whereby short interfering RNA (siRNA)-mediated knockdown of NELF lead to the downregulation of the vast majority of NELF-regulated genes, most of which are involved in cell cycle progression (Sun & Li, 2010). Taken together, this indicated the importance of NELF

in maintaining the transcription of its target genes and challenged the prior view of RNAPII stalling being a strictly transcriptional repressor.

1.2.3 Nuclear Receptors

Upon binding of estrogen to ER α , the receptor undergoes conformational changes and becomes activated. An activated ER α will then bind to estrogen-responsive elements present in the promoters of estrogen-responsive genes to enhance their transcription. ER α represents one of the mechanisms by which estrogen is able to mediate its widespread biological effects, under both physiological and pathological conditions (Aiyar *et al.*, 2004).

Different mechanisms are normally in place in order to allow for such a process to be tightly regulated and readily reversible. In 2004, Aiyar *et al.* demonstrated a novel role for COBRA1 as a transcriptional corepressor in the repression of estrogen-responsive transcription. In response to estrogen, COBRA1 is recruited along with the rest of the NELF complex, to the promoters of a subset of estrogen-responsive genes where it acts to stall RNAPII and repress ER α -mediated transcription. Given the positive role ER α -mediated signaling has on the development of breast cancer (Foster *et al.*, 2001; Persson, 2000), a physiological role for COBRA1 as an inhibitor of estrogen-dependent growth of breast cancer cells was established (Aiyar *et al.*, 2004).

In addition to ER α , COBRA1 was also shown to bind with various degrees of affinity to other NRs such as PRB and GR. In particular, COBRA1 was reported to bind strongly to the AR and repress AR-mediated transcription. Of the NRs examined in the study (GR, PR and AR), AR was shown to exhibit the strongest affinity for COBRA1 and thus, the most susceptible to COBRA1-mediated repression (Sun *et al.*, 2007). COBRA1's interaction with a number of nuclear receptors goes in agreement with its previously characterized protein structure, which contains multiple copies of NR-binding "LXXLL" motifs (Ye *et al.*, 2001).

1.2.4 AP-1 Complex

The biological roles of COBRA1 is yet to be fully elucidated. With no DNA binding domain of its own, its ability to regulate genes is mediated via its interaction with different DNA transcription factors, as seen with both BRCA1 and the NELF complex. COBRA1 was shown to interact with yet another widely recognized transcription factor, known as AP-1 (Zhong *et al.*, 2004). AP-1 is a complex that regulates the expression of a large number of genes; genes involved in mediating cellular responses to stimuli, including growth factors, cytokines, stress, bacterial and viral infections as well as oncogenic stimuli (Hess, Angel,

& Schorpp-Kistner, 2004). The AP-1 signaling pathway, while essential for cell survival, extensive literature exists to support its role in inducing apoptosis. The role of AP-1 in determining cellular fate (life or death) appears to largely depend on cellular context. Its opposing nature on cell fate has interestingly triggered some to refer to it as a “nuclear decision-maker” (Ameyar, Wisniewska, & Weitzman, 2003).

COBRA1 was shown to act as an AP-1 cofactor, where it was shown to physically bind to AP-1 family members and inhibit AP-1 transcriptional activity of downstream targets. It has been proposed that COBRA1 may utilize its ability to recruit the NELF complex in order to attenuate AP-1 transcriptional activity; however, this has not been shown and renders further investigation. Given COBRA1’s ability in affecting the AP-1 pathway, COBRA1 has also been proposed to have an identical role in determining cell fate decisions, such as proliferation, apoptosis and differentiation (Zhong *et al.*, 2004).

1.3 COBRA1 in Cancer

The pathological role of NELF-B or COBRA1 in cancer is not yet fully understood and is of current interest. To date, it has only been studied in breast and gastrointestinal cancer.

In breast cancer, several lines of evidence highlight COBRA1 as a tumor suppressor with a major role in the tumorigenicity of the cancer. In a study by Sun *et al.* (2008), COBRA1 expression was demonstrated lower in breast cancer when compared to normal mammary epithelium. In addition, the expression of COBRA1 mRNA was also found to be inversely correlated with breast cancer progression, with significantly lower expression in patients with distant metastasis and local recurrence (Sun *et al.*, 2008).

COBRA1-regulated genes were found to enrich pathways involved in cell cycle control, metabolism, cell proliferation and DNA repair. In addition, a significant number of these identified genes had wide implications in cancer. The direction of COBRA1’s regulation of these genes was found to support its role in suppressing breast cancer development (Aiyar, Blair, Hopkinson, Bekiranov, & Li, 2007; Aiyar, Cho, et al., 2007). This goes in line with the previously reported role of COBRA1 in the proliferation and tumorigenesis of breast cancer. In that study, the authors demonstrated a significant reduction in the rate of proliferation of T47D cells upon the ectopic expression of COBRA1 (Aiyar *et al.*, 2004). Similarly, siRNA knockdown of COBRA1 resulted in an increase in cellular proliferation as assessed by both two- and three-dimensional tissue culture systems. Surprisingly however, the increase in cellular proliferation was evident only when exogenous estrogen was added to the culture media, indicating thus a physiological role for COBRA1 in repressing the estrogen-dependent growth of breast cancer cells (Aiyar *et al.*, 2004). The absence of estrogen

might explain why COBRA1 knockdown in a later study by Sun & Li (2010) was shown to have no effect on the cell proliferation of T47D. In agreement, subsequent findings associated the low expression of COBRA1 with metastasis and recurrence in breast cancer (Sun *et al.*, 2008).

In contrast to breast cancer, significant overexpression of COBRA1 was reported in 79% of upper gastrointestinal cancers (UGCs), suggesting COBRA1 as a novel oncogene in UGC. Interestingly, COBRA1 overexpression was found associated with an attenuated expression of trefoil factor 1 (TFF1) (McChesney *et al.*, 2006) – a well-established UGC tumor suppressor (Im *et al.*, 2012; Tanaka *et al.*, 2013). TFF1 is a member of the trefoil factor family of secreted peptides that is normally expressed and secreted in the upper gastrointestinal tract, with several protective and healing roles (Emami *et al.*, 2004). While COBRA1 knockdown led to an increase in TFF1 expression, overexpression of COBRA1 had an opposite effect, thus highlighting the physiologic role of COBRA1 as an inhibitor of TFF1 expression in UGC (McChesney *et al.*, 2006). This evidence further supported the oncogenic role for COBRA1 in UGCs.

Even though COBRA1 has only been studied in breast and gastrointestinal cancer only, findings seem to highlight a cancer type-dependent role for COBRA1. It is also important to mention that COBRA1 was recently identified in a proteomics study in lung cancer as a novel target of the oncogenic Ras/MAPK signaling pathway (Sudhir *et al.*, 2011). This was consistent with prior findings reported by Pohl *et al.* (2005), where COBRA1 was one of the major downregulated genes upon the inactivation of the MAPK pathway in ovarian cancer. Interestingly, this was accompanied by a profound decrease in the growth of the ovarian cancer cells. Taken together, these data suggest an important role for COBRA1 in cancer.

1.4 COBRA1 in Hepatocellular Carcinoma

In hepatocellular carcinoma, the role of COBRA1 remains unclear. To date, only one study has analyzed the expression pattern of COBRA1 in HCC. This study explored the differential expression of COBRA1 at both the RNA and protein level in HCC tumor samples relative to noncancerous peri-tumor tissue from the same patient. Samples were collected from Egyptian patients undergoing either surgical resection or transplantation from different hospitals based in Cairo and an upregulated expression pattern was reported implicating COBRA1 as an oncogene in HCC (Kamel, 2012). However, the biological role that COBRA1 plays in the progression of HCC still remains unclear.

HYPOTHESIS AND SPECIFIC OBJECTIVES

As a functional component of major transcriptional complexes and by acting as a cofactor for several nuclear receptors, COBRA1 can regulate a substantial number of genes (Aiyar, Blair, *et al.*, 2007; Aiyar, Cho, *et al.*, 2007). Based on previous preliminary data from our lab implicating COBRA1 as a potential oncogene in HCC (Kamel, 2012), a deregulation in the expression pattern of COBRA1 will imply aberrant expression of all its regulated genes. Hence, our hypothesis for a role for COBRA1 in the pathogenesis of HCC. To address this hypothesis, our study had three main objectives.

1. To expand on the lab's prior findings and further explore on a larger scale the expression pattern of COBRA1 in HCC expression array data available online.
2. To optimize and establish a siRNA-mediated COBRA1 knockdown in the well differentiated HCC cell line, HepG2. The HepG2 cell line is a non-tumorigenic cell line derived from a 15-year old Caucasian, American male with early stage HCC. The cells have a characteristic cobblestone-like, epithelial appearance with no detectable HBV surface antigens (Costantini, Di Bernardo, Cammarota, Castello, & Colonna, 2013).
3. To analyze the effect of COBRA1 silencing on cellular proliferation and migration.

CHAPTER 2. MATERIALS AND METHODS

2.1. Cell lines and Culture

The well-differentiated human hepatocellular carcinoma cell line, HepG2, was a generous gift from Dr. Mehmet Ozturk from the Department of Molecular Biology and Genetics, Bilkent University, Turkey. Cells in the logarithmic phase of growth from passage numbers 6-20 were used in all experiments described below. They were routinely cultured in complete media composed of RPMI 1640 (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA), 100 units/ml penicillin and 100 mg/ml streptomycin (Invitrogen, USA). Cells were cultured in a humidified incubator at 37°C and 5% CO₂ and propagated at 70-80% confluence according to the American Type Culture Collection protocol. Cells were observed using an inverted microscope (Olympus IX70, USA).

2.2. Viable Cell Count

The viable cell count was performed using the trypan blue exclusion method. The cell suspension was mixed well by repeated pipetting to disperse any cell clumps. In a 1.5 ml Eppendorf tube, a fifty µl aliquot of this cell suspension was mixed with 50 µl 0.4% trypan blue in phosphate-buffered saline (PBS). Ten microliters (µls) from this suspension was then loaded into each chamber of a hemocytometer (Hausser Scientific, USA). The number of cells in each of the four outer squares in the two chambers were counted. To calculate the number of cells per ml, the following equation was used:

Number of cells /ml = Total no. of cells / total no. of squares x dilution factor x 10,000.

2.3. RNA Interference

To achieve knockdown of COBRA1 (NCBI Genebank: NM_015456), a pool of 4 different siRNAs targeting different regions of the COBRA1 mRNA (siGENOME SMARTPool; M-015839-00) was used, which was purchased from Dharmacon. Target sequences and exons of the COBRA1 siRNAs are shown in Table 1. Positive Control GAPDH siRNA (SI02653266) and AllStars Negative Control siRNA (SI03650318) were purchased from Qiagen. All siRNAs were resuspended in RNase-free water to a final concentration of 20 µM, according to the manufacturer's instruction. Positive Control GAPDH siRNA was used to ensure the efficiency of the transfection procedure in achieving siRNA delivery to the cells. AllStars Negative Control siRNA is a validated and tested siRNA with no homology to any known mammalian gene ("AllStars Negative Control siRNA - QIAGEN - AllStars Negative Control siRNA," n.d.), that was used to control for any changes induced by the siRNA transfection procedure itself.

2.4 siRNA Transfection

Reverse transfection of COBRA1 siRNA into HepG2 cells was carried out in 6-well plates using Lipofectamine RNAiMAX (Life Technologies). An RNase-free environment was maintained at all times. As controls, cells were either left untreated (blank) or treated with Lipofectamine only (mock). The optimized transfection conditions mentioned below were maintained in all experiments. Approximately 2.5×10^5 cells were reverse transfected with 25 nM of siRNA using 5 μ l of Lipofectamine RNAiMAX according to the manufacturer's protocol in a final volume of 2 ml. In a 1.5 ml Eppendorf tube, 2.5 μ l of the siRNA (20 μ M) was mixed with 500 μ l of serum-free DMEM. After 5 minutes (mins) incubation at room temperature (RT), 5 μ l of Lipofectamine was added and then left for another 10-20 mins to allow for complexation. The siRNA-Lipofectamine mix was then transferred to one of the wells of a 6-well plate, after which 2.5×10^5 cells were added in 1.5 ml complete RPMI media without antibiotics. Growth media was replenished 24 h after transfection. Cells were cultured for 72 hours (hrs) after which they were harvested for RNA and protein analysis. Knockdown efficiency was analyzed using Western blot analysis.

Table 1. Target sequences and the corresponding exon locations of COBRA1 siRNAs (NCBI Genebank: NM_015456) used in the study.

siRNA Identifier		siRNA Target Sequence (5'-3')	Target Exon
siGENOME SMARTPool (M-015839-00)	1	CCGAAAGCUUCACUAAGUU	9 & 10
	2	GCGACUUGGCCUUUGGCGA	11
	3	GAGCCUGGGACAUGAUCGA	8
	4	CGUCUAAGCUGGAGGCGUU	12

2.5. RNA Extraction

Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. An RNase-free environment was maintained at all times. Extracted RNA was dissolved in diethylpyrocarbonate-treated (DEPC) water. The quantity and quality of the RNA were verified by measuring the absorbance at both 260 and 280 nm using a UV spectrophotometer (Shimadzu, Japan).

2.6. Primer Design

All used forward and reverse primers are located on two different exons. The sequences of these primers as well as their amplicon size and PCR conditions are listed in Table 2.

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

Gene Name	Primer Sequence (5'-3')	Amplicon size (bp)	PCR conditions
B-ACTIN	F: GCAAAGACCTGTACGCCAAC R: GAGACCAAAAGCCTTCATACATCTC	777	58°C 27 cycles
COBRA1	F: ACATCACCAAGCAGAGGAA R: GATCCAGCTGTTCCAGCTTC	366	59.5°C 32 cycles
Survivin	F: TTGAATCGCGGGACCCGTTGG R: CAGAGGCCTCAATCCATGGCA	Isoform 1: 477 Isoform 2: 359 Isoform 3: 546	61°C 27 cycles
NELF-A	F: GTCGGCAGTGAAGCTCAAGT R: TTCACACTCACCCACCTTTTCT	250	60°C 35 cycles
NELF-C/D	F: GAAGAAGGAGAGACCCCAGC R: GTGCCCAAGGCTAGTGTGAT	443	56°C 28 cycles
NELF-E	F: TGGTGAAGTCAGGAGCCATCAG R: CGCCGTTCCAGGGAATGAATC	565	63°C 28 cycles
Ki-67	F: CTTTGGGTGCGACTTGACG R: GTCGACCCCGCTCCTTTT	199	60°C 28 cycles

2.7. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Analysis of differential gene expression was performed using semi-quantitative RT-PCR. Total RNA (1 µg) was reverse transcribed using random primers in a final volume of 20 µl, using a RevertAid First Strand cDNA synthesis Kit (Thermo Scientific, USA) according to the manufacturer's protocol.

The PCR amplification was performed in a final volume of 25 µl, consisting of 1 µl cDNA template, 2.5 µl 10X DreamTaq Green Buffer (includes 20 mM MgCl₂) (Thermo Scientific), 0.5 µl dNTP mix (10 mM each) (Thermo Scientific), 0.25 µl DreamTaq DNA Polymerase (Thermo Scientific) and 0.75 µl of each primer (forward and reverse). B-ACTIN was used as the internal control. PCR conditions were the same for all except for annealing temperatures and cycle numbers, which are mentioned in Table 2: 95°C for 5 mins, followed by cycles of (95°C for 30 secs, annealing temp for 30 secs and 72°C for 45 secs), before a final extension at 72°C for 7 mins. The amplified PCR products were run on 1.5-2% agarose gel according to the size of the PCR amplicon and then visualized using Gel Doc EZ System (Bio-Rad, USA).

2.8. Immunoblotting

Unless stated otherwise, all steps were performed at 4°C. Cells were rinsed with ice cold PBS and lysed by repeating pipetting in ice-cold Laemmli Lysis Buffer (50mM Tris pH6.8,

2% sodium dodecyl sulfate (SDS), and 10% glycerol), supplemented with 1X Halt Protease Inhibitor Cocktail (ThermoScientific, USA). Samples were incubated at 4°C for 60 min with shaking followed by centrifugation at 12,000 rpm at 4°C for 20 minutes. The protein-containing supernatant was then collected and quantified using BCA Protein Assay Kit (Pierce Biotechnology, USA) according to the manufacturer's protocol.

Equal amounts of whole cell lysates (20-50 µg) were mixed with 6X SDS-Laemmli loading dye (60% Glycerol, 360 mM Tris-HCl pH 6.8, 12% SDS, 0.06% bromophenol blue, 30% beta-mercaptoethanol) in a final volume of 24 µl. Samples were boiled at 99°C for 10 mins before loading them onto a 12% SDS–polyacrylamide gel. The protein samples were then run at 120V along with a pre-stained protein ladder until the tracking dye ran out. Resolved proteins were transferred from the gel onto a nitrocellulose membrane (GE Healthcare) using a wet transfer apparatus (120 V, 90 mins). The membrane was blocked for 1 hr using 5% non-fat dry milk in 1X PBST (0.01% Tween-20 in PBS), then incubated with a primary antibody overnight at 4°C. After 3 washings in 1X PBST (5 mins each), the membrane was incubated 1 hr at RT with an alkaline phosphatase conjugated secondary antibody (either goat anti-rabbit IgG (KPL) or goat anti-mouse (KPL)) at 1:10,000 dilution in 5% non-fat dry milk in 1X PBST. After 3 washes in 1X PBST (5 mins each) and two in 0.2 M Tris base (pH 9-9.5), the membrane was incubated with the chemiluminescent PhosphoGLO Substrate (55-60-04, KPL) for 1 min and then exposed to a film that was manually processed. Films were then visualized using a Gel Doc EZ System (Bio-Rad, USA). Primary antibodies used in this study were as follows; anti-B-TUBULIN (Sigma, T7816) (1:20,000 in 5% non-fat dry milk), anti-COBRA1 (AbCam, ab167401) (1:1000 in 5% non-fat dry milk).

To incubate the same membrane with another primary antibody, after detection membranes were stripped using a homemade harsh stripping buffer (0.5 M Tris-HCl, 10% SDS and beta-mercaptoethanol). The membrane was incubated in the buffer for 15 minutes at 65°C after which it was washed thoroughly with water to remove any traces of beta-mercaptoethanol and re-blocked with 5% nonfat dry milk in 1X PBST before incubating it with another primary antibody.

2.9. Scratch Wound Healing Assay

Differences in cell migration were assessed using a classical scratch wound healing assay as previously described (Qin & Cheng, 2010; Yu et al., 2014). Twenty-four hours post-transfection, cells were reseeded in 24-well plates. At 72 hr post-transfection, the cell monolayer was carefully scraped using a 20 µl pipette tip, once horizontally and once vertically to create a cross in the center of the well. The cells were then washed with PBS to remove any floating cells and then incubated for another 24 hrs. The migration of the

cells was monitored with an inverted microscope. Images were taken with phase contrast at 10X magnification power at the start (0 hr) and at the end (24 hr) of the incubation. Three different wound locations were chosen per well. The wound area was analyzed using the TScratch software (Gebäck, Schulz, Koumoutsakos, & Detmar, 2009) and the percentage wound closure was then calculated as follows:

$$\text{Percentage wound closure} = [(\text{wound area}^{24 \text{ hr}}/\text{wound area}^{0 \text{ hr}})/ \text{wound area}^{24 \text{ hr}}] \times 100$$

2.10. Data Analysis

Densitometric analysis was performed using ImageJ Software (National Institute of Health, USA, <http://www.imagej.nih.gov/ij>). For both the PCR and Western Blot, quantified band intensities were normalized to the loading control B-ACTIN or B-TUBULIN, respectively. Relative changes in gene expression are expressed as fold change from the negative siRNA-transfected cells (control), unless specified otherwise.

Graphical representations and statistical analyses were performed using GraphPad Prism 5.0 (GraphPad, San Diego California USA, <http://www.graphpad.com/>). All values represent the mean \pm standard deviation (SD) from three independent experiments, unless specified otherwise. For pairwise comparisons, statistical significance was assessed using an unpaired student's t-test (two-tailed). One-way ANOVA, followed by a Bonferroni post-test was used to analyze the difference between multiple experimental groups in case of a single variable, while a two-way ANOVA was used in cases of two variables. A *p*-value less than 0.05 was considered significant (**p* < 0.05; ***p* value < 0.01; ****p* value < 0.001).

2.11. Data Mining

The Oncomine cancer microarray database (<http://www.oncomine.org/>, last accessed July 18, 2015) (Rhodes *et al.*, 2004) was used to query the expression profile of COBRA1 mRNA in HCC tissues versus their normal counterparts. Statistical significance was automatically computed by the default Oncomine algorithms using a two-tailed Student's t-test. HCC microarray datasets utilized in this study are summarized in Table 3.

Table 3. Oncomine microarray datasets used in this study.

Study	Sample Type	No. of Patients (n)	Patients /Sample	Year of Study	Reference
Mas Liver	Normal Liver Cirrhotic Hepatocellular Carcinoma	115	19 58 38	2009	(Mas <i>et al.</i> , 2009)
Roessler Liver	Paired Non-Tumor Hepatocellular Carcinoma	43	21 22	2010	(Roessler <i>et al.</i> , 2010)
Roessler Liver 2	Paired Non-Tumor Hepatocellular Carcinoma	445	220 225	2010	(Roessler <i>et al.</i> , 2010)
Wurmbach Liver	Normal Liver Cirrhotic Liver Cell Dysplasia Hepatocellular Carcinoma	75	10 13 17 35	2007	(Wurmbach <i>et al.</i> , 2007)

CHAPTER 3. RESULTS

3.1. Expression of COBRA1 mRNA in human hepatocellular carcinoma tissues relative to normal liver tissue

All publicly available HCC microarray data in the OncoPrint® Cancer Microarray database (<http://www.oncoPrint.org/>) were analyzed in order to explore the differential expression of COBRA1 at the RNA level. A query search on the differential expression of COBRA1 in HCC tumor tissues versus normal liver tissue yielded results from 4 different datasets, from 3 independent studies (Mas *et al.*, 2009; Roessler *et al.*, 2010; Wurmbach *et al.*, 2007). In both the Mas and Wurmbach study, samples were from patients with an HCV etiology with no other known HCC etiologies or CLD causes (Mas *et al.*, 2009; Wurmbach *et al.*, 2007). However, the vast majority of the samples in the Roessler study were from patients with a history of HBV infection or HBV-related cirrhosis (Roessler *et al.*, 2010).

The analysis of these datasets revealed a significant overexpression of COBRA1 mRNA in HCC tumor tissues versus their normal tissue counterparts ($p \leq 0.05$) (Figure 1). Although 1 of the 4 datasets (Mas *et al.*, 2009) found that COBRA1 expression did not differ significantly between normal liver tissue and either cirrhotic or HCC tissue, the remaining 3 datasets (Roessler *et al.*, 2010; Wurmbach *et al.*, 2007) demonstrated otherwise. All 3 datasets show a significant overexpression of COBRA1 mRNA in HCC tumor tissues versus normal or non-tumor liver tissue. Both the Roessler Liver 2 and Liver datasets (Roessler *et al.*, 2010) show comparable results, with a fold change of 1.614 ($p=2.61E-43$) and 1.635 ($p=5.29E-6$), respectively (Figure 1). In agreement with the Roessler datasets, the Wurmbach *et al.* (2007) study shows a similar result as well with a fold change of 1.402 ($p=0.002$). In the latter, no significant differences in expression were observed between normal tissue & either cirrhotic or dysplastic tissue.

3.2. siRNA-mediated knockdown of COBRA1 in the HCC cell line, HepG2

Having noted an upregulated expression pattern for COBRA1 in HCC tumors from both our previous lab findings (Kamel, 2012) as well as from our *in silico* study (Roessler *et al.*, 2010; Wurmbach *et al.*, 2007), we analyzed the effect of siRNA-mediated knockdown of COBRA1 on the HCC cell line, HepG2. To efficiently knockdown COBRA1, the different transfection conditions (siRNA concentration, volume of the lipid-based reagent, in this case, Lipofectamine and the duration of incubation) had to be initially optimized following the supplier's recommendations, in order to achieve the highest knockdown efficiency.

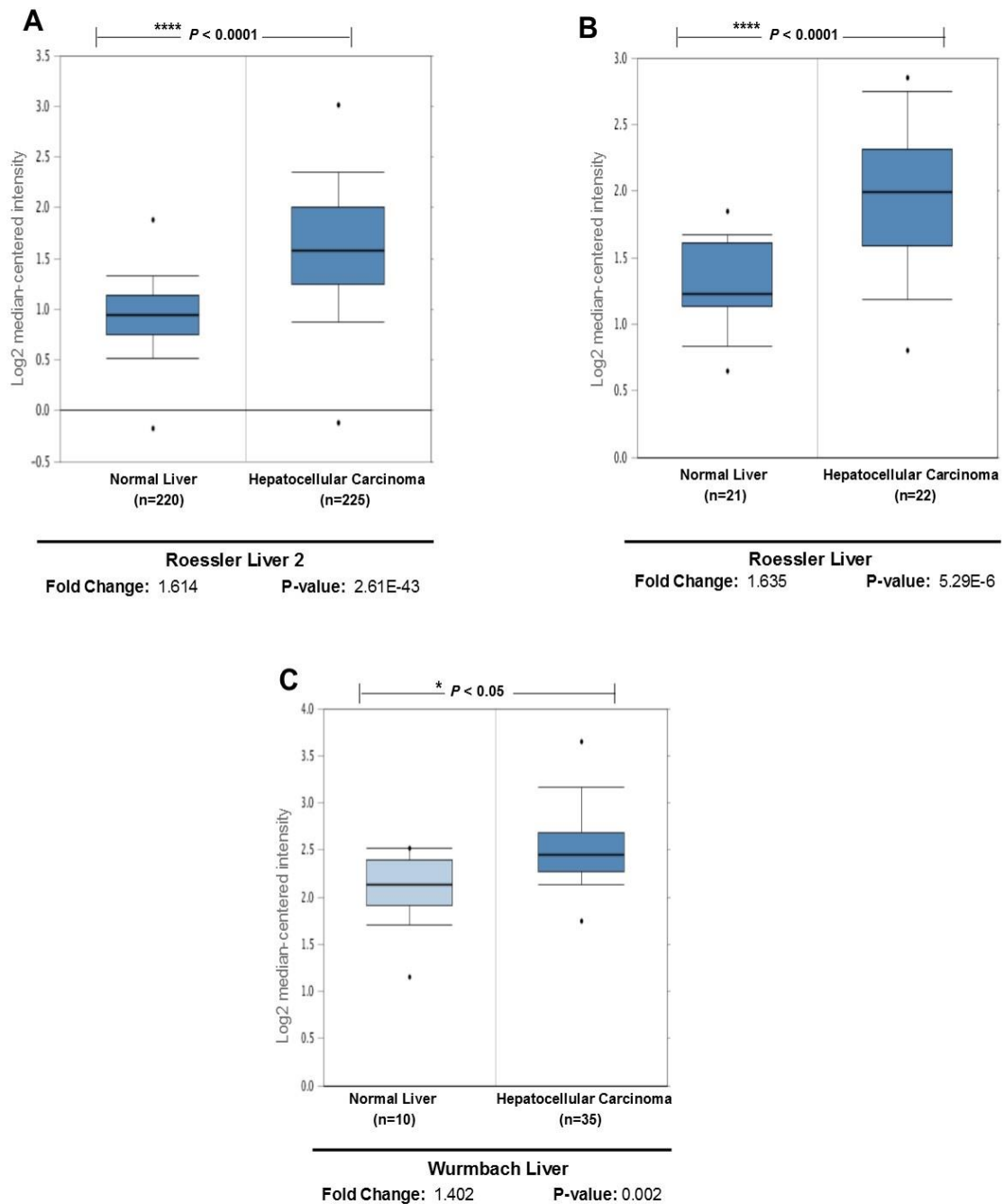


Figure 1. Overexpression of COBRA1 mRNA in hepatocellular carcinoma versus normal liver tissue The OncoPrint™ cancer database of publicly available microarray data was queried to examine the differential expression of COBRA1 in hepatocellular carcinoma versus normal liver tissue. Three datasets from 2 independent studies (Roessler *et al.*, 2010; Wurmbach *et al.*, 2007) were analyzed (A) Roessler Liver 2 dataset (B) Roessler Liver dataset (C) Wurmbach Liver dataset. OncoPrint™ results are illustrated as boxplots, with the top and bottom of the box representing the lower quartile and upper quartile, respectively and the bar across the box, the median. The bars represent the 10th and 90th percentile and dots represent the minimum and maximum values (range of data within each category). For each study, the number of patients in each category is indicated in brackets as well as the fold change and *p* value as determined by the Student's *t*-test. * $p < 0.05$; **** $p < 0.0001$.

3.2.1. siRNA concentration

The concentration of siRNA is an important transfection parameter. Concentrations that are too high can risk off-target activity, where the siRNA will not only result in the degradation of the mRNA of interest but will degrade other mRNAs that are partially homologous or even non-homologous (Semizarov *et al.*, 2003). On the other hand, siRNA concentrations that are too low will result in low knockdown efficiencies. Different concentrations across the range recommended by the supplier were tested; 25 nM, 50 nM, and 100 nM. Knockdown efficiency was analyzed 96 hr following transfection by a Western blot, and as shown in Figure 2A, all tested siRNA concentrations resulted in comparable knockdown efficiencies. Being the lowest concentration to result in an efficient protein knockdown, the 25 nM was therefore used for all subsequent siRNA transfections. It is important to mention that cell viability as observed by microscopy, was jeopardized in all siRNA and mock-treated cells. However, this was not the case in untreated cells. This is usually associated with most commonly used lipid-based transfection reagents and was addressed by optimizing the volume of Lipofectamine.

3.2.2. Incubation duration

RNAi-mediated knockdown is known for being a transient knockdown and not a stable one. To achieve maximal protein knockdown while allowing at the same time sufficient time for phenotypic changes to be visible, time points for analysis should typically range from 48-96 hrs. To identify the optimum time for analysis, knockdown efficiency was analyzed at both 72 hr and 96 hr post-transfection. As shown in Figure 2B, at both time points, knockdown efficiency was comparable showing no significant difference. Therefore, all subsequent siRNA transfections in the HepG2 cell line were performed at 72 hrs.

3.2.3. Lipofectamine volume

The volume of Lipofectamine is also an important parameter that has to be optimized. Too much will expose the cells to unnecessary stress, while too little will jeopardize transfection efficiency. Here, two different strategies recommended by the supplier were analyzed. First, the titration of the volume of Lipofectamine within the range recommended by the supplier (4-6 μ l) to determine the lowest volume of Lipofectamine required to ensure an efficient knockdown. Second, the addition of more FBS-supplemented media 6-8 hr from transfection while keeping the same established Lipofectamine volume (6 μ l). The cells transfected with 6 μ l of Lipofectamine (with or without 8 hr media addition) showed the highest knockdown, with an almost complete knockdown. Similarly, cells transfected with

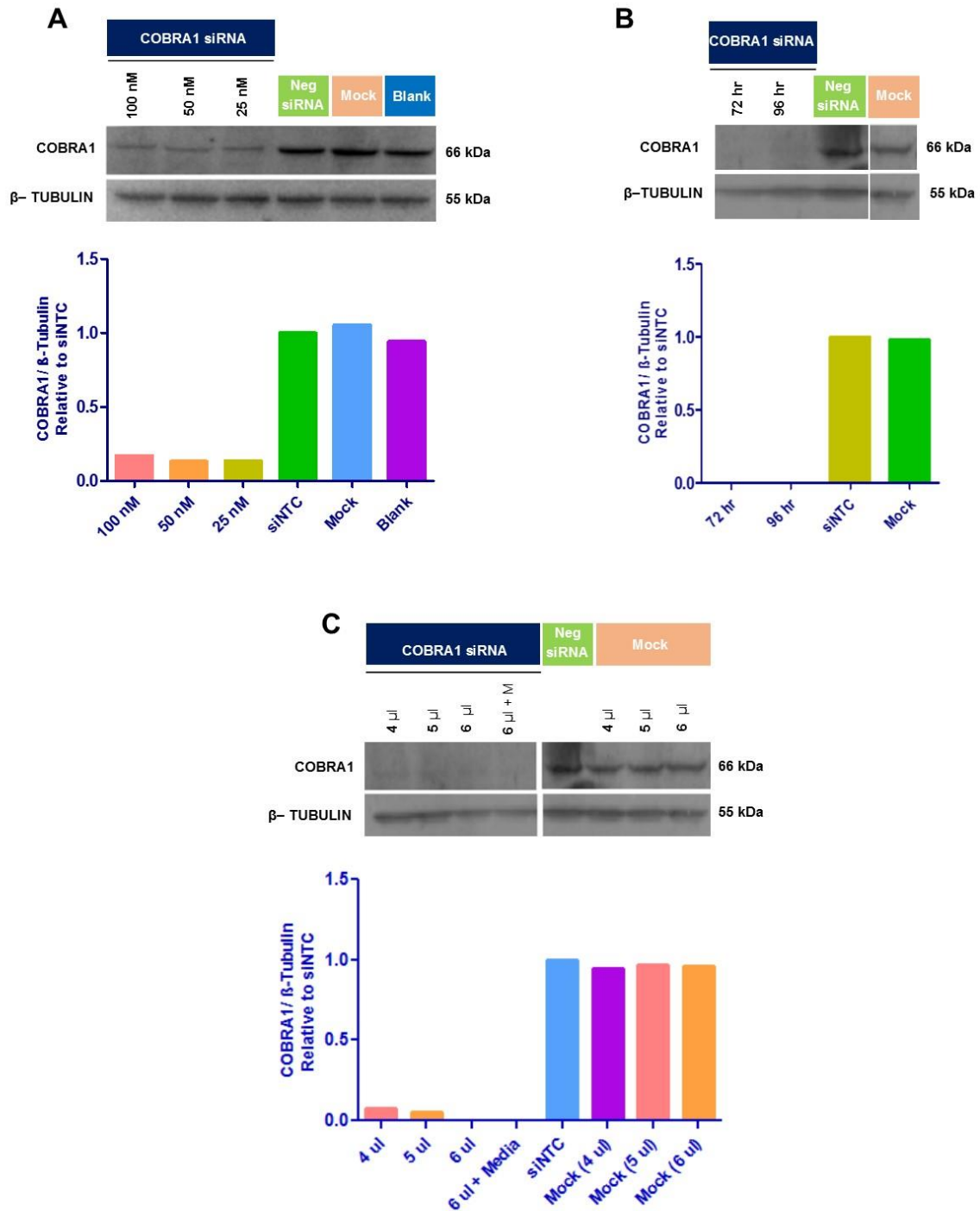


Figure 2. Optimization of transfection conditions of COBRA1 siRNA in HepG2. For each transfection parameter, different conditions were compared with respect to their knockdown efficiency, which was analyzed by Western blot analysis. Optimization of (A) *siRNA concentration*. Different concentrations (25 nM, 50 nM and 100 nM) were analyzed. For all tested concentrations, volume of Lipofectamine (6 μl) and incubation time (96 hr) were kept constant. (B) *Incubation duration*. Knockdown efficiency was analyzed at both 72 hr and 96 hr post-transfection. siRNA concentration (25 nM) and volume of Lipofectamine reagent (6 μl) were kept constant. (C) *Lipofectamine volume*. Lipofectamine was titrated within the range recommended by the supplier (4, 5, 6 μl) and compared with respect to their knockdown efficiency. In one condition, the 6 μl Lipofectamine volume was maintained and more FBS-supplemented media was added 8 hr post-transfection. The bands intensities were quantified by ImageJ and normalized to the loading control B-TUBULIN. Relative expression is expressed as fold change to the siNTC. siNTC - Negative siRNA, 6 μl + M - 6 μl plus media addition 8 hr post-transfection.

4 μ l and 5 μ l of Lipofectamine effectively decreased COBRA1 expression by 92% and 95%, respectively (Figure 2C). The previously observed cytotoxicity was no longer visible in all of the newly tested conditions. Taking into consideration knockdown efficiency as well as cell viability, the 5 μ l was chosen as the optimal volume of reagent required for transfection. All subsequent siRNA transfections were thus performed as such.

3.3. SMARTPool siRNA effectively silences COBRA1 expression in HepG2

The COBRA1 siRNA was successful in silencing COBRA1 expression, resulting on average in a 93% COBRA1 knockdown at the protein level, relative to the negative siRNA (Figure 3A). To determine whether protein knockdown correlated with mRNA knockdown, the effect of siRNA-mediated silencing of COBRA1 on the levels of COBRA1 mRNA was analyzed by semi-quantitative PCR. As shown in Figure 3B, the pool significantly silenced COBRA1 expression at the RNA level, which is consistent with the knockdown observed at the protein level. No significant difference was found between the different control groups (siNTC, mock-treated and untreated cells). It is important to mention that knockdown of COBRA1 had no significant effect on the overall patterns of cell morphology, as observed under the inverted microscope (40X) (Figure 4). To investigate whether COBRA1 silencing affects the expression of the other NELF subunits as well, the mRNA expression patterns of NELF-A, NELF-C/D, and NELF-E subunits were compared prior and after COBRA1 knockdown. Interestingly, COBRA1 knockdown did not significantly affect their expression ($p > 0.05$) (Figure 5).

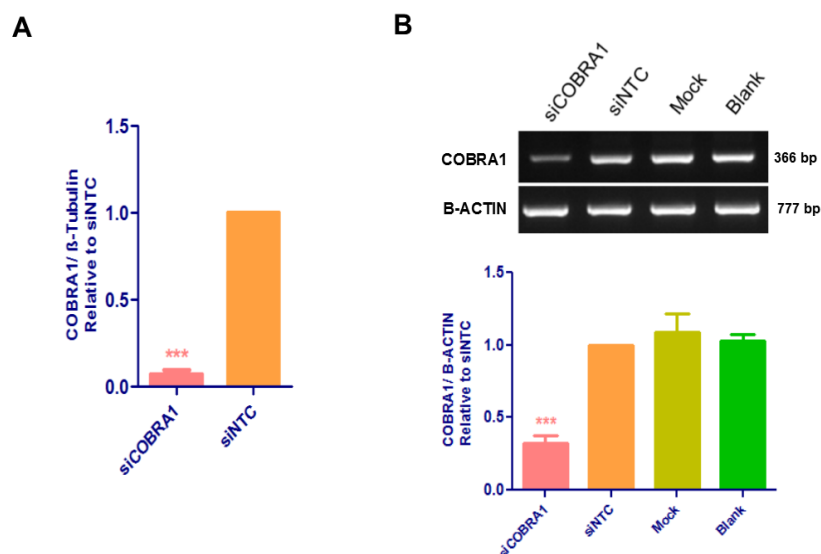
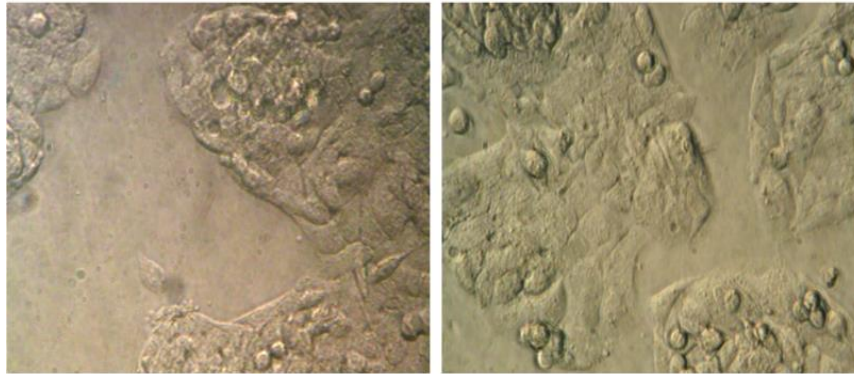
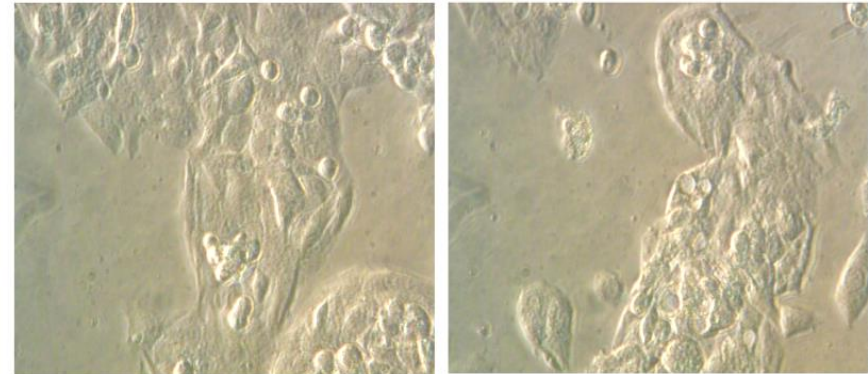


Figure 3. COBRA1 siRNA effectively silences COBRA1 expression at both the RNA and protein level. (A) Knockdown efficiency of COBRA1 siRNA in silencing COBRA1 protein levels, relative to the negative siRNA (B) RNA expression was analyzed by semi-quantitative RT-PCR. The band intensities were quantified by ImageJ and normalized to the internal control B-ACTIN. Relative expression is expressed as fold change to siNTC. Data represents the mean \pm SD of three independent experiments ($n = 3$). Statistically significant at *** $p < 0.001$. siCOBRA1 - COBRA1 siRNA, siNTC - Negative siRNA.

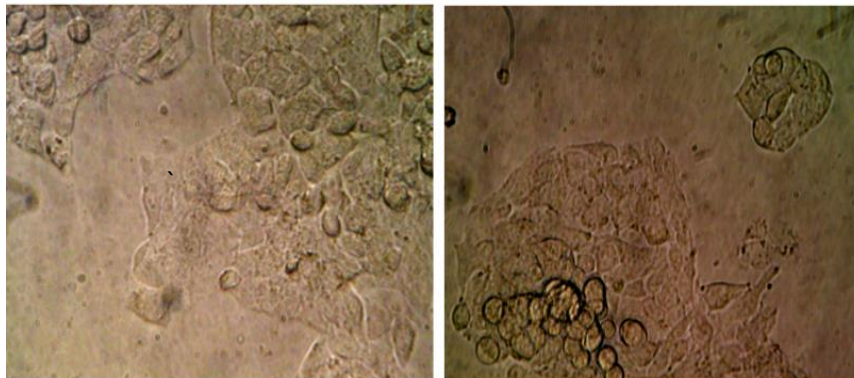
COBRA1 siRNA



Negative siRNA



Blank



Mock

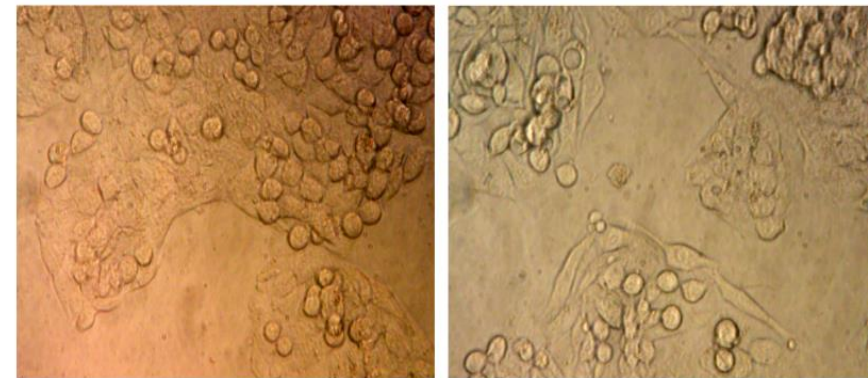


Figure 4. No significant morphology changes associated with COBRA1 knockdown observed in HepG2 cells (40X). Photos were taken at 40X magnification power 72 hr post-transfection for cells transfected with either COBRA1 siRNA or Negative siRNA. Cells that were either left untreated or mock-treated with Lipofectamine only were used as controls.

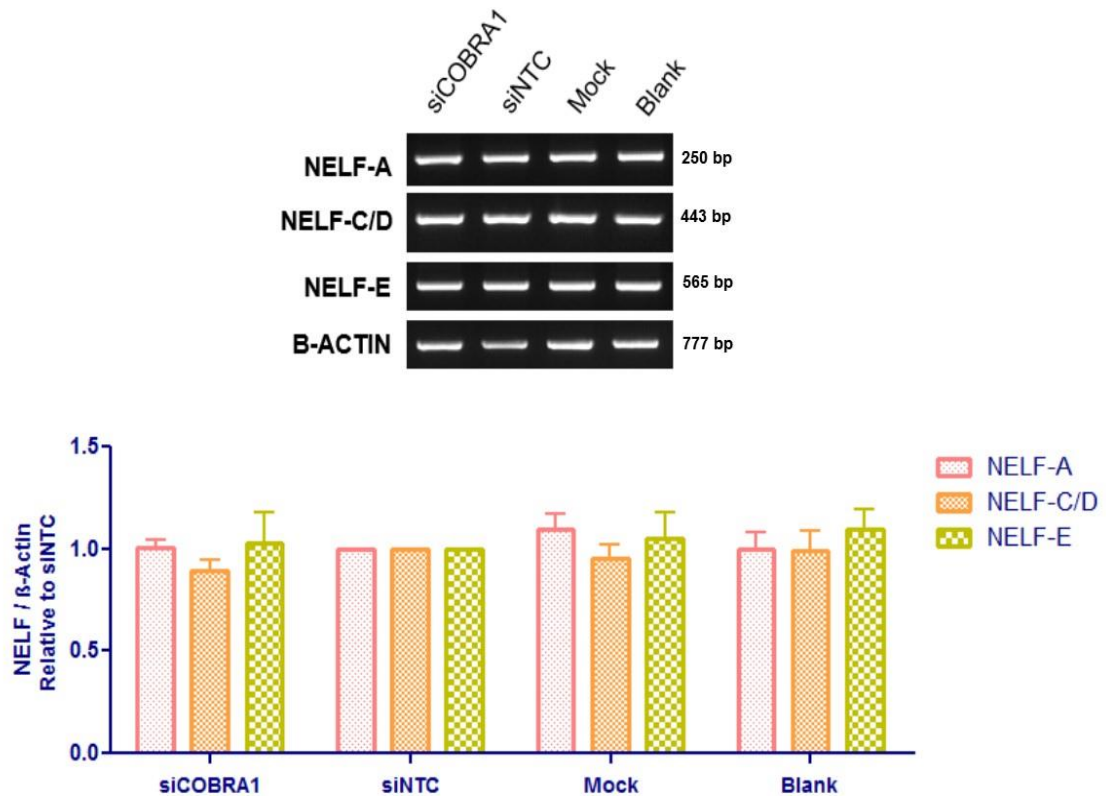


Figure 5. COBRA1 knockdown does not affect the expression of the remaining NELF subunits. Semi-quantitative RT-PCR analysis of NELF-A, NELF-C/D and NELF-E transcripts prior and following COBRA1 knockdown. The band intensities were quantified by ImageJ and normalized to the internal control B-ACTIN. Relative expression is expressed as fold change to siNTC. Data represents the mean \pm SD of three independent experiments (n = 3). No statistically significant differences were observed ($p > 0.05$) (one-way ANOVA, Bonferonni's post-test). siCOBRA1 - COBRA1 siRNA, siNTC - Negative siRNA.

3.4. Silencing of COBRA1 suppresses cell proliferation of HepG2.

To examine the effect of COBRA1 knockdown on cellular proliferation, HepG2 cells, were transfected with either 25 nM COBRA1 siRNA or Negative siRNA and harvested at different time points. Cell counting was performed to construct a growth curve and assess the rate of cell proliferation. Significant suppression was observed in the growth of cells transfected with COBRA1 siRNA at day 3 and 4 post-transfection, when compared to control cells (Figure 6).

In order to further investigate the effect of COBRA1 knockdown on cell proliferation, the expression of an important marker of cellular proliferation, Ki-67, was examined. Ki-67 is a nuclear protein that is present in all active phases of the cell cycle (G1, S, G2, and mitosis), with the exception of the resting (G0) phase (Scholzen & Gerdes, 2000). Even though little is known about the function of Ki-67, its presence is believed to be an absolute requirement

for cell cycle progression and the maintenance of cell proliferation (Schlüter et al., 1993). Recently, it was shown to play an important role in the stabilization and maintenance of mitotic spindles (Vanneste, Takagi, Imamoto, & Vernos, 2009). Consistent with the previous cell count data, semi-quantitative PCR revealed a significant reduction of ~20% in the expression of Ki-67 mRNA upon COBRA1 knockdown, when compared to control cells ($p < 0.01$; Figure 7). No significant difference in the expression of Ki-67 was found between the different control groups.

3.5. Silencing of COBRA1 inhibits cell migration of HepG2.

The effect of COBRA1 knockdown on the migratory potential of HepG2 cells was analyzed using a classical scratch wound healing assay (Qin & Cheng, 2010; Yu et al., 2014). After 24 hr from inflicting the wound in the cell monolayer, the wound area in the transfected cells was compared to the initial wound area and used to calculate the percentage wound closure. Visual assessment of each well revealed a noticeable difference in the area of the wounds; the wound area in COBRA1 knockdown cells was wider than that of the negative control cells (Figure 8A). Wound areas were automatically analyzed by the TScratch software and revealed a ~60% decrease in the migration rate following COBRA1 knockdown ($p < 0.001$; Figure 8B). While the control cells showed a 31.86% wound closure after 24 hrs, COBRA1 knockdown cells showed a delayed wound closure rate at 13.12%.

3.6. Silencing of COBRA1 significantly suppresses survivin gene expression

Deregulation of cell proliferation and migration in cancer is usually associated with the deregulation of many key signaling pathways (Hanahan & Weinberg, 2011; Malumbres & Carnero, 2003). To explore the underlying mechanisms by which COBRA1 regulates the proliferation and migration of HepG2 cells, semi-quantitative PCR was utilized to examine the expression of genes commonly reported misregulated in cancer and play key roles in the proliferation as well as survival of cancer such as survivin. Of the 3 transcript variants analyzed, the wild-type survivin transcript represents the dominant form of survivin in HepG2. As shown in Figure 9A, knockdown of COBRA1 significantly suppresses the expression of the 3 different variants of survivin compared with cells transfected with control siRNA. While survivin-2B and survivin-deltaex3 were downregulated by 21.5% and 16.4%, respectively, the wild type (WT) survivin was the one most affected, with 40% downregulation in the knockdown cells when compared to the control siRNA (Figure 9B).

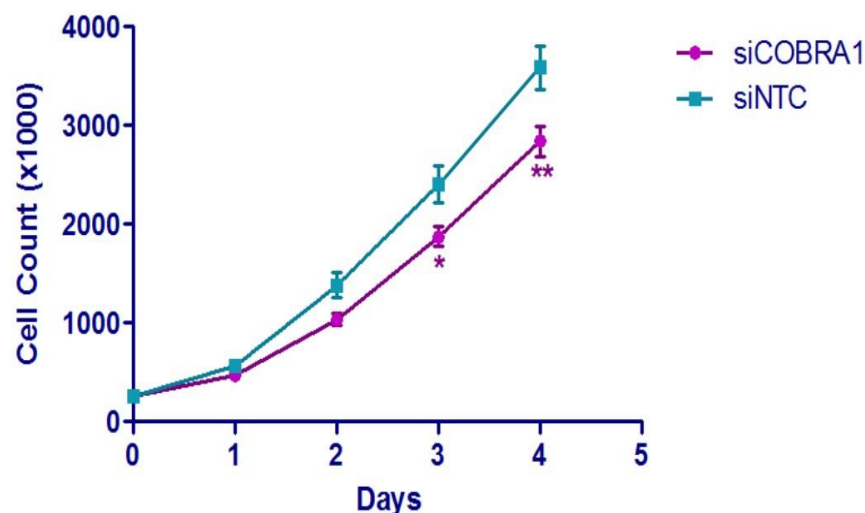


Figure 6. Knockdown of COBRA1 inhibits the cell proliferation of HepG2 cells. (A) The growth of cells transfected with either COBRA1 siRNA or Negative siRNA were monitored for 4 days post-transfection in order to analyze the growth rate. Cells were harvested at the indicated time points following transfection and counted using a hemocytometer. Data represents the mean \pm SD of at least two independent experiments ($n = 2$). Statistically significant at ** $p < 0.01$, * $p < 0.05$ (two-way ANOVA, Bonferonni's post-test)

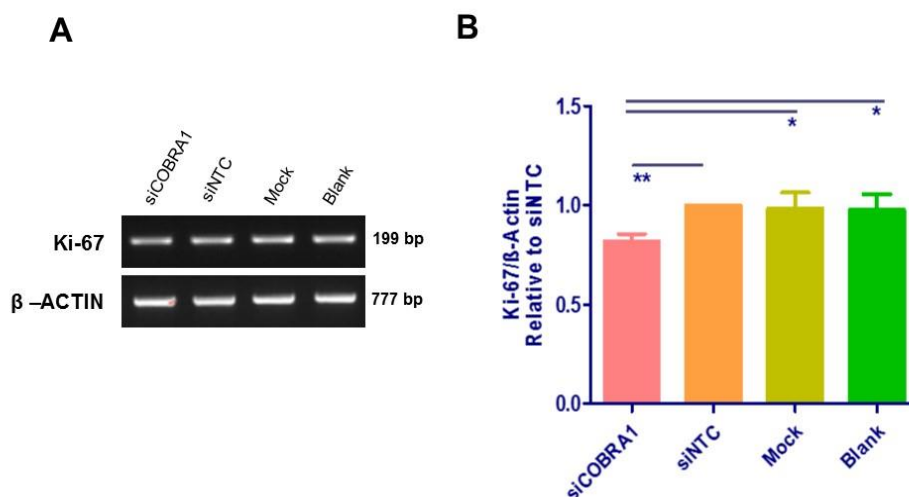


Figure 7. Effect of COBRA1 knockdown on the expression of the cellular proliferation marker, Ki-67. Effect of COBRA1 knockdown was analyzed by semi-quantitative RT-PCR. The bands intensities were quantified by ImageJ and normalized to the internal control B-ACTIN. Relative expression is expressed as fold change to the siNTC. A significant reduction was observed in the expression of Ki-67 following COBRA1 knockdown, relative to siNTC. Data represents the mean \pm SD of 2 independent experiments ($n = 3$). Statistically significant at ** $p < 0.01$, * $p < 0.05$ (one-way ANOVA, Bonferonni's post-test). siCOBRA1 - COBRA1 siRNA, siNTC - Negative siRNA.

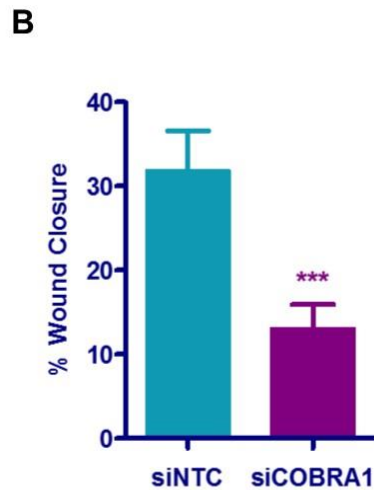
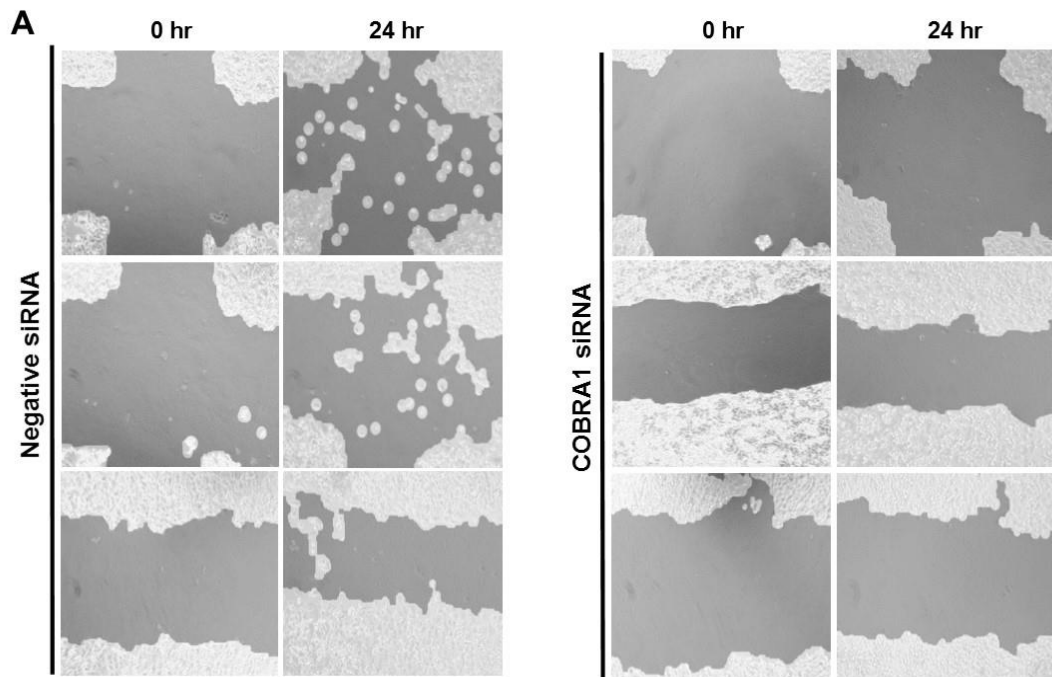


Figure 8. Silencing of COBRA1 impairs HepG2 cell migration. (A) Wound healing assay was utilized to analyze the migration of HepG2 cells after knockdown of COBRA1. Seventy-two hrs following transfection, the cell monolayer was scratched by a sterile pipette tip and migration monitored for the following 24 hrs. (B) Quantitative analysis of wound closure in COBRA1-knockdown cells versus negative siRNA-transfected cells. Automated analysis of the wound area was performed using TScratch software (Gebäck *et al.*, 2009). Wound areas were then used to calculate the percentage wound closure. Data represents the mean \pm SD from three independent experiments ($n = 3$). Statistically significant at *** $p < 0.001$ (Student t-test, two-tailed). siCOBRA1- COBRA1 siRNA, siNTC - Negative siRNA.

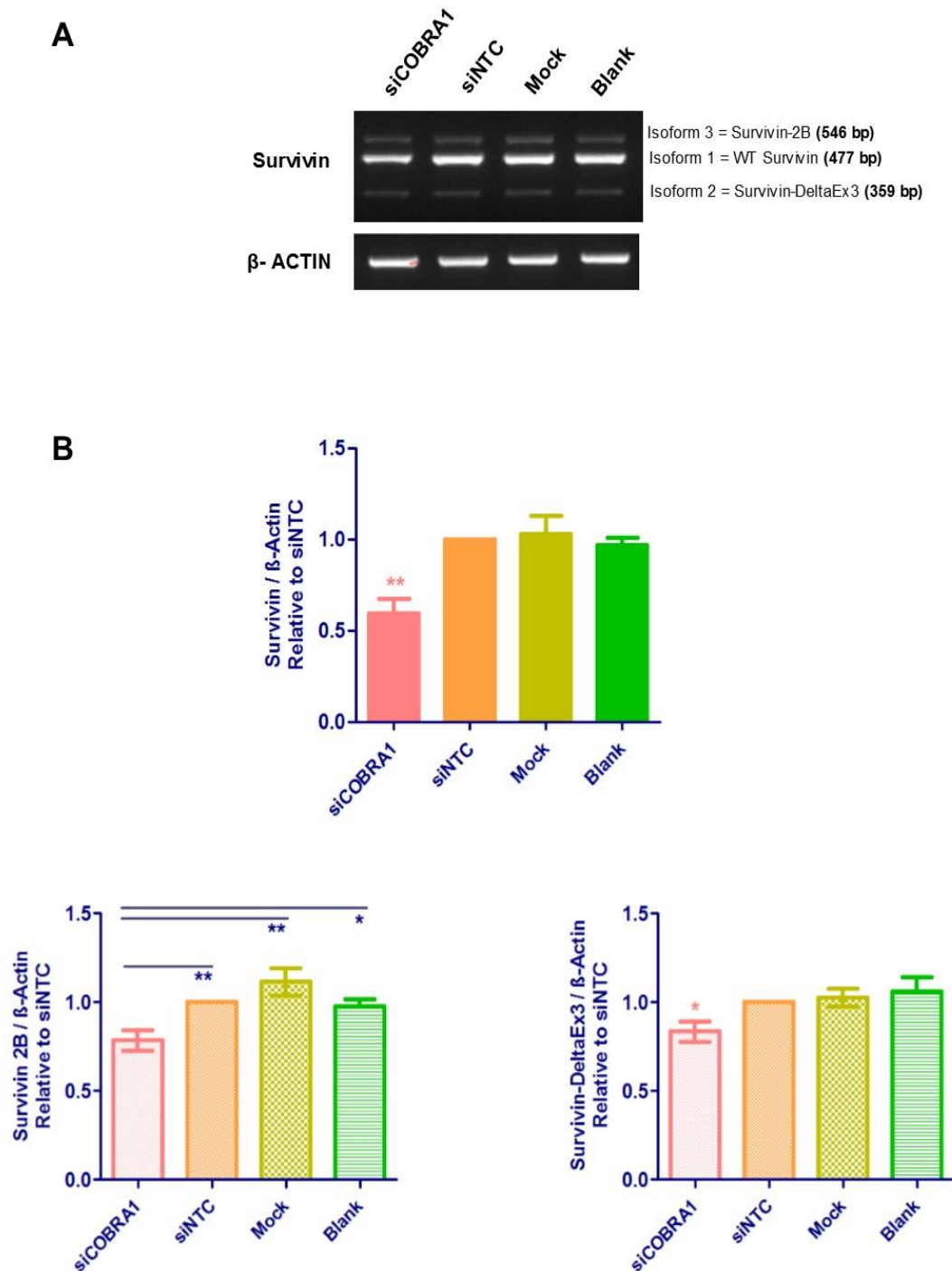


Figure 9. Silencing of COBRA1 suppresses Survivin expression. (A) At 72 hr post-transfection of COBRA1 or Negative siRNA in HepG2 cells, the expression of Survivin was analyzed by semi-quantitative RT-PCR. B-ACTIN was used as a loading control. Primers used detect 3 different isoforms of Survivin; Wild-type Survivin, Survivin-2B and Survivin-DeltaEx3. (B) The bands intensities were quantified by ImageJ and normalized to B-ACTIN. Relative expression is expressed as fold change to the siNTC. A significant reduction was observed in the expression of all 3 isoforms of Survivin following COBRA1 knockdown, relative to siNTC. Data represents the mean \pm SD of 3 independent experiments (n = 3). Statistically significant at ** $p < 0.01$, * $p < 0.05$ (one-way ANOVA, Bonferonni's post-test). siCOBRA1- COBRA1 siRNA, siNTC - Negative siRNA.

CHAPTER 4. DISCUSSION

Despite the different treatment regimens available, prognosis of HCC remains very poor with an overall 5-year survival rate of less than 5% (X. Huang *et al.*, 2013), making it the second most common cause of cancer-related mortality worldwide (Ferlay *et al.*, 2013). The lethal nature of HCC stems from the aggressive course of the disease combined with the high risk of tumor recurrence (Capece *et al.*, 2013). In addition, due to the lack of biomarkers that can detect the disease in its early stages, the majority of HCCs are diagnosed at advanced stages when curative surgical treatment options are no longer available. This makes their treatment very difficult (Farazi & DePinho, 2006).

It is believed that proper molecular markers will not only aid in the early diagnosis of HCC, but also in the prognosis and therapy monitoring of the disease. Therefore, research is currently directed towards identifying molecular markers that are sensitive and specific enough to allow for efficient patient management. In addition, due to the heterogeneity and underlying complexity of HCC, the molecular mechanisms lying behind the development and progression of HCC remain largely unknown. A comprehensive understanding of the multiple layers of genetic and epigenetic changes associated with this disease will provide a platform for the discovery of novel treatment strategies (Cornellà *et al.*, 2011).

In this study, our interest was in one of the genes recently implicated as a potential player in the development and progression of several cancers, known as COBRA1 (McChesney *et al.*, 2006; Sun *et al.*, 2008). COBRA1 is part of the transcriptional regulatory machinery of the cell, by which the cell is able to regulate a substantial number of genes (Aiyar, Blair, *et al.*, 2007; Aiyar, Cho, *et al.*, 2007; Aiyar *et al.*, 2004; Sun & Li, 2010). Even though it has been extensively studied over the years, the majority of these studies have mostly reported roles for COBRA1, either as a functional component (Narita *et al.*, 2003) or as a cofactor for site-specific transcription factors (Aiyar *et al.*, 2004; Sun *et al.*, 2007; Zhong *et al.*, 2004). In the field of cancer, the role of COBRA1 is not yet fully understood and much of the data that currently exists regarding its role is conflicting. Nonetheless, data that exists does demonstrate an important role for COBRA1 in cancer and the fact that much of it is conflicting highlights the underlying complexity of COBRA1's role. Here, we provide several lines of evidence for the first time to support a critical role for COBRA1 in supporting HCC.

4.1. Overexpression of COBRA1 in HCC tumor tissues versus normal liver tissue

To date, little is known about COBRA1 in HCC. Previous research in Amleh's Lab (Kamel, 2012) provides preliminary data supporting an upregulated expression pattern for COBRA1

mRNA (68%) and protein (50%) in HCC tumor tissues, when compared to peri-tumor tissues collected from the same patient. However, the small sample size was a major limitation of the study. In the current study, we extended on the lab's previous data findings and utilized microarray data publicly available online to further explore this variation in expression on a larger scale. Analysis of HCC microarray data available in the OncoPrint database revealed 3 datasets that collectively demonstrated a significant overexpression of COBRA1 mRNA (1.4-1.6 folds) in HCC tumor tissues versus normal liver tissues.

Chronic HBV and HCV infection have together the majority of the share in the incidence of HCC. Similar to previous research in Amleh's lab, samples in the Wurmbach study were from patients with an HCV etiology. In contrast, the large majority of the samples in the Roessler study were from patients with a history of HBV infection or HBV-related cirrhosis. This strongly suggests that the overexpression of COBRA1 is a feature associated with both etiologies of HCC. It is important to mention that even though 3 datasets were in agreement with regards to the differential expression of COBRA1 between HCC tissue and either normal liver or paired non-tumor tissue, one dataset (Mas Liver dataset) did not show any significant difference. However, this might be attributed to experimental factors related to variabilities in RNA handling, used probe sequences or differences in the computational methods used. Therefore, further meta-analysis of data from these datasets is necessary to gain a more reliable insight.

Furthermore, the Mas Liver dataset does not show any difference in the expression of COBRA1 between normal tissue and cirrhotic tissue. In agreement, the Wurmbach dataset also shows no difference between normal liver tissue & either cirrhotic or dysplastic tissue. This data suggests the potential of COBRA1 as a biomarker in differentiating HCC from other chronic liver disorders. However, this still requires further study on a wider range of samples. Taken together, data presented here demonstrates the frequent overexpression of COBRA1 in HCC tumors versus their normal counterparts and suggests a potential oncogenic role for COBRA1.

4.2. Efficiency of SMARTPool siRNA in the Silencing of COBRA1

To clarify a role for the frequent overexpression of COBRA1 in HCC, RNA interference (RNAi) was used to silence COBRA1 expression. RNAi is the cellular mechanism by which double stranded RNAs trigger silencing of gene expression, by targeting complementary mRNA sequences for degradation (Echeverri & Perrimon, 2006). With a higher expression of COBRA1 mRNA and protein relative to the normal human hepatocyte cell line MIHA, the HepG2 cell line (as observed by Amleh Lab) was chosen as our cell model.

Despite its power, off-target activity is inherent to the siRNA technology and complicates the interpretation of all knockdown experiments. Attempts so far have only been able to reduce but not eliminate off-target activity (Echeverri *et al.*, 2006; Jackson & Linsley, 2010). Due to the concerns over off-target activity commonly associated with siRNAs combined with the large variabilities that exist in the knockdown efficacies of individual siRNAs, it has become standard practice in siRNA experiments to analyze different siRNAs per target (at least 2-3). There are 2 common approaches by which these siRNAs can be delivered; either individually or as pools. Conflicting data exists regarding the pros & cons associated with using either. Nonetheless, both remain standard knockdown strategies (Echeverri *et al.*, 2006; Parsons, Schindler, Evans, & Foley, 2009).

Here, a siRNA pool was utilized to establish a transient COBRA1 knockdown in the HepG2 cell line. The strong, near complete knockdown observed indicated that the siRNAs were successful in silencing COBRA1 expression at the protein level. This was important for our study as it was previously reported that a silencing threshold must sometimes be reached before a detectable loss-of-function (LOF) phenotype can be observed. In fact, the authors reported that sometimes a complete protein knockdown is essential in order to make an accurate assessment of gene function (F. Huang, Khvorova, Marshall, & Sorkin, 2004). In support, a recent study reported experiments performed with pooled siRNAs to be more likely associated with LOF phenotypes than single siRNAs (Parsons *et al.*, 2009).

In addition, Dharmacon RNA Technologies (<http://www.dharmacon.com/>) have shown that their pooling strategy of 4 different siRNAs superior to individual siRNAs in diluting off-target effects. With each siRNA having a distinct off-target signature of its own, the overall off-target signature of the entire pool is less than any of the individual siRNAs. Furthermore, pooling siRNAs is known to reduce the contribution of each of the single siRNAs to the final pool and thus perceived to reduce off-target activity. In this respect, Semizarov *et al.* (2003) reported siRNA concentrations ≤ 20 nM sufficient to eliminate off target activity. In our study, 25 nM of the pool was used. Thus, ~ 6.25 nM from each of the 4 single siRNAs. Nonetheless, it is still important to mention that off-target activity is impossible to rule out and much of the data that exists regarding how it should be minimized is conflicting. Therefore, to ensure a more rigorous study, further analysis of the single siRNAs that constitute the pool is necessary.

4.3. Knockdown of COBRA1 Inhibits Cellular Proliferation of HepG2

Deregulation of cellular proliferation is one of the major hallmarks of cancer (Hanahan & Weinberg, 2011; Malumbres & Carnero, 2003). Here, our data indicates that COBRA1 is

essential for maintaining the cellular proliferation rates of the HepG2 cell line. This positive effect that COBRA1 has in mediating the growth of HCC goes in line with its previously reported role in maintaining the transcription of a large number of genes involved in cell cycle control, cell proliferation, cell death and DNA repair (Aiyar, Blair, *et al.*, 2007; Aiyar, Cho, *et al.*, 2007). In fact, this is not the first time COBRA1 has been implicated in the proliferation of cancer. Aiyar *et al.* (2004) reported a significant reduction in the rate of proliferation of T47D cells upon the ectopic expression of COBRA1. Similarly, knockdown of COBRA1 resulted in an increase in the estrogen-dependent growth of the cells, thereby establishing an inhibitory role for COBRA1 in breast cancer tumorigenesis. However, in contrast to breast cancer, COBRA1 knockdown significantly inhibited HepG2 proliferation as shown by both the reduced count and decrease in the expression of Ki-67. Taken together, data presented here supports a positive role for COBRA1 in the growth of HCC.

These findings go in line with other studies that also suggest a positive role for COBRA1 in the growth of both ovarian and lung cancer. In both cancers, COBRA1 was shown a novel target of the widely established oncogenic Ras/MAPK pathway (Pohl *et al.*, 2005; Sudhir *et al.*, 2011). Chemical inactivation of the pathway resulted in a profound decrease in the proliferation of different ovarian cancer cell lines accompanied by a substantial downregulation (>3-fold) in the expression of COBRA1 (Pohl *et al.*, 2005). It is important to mention that the contribution of COBRA1's downregulation towards the observed phenotype is still not known. Nonetheless, the fact that COBRA1 was identified as a target of the pathway and the impact of this pathway in supporting carcinogenesis, strongly suggest a positive role for COBRA1 in mediating the effects of this pathway.

4.4. Knockdown of COBRA1 Inhibits Migratory Potential of HepG2

Local invasion and distant metastasis is another key cancer hallmark and an indicator of poor prognosis (Hanahan & Weinberg, 2011). In line with COBRA1's established role in breast cancer as a tumor suppressor, lack of COBRA1 in cancerous tissues is associated with distant metastasis and recurrence in patients and thus considered as an indicator of poor prognosis (Sun *et al.*, 2008). In agreement, the expression pattern of many genes reported earlier to be regulated by COBRA1 have been associated with advanced and/or metastatic breast cancer (Aiyar, Blair, *et al.*, 2007; Aiyar, Cho, *et al.*, 2007; Aiyar *et al.*, 2004). In contrast to breast cancer, where COBRA1's expression is inversely correlated with metastasis, our data findings suggests a positive role for COBRA1 in metastasis. The molecular basis behind the tissue-specific nature of COBRA1's functions remain unknown and warrants further study. It is also important to mention that this study has to be further extrapolated to other cell lines to gain a comprehensive understanding of COBRA1 in HCC.

4.5. Interdependent Nature of NELF Subunits

COBRA1 is a functional component of the NELF complex. It has been reported that upon knockdown of any NELF subunit, co-depletion of the remaining subunits occur almost simultaneously (Narita *et al.*, 2007; Sun & Li, 2010; Sun *et al.*, 2008). Even though our results show otherwise, findings from these studies have shown that the changes observed in the expression levels of the NELF subunits occurred exclusively at a post-transcriptional level (Narita *et al.*, 2007; Sun & Li, 2010; Sun *et al.*, 2008). Therefore, further examination is required before a conclusion can be drawn here.

Similar to COBRA1, the rest of the NELF subunits (NELF-A, NELF-C/D and NELF-E) have also been implicated in different cancers (Mehra *et al.*, 2011; Oka, 2012; Zou *et al.*, 2010). Given the previously established interdependent nature of the NELF subunits (Narita *et al.*, 2007; Sun & Li, 2010; Sun *et al.*, 2008), a disease-based deregulation in the expression pattern of one subunit is expected to equally reflect in the remaining subunits. It is therefore tempting to speculate overlapping roles for the different NELF subunits in the same cancer. Limited with the small number of studies, this pattern has been observed so far in breast cancer. Both NELF-B (COBRA1) and NELF-C/D were reported independently to have negative roles in the growth and progression of breast cancer. In addition, the expression of either negatively correlated with the aggressiveness of breast cancer (Sun *et al.*, 2008; Zou *et al.*, 2010). Here, we believe our results follow the same pattern and goes in line with previous data published regarding the role of other NELF subunits in HCC. NELF-E (also known as RDBP) was shown to have a higher expression pattern in HCC tumors versus their paired non-HCC tissues. In line with its preferential overexpression in tissues with portal vein invasion, NELF-E expression was also reported an independent risk factor for intrahepatic recurrence. Furthermore, knockdown of NELF-E resulted in a decrease in cell proliferation rate of the hepatoma cell line, HLE, with no significant changes observed in the cell cycle distribution (Oka, 2012). This was also earlier established by Midorikawa *et al.* (2002) who reported the dedifferentiation process of HCC, one of the events that define the multi-step progression of hepatocarcinogenesis, associated with the overexpression of RDBP, among others.

4.6. Silencing of COBRA1 Suppresses Survivin Expression

Survivin represents one of the genes that is commonly upregulated in almost all human malignancies including HCC and known to play key roles in cellular proliferation and survival (Fukuda & Pelus, 2006). It is a member of the inhibitor of apoptosis (IAP) family of proteins that play key roles in inhibiting different pathways of programmed cell death

(Elmore, 2007). While in normal tissue survivin has minimal expression levels, in cancer its expression is upregulated and correlates with a more aggressive disease and a poor prognosis (Jaiswal, Goel, & Mittal, 2015). Survivin expression is cell-cycle dependent, being low in the G1 phase, high in the S phase (6 times higher) and at its peak in the G2/M phase (40 times higher) (Boidot, Végran, & Lizard-Nacol, 2014). In line with its cell cycle-dependent expression, survivin functions mainly as both a regulator of cell division and an inhibitor of apoptosis (Mita, Mita, Nawrocki, & Giles, 2008).

Besides the 4-exon WT survivin transcript, at least 6 alternatively spliced variants have been identified to date: WT survivin, survivin-2b, survivin- Δ Ex3, survivin-3b, survivin-2a, survivin-2b+32 and survivin-image (Pavlidou, Kroupis, & Dimas, 2014). Of those, only 3 are well established and have been extensively studied. These are the WT survivin, survivin-2b and survivin- Δ Ex3. In one study, the 3 variants were shown to represent nearly 98% of the mRNA expression from the survivin gene (Mull, Klar, & Navara, 2014). While survivin-2b arises from the inclusion of a cryptic exon that lies within intron 2, survivin- Δ Ex3 arises from the removal of exon 3 and a frameshift that results in the inclusion of part of the 3'-untranslated region (UTR) (Li, 2005; C Mahotka, Wenzel, Springer, Gabbert, & Gerharz, 1999) (Figure 10).

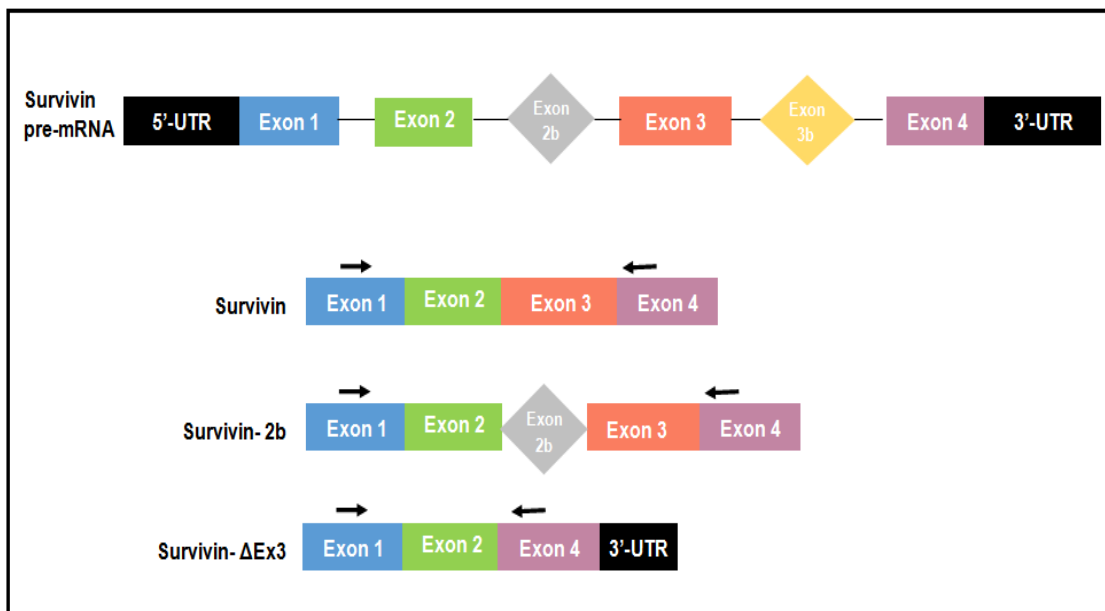


Figure 10. Alternatively Spliced Survivin Transcripts. The survivin pre-mRNA includes four exons (exon 1-4) and two cryptic exons (exon 2b, 3b). The survivin pre-mRNA generates at least seven alternatively spliced variants identified to date. The forward and reverse primers used in this study pick up only three of these survivin transcripts: WT survivin, survivin-2b and survivin- Δ Ex3. WT survivin is derived from exons 1-4. Survivin-2b arises from the inclusion of a cryptic exon referred to as exon 2b, located within intron 2. Survivin- Δ Ex3 arises from the removal of exon 3 and inclusion of part of the 3'-UTR. Black arrows indicate the positions of PCR primers (Exon 1 and exon 4). WT: wild type, UTR: untranslated region (Mokuda et al., 2015; Turkkila et al., 2015)

Even though, these 3 variants have been extensively studied, little is known about their functions. In contrast to WT survivin, survivin-2b and survivin- Δ Ex3 do not seem to have a role in the regulation of cell division (Noton *et al.*, 2006). Instead, they have only been shown until now to play a role in the regulation of apoptosis; while survivin- Δ Ex3, similar to WT survivin has an anti-apoptotic function, survivin-2b is believed to have a pro-apoptotic function (Pavlidou *et al.*, 2014). Even though this is the case, reported data for survivin-2b sometimes suggest otherwise (Y. Huang *et al.*, 2011; Nakano *et al.*, 2008; Vivas-Mejia *et al.*, 2011).

Our results show that of the 3 transcripts examined, WT survivin represents the dominant splice variant expressed in HCC which goes in line with previous literature (Kannangai, Wang, Liu, Sahin, & Torbenson, 2005). In fact, it is worth to mention that the predominant expression of WT survivin has been reported in many other cancers as well (Fangusaro *et al.*, 2005; Krieg *et al.*, 2002; Csaba Mahotka *et al.*, 2002; Ryan *et al.*, 2005; Taubert *et al.*, 2005). In HCC, Takashima *et al.* (2005) reported that that high levels of WT survivin mRNA correlated with a more malignant cancer. In addition, it was shown that while the mRNA expression levels of WT survivin and survivin- Δ Ex3 correlated with high proliferative activity, that of survivin-2b did not (Takashima *et al.*, 2005). Even though not enough data is available to draw a conclusion for the role of survivin-2b in HCC, the overexpression of survivin transcripts observed in HCC compared to normal tissue is believed to be associated with hepatocarcinogenesis (Kannangai *et al.*, 2005; Takashima *et al.*, 2005). Therefore, taken together, the suppression of survivin expression upon COBRA1 knockdown suggests that survivin might be one of the mechanisms by which COBRA1 mediates its involvement in HCC growth and migration. It is also worth to mention that this finding is in line with a previously published microarray study in breast cancer that also demonstrated survivin to be downregulated upon COBRA1 knockdown (Aiyar, Cho, *et al.*, 2007).

In summary, COBRA1's role in cancer seems to be highly cancer type-dependent. While previously reported a tumor suppressor in breast cancer with an expression pattern directly correlated with prognosis, data in UGCs seems to support its role as an oncogene (McChesney *et al.*, 2006; Sun *et al.*, 2008). Here, in HCC, our data findings collectively highlight an oncogenic role for COBRA1 in HCC, similar to that in UGC.

CONCLUSION

To the best of our knowledge, our study provides several lines of evidence for the first time to support a positive role for COBRA1 in the growth and migration of HCC.

First, we show through *in silico* analysis of publicly available gene expression data on a large number of tissue samples, the frequent overexpression of COBRA1 in HCC tumors versus their normal counterparts. In addition, we highlight the potential of COBRA1 as a selective HCC biomarker, given no differences in COBRA1 gene expression were found between normal tissue and tissue from other non-HCC chronic liver diseases. Second, we show that COBRA1 expression is important for the proliferation and migration of HCC cells. Finally, the deregulation of cell proliferation and migration observed in cancer is usually associated with the deregulation of multiple mechanisms that normally exist to suppress tumor formation and metastasis. Here, data findings from our study also show that one of the possible mechanisms by which COBRA1 mediates its positive role on the growth and migration of HCC might be through the upregulation of survivin expression.

FUTURE RECOMMENDATIONS

Microarray data remains to date a rich, untapped source of knowledge that remains largely underutilized. Further analysis of publicly available HCC gene expression datasets should be done, to study the correlation between COBRA1 expression and clinicopathological parameters. This will help clarify the potential of COBRA1 mRNA as a prognostic factor, in predicting the aggressiveness of HCC. Analysis should also be extrapolated to the protein level via immunohistochemistry of tissue arrays.

As earlier discussed, off-target effects are inherent to the siRNA technology and attempts have so far have only been able to reduce but not eliminate off-target activity (Echeverri *et al.*, 2006). Even though the utilized pooling strategy has been shown by Dharmacon RNA Technologies (<http://www.dharmacon.com/>) to dilute off-target effects observed with single siRNAs, the superiority of pooled siRNAs to individual siRNAs in this respect remains controversial among researchers (Echeverri & Perrimon, 2006; Smith, 2006). Confirmation with redundant silencing reagents remains the gold standard for demonstrating siRNA specificity (Echeverri *et al.*, 2006). Therefore, our data should be further confirmed with at least 2-3 individual siRNAs. In addition, analysis of the effect of COBRA1 overexpression in HepG2 will further validate the specificity of our data findings.

Extrapolation of the methodology to include more HCC cell lines, representing different stages of HCC, will ensure a more reliable assessment of COBRA1's function. In addition, further analyses of COBRA1-knockdown cells should be done for a more comprehensive understanding. For example, cell cycle analysis can be performed to further investigate the molecular mechanisms underlying the decrease in proliferation by analyzing both the cell cycle distribution (G1, G2 and S phases) and the apoptotic cell fraction upon knockdown.

The frequent overexpression of COBRA1 mRNA in HCC tumors raises the question of as to how this upregulation is achieved and the molecular mechanisms behind it. Further studies should investigate whether the oncogenic Ras/MAPK pathway, as an upstream regulator of COBRA1, plays a role in mediating COBRA1's positive role in HCC proliferation & migration. In addition, given COBRA1's role in suppressing AR-signaling pathway and the impact of this pathway in mediating carcinogenesis of HCC, it would be interesting also to find out the functional link between both in HCC.

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