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School of Sciences and Engineering

HELA CELL LINE, A MODEL TO STUDY THE ROLE OF COFACTOR OF BRCA1 IN CERVICAL CANCER

A Thesis Submitted to the
Biotechnology Master's Program

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

By
Noha Saad Abd Elhamied
Bachelor of Pharmaceutical Sciences

Under the supervision of
Dr. Asma Amleh
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May 2018

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

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Has been approved by

Thesis Committee Supervisor/Chair

Affiliation _____

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_____	_____	_____	_____
Dept. Chair/Director	Date	Dean	Date

DEDICATION

To my parents, *Saad Abd Elhamied Mohamed* and *Faten Ahmed Mosleh*; this is
because of you.

Thank you for never letting me give up

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All praise be to Allah

I would like to express my deepest gratitude to my supervisor Dr. Asma Amleh for her continuous guidance and support during my thesis work. I will always be grateful for the time and effort she spent on putting me on the right path of scientific research. Her valuable discussions and suggestions were essential for the progress of the project. I will always be motivated by her dedication to her work and to science.

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HELA CELL LINE, A MODEL TO STUDY THE ROLE OF COFACTOR OF BRCA1 IN CERVICAL CANCER

ABSTRACT

Being one of the four subunits that makes up the Negative Elongation Factor Complex (NELF), Cofactor of BRCA1 (COBRA1); also known as NELF-B, is able to regulate a number of genes involved in cellular proliferation, metabolism, cell cycle progression and DNA repair. In addition, COBRA1 was shown to interact with other transcription factors such as BRCA1, AP-1 complex and several nuclear receptors. Despite the evidences that suggest COBRA1 as a potential player involved in the progression of a number of cancers, its role in cervical cancer has not been previously investigated. To date, it has been studied in breast, upper gastrointestinal and liver cancers. The main objective of our study was to investigate the potential involvement of COBRA1 in cervical cancer progression. We first did *in-silico* analysis of the expression patterns of COBRA1 in cervical cancer tissues relative to normal cervical tissues using the publicly available Oncomine Cancer Microarray Database. Search results revealed a significant upregulation of COBRA1 in two mRNA microarray datasets. RNA interference technique was then used to knockdown COBRA1 expression in cervical cancer cell line, HeLa. Once a successful siRNA mediated silencing at the RNA and protein levels of COBRA1 was established and confirmed through semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Western Blot, we investigated its consequences on proliferation, migration and survival of HeLa cells. Interestingly, COBRA1 depletion resulted in a significant increase in the mRNA expression of Trefoil Factor 1 (TFF1) accompanied by a subsequent decrease in the β -catenin mRNA levels. These findings suggests an effect for COBRA1 on the Wnt/ β -catenin signalling pathway, which could be mediated through TFF1. In addition, COBRA1 silencing resulted in significant decrease in the expression of survivin 2B and survivin DeltaEX3 isoforms while the observed decrease in survivin wild type form was found to be statistically insignificant. Survivin is known to play a major role in cancer cells proliferation and survival. Yet, the finding that the noted decrease in β -catenin and survivin expression was not reflected on the proliferation and migration abilities of HeLa is not conclusive and requires further investigations. Taken together, these findings could help as an initial step in identifying the role of COBRA1 in cervical cancer tumorigenesis.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
AP-1	Activator Protein-1
AR	Androgen receptor
Bps	Base pairs
BRCA1	Breast cancer type 1 susceptibility protein
CC	Cervical cancer
ChIP	Chromatin Immunoprecipitation
COBRA1	Cofactor of BRCA1
Co-IP	Co-Immunoprecipitation
DRB	5,6-Dichloro-1- β -D-ribofuranosylbenzimidazole
DSIF	DRB sensitivity-inducing factor
ER	Estrogen receptor
ER- α	Estrogen receptor-alpha
ESCs	Embryonic stem cells
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FIGO	The International Federation of Gynecology and Obstetrics
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GR	Glucocorticoid receptor
GSK-3 β	Glycogen synthase kinase 3 β
HCC	Hepatocellular carcinoma
HepG2	Well differentiated human hepatocellular carcinoma cell line
HPV	Human papilloma virus
HR-HPV	High risk human papilloma virus
Hrs	Hours
IAP	Inhibitor of apoptosis
IARC	International Agency for Research on Cancer
ICO	Catalan Institute of Oncology
MAPK	Mitogen activated protein kinase
Mins	Minutes

MI	Millilitres
µls	Microliters
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NELF	Negative elongation factor
NR	Nuclear receptor
PAGE	Polyacrylamide gel electrophoresis
Pap	Papanicolaou
PBS	Phosphate-buffered saline
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 sulphate
siCOBRA1	COBRA1 siRNA
SiHa	Squamous cervical carcinoma cell line
siNTC	Non Targeting siRNA (Negative Control siRNA)
siRNA	Short interfering RNA
T47D	Metastatic human breast cancer cell line
TBS	Tris-buffered saline
TBST	0.01% Tween-20 in 1X TBS
TFF1	Trefoil Factor 1
UGCs	Upper gastrointestinal cancers
WHO	World Health Organization
WT	Wild type

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1. LITERATURE REVIEW

1.1 Cervical Cancer

1.1.1 Incidence and Epidemiology

Cervical cancer is the fourth most frequent cancer among women worldwide with 527,600 new cases being diagnosed annually. It is the fourth cause of cancer related deaths and responsible for around 265,700 deaths globally per year. Cervical cancer primarily affects developing countries, being the second most commonly diagnosed type of cancer and third leading cause of cancer death among females in those countries (**Arbyn et al., 2011; Torre et al., 2015**). Over 85 % of all cervical cancer cases and related death occurs in developing countries. The incidence rates are lowest in Australia/New Zealand, Northern America and Western Europe while the highest rates are in Eastern, Western and Southern Africa, South America, South Central Asia together with Melanesia. Notably, the most affected are socio-economically active young women who are in the prime of their lives (**Arbyn et al., 2011**). This variation is mainly attributed to differences in the availability of efficient screening tools allowing for the early detection and removal of precancerous lesions. (**Arbyn et al., 2011; Torre et al., 2015**).

Based on the information available from ICO/IARC Information Centre on HPV and Cancer, cervical cancer is the 13th most frequent cancer among women in Egypt and ranks as the 10th most frequent cancer among women between the ages of 15 and 44 years. It accounts for a total number of 866 cancer new cases and 373 cancer related death annually (<http://www.hpvcentre.net>, last accesses April 2018) (**Bruni et al., 2017**).

1.1.2 Aetiology

Almost all cervical tumours (99%) develop as a result of persistent human papilloma virus (HPV) infection (**Colombo et al., 2012**). Human papillomaviruses are DNA viruses that infect and replicate in cutaneous and mucosal epithelia. Oncogenic subtypes such as HPV 16 and 18 account for 70% of all cervical malignancies. Other oncogenic subtypes include

HPV 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82. Studies suggest that genetic sequences variations among different HPV subtypes could produce differences in their oncogenic potentials and hence different clinical outcomes among CC patients. **(Dasari, Wudayagiri, & Valluru, 2015).**

When HPV infection occurs, it is usually eliminated by the immune system. If viral elimination does not happen, prolonged presence of the oncogenic HPV subtype and increased viral load occur leading to higher chances of developing a precancerous cervical intraepithelial neoplastic (CIN) lesion, which can progress to invasive cervical tumors. Risk cofactors that can halt the process of HPV elimination include the presence of defective immune response as with human immunodeficiency virus (HIV) infection and the use of organ transplant rejection drugs. Smoking can also be a cofactor for the development of chronic HPV infection **(Newton & Mould, 2017; Uyar & Rader, 2014)**. It is worth mentioning that also estrogen contributes to HPV infection persistence and the resulting neoplastic progression via increasing viral oncogenes expression **(S. H. Chung, Franceschi, & Lambert, 2010; Nair et al., 2005)**.

Cervical cancer is more common than other HPV derived cancers (vagina, vulva, penile, anal and oropharynx) due to the presence of a transformation zone on the cervix. This is mainly an area of exposed columnar tissue on the cervix known as the ectropion which undergoes squamous metaplasia and transforms into squamous tissue, hence the label transformation zone. This area is highly susceptible to the effects of oncogenic HPV **(Newton & Mould, 2017)**.

1.1.3 Surveillance and Diagnosis

Cervical epithelial tumors are classified by WHO into three main categories; squamous (70-80%), adenocarcinoma (10-15%) and other epithelial tumors (including neuroendocrine tumors and undifferentiated carcinoma) **(Colombo et al., 2012)**. Despite the decline in the incidence of CC in developed countries, several reports during the past 40 years have documented an increase in relative distribution of the adenocarcinoma subtype compared to squamous cell carcinoma in these countries **(Gien, Beauchemin, & Thomas, 2010; Hildesheim & Berrington de González, 2006)**.

Symptoms associated with early stages of CC are usually non-specific and are common with other clinical conditions. The most common sign suggesting the presence of cervical cancer is bleeding; which could be postcoital, intermenstrual or postmenopausal bleeding. Other symptoms include blood stained vaginal discharges, pelvic pain and suspicious cervix on examination. The lateral spread of the tumor which is present in advanced stages of CC can result in the occurrence of loin pain caused by hydro-nephrosis, sciatica with tumor compressing nerve roots and deep vein thrombosis causing swollen legs (**Newton & Mould, 2017**).

Screening techniques for CC mainly include cell cytology and HPV-DNA testing. The basic widely used cytology screening test for CC is the Papanicolaou (Pap) smear. It involves sampling the epithelial cells from the surface of cervix and assessing their morphology. Implementing this screening method in some parts of the world has resulted in a significant decrease in the incidence of CC. Still, the Pap smear test suffers from lack of sensitivity, being of subjective nature and it might not be as efficient for the detection of adenocarcinoma subtype. In 2008, molecular HPV-DNA testing has been employed as a new screening approach with greater diagnostic sensitivity and reproducibility especially when detecting precancerous stages (**Colombo et al., 2012**).

Although cervical cancer can be detected in its early stages by the above mentioned techniques, hence can be successfully eradicated through surgery, curative treatments do not yet exist for advanced, recurrent or metastatic stages of the disease (**Cao, Liu, Yang, Chen, & Zheng, 2017**).

1.1.4 Vaccination

The notion that HPV infection is a main contributor in the development of CC has facilitated the measures taken for CC prevention through the development of HPV vaccines. HPV vaccination has been the primary prophylactic strategy for CC with a number of vaccines being developed and are of wide use in some countries across the world. In 2006, FDA approved Gardasil [Merck and Company, Whitehouse Station, NJ], a quadrivalent vaccine developed for the prevention of diseases associated with infection with different HPV types (6, 11, 16 and 18). Two additional vaccines were approved for use in 2009 and 2014: Cervarix [GlaxoSmithKline, Brentford, UK], a bivalent vaccine against HPV types 16 and

18, and Gardasil 9 [Merck], the most advanced prophylactic vaccine to date, which protects against nine HPV types: 6, 11, 16, 18, 31,33, 45, 52 and 58 (**Newton & Mould, 2017**). Some concerns have been raised regarding the use of vaccines which are mainly that they were tailored towards the population within the developed countries with decreased potency in developing countries; where they are needed most. In addition, the high cost stands against the wide scale use of the vaccines in the developing parts of the world.

It is highly recommended that all women; including those previously vaccinated, continue to be screened. This is mainly because HPV vaccines do not have any therapeutic effects so they cannot protect against already established infections, together with the fact that they do not protect against all of the other types of high risk HPVs that can cause cervical cancer. Unfortunately, many low-resource countries do not have the technical and public health infrastructure needed to support Pap smear testing; the most common screening tool for cervical cancer in more developed countries (**Pyeon et al., 2007; Torre et al., 2015**).

1.1.5 Prognosis and Available Treatments

Prognostic factors for survival rate in CC patients include the clinical stage, lymph node state, size of the tumor and grade (**Gien et al., 2010**). The cancer stage and tumour size are currently the basis for choosing suitable treatment. Surgical options for patients with early stage disease (FIGO stage IA- IB1, in which the lesion is confined to the cervix) include cervical conisation, hysterectomy and pelvic trachelectomy, with 5 years survival rates that exceeds 90%. In stages IIB2- IIIB, it becomes more challenging to obtain negative surgical margins; in these instances surgery, chemotherapy and/or radiation is given in combination aiming to improve treatment results (**Colombo et al., 2012; Newton & Mould, 2017**).

For women with cancers that have spread within the region, the 5-years survival rates falls to 57% and for cancers which have metastasised to distant organs survival is estimated at only 17%. For the more advanced stages of the disease (stage IV), surgery could not remain an available option and the treatment is usually palliative with the objective of controlling symptoms and improving quality of life (**Colombo et al., 2012; Newton & Mould, 2017**).

1.1.6 Molecular Biology of CC Progression

Identifying the molecular mechanisms involved in cervical carcinogenesis is essential for developing therapeutic targets and biomarkers that would allow for better management of the disease. The fact that not all women infected with HPV will develop CC has shed the light that other factors in addition to HPV infection are involved. HPV DNA integration into the host chromosomal genome is a crucial step in cervical cancer progression and is almost present in all invasive CC. This integration usually occurs at common fragile sites in the human genome. During this process, cleavage occurs at the E1/E2 genes cleavage site leading to deletion of E2 gene, thus leading to overexpression of E6 and E7 viral oncoproteins whose expression is repressed by E2. This in turn promotes genomic instability, accumulation of secondary mutations, and malignant transformation of the host cells. In addition, the virus integrates into host genes and regulatory elements with the possibility of inducing structural alteration of the host genome and transcriptional deregulation of normal genetic expression (**Pérez-Plasencia, Dueñas-Gonzalez, & Bustos-Martínez, 2008; Uyar & Rader, 2014**).

The expression of certain proteins of the host cells such as those involved in cell division and apoptosis are altered by the viral oncoproteins. The E7 protein can bind to tumor-suppressor proteins of the retinoblastoma (pRB) family, and degrades them leading to uncontrolled activation of E2F transcription factors which in turn stimulate expression of genes involved in S phase of the cell cycle. In addition, E7 interacts with the cyclin-dependent kinases (CDK) inhibitors, p21 and p27, promoting the transition from G1 to S phase. The E6 protein in turn halts the pro-apoptotic effect of p53, BCL2 Associated X protein (BAX), BCL2 Antagonist /Killer (BAK) and c-Myc. The E5 viral protein is mainly active during early stages of the disease before viral DNA integration occurs. One of its main activities is the interaction with epidermal growth factor receptor (EGFR) leading to increased cellular proliferation rate (**De Freitas, Coimbra, & Leitão, 2014**).

Improved understanding and increased knowledge of the molecular biology events associated with CC progression will help develop novel treatment strategies. The main interest of this study is COBRA1 gene, which have been recently identified for having a potential role in the progression of several cancers.

1.2 Cofactor of BRCA1

1.2.1 Identification

Cofactor of BRCA1; also known as NELF-B, was first isolated from a human ovary cDNA library. It is located on chromosome 9 and encodes a 580 amino acid protein with 3 repeats of the LXXLL motif, where L-leucine and X-any amino acid. It has been first identified as a BRCA1 interacting protein via a yeast two-hybrid assay. Results suggested that BRCA1-dependent unfolding of higher levels of chromatin structure is likely to be partially mediated through recruitment of COBRA1 to the BRCT1 domain of BRCA1 (Ye et al., 2001). Later in 2003, COBRA1 was found to be the same as NELF-B, which is one of the four subunits that constitute the negative elongation factor (NELF) complex (Narita et al., 2003). It has been proved that COBRA1 is involved in the transcriptional regulatory machinery of the cells and is involved in the regulation of multiple considerably important genes (Aiyar et al., 2004; Aiyar, Blair, Hopkinson, Bekiranov, & Li, 2007; Aiyar, Cho, Lee, & Li, 2007). In addition, *in-vivo* studies by Amleh et al., (2009) shed the light on an important role for COBRA1 in early embryogenesis where the general knockout of COBRA1 in murine embryos was found to be lethal. COBRA1 was also found to be involved in maintaining the undifferentiated state of mouse ESCs (Amleh et al., 2009).

1.2.2 COBRA1 and Transcription factors

With no DNA binding domain of its own, COBRA1 was found to regulate the transcription of its downstream target genes via interacting with other transcription factors including BRCA1, nuclear receptors and AP-1 complex (Aiyar et al., 2004; Sun, Blair, Aiyar, & Li, 2007; Ye et al., 2001; Zhong et al., 2004). This network of interactions suggests COBRA1 being involved in the regulation of multiple cellular processes as proliferation, cell survival and tumorigenesis. An overview of the available knowledge on COBRA1 interactions with various transcription factors will be discussed next.

1.2.2.1 BRCA1

The Breast Cancer Type 1 susceptibility protein (BRCA1) gene; encoding a 1,863 amino acid protein, was identified and cloned in 1994 (**Rosen, Fan, Pestell, & Goldberg, 2003**). It has been linked to breast and ovarian cancers, with mutations in BRCA1 accounting for only 2-3% of all breast cancers; yet its expression is frequently reduced or absent in sporadic cancers. The tumor suppressor activity of BRCA1 has been extensively investigated and have been attributed to its involvement in several molecular functions including DNA repair, DNA damage response, cell cycle check points, regulation of certain transcriptional pathways and apoptosis (**Rosen et al., 2003**).

In accordance with BRCA1's reported role as tumor suppressor in breast cancer, several studies had shed the light on the role of COBRA1 as being a breast cancer tumor suppressor as well. Aiyar *et al.*, 2007 used gene expression profiling in T47D breast cancer cell line to uncover a significant overlap of the genes that are regulated by COBRA1 and BRCA1, of which multiple genes are known to be involved in breast cancer progression (**Aiyar, Cho, et al., 2007**).

1.2.2.2 NELF Complex

COBRA1 was identified as being the same as NELF-B, one of the four subunits that makes up the negative elongation factor (NELF) complex (**Narita et al., 2003**). This complex is made up of NELF-A (66 kDa), NELF-B (62 kDa), NELF-C (60 kDa) or NELF- D (59 kDa) and NELF-E (46 kDa). Being translational variants of a common mRNA, either NELF-C or NELF-D will be present at a certain point within the NELF complex. The core of the complex consists of NELF-C/D together with NELF-B bridging NELF-A; having the RNAPII binding domain and NELF-E; having the RNA binding domain (**Narita et al., 2003; Sims, Belotserkovskaya, & Reinberg, 2004**).

NELF complex together with DRB- sensitivity inducing factor (DSIF) have been associated with a process known as promoter proximal pausing, in which the RNA polymerase II (RNAPII) is stalled 30-50 bps downstream of the transcription initiation site (**Yamaguchi et al., 1999**). The kinase activity of the positive transcription elongation factor b (P-TEFb) causes the release of paused polymerase into productive elongation phase again by

phosphorylating the serine 2 position of the C-terminal domain in the largest subunit of RNAPII (**Sun & Li, 2010**).

It is worth mentioning that all the four subunits; NELF-A, NELF-B, NELF-C/D and NELF-E, are required for the assembly of a functional NELF complex (**Narita et al., 2003**). This was found consistent in several studies, where the knockdown of any of the four subunits resulted in co-depletion of the other ones at the protein level but not at the mRNA level (**Narita et al., 2003; Sun et al., 2008; Sun & Li, 2010**).

The NELF complex mediated stalling of RNAPII has been recently found to have functional consequences other than transcription inhibition. Studies done in *Drosophila* revealed that stalled RNAPII was associated with the promoters of around 50% of most of its highly expressed genes indicating a role in fine tuning of gene expression rather than inhibiting it, thus potentiating genes for future activation (**Gilchrist et al., 2008, 2010**). In addition, recent studies in human cells have been proposing a positive role for NELF Complex in the transcription activation of a large number of genes particularly those involved in cell cycle regulation (**Sun & Li, 2010**). Based on results obtained from gene expression profiling analysis following depletion of NELF subunits in T47D breast cancer cell line, it was suggested that NELF complex mainly functions in sustaining active expression of target genes rather than inhibiting it through promoting association of RNAPII with the actively transcribing genes rather than preventing the active transcription elongation. This was thought to be mediated through maintaining a permissive chromatin structure and preventing nucleosomal encroachment at promoters of target genes (**Sun et al., 2011; Sun & Li, 2010**).

1.2.2.3 AP-1 Complex

COBRA1 has no DNA binding domains, hence its ability to regulate genetic expression is mainly through interaction with other transcription factors. In addition to its roles with BRCA1 and as a functional component of NELF complex, it was shown to act as a regulatory transcription cofactor for Activator Protein-1 complex (AP-1), whose signalling pathways plays a major role in determining the cellular fate (**Zhong et al., 2004**).

The activating protein1 (AP-1) is a transcription factor which regulates basal and inducible transcription of multiple genes having the consensus AP-1 sites. Within the complex cellular context, AP-1 activity is regulated in response to several stimuli including cytokines, stress

signals, growth factors, and infections in addition to oncoproteins (**Hess, Angel, & Schorpp-Kistner, 2004**)

AP-1 have been found to be implicated in the control of both cell survival and apoptotic pathways (**Shaulian & Karin, 2001**). The modulation of this decision was found to be dependent on cell lineage, abundance of different AP-1 complex members, microenvironment, type of stimulus and the presence of regulatory transcription cofactors (**Hess et al., 2004; Shaulian & Karin, 2001**).

COBRA1 has been proposed as one of the cofactors affecting AP-1 activity. In 2004, a study by Zhong *et al.* revealed that the overexpression of COBRA1 inhibited AP-1 mediated transcriptional activation in transfected cells with the opposite effect observed when silencing of COBRA1 with small interfering RNA was applied. This effect was attributed to a physical interaction between COBRA1 and AP-1 family members' c-Jun and c-Fos causing an inhibition of the AP-1 transactivation of target genes. Given that the activity of AP-1 is effective on the cellular proliferation, differentiation, survival, apoptosis and malignant transformation, it was proposed that COBRA1; by acting as a cofactor in AP-1 transactivation, is involved in the regulation of these cellular processes as well (**Zhong et al., 2004**).

1.2.2.4 Nuclear receptors

The ligand binding activation of ER induces conformational changes and activation of the receptor and its subsequent binding to the estrogen responsive elements in promoters of target genes (**Aiyar et al., 2004; S. Chung, Franceschi, & Lambert, 2010**).

A novel role for COBRA1 in the regulation of hormone-responsive transcription was identified by Aiyar *et al.*, (2004). It was found that COBRA1 can bind directly to the activated ER- α and repress genetic transcription mediated through ER- α . These findings supported a role for COBRA1 in suppressing estrogen-dependent growth of breast cancer cells (**Aiyar et al., 2004**). This was attributed to the previously identified 3 repeats of the LXXLL motif present in COBRA1 protein needed for NR-binding (**Ye et al., 2001**).

COBRA1 was found to interact with variable affinities to other nuclear receptors as the progesterone receptor B (PRB), androgen receptor (AR) and glucocorticoid receptor (GR). The strongest binding affinity was found to be related to the AR resulting in the subsequent regulation of the AR-dependant transcription (**Sun et al., 2007**).

1.3 Role of COBRA1 in Cancer

The biological role of COBRA1 in cancer pathogenesis is not fully understood but there is a growing body of evidences which suggests that COBRA1 plays a role in the malignant transformation, proliferation and invasion of cancer cells. Previous findings are suggesting a cancer-type dependent role for COBRA1, with different expression patterns in different cancers. To date it has been studied in breast, UGCs and liver cancers.

1.3.1 Breast Cancer

The identification of COBRA1 as a BRCA1 interacting protein has highlighted the possibility of COBRA1 having a role in breast cancer. Several studies have previously identified COBRA1 to act as a tumor suppressor in breast cancer. In a study by Zhu *et al.* (2004), COBRA1 mRNA and protein levels were found to be differentially expressed in different breast cancer cell lines. Its role in the regulation of breast cancer growth was proposed based on the identified physical interaction between endogenous COBRA1 and BRCA1 within the examined cells. In addition, both ER- positive and ER-negative cell lines were found to express COBRA1 suggesting absence of correlation between ER and COBRA1 expression (**J. Zhu et al., 2004**). Another study in 2004 done by Aiyar *et al.* on breast cancer cells showed that COBRA1; as being the NELF-B subunit of NELF complex, binds to the activated ER- α causing the RNA polymerase II to pause at the promoter proximal region and thus attenuate the ER α mediated transcription activation. The use of siRNAs targeting COBRA1 in T47D breast cancer cells resulted in increased cellular proliferation. However this increase was observed only when exogenous estrogen was added to culture, indicating a possible role for COBRA1 in suppressing the estrogen-mediated growth of breast cancer and expression of genes such as the Trefoil Factor 1(TFF1) known to be associated with breast cancer progression and metastasis (**Aiyar et al., 2004**). This was confirmed in another study where COBRA1 knockdown did not affect the cellular proliferation of T47D cells in the absence of estrogen (**Sun & Li, 2010**). In accordance, COBRA1 expression was found to be reduced in patients with metastatic breast cancer and local recurrence (**Sun et al., 2008**).

1.3.2 Upper Gastrointestinal Cancers

COBRA1 has been identified as a novel oncogene in UGCs with high expression levels of COBRA1 mRNA and protein observed in tumor samples compared to normal (**McChesney et al., 2006**). The overexpression of COBRA1 was found to be accompanied by

downregulation of TFF1 expression, which has several protective and healing roles in upper gastrointestinal tract and has been reported to be a tumor suppressor in UGCs (**Tanaka et al., 2013**). This was found contradictory to its previously reported role as tumor suppresser in breast cancer (**Aiyar et al., 2004**). In addition, examining the regulatory effects of COBRA1 on TFF1 gene expression in gastric adenocarcinoma cell lines revealed its role as inhibitor of TFF1 expression was estrogen independent and NELF-E independent. It was found to occur by COBRA1 inhibiting the AP-1 complex activation of the TFF1 gene in UGC, while in breast cancer, COBRA1 inhibits hormone-dependent activation of TFF1 through recruitment to the activated ER- α . The results highlighted the ability of COBRA1 to regulate definite transcription processes in different cells and tissue types via interaction with different transcription factors (**McChesney et al., 2006**).

1.3.3 Liver Cancer

Studies done in Dr. Amleh's lab have revealed a potential involvement of COBRA1 in HCC pathogenesis. Initially, Kamel (2012) has observed frequent overexpression of COBRA1 in HCC tumor tissues relative to their corresponding non-tumor specimens. Yet this study was limited with the small sample number which made it difficult to statistically correlate COBRA1 expression to clinicopathological parameters (**Kamel, 2012**). To further explore the role of COBRA1 as a potential prognostic marker for HCC, Youssef *et al.* (2016) investigated the protein and mRNA expression of COBRA1 across four different cell lines representing different grades of HCC. Results revealed a gradual decrease in expression of COBRA1 with increased HCC aggressiveness. The highest expression was observed in the low grade HepG2 cell line and the lowest expression in the high grade SNU-387 (**Youssef, Shawer, Afify, & Amleh, 2016**). In 2017, Silencing of COBRA1 expression in HepG2 cell line using RNA interference resulted in a significant decrease in the cellular proliferation and migration rates of HepG2. This was associated with a significant decrease in the mRNA expression levels of the proliferation marker Ki-67 and the proto-oncogene survivin (**El Zeneini, Kamel, El-Meteini, & Amleh, 2017**).

1.3.4 Other Cancers

In a study done on ovarian cancer, COBRA1 was found to be a downstream target of the RAS/MAPK signaling pathway. The inactivation of this signaling pathway in ovarian cancer cell lines revealed a profound downregulation of COBRA1 expression, which was

accompanied by decreased cellular proliferation (**Pohl, 2005**). This finding was consistent with another study done on Lung cancer (**Sudhir et al., 2011**).

All previous findings provide conflicting data regarding COBRA1 and suggest it having a cancer-type dependent role, which in turn highlights the complexity of this molecule. As to our knowledge, the role of COBRA1 in cervical cancer progression has not been studied before.

HYPOTHESIS AND SPECIFIC OBJECTIVES

Previous studies on the role of COBRA1 in cancer progression have revealed a cancer-type dependant role. It has been previously implicated in restraining breast cancer while promoting UGCs development. Studies from our group indicated a potential role for COBRA1 in HCC (**El Zeneini et al., 2017; Youssef et al., 2016**). Very little is known about the role of COBRA1 in CC, hence we decided to extend the scope of our group's research from HCC to CC. We hypothesize that COBRA1 could be one of the players involved in the pathogenesis of CC. To address this hypothesis, our study had the following four main objectives:

1. *In-silico* analysis of the expression pattern of COBRA1 in cervical carcinoma versus normal cervical tissues using online publicly available microarray database.
2. To achieve an efficient COBRA1 knockdown in the cervical cancer cell line, HeLa. The HeLa cell line is an adenocarcinoma cell line derived from Ms. Henrietta Lacks, a 31-years old African-American female. The cells have a characteristic cobblestone-like, epithelial appearance and has been reported to contain human papilloma virus 18 (HPV-18) sequences (**Lucey, Nelson-Rees, & Hutchins, 2009**).
3. To investigate the effect of COBRA1 Knockdown on cellular proliferation and migration of HeLa.
4. To examine the expression pattern of NELF complex subunits and some cancer related genes following COBRA1 silencing.

2. MATERIALS AND METHODS

2.1 Cell lines and culture

Human cervical adenocarcinoma cell line HeLa was grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, USA) supplied with 10% fetal bovine serum (GIBCO, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (GIBCO, USA). Cells were maintained in a humidified incubator supplied with 5% CO₂ at 37 °C. In all below-described experiments, cells in the logarithmic phase of growth from passage numbers 6-20 were used and propagated at 70-80% confluency according to the American type culture collection protocol. An inverted microscope (Olympus IX70, USA) was used to observe the cells. HeLa cells doubling time has been found to be approximately 25 hrs.

2.2 Viable Cell Count

Trypan Blue exclusion method was used to obtain the viable cell count prior to each experiment. Cells were harvested and re-suspended in fresh media by pipetting up and down until a homogenous cell suspension with no cell clumps was obtained. Fifty microliters (µl) from this cell suspension was mixed with 50 µl of 0.4% trypan blue in PBS. In each chamber of a haemocytometer (Hausser Scientific, USA) 10 µl of the trypan blue-cell suspension was loaded. The cells in each of the outer four squares in the two chambers were counted and the following equation was used to calculate the number of cells per 1 ml of cells suspension:

$$\text{Number of cells/ml} = (\text{Total numbers of viable cells in all counted squares} / \text{total number of counted squares}) \times \text{dilution factor} \times 10000$$

2.3 RNA Interference

Silencing of COBRA1 (NCBI: NM_015456) was achieved by using siGENOME SMARTpool (Dharmacon, M-015839-00). It is a pool of four different siRNAs targeting different regions of COBRA1 mRNA. Table1 includes the siRNAs target sequences and

corresponding exon locations of COBRA1. Other siRNAs used in this study include GAPDH siRNA (SI02653266) and ALLStars Negative control siRNA (SI03650318), both purchased from Qiagen. GAPDH siRNA was used as a positive control for the transfection procedures and its efficiency in delivering the siRNAs into HeLa cells. ALLStars Negative control siRNA has no homology to any known mammalian gene and it was used to control for any nonspecific effects on gene expression and phenotype that can happen as a result of the transfection procedure itself. All siRNAs were received as lyophilized powders and were re-suspended in RNase-free water as per the manufacturers' instructions to obtain working solutions of final concentrations of 20 μ M.

Table 1. COBRA siRNAs target sequences with their corresponding exons locations

	siRNA	Target sequence	Target Exon
siGENOME	1	CCGAAAGCUUCACUAAGUU	9&10
SMARTPool	2	GCGACUUGGCCUUUGGCGA	11
M-015839-00	3	GAGCCUGGGACAUGAUCGA	8
	4	CGUCUAAGCUGGAGGCGUU	12

2.4 siRNA Transfection

An RNase-free environment was strictly maintained throughout all experimental procedures. The transfection of siRNAs (COBRA1 siRNA, ALLStars Negative control siRNA and GAPDH siRNA) into HeLa cells was performed in 6-well plates using Lipofectamine 3000 (Life Technologies). The efficiency of Lipofectamine 3000 in HeLa cells was assessed via forward transfection of 25nM of GAPDH siRNA for 72 hrs. Transfection procedures were optimized regarding the transfection protocol (forward versus reverse), siRNAs final concentration/well and duration of transfection. The following optimized conditions were maintained in all experiments. Approximately 1.5×10^5 cells were reverse transfected with 35 nM of siRNA using 3.75 μ l of Lipofectamine 3000 according to the manufacturer's protocol in a final volume of 2 ml/ well. In one well of a 6-well plate, 3.5 μ l of COBRA1 siRNA solution (20 μ M) was mixed with 500 μ l of low serum opti-MEM media (Gibco). After 5 minutes, 3.75 μ l of Lipofectamine was added to the well and left for 15-20 minutes at room temperature to allow for the formation of siRNA- Lipid

complex. Following this short incubation, 1.5 ml of HeLa cells suspension (approximately 1.5×10^5 cells) in antibiotic free complete DMEM media were added to the well. The same procedures were repeated for the negative control siRNA. As additional controls, cells were either left un-transfected (Untreated) or treated with Lipofectamine only (Mock). Cells were maintained in culture for 72 hrs post transfection followed by harvesting for RNA and protein analysis.

2.5 RNA Extraction

Trizol reagent (Invitrogen, USA) was used to extract total RNA from HeLa cells according to the manufacturer's instructions. An RNase-free environment was maintained during all extraction procedures. The extracted RNA was dissolved in nuclease-free water (Thermo Scientific, USA). The concentration and purity of the RNA were measured at both 260 and 280 nm using a SPECTROstar Nano Absorbance plate reader (BMG Labtech). RNA concentration was automatically computed by the device using the following equation:

$$[\text{RNA concentration } (\mu\text{g/ml}) = \text{OD at A260} \times \text{Dilution Factor} \times 40].$$

2.6 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Semi quantitative RT-PCR was used to determine the differential gene expression at the messenger RNA level (mRNA) among tested conditions. Total RNA (0.5 μg) was reverse transcribed in a final volume of 20 μl using Revert Aid First strand cDNA synthesis Kit (Thermo Scientific, USA) according to the manufacturer's protocol.

All primers used for the amplification of the selected genes are listed in Table 2. PCR amplifications conditions were programmed for 5 minutes at 94°C, followed by cycles of (denaturation at 94°C for 30 seconds, annealing at the specified temperature for each of the used primers sets for 30 seconds, extension at 72°C for 45 seconds) and ending the process with 10 minutes at 72°C. The PCR products were then separated on a 1.5 - 2 % agarose gel depending on product size and visualized using Gel Doc EZ System (Bio-Rad, USA)

Table 2. RT-PCR primer sequences, PCR amplification conditions and amplicon sizes (F: forward primer, R: reverse primer, bp: base pair)

Gene	Primer Sequence	PCR Conditions	Amplicon Size
GAPDH	F : 5'-CCACCCATGGCAAATTCCATGGCA-3' R: 5'-TCTAGACGGCAG GTCAGGTCCACC-3'	60.5°C 27 Cycles	598 bp
β-ACTIN	F: 5'-GCAAAGACCTGTACGCCAAC-3' R: 5'-GAGACCAAAAGCCTTCATACATCTC-3'	58°C 27 Cycles	777bp
COBRA1	F: 5'-ACATCACCAAGCAGAGGAA-3' R: 5'-GATCCAGCTGTTCCAGCTTC-3'	59.5°C 32 Cycles	366 bp
NELF-A	F: 5'-GTCGGCAGTGAAGCTCAAGT-3' R: 5'-TTCACACTCACCCACCTTTTCT-3'	60°C 35 Cycles	250 bp
NELF-C/D	F: 5'-GAAGAAGGAGAGACCCCAGC-3' R: 5'-GTGCCCAAGGCTAGTGTGAT-3'	56 °C 28 Cycles	443 bp
NELF-E	F: 5'-TGGTGAAGTCAGGAGCCATCAG-3' R: 5'-CGCCGTTTCAGGGAATGAATC-3'	63 °C 28 Cycles	565 bp
Ki67	F: 5'-CTTTGGGTGCGACTTGACG-3' R: 5'-GTCGACCCCGCTCCTTTT-3'	60 °C 28 Cycles	199 bp
β-catenin	F: 5'-ACTGGCAGCAACAGTCTTACC-3' R: 5'-TTTGAAGGCAGTCTGTCGTAAT-3'	61 °C 30 Cycles	837 bp
survivin	F: 5'-TTGAATCGCGGGACCCGTTGG -3' R: 5'-CAGAGGCCTCAATCCATGGCA-3'	61 °C 28 Cycles	Isoform 1: 477 bp Isoform 2: 359bp Isoform 3: 546bp
TFF1	F: 5'-TTTGGAGCAGAGAGGAGGCAATGG-3' R: 5'-TGGTATTAGGATAGAAGCACCAGGG-3'	64 °C 32 Cycles	240 bp

2.7 Western Blot Analysis

Cells were harvested at the specified time point and washed with ice cold PBS before they were lysed in 1X ice-cold Laemmli Lysis Buffer (50mM Tris Ph6.8, 2% sodium dodecyl sulphate (SDS) and 10% glycerol). For each 1 ml of Lysis buffer, 10 μ l of 100X Halt Protease Inhibitor Cocktail (ThermoScientific, USA) was added. Cells in Lysis buffer were incubated at 4°C for 40 minutes with vortexing at 10 minutes intervals followed by centrifugation at 14000 rpm and 4°C for 15 minutes. Supernatants were collected and were used to determine protein content using BCA Protein Assay Kit (Pierce Biotechnology, USA) according to the manufacturer's protocol.

Equivalent amounts (20-40 μ g) of proteins diluted in lysis buffer were mixed with 4X 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 28 μ l. Samples were boiled at 100°C for 5 minutes prior to being loaded onto a 12 % SDS-polyacrylamide gel and separated by electrophoresis at 120V. Consequently, they were blotted to a nitrocellulose membrane (GE Healthcare). Blotted membrane was blocked with 5% non-fat dry milk in 1X TBST (0.01% Tween-20 in TBS) at room temperature for 2 hrs, after which the membrane was incubated with primary antibodies at 4 °C overnight. Following three times washing (5 minutes each) with the wash solution (1 X TBS with 0.01% 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 2 hrs. Three times of washing to remove excess secondary antibody with the wash solution was performed. For detection, the membrane was incubated with chromogenic substrate BCIP/NBT (KPL) until signals became visible with naked eye. Anti-GAPDH (Abcam, ab8245) (1:10,000 in 5% non-fat dry milk), Anti- β -tubulin (Sigma, T7816) (1:20,000 in 5% non-fat dry milk) and anti-COBRA1 (Abcam, ab167401) (1:1000 in 3% non-fat dry milk) were used as primary antibodies in this study.

2.8 MTT Assay

The effect of COBRA1 silencing on the viability and proliferation of HeLa cells was analysed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described (**Chai & Yang, 2014; Yang et al., 2009; K. Zhu, Chen, Han, Wang, & Wang, 2012**). Briefly; for reverse transfection in 96 well plates, 0.175 μ l of the 20 μ M siRNA solutions and 0.15 μ l Lipofectamine 3000 were diluted with 20 μ l opti-MEM media and mixed with 6000 cells suspended in 80 μ l antibiotic free complete DMEM media to reach a final concentration of 35 nM of siRNAs/well. Cells were maintained in culture for 72h, after which media was replaced with 100 μ l of fresh opti-MEM and 10 μ l of 5 mg/ml MTT solution (Serva) was added to each well. The cells were incubated for 3 hr, media with MTT removed and the resulting intracellular purple formazan was solubilized using 100 μ l of dimethylsulphoxide (DMSO, Sigma). Cells were incubated with DMSO for 5-10 min, then shaken for 2 min in dark. Absorbance was measured at 490 nm using SPECTROstar Nano microplate reader (BMG Labtech). The assay was run in triplicates for each condition (Untreated cells, Mock-treated, Negative siRNA and COBRA1 siRNA treated cells). Three control wells of used medium alone were used to provide the blanks for absorbance readings. The average of the obtained triplicate absorbance readings for each tested condition was used to calculate percentage cell viability as follows;

$$\text{Percentage Cell Viability} = (\text{Absorbance of Test} / \text{Absorbance of Control}) \times 100$$

2.9 Wound Healing Assay

The scratch wound healing assay was used to investigate differences in cell migration abilities that might occur as a result of COBRA1 silencing in HeLa cells. The assay was performed as previously described (**El Zeneini et al., 2017; Youssef et al., 2016**). In brief, reverse transfection of COBRA1 siRNA into HeLa cells was carried out in 12 well plate. Approximately 7.5×10^4 cells were reverse transfected with 35nM of siRNA using 1.5 μ l of Lipofectamine 3000 according to the manufacturer's protocol in a final volume of 1ml. At 72 hours post-transfection, the cell monolayer was carefully scraped using a sterile 20 μ l pipette tip, once vertically and once horizontally creating a cross in the center of each well (0 hr). Floating cells were removed by washing with media. Cells were monitored for additional 24 hours (24 hrs). Images were taken using phase contrast at 10X magnification

power at 0 hr and 24 hrs. The wound area was analysed using ImageJ software and percentage wound closure was calculated as follows:

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

2.10 Data Mining

The Oncomine cancer microarray database and integrated data-mining platform (<http://www.oncomine.org/>, last accessed April 2018) (Rhodes et al., 2004) was used to investigate the expression profile of COBRA1 mRNA in cervical carcinoma versus respective normal samples. The used filter criteria included COBRA1 gene in cancer versus normal analysis, cancer type was restricted to cervical cancer and data type was limited to mRNA microarrays. Statistical significance was automatically computed by the default Oncomine algorithms using a two-tailed Student's t-test. Table 3 includes cervical cancer microarray datasets utilized in this study.

The Human protein Atlas (<https://www.proteinatlas.org/>, last accessed April 2018) was used to query the expression pattern of COBRA1 in HeLa relative to other cervical carcinoma cell lines, in addition to HepG2 cells previously used by our group for COBRA1 silencing.

2.11 Statistical Analysis of Data

Image J software (National Institute of Health, USA, <http://www.imagej.nih.gov/ij/>) was used for quantifying the bands intensities for PCR and Western Blot analyses. Bands were quantified and normalized per the used internal control. Relative differences in gene expression are described as fold change relative to negative siRNA transfected cells unless specified otherwise. Statistical analyses and graphical representations were performed using GraphPad Prism 5.0 (GraphPad, San Diego California USA, <http://www.graphpad.com/>). All the data represent the average \pm standard deviation (SD) from three independent experiments unless specified otherwise. One-way ANOVA, followed by a Bonferroni post-hoc test, was used to determine the statistical significance among multiple different experimental groups in case of single variable. For comparisons made between two different groups, statistical significance was assessed using an unpaired student's t-test (two-tailed). P-value less than 0.05 was considered to be statistically significant (* p <0.05, ** p <0.01, *** p <0.001).

Table 3. Oncomine Microarray datasets utilized to query the expression profiles of COBRA1 in cervical carcinoma compared to normal cervix samples.

Study	Total No. of Samples/Microarray	Type of sample	Number of samples/Type	Reference
Scotto Cervix 2	66	<ul style="list-style-type: none"> • Normal Cervix • Cervical Squamous Cell Carcinoma 	24 32	Scotto et al., 2008
Pyeon Multi-cancer	84	<ul style="list-style-type: none"> • Normal Cervix • Cervical Cancer 	8 20	Pyeon et al., 2007
Zhai Cervix	41	<ul style="list-style-type: none"> • Normal Cervix • Cervical Squamous Cell Carcinoma • High Grade Cervical Squamous Intraepithelial Neoplasia 	10 21 7	Zhai et al., 2007
Biewenga Cervix	45	<ul style="list-style-type: none"> • Normal Cervix • Cervical Squamous Cell Carcinoma 	5 40	Biewenga et al., 2007

3. RESULTS

3.1 Expression of COBRA1 mRNA in cervical cancer tissues relative to normal cervical tissues

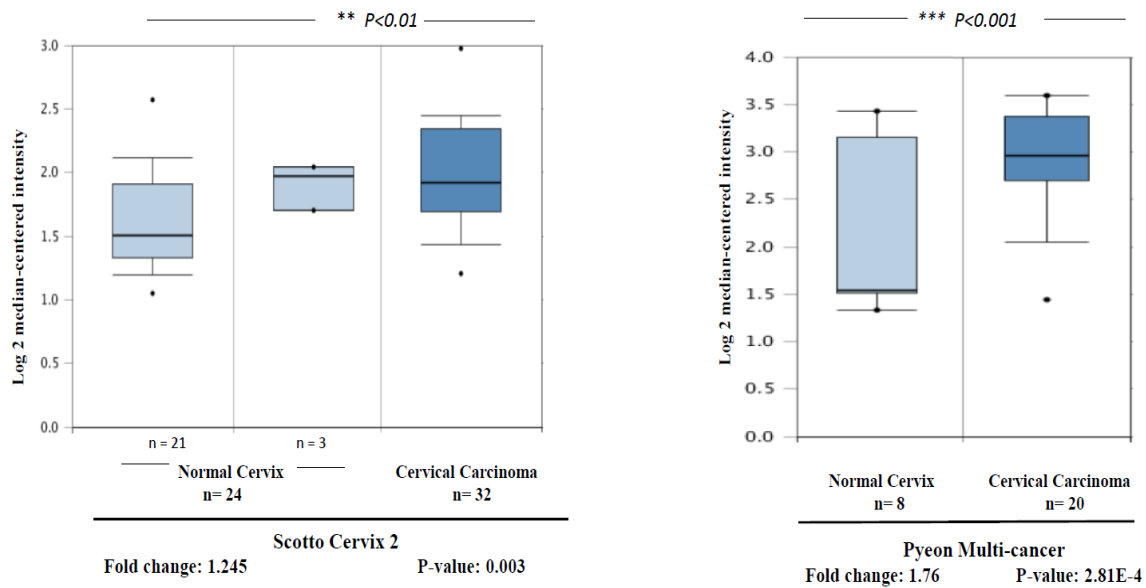
Publicly available microarray datasets in the Oncomine Cancer Microarray database were queried to investigate the expression pattern of COBRA1 in cervical cancer versus normal counterparts at the RNA level. The search yielded results from four different datasets, from four independent studies (**Biewenga et al., 2008; Pyeon et al., 2007; Scotto et al., 2008; Zhai et al., 2007**). As shown in Figure 1, COBRA1 was found to be significantly overexpressed in cervical cancer relative to normal cervix tissues in each of the Scotto Cervix and Pyeon multi-cancer studies with a fold change between 1.2 - 1.8 ($p < 0.05$). In Zhai Cervix dataset, COBRA1 was found to be slightly higher than normal with a fold change of 1.022 but with a statistically non-significant p-value ($p > 0.05$). On the contrary, in Biewenga cervix, COBRA1 was shown to be downregulated relative to normal tissues, but again this was found to be statistically non-significant ($p > 0.05$).

Results obtained from Human Protein Atlas (HPA) revealed comparable expression pattern of COBRA1 in each of HeLa (cervical adenocarcinoma), SiHa (cervical squamous carcinoma) and HepG2 (Liver cancer) cell lines. HPA results are represented as the transcript abundance for each protein-coding gene. It is worth mentioning that HepG2 was previously used by our group for successful silencing of COBRA1. Since successful siRNA mediated knockdown depends; among other factors, on target gene abundance (**Hong, Jiang, Kim, Li, & Lee, 2014**), HPA results suggested that HeLa could serve as a suitable model for COBRA1 silencing (Figure 2).

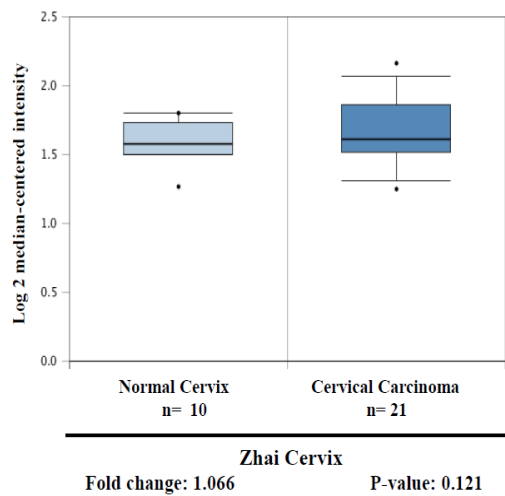
3.2 Knockdown of COBRA1 in CC cell line, HeLa

Based on the finding of an upregulated expression pattern for COBRA1 in CC observed from our *in silico* studies, we hypothesized that COBRA1 could have a role in CC tumorigenesis. Hence, we analysed the effect of COBRA1 gene silencing via siRNA-mediated RNA interference (RNAi) in HeLa cells. For achieving an efficient siRNA

A



B



C

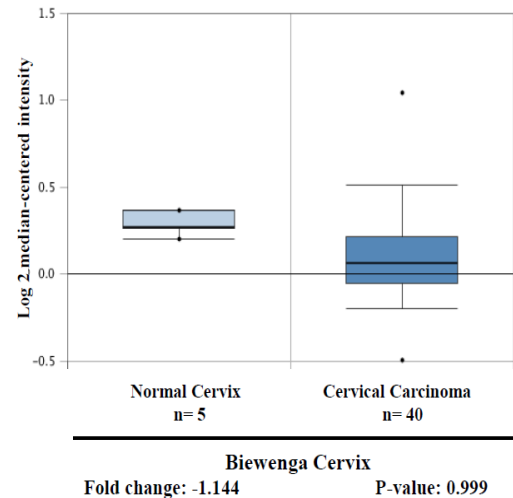


Figure 1. Overexpression of COBRA1 in cervical carcinoma tissues versus normal samples. Publicly available microarray data in Oncomine™ cancer database was searched to examine the expression of COBRA1 in cervical carcinoma versus normal cervix samples. Four datasets from 4 independent studies were analysed. A] Studies showing significant overexpression of COBRA1 in cervical carcinoma B] Study showing slightly higher COBRA1 expression but statistically non-significant. C] Study showing downregulation in COBRA1 expression. Oncomine™ results are illustrated as boxplots, with the top and bottom of the box representing the upper and lower quartiles, 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 within each category. Number of patients in each category (n), fold change and P-value are indicated for each study ** P<0.01, *** P<0.001. In Scotto Cervix, normal samples included 21 normal cervix tissue specimens and 3 commercial RNA from cervix uteri.

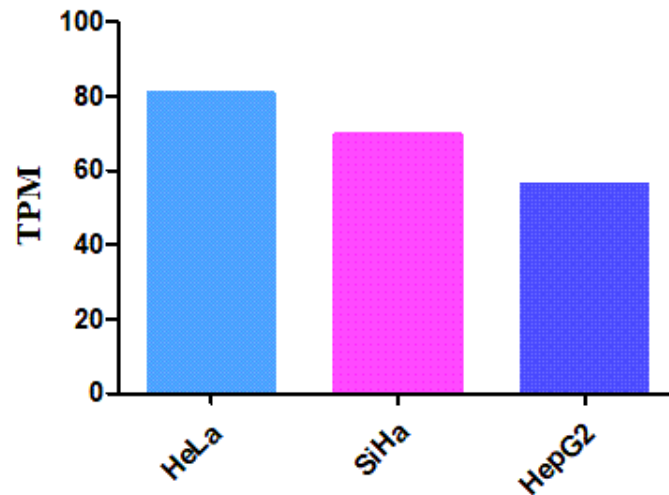


Figure 2. The abundance of COBRA1 in cancerous cell lines. Human protein Atlas was queried to investigate the abundance of COBRA1 mRNA in HeLa cells compared to SiHa and HepG2 cancer cell lines. The RNA-sequencing results generated in the HPA are reported as the number of Transcripts per Million (TPM) of the total protein-coding transcripts.

mediated transient knockdown, optimization of the transfection protocol regarding efficiency of Lipofectamine 3000 in HeLa cells, siRNA concentration, transfection method and transfection duration had to be first performed according to the manufacturers' protocols. This optimization process was aimed at obtaining the highest possible knockdown efficiency at the COBRA1 protein and mRNA levels, with minimal effects on cell viability.

3.2.1 Lipofectamine 3000 knockdown efficiency in HeLa cells

To investigate the possibility of performing COBRA1 knockdown using Lipofectamine 3000 reagent, knockdown of the house-keeping gene GAPDH with 25 nM of GAPDH specific siRNA using 3.75 μ l Lipofectamine 3000 was first performed via forward transfection protocol. Cells were harvested 72 hrs post-transfection and results were evaluated at the mRNA level by semi-quantitative RT-PCR. As shown in Figure 3, Lipofectamine 3000 resulted in ≈ 80 % silencing of GAPDH mRNA relative to the negative control.

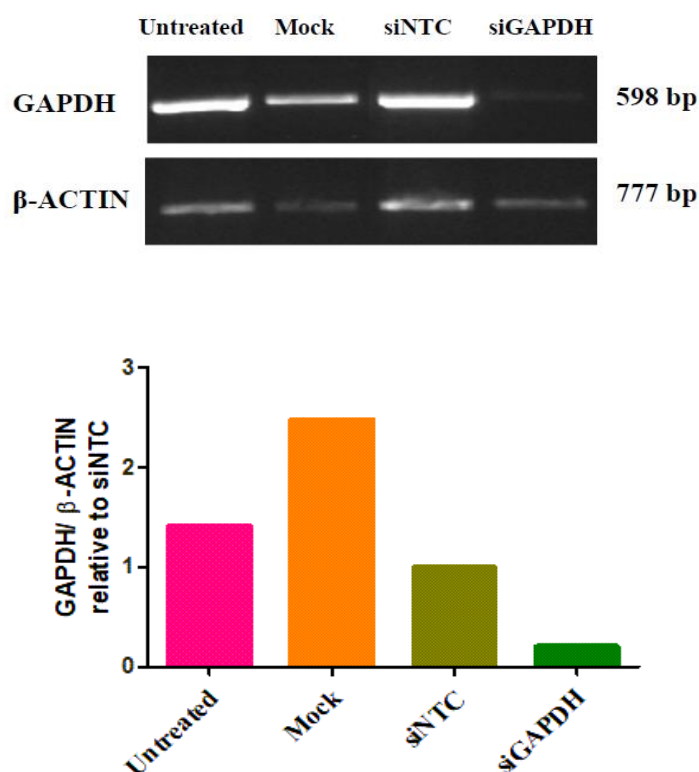


Figure 3. Knockdown of GAPDH in HeLa cells using Lipofectamine 3000. RNA expression was analysed by semi-quantitative RT-PCR. Band intensities were quantified by ImageJ and normalized to the internal control β -ACTIN. Relative expression is expressed as fold change to siNTC. siGAPDH: GAPDH siRNA, siNTC: Negative siRNA.

3.2.2 SiRNA Concentration

Different concentrations of siRNAs within the range recommended by the supplier; 25 nM, 35 nM, and 45 nM, were tested using forward transfection protocol. Knockdown efficiency was assayed 72 hrs following transfection by semi-quantitative RT-PCR and Western Blot. As shown in Figure 4, transfecting the cells with a final concentration of 35 nM COBRA1 siRNA/well resulted in achieving a knockdown of $\approx 90\%$ at the mRNA level and the highest observed knockdown of $\approx 80\%$ at the COBRA1 protein level. Hence, the 35 nM concentration was used for all subsequent siRNA transfections. Cell viability and morphology were observed by phase contrast microscopy 10X and were found to be comparable in all siRNAs treated, mock-treated and untreated cells with the volume of Lipofectamine used (3.75 μ l).

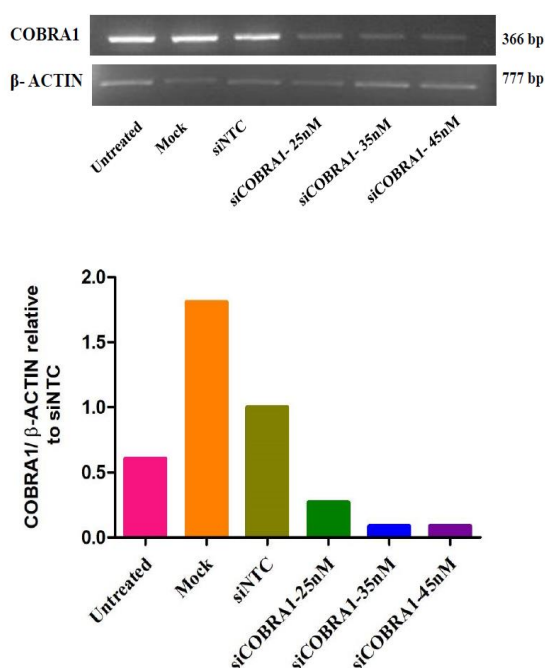
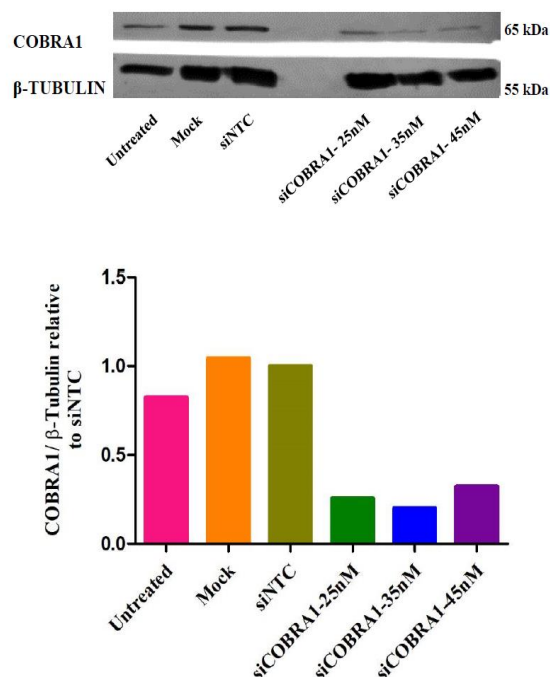
A**B**

Figure 4. Optimization of siRNA concentration via forward transfection. A) RNA expression was analysed by semi-quantitative RT-PCR. Band intensities were quantified by ImageJ and normalized to the internal control β -ACTIN. B) Protein expression analysed by Western Blot. Band intensities were quantified by ImageJ and normalized to the internal control β -TUBULIN. Relative expression is expressed as fold change to siNTC. siCOBRA1: COBRA1 siRNA, siNTC: Negative siRNA.

3.2.3 Transfection Method

Successful gene silencing in siRNA-mediated RNA interference (RNAi) experiments requires efficient uptake of siRNA into the cells. Two variations of transfection were tested: standard forward and reverse transfections. They mainly differ in the order and timing of the addition of the three necessary components of transfection: siRNA, lipid-based transfection reagent, and cells. In forward transfection, siRNA and Lipofectamine reagent are complexed in low serum media and added to pre-seeded cells that are 70-80% confluent. In reverse transfection, all three components are added to the wells at essentially the same time. Reverse transfection reduces the time for transfection by one day compared to forward transfection. As shown previously (Figure 4), forward transfection of HeLa cells with 35 nM COBRA1 siRNA resulted in $\approx 80\%$ knockdown on the COBRA1 protein level. For the

aim of selecting the method that could achieve the highest possible depletion Of COBRA1 protein, reverse transfection method was performed and the transfection efficiency between both the reverse and forward transfections was compared. As shown in Figure 5, 72 hr post-transfection, the reverse transfection resulted in a higher efficiency (93%) than that observed with the forward transfection. Accordingly, the reverse transfection protocol together with the 35nM concentration of siRNAs was used in the subsequent transfection experiments.

3.2.4 Knockdown Duration

The siRNA mediated COBRA1 used in this study cause transient silencing of the expression of the target gene. To identify the optimum time point at which maximum knockdown of the effective protein levels occurs, knockdown efficiency was analysed at 24 hr, 48 hr and 72 hr post-transfection by Western Blot and RT-PCR. As shown in Figure 5, incubating the cells for 72 hrs resulted in the highest silencing effect (93%) at the COBRA1 protein level, compared to (86%) at 48 hrs and (28%) at 24 hrs. On the other hand, the highest knockdown at the mRNA level (94.2%) was observed at 24 hrs post transfection compared to $\approx 80\%$ at 72 hrs. Since the goal is achieving an efficient knockdown at the protein level, all subsequent siRNA transfections in the HeLa cell line were performed at 72 hrs.

3.3 COBRA1 SMARTPool siRNA Knockdown efficiency in HeLa cells

To confirm the efficiency of SMARTPool siRNA in HeLa cells, transfection was repeated using the finally chosen optimized conditions; reverse transfection, 35Nm siRNA, 72 hrs and 3.75 μ l Lipofectamine 3000. The expressions of COBRA1 mRNA and protein levels were assessed post-transfection in each of the tested groups by semi-quantitative RT-PCR and Western Blot respectively. Figure 6 shows that both mRNA and protein levels were significantly down regulated in COBRA1 siRNA HeLa treated cells compared to the Negative siRNA treated and the untreated cells. Data from three independent experiments were collected and statistical significance was computed. The COBRA1 silencing effect was found to be $\approx 79\%$ at the mRNA levels ($P<0.01$) and 95.5% at the protein level ($P<0.001$).

It is worth mentioning that the COBRA1 knockdown did not have any significant impact on the overall cellular morphology of HeLa cells as observed under the inverted microscope (10X) when compared to the untreated or negative siRNA treated cells (Figure 7).

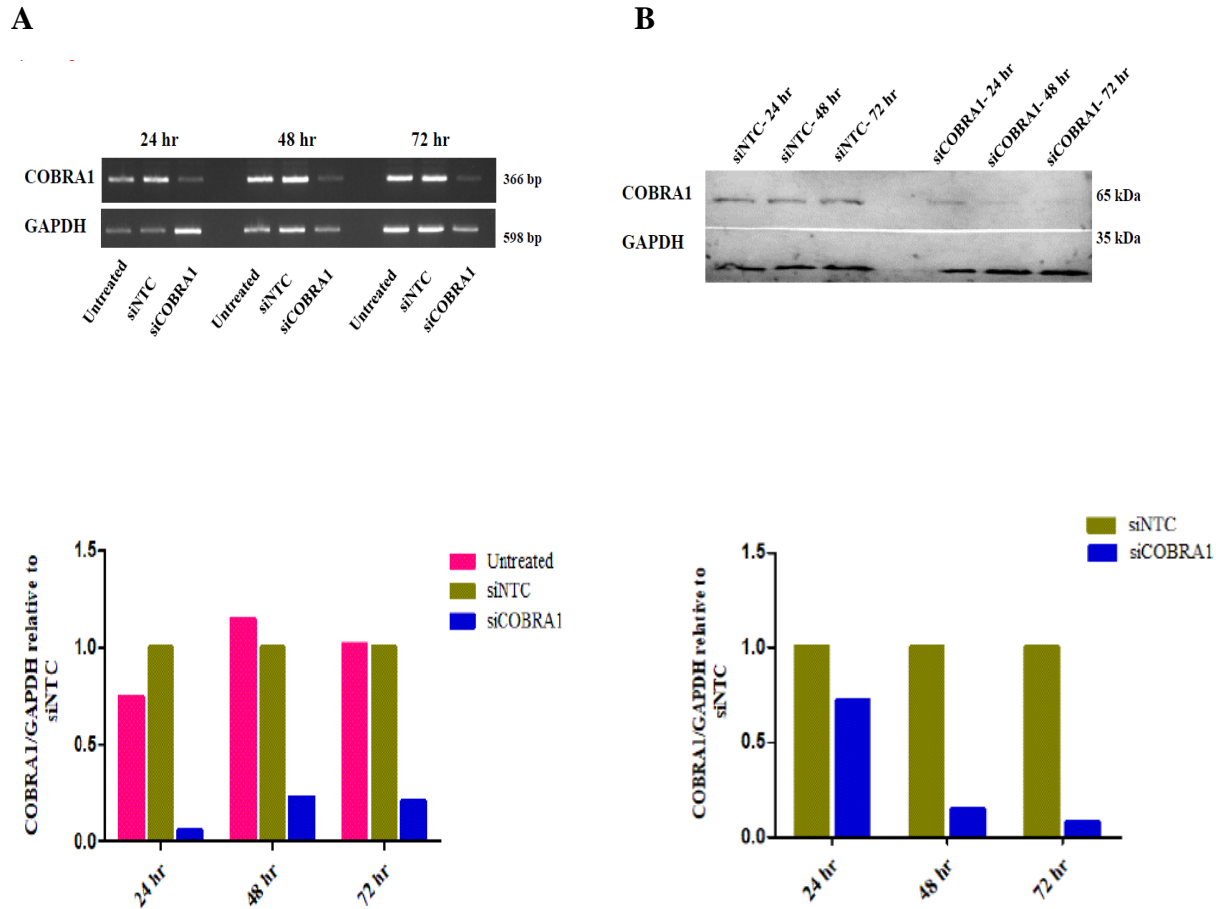
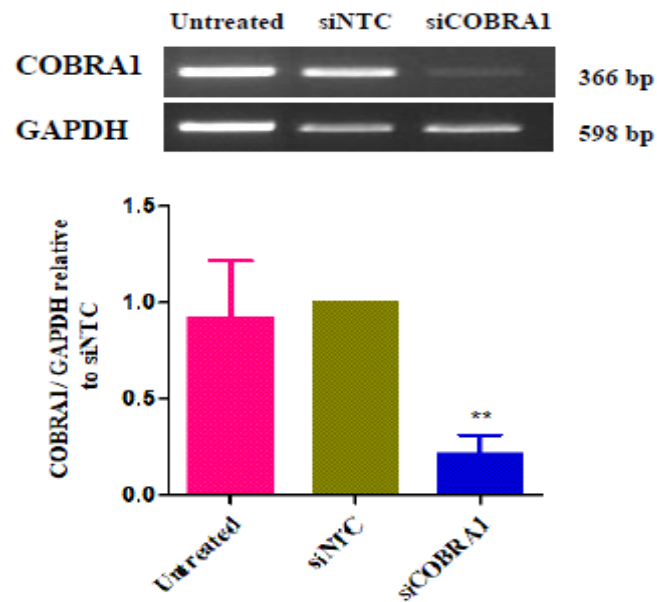


Figure 5. Optimization of knockdown duration via reverse transfection. Knockdown efficiency was analysed at 24, 48 and 72 hours post-transfection. A) RNA expression was analysed by semi-quantitative RT-PCR. B) Protein expression analysed by Western Blot. Bands intensities were quantified by ImageJ and normalized to the internal control GAPDH. Relative expression is expressed as fold change to siNTC. siCOBRA1: COBRA1 siRNA, siNTC: Negative siRNA.

A



B

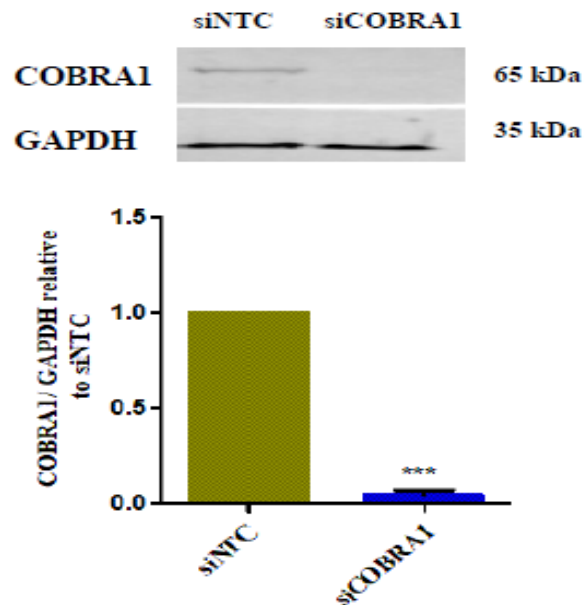


Figure 6. COBRA1 SMARTPool siRNA effectively silences COBRA1 expression using the optimized transfection conditions. A) mRNA expression was analysed by semi-quantitative RT-PCR. B) Protein expression analysed by Western Blot. Bands intensities were quantified by ImageJ and normalized to the internal control GAPDH. Relative expression is expressed as fold change to siNTC. Data represent the means \pm SD of three independent experiments (n=3). Statistically significant at **p<0.01, ***p<0.001(one-way ANOVA, Bonferroni's post-test (A) and unpaired Student's t-test, two tailed (B)). siCOBRA1: COBRA1 siRNA, siNTC: Negative siRNA.

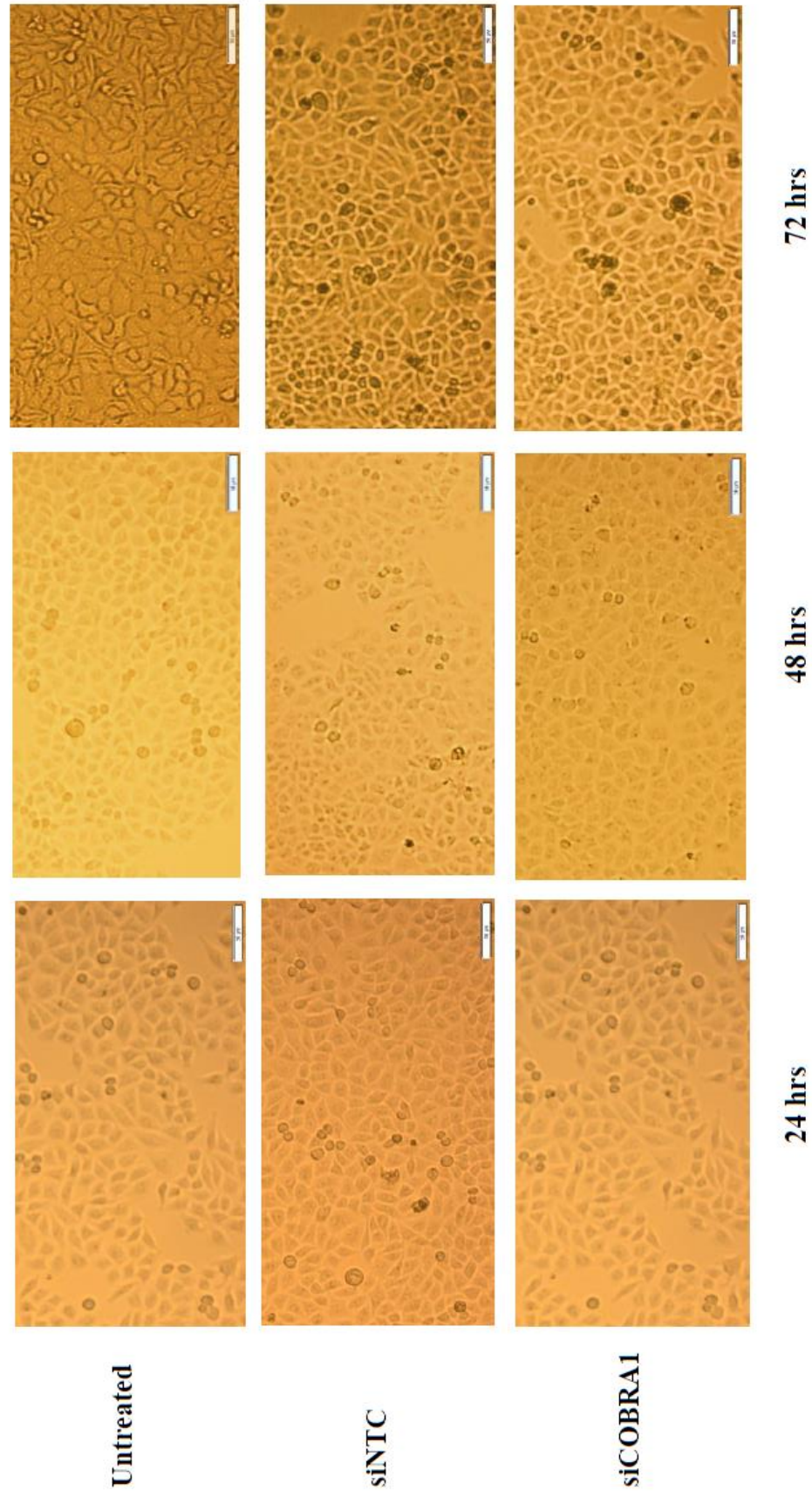


Figure 7. Morphological examination of HeLa cells post-transfection. Photos were taken with 10X magnification power at 24, 48 and 72 hours post-transfection. Cells were either reverse transfected with COBRA1 siRNA or Negative siRNA. As control, cells were left untreated

3.4 Effect of COBRA1 silencing on the expression patterns of NELF complex subunits

The mRNA expression levels NELF-A, NELF- C/D and NELF-E subunits were analysed using semi-quantitative RT-PCR in all of the tested conditions (Untreated and Negative siRNA compared to COBRA1 siRNA transfected cells). It was found that none of the other three subunits was affected by COBRA1 (NELF-B) knockdown and there was no statistically significant difference at the mRNA expression level among any of the tested conditions ($P > 0.05$) (Figure 8).

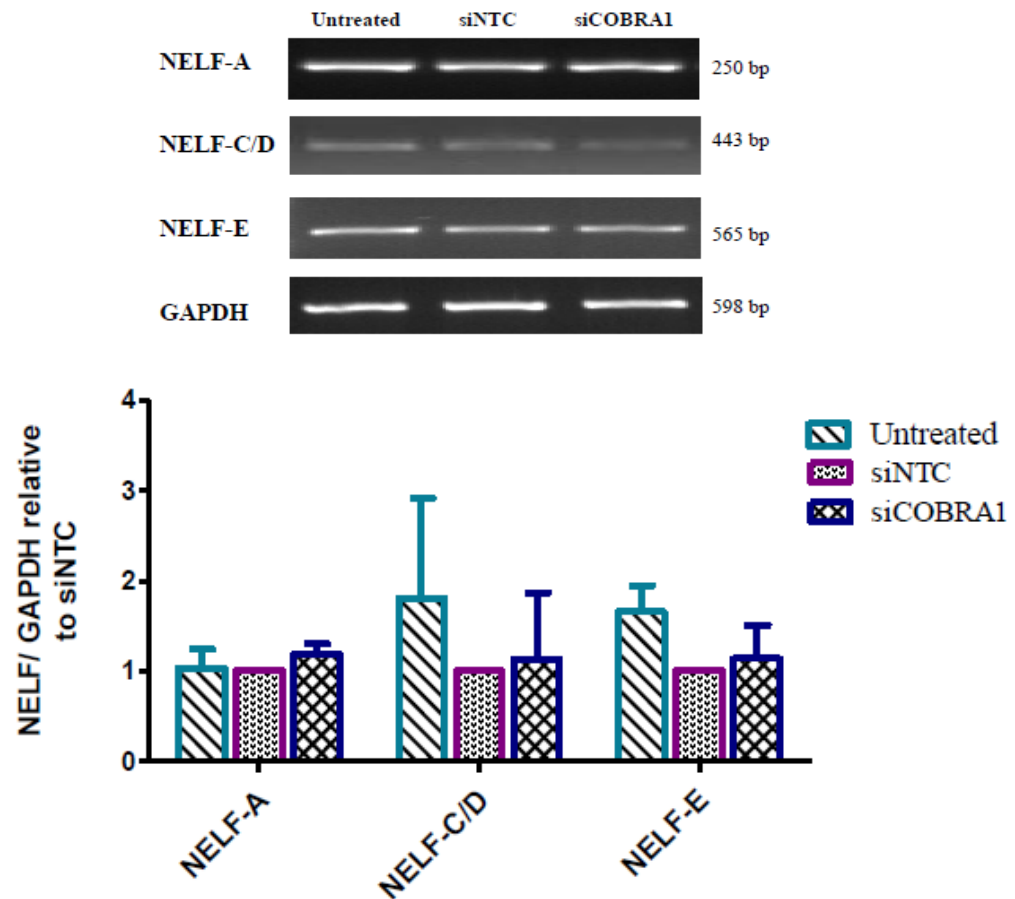


Figure 8. COBRA1 silencing does not alter the mRNA steady-state expression of other NELF complex subunits. NELF-A, NELF-C/D and NELF-E RNA expression was analysed by semi-quantitative RT-PCR 72 hrs post-transfection in each of the tested groups. Bands intensities were quantified by ImageJ and normalized to the internal control GAPDH. Relative expression is expressed as fold change to siNTC. Data represent the means \pm SD of three independent experiments ($n=3$). Results were found to be statistically non-significant at $p > 0.05$ (one-way ANOVA, Bonferroni's post-test). siCOBRA1: COBRA1 siRNA, siNTC: Negative siRNA.

3.5 COBRA1 Silencing effect on the proliferation of HeLa cells

The effect of COBRA1 on cellular proliferation was first examined via the methyl tetrazolium (MTT) assay. Reverse transfection of HeLa cells with 35 nM of either the COBRA1 siRNA or Negative siRNA using Lipofectamine 3000 was performed in 96 well plates according to the manufacturers' protocols. Percentage cell viability was used as an indication for the cellular proliferation. As shown in Figure 9A, there was a statistically significant decrease in the cellular proliferation of the COBRA1 siRNA treated cells compared to the untreated and mock controls but not to the negative siRNA treated cells.

Further investigation of the effect of silencing on HeLa cells proliferation was done by examining the expression profile of Ki-67, an important marker for cellular proliferation. Ki-67 protein is absent in the resting phase of cell cycle (G0) while it is present in all active phases (G1, S, G 2, and mitosis) proving that its expression is essential for cell cycle progression (**Scholzen & Gerdes, 2000**). In accordance with the previous results from the MTT assay, no statistically significant difference in the mRNA level of Ki-67 was observed compared to the negative control but there was significant decrease compared to the untreated cells (Figure 9B).

3.6 COBRA1 Silencing effect on the migration potentials of HeLa cells

The classical wound healing assay was used to investigate the effect of COBRA1 silencing on the migratory potential of HeLa cells (**El Zeneini et al., 2017; Youssef et al., 2016**). The cell monolayer was scratched post-transfection at the optimum knockdown time point (72 hrs) and images at this initial timing were compared to those taken after 24 hrs (Figure 10 A). Wound areas were analysed using ImageJ software and percentage wound closure was calculated based on the given areas. Results have shown that there was no statistically significant difference in the wound closure ability among tested conditions ($p > 0.05$; Figure 10 B). The COBRA1 knockdown cells showed approximately 49.7 % wound closure after 24 hrs and the untreated, Mock-treated and siNTC treated cells showed 49.74%, 50.8% and 56.14 % wound closure respectively.

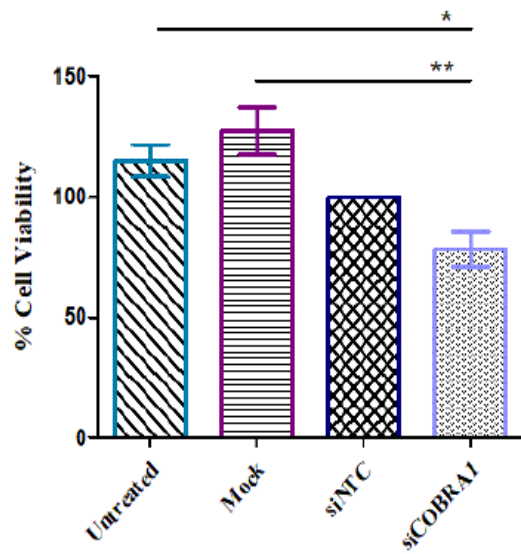
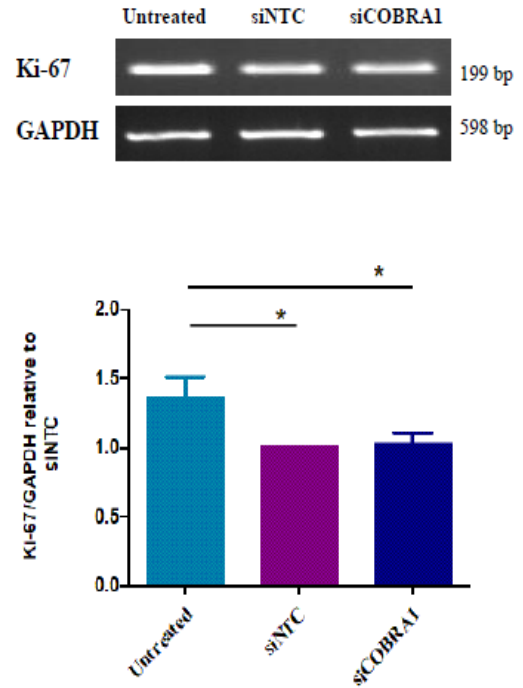
A**B**

Figure 9. Effect of silencing of COBRA1 on HeLa cells proliferation. A) MTT assay was used to analyse the viability of HeLa cells following COBRA1 knockdown compared to the control groups. B) RT-PCR of the proliferation marker Ki-67. Bands intensities were quantified by ImageJ and normalized to the internal control GAPDH. Relative expression is expressed as fold change to siNTC. Data represent the mean \pm SD of three independent experiments (n=3). Statistically significant at * $p < 0.05$, ** $p < 0.01$ (one-way ANOVA, Bonferroni's post-test). siCOBRA1: COBRA1 siRNA, siNTC: Negative siRNA.

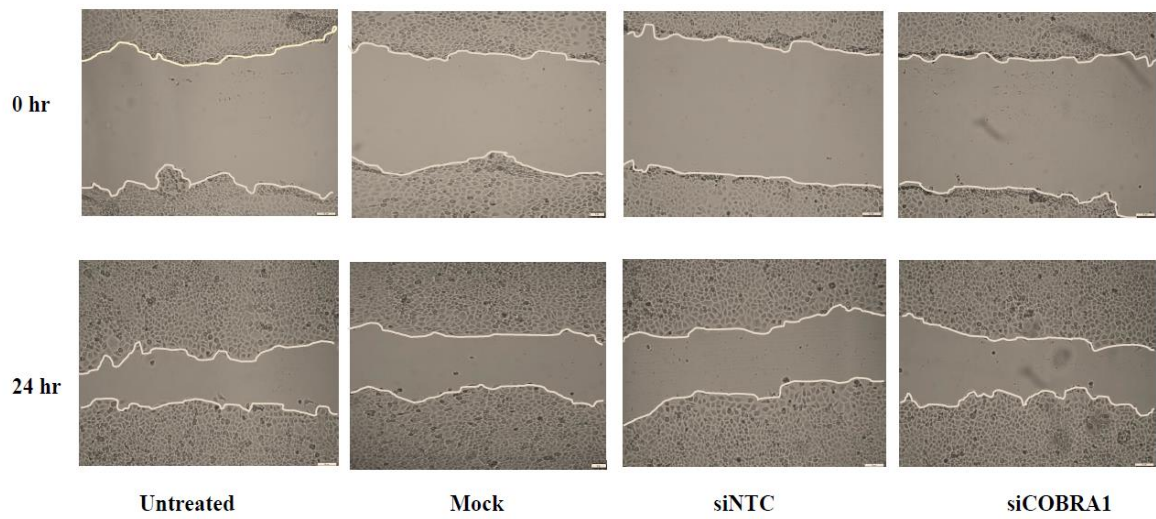
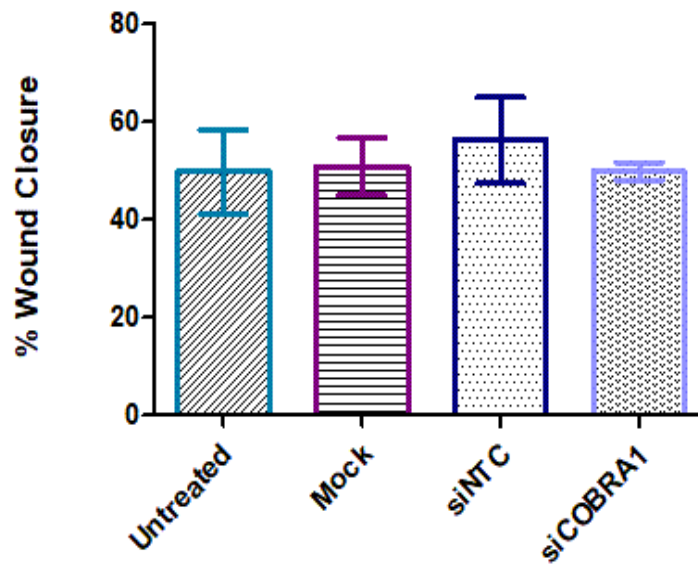
A**B**

Figure 10. Effect of silencing of COBRA1 on HeLa cells migration. Wound healing assay was used to analyse the migration rate of HeLa cells following COBRA1 knockdown. A) Images of the wound areas taken at 0 and 24 hrs times using phase contrast microscopy at 10X magnification. B) Bars are representing Percentage of wound closure in each of the tested conditions. The open areas were measured using ImageJ software followed by the calculation of the percentage of wound closure. Data represent the mean \pm SD of two independent experiments (n=2). (one-way ANOVA, Bonferroni's post-test) ($P > 0.05$). siCOBRA1: COBRA1 siRNA, siNTC: Negative siRNA.

3.7 COBRA1 Silencing effect on some cancer related genes

The differential expression of a number of genes that have been previously reported to be commonly deregulated in cancer were examined at the mRNA level post COBRA1 siRNA transfection.

3.7.1 β - Catenin

Recent studies has suggested the Wnt/ β -catenin signalling pathways as one of the pathways involved in CC (**Pérez-Plasencia et al., 2008**). Semi-quantitative RT-PCR was carried out to examine the mRNA expression of the β -catenin gene following COBRA-1 silencing. As shown in Figure 11A, upon COBRA1 knockdown, there was a significant decrease in the mRNA levels of β -catenin in COBRA1 siRNA treated cells relative to the negative control.

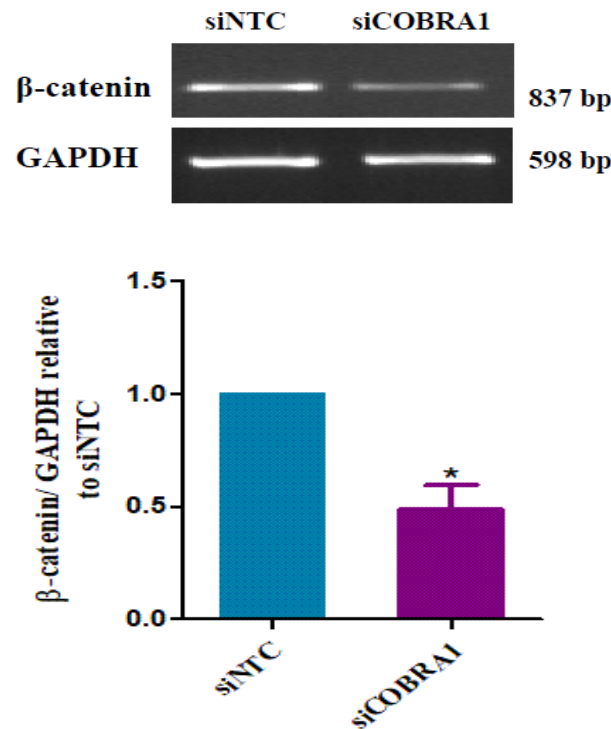
3.7.2 Survivin

Survivin is one of the genes known to be commonly deregulated in cancer (**Garg, Suri, Gupta, Talwar, & Dubey, 2016; Jaiswal, Goel, & Mittal, 2015**). RT-PCR was carried out to examine the mRNA expression of the survivin gene following COBRA-1 silencing. As shown in Figure 11B, upon COBRA1 knockdown , there was an observable decrease in the levels of each of the three detected isoforms of survivin (survivin-2B, survivin-DeltaEx3 and the wild-type survivin) but it was found to be statistically significant only in survivin-2B and survivin-DeltaEX3 isoforms levels compared to the negative control group with a p-value<0.05.

3.7.3 TFF1

TFF1 is one of a family of highly conserved, secreted trefoil peptide proteins. It has been previously reported that TFF1 expression is negatively regulated by the NELF complex in breast cancer (**Aiyar et al., 2004; Aiyar, Cho, Lee, & Li, 2007**). In upper gastrointestinal tract cancers (UGC), overexpression of COBRA1 was associated with down regulation of TFF1 (**McChesney et al., 2006**). To determine the effect of silencing COBRA1 on the mRNA levels of TFF1, semi-quantitative RT-PCR was performed. As shown in Figure 12, there was a statistically significant increase in the TFF1 levels following COBRA1 siRNA transfection compared to the negative siRNA treated cells (p <0.001).

A



B

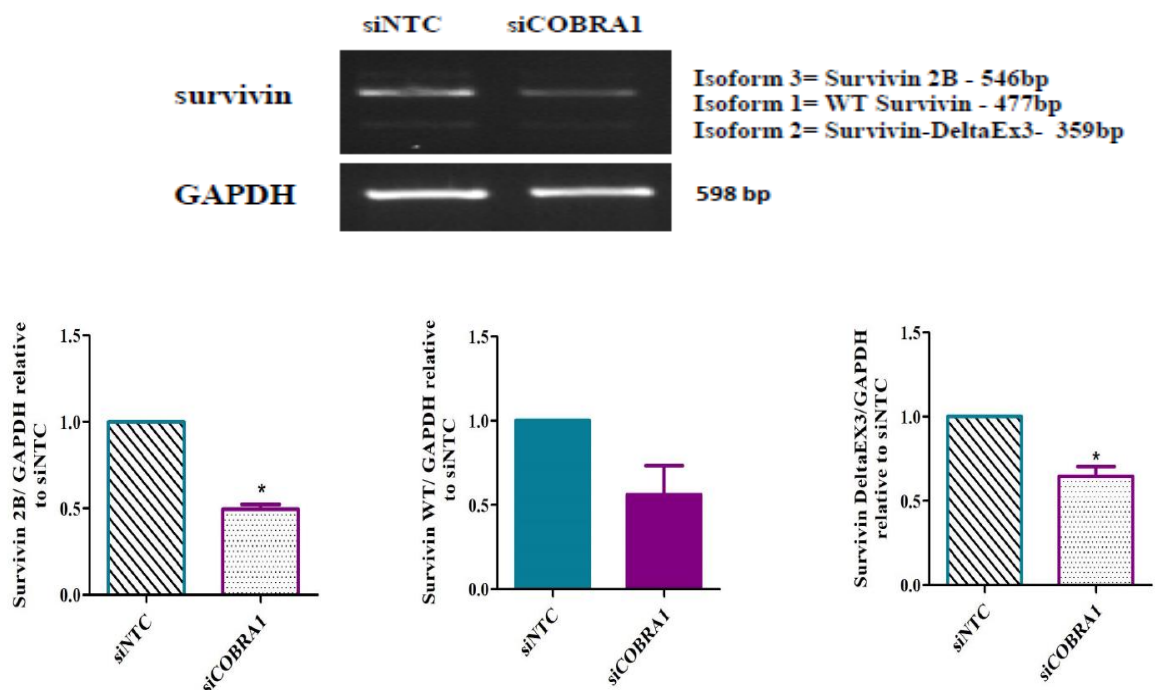


Figure 11. Effect silencing of COBRA1 on the expression of B-catenin and survivin. A) Effect of COBRA1 knockdown on β -catenin. B) Effect of COBRA1 knockdown on survivin. mRNA expression was analysed by semi-quantitative RT-PCR. Bands intensities were quantified by ImageJ and normalized to the internal control GAPDH. Relative expression is expressed as fold change to the siNTC. Data represent the mean \pm SD of 2 independent experiments (n = 2). Statistically significant at *p < 0.05 (unpaired Student t-test, two tailed). siCOBRA1: COBRA1 siRNA, siNTC: Negative siRNA.

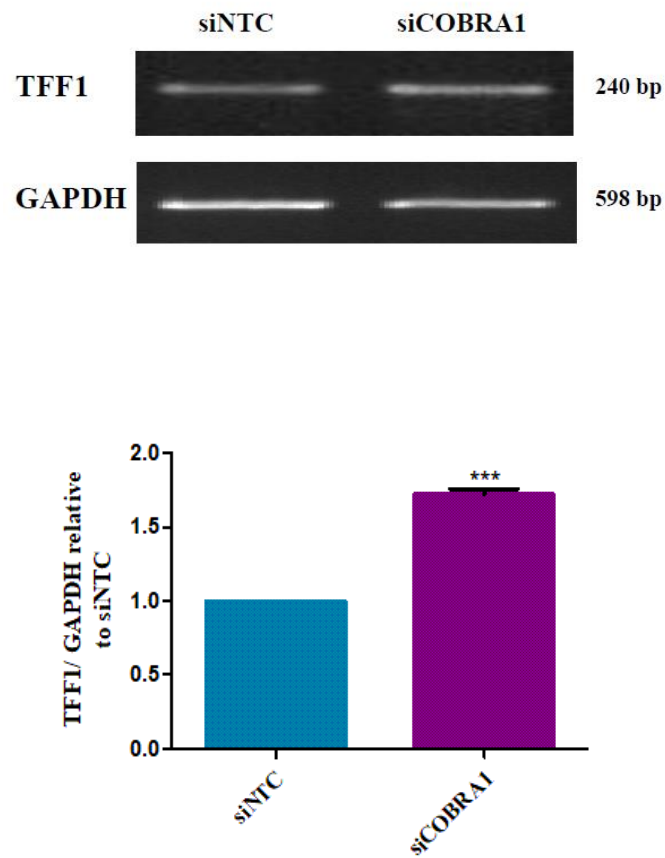


Figure 12. Effect of silencing of COBRA1 on the expression of TFF1. Effect of COBRA1 knockdown on TFF1 mRNA levels was analysed by semi-quantitative RT-PCR. Bands intensities were quantified by ImageJ and normalized to the internal control GAPDH. Relative expression is expressed as fold change to the siNTC. A significant upregulation was observed in the expression of TFF1 following COBRA1 knockdown, relative to siNTC treated group. Data represents the mean \pm SD of 2 independent experiments (n=2). Statistically significant at *** $p < 0.001$ (one-way ANOVA, Bonferroni's post-test). siCOBRA1: COBRA1 siRNA, siNTC: Negative siRNA.

4. DISCUSSION

Cervical carcinoma (CC) is the fourth leading cause of cancer-related deaths among women worldwide and the second most commonly diagnosed cancer in developing countries, accounting for an estimated 527,600 new cervical cancer cases and 265,700 deaths worldwide in 2012 (**Torre et al., 2015**). Improved understanding and increased knowledge of the molecular biology events associated with cervical carcinogenesis will help in developing more specific biomarkers and targeted therapeutics for better control of CC.

Cofactor of BRCA1 (COBRA1) has been shown to be involved in many types of cancers with previous studies by our group have identified a potential role for COBRA1 in HCC progression (**El Zeneini et al., 2017; Youssef et al., 2016**). In this study, we extended the scope of our group's research to investigate the possibility of COBRA1 having a potential role in CC as previously detected in HCC. This was performed through analysing the consequences of COBRA1 Knockdown on the cellular behaviour of HeLa cells and on the expression pattern of some cancer-related genes.

4.1 Data mining results

To explore the expression pattern of COBRA1 in cervical cancer, analysis of the microarray data publicly available on the Oncomine cancer microarray dataset was first performed. The used search filter criteria resulted in the identification of four datasets from four different independent studies; Pyeon Multi-cancer, Scotto Cervix 2, Zhai Cervix and Biewenga Cervix (**Biewenga et al., 2008; Pyeon et al., 2007; Scotto et al., 2008; Zhai et al., 2007**).

These studies were directed at investigating different aspects related to cervical cancer. The Scotto Cervix study mainly aimed at identification of the genetic expression profiles associated with the gain in long arm of chromosome 20 in various stages of progression of CC [Stages I, II, III and IV]. Pyeon Multi-cancer study goal was defining similarities and differences of HPV-positive versus HPV-negative cancers arising in the same tissue, this study included samples for head and neck cancers in addition to cervical cancers [FIGO stage IB (80%), II/III (15%) and IV (5%)]. The Zhai cervix study aim was to test differential genetic expression profiles in human pre-invasive, invasive cervical squamous cell carcinomas and normal cervical epithelia, the stages of disease for the tumor specimens used

were not specified. As for Biewenga cervix study, its main objective was first to test whether there is a differential gene expression profile for early stage cervical cancer tumours [FIGO stage IB1, IB2 and IIA] with and without lymph node metastasis, as a means for predicting the presence of lymph node metastasis before treatment. Subsequently, the authors compared gene expression profiles between healthy cervical tissue and early stage cervical cancer tissue.

In two of these studies, Scotto Cervix and Pyeon multi-cancer, COBRA1 mRNA was found to be significantly overexpressed in CC tissues versus normal with a fold change of 1.245 ($p=0.003$) and 1.76 ($p=2.81E-4$) respectively. As for Zhai cervix study, COBRA1 was found to be slightly upregulated with a fold change of 1.066 but this was found to be statistically insignificant ($p=0.121$). In contrast to the first three studies, in the Biewenga Cervix study COBRA1 was found to be downregulated with -1.1441 fold change but again this was found to be insignificant with a very high p-value of 0.999.

The observed variations in microarrays data might be referred to differences in the experimental factors associated with each study. This could include variations in samples handling, RNA handling, used microarray platforms, probes sequences and differences in used computational methods for data analysis and validation. It is worth noting that in each of the Pyeon Multi-cancer, Scotto cervix and Zhai Cervix studies, studies used microdissection to enrich for the epithelial tumor cells before RNA extraction for microarray analysis, while in Biewenga RNA was extracted from tissues of samples having >70% tumors thus including normal cells in addition to the cancerous ones. In addition each of the first three studies Affymetrix U133A oligonucleotide microarrays were used (containing the whole genome, 14,500 and 22,283 probe sets respectively), while in Biewenga the whole genome human oligonucleotide microarrays (44K Agilent) were used. The staging of the tumors also might contribute to the results. In each of Pyeon multi-cancer and Scotto Cervix, samples included in the microarray analysis represented different stages of CC [Stages I, II, III and IV]. On the other hand, Biewenga Cervix focused on early stages of CC [stages I and II]. Tumor stages were not specified in Zhai Cervix and were referred to collectively as being invasive cervical cancers.

Altogether, the *in-silico* analysis results suggests that there is an overexpression pattern for COBRA1 in CC. Further meta-analysis of these datasets will help gain more necessary

information through correlating COBRA1 expression with the clinic-pathological features as the HPV-subtype, stage and size of the tumor and treatment outcome.

4.2 Expression of NELF complex subunits

The assembly of the four subunits of the NELF complex (NELF-A, NELF-B, NELF-C/D and NELF-E) is required for the formation of a functional complex. The core of the complex; which consists of NELF-B (COBRA1) and NELF-C/D, binds each of the NELF-A having the RNAPII binding domain and NELF-E having the RNA binding domain (**Narita et al., 2003**). This interdependent nature of the NELF subunits was reported in 2004 by Aiyar *et al.*, where the binding of the COBRA1 to the activated ER α was followed by the recruitment of the other NELF subunits and the formation of a functional NELF complex at the promoters of estrogen responsive target genes (**Aiyar et al., 2004**).

In this study, the knockdown of COBRA1 had no effect on the expression pattern of the other NELF subunits at the mRNA level. This goes in accordance with previous results from studies done on HCC by our group (**El Zeneini et al., 2017**) and other groups on UGC and breast cancer (**McChesney et al., 2006; Sun et al., 2008**). It has been previously reported that the depletion of one or more subunits of the NELF complex affects the other subunits at the protein level (**Aiyar et al., 2004; Kininis, Isaacs, Core, Hah, & Kraus, 2009; Sun et al., 2008**), which suggests the presence of post-transcriptional regulatory mechanisms that governs the interdependent expression of NELF subunits . It will be interesting to further investigate the protein levels of other subunits using Western Blot following silencing of COBRA1 in HeLa cells to confirm this interdependency in cervical cancer.

4.3 Effect on HeLa cells proliferation and migratory abilities

The ability of cancer cells to maintain a state of chronic proliferation and to metastasize from their initial primary tumor sites to secondary locations within the human body are considered to be main hallmarks of cancer (**Hanahan & Weinberg, 2011**). Previous studies related to COBRA1 and its role in cellular proliferation show some conflicting results. In 2004, Aiyar *et al.* research using breast cancer cells postulated a role for COBRA1 as an inhibitor of estrogen mediated growth of T47D cancer cells (**Aiyar et al., 2004**). On the

other hand COBRA1 has been found to support the proliferation of HCC with results showing decreased cell count and Ki-67 mRNA expression in HepG2 cells upon depletion of COBRA1 compared to negative control (**El Zeneini et al., 2017**).

In the present study, the role of COBRA1 in HeLa cells proliferation has been investigated post-transfection first by measuring cell viability using MTT assay and second by examining the expression pattern of the cellular proliferation marker Ki-67 at the mRNA level across different tested groups. Even though there was a significant difference in percentage cell viability of the COBRA1 siRNA treated cells compared to the untreated ($p < 0.05$) and mock-treated cells ($p < 0.01$), there was no significant difference in cell viability when compared to the Negative siRNA treated cells. These results were also reflected at the Ki-67 levels, where there was a significant difference in the expression between the untreated cells compared to the siRNA treated cells but again no significant difference was observed in the COBRA1 siRNA relative to the negative siRNA treated cells. This decrease might be attributed to the effects on cell viability that are known to be associated with the transfection process and not due to the knockdown of COBRA1 itself.

It is important to mention that under the applied experimental conditions in this study, no exogenous estrogen was supplied to any of the tested groups. Growing body of evidences suggests a role for estrogen in the development of cervical carcinogenesis. Studies done on HPV transgenic mice revealed that despite the fact that those mice could develop spontaneous tumors, mainly in the skin, they rarely develop cervical cancers spontaneously except when they are treated with exogenous 17 β -estradiol (**S. Chung et al., 2010**). In accordance, the findings of a recent study in 2017 showed that estradiol could enhance the proliferation and inhibit the apoptosis of Hela cervical adenocarcinoma cell line (**Liu, Tian, Yang, & Zhang, 2017**). Hence, no conclusive results can be obtained from our findings with regard to the role of COBRA1 in HeLa cells proliferation unless the transfection was further performed with exogenous estrogen added to the media.

Regarding the effect of COBRA1 silencing on the migratory abilities of HeLa cells post COBRA1 siRNA silencing, no significant differences in the percentage wound closure across 24 hrs interval were noticed among any of the tested groups. This suggests that the effect of COBRA1 with respect to proliferation and migration of HeLa cells might be governed by the same above mentioned stimulus.

4.4 Effect on mRNA expression level of TFF1

TFF1 (also known as pS2) is one of a family of highly conserved, secreted trefoil peptide proteins. It is expressed mainly in the gastric epithelium as a component of the mucus layer protecting the stomach against mucosal injury (Soutto et al., 2015). Initially, TFF1 was found to be one of the estrogen inducible genes in the breast cancer cell line MCF7 (Masiakowski et al., 1982). In 2002, a study by Leung *et al.* suggested that there was a gradual loss of TFF1 expression along the process of gastric tumorigenesis (Leung et al., 2002).

It has been previously reported that TFF1 expression is negatively regulated by COBRA1 in both breast cancer and upper gastrointestinal tract cancers (Aiyar et al., 2004, 2007; McChesney et al., 2006)

Notably, Aiyar *et al.*, studies in breast cancer cell line T47D have shown that COBRA1 causes RNAPII pausing at the promoter proximal regions of TFF1 in response to the ligand-dependent activation of ER α (Aiyar et al., 2004). This was contradictory to the results obtained by Kininis *et al.*, 2009 in MCF7 cells where the depletion of COBRA1 did not alter the expression of TFF1 suggesting a cell-type specific effect for COBRA1 (Kininis et al., 2009).

In this study, examining the TFF1 mRNA levels in HeLa cell line upon COBRA1 silencing revealed an inverse correlation between COBRA1 and TFF1. There was a significant increase in TFF1 expression in COBRA1 siRNA treated cells compared to the negative siRNA treated cells ($p < 0.001$). This finding suggests that COBRA1 negatively regulates TFF1 in HeLa cells as was previously reported in breast and UGCs.

4.5 Effect on mRNA expression level of β -catenin

Wnt/ β -catenin signaling is involved in the control of a number of biological processes as cellular proliferation, fate specification, migration, cell adhesion, tissue architecture, and organogenesis. After Wnt couples to its receptors, one of the pathways that becomes activated is the canonical pathway which induces the entry of β -catenin to the nucleus where it acts by affecting the transcription of target genes (Pérez-Plasencia et al., 2008). Recent studies have suggested the Wnt/ β -catenin signaling as one of the main pathways deregulated

in CC (**Kwan et al., 2013; Pérez-Plasencia et al., 2008**). Aberrant activation of Wnt/ β -catenin signaling is mainly caused by the accumulation of β -catenin, which is closely associated with cervical carcinogenesis (**Kwan et al., 2013**). Hence, targeting this pathway might be a promising molecular therapeutic approach for CC.

A recent study proposed that in gastric cancer, TFF1 depletion induces cellular proliferation via β -catenin signaling. TFF1 induced the activation of protein phosphatase 2A (PP2A) and glycogen synthase kinase 3 (GSK3 β) which in turn were reported to regulate the AKT- β -catenin signaling negatively. This was reflected in the expression levels of downstream targets c-MYC and Cyclin D1 (**Soutto et al., 2015**). In line with these results, we observed decreased expression of β -catenin in COBRA1 siRNA treated cells compared to negative siRNA treated cells. This decrease was found to be statistically significant at a p-value <0.05. Thus, the upregulation of TFF1 upon COBRA1 silencing was associated with decreased β -catenin expression.

4.6 Effect on mRNA expression of survivin

Survivin is a member of the inhibitor of apoptosis proteins (IAP) family with its expression known to be associated with cellular proliferation, angiogenesis and inhibition of apoptosis (**Fan & Chen, 2017**). Identifying the role of survivin in cancer emerged from the findings that it is highly expressed in most human cancers as well as during development while it is undetectable in non-proliferating differentiated adult tissues (**Li, 2005**). Survivin gene is located on chromosome 17q25 and it encodes multiple splice variants which include wild-type survivin, survivin 2B, survivin DeltaEx3, survivin 3B, survivin 2 α and survivin 3 α (**Garg et al., 2016; Jaiswal et al., 2015**). In cancer cells, it was reported that all these isoforms are expressed at very high levels compared to normal tissues and was found to be correlated to tumor aggressiveness and treatment resistance (**Jaiswal et al., 2015**). The role of survivin, survivin 2B and survivin DeltaEx3 in cancer has been extensively studied with results indicating an anti-apoptotic effect for both wild-type survivin and survivin DeltaEx3 while survivin 2B was shown to have pro-apoptotic action. The fact that there are many splice variants of survivin with even different subcellular pools adds to the complexity of survivin's biological functions (**Garg et al., 2016; Li, 2005**). It is worth mentioning that the expression of survivin has been reported to be regulated by the Wnt signalling pathway (**Garg et al., 2016; Jaiswal et al., 2015**). It has been proposed that the alternative splicing

of survivin might serve as a mechanism by which cancer cells maintain their proliferation, yet this requires further investigations (**Garg et al., 2016**). Notably, NELF complex; having the RNA recognition motif present in NELF-E subunit, was found to possess a dual function of transcription regulation and RNA processing. With COBRA1'S role in attenuating steroid hormones regulated transcription via recruitment of NELF complex to the promotor of target genes, it was also found to mediate alternative splicing by promoting inclusion of exons and attenuating skipping (**Sun et al., 2007**).

In 2017, a systematic meta-analysis was done by Fan and Chen to evaluate the clinical significance of survivin expression in CC using data from 18 studies with 791 CC patients, 1013 CIN lesions, 199 normal cervical tissues and 95 chronic cervicitis samples. Results indicated the presence of higher expression levels of survivin in CC relative to other tested groups. In addition, expression was elevated in high-grade than in low-grade patients, in advanced stage than in early stage patients and in patients with lymph node metastasis relative to those without. Also, higher expression was noted in SCC than in AC, yet this specific result has to be carefully considered as only 55 AC patients were included in this study compared to 302 SCC patients (**Fan & Chen, 2017**).

In the present study, we observed decreased expression of β -catenin in COBRA1 siRNA treated cells compared to negative siRNA treated cells. This decrease was found to be statistically significant at a p-value <0.05. The results obtained at the β -catenin mRNA levels were in accordance with results obtained when investigating the expression levels of survivin, where a decreased expression of the three isoforms; wild-type survivin, survivin 2B and survivin DeltaEx3 was observed. Yet, this decrease was found to be statistically significant except for wild-type survivin. This might account for the insignificant effect of COBRA1 on the proliferation and migration of HeLa since the wild-type survivin, which is the predominantly expressed variant in HeLa, was the least to be affected by COBRA1 depletion. Still, it will be interesting to further test the transduction of this decrease along the apoptosis signaling pathway by examining the protein levels of caspases 3 and 9 which are known to be inhibited by survivin.

Taken together, COBRA1 depletion resulted in an increase in the expression of TFF1 accompanied by a subsequent decrease in the β -catenin and survivin expressions at the mRNA level suggesting that COBRA1 might have an effect on the Wnt/ β -catenin signaling pathway (Figure 13). It will be interesting to investigate the protein levels of the examined

genes using Western Blot. This could validate if the observed alterations at the mRNA levels were transmitted to the protein levels of the tested genes.

Since the effect of COBRA1 on TFF1 in gastric cancer was suggested to be through the inhibition of the AP-1 complex transactivation of TFF1, performing Co-IP and ChIP assays will be of value in examining the binding of COBRA1 to AP-1 and their possible interaction with the TFF1 promotor.

The observed effect of COBRA1 on the TFF1/ β -catenin signaling axis may represent one of the molecular mechanisms underlying the potential role of COBRA1 in cervical cancer. Yet, the finding that the noted decrease in β -catenin and survivin was not reflected on the proliferation and migration abilities of HeLa is not conclusive and requires further investigations.

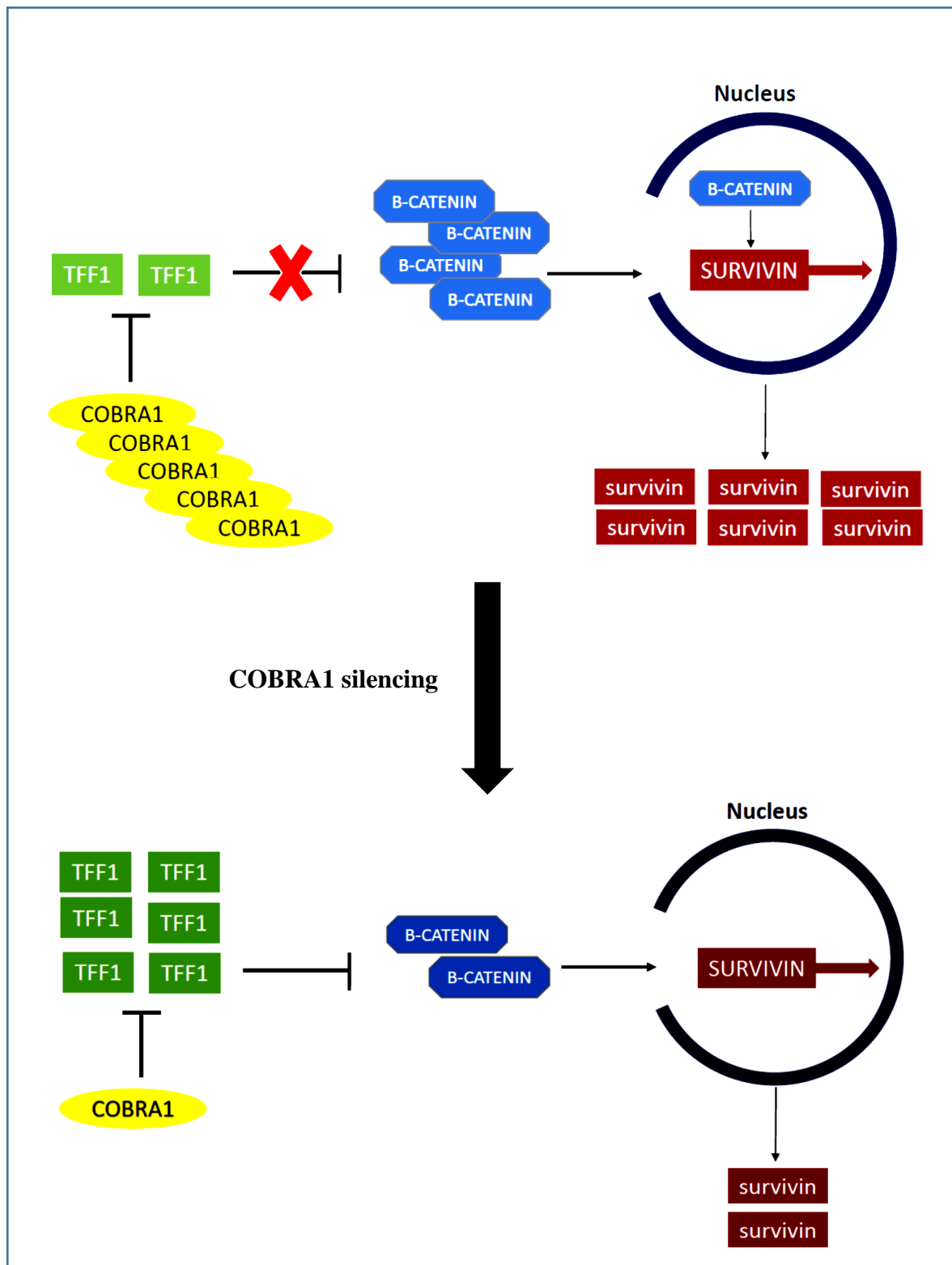


Figure 13. Schematic diagram illustrating the consequences of COBRA1 silencing on mRNA expression levels of examined genes. Successful COBRA1 depletion in HeLa cells caused a significant increase in the expression of TFF1 leading to decreased expression levels of the β -catenin and its decreased entry to the nucleus. This resulted in decreased levels of β -catenin downstream target gene, survivin.

5. CONCLUSION

As to our knowledge, this is the first study addressing the potential role of COBRA1 in CC. In summary, our data mining results showed that a statistically significant overexpression of COBRA1 in cervical carcinoma tissues versus normal cervical tissues was observed and found to be consistent in two microarray datasets out of the four identified by searching the publicly available Oncomine cancer microarray dataset. Results from the other two datasets were found to be contradictory and statistically insignificant.

Results obtained from semi-quantitative RT-PCR analysis of the NELF complex subunits showed no significant difference in their mRNA levels upon COBRA1 knockdown which is consistent with previous results suggesting the presence of tight post-transcriptional regulation of the NELF complex.

The insignificant effect of COBRA1 silencing on the proliferation and migration abilities of HeLa cells under the applied experimental conditions cannot be conclusive and requires further investigations.

The observed decrease in the β -catenin expression in COBRA1 siRNA treated cells compared to the negative siRNA treated cells was found to be statistically significant and was accompanied by comparable decrease in survivin expression suggesting an effect for COBRA1 depletion on the Wnt/ β -catenin signaling pathway. COBRA1 silencing resulted in a significant increase of TFF1 which could be the modulator of the effects observed on the β -catenin expression.

Altogether, this study could help as an initial step in identifying the role of COBRA1 in cervical cancer tumorigenesis.

6. FUTURE DIRECTIONS

More research is needed to further characterize the role of COBRA1 in cervical cancer and help clarify the possibility of it being a suitable prognostic and therapeutic target in CC.

Since this study was limited by the use of one type of CC cell lines; HeLa, therefore extending the methodology to include more CC cell lines representing the squamous cell cervical carcinoma which is the most common subtype will help provide a more reliable evaluation of COBRA1's role in CC.

Further analyses of COBRA1 knocked cells relative to control cells using mRNA microarrays will help provide a larger view of the involved genetic pathways and their possible role in CC progression. In addition, studying the effect of COBRA1 knockdown on cell cycle distribution using flow cytometry could also help clarify the role of COBRA1 mediated regulation of gene expression in HeLa cells. It will be also interesting to investigate the effect of complete Knockout of COBRA1 in HeLa using Crisper/Cas systems.

It is also worth mentioning that under the applied experimental conditions, none of the tested conditions was supplied with exogenous estrogen during the transfection process. Hence, the consequences of adding exogenous estrogen together with the silencing process could be worth testing.

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