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Overexpression of cofactor of BRCA1 in HepG2 cells: A step towards understanding the role of COBRA1 in hepatocellular carcinoma

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Overexpression of Cofactor of BRCA1 in HepG2 Cells: A Step Towards Understanding the Role of COBRA1 in Hepatocellular Carcinoma

A Thesis Submitted to the
Biotechnology Master's Program

In partial fulfilment of the requirements for the

Degree of Master of Science

By: Razan Jamil Masad

(Under the supervision of Dr. Asma Amleh)

December/2016

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DEDICATION

To my dear mother, father and my beloved family for their continuous support, love and prayers throughout the journey. To my dearest friends for being always by my side and for being my stress release and laughter when needed. Without all of your care and support, I wouldn't have been able to complete this process. I love you all

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ABSTRACT

The American University in Cairo

Overexpression of Cofactor of BRCA1 in HepG2 Cells: A Step Towards Understanding the Role of COBRA1 in Hepatocellular Carcinoma

By Razan Jamil Masad

Under the supervision of Dr. Asma Amleh

Cofactor of BRCA1 (COBRA1) is a BRCA-1 interacting protein that represents one of the four subunits of the negative elongation factor (NELF) complex. NELF is known by its ability to stall RNA Polymerase II during the early phase of transcription elongation, resulting in repressed transcription of several genes including ones associated with tumorigenesis of different cancer types. While it was found to be down-regulated in breast cancer, COBRA1 was found to be up-regulated in the upper gastrointestinal carcinoma. Up to date, the role of COBRA1 in hepatocellular carcinoma (HCC) is unclear. We have previously demonstrated that silencing of COBRA1 in the HCC cell line HepG2, significantly inhibited the proliferation and migration potentials of the cells. Here, we investigated the effect of ectopic expression of COBRA1 on HepG2 cells proliferation and migration. Lipofectamine 3000 was used to transfect HepG2 cells with a pCMV5-HCOBRA1 plasmid. The transfection efficiency was determined by the percentage of EGFP positive cells (pEGFP-N1+) via fluorescent microscope, semi-quantitative RT-PCR as well as western blot analysis. The cells proliferation and migration following COBRA1 overexpression were assessed using the trypan blue dye exclusion method and the wound-healing assays respectively. The semi-quantitative RT-PCR was used to analyse the mRNA expressions of the other NELF subunits, TFF1 and TFF3 genes, which are known to be regulated by the NELF complex, as well as other tumorigenesis related genes. Our results revealed that COBRA1 transfected cells exhibited a comparable proliferation and migration rates to non-transfected cells. These results were accompanied by an insignificant effect of COBRA1 overexpression on the levels of the proliferation marker; Ki-67 and the anti-apoptotic gene; survivin. Also, the mRNA levels of the other NELF subunits, TFF1 and TFF3 were found to be comparable among all the tested groups. Collectively, our results suggest that the proposed involvement of COBRA1 in HCC is supported by and dependent on the assembly of the active NELF complex, which requires the expression of all four NELF subunits. Moreover, COBRA1 mediated role in HCC tumorigenesis might be due to mechanisms and regulatory pathways other than the ones examined here. However, further studies are required to confirm these notions.

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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 Receptor1
ARID1	AT-Rich Interaction Domain 1
ARID2	AT-Rich Interaction Domain 2
BIRC5	Baculoviral Inhibitor of Apoptosis Protein Repeat- Containing 5
Bps	Base Pairs
BRCA1	Breast Cancer Type 1 Susceptibility Protein
BRCT1	BRCA1 C-terminal 1
BRCT2	BRCA1 C-terminal 2
CAP2	Cyclase-Associated Protein2
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
ChIP	Chromatin Immunoprecipitation
COBRA1	Cofactor of BRCA1
CTD	C-Terminal Domains
CTNNB1	Catenin Beta 1
DEC1	Deleted in Esophageal Cancer 1
DEPC	Diethylpyrocarbonate
DSIF	DRB Sensitivity-Inducing Factor
E-cadherin	Epithelial Cadherin
EGF	Epidermal Growth Factor
ERα	Estrogen Receptor-Alpha
ESCs	Embryonic Stem Cells
ESMO	European Society for Medical Oncology
FBS	Fetal Bovine Serum
GFP	Green Florescent Protein
GPC3	Glypican-3
GR	Glucocorticoid Receptor
HBV	Hepatitis B Virus

HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HepG2	Well Differentiated Human Hepatocellular Carcinoma- Cell Line
Hr	Hour
Hsp70	Heat Shock Protein 70
HTA	Hepatoma-Associated Gene
IAP	Inhibitor of Apoptosis
IFN-a	Interferon Alpha
KLF5	Kruppel-Like Factor 5
LBD	Ligand Binding Domain
LT	Liver Transplantation
MAPK	Mitogen-Activated Protein Kinases
MECC	Middle East Cancer Consortium
MELD	Model for End-stage Liver Disease
Mins	Minutes
MLL	Mixed-lineage Leukemia Protein 2
NAFLD	Nonalcoholic Fatty Liver Disease
NELF	Negative Elongation Factor
NR	Nuclear Receptors
PBS	Phosphate-Buffered Saline
PBST 0.01%	Tween-20 in 1X PBS
PCR	Polymerase Chain Reaction
PDGFRs	Platelet-derived Growth Factor Receptors
PI3K	Phosphoinositide 3-kinase
PRB	Progesterone Receptor B
P-TEFB	Positive Transcription Elongation Factor B
RFA	Radiofrequency Ablation
RNAPII	RNA Polymerase II
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SEER	Surveillance, Epidemiology and End Results Populations
Sox2	SRY (sex determining region Y)-box 2

SK-BR-3	Metastatic Human breast Carcinoma Cell Line
SRPK	SR Protein-Specific Kinase
STAT3	Signal Transducer and Activator of Transcription 3
T47D	Metastatic Human Breast Carcinoma Cell Line
TACE	Transarterial Chemoembolization
TFF2	Trefoil Factor 2
TFF3	Trefoil Factor 3
TGF-β1	Transforming Growth Factor-β1
UCSF	University of California San Francisco
UGCs	Upper Gastrointestinal Cancer
VEGF	Vascular Endothelial Growth Factor
WHSC2	Wolf-Hirschhorn Syndrome Candidate 2
WT	Wild Type
µls	Microliters

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CHAPTER 1. LITERATURE REVIEW

1.1. Hepatocellular Carcinoma

1.1.1 Incidence and epidemiology

Hepatocellular carcinoma (HCC) is the most frequent type of liver cancer, accounting for more than 90% of primary liver cancer cases (Bodzin & Busuttil, 2015; Liver, 2012). Currently, HCC ranks the sixth most frequent cancer and the second leading cause of cancer-related deaths worldwide, where almost 746000 people die from HCC each year(Ashtari, Pourhoseingholi, Sharifian, & Zali, 2015; Flores & Marrero, 2014).

The frequency of HCC increases with age. In fact, most of the reported cases are diagnosed at the age of 65 years or more (Waghray, Murali, & Menon, 2015). Having a three times higher incidence rate in men than in women, HCC is considered a male predominant cancer (Howlader, Noone, & Krapcho, 2015).

An extensive variation in the world-wide distribution of HCC has been remarkably observed. While 3 out of 100 000 are infected with HCC in the western countries, more than 15 out of 100,000 are infected in other regions of the world, most of which are noticed in the developing countries (Siegel, Miller, & Jemal, 2015). In Egypt, HCC incidence is very high compared to other countries. According to the MECC (Middle East Cancer Consortium), HCC rate among Egyptian men was 7 times higher than other MECC countries (Cyprus, Palestine & Jordan) and 3 times higher than the US SEER (Surveillance, Epidemiology and End results populations). Among females, however, HCC rate was 3 times higher than other MECC countries and more than twice the US

SEER rate (Freedman, Edwards, Ries, & Young, 2006). The high occurrence of HCC in Egypt is due to the high number of HCV infections, where almost 14% of the Egyptian population is HCV-infected(Gomaa, Hashim, & Waked, 2014; Shelbaya, Kuznik, Salem, Mankola, & Sadik, 2015)

1.1.2 Risk factors and prevention

Several factors have been reported to be involved in HCC development. These factors include; liver cirrhosis, chronic hepatitis B virus (HBV) infection, chronic hepatitis C virus (HCV) infection, hereditary hemochromatosis, aflatoxin consumption, alcoholism, diabetes as well as obesity (Flemming, Yang, Vittinghoff, Kim, & Terrault, 2014).

Being presented in almost (80-90%) of HCC-infected individuals, liver cirrhosis is considered as the main risk factor for HCC (El-Serag, 2012a). Mainly, liver cirrhosis results from viral hepatitis infection, Alcohol-related liver disease**,** Nonalcoholic fatty liver disease (NAFLD) as well as aflatoxin consumption (Flemming et al., 2014).

Despite the wide regional variations in HCC etiology and distribution, chronic infections with hepatitis B and C represent the majority of HCC cases globally (El-Serag, 2012a). HBV infection results in acute and chronic liver disease, increasing by this the risk of developing HCC(El-Serag, 2012a). Several mechanisms for the progression from HBV to HCC have been proposed. For instance, both the inflammation and necrosis that result from HBV may cause alterations in the hepatocyte's genetic expressions, resulting in induction of the malignancy(Rossner, 1992). In addition, viral integration in to the liver cells results in a chromosomal instability and defected cellular replication leading to the development of HCC(Brechot, Pourcel, Louise, Rain, & Tiollais, 1980; Kremsdorf, Soussan, Paterlini-Brechot, & Brechot, 2006).

Upon introducing the obligatory universal HBV vaccination at birth, a decline of the HBV-related HCC cases was observed. For instance, following implementation of the Taiwan universal vaccination program, a significant reduction in the HCC annual incidence from 0.70 to 0.36 per 100,000 children was reported within a 10-year period (Ni et al., 2007). However, since this mandatory vaccination was implemented in the early 1980s, most of the adults over the age of 30 are not vaccinated and are at high risk of developing HCC(Pol, 2015). Thus, HBV prevention among adults is carried out through focusing on anti-viral treatments such as interferon alpha (IFN-a). In fact, IFN-a was shown to decrease the risk of developing HCC by a percentage of 6.4% (Cammà, Giunta, Andreone, & Craxì, 2001).

HCV infection leads to an inflammatory hepatocyte damage from oxidative stress, indorsing by this liver cirrhosis and resulting in HCC development (Parola & Robino, 2001). Currently, almost 40-50% of HCC cases are attributed to chronic HCV infections (Bruix & Sherman, 2005; Omar, Abou-Alfa, Khairy, & Omar, 2013). In fact, HCV-infected individuals have 17-times higher risk of developing HCC compared to HCV-negative individuals (Tanaka et al., 2006). Usually, HCV antiviral treatments are used to decrease the risk of the development of HCV-based HCC. Many studies have shown that responders to treatment with interferon (IFN) have a lower risk of developing HCC compared to non-responders and not-treated patients (Yoshida et al., 1999).

Alcohol related liver disease and Nonalcoholic fatty liver disease (NAFLD) also contributes to the development of HCC(Waghray et al., 2015). The molecular strategy by which alcoholism leads to HCC is not well addressed. However, several evidences indicate that it might be through indorsing altered DNA methylation, reduction in the

liver's retinoic acid level, oxidative stress as well as chromosomal loss (Bruix, Gores, & Mazzaferro, 2014).

NAFLD incidence is increasing due to the increasing numbers of obesity and diabetes worldwide. NAFLD is characterized by the fatty livers of infected individuals as well as inflammation and fibrosis(Petta et al., 2015; Pinzani, 2015). Almost 40% of NAFLD patients develop fibrosis, in which 9% of them progress to cirrhosis, resulting in the development of HCC as a complication (Adams et al., 2005; J. M. Hui et al., 2003).

Alfatoxin-B1 consumption is also considered as one of the major contributors to HCC development. Aflatoxins are mycotoxins produced by the Aspergillus fungus and Aspergillus parasiticus. They are mostly found on improperly stored food including rice, corn, peanuts and wheat (El-Serag, 2012a). The ability of aflatoxins to induce hepatocarcinogenesis was shown in several animal experiments. One study has revealed that when ingested, aflatoxin metabolizes to an active aflatoxin B1-exo-8, 9-epoxide. As a result, it binds to the DNA and causes DNA damage. This eventually results in the development of mutations in the tumor suppressor gene p53 (p53 249 ser), which will in turn induce the development of HCC (Garner, Miller, & Miller, 1972). In fact, these mutations have been observed in almost 30-60 % of HCC patients in aflatoxin-endemic regions (TURNER et al., 2002). In Egypt, Aflatoxin B1 consumption is considered as one of the main risk factors of HCC development (Omar et al., 2013).

Both obesity and diabetes are common risk factors of HCC. In fact, obese patients are100-times at risk of developing HCC than non-obese patients (Chen et al., 2008). Likewise, Diabetes is related to a 2-3 fold increase in the risk of developing HCC(J. Davila, Morgan, Shaib, McGlynn, & El-Serag, 2005).

Other less common risk factors for HCC development include; Wilson's disease, glycogen storage disease, autoimmune hepatitis, hereditary hemochromatosis and alpha 1-anti trypsin deficiency (Waghray et al., 2015).

1.1.3 Surveillance and diagnosis

HCC patients diagnosed at early stages have higher survival rates compared to those diagnosed with advanced stages (Bruix & Llovet, 2009). Therefore, standardized surveillance is recommended for patients at risk of HCC development. Surveillance involves a repeated screening of patients at high risk of HCC. The effectiveness of surveillance depends on the incidence of HCC in the target population as well as the availability of effective diagnostic tests and treatments (Verslype, Rosmorduc, Rougier, & Group, 2012).

Previous studies have indicated that surveillance increases the survival when the yearly rate of HCC is more than 1.5% of the target population (Sarasin, Giostra, & Hadengue, 1996). According to the guidelines indorsed by the American association for the study of liver diseases (AASLD) and the European society for medical oncology (ESMO), HCC Surveillance includes a mandatory abdominal ultrasound screening every 6 months (El-Serag, 2012b; Verslype et al., 2012).

1.1.4 Staging and therapeutic management

Currently, there are several treatment options available for HCC patients. These treatments could be either curative or palliative. The curative treatment options include; liver transplantation, surgical resection and local ablation. These options have shown a 5-year survival rates of up to 75% (El-Serag, Marrero, Rudolph, & Reddy, 2008; Lin, Hoffmann, & Schemmer, 2012). Nonetheless, due to the small number of eligible patients to such treatments (less than 20%), most of the patients are exposed to

palliative treatments such as transarterial chemoembolization as well as chemotherapy (Clark et al., 2005; J. A. Davila, Duan, McGlynn, & El-Serag, 2012).

The choice of the optimal therapy is decided according to the tumour stage. Currently, several staging systems have been developed to assess the stage of HCC. Both the Child-Pugh system and the model for end-stage liver disease (MELD) are used to evaluate the disease's severity without dealing with the patient's symptoms or performance (Bruix & Sherman, 2011; Jelic, Sotiropoulos, & Group, 2010).

The Barcelona Clinic Liver Cancer (BCLC) staging system aims to link the disease stage with the patient's performance, and it is the best to be used for selection of the proper therapy for HCC patients (Bruix & Sherman, 2011; J. Llovet & Ducreux, 2012). Usually, this system is used to identify patients with early-stage HCC (stage 0 and A) who are suitable for curative therapies, patients at intermediate or advanced stages (stages B&C) who are suitable for palliative treatments as well as patients with stage D HCC who have a very poor life expectancy (Lencioni, Chen, Dagher, & Venook, 2010).

Liver transplantation (LT) is the best possible curative treatment choice for HCC as well as the underlying cirrhosis(de Lope, Tremosini, Forner, Reig, & Bruix, 2012). It was shown that liver transplantation has resulted in a better overall survival rate compared to other options (Waghray et al., 2015). Thus, HCC patients with complicated cirrhosis should be assessed for their eligibility for such treatment. To assess the patient's eligibility to liver transplantation, three selection criteria are used [Milan Criteria, University of California San Francisco (UCSF) and MELD (Mazzaferro et al., 1996; Pelletier et al., 2009). According to the Milan criteria, patients that have a single lesion(< 5 cm) or a maximum of 3 lesions (with each < 3cm), without any metastasis or vascular invasion are good candidates for LT (Mazzaferro et al.,

2011). Patients meeting these criteria have shown a 4-year overall survival rate of 85% and a recurrence rate of less than12% (Kim & Hemming, 2009; Mazzaferro et al., 2011).

In 2011, the UCSF criteria has expanded the eligibility requirements, and included patients present with a single lesion (< 6.5 cm) or 3 lesions (with each one < 4.5 cm), and a total tumor diameter that is equal to or less than 8 cm. This selection criterion has shown comparable survival rates to the Milan criteria (Leung et al., 2004).

The MELD score system aims to prioritize the patient's assignments to the LT waiting list. Each HCC patient has a MELD score of 22 which increases every 3 months. The MELD score is able to expect the mortality rates among patients with cirrhosis. However, due to its inability to predict the mortality rates among non-cirrhotic HCC patients, an exception criteria was created to give extra points for HCC patients. In fact, applying this criteria have resulted in a rise of the number of HCC patients undergoing LT (Taniguchi, 2011).

Surgical resection is considered as the best treatment choice for early stage-HCC patients having single lesions without any underlying cirrhosis (Vennarecci et al., 2007). However, despite having a five-year survival rate of 70%, patients undergoing surgical resection have a high recurrence risk. In fact, an early recurrence within two years of surgery usually occurs due to an intrahepatic metastasis and local invasion. (Cucchetti et al., 2009; Xia et al., 2010).

In the last decade, tumor ablation was used in treating HCC and has resulted in highly satisfactory results. Radiofrequency ablation (RFA) is considered as the best ablative therapy used for early stage HCC patients that are unsuitable for liver transplantation nor surgical resection (Nishikawa, Kimura, Kita, & Osaki, 2013). Patients with tumors of less than two cm diameter were reported to have the best results

when treated with RFA. In fact, these patients showed 90% chance of complete ablation upon receiving this treatment (Nishikawa et al., 2013).

Transarterial chemoembolization (TACE) is the best therapy choice for patients with intermediate-stage HCC with a single lesion of less than 5 cm without any vascular invasion or hepatic metastasis (Forner, Llovet, & Bruix, 2012). It is based on the obstruction of blood supply to the tumor by introducing several chemotherapeutic agents such as doxorubicin, cisplatin and epirubcin (Forner et al., 2012). TACE- based therapy has resulted in a 25% decrease in tumor size in almost 40% of the patients(J. M. Llovet & Bruix, 2003).

Systemic chemotherapy using cytotoxic drugs such as tamoxifen, cisplatin and everolimus have shown low response rates (Verslype et al., 2012). In fact, until now, Sorafinib was shown to be the only effective systemic therapy in advanced HCC (Bolondi et al., 2015). Sorafinib is an oral multi kinase inhibitor that works by blocking several cellular kinases involved in tumorigenesis such as vascular endothelial growth factor (VEGF) receptors, platelet-derived growth factor receptors (PDGFRs), c-KIT and RaslMAPK signalling pathways (S. Wilhelm et al., 2006; S. M. Wilhelm et al., 2004). In a 2- phase III randomized placebo-controlled trials conducted in America, Europe and Asia, Sorafinib was found to have an anti-proliferative, anti-angiogenic as well as pro-apoptotic activity. In addition, using Sorafinib has resulted in 44% increase in the overall survival and a 3 months increase in the median survival among HCC patients (Cheng et al., 2009; J. M. Llovet et al., 2008).

1.1.5 Molecular alterations in HCC

Regardless of the availability of several surveillance and management tools of HCC, the exact molecular pathogenesis behind HCC remains unclear (Aravalli, Steer, & Cressman, 2008). Thus, research is focusing currently on identifying the key molecular

mechanisms underlying the development of HCC with the hope of developing new novel therapeutic strategies.

Several oncogenes and tumor suppressors involved in the cell cycle and apoptosis are known to be associated with HCC development as a result of their mutations. p53 gene is the most commonly mutated gene in HCC, where it was found to be mutated at advanced HCC stages compared to earlier ones (Oda, Tsuda, Sakamoto, & Hirohashi, 1994; Qin et al., 2002). The p16 (CDKN2A) tumor suppressor gene is known to be involved in the cell cycle regulation, and was shown to be suppressed in HCC due to its hypermethylation. Mainly, p16 suppression occurs more frequently in advanced HCCs (40%) than in early HCC cases(A. Hui et al., 1996). Other HCC-associated deregulated oncogenes and tumor suppressors include β-catenin, CTNNB1, ARID2, ARID1A, MLL, ErbB family members and E-cadherin (Farazi & DePinho, 2006; Fujimoto et al., 2012).

Deregulation of several signal transduction pathways also contributes to the development of HCC. Among these signalling pathways is the Wnt/β-catenin, RaslMAPK/EGFR,IGF and AKT/PKB(Guichard et al., 2012; Wong & Ng, 2008).

All of the above mentioned molecular alterations are more frequent in advanced stages of HCC than in early stages. Thus, currently, the research is directed towards identifying new molecular markers associated with early stages of HCC. Among these are the heat-shock 70 (hsp70) and adenylate cyclase-associated protein 2(CAP2). In fact, both these genes were found to be detected in early HCC stages in addition to their high expression in advanced stages(Chuma et al., 2003; Shibata et al., 2006).

Other molecular markers detected in the blood are used for HCC diagnosis. Alpha-fetoprotein (AFP) is the most commonly used HCC tumour marker (Debruyne & Delanghe, 2008). Among its three forms (AFP-L1,AFP-L2 and AFP-L3), AFP-L3 has

the highest sensitivity and specificity percentages in HCC detection (96.9%, 92% respectively) (Hiraoka et al., 2015). In addition to AFP, Glypican-3 (GPC3), Transforming growth factor-β1 (TGF-β1), Hepatoma-associated gene (HTA), AFP mRNA and miRNAs were also described as crucial blood diagnostic markers of HCC. (Liu et al., 2010; Lun, Ai-Qin, & Xuan, 2014; Zhi, Zhan, Deng, & Huang, 2007).

1.2. Cofactor of BRCA1

1.2.1 Identification, Structure and Function

Cofactor of BRCA1 (COBRA1) was first isolated from a human ovary cDNA library and identified as a novel interacting protein with the breast cancer susceptibility gene (BRCA1)(Ye et al., 2001). Previous studies have identified the role of BRCA1 in regulating several nuclear processes such as transcription, recombination and DNA repair (Chapman & Verma, 1996) (Lee, Collins, Brown, Lee, & Chung, 2000). These nuclear functions are believed to be mediated by the chromatin de-condensation activity of BRCA1, which occurs in the two BRCT domains required for interacting with several transcription-regulatory factors such as p53, RNA helicase A and CBP/p300 (Ye et al., 2001; Zhong et al., 2004).

In a study aiming to identify cofactors recruited by BRCA1 to mediate chromatin de-condensation, a novel cofactor of BRCA1 (COBRA1) was identified and found to be bound to the first chromatin de-condensation domain (BRCT) of BRCA1 (Ye et al., 2001). The results of the study revealed that BRCA1-mediated chromatin de-condensation is partially dependent on its recruitment of COBRA1. Remarkably, COBRA1 was found to have a sufficient ability to induce a large scale of chromatin de-condensation by itself. Moreover, BRCA1 mutations involved in the enhancement of chromatin de-condensation were found to increase the affinity and recruitment of BRCA1 to COBRA1 (Ye et al., 2001). These results indicated that chromatin

remodelling is a significant step in both BRCA1 and COBRA1-mediated nuclear activities.

COBRA1 encodes a 580-amino acid protein, in which 17% of its constituents are rich in leucine residues(Ye et al., 2001). It contains three repeats of the LXXLL motif. In fact, this motif is a main part of many transcription coactivators, due to its ability of mediating the ligand-dependant interactions with several steroid hormone receptors (Heery, Kalkhoven, Hoare, & Parker, 1997; Ye et al., 2001).

In 2003, COBRA1 was found to represent the β-subunit of the negative transcription elongation factor complex (NELF), a 4-subunit complex that is known by its ability to induce the stalling of RNA polymerase II during the early phase of transcription elongation. The NELF complex is reported to regulate many genes known to be involved in cellular mechanisms such as the cell cycle, differentiation, proliferation and metabolism (Yamaguchi et al., 1999). The role of COBRA1 in early embryogenesis was identified later through *in vivo* studies, where it was found that knocking out COBRA1 in the mouse leads to early embryonic lethality (Amleh et al., 2009).

Having no DNA binding domain, COBRA1 could repress the transcription of its target genes through interacting with other DNA-binding transcription factors such as the remaining members of the NELF complex, AP1 complex, ERα as well as the androgen receptor (AR)(S. E. Aiyar et al., 2004; J. Sun, Blair, Aiyar, & Li, 2007; Zhong et al., 2004). This ability of COBRA1 to interact with different transcription complexes, suggests its involvement in regulating different cellular processes including transcription, gene expression and tumorigenesis. Below is an overview of some of the main roles of COBRA1 in transcription regulation and tumorigenesis.

1.2.2 COBRA1 role in transcription regulation

The transcription process (controlled by RNA polymerase II) is a crucial step by which several genes and biological processes are controlled (Narita et al., 2003). During the elongation phase, shortly after the transcription initiation, RNAPII escapes from the promoter-proximal region and pauses 20-45 nucleotides downstream the transcription start site, influencing by this, the efficiency of transcription and hindering the transition to the productive elongation phase. This process is known as 'promoter proximal pausing or RNAPII stalling', and was found to be associated with a wide variety of biological systems (Adelman & Lis, 2012; Gilchrist et al., 2008; Narita et al., 2003). The regulation of RNA stalling requires the involvement of negative and positive transcription elongation factors.

DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole) sensitivity-inducing factor (DSIF) is a heterodimeric complex composed of p14 and p16. Along with the NELF complex, it is responsible for the transcriptional pausing process (Narita et al., 2003; Wada et al., 1998). Previous studies have revealed that DSFI doesn't have an effect on the catalytic activity of RNAPII upon binding to it (Yamaguchi et al., 1999). Similarly, NELF doesn't have a binding affinity to either DSIF or RNAPII alone. Instead, the DSIF/RNAPII complex is required for such binding to occur. This association is believed to activate the transcriptional pausing process(Yamaguchi, Inukai, Narita, Wada, & Handa, 2002).

P-TEFB, a protein kinase composed of Cdk9 and one of the cyclin complexes T1, T2a, T2b and K, is believed to reverse the transcription elongation inhibition by preventing the action of both DSIF and NELF(Price, 2000; Yamaguchi et al., 1999). It is believed that the kinase activity of P-TEFB is crucial for stimulating the transcriptional elongation. Through phosphorylating the C-terminal domains of

RNAPII (CTD), release of both DSIF and NELF from RNAPII is likely to occur. As a result, the binding of 3' elongation factors is facilitated, resulting in stimulation of the elongation process (Bourgeois, Kim, Churcher, West, & Karn, 2002; Ping & Rana, 2001; Price, 2000).

Despite the involvement of the RNAPII stalling process in a variety of biological mechanisms, at first, only few genes were reported to be regulated by this process such as the mammalian proto-oncogene *junB*, Drosophila *Hsp70*, and HIV RNA(Wu et al., 2005). The fact that only few genes were associated with the RNA stalling process has led many to view it as a rare phenomenon. However, a genome-wide analysis of Drosophila genes has changed this view by identifying more than 18000 Drosophila genes associated with RNA stalling in their promoter regions(Muse et al., 2007; Zeitlinger et al., 2007). Similarly, a study of RNAPII distribution in human cells has identified the importance of the RNA stalling step in regulating the expression of many genes known to have a role in the development (Guenther, Levine, Boyer, Jaenisch, & Young, 2007; Muse et al., 2007; Zeitlinger et al., 2007).

All of these studies have increased the interest into understanding and identifying the mechanisms by which the RNAPII stalling process is regulated. In a microarray analysis of NELF regulated genes in drosophila, NELF was found to affect the expression of many genes involved in a variety of cellular processes (Gilchrist et al., 2008). Unexpectedly, most of the target genes have shown reduced levels upon NELF knockdown, suggesting a role of NELF-dependent RNA stalling in enhancing the gene expression in addition to suppressing it. This observation was explained by the ability of the RNA stalling process of sustaining the chromatin architecture at the promoters regions of these genes (Gilchrist et al., 2008).

1.2.3 COBRA1 interaction with other transcription factors

Having no DNA binding domain, COBRA1 could repress the transcription of its target genes through interacting with other DNA-binding transcription factors such as the remaining members of the NELF complex, the Estrogen receptor α (ERα), the Androgen receptor (AR) as well as the activator protein-1 (AP-1) complex (S. E. Aiyar et al., 2004; J. Sun et al., 2007; Zhong et al., 2004).

In 2003, COBRA1 was found to represent the β-subunit of the negative transcription elongation factor complex (NELF)(Narita et al., 2003). The negative elongation factor (NELF) is a transcription regulatory complex that was identified by its ability to induce stalling of RNA polymerase II (RNAPII) with the assistance of the DRB sensitivity-inducing factor (DSIF) during the early phase of transcription elongation (Yamaguchi et al., 1999).

The NELF complex consists of four subunits; NELF-A (66kDa), NELF-B (62kDa) NELF-C/D (60kDa) and NELF-E (46kDa). NELF-A gene (WHSC2), which was identified as a potential participant in the Wolf-Hirschhorn syndrome, contains an RNA polymerase II binding domain, through which the NELF complex binds to RNA polymerase II. NELF E, which has an RNA recognition domain, is responsible for binding to the emerging RNAs arising from polymerase II. NELF-C/D, which are homologous to the TH1-like mammalian protein, are believed to arise from a common mRNA through alternative usage of translation initiation codons. Hence, either a C or a D subunit is present in the NELF complex (Gilchrist et al., 2008; Narita et al., 2003).

Along with the NELF B subunit (COBRA1), all of these subunits are required for the assembly of a functional NELF complex. In fact, several studies have identified the interdependent manner by which the NELF subunits are regulated, where it was

reported that a depletion in one of these subunits results in a similar effect on the remaining subunits (Narita et al., 2003; J. Sun et al., 2008).

Both Estrogen receptor α (ERα) and androgen receptor (AR) are DNA-binding transcription factors that belong to the steroid hormone receptor family. These factors are believed to control the transcription of genes by either a ligand-dependent or independent means (Nilsson et al., 2001). The strength of such a transcription process is highly influenced by the availability of cognate hormones in addition to the recruitment of several transcription co-regulators (Glass & Rosenfeld, 2000; Shang, Hu, DiRenzo, Lazar, & Brown, 2000). Most of which are believed to influence the rate of transcription by modifying the chromatin structure as well as facilitating the hormone receptors communication with the transcription machinery(Glass & Rosenfeld, 2000; McKenna & O'Malley, 2002).

In addition to these regulatory processes, transcription elongation and RNA processing are also linked with gene expression control (Auboeuf et al., 2004; Orphanides & Reinberg, 2000; Zorio & Bentley, 2001). Previous *in vivo* studies have indicated a novel role of COBRA1 as a corepressor of the ER-α dependent gene expression through stalling of RNAPII (S. E. Aiyar et al., 2004). Upon Estrogen stimulation, COBRA1 along with the other NELF subunits binds to ERα in breast cancer cells and is recruited to the promoters of ER target genes, resulting in RNAPII stalling and suppression of the ERα-mediated transcription(S. E. Aiyar et al., 2004). This regulation of the transcription has introduced a unique level of hormonal gene regulation which is diverse from most of the known steroid receptors co-regulators actions(J. Sun et al., 2007).

The majority of nuclear receptors (NR) co-regulators have the ability of binding to several receptors as well as mediating the regulation of transcription. This capability is

mainly explained by the presence of many NR-binding motifs 'LXXLL' within their structures(Heinlein & Chang, 2002; McKenna & O'Malley, 2002). Having this structural feature, COBRA1 was found to bind to several steroid hormone receptors with diverse affinity degrees. In fact, COBRA1 was found to bind to the androgen receptor (AR), progesterone receptor B (PRB) and glucocorticoid receptor (GR). Among these three nuclear receptors, AR was reported to have the strongest binding affinity to COBRA1. In fact, through its binding to the ligand binding domain (LBD) of AR, COBRA1 was found to regulate the AR-dependent transcriptional processes.

In addition to binding to the NELF subunits and steroid hormone receptors, COBRA1 was also found to interact with the transcription factor activator protein 1(AP-1), a heterodimer composed of c-Fos and c-Jun family proteins (Hess, Angel, & Schorpp-Kistner, 2004). The role of COBRA1 in regulating the AP-1 transcriptional activity was investigated by Zhong et al in 2004, where it was proved for the first time that overexpressing COBRA1 represses the activator protein 1(AP1) transcriptional activity. On the other hand, reduction of COBRA1 via siRNA enhances the AP-1 transcriptional activity. This action of COBRA1 was attributed by its ability to bind to both c-Fos and c-Jun family members. Particularly, the interaction with the c-Fos members is essential for this inhibitory action, where the lack of the c-Fos binding site in COBRA1 completely stops the inhibitory effect of COBRA1 on the AP1-mediated transcriptional activity (Zhong et al., 2004). Given that AP-1 is known to be involved in many biological processes including cell fate and oncogenesis, COBRA1 was proposed to have a function in regulating these processes through AP-1 signalling.

1.2.4 Role in Cancer

The role of COBRA1 in cancer was first driven by its identification as a BRCA1-interacting nuclear protein, and its potential role in regulating nuclear events such as transcription, cell cycle, DNA repair and cell proliferation(Zhu et al., 2004). In addition, the role of COBRA1 in regulating the AP1- mediated transcriptional activity and ligand-dependant gene expression, which are both involved in cancer development, has raised the possibility of its involvement in cancer (S. E. Aiyar et al., 2004; J. Sun et al., 2007; Zhong et al., 2004).

To date, COBRA1 role in the tumorigenesis of breast and gastrointestinal carcinoma has been investigated and identified. In breast cancer, previous studies have identified COBRA1 as a tumor suppressor. Upon the identification of its ability to interact with BRCA1 in 2001, the mRNA and protein levels of COBRA1 were found to be reduced in different breast cancer cell lines (Zhu et al., 2004). In addition, a physical interaction between BRCA1 and COBRA1 was observed in these cells, indicating its possible involvement in the regulation of breast cancer growth (Zhu et al., 2004). Moreover, COBRA1 was found to physically bind to the ligand binding domain (LBD) of ERα and inhibits the estrogen-dependent transcription in breast cancer cells (S. E. Aiyar et al., 2004).

In line with the effect of COBRA1 in the regulation of breast cancer growth, another study done by Sun et al., (2008) has demonstrated a tumor-suppression role of COBRA1 in breast cancer. In this study, it was found that COBRA1 expression is significantly reduced in metastatic breast cancer samples (J. Sun et al., 2008). These observations go in line with another study that revealed that COBRA1 can regulate the expression of genes associated with breast cancer progression and metastasis such as the trefoil factor1 gene (TFF1) (S. E. Aiyar et al., 2004; Smid et al., 2006).

Contrasting its role in breast cancer, COBRA1 was found to act as an oncogene in the upper gastrointestinal carcinomas (UGCs). In fact, COBRA1 was found to be overexpressed in 60% of primary UGG samples compared to normal samples (McChesney et al., 2006). Unlike breast cancer, COBRA1 overexpression was accompanied by down regulation of TFFI (McChesney et al., 2006). This contradictory role of COBRA in breast and gastrointestinal carcinoma is believed to be due to its interaction with different site-specific transcription factors. Thus, regulating distinct transcription processes in different cell and tissue types (Adams et al., 2005; McChesney et al., 2006; J. Sun et al., 2008).

The role of COBRA1 in other cancer types has not been identified yet. However, it's worth mentioning that a previous tissue array study has indicated a significant expression of COBRA1 in the epithelial of many tissues, indicating the possibility of its involvement in the tumerogenousis in tissues other than breast and upper gastrointestinal tract (J. Sun et al., 2008). Given the role of COBRA1 in influencing the alternative splicing pattern of receptor genes, current research is directed towards identifying cancer-associated alternative splicing patterns of COBRA1-regulated genes. Moreover, due to its role as a modulator of androgen-dependent transcription in addition to the importance of androgen in the development of prostate cancer, COBRA1 was suggested to have a role in the tumorigenesis of prostate cancer. However, further studies to confirm these notions are required.

1.2.5 Role in Hepatocellular Carcinoma

In hepatocellular carcinoma, Previous studies have revealed a potential role for COBRA1 in HCC pathogenesis (Youssef, Shawer, Afify, & Amleh, 2016). In addition, other studies have established a possible link between COBRA1 and several proteins known to be involved in tumorigenesis and anti-apoptotic pathways (El Zeneini et al., 2016). In this study, COBRA1 was knocked-down. As a result, the growth and migration of HCC cells were significantly decreased. In addition, a decrease in the expression of the proliferation marker; Ki-67 as well as the anti-apoptotic gene; survivin was also observed (El Zeneini et al., 2016).

HYPOTHESIS AND SPECIIC OBJECTIVES

The above-mentioned data obtained from ongoing research suggests strongly a positive role of COBRA1 in the migratory and proliferation potentials of HCC. Therefore, we hypothesize that COBRA1 overexpression will have a role in both the proliferation and migration potentials of HCC cells. To address this hypothesis, our study had the following three objectives:

1. To establish a successful COBRA1-overexpression in the HCC cell line, HepG2. HepG2 represents a pure cell line of Hepatocellular Carcinoma. It is derived from a 15-year old Caucasian, American male with early stage HCC (Costantini, Di Bernardo, Cammarota, Castello, & Colonna, 2013; Fornari et al., 2010; Yie et al., 2015).
2. To examine the levels of the NELF complex subunits as well as the NELF complex-regulated genes, TFF1 and TFF3 following COBRA1 overexpression.
3. To investigate the effect of COBRA1 overexpression on the proliferation and migration of HepG2 cells as well as the expression of tumorigenesis-related genes.

CHAPTER 2. MATERIALS AND METHODS

2.1. Cell Culture

The human hepatocellular carcinoma cell line, HepG2, was kindly provided by Dr. Mehmet Ozturk from the Department of Molecular Biology and Genetics, Bilkent University, Turkey. The cells were cultured in RPMI 1640 (Lonza, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, USA) and 5% Penicillin-streptomycin antibiotic (Invitrogen, USA). Cells were incubated at 37°C in a humidified atmosphere of 5% CO2 and used when in the log phase of growth. Cells were regularly observed by the inverted microscope (Olympus IX70, USA) for their morphology, growth, lack of any biological contamination and the percentage of confluence.

2.2. Viable Cell Count

The cells viability was estimated using the trypan blue dye-exclusion method, where an aliquot of the cells was two-fold diluted using 0.4% w/v trypan blue. 10 ul of the diluted cell suspension was loaded into the haemocytometer's chamber. The number of cells in the chamber's four outer squares was then counted. To determine the cell count, the following formula was applied:

[Viable cell count (live cells/ml) = (Number of live counted cells x dilution factor x 10,000) / Total number of counted squares].

2.3. Plasmid constructs

2.3.1 pCMV5-HCOBRA1Plasmid

The pCMV5-HCOBRA1 expression plasmid was generously provided by Dr.Rong Li, University of Texas Health Science Centre, San Antonio-USA. To generate pCMV5-HCOBRA-1, EcoRI and SalI enzymes were used to clone the 5`UTR+CDS of

HCOBRA1+flag to the 4.7 Kb sized pCMV5 vector, resulting in a plasmid of the size of 6.6 Kb. The plasmid was shipped on a filter paper and recovered by immersing in TE buffer followed by transforming into Top10 competent bacterial cells.

2.3.2 pCMV5-empty plasmid

In order to be used as a negative control in the transfection process, an empty pCMV5-plasmid was prepared. Briefly, pCMV5-HCOBRA1 was digested with EcoRI and SalI enzymes. The empty vector (4.7 fragment) was purified using QIAquick Gel Extraction Kit. The 5` overhangs resulted from the digestion were blunted by filling in with the Klenow fragment of DNA polymerase I. One unit of klenow was added per 1 µg of DNA, followed by incubation at 25°C for 15 minutes and heating at 67 °C for 20 minutes. The blunted vector was then re-ligated using T4 DNA ligase. Correct ligation was confirmed by restriction linearization using BamH1 enzyme as well as the colony PCR technique. The ligation reaction was then transformed into Top10 competent cells, and glycerol stocks of the vector were prepared.

2.3.3 pEGFP-N1 Plasmid

The pEGFP-N1expression plasmid was provided by Dr. Ahmed Osman, Ain Shams University, Egypt. The expression vector was originally purchased from Clontech (http://www.clontech.com).

2.4. Transformation

In order to have larger amounts of plasmid DNA for further experiments, bacterial cells were transformed with the acquired plasmids and propagated in bacterial medium prior to preparation of glycerol stocks and harvesting for DNA.

Top10 competent cells were transformed with the suitable plasmids. 50 µl of the cells were first thawed on wet-ice. 1-5 µl of the plasmid-DNA were added to the competent cells and mixed gently. The mixture was left on ice for 30 minutes followed

by an immediate heat-shock at a temperature of 42 ºC for 30 seconds. Following this, 900 µl of pre-warmed LB media were added to the mixture. The vial containing the cells was then incubated at 37 ºC for 1 hour. 50 µl of the transformation mixture were plated on LB agar plates that contain 100 µg/ µl ampicillin. The plates were then incubated at 37 ºC overnight. A single colony was selected the next day and placed in a culture containing 5 ml of LB media with 100 µg/µl ampicillin. Following an overnight incubation in a 37 ºC shaker, the cells were harvested and the plasmid DNA was extracted using Mini Prep Kit (Qiagen).

To prepare glycerol stocks, 600 µl of the liquid culture were added to 400 µl of 50% glycerol and stored at -80 ºC for further use.

2.5. Restriction digestion

Restriction enzymes were used to confirm the identities of the newly obtained plasmids. EcoR1 and SalI restriction enzymes were used to digest the pCMV5-HCOBRA1 vector. EcoRI was used to linearize the pCMV5-COBRA1 and the pEGFP.N1vectors. SalI was used to linearize the pEGFP.N1vector. BamH1was used to linearize the pCMV5-empty vector. The digestion reaction was performed in a final volume of 20 µl, consisting of 15 µl distilled water, 2 µl of 10 X NEBuffer 3.1to a final concentration of 1X, 0.25 µg of the extracted plasmid DNA and 1 µl of each enzyme. The linearization reaction was performed in a final volume of 20 µl, consisting of 15 µl distilled water, 2 µl of 10X NEBuffer EcoRI or 10 X NEBuffer 3.1 to a final concentration of 1X, 0.25 µg of the extracted plasmid DNA and 1.8 µl of EcoRI, SalI or BamH1 enzyme. The reaction was gently mixed and incubated for 1 hour at 37 ºC. The undigested plasmids, linearized plasmids and digested plasmids were run on a 1% agarose gel and visualized using Gel Doc EZ System (Bio-Rad, USA).

As EcoRI cuts the pCMV5-HCOBRA1 plasmid once at the 0.92 Kb position, a 6.557 Kb fragment will be expected. As SalI enzyme cuts the plasmid once at the 0.968 position, when used together with EcoRI, two bands of the sizes 4.609 Kb and 1.9 Kb will be expected on the agarose gel. As BamH1 cuts the plasmid once at the 0.98 Kb position, a 4.657 Kb fragment will be expected. As EcoRI cuts the pEGFP.N1 plasmid once at the 0.629 Kb position, a 4.733Kb fragment will be expected. As SalI enzyme cuts the plasmid at 0.639 Kb position, a 4.733 Kb fragment will be expected.

2.6. Colony PCR

The identity of the pCMV5-HCOBRA1 and pCMV5-empty vector was further confirmed by the colony PCR technique. A single colony obtained following an overnight culture of pCMV5-HCOBRA1 and/or pCMV5-empty transformed bacterial cells was suspended in 20 µl distilled water and boiled at 95 ºC. The PCR reaction for colony analysis was prepared in a final volume of 25 µl consisting of the followings: 2.5 µl of 10X DreamTaq Green Buffer(Thermo Scientific), 0.5 µl dNTPs mix (Thermo Scientific), 0.2 µl Dream *Taq* DNA Polymerase (Thermo Scientific), 1 µl of forward and reverse specific primers, 1 µl of the colony suspension as well as H2O. The PCR conditions were as follows: initial denaturation at 95ºC for 4 minutes, followed by 32 cycles of (denaturation at 95ºC for 30 seconds, annealing at 59.5ºC for 30 seconds and extension at 72ºC for 45 seconds) before finishing with a final extension at 72ºC for 7 minutes. The amplified PCR products were run on a 1.5% agarose gel and visualized using Gel Doc EZ System (Bio-Rad, USA).

2.7. Cell transfection

Transient transfection of HepG2 cells with pCMV5-HCOBRA1 was performed using the lipofectamine 3000 reagent (Life Technologies) according to the manufacturer's protocol. Briefly, HepG2 cells were seeded in six-well culture plates,

and incubated for 24 hours until reached 70-90% confluence. Before transfection, 3.75 µl of lipofectamine 3000 were added to 125 µl of Opti-MEM reduced serum medium, and incubated for 5 minutes at room temperature. Meanwhile, 2.5 µg pCMV5-HCOBRA1 was diluted in 125 Opti-MEM reduced serum medium and 5 µl of P3000 reagent. Following this, the two dilutions were mixed and incubated for 20 minutes to allow formation of the complex. The DNA-lipofectamine complexes (250 µl) were then added to each well of the 6-well plate. Media was changed 8 hours post transfection. 48 hours following transfection, cells were harvested for RNA and protein analysis.

As controls, cells were either left un-transfected (blank) or transfected with an empty vector (pCMV5-empty vector). The pEGFP.N1 vector was used as a positive control to determine the transfection efficiency. Forty eight hours following transfection, the florescent cells were visualized by the Olympus IX70 fluorescence microscope using the GFP filter set. Likewise, the total number of cells in the same field were visualized using the bright filter set. Photo-microscopy was done using cellsens imaging software (Olympus IX70). Cell counts were performed using ImageJ Software (National Institute of Health, USA, http://www.imagej.nih.gov/ij). The transfection efficiency was then estimated according to the following formula:

Tansfection efficiency = (florescent cells per field /total number of cells per field) X 100'

2.8. RNA Extraction

Total RNA was extracted from HepG2 cells using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The extracted RNA was suspended in diethlpyrocarbonate –treated (DEPC) water. Following this, the concentration of the extracted RNA was measured at 260 nm using an UV spectrophotometer (Shimadzu, Japan).

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

Total RNA (0.5 µg) was reverse transcribed using a Revert Aid First strand cDNA synthesis Kit (Thermo Scientific, USA). Messenger RNA expressions were determined using semi-quantitative RT-PCR. The PCR reaction was prepared by mixing the following reagents: 10X DreamTaq Green Buffer (Thermo Scientific), 10 mmol/L dNTPs mix) (Thermo Scientific), 1X Dream *Taq* DNA polymerase (Thermo Scientific), forward and reverse specific primers and water. β-actin was used as an internal control. The primers used for the amplification of the genes are listed in Table (1). PCR amplifications conditions were programmed for 5 minutes at 95°C, followed by cycles of (denaturation at 95ºC for 30 seconds, annealing at 56ºC-63ºC depending on the used primers for 30 seconds, extension at 72ºC for 45 seconds) and finishing with 7 minutes at 72ºC. The amplification process was carried out for a number of cycles depending on the used primers (Table 1). The PCR products were then separated on a 1.5% agarose gel and visualized using Gel Doc EZ System (Bio-Rad, USA).

Table 1. RT-PCR primer sequences, annealing temperatures, cycle numbers and amplicon sizes (F: forward primer, R: reverse primer, bp: base pair)

Gene	Primer Sequence	Annealing Temp.	Cycle number	Amplicon size (bp)
B-ACTIN	F: GCAAAGACCTGTACGCCAAC R: GAGACCAAAAGCCTTCATACATCTC	58°C	27 cycles	777
COBRA1	F: ACATCACCAAGCAGAGGAA R: GATCCAGCTGTTCCAGCTTC	59.5°C	32 cycles	366
Survivin	F: TTGAATCGCGGGACCCGTTGG R: CAGAGGCCTCAATCCATGGCA	61˚C	32 cycles	Isoform 1: 477 Isoform 2: 359 Isoform 3: 546
NELF-A	F: GTCGGCAGTGAAGCTCAAGT R: TTCACACTCACCCACCTTTTCT	60˚C	35 cycles	250
NELF-C/D	F: GAAGAAGGAGAGACCCCAGC R: GTGCCCAAGGCTAGTGTGAT	56˚C	28 cycles	443
NELF-E	F: TGGTGAAGTCAGGAGCCATCAG R: CGCCGTTCAGGGAATGAATC	63˚C	28 cycles	565
Ki-67	F: CTTTGGGTGCGACTTGACG R: GTCGACCCCGCTCCTTTT	60˚C	28 cycles	199
TFF1	F: TTTGGAGCAGAGAGGAGGCAATGG R: TGGTATTAGGATAGAAGCACCAGGG	60˚C	32 cycles	240
TFF3	F: GTGCCAGCCAAGGACAG R: CGTTAAGACATCAGGCTCCAG	58˚C	35 cycles	302

2.10. Western blot Analysis

Total cell lysates were prepared using ice-cold PBS and laemmli lysis buffer supplemented with 1X Halt Protease Inhibitor Cocktail (Thermo Scientific, USA). The concentrations of proteins were determined using Thermo Scientific Pierce BCA protein kit (Pierce Biotechnology, USA) according to the manufacturer's protocol.

Equivalent amounts (20-50 μg) of proteins diluted in a lysis buffer were mixed with the loading dye and separated on a 12 % SDS-PAGE. Consequently, they were blotted to a nitrocellulose membrane. Following blocking with 5% non-fat dry milk in 1X PBST

(0.01% Tween-20 in PBS) at room temperature for 1 hour, the membrane was incubated with the primary antibody at 4 ºC overnight. Following three times washing (5 minutes each) with the wash solution (1 X PBS and 0.1% Tween), the membrane was incubated with alkaline phosphatase conjugated secondary antibody (either goat anti-rabbit IgG (KPL) or goat anti-mouse (KPL) diluted as 1:20,000 in 5% non-fat milk) at RT for two hours, and washed 3 times with the wash solution. For detection, the membrane was incubated with the chemiluminescent PhosphoGLO Substrate (55-60-04, KPL) for 5 minutes prior to exposing it to an X-ray film in the dark. Signals were then detected by developing the film in developer and fixer solutions. Anti-β-tubulin (Sigma, T7816) (1:20,000 in 5% non-fat dry milk), anti-COBRA1 (Abcam, ab167401) (1:1000 in 5% non-fat dry milk) and anti-NELF-E (Abcam ab170104) (1:1000 in 5% non-fat dry milk) were used as primary antibodies in this study.

In order to use the same membrane for detection of other proteins, the membranes were incubated in the stripping buffer (0.5 M Tris-HCl,10% SDS and beta-mercaptoethanol) at 55 °C for 30 minute prior to excessive washing with water to remove any beta-mercaptoethanol traces. The membranes were then blocked with 5% non-fat dry milk in 1X PBST before incubating with another primary antibody.

2.11. Wound-healing assay

The wound-healing assay was used to evaluate the migratory potentials of HepG2 cells. Forty-eight hours post transfection, the cells monolayer was scraped by a yellow pipette-tip creating a cross-shaped wound. The cells were then washed with PBS to remove any detached cells and incubated for 24 hours. Images of the same wound location were obtained at both the (0 hr) and the (24 hr) times. The open wound area was analysed using the TScratch software (Gebäck, Schulz, Koumoutsakos, &

Detmar, 2009), and the percentage wound closure was calculated according to the formula:

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

2.12. Statistical analysis

For PCR and Western blot analysis, the bands intensities were quantified and normalized per the used internal control using Image J software (National Institute of Health, USA, http://www.imagej.nih.gov/ij). Relative differences in gene expression are described as fold change to the empty vector-transfected cells.

All statistical analysis were performed using GraphPad Prism 7.0 (GraphPad, San Diego California USA, http://www.graphpad.com/). All the data represent the average ± standard deviation (SD) from three independent experiments. When comparison was made between two different groups, statistical significance was determined using an unpaired student's t-test (two-tailed). One-way ANOVA (Analysis of Variance), followed by a Bonferroni post-test was used to determine the statistical significance among multiple different experimental groups. In all analyses, $P < 0.05$ was considered statistically significant (* p <0.05; ** p value <0.01; *** p value <0.001).

CHAPTER 3. RESULTS

3.1. Confirmation of plasmids identities

Before using the plasmids in the transfection assays, their identities were confirmed by performing restriction digests on the plasmids and comparing the obtained fragments sizes with those expected from the corresponding plasmid restriction map. The pcMV5-HCOBRA1 plasmid restriction digestion by both EcoRI and SalI resulted in two bands of the sizes 4.609 and 1.9 Kb as expected from the restriction map. EcoRI digestion resulted in a linearized plasmid with the expected band size of 6.6 Kb (Figure 1A). The pCMV5-empty plasmid restriction digestion by BamH1 resulted in a linearized plasmid with the expected band size of 4.657 Kb (Figure 1B). The pEGFP.N1 plasmid restriction digestion by EcoRI resulted in a linearized plasmid with the expected band size of 4.7 Kb. As such, pEGFP.N1 plasmid restriction digestion by SalI resulted in a linearized plasmid with the expected band size of 4.7 Kb (Figure 1C).

The pCMV5-HCOBRA1 and the pCMV5-empty plasmids identities were further confirmed through colony PCR. Amplification of the colony obtained from the pCMV5-HCOBRA1 transformed cells resulted in an amplicon of the expected size of 366 bp. On the other hand, amplification of the colony obtained from pCMV5-empty plasmid resulted in no bands (Figure 1D).

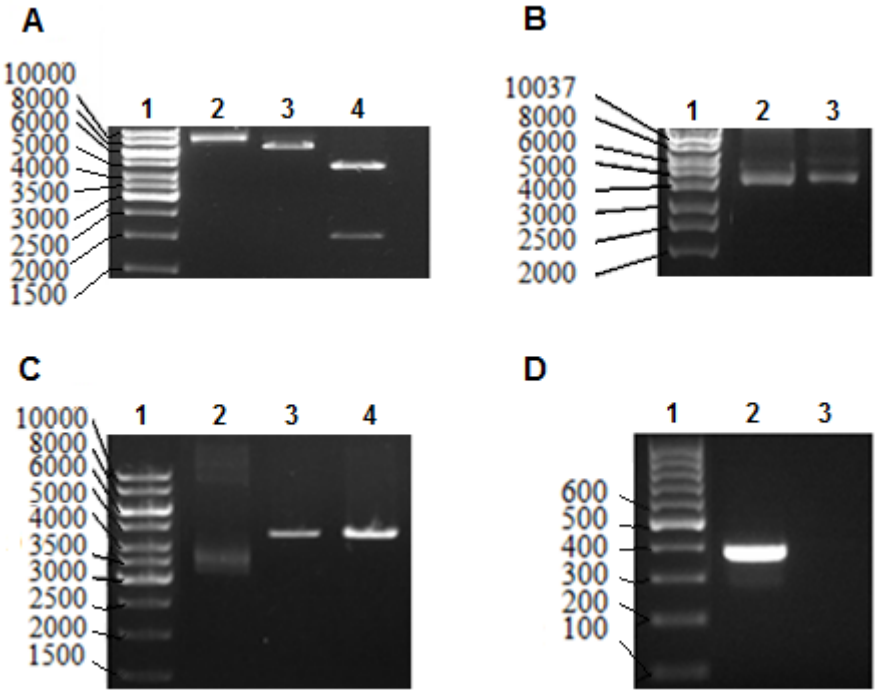


Figure 1. Confirmation of plasmids identities
Lane 1 shows GeneRuler 1 Kb ladder (A) pCMV5-HCOBRA1 restriction digests: Lane 2 shows the uncut plasmid. Lane 3 shows an EcoRI digestion resulting in a band of the approximate size of 6.6 Kb. Lane 4 shows EcoRI & SalI digestion resulting in two bands of the sizes of 4.609 and 1.9 Kb. (B) The pCMV5-empty plasmid restriction digests: Lane 2 shows the uncut plasmid. Lane 3 shows BamH1 digestion resulting in a band of the size of 4.657 Kb. (C) pEGFP-N1 restriction digests: Lane 2 shows the uncut plasmid. Lane 3 shows EcoRI digestion resulting a band of the size of 4.7 Kb. Lane 4 shows SalI digestion resulting in a band of the size of 4.7 Kb. (D) Colony PCR: colonies obtained from pCMV5-HCOBRA1 transformed cells resulted in a 366 bp amplicon (Lane 2). Colonies obtained from pCMV5-empty plasmid resulted in no bands.

3.2. Optimal protocol for HepG2 cells transfection

To determine if lipofectamine 3000 is effective in overexpressing of COBRA1 in HepG2 cells, we first optimized the best protocol to be used in transfecting HepG2 cells. A series of optimizations were conducted per the manufacturer's recommendations. We first compared the transfection efficiency between both the reverse and forward transfections. Then, we attempted to determine the most suitable lipofectamine volume to be used in the transfection. Finally, we examined whether increasing the incubation time of the cells following transfection can further enhance the transfection efficiency.

3.2.1. Reverse transfection versus Forward transfection

Both reverse and forward transfections were used to overexpress COBRA1 in HepG2 cells. Usually, when using the forward transfection, cells are seeded 24 hours prior to transfection. On the other hand, in the reverse transfection, the cells are added directly to the transfection mix(Erfle et al., 2007).

Overexpression efficiency was compared between both reverse and forward transfections based on the percentage of the green florescent (pEGFP.N1+) cells. As shown in figure 2 A, the forward transfection resulted in a higher efficiency (45%) than that observed with the reverse transfection (20%). Accordingly, the forward transfection was used in the subsequent transfection experiments.

3.2.2. Lipofectamine volume

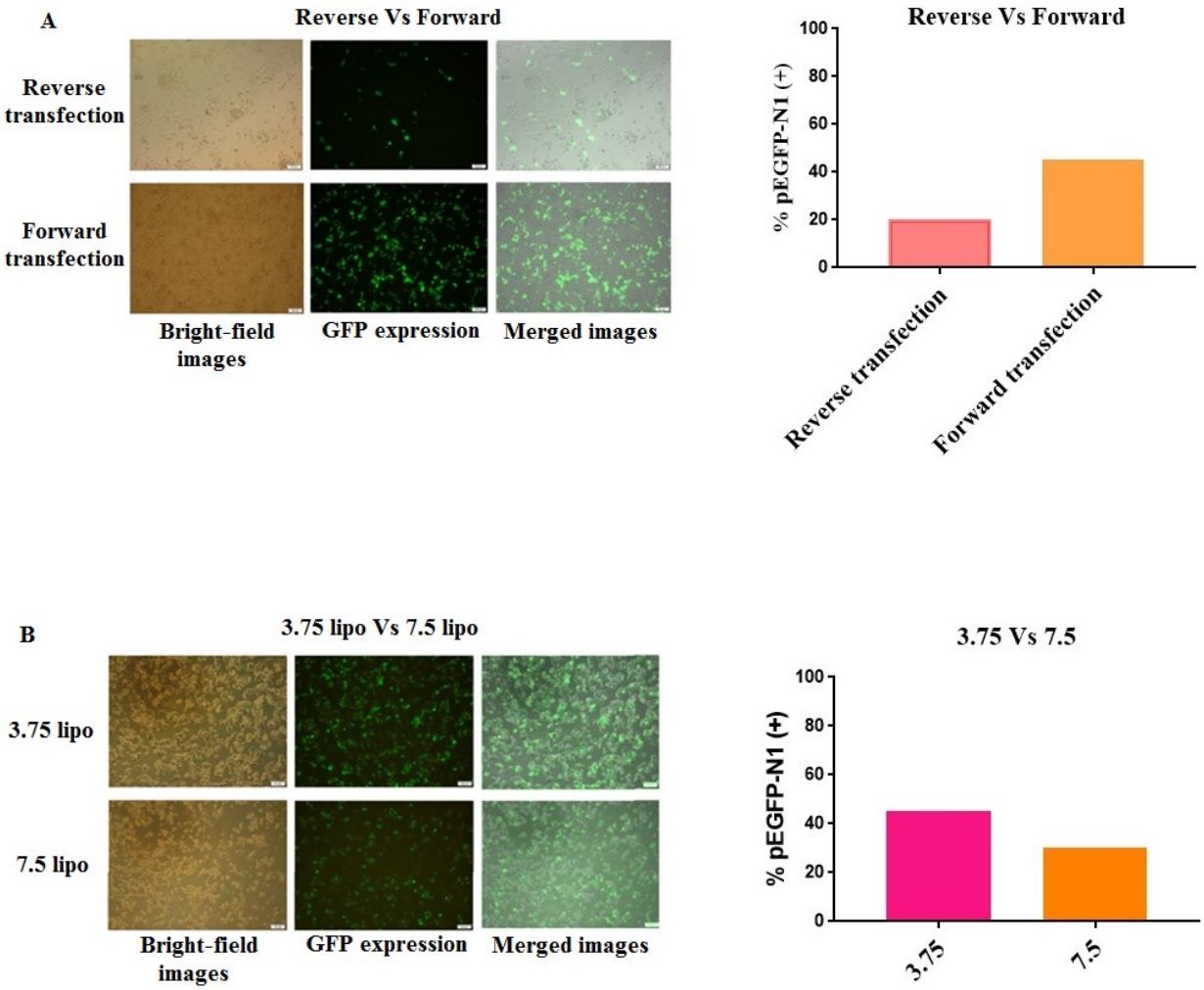
Two lipofectamine volumes (3.75 µL and 7.5 µL) were used to form lipofectamine-DNA complexes as recommended by the supplier. Transfection using 3.75 µL lipofectamine resulted in a transfection efficiency of 45 %. On the other hand, transfection using 7.5 µL lipofectamine resulted in a transfection efficiency of 30%. Using this volume, detrimental cytotoxicity was observed following the transfection when compared to un-transfected cells (Figure 2 B).

Beside the GFP expression, COBRA1 overexpression was also compared between the two tested volumes. As shown in figure 2 C&E, cells transfected with 3.75 µL of lipofectamine showed the highest overexpression on both the mRNA steady state expression and protein levels. Due to its higher transfection efficiency as well as low cytotoxic effects, the 3.75 µL lipofectamine was used in the subsequent transfections.

3.2.3. Incubation duration

We further optimized our protocol by examining the effect of increasing the incubation duration of cells following transfection. Transfection efficiency was

analysed at both 24 hr and 48 hr post-transfection. As shown in figure 2 E, incubating the cells for 48 hours resulted in a higher transfection efficiency (60%), compared to (45%) observed after 24 hours incubation. Accordingly, cells were incubated for 48 hours in the following transfections.



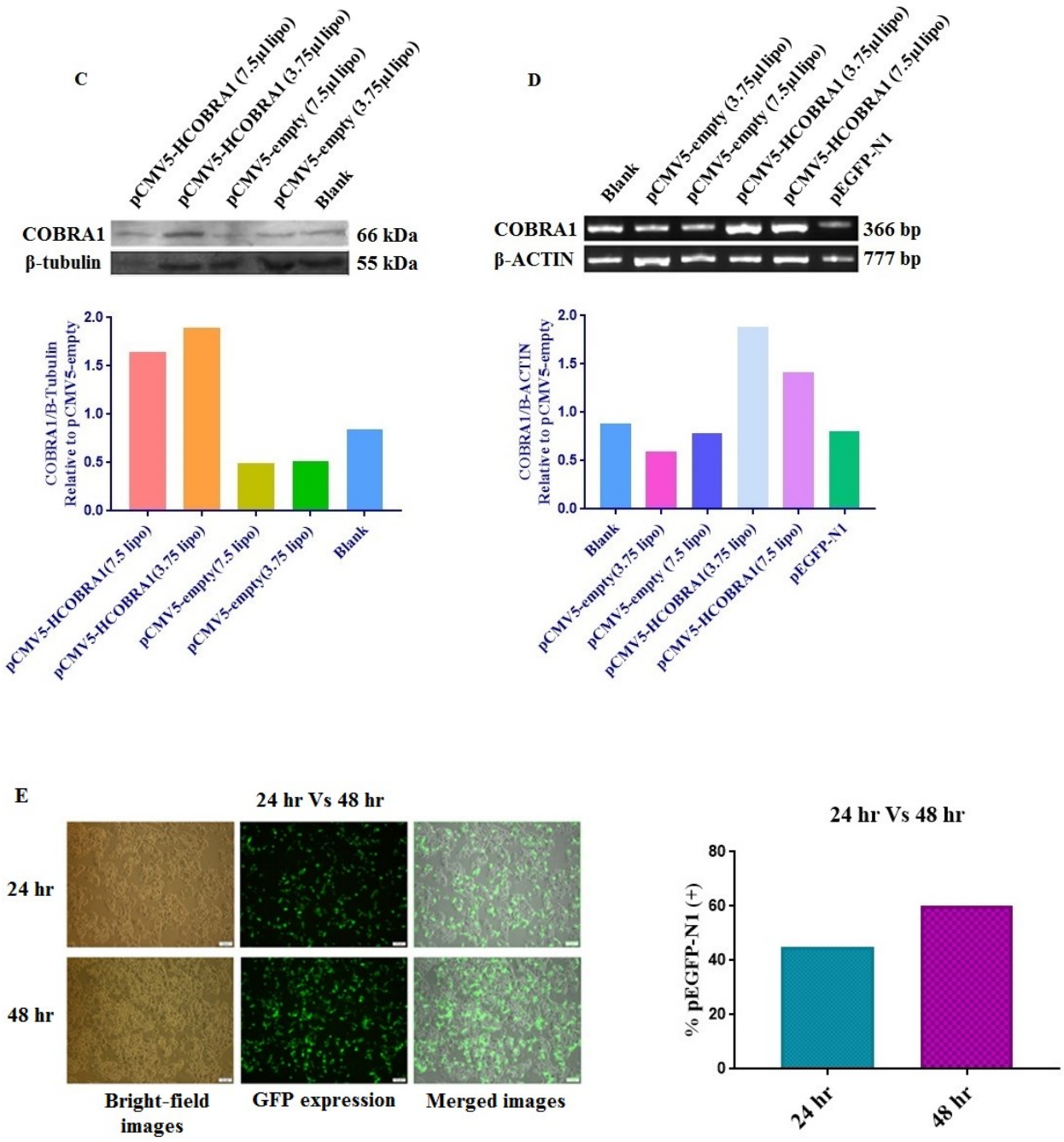


Figure 2. Optimization of COBRA1 transfection in HepG2 cells

The optimization of the transfection process is based on the percentage of the green florescent (pEGFP-N1+) cells, RT-PCR and western blot analysis (A) Comparison between the transfection efficiency (pEGFP-N1+ cells) of reverse and forward transfection 24 hrs post transfection. The forward transfection resulted in an efficiency of 45% compared to 20% observed with the reverse transfection. (B) Lipofectamine volume. Two lipofectamine volumes of (3.75µl and 7.5 µl) were used and compared to their transfection efficiency based on the green florescent (pEGFP-N1+) cells. 45% transfection efficiency was obtained by using 3.75 µl lipofectamine in comparison to 30% obtained with 7.5 µl lipofectamine. (C) Comparison between the two lipofectamine volumes based on western blot analysis. (D) Comparison between the two lipofectamine volumes based on COBRA1 mRNA expression using semi-quantitative RT-PCR. The bands intensities in (C) and (D) were measured and normalized to their corresponding bands of the internal control (β-actin, β-tubulin) using image J software. (E) Incubation duration. Transfection efficiency was analysed at both 24 hr and 48 hrs post transfection and compared based on the green florescent (pEGFP-N1+) cells. Incubating the cells for 48 hrs resulted in a higher transfection efficiency (60%) compared to (45%) observed after 24 hrs incubation.

3.3. COBRA1 transfection efficiency and effect on cells morphology

Upon using lipofectamine 3000 in the transfection, cells transfected with the pCM V5-HCOBRA1 plasmid showed a significant higher COBRA1 protein expression ($P < 0.001$) compared to the control groups (Figure 3 A). Similarly, COBRA1 mRNA steady state expression was also significantly increased compared to the control groups as evidenced by the semi-quantitative RT-PCR ($P<0.001$) (Figure 3B).

To determine whether COBRA1 overexpression alters the morphology pattern of HepG2 cells, photos of the pCMV5-HCOBRA1- transfected cells were compared to those obtained from the other control groups (pCMV5-empty-transfected, pEGFP.N1 transfected and un-transfected cells). No significant difference in the cell morphology among all the tested groups was observed, indicating that COBRA1 doesn't induce any morphological changes in HepG2 cells (Figure 4).

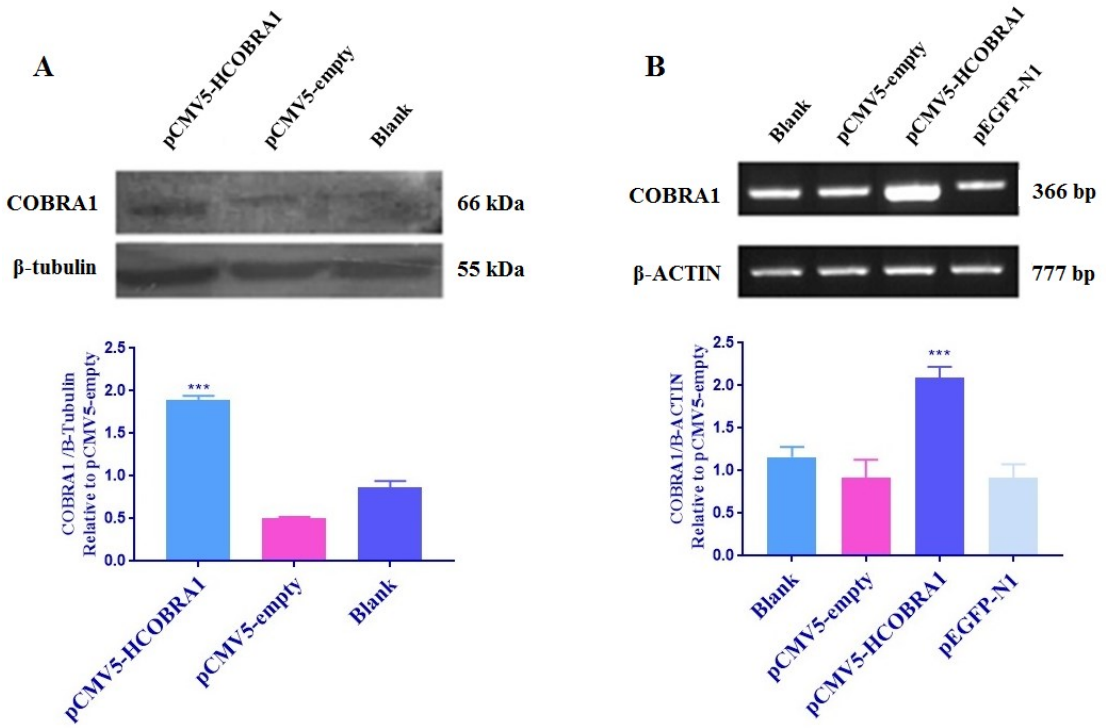


Figure 3. COBRA1 significantly overexpressed at both mRNA and protein levels
(A) Western blot analysis of COBRA1 protein expression in pCMV5-HCOBRA1-transfected cells, relative to pCMV5-empty vector-transfected cells (B) Semi-quantitative RT-PCR analysis of COBRA1 mRNA steady state expression in pCMV5-HCOBRA1- transfected cells, relative to pCMV5-empty-transfected cells. A significant overexpression of COBRA1 on both mRNA and protein levels was observed. The intensities of the bands in (A) and (B) were measured then normalized to the loading control by image J software. Data represents the average ± SD of three independent experiments. Statistically significant at *** $p < 0.001$. (One-way ANOVA, Bonferonni's post-test).

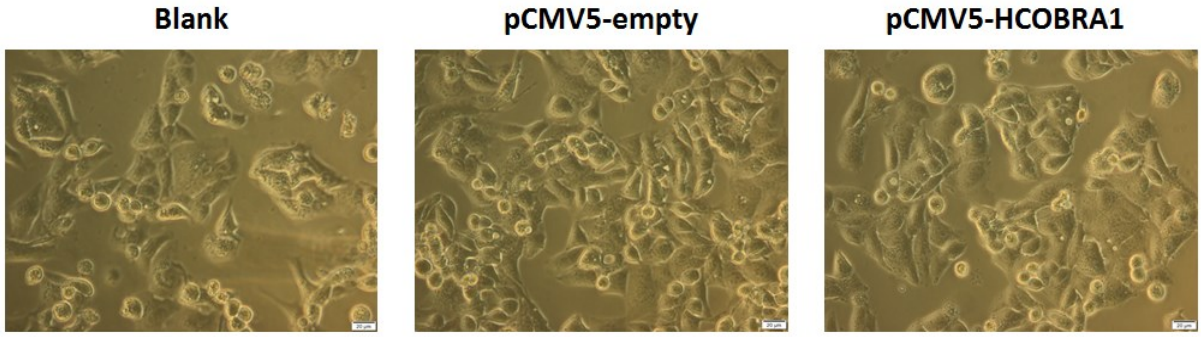


Figure 4. The morphology pattern of HepG2 cells following COBRA1 overexpression.
No changes on the cells morphology were observed following COBRA1 overexpression. Photos were obtained using phase contrast at 40X magnification.

3.4. mRNA and protein levels of the NELF subunits following COBRA1 overexpression

To determine whether COBRA1 overexpression influences the expression of the other NELF subunits, the mRNA levels of NELF-A, NELF-C/D and NELF-E were compared between COBRA1 transfected cells and the negative controls using RT-PCR. As shown in figure 5 A, the mRNA levels of the three subunits showed comparable levels with no statistically significant difference noticed among them (P> 0.05) (Figure 5A). Similarly, when examined by western blot analysis, no significant difference in NELF-E protein levels was noticed in pCMV5-HCOBRA1-transfected cells compared to the control groups (p > 0.05) (Figure 5B).

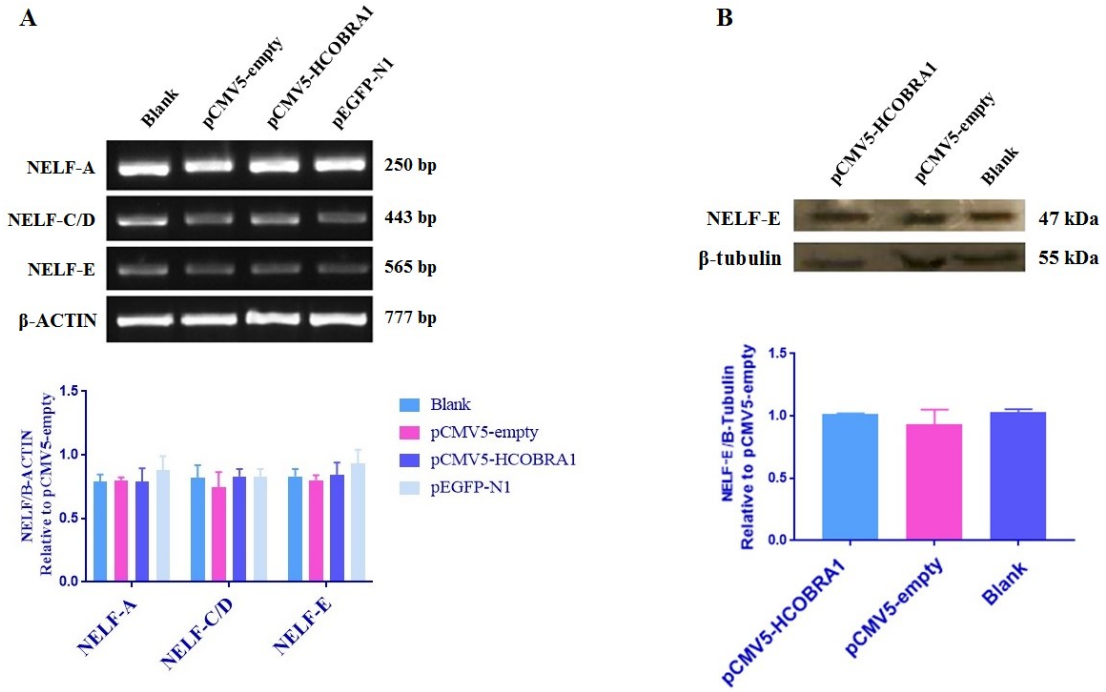


Figure 5. The expression of NELF-A, NELFC/D and NELF-E subunits upon COBRA1 overexpression

HepG2 cells were transfected with pCMV5-empty vector, pCMV5-HCOBRA1, pEGFP-N1 or left un-transfected. 48 hrs post transfection, cells were harvested and analysed for mRNA expressions of NELF-A, NELF-C/D and NELF-E by the semi quantitative RT-PCR. (B) HepG2 cells were transfected with pCMV5-empty vector, pCMV5-HCOBRA1 or left un-transfected. 48 hrs post transfection, cells were harvested and analysed for the protein levels of NELF-E by western blot analysis. The intensities of the bands in (A) and (B) were measured then normalized to the loading control by image J software. Data represents the average ± SD of three independent experiments (one-way ANOVA, Bonferonni's post-test) (P>0.05).

3.5. TFF1 and TFF3 mRNA levels following COBRA1 overexpression

Both TFF1 and TFF3 are known to be regulated by the NELF complex (S. Aiyar, Blair, Hopkinson, Bekiranov, & Li, 2007; S. E. Aiyar et al., 2004; Kininis, Isaacs, Core, Hah, & Kraus, 2009). To determine the effect of overexpressing COBRA1 on the levels of TFF1 and TFF3, the mRNA steady state levels of these genes were compared between the COBRA1 transfected cells and the non-transfected cells. Notably, no statistically significant difference in the mRNA levels of both TFF1 and TFF3 was noticed among all the groups (Figure 6).

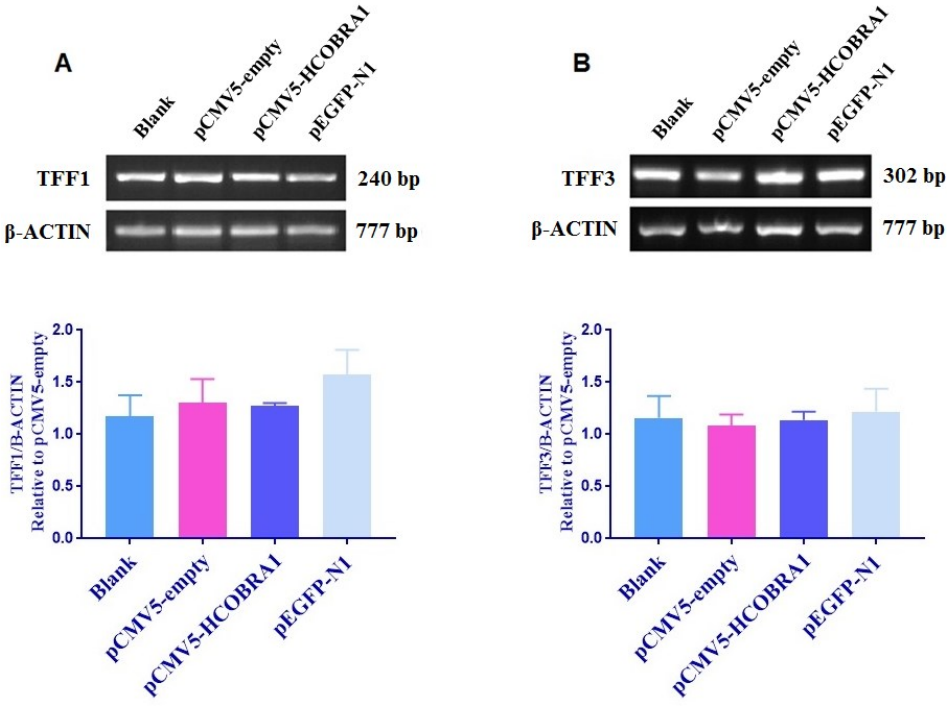


Figure 6. TFF1 and TFF3 expressions following COBRA1 overexpression
(A) Semi-quantitative RT-PCR analysis of TFF1 mRNA levels in pCMV5-empty-transfected cells, pCMV5-HCOBRA1-tranfected cells, pEGFP-N1-tranfected cells and un-transfected cells. (B) Semi-quantitative RT-PCR analysis of TFF3 mRNA levels in pCMV5-empty-transfected cells, pCMV5-HCOBRA1-tranfected cells, pEGFP-N1-tranfected cells and un-transfected cells. The intensities of the bands in (A) and (B) were measured then normalized to the loading control by image J software. Data represents the average ± SD of three independent experiments. (One-way ANOVA, Bonferonni's post-test). ($P>0.05$).

3.6. COBRA1 overexpression effect on the proliferation potential of HepG2 cells

To investigate if overexpressing COBRA1 can promote the cellular proliferation, the growth of cells transfected with either pCMV5-COBRA1 or pCMV5-empty vector was monitored for 4 days following transfection.

COBRA1 transfected cells showed comparable proliferation rate compared to that of those transfected with the empty vector (Figure 7A). These results suggested that COBRA1 overexpression doesn't have a proliferation endorsing role in HepG2 cells. To confirm this notion, we examined the expression of the proliferation marker, ki-67 following COBRA1 overexpression. Ki-67 is known to be associated with cell proliferation. The presence of this protein in the nuclei of cells in the active phases of the cell cycle (G1, S, G2, and mitosis), in addition to its absence from the resting phase (G0), makes of it a good indicator of the cells growth and proliferation (Gerdes & Scholzen, 2000). No significant difference in ki-67 mRNA levels was observed in COBRA1-trnasfected cells when compared to the control cells as evidenced by semi-quantitative RT-PCR ($P > 0.05$) (Figure 7 B).

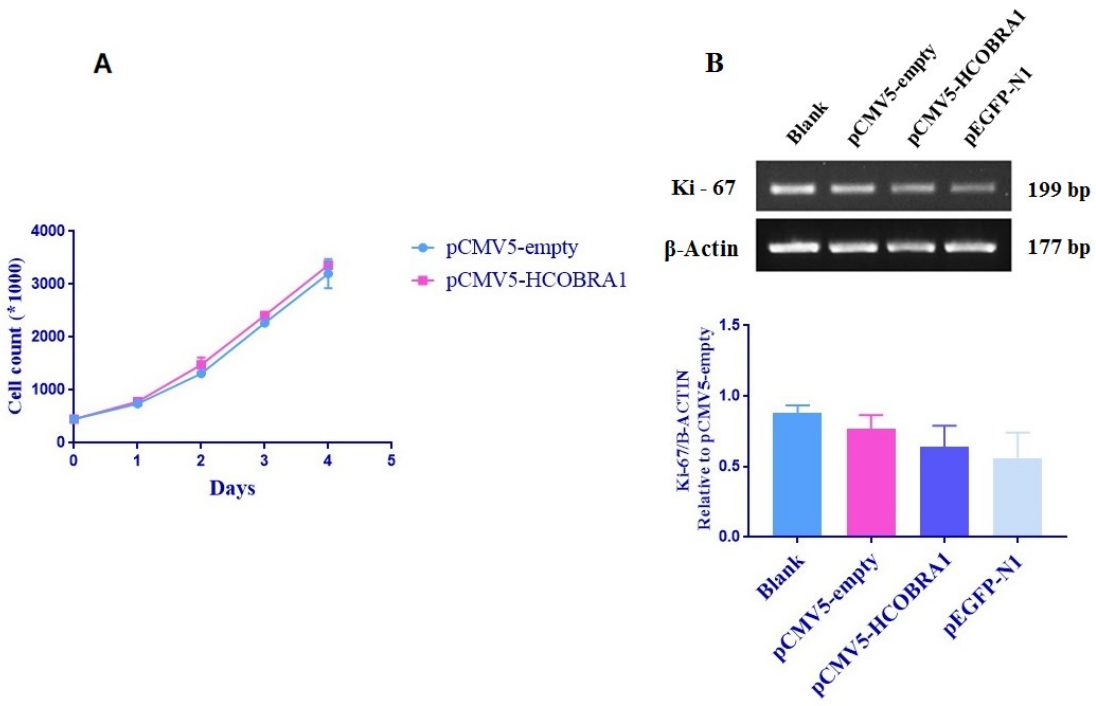


Figure 7. The proliferation potential of HepG2 cells following COBRA1 overexpression
(A) Cell growth curve representing the growth of cells transfected with either pCMV5-HCOBRA1 or pCMV5-empty vector. HepG2 cells were transfected with the pCMV5-empty or pCMV5-HCOBRA1 vector, harvested at 24, 48, 72 and 96 hrs post transfection, and counted using the trypan blue dye-exclusion method. Data represents the average ± SD of three independent experiments (two-way ANOVA, Bonferonni's post-test) (B) Semi-quantitative RT-PCR analysis of Ki-67 mRNA expression in pCMV5-empty-transfected cells, pCMV5-HCOBRA1-tranfected cells, pEGFP-N1-tranfected cells and un-transfected cells. The intensities of the bands were measured then normalized to the loading control by image J software. Data represents the average ± SD of three independent experiments. (One-way ANOVA, Bonferonni's post-test) (P>0.05).

3.7. COBRA1 Overexpression effect on the migratory potential of HepG2 cells

To examine if overexpressing COBRA1 can enhance the migration of HepG2 cells, the wound-healing assay was applied. Both pCMV5-HCOBRA1 and pCMV5-empty plasmids-transfected cells were compared according to their migration rates. Cells transfected with pCMV5-HCOBRA1 closed the scratch wounds at a higher rate than the cells transfected with the pCMV5-empty plasmid. However, the wound closure of both groups showed no statistically significant difference (P>0.05), with a wound closure percentage of 11.3% obtained for pCMV5-empty plasmid-transfected cells and 15.5 % for pCMV5-HCOBRA1plasmid-transfected cells (Figure 8).

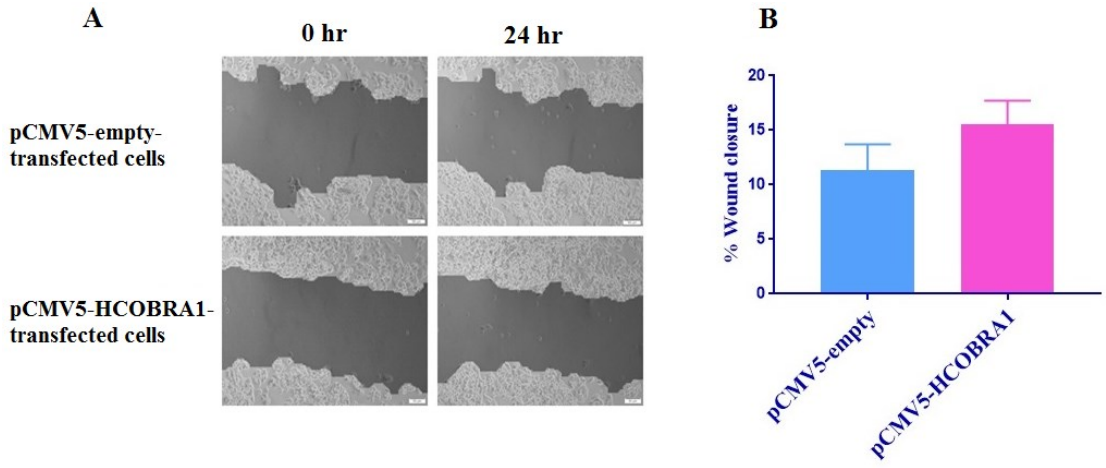


Figure 8. The migratory potential of HepG2 cells following COBRA1 overexpression.
The wound healing assay was used to analyse the migration rate of HepG2 cells following COBRA1 overexpression (A) Images of the wound areas taken at both 0 and 24 hrs times using phase contrast at 10X magnification (B) The percentage of wound closure in pCMV5-HCOBRA1 transfected cells versus pCMV5-empty transfected cells. The open areas were measured using TScratch software followed by calculation of the percentage of wound closure. Data represents the average ± SD of three independent experiments. (Student t-test, two-tailed) ($P>0.05$).

3.8. COBRA1 Overexpression effect on the survivin gene expression

To determine if overexpressing COBRA-1 could affect the expression of genes known to be deregulated in cancer, RT-PCR was carried out to examine the mRNA expression of the survivin gene following COBRA-1 overexpression. The survivin gene is known to be deregulated in cancer, and was shown to play key roles in the survival and proliferation of cancer cells (Fukuda & Pelus, 2006). As shown in Figure 9, upon COBRA1 overexpression, no statistically significant difference in the levels of the 3 survivin transcripts (survivin-2B, survivin-deltaex3 and the wild type survivin) was noticed compared to the control groups ($P>0.05$).

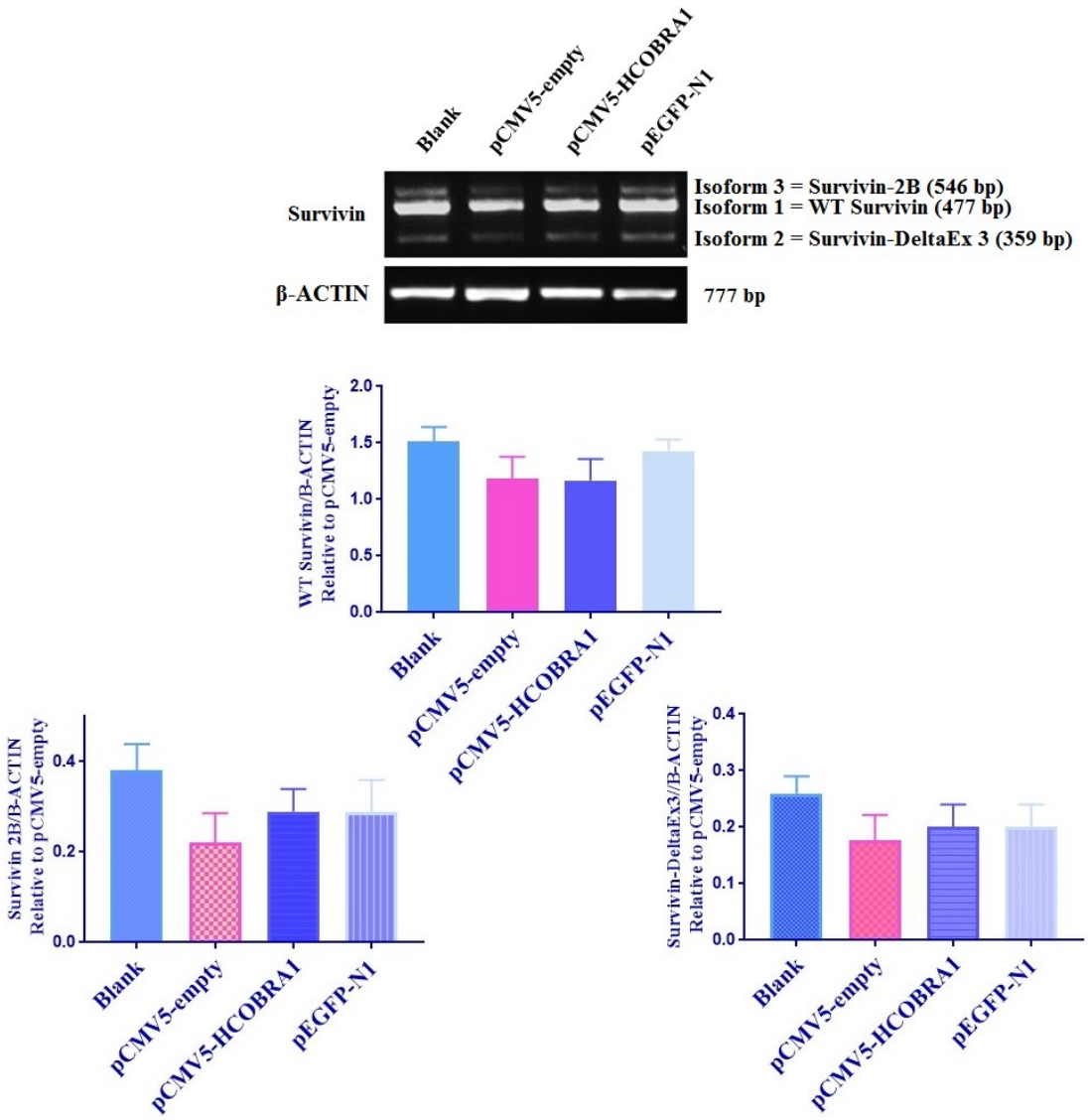


Figure 9. survivin mRNA expression following COBRA1 overexpression.
HepG2 cells were transfected with pCMV5-empty, pCMV5-HCOBRA, pEGFP-N1 or left un-transfected. 48 hrs post transfection, total RNA was extracted and analysed for survivin mRNA expressions using semi quantitative RT-PCR. The intensities of the bands were measured then normalized to the loading control by image J software. Data represents the average ± SD of three independent experiments. (One-way ANOVA).

CHAPTER 4. DISCUSSION

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths worldwide, accounting for around 746,000 deaths annually(Flores & Marrero, 2014). Despite the improvements in HCC therapy, almost 80% of the patients were reported to proceed to advanced stages with poor prognosis(C. Sun et al., 2013; Yin et al., 2012). Therefore, identifying the molecular mechanisms and biomarkers involved in HCC pathogenesis is essential for a better management of the disease.

One of the biomarkers that was shown to be involved in HCC is Cofactor of BRCA1 (COBRA1). Previous studies have demonstrated a potential role for COBRA1 in HCC pathogenesis (Youssef et al., 2016), and established a conceivable link between COBRA1 and several proteins known to be involved in tumorigenesis including cell proliferation and anti-apoptotic pathways (ElZeneini et al., 2016). In the current study, we extended the previous studies by elucidating the link between COBRA-1 overexpression and HCC tumerogenesis.

4.1. Co-dependent stability of the NELF subunits

The multi-subunit negative elongation factor (NELF) complex consists of four subunits (NELF-A, NELF-B, NELF-C/D and NELF-E). It was reported that all of the four subunits are essential for the formation of the functional complex. Along with NELF C/D, NELF-B (COBRA1) acts as a core of the NELF complex to bring both NELF-A and NELF-E together, resulting in a functional complex (S. E. Aiyar et al., 2004; Narita et al., 2003). This explains the interdependent manner by which the NELF complex works. In fact, several studies have previously reported a simultaneous recruitment of the NELF subunits upon the binding of COBRA-1 to its ER-α regulated target genes(S. E. Aiyar et al., 2004).

Our RT-PCR analysis results showed comparable mRNA expressions of the NELF subunits following COBRA1 overexpression. These results correlates with the previously suggested involvement of post transcriptional mechanisms in the regulation of the NELF complex interdependent expression(J. Sun et al., 2008).

Following COBRA1 overexpression, the protein levels of NELF-E showed similar expressions with those observed in the control groups. These results are supported by another study, in which ectopic expression of COBRA-1 in T47D breast cancer cells resulted in an increase in the protein levels of COBRA1, with no change observed in the expression of the other NELF subunits(J. Sun et al., 2008). These findings indicate the tight control that governs the stability of the NELF complex under normal cellular perspectives.

In light with the previously reported co-dependent regulation of the NELF complex, previous studies have reported a similar expression of different NELF subunits in the same cancer type. This was reported in breast cancer, where both NELF-B and NELF-C/D were found to have decreased levels in the advanced breast cancer cells(J. Sun et al., 2008). This pattern was also observed in another study, in which both NELF-E and NELF-B were reported to be associated with the tumorigeneses of gastrointestinal carcinoma (McChesney et al., 2006). Hence, it would be of interest to examine the roles of different NELF subunits in HCC development.

4.2. Trefoil factors (TFF1 & TFF3) expressions relative to COBRA1 overexpression

Trefoil factor family (TFF) members, TFF1 (pS2), TFF2 (SP) and TFF3 (ITF) are small mucin associated proteins that are highly conserved and tandemly clustered within a region of 55 kb on the human chromosome 21(Terada, Sakagami, Tabuchi, & MAEDA, 2001). They are normally expressed in the upper gastrointestinal tract. In fact, several studies have indicated the involvement of the trefoil factor family (TFF) genes in the gastrointestinal tract repair and defence mechanisms(Taupin, Kinoshita, & Podolsky, 2000; Terada et al., 2001).

It was reported that both TFF1 and TFF3 are regulated by the NELF complex (S. Aiyar et al., 2007; S. E. Aiyar et al., 2004). In fact, previous studies have indicated that NELF-B (COBRA1) overexpression negatively regulates the expression of these genes (S. Aiyar et al., 2007; McChesney et al., 2006). Our findings showed a comparable mRNA levels of both TFF1 and TFF3 between COBRA1-transfected cells and non-transfected cells. In this regard, it is vital to point out the functional entity by which the NELF complex modulate the gene expression. Previously, it was reported that COBRA1 mediated repression of ER-α controlled genes is dependent on the presence of other NELF subunits (S. E. Aiyar et al., 2004). Our results didn't reveal an increase in the expression of the other NELF subunits upon COBRA1 overexpression. This suggests that COBRA1 mediated suppression of TFF1 and TFF3 expression is dependent on the expression of the other NELF subunits. Thus, we speculate that overexpressing the other NELF subunits is likely to significantly affect TFF1 and TFF3 genes expression.

Since there are many transcription-binding sites in the promoters of both TFF1 and TFF3 genes, it is also possible that the expression of these genes is influenced by other transcription factors beside the NELF complex. This notion is supported by another

study done on UGC cells. In this study, site-directed mutagenesis of the AP-1 complex binding sites, to which COBRA1 binds and controls the expression of TFF1, didn't alter the expression of TFF1, indicating that TFF1 expression regulation is not exclusive to COBRA1 (McChesney et al., 2006). Furthermore, it is crucial to mention that in hepatic cancer, the regulation of TFF1 expression was reported to be mediated by estrogen stimulation (Barkhem, Haldosén, Gustafsson, & Nilsson, 2002; Jakacka et al., 2001). In fact, this regulation depends on the interaction between both the ER and the AP-1 complex. Thus, it is possible that COBRA1 regulation of TFF1 is dependent on both estrogen stimulation and AP-1 complex. Accordingly, we hypothesize that, because under the applied experimental conditions, estrogen was at its basal levels, COBRA1 regulation of TFF1 expression was altered, leading to the observed results. However, in order to confirm this notion, further studies should investigate whether estrogen treatment will enhance COBRA1 control of TFF expression.

4.3. COBRA-1 overexpression effect on the proliferation potential of HepG2 cells

One of the essential traits of cancer cells is their ability to sustain chronic proliferation. Hence, proliferation is considered as one of the main trademarks of cancer (Hanahan & Weinberg, 2011). Previously, COBRA1 involvement in the proliferation of different cancer types was reported. In breast cancer, a significant reduction of the proliferation of breast cancer cells was noticed following COBRA1 Overexpression (S. E. Aiyar et al., 2004). In addition, the role of COBRA1 in ovarian cancer proliferation was reported. Pohl et al, 2005 have reported a noteworthy decrease in the proliferation of ovarian cancer cells upon the inactivation of the Ras/MAPK pathway. Being identified as one of the targets of this oncogenic pathway, COBRA1 expression was also significantly downregulated (>3-fold)(Pohl et al., 2005).

Our results haven't indicated any significant role of COBRA1 overexpression in enhancing the proliferation of HepG2 cells, as evidenced by both the cell growth and the ki-67 mRNA expression. These findings might appear somewhat unexpected, given that our group has previously demonstrated a significant decrease of HepG2 cells proliferation following COBRA1 knock-down (El Zeneini, 2016). This is most likely explained by the tight regulation of the NELF complex. In fact, previous studies have demonstrated an interdependent regulation of the NELF subunits, where a knockdown of any of the NELF subunits results in a co-depletion of the other NELF subunits(Narita et al., 2007; J. Sun et al., 2008). Thus, it is possible that upon knocking down NELF-B, the proper formation of the NELF complex was hindered, resulting in an altered function of the complex and leading to the observed effect on the cells proliferation.

This raises the possibility that COBRA1 role in the proliferation is dependent on its cooperation with the other NELF subunits, and that overexpressing NELF B is only part of the process of promoting the cells proliferation. Thus, it is conceivable that overexpressing the other NELF subunits would enhance the proliferation-activation ability of COBRA1. In fact, previous studies done on proteins that function within complexes, have indicated the importance of the assembly of all the complex subunits for a proper function of the protein. Wang et al. (2014) reported a similar proliferative phenotype upon both the knock-down and overexpression of one of the three subunits of the SR protein-specific kinase (SRPK) complex. This similar phenotype was explained by the improper assembly of the complex that has resulted in both cases(Wang et al., 2014). Taken together, our data support the notion that COBRA1 overexpression alone is inadequate to promote HepG2 cells proliferation.

It is also worth pointing out that other regulatory pathways might be involved in COBRA1 regulation of the expression of proliferation markers like Ki-67. Nonetheless, further examinations are required before a conclusion could be drawn here.

4.4. COBRA1 overexpression effect on the migratory potential of HepG2 cells

Another fundamental trait of cancer cells is their ability to migrate from the primary tumour to other parts of the body, providing by this a good indicator of cancer progression and metastasis. Thus, studying the migratory potential of cancer cells is usually carried out to obtain a clear picture of the state of cancer progression and prognosis (Hanahan & Weinberg, 2011).

The role of COBRA1 in the migration was previously shown in many cancer types including breast cancer. Sun et al, 2008 have reported a significant low levels in the advanced breast cancer cell lines, suggesting a strong association with metastatic breast cancer. These results go in line with the established role of COBRA-1 as a tumor suppressor in breast cancer(J. Sun et al., 2008).

Here, the wound-healing assay was carried out to investigate COBRA1 overexpression role in the migratory potential of HepG2 cells. Our results showed comparable migratory ability of COBRA1-transfected cells with that observed with the empty plasmid-transfected cells. These results oppose those made previously by our group, in which COBRA1- knockdown was found to significantly decrease the migratory potential of HepG2 cells (El Zeneini, 2016). This could be explained by the previously mentioned tight regulation of the NELF complex, which is likely to function properly within an interdependent manner. Thus, overexpression of COBRA1 alone is not enough to induce the migration of the cells.

It is crucial to point out that our study was done using HepG2 cells, which represent early stages of HCC. Given that the migratory potential of cancer cells indicates the

metastasis and progression state of cancer, it will be worthwhile to include more cells that represent advanced stages of HCC. In this regard, it is worth mentioning that previously, our group have reported a higher migratory ability of the advanced stage-HCC cells; SNU-449 compared to HepG2 cells(Youssef et al., 2016).

4.5. COBRA1 overexpression effect on the mRNA expression of survivin

survivin, one of the members of the inhibitor of apoptosis family (IAP), is known to have high expression levels in cancer compared to normal tissues (Ambrosini, Adida, & Altieri, 1997; Jaiswal, Goel, & Mittal, 2015). The high expression of survivin in cancer is mainly due to its anti-apoptotic activity as well as its involvement in the cell cycle progression. In fact, the expression of survivin is cell cycle-dependant. It has weak expression at the G1 phase, multiplied by 6 in the S phase and exceeding 40 in the G2/M phase (Boidot, Végran, & Lizard-Nacol, 2014).

survivin is encoded by *BIRC5*, a 14.5 Kb gene that consists of 3 introns and 4 exons located in chromosome 17 (Altieri, 2003; Ambrosini et al., 1997). Beside encoding survivin, *BIRC5* encodes four additional splice variants (survivin-2b, survivin-ΔEx3, survivin-3b and survivin-2a), resulting in variants with different functions (Caldas, Honsey, & Altura, 2005; Mahotka, Wenzel, Springer, Gabbert, & Gerharz, 1999). Until now, only WT survivin, survivin-ΔEx3 and survivin-2b functions have been identified. Both WT survivin and survivin-ΔEx3 were reported to have anti-apoptotic activity, In contrast, survivin-2b was found to have a pro-apoptotic activity (Conway et al., 2000). While WT survivin was found to have a role in the cell cycle regulation, both survivin-2b and survivin-ΔEx3 weren't found to have such a role (Noton et al., 2006).

Our findings indicate that all of the three variants of survivin were detected, with a dominant expression observed in the WT survivin. These results are consistent with a previous publication, in which the WT survivin was the dominant mRNA transcript

among the other variants in HCC (Kannangai, Wang, Liu, Sahin, & Torbenson, 2005). In fact, this pattern of expression was also observed in other cancer types such as stomach carcinoma as well as cervical cancer(Krieg et al., 2002; Li, 2003).

Previously, our group has demonstrated a suppression of the expression of survivin upon COBRA1 silencing. Here, our findings didn't demonstrate any significant difference in the expression of survivin among all the tested groups. In this regard, it is valuable to point out that the activation of survivin is mediated by several transcription factors such as STAT3, KLF5 and DEC1(Boidot et al., 2014). In addition to these transcription factors, many signalling pathways were found to be involved in the regulation of survivin expression. Among these are the Phosphoinositide 3-kinase(PI3K)/Akt pathway and the mitogen-activated protein kinase (MAPK) pathway (Dan et al., 2004). In fact, these regulators are activated by different stimuli. For instance, it was reported that the treatment of the SK-BR-3 breast cancer cells with EGF results in induction of the MAPK pathway, increasing by this the expression of survivin (Peng et al., 2006). Thus, it is possible that COBRA1 regulation of survivin is mediated by several stimuli. Given this, we speculate that treating the cells with certain stimuli might induce COBRA1 regulation of survivin. Also, it would be of value to do a microarray analysis to identify COBRA1- regulated genes and specify their relation to survivin regulatory pathways.

In light with the established notion that the expression of survivin is correlated with higher tumor grades(Takashima et al., 2005), it will be interesting to examine COBRA1 effect on the survivin gene using advanced stages- HCC cells. It is also worth mentioning that there is an increasing evidence suggesting the involvement of post-transcriptional pathways in regulating the expression of survivin (Li, 2003). Thus, it is possible that detecting survivin on the protein level might lead to more valuable results.

CONCLUSION

As far as we know, this is the first study to examine the role of COBRA1 overexpression in HCC. In summary, our findings showed that COBRA1 overexpression didn't alter the expression of the other NELF subunits, indicating a tight regulation of the NELF complex under normal cellular circumstances.

The insignificant effect of COBRA1 overexpression on the expression levels of both TFF1 and TFF3 supports the involvement of the other NELF subunits in this regulation, and raises the possibility of the contribution of other transcription factors and regulatory pathways as well.

Furthermore, our findings didn't reveal a significant role of COBRA1 overexpression on the proliferation and migration rates of HepG2 cells. These results suggest that COBRA1 role in mediating the growth and migration of HCC cells is dependent on the expression of the other NELF subunits, indicating the functional entity by which the NELF complex work.

Taken together, this study might serve as a base for further investigation of the role of the NELF complex in the tumorigenesis of HCC.

FUTURE DIRECTIONS

This study was limited by testing one type of HCC cells. Hence, in order to gain a complete picture of the role of COBRA1 in HCC, it will be of value to include more cells that represent different stages of HCC. In addition, analysing the cell cycle distribution as well as the apoptotic index will give more insight about the molecular mechanisms by which COBRA1 overexpression affect HCC. Moreover, Microarray analysis of COBRA1-regulated genes in HCC will assist in determining the regulatory pathways by which COBRA1 control HCC.

Under the applied experimental conditions, the GFP-expressing vector used to determine the transfection efficiency was separate from the COBRA1-expressing vector. Thus, it is recommended to clone COBRA1 into the GFP-expressing vector to gain a more reliable view of the transfection efficiency.

In light of the observation of the importance of the expression of all the NELF subunits in inducing the function of COBRA1, it will be worthy to examine the effect of overexpressing the other NELF subunits beside COBRA1 on both of the growth and migration of HCC cells.

To further investigate the role of COBRA1 in the tumorigenesis of HCC, it will be of interest in the future to include *invivo* experiments.

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