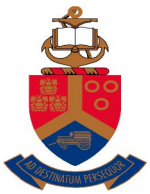

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Investigating the effects of RBBP6 gene expression on telomerase activity in cervical cancer cells

Mpho Mosweu

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Submitted in partial fulfilment of the requirements for the degree:

MSc Genetics

In the faculty of Natural and Agricultural Sciences

Department of Biochemistry Genetics and Microbiology

University of Pretoria

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Abbreviations

A	Absorbance
AIDS	Acquired immune deficiency syndrome
Ago-2	Argonaute 2
AP	Alkaline phosphatase
BCA	Bicinchoninic acid assay
BSA	Albumin standards
BrdU	Thymidine analog 5'-bromo-2'-deoxyuridine
CaSki	Epidermoid cervical carcinoma cells
CDK	Cyclin-dependent kinases
cDNA	Complementary DNA
CI	Cell index
CHAPS	((3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate))
ChIP	Chromatin Immunoprecipitation
DAPI	4', 6'-diamidino-2-phenylindole
DMEM	Dulbecco's modified medium
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
DWNN	Domain with no name
E1, E2, E3	Ubiquitin enzymes
E2F	E2 transcription factor
E6AP	E6-associated protein
FBS	Fetal bovine serum
G1	Gap1
G2	Gap 2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLOBOCAN	Global cancer incidence mortality and prevalence
HEK 293	Human embryonic kidney 293 cells
HeLa	Henrietta Lacks
HIV	Human immunodeficiency virus

HPV	Human papillomavirus
HRP	Horseradish peroxidase
hTERT	Human telomerase reverse transcriptase
ICC	Invasive cervical cancer
kDa	Kilo Dalton
L1 and L2	Late proteins 1 and 2, respectively
LCR	Long control region
M	Mitotic phase
Min	Minutes
MDM2	Mouse double minute 2 homolog
MRC-5	Medical Research Council cell strain 5
mRNA	Messenger RNA
ORF	Open reading frame
P2P-R	Proliferation potential protein-related
P53	Protein 53
PACT	P53-associated cellular protein testis-derived
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
pRB	Retinoblastoma protein
PVDF	Polyvinylidene difluoride
qPCR	Quantitative polymerase chain reaction
RBBP6	Retinoblastoma binding protein 6
RISC	RNA-induced silencing complex
RING	Really Interesting New Gene
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Real time polymerase chain reaction
RBQ-1	Q protein 1
s	Seconds
S	Synthesis phase

SA	South Africa
SCC	Squamous cell carcinoma
sihTERT	siRNA targeting hTERT
SIL	Squamous intraepithelial lesions
siRBBP6	siRNA targeting RBBP6
siRNA	Short interfering RNA
SDS	Sodium dodecyl sulfate
TERC	Telomerase RNA component
TRAP	Telomeric repeat amplification protocol
Trf1	Telomeric repeat binding factor 1
USA	United States of America
VIA	Visual inspection by acetic acid

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Acknowledgements

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Abstract

Cancer remains a major health problem. It is ranked the second most common cause of death to cardiovascular diseases worldwide, with about 9.6 million deaths annually. This burden is greatly carried by developing countries, which are accountable for about 65% of cancer death worldwide. Retinoblastoma binding protein 6 (RBBP6) is one of the genes identified as proliferative genes that play a role in cancer development. It has been shown, as well as telomerase activity to be highly increased in various cancer cells. E6 protein of human papillomavirus (HPV) and RBBP6 are known to enhance the progression of cancer cells by interacting with p53 and presenting it for ubiquitination by the proteasome, thereby promoting cell proliferation and preventing apoptosis. Studies also show that HPV E6 protein has the ability to increase telomerase activity by activating the expression of human telomerase reverse transcriptase (hTERT) thus, enabling the immortalization of the cells. With RBBP6 and hTERT sharing similar functions, here we seek to investigate possible effect of RBBP6 expression on telomerase activity. Using real time qPCR and TRAPeze RT Telomerase detection kit (Merck, United states), CaSki cells showed that the expression of hTERT and telomerase activity, respectively, were decreased significantly in RBBP6-knockdown cells. However, HeLa cells showed non-significant changes in hTERT expression or telomerase activity in response to RBBP6 silencing. Real-time cell analysis assay revealed a significant cell growth reduction in cells co-silenced for RBBP6 and hTERT, thus substantiating our speculation that RBBP6 and hTERT play an additive role in regulating cell proliferation. Taken all together, RBBP6 expression might be altering telomerase activity in a cell line dependent manner and RBBP6 and TERT co-silencing has an effect on cell proliferation.

Chapter 1: Introduction and Literature Review

Cervical cancer continues to be the highest cause of gynaecological cancer deaths among females in developing countries (Iyoke and Ugwu 2013). It is a highly preventable disease, however due to factors such as lack of early and regular screening, most females remain at a higher risk of acquiring HPV or HIV, consequently leading to mortality rates being on the rise. These mortality rates are often seen in low-to-middle income countries that cannot afford to implement stringent formal-screening programmes and therefore end up carrying the burden of advanced cervical cancer. Advanced cervical cancer presents many challenges when it comes to treatment due to its heterogeneous nature. Heterogeneity in cancer means that the tumour might include a diverse collection of cells harbouring distinct molecular signatures with differential levels of sensitivity to treatment, therefore necessitating the need for targeted personalized therapy. However, in order to come up with personalized rather than a one-fits-all treatment strategy for cervical cancer, it is paramount to thoroughly understand the mechanisms behind cancer development and progression. It is for this reason that we want to further understand the mechanism of action of RBBP6 in cervical cancer by probing a possible interaction with telomerase. Knowledge generated from this study will contribute to the development of personalized medicines for patients with advanced cervical cancer.

1.1 Cervical cancer statistics

Cancer is a disease that is identified by an uncontrolled and abnormal division of cells and the spread of malignant tumour cells to other organs of an organism (Russell 2010). Cancer is a major health problem that ranks as the second most common cause of death in the world after cardiovascular disease (Siegel, Miller et al. 2018). According to the global cancer (GLOBOCAN) reporting, there were 18.1 million cancer cases and 9.6 million deaths in 2018 worldwide (Bray, Ferlay et al. 2018). The most common cancer cases and deaths worldwide among males and females combined are attributed to lung cancer, followed by breast, colorectum and prostate cancer (Fitzmaurice, Dicker et al. 2015, Bray, Ferlay et al. 2018). The most common cancer sites among males diagnosed are lungs, prostate, colorectum, however the leading cause of cancer deaths among males are the lung, liver and stomach cancers.

The most common cancer sites among females worldwide are breast, colorectum, and lung, which are the leading cause of cancer deaths (Torre, Bray et al. 2015, Stewart and Wild 2017). On the contrary, in developing countries the leading cancer mortality among females is breast cancer followed by cervical cancer (Torre, Bray et al. 2015, Bray, Ferlay et al. 2018). It has been predicted that the burden of the disease will continue to shift from developed countries to developing countries, where the latter is responsible for more than 57% of the new cancer cases and results in 65% of cancer death worldwide (Iyoke and Ugwu 2013). These developing countries mainly consist of Sub-Saharan Africa, Latin America and South-Eastern Asia (Torre, Bray et al. 2015, Stewart and Wild 2017). In developing countries, cervical cancer accounts as the second most commonly diagnosed cancer and almost 70% of the cervical cancer deaths. For example, there was an estimated 570 000 new cervical cancer cases and 311 000 deaths worldwide in 2018 (Torre, Bray et al. 2015, Bray, Ferlay et al. 2018). Furthermore, cervical cancer has been reported to account for more than 60% of gynaecological cancer cases diagnosed in developing countries (Iyoke and Ugwu 2013).

In Africa, it is estimated that on an annual basis, 78 897 women are diagnosed with cervical cancer and 78% will die from the disease if left untreated (Stewart and Wild 2017). Current data on cervical cancer incidence in South Africa is non-existed due to a lack of maintenance of the pathology based cancer registry (Jordaan, Michelow et al. 2016). However, in South Africa according to Jordaan et al. (2017), about 7 735 new cases are diagnosed and an average of 4248 women die from this disease annually. Therefore, cervical cancer is a serious threat to South African females (Jordaan, Michelow et al. 2017). Although cervical cancer is a highly preventable disease, alarming mortality rates continue to be reported as a result of factors such as late diagnosis, lack of organized screening programmes, lack of highly skilled medical personnel and improper infrastructure (Jordaan, Michelow et al. 2017).

1.2 Cervical Cancer Risk Factors

1.2.1 Human Papillomavirus (HPV)

Human papillomavirus (HPV) is the major causative factor of cervical cancer and it belongs to the family of DNA tumour viruses. DNA tumour viruses have been found to be oncogenic and to induce cell proliferation (Russell 2010). HPV is divided into two groups: low-risk and high-risk HPV. Low-risk HPV does not cause or lead to malignancy, while high risk HPV are associated with causing malignant cervical lesions (Bosch, Burchell et al. 2008). E6 and E7 are HPV viral oncoproteins known to be involved in the initiation and progression of cervical cancer.

The structure of HPV

HPV is a small non-enveloped virus that consists of a double-stranded circular DNA genome of approximately 8 kb in size (Cooper and Hausman 2007). This genome is associated with histone-like proteins and it is protected by an icosahedra capsid that is formed by late proteins, L1 and L2 (figure 1) (Fernandes and de Medeiros Fernandes 2012). The viral genome can be divided into three regions: non-coding region (long control region (LCR)), early region and late region. The LCR located upstream is a regulatory region with regulatory function for E6 and E7 viral genes (Fehrmann and Laimins 2003). The early region consists of six open reading frames (ORFs) E1, E2, E4, E5, E6 and E7, which are non-structural regulatory proteins but involved in viral replication and to induce cell proliferation (Zheng and Baker 2006). The late region consists of two ORFs, which encodes for the structural proteins, L1 and L2 (Sapp, Volpers et al. 1995).

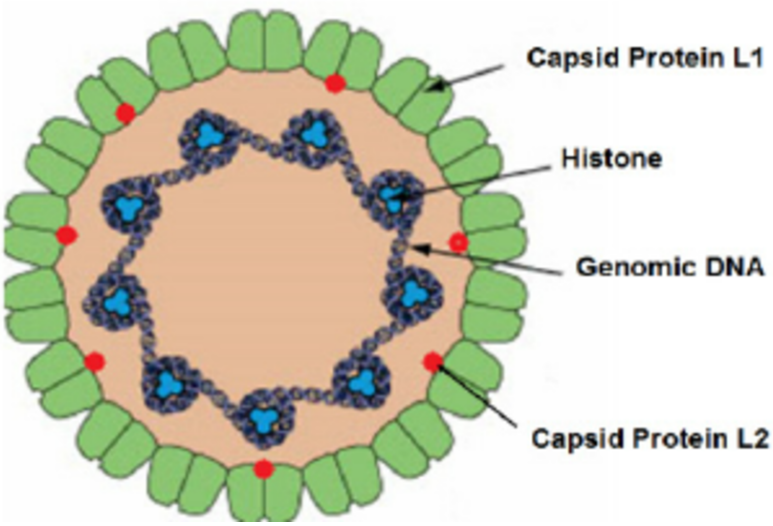

Figure 1: The Structure of HPV. (Adapted from Swiss Institute of Bioinformatics, Viral Zone. Available in http://viralzone.expasy.org/all_by_species/5.html). (Fernandes and de Medeiros Fernandes 2012).

Each capsid is made up of 72 capsomeres which is composed of five monomeric units that combine to form a pentamer corresponding to the major capsid protein (L1) (Sapp, Volpers et al. 1995). L2 is the secondary capsid protein (also known as minor capsid protein) with about 75KDa in size, is found within the virion (Fernandes, Araújo et al. 2013). In order to assemble the capsid, the L1 pentamers join to a number of L2 proteins, thus forming a particle of about 55 nm in diameter (Buck, Cheng et al. 2008).

Human papillomavirus infection

HPV is known to be transmitted sexually through a mechanical abrasion of an infected epithelial cells to uninfected epithelial cells (Burd 2003). HPV can be divided into two categories of how it can infect an individual either through a mucosal type or a cutaneous type: in which one way is to infect the mucosa of the mouth, the throat, respiratory tract and anogenital tract and another way is to infect the keratinized surface of the skin, respectively (Burd 2003, Mistry, Wibom et al. 2008). Over 150 different HPV types have been identified and only 40 can infect the epithelial lining of anogenital tract and other mucosal areas of the body (Doorbar, Quint et al. 2012, Fernandes and de Medeiros Fernandes 2012). HPV is also classified as either low-risk or high-risk HPV. A low-risk HPV is responsible for common genital warts, which

are mostly caused by HPV 6 and 11, and can cause benign lesions with a low chance of developing into a malignant carcinoma (Bosch, Burchell et al. 2008). High risk HPV are associated with causing pre-malignant and malignant cervical lesions, often caused by HPV type 16 and 18 (Bernard, Burk et al. 2010, Denny 2010).

The life cycle of HPV

The life cycle of HPV can be divided into two phases for better understanding: the maintenance phase and the differentiation-dependent phase. In summary, the maintenance phase is where the viral genome is established in a host and in the differentiation-dependent phase is where the viral genome replication is greatly increased and the viral particles are released (figure 2) (Fernandes and de Medeiros Fernandes 2012).

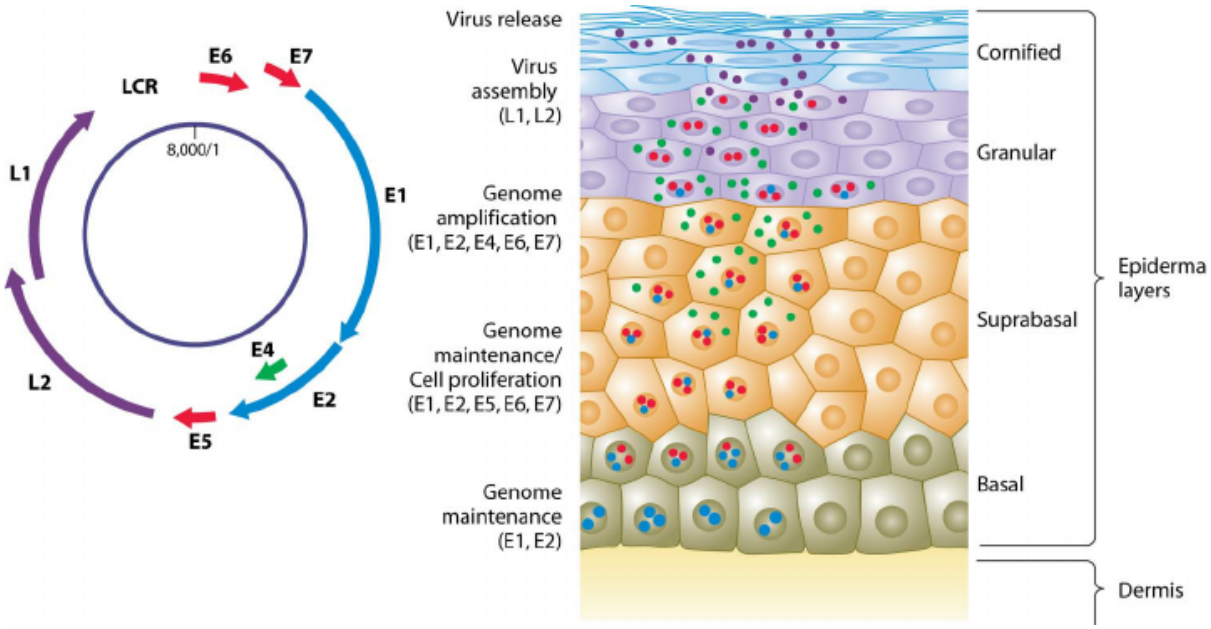

Figure 2: HPV life cycle. The maintenance phase starts from the basal epithelial layer to the suprabasal epithelial layer and the differentiation-dependent phase, consists of the granular layer to the cornfield layer (Lazarczyk, Cassonnet et al. 2009).

The maintenance phase

The purpose of the maintenance phase of the viral cycle is to ensure the maintenance of the viral episome. The life cycle begins with an infection of stem cells of the basal epithelium (Lazarczyk, Cassonnet et al. 2009). The expression of E1 and E2 genes

occurs and their proteins bind to the viral origin of replication as well as to recruit the proteins needed for DNA replication such as DNA polymerases. At this point, the viral genome is replicated but at a relatively low copy number (Frattini and Laimins 1994). In the suprabasal epithelial layer, the expression of E6, E7, E1 and E2 follows to ensure a continued episomal maintenance and to induce cell proliferation (Fernandes and de Medeiros Fernandes 2012). After the establishment phase, the viral DNA is replicated with host cell and are distributed to the daughter cells. The virus switches to a rolling-circle mode of DNA replication, which enables the amplification of the viral DNA to high copy number (Flores, Allen-Hoffmann et al. 1999).

Differentiation-dependent phase

The persistent infection of HPV is dependent upon the differentiation of the host cell (Stubenrauch and Laimins 1999). After the initial infection of the stem cells occurring in the basal epithelial layer, the stem cells divide into transit-amplifying cells. The transit-amplifying cells produce daughter cells, which withdraw from the cell cycle and migrate away from the basal layer and become committed to differentiation (figure 2) (Stubenrauch and Laimins 1999). The infected cells undergo differentiation after they leave the basal layer and high levels of viral proteins synthesis are induced (Frazer 2009). Upon differentiation the late viral function become activated by viral proteins such as E1, E4 and E5 (Wilson, Fehrmann et al. 2005). The late product genes L1 and L2 gather to form viral capsid and form virions, which reach the cornfield layer of the epithelium and the particles are released (Fernandes and de Medeiros Fernandes 2012).

The role of HPV E6 and E7 in Cervical Cancer Development

Human papillomavirus (HPV) is a leading risk factor of cervical cancer with about 90% of invasive cervical cancer (ICC) cases associated with HPV type 16 and 18 (Walboomers, Jacobs et al. 1999, Burd 2003). E6 and E7 are HPV viral oncoproteins known to be involved in the initiation and progression of cervical cancer (figure 3). The activation of E6 and E7 is made possible by an E2 gene, which serves as an integration site where the viral genome of E6 and E7 are inserted leading to the events depicted in figure 3. After the integration, the E2 gene is deactivated (Janicek and

Averette 2001). E7 viral protein plays a role in the interruption of the interaction between E2F (a family transcription factor) and retinoblastoma protein (pRB) by binding to the latter protein. This results in an ongoing expression of E2F-responsive genes such as cyclin A and E, and these promote DNA (deoxyribonucleic acid) synthesis, the progression of cell cycle, and a premature S phase entry. At this stage, the G1/S checkpoint control is lost and the cell will continues with the cell cycle causing an uncontrolled cellular proliferation (Cobrinik, Dowdy et al. 1992).

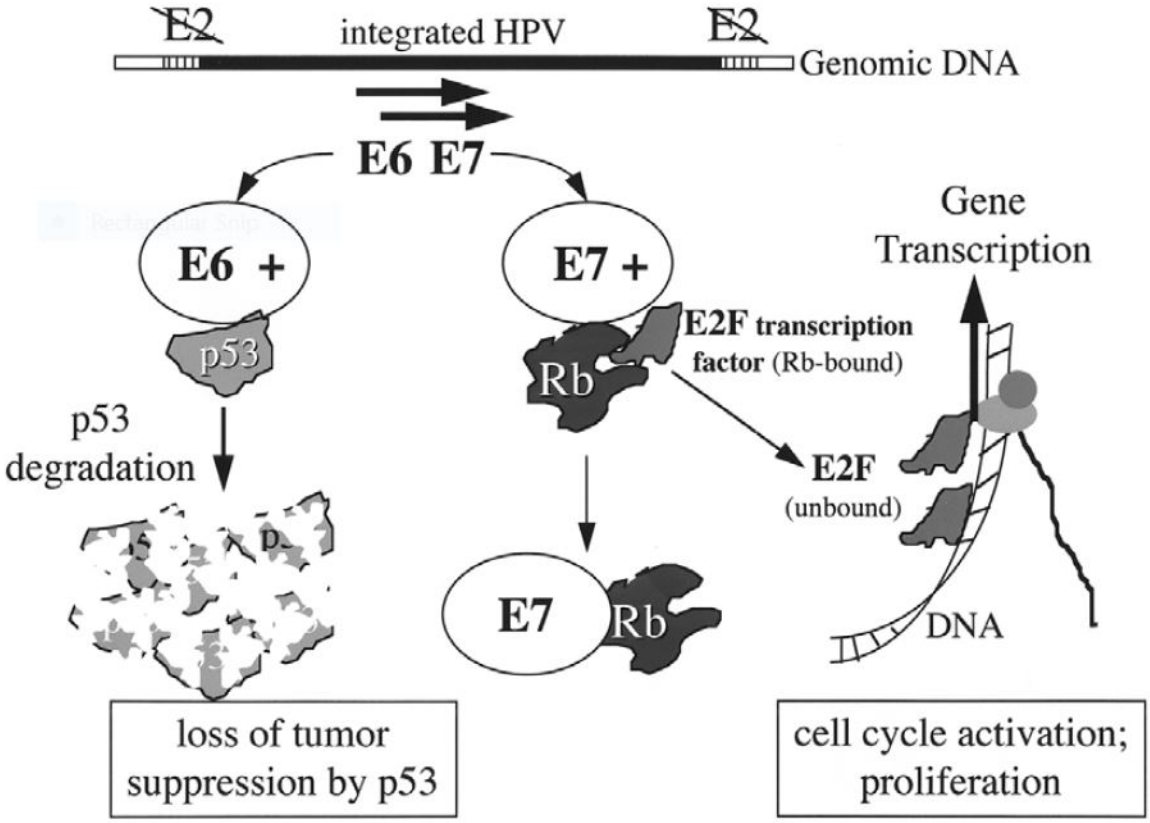

Figure 3: The role of HPV E6 and E7 in cervical cancer development (Janicek and Averette 2001).

On the other hand, E6 protein along with an E6-associated protein (E6AP) of an E3 ligase activity, have the ability to bind to and degrade p53 through the proteasome pathway, thus enabling cell cycle arrest and apoptotic signals carried out by p53 to be overridden. In addition, when p53 function is inhibited, the apoptotic signalling is not there to enable elimination of HPV infected cell, which leads to malignant HPV lesion (Fernandes, Araújo et al. 2013).

1.2.2 Human Immunodeficiency Virus (HIV)

Another leading risk factor of cervical cancer that is of great concern especially in South Africa is HIV/AIDS being one of the largest epidemics in the world (Pillay-van Wyk, Msemburi et al. 2019). Studies show that repression of the body's immune system as a result of HIV infection causes patients to be highly susceptible to persistent HPV infection (type 16 &18), which is highly likely to escalate the prevalence of cervical lesions and a lead to an increased rate of progression to advanced cervical cancer. HPV/HIV co-infection results in dysfunction of cellular and hormonal arms of the local and systemic immune system and a subsequent disease progression (Massad, Ahdieh et al. 2001, Denny, Franceschi et al. 2012, Keller 2015). HIV-infected women with decreased CD4 counts show a two-fold increase in the prevalence of squamous intraepithelial lesions (SIL) when compared to women with higher counts meaning higher immunosuppression results in high HPV viral load (Keller 2015, Konopnicki, Manigart et al. 2016)

1.2.3 Other Risk Factors

Other risk factors that contributes to cervical cancer in low to middle income countries are found to be behavioural and environmental, which includes the number of sexual partners and the early age of commencement of sexual activity in females, long term use of oral contraceptives, alcohol, a lack of a healthy diet, and cigarette smoking (Brinton, Hamman et al. 1987, Bosch, Munoz et al. 1992, Danaei, Vander Hoorn et al. 2005)

1.3 Prevention and Treatment of Cervical Cancer

1.3.1. Prevention

Primary prevention of cervical cancer includes abstinence, safe sex practices and HPV vaccination. Two vaccines have been designed and used against the types of HPV that is Cervarix™ (GlaxoSmithKline Biologicals, Belgium) and Gardasil® (Merck and Co., United States of America (USA)) (Einstein, Baron et al. 2009). The limitation of these vaccines is that they are not efficient when administered to an individual who is

infected with HPV already. It was also suggested it should be administered to school girls between the ages of 9-12 years as they are most likely not to be sexually active at that age (Snyman 2013, Aggarwal 2014). This, however, leaves a great margin of women who are most likely to be infected with HPV or even HIV in their lifetime contributing vastly to the mortality rates. In addition, the costs of these vaccines is not something that can be afforded by families in low-to-middle income countries (Moodley 2009).

Secondary prevention includes the detection of pre-invasive lesions and early diagnosis of invasive carcinoma using papanicolaou smear (pap smear) and cervical cytology methods such as visual inspection by acetic acid (VIA) or lugol's iodine (Snyman 2013). Prevention of cervical cancer at tertiary level involves the diagnosis and treatment of confirmed cases of cervical cancer (Denny 2010). A significant decrease in the number of cases diagnosed in developed countries is as a result of a proper population-based screening. However, in South Africa this type of screening has not been formally implemented countrywide because the current methods are found to be highly expensive (Snyman 2013). It is for this reason that cervical cancer cases continue to be on the rise not only in South Africa but all other developing countries as well. This therefore calls for a need to implement treatment and diagnostic strategies that are cost-effective and easily accessible to people in our country and other developing countries.

1.3.2 Treatment of cervical cancer

Overtime researches are more and more drawn to the use of gene-based therapies, which includes immunotherapy, a boosting of the immune system so it can destroy and target cancer cells but this has not been successful. Another area of therapy is to use viruses that have been engineered to destroy and target cancer cells and not normal cells. However, limitation to this method is that people would have antibodies that would destroy the viral agent before it can reach the cells (Cross and Burmester 2006). More recent years, siRNA-based treatment (tautology of therapy), which works by delivering double stranded siRNA (short interfering ribonuclease acid) to the cytoplasm of the cells has been an attractive target. RNA interference (RNAi) is a specific post-transcriptional pathway that results in silencing gene function (Deng,

Wang et al. 2014). It occurs in eukaryotic organisms such as plants and animals and it is triggered by double stranded precursors that differ in length and origin (Meister and Tuschl 2004). It uses small RNA molecules of approximately 21-28 nucleotides to target and degrade a specific mRNA and silence the gene activity (Almeida and Allshire 2005). The siRNAs are produced by cleavage of a longer double stranded RNA (dsRNA) precursor by the DICER enzyme (Aagaard and Rossi 2007). The TAR-RNA binding protein gets complexed with the DICER enzymes and it hands off the siRNA to the RNA-induced silencing complex (RISC).

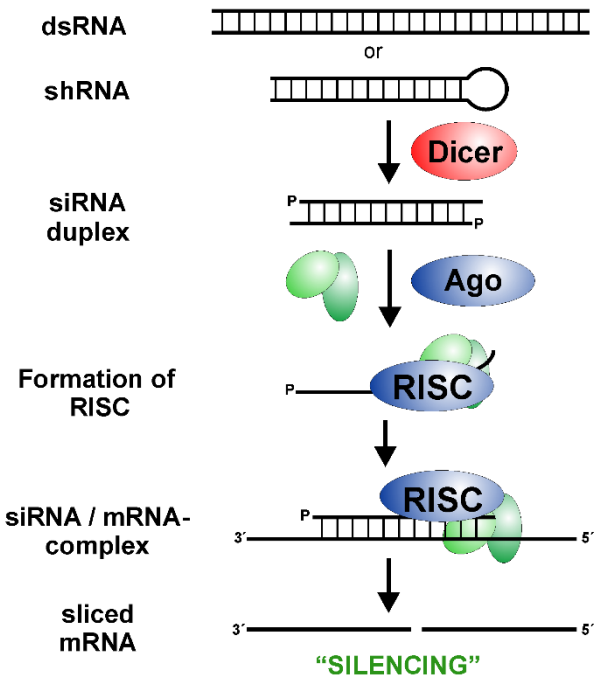

Figure 4: Schematic diagram showing siRNA silencing pathway. [accessed http://www.gene-quantification.net/si-rna.html]

RISC consist of Argonaute 2 (Ago-2) that cleaves the target mRNA as it is the only on with catalytic domain. As the double stranded siRNA is loaded to RISC, Ago-2 cleaves and releases the passage strand, which is immediately degraded and the remaining strand called the leading strand, is used to direct the specificity of the target mRNA recognition by base-pairing. This induces cleavage of the mRNA target strand thus leading to the silencing of that mRNA. This strategy has been adapted and implemented in biological studies to achieve the silencing or inactivation of gene expression both *in vitro* and *in vivo* by delivering the chemically synthesized siRNA to

the desired cell type or tissue or organ. The most common strategy of introducing siRNA to cell types is through liposome based strategies. Various encapsulation has been refined for efficiencies from basic liposomes to triblock micelles (Resnier, Montier et al. 2013). One study by Landen, Chavez-Reyes et al has proven to be a success, in which siRNA was incorporated into the neutral liposome and was efficiently delivered *in vivo* (Landen, Chavez-Reyes et al. 2005).

1.4 Telomeres and Telomerase

1.4.1 Structure of Telomeres

Telomeres are long repetitive sequences found at the ends of eukaryotic chromosomes (Russell 2010). The human telomere length is about 5-15 kb in size and consist of the repetitive sequence 5'-TTAGGG-3' (Shay, Zou et al. 2001). The telomeric DNA was found to be fold in a T-loop structure, which is believed to be a mechanism for telomere capping (Pfeiffer and Lingner 2013) . In order for the ends of the chromosomes not to be mistaken as a damaged or broken DNA, telomeric DNA is protected by six sequence-specific DNA binding proteins forming a capping structure called shelterin complex (figure 5) (de Lange 2009). Three sheltering proteins (Trf1, Trf2 and Pot1) directly bind to the double stranded TTAGGG repeats and they are interconnected by the additional sheltering proteins (Tin2, Tpp1 and Rap1) (De Lange 2005). The first human telomere-binding protein, Trf1 (telomeric repeat binding factor) binds to the double-stranded telomeric DNA (Chong, van Steensel et al. 1995). This is followed by a second Trf2, homologous protein which also binds directly to the double-stranded telomeric DNA as a dimer (Broccoli, Smogorzewska et al. 1997). Both Trf1 and Trf2 binds to Tin2, which in turn binds Tpp1. Tpp1 then binds to Pot1 (De Lange 2005). In addition, a human CST complex has been identified to be present at telomeres as well as other regions in the genome (Miyake, Nakamura et al. 2009).

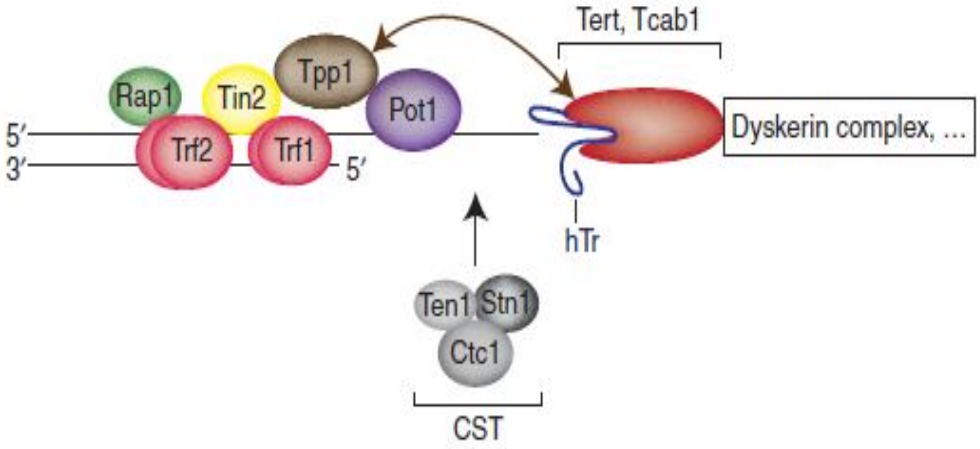

Figure 5: Graphical representation of telomeric shelterin complex (Pfeiffer and Lingner 2013).

1.4.2 Function of Telomeres

The function of telomeres is to prevent DNA end fusion, to prevent chromosome degradation, chromosome loss and instability (Takakura, Kyo et al. 1998, de Lange 2002, De Lange 2005). For every normal successful cell cycle, the length of the telomeres shorten because the DNA polymerase cannot continue DNA synthesis on a 3' single-stranded overhang (Shay, Zou et al. 2001). This is called end-replication problem that can be solved by telomerase, an enzyme that can synthesize telomeric DNA *de novo* (Allsopp, Chang et al. 1995). When the telomeres becomes short enough, the cell stops dividing and enters in a state called cellular senescence whereby the growth of a cell is arrested (Russell 2010). It is thought as an anti-cancer mechanism, where a permanent cell cycle arrest occurs before the cells could accumulate mutations that could lead to cancer (Shay, Zou et al. 2001).

1.4.3 Telomerase

Human telomerase is a ribonucleoprotein complex that enables the stability and maintenance of telomere length in cancer cells, human stem cells and reproductive cells, by adding TTAGGG repeats onto telomeres and overriding the cellular senescence state (Morin 1989).

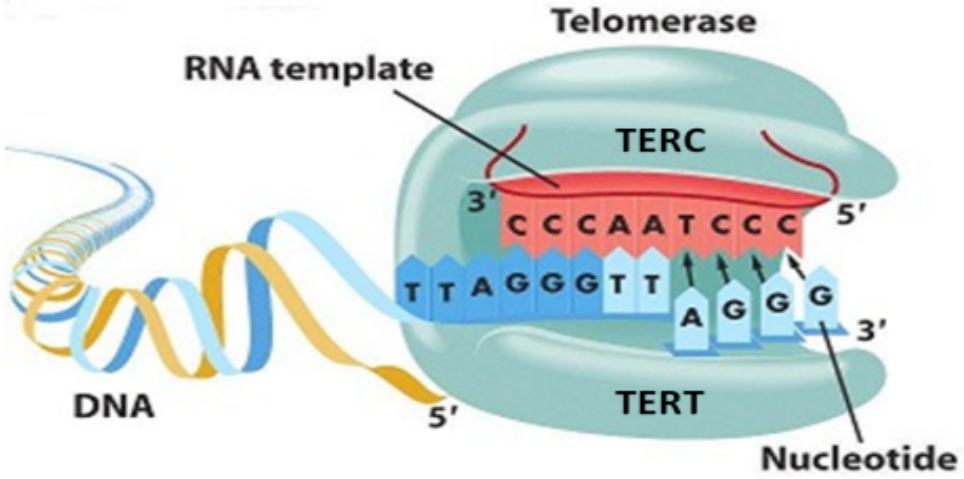

Figure 6: Schematic representation of the telomerase enzyme. The TERC subunit (red) serving as a template region for hTERT (blue) to add telomeric repeats at the end of the chromosome.

Studies have shown that the human telomerase complex is a dimeric structure that consists of two TERC (153 kDa) and two TERT (127 kDa) subunits, which are linked by a flexible hinge domain (Sandin and Rhodes 2014). TERT is the catalytic subunit of the enzyme also known as human telomerase reverse transcriptase (hTERT) (Meyerson, Counter et al. 1997). TERC subunit is the telomerase RNA component and functions as a template region for telomeric repeat synthesis (figure 6) (Sandin and Rhodes 2014). To synthesize telomere sequence, telomerase uses the DNA 3' single-stranded overhang at the end of the chromosome and positions it to the active site of the enzyme (TERT) and aligning it to the RNA template in TERC through base-pair formation. Second step, which is the elongation step involves the addition of nucleotides for telomeric DNA repeat. The third and final step, the telomerase shifts and restarts the cycle again (Blackburn and Collins 2011, Pfeiffer and Lingner 2013).

1.4.4 Role of telomerase in cancer

In a circumstances where p53 has been mutated that is ~50% of cancers, the cell cannot enter cellular senescence (Hollstein, Sidransky et al. 1991). This promotes cancer progression, where telomere protection has been lost this leads to a telomere crisis, which is a state whereby the genome is unstable (Maciejowski and de Lange

2017). In order for cancer cells to continue to proliferating telomeres must be maintained through: upregulation of telomerase, which is responsible for 90% cancers or through alternative lengthening of telomeres (occurring in ~10% cancers). Making study of telomerase highly important. Telomerase have been found to be active in cancer cells and not active in normal cells (Snijders, van Duin et al. 1998, Maciejowski and de Lange 2017).

The level of mRNA expression of hTERT has been found to increase with telomerase activity in cervical cancer progression (Takakura, Kyo et al. 1998). Moreover, studies prior to that show in most cancer cells the level of telomerase activity often correlates with the proliferation state of the cells (Shay and Bacchetti 1997). E6 of HPV is a multifunctional protein with many cellular protein targets besides p53. Among the crucial functions of E6 oncoprotein is the ability of using E3 ligase activity (E6AP) to interact with transcription factors in hTERT promoter region, which will induce hTERT expression and thereby inducing telomerase activity (James, Lee et al. 2006).

1.5 Retinoblastoma binding protein 6 (RBBP6)

Retinoblastoma binding protein 6 (RBBP6) gene is situated on chromosome 16p11.2-p12. Its full length transcript is made from three partial transcripts namely: Q protein 1 (RBQ-1, 6.1 kb), P53-associated cellular protein testis-derived (PACT, 6.0 kb) and proliferation potential protein-related (P2P-R, 1.0 kb) which occur by a combination of alternative splicing (Sakai, Saijo et al. 1995, Simons, Melamed-Bessudo et al. 1997, Yoshitake, Nakatsura et al. 2004, Pugh, Eiso et al. 2006). These transcripts encode RBBP6 protein isoform 1, 2, 3, and 4, which consists of 1792, 1758,118, 952 amino acids, respectively. RBBP6 is a 250 kDa nuclear protein, which consists of 3 N-terminal domains namely; DWNN (domain with no name) domain, a zinc knuckle domain and RING-finger (Really Interesting New Gene) domain (Figure 7) (Pugh, Eiso et al. 2006).

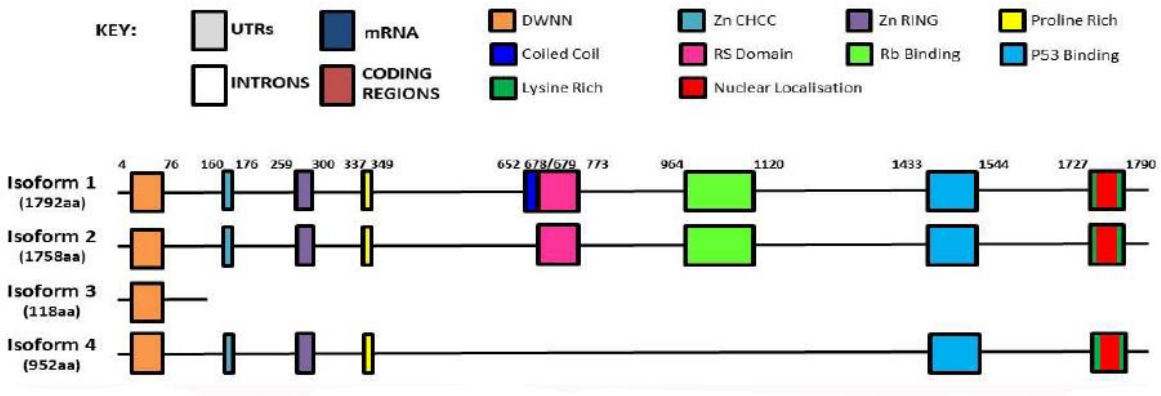

Figure 7: The domain structure of RBBP6 in humans (Ntwasa, Nweke et al. 2018).

DWNN is an ubiquitin-like domain that is found only at the N-terminus of the RBBP6 of Homo sapiens and it is conserved in other eukaryote organism such as plants and animals (Mather, Rakgotho et al. 2005, Pugh, Eiso et al. 2006). The ZINC finger is a small protein domain that plays a structural role to stabilize the domain (Krishna, Majumdar et al. 2003). RING finger domain serve as a mediator for E3-ubiquitin ligases (Joazeiro and Weissman 2000). In the RBBP6 domain structure, the retinoblastoma protein (pRB) binding domain is located near the C-terminus of the protein with p53 binding domain found upstream of this domain (Motadi, Bhoola et al. 2011).

Ubiquitination is a process that is completed using three enzymatic steps and it occurs at a post-translational level. The first step involves an activation of free ubiquitin using E1 (ubiquitin-activating enzyme). This is followed by E2, which is called a conjugating enzyme as it will form thioester bond with ubiquitin that was initially activated. Final step, the E3 will then label the target protein with the activated ubiquitin (Mather, Rakgotho et al. 2005). It has been reported that the DWNN domain together with the RING-finger domain has E3 ubiquitin ligase activity, which enables RBBP6 to interact with both p53 and pRB tumour suppressor proteins (Motadi, Bhoola et al. 2011). Tumour suppressor protein pRB's function is known to bind to the transcription factor E2F and inhibit cellular proliferation (Weinberg 1992) and p53 in response to DNA damage or cellular stress, it activates cell cycle arrest or apoptosis (Mather, Rakgotho et al. 2005).

Among the many structural and functional features of RBBP6, it is known to play an important role in degrading p53 in the proteasome, which causes an unregulated increase in cell proliferation. However, the mechanism which RBBP6 degrades p53 is unknown. Extensive studies of RBBP6 isoforms and its expression in cancer has been done and following are examples of these studies that show that RBBP6 is a potential marker for cancer therapeutics. Yoshitake et al showed that RBBP6 homologue not only was it highly expressed but that it was involved in the progression of oesophageal cancer tissues (Yoshitake, Nakatsura et al. 2004). According to Motadi et al RBBP6 has been up-regulated in tumours of the lung (Motadi, Bhoola et al. 2011). Furthermore in recent studies it was shown that silencing RBBP6 in breast cancer cells induced apoptosis (Moela, Choene et al. 2014).

Finally, Moela et al had interesting insight that RBBP6 was also highly expressed in cervical cancer tissue sections particularly cervical sections that were in stage II and stage III of development and they also suspected that RBBP6 seem to be promoting S-phase entry in cell cycle and cell proliferation (Moela and Motadi 2016). Therefore supporting previous findings that RBBP6 has been shown to be involved in the cell cycle (Yoshitake, Nakatsura et al. 2004, Ntwasa 2008). In summary, RBBP6 has been shown to be highly expressed in different cancers such as oesophageal, lung, colon, breast & cervical cancer, which shows that it is a potential biomarker in cancer therapeutics (Pugh, Eiso et al. 2006, Motadi, Bhoola et al. 2011, Moela, Choene et al. 2014, Moela and Motadi 2016). The similar functions between RBBP6 and HPV E6 with E3 ligase activity is the reason behind the basis of this study.

Chapter 2: Aim and Objectives

The involvement of both telomerase and RBBP6 in cancer, especially the fact that the two proteins play a significant role in cancer cell proliferation is sufficiently studied. However, the mechanism of action of RBBP6 in cancer progression is not well understood. Although previous studies have shown a strong link between RBBP6 and apoptosis, the mechanism behind RBBP6-mediated cell proliferation remains unclear. Therefore, we sought to explore a possible relation between RBBP6 and telomerase as a way of promoting increased cancer cell proliferation. The effect of RBBP6 expression on telomerase activity in cervical cancer cells will therefore be investigated. The aim of this study is to investigate the effects of RBBP6 gene expression on telomerase activity in cervical cancer cells.

Objective 1: To investigate the expression of hTERT following silencing of RBBP6 at RNA and protein level using qPCR and western blot, respectively.

Objective 2: To investigate the expression of telomerase activity following silencing of RBBP6 using telomerase activity (TRAP) assay.

Objective 3: To analyse cell growth after co-silencing RBBP6 and hTERT using xCELLigence system.

Chapter 3: Materials and methods

3.1. Materials

3.1.1. Cell lines

Normal cell line and different tumorigenic cell lines were used in this study as the main source of mRNA and protein. Epidermoid cervical carcinoma cell (Ca-Ski) is a squamous cell carcinoma(SCC)-derived cervical cancer line, which is known to be integrated with HPV 16. Henrietta Lacks (HeLa) is an adenocarcinoma-derived cell line, which contains HPV 18. Human embryonic kidney 293 cells (HEK 293) is derived from a human embryonic kidney cells and is a positive telomerase cell line. MRC-5 (Medical Research Council cell strain 5) is a normal cell line derived from a lung fibroblast and is a negative telomerase cell line. These cell lines were purchased from National Institute of Biomedical Innovation, Health and Nutrition (Japan).

3.1.2. Primers

Pre-designed qPCR primers for GAPDH, and RBBP6 were obtained from (Integrated DNA technologies, Whitehead Scientific (Pty) Ltd, South Africa (SA)) IDT Prime Time® while hTERT primers sequence were obtained from a published article (Widschwendter, Müller et al. 2004), which were synthesised by Inqaba® (SA). The primers were used to amplify the corresponding genes (Table 1):

Table 1: Primer sequences used in this study

Primers	Sequences
RBBP6	Forward primer: 5' CAGCGACGACTAAAAGAAGAG 3' Reverse primer: 5' GAGCGGCTGAATGATCGAGA 3'
GAPDH	Forward Primer: 5' CAGCCGCATCTTCTTTTGCG 3' Reverse Primer: 5' TGGAATTTGCCATGGGTGGA 3'
hTERT	Forward Primer: 5' TGACACCTCACCTCACCCAC 3' Reverse Primer: 5' CACTGTCTTCCGCAAGTTCAC 3'

3.1.3. RNAi Oligonucleotides

Silencing was achieved using Ambion's silencer® select pre-designed siRNAs supplied by Life Technologies™ (USA) which targeted the RBBP6 and hTERT genes. The Ambion silencer incorporate short interfering (siRNAs) that are strategically chemically modified to ensure that the correct siRNA strand is taken up by the RISC complex that is found in the cytoplasm of mammalian cells. In order to deliver the Ambion's silencer® select pre-designed siRNAs into the mammalian adherent cells, a lipid-based Lipofectamine® 3000 transfection agent (Thermo Fischer Scientific, USA) was used. Oligonucleotide targeting Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used to serve as a positive control and a non-targeting oligonucleotide as a negative control.

3.2. Methods

3.2.1. Cell culture

A normal cancer line MRC-5 and cervical cancer cell lines such as Ca-Ski, and HeLa were grown in Dulbecco's modified medium (DMEM) (Lonza Biosciences, USA). To make the growth medium complete the following components were added to a final concentration: 10% fetal bovine serum (FBS) (Biowest, SA), and 1% antibiotic (penicillin/streptomycin) (Lonza Biosciences, USA). The medium was changed two times per week by discarding the old media, washing the cells with 1X phosphate buffered saline (PBS) and replace it with fresh media. The cells were maintained at 37°C in a 5% CO_2 incubator. Monolayer cells were used when they reach 70-90% confluency.

3.2.2. Gene silencing by short interfering RNAs (siRNAs)

Principle

siRNA transfection is a strategy that has been artificially adopted by researches to achieve inactivation or inhibition of gene expression in cultured cells and some living organisms (Meister and Tuschl 2004). Transfection can be achieved using either of the two methods: short hairpin RNAs (shRNAs) or siRNAs. The difference between the two is that shRNAs consist of about 80 base pairs including a hairpin structure. It is also called a DNA-based approach because the siRNAs are produced within the cell (Aagaard and Rossi 2007). RNA-based approach or siRNA method, it consists of approximately 21-28 nucleotides that are delivered to target and degrade the targeted mRNA. The advantages of using shRNAs is that it is a long term or stable strategy but it is labour intensive. On the other hand, the use of siRNA is simple and it results in an efficient silencing the disadvantage is that the effect is short-term or transient (Almeida and Allshire 2005, Curtis and Nardulli 2009).

Technique

Cells were seeded to be 70-90% confluent at the time of transfection. The cancer cell lines (CaSki & HeLa) were transfected at 60 pmol using siRBBP6 or sihTERT pre-designed Ambion Silencer with lipofectamine® 3000 transfection agent (Thermo Fischer Scientific, USA). Transfection was carried out according to the manufacture's instruction with minor modification. The lipofectamine™ 3000 reagent and siRBBP6 (or sihTERT) were diluted separately in Opti-MEM® reduced serum medium (Thermo Fischer Scientific, USA). Then siRNAs were mixed with lipofectamine™ 3000 reagent in a 1:1 ratio and incubated for 10-15 minutes to form a DNA-lipid complex. The DNA-lipid complex was added to cells in a 6 well plate. The plate was incubated for 48 hours at 37°C incubator (Forma Steri-cycle i160, Thermo Scientific, USA) in the presence of CO_2.

3.2.3. RNA extraction

Principle

This process of RNA extraction is important because it is the starting material for the following study to be possible i.e. reverse transcriptase real time-PCR. On its own RNA is a very unstable molecule in a sense that during the procedure one has to make sure that the RNA will not be degraded by RNases which are present in all biological molecules. The RNA isolation kit that was used in this study is called RNeasy® mini kit (Qiagen, Germany). To ensure a good recovery of the RNA material and without RNases interfering in the process. The RNeasy® mini kit consists of guanidine-thiocyanate–containing buffer, which inactivates the RNases when cells are being lysed. This kit is also unique by having a silica membrane to absorb RNA to it and contaminating DNA that is quickly removed by DNases that is in the solution which is directly applied in the silica membrane during preparation.

Technique

Before RNA isolation, cells were transfected with siRBBP6 or sihTERT at 60 pmol. The 6-well plate with transfected cells that was incubated for 48 hours was trypsinized with 500 µl of Gibco® trypsin-EDTA (Thermo Fischer Scientific, USA) into each well

for 1 minute or before cells could detach from the well. The cultured cells were collected by centrifugation and washed with 1X PBS. RNA isolation was followed according to the manufacturer's instructions. Briefly, cells were lysed with RLT lysis buffer combined with (10%) ß-mercaptoethanol and one volume of 70% ethanol was added to the homogenized lysate. The lysate was placed onto a spin-column and centrifuged for 15 s at 8000 x g (Eppendorf 5415D centrifuge, Germany) and flow-through was discarded. RW1 wash buffer was added to the spin-column and centrifuged at 8000 x g and the flow-through was discarded. Buffer RPE was added to a spin-column for 15 s at 8000 x g. The column was placed into a new nuclease-free collection tube and RNase-free H_2O was added and centrifuged for 1 min at 8000 x g where a pure RNA was eluted. Quantification of RNA was done using a Nanodrop spectrophotometer 1000 (NanoDrop technologies, USA). Readings taken at an Absorbance 260/280 nm and a ratio of 1.8 or more was regarded as pure quality. The RNA was either used for reverse transcription or it was stored at -80ºC in aliquots of 5 µl for future use. The RNA integrity was confirmed by assessing RNA bands (18S and 28S) using 1.5% agarose gel stained with ethidium bromide.

3.2.4. Reverse transcription

Technique

Before performing the real time PCR the isolated RNA template was converted into a complementary DNA (cDNA) using the reverse transcriptase enzyme. Reverse transcription was performed using the ImProm-II-Reverse Transcription System (Promega, USA). The manufacturer's instructions were followed. cDNA synthesis reaction master mix was prepared by adding the components as follows: ImProm-II™ 5X reaction buffer, $MgCl_2$, dNTP Mix 0.5mM each, RNasin Ribonuclease Inhibitor, ImProm-II™ reverse transcriptase, 1 µg of total RNA sample, 0.5 µg/reaction oligo (dT)15 or random primers, and nuclease-free water (to a final volume of 20µl). The reaction mix was placed in a thermal cycler (Applied Biosystems™ 2720 thermo cycler, USA). The set conditions were followed according to the manufacturer's recommendations (Table 2). The samples were either used immediately for real-time PCR or stored at -20°C. Quantification of cDNA was done using a Nanodrop

spectrophotometer 1000 (NanoDrop technologies, USA). Readings taken at Absorbance 260/280 nm and a ratio of 1.8 or more was regarded as pure quality.

Table 2: PCR thermal cycling conditions for reverse transcription

Step	Temperature (°C)	Time
Anneal	25	5 min
Extension	42	1 hour
Inactivate transcriptase	70	5 min

3.2.5 Real time Polymerase chain reaction (RT-PCR)

Principles

In order to measure the relative expression of hTERT after silencing of RBBP6, real time PCR was used. The Luminaris™ Color HiGreen quantitative polymerase chain reaction (qPCR) master mix (Thermo Fischer Scientific, USA) was used to achieve this goal. Similarities between real time PCR and conventional PCR is that the cDNA is denatured and primers anneal to the strands and are extended by the thermostable Taq DNA polymerase. However, unlike the conventional PCR, RT-PCR contains SYBR Green in its reaction mixture. SYBR Green is an inexpensive yet highly sensitive fluorescence dye that can bind to double-stranded DNA. During extension of the DNA, SYBR Green binds to the double strands and as the extension continues more SYBR Green molecules will bind and increase fluorescence more and more. With RT-PCR the level of expression of the genes would be able to be quantified in real time at the end of every cycle. On the other hand, with conventional PCR the level of gene expressed will be quantified at the end of the whole reaction.

Technique

The following components were added to a total 20 µl reaction volume: Luminaris Color HiGreen qPCR master mix (contains SYBR Green, $MgCl_2$, Taq polymerase, and dNTPs), primers of RBBP6 or hTERT or GAPDH, cDNA template and nuclease free-water (to a volume of 20 µl). The reaction was mixed thoroughly and dispensed into the PCR tubes. The PCR tubes were placed in the real-time cycler and thermal condition was set as indicated in Table 3 (BioRad CFX96 Connect™, USA). After obtaining standard curves generated by 1:10 serial dilutions for GAPDH (reference gene) and hTERT or RBBP6 (target gene) see appendix A, the relative gene

expression of hTERT or RBBP6 was generated using BioRad CFX Maestro version 1.1 and bar charts were generated by GraphPad Prism 7.0.

Table 3: Three-step thermal cycling condition for qPCR

Step	Temperature (°C)	Time	Number of cycles
UDG pre-treatment	50	2 min	1
Initial denaturation	95	10 min	1
Denaturation	95	5 s	39
Annealing	55-58	30 s	
Extension	72	30 s	

3.2.6 Protein extraction and quantification

Before starting with western blotting procedure, whole cell protein was extracted after seventy-two hours of post transfection. Cells were washed with cold 1X PBS and discarded. Then the cells were lysed with Pierce® RIPA buffer (Thermo Fischer Scientific, USA) and collected by scraping. The lysate was transferred to microcentrifuge tubes and placed for 20 minutes on ice with gentle shaking. The total protein was separated from cell debri by centrifugation at 14000 x g for 20 minutes. The supernatant was transferred to a new microcentrifuge tube and the pellet was discarded. The protein was quantified with Pierce® bicinchoninic acid assay (BCA) Protein Assay Kit (Thermo Fischer Scientific, USA), following the manufacturer's instructions. Briefly, Albumin standards (BSA) were prepared as indicated in appendix B1. A working reagent was prepared by mixing BCA reagent A with BCA reagent B (50:1 ratio).

A volume of 25 µl of each standard and unknown protein samples was placed in a 96-well plate in triplicate. Working reagent of 200 µl was added into each well with samples. The plate was covered with foil and placed in a 37°C incubator (Forma Steri-cycle i160, Thermo Scientific, USA) for 30 minutes. The plate was cooled to room temperature before the absorbance was read at 562 nm using SpectraMax® Paradigm Multi-Mode Microplate Reader (Molecular devices, USA). Standard curve (see

appendix B2) was generated from the absorbance values obtained (minus blank) versus the BSA standards concentration using Microsoft excel (2016). The unknown protein samples concentration values were extrapolated from the standard curve (minus blank). For western blotting only 30 µg of protein was loaded for each samples (see appendix B2 for calculations).

3.2.7 Western blotting

Principle

To check what happens to the protein expression of hTERT when RBBP6 is silenced western blotting technique was used to achieve this goal. Western blotting also known as protein blotting is a technique that allows the transfer of proteins from a sodium dodecyl sulfate polyacrylamide gel (SDS PAGE) to an absorbed membrane (Towbin, Staehelin et al. 1979, Kurien and Scofield 2006). After proteins have been separated by SDS-PAGE, the proteins are blotted onto a membrane either a nitrocellulose or polyvinylidene difluoride (PVDF) or activated nylon paper. The most commonly used membrane is the nitrocellulose membrane because of its mechanical strength. The membrane was blocked with a non-fat milk buffer to avoid the nonspecific binding of antibodies to the membrane then incubated with a primary antibody.

There are two methods that are commonly used to detect proteins after the addition of primary antibody to blocked protein blot: it is by radioactive or enzyme-linked reagents. In radioactive method, the radiolabelled protein A binds to the primary antibody that is bound to the antigen on the membrane blot and this enables the detection of the antigen of interest. There are two distinct advantages in using radioactive detection methods. First, radioactivity in a band is easy to quantitate by means of timed exposure to film and densitometry or by directly excising the band from the membrane and counting using a scintillation counter. Second, the autoradiography image can be reproduced accurately and easily for publication purposes.

The enzyme-linked method uses the alkaline phosphatase (AP) or horseradish peroxidase-coupled (HRP) antibodies along with soluble substrates that yield insoluble coloured products. Chemiluminescence is the improved version of the

original enzyme-linked method and it is currently used due to its sensitivity and selectivity. It has been used for the detection of protein bands on various membranes (nitrocellulose or PVDF). In the chemiluminescence method the secondary antibody is conjugated with either HRP or AP then substrates are added. AP enzyme dephosphorylates the chemiluminescent substrate, which results in the emission of light. On the other hand, HRP catalyses the oxidation of luminol in the presence of hydrogen peroxide. The luminol, which is in an excited state immediately following the reaction, it decays via a light-emitting pathway. The image of the blot is then visualized by exposing the blot to film.

Technique

In order to achieve electrophoretic separation of proteins, a 40 % acrylamide-bis gel was prepared as outlined in Table 4. After mixing 2X Laemmli sample buffer (BioRad, USA) with protein sample, the samples were heated at 95°C for 5 min and then were loaded per well. The gel was run at 130V for 1,5 hours. The proteins were then transferred onto a nitrocellulose membrane using wet electro-transfer method for 1 hour at 100V. This was followed by blocking the membrane with 5% non-fat milk for an hour. The membrane was then incubated with a primary antibody (see Table 5) with agitation overnight. The next day, the blot was washed with 1XPBS and incubated with HRP-linked secondary antibody for an hour. The light signal produced from the secondary antibody was detected and enhanced using the Clarity™ Western ECL Chemiluminescence Substrate (BioRad laboratories, USA) and the blots were imaged by the CCD-based ChemiDoc™ MP system (BioRad, USA). The blots or band intensities were quantified using Image Lab 4.1 software (BioRad, USA). Densitometric analysis (see appendix B3) evaluating the relative quantity of each band using untreated cell bands as reference. The data was exported to excel in order to calculate the band intensities percentages.

Table 4: Gel composition of separating and stacking gel for SDS-PAGE

	10%	
Separating gel	**Mini gel**	**Large gel or** 2 mini gels
30% Acrylamide/ 0.8% Bisacrylamide (40%)	2.50 ml	5.00 ml
4X Separating Buffer (pH8.8) (25%)	1.87 ml	3.75 ml
dH_2O (33%)	3.07 ml	6.15 ml
10% Ammonium persulphate (AP)	0.10 ml	0.10 ml
TEMED	0.01 ml	0.01 ml
	7.5 ml	15.01 ml

5%		
Stacking gel	**Mini gel**	**Large gel** or 2 mini gels
30% Acrylamide/ 0.8% Bisacrylamide (17%)	0.5 ml	1.0 ml
4X Stacking Buffer (pH6.8) (25%)	0.75 ml	1.5 ml
dH_2O (57%)	1.7 ml	3.4 ml
10% Ammonium persulphate (AP)	0.1 ml	0.1 ml
TEMED	0.01 ml	0.01 ml
	3.06 ml	6.01 ml

Table 5: Antibodies used in this study and dilutions prepared

Name of Antibody	**Dilution**	**Supplier**
Anti-GAPDH rabbit monoclonal (1°Ab)	1: 1000	Abcam®, UK
Anti-TERT rabbit monoclonal (1°Ab)	1: 500	Abcam®, UK
Anti-RBBP6 rabbit monoclonal (1°Ab)	1: 500	Santa Cruz Biotechnology, USA
Goat anti-rabbit IgG (peroxidase/HRP conjugate) (2°Ab)	1: 2000	Elabscience®, USA

3.2.5. Telomerase activity assay

Principle

In order to measure the expression of telomerase activity against siRBBP6 the TRAPeze® RT telomerase detection kit (Merck, USA) was used. The fluorometric

detection of telomerase activity is made successful by use of ampliflour primers. These fluorophores conjugated primers normally exist in hairpin configuration where the 5' end of the of the hairpin structure consists of the fluorophore and the 3' end (quencher) has the complementary sequence to the DNA template (target sequence). In its hairpin form the fluorophore is in close proximity with its quencher on the 3'end making it easy for the quencher to absorb the fluorescence signal. As soon as the quencher binds to the target sequence the distance between fluorophore and quencher increases and a fluorescence signal is generated. The assay is a two-step reaction where: the reaction starts when the telomerase adds telomeric repeats (GGTTAG) onto the 3' end of a TS primer (telomere imitating sequence). In the second step, the Taq polymerase will then amplify the newly formed telomere sequence after incorporation of the fluorophore conjugated primers. This will generate a ladder of fluorescent products with 6 base increments starting at 61 nucleotides, then 67, 73 and so forth.

Technique

Telomerase activity was evaluated from cells that had been transfected for seventy-two hours. The procedure was followed according to the manufacturer's protocol with minor modifications. Cells were trypsinized with 500 µl of Gibco® trypsin-EDTA (Thermo Fischer Scientific, USA) into each well for 1 minute or before cells could detach from the well. Cells were collected by centrifugation and then washed with 1XPBS and re-pelleted. At this point, one could either store the pellet at -80°C for future use or continue with extraction of telomerase. Telomerase was extracted from the tumorigenic and non-tumorigenic cells by lysing the cells with the CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate)) lysis buffer (provided in kit). The suspension was kept on ice for 30 min before centrifugation at 12,000 x g (Eppendorf 5415D centrifuge, Germany) for 20 minutes at 4°C. The supernatant was collected into a new tube and the protein concentration measured using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, USA).

Protein concentrations obtained by NanoDrop was normalized to 500 ng/µl for each sample (see appendix C1). A master mix was prepared by mixing the following reagents: 5X TRAPEZE® RT reaction mix, Titanium™ Taq polymerase (clontech laboratories, USA), and nuclease free water. The reaction mix was aliquoted into

respective PCR tubes and the different cell extract were added accordingly. The thermo cycler (BioRad CFX96 Connect™, USA) was initiated with the following conditions (Table 6). The data was analyzed with BioRad CFX Maestro software version 1.1. Telomerase activity values were extrapolated from the standard curve of TSR8 control generated by 1:10 serial dilutions (20-0.2 amoles) see appendix C2. The bar charts were generated using GraphPad Prism 7.0.

Table 6: Telomerase thermal cycling conditions

Step	Temperature (°C)	Time	Number of cycles
Telomerase extension	30	30 min	1
Initial denaturation	95	2 min	1
Denaturation	95	15 s	45
Annealing	59	1 min	
Extension	45	10s	

3.2.6. xCELLigence real-time cell analysis system

Principle

Cell growth was analysed after co-silencing RBBP6 and hTERT using the xCELLigence real time cell analysis system (RTCA) (ACEA Biosciences, USA). xCELLigence is a device that uses a non-invasive electrical impedance in order to measure or assess morphology change, cell proliferation as well as the attachment quality of the cell in a label free manner. This system differs from end-point assays, where one cannot determine the point at which the cells being analysed changed in morphology, biochemistry or even a point at which the cells reached cell death. Therefore, it eliminates the guess work and labour intensive steps that comes with end point assays by allowing monitoring of the cell in real-time. The xCELLigence system functions as cellular impedance assay that involves a set of gold microelectrodes that are fixed to the microtiter plate well.

Cell index (CI) is a unitless parameter that is used to report the impedance of electron flow caused by adherent cells. When medium or a buffer solution is added to the well there is a complete circuit due to the electrodes passing from the negative terminal to the positive terminal. When cells are added to the suspension, there is an increase in

impedance because of cells falling out of suspension and adhering to the electrodes. This is followed by a steady increase in CI when cells proliferate and when the cells reach confluency the CI value plateaus. At this point the cells can be introduced to an apoptosis inducer or a drug, which would result in the cells rounding and detaching from the well bottom. However, for this study the focus was if the cells were transfected with RBBP6 and hTERT for 48 hours, what is the response in terms of cell growth.

Technique

Before the start of the experiment, the xCELLigence instrument (RTCA S16, ACEA Biosciences, USA) was placed in a 37°C incubator (Forma Steri-cycle i160, Thermo Scientific, USA) then a volume of 100 µl of antibiotic-free culture medium was added onto the 16-well E-Plate and the plate was placed on the xCELLigence instrument in order to record the background reading. 1.5 x 10^4 cells were seeded onto the well plates and the E-plate was placed back to the current flow of the instrument. The next day, cells were transfected with both siRBBP6 and sihTERT and cell growth was monitored after 48 hours. Cell index values were recorded at 15 minutes' interval sweeps until the end of the experiment. The xCELLigence parameters were as follows: Step 1: 1 sweep, 1 minute, 00:00:01 total time to measure background. Step 2: 100 sweeps, 15 minutes' interval, 24:45: 01 total time to measure cells impedance. Step 3: 25 sweeps, 15 minutes' interval, 30:45:01 total time to add siRNA and stop after 6 hours. Step 4: 193 sweeps, 15 minutes' interval, 79:00:00 total time to monitor cell.

Chapter 4: Results

The primary hypothesis of this study is that there is possible correlation between RBBP6 expression and telomerase activity in cervical cancer. Both RBBP6 and telomerase are highly expressed in cervical cancer. RBBP6 promotes cancer progression through cell cycle acceleration and apoptosis reduction. Human telomerase reverse transcriptase (hTERT), an essential subunit responsible for the main function of telomerase, is overexpressed in ~90% of cervical cancer cases. Increased expression of hTERT and high telomerase activity strongly associate with advanced cancer. Given that RBBP6 and telomerase both play a role in cervical cancer progression, we seek to probe a possible relation between RBBP6 and telomerase activity. To address this question, RBBP6 was knocked down in cervical cancer cells using RNA interference, followed by analysis of hTERT expression at both RNA (qPCR) and protein level (western blotting). Furthermore, telomerase activity was analysed in RBBP6-knockdown cells using TRAPeze RT Telomerase detection kit (Merck). Lastly, cell proliferation in response to RBBP6/hTERT co-silencing was monitored in real time using the xCELLigence system.

4.1. Confirmation of siRNA-mediated RBBP6 knockdown

Prior to conducting our investigation on whether there is a possible correlation between RBBP6 and telomerase in cervical cancer progression, we first had to verify if silencing was successful. RBBP6 silencing was confirmed at both RNA and protein level using real time quantitative PCR and western blotting. RBBP6 gene silencing was significantly high ($P < 0.05$) in both CaSki and HeLa cells, with mRNA relative ratios of 0.3 and 0.5, respectively (figure 8 A & B). Quantification at protein level using western blotting further confirmed the observed gene silencing (figure 8 C). In CaSki cell line, RBBP6 protein expression was knocked down almost completely, as can be seen by the faded band intensity (~ 5% band intensity) in transfected cells compared to non-transfected cells (figure 8 C & D). A similar trend was observed in HeLa cell line where ~ 25% band intensity was recorded in cells transfected with siRBBP6 (figure 8 D).

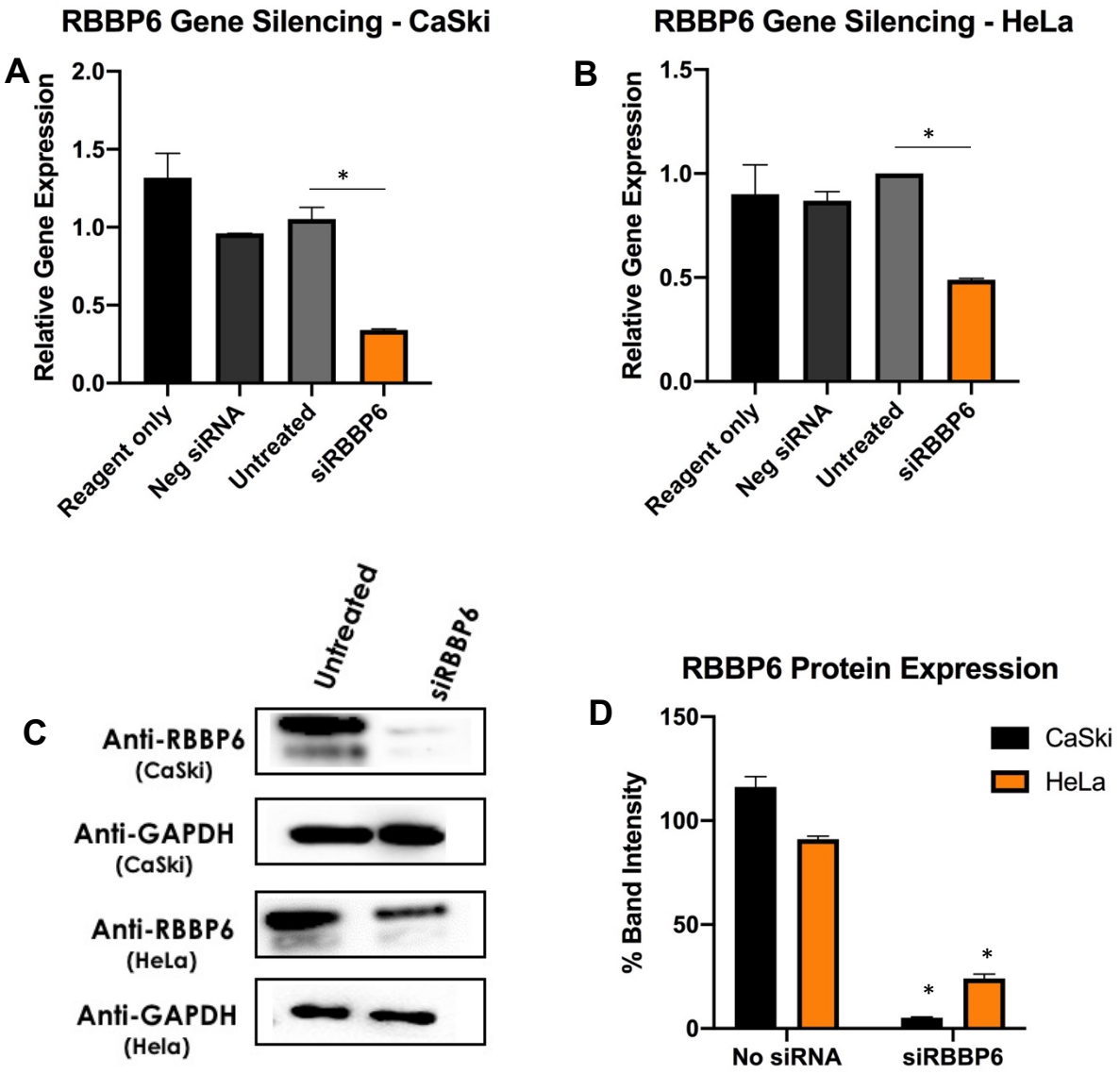

Figure 8: Confirmation of successful RBBP6 gene silencing in CaSki and HeLa cell lines RBBP6 expression was measured at mRNA and protein level after 48 and 72 hour transfection, respectively, with 60 pmol siRNA targeting RBBP6. The experiments were done in duplicates and two independent biological repeats (n=2). **p< 0,05, **p<0,01, ***p<0,001, p>0,05 (ns).*

4.2. hTERT gene expression in RBBP6-knockdown cells

In this section, we examined the effects of RBBP6 knockdown on hTERT expression in cervical cancer cell lines. In order to examine a possible relation between RBBP6 and hTERT in cervical cancer, cells were transfected with siRBBP6 for 48 and 72 hours followed by measurement of hTERT expression at mRNA and protein level, respectively. In CaSki cell line, hTERT mRNA expression was significantly ($P < 0.05$) decreased (0.4) relative to untreated cells (figure 9 A). On the contrary, in HeLa cells there was a non-significant ($P > 0.05$) increase (1.3) in hTERT expression was observed (figure 9 B). At protein level, the effect of RBBP6 silencing on hTERT in CaSki cells conformed to that observed at RNA level, although the decrease in hTERT protein expression was minimal, as shown by the band intensity (figure 9 C & D). Interestingly, there was no change in hTERT protein expression in HeLa cells compared to untreated cells (figure 9 B).

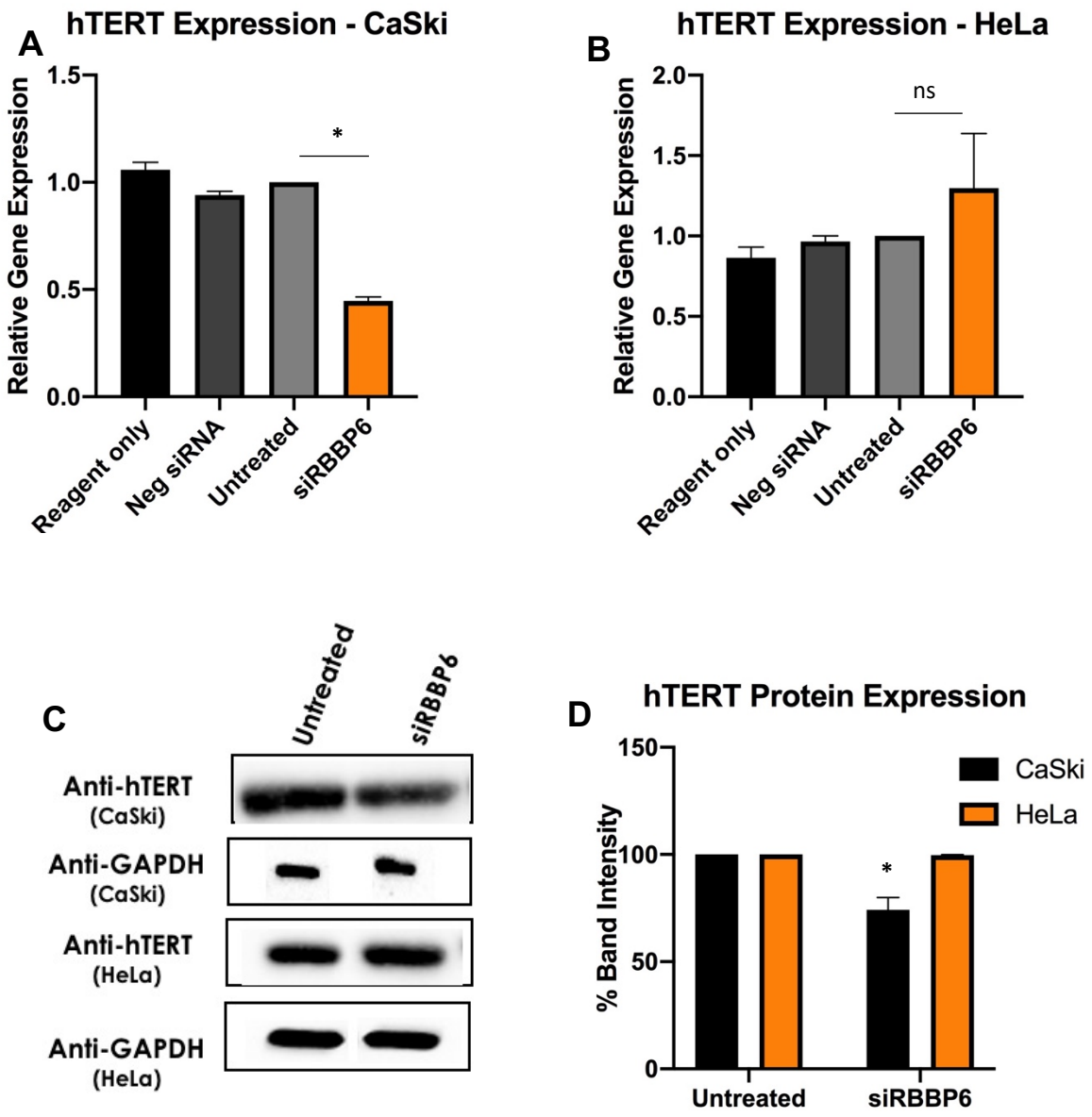

Figure 9: hTERT gene expression in response to RBBP6 knockdown in CaSki and HeLa cell lines. hTERT expression was measured at mRNA and protein level after 48 and 72 hours transfection, respectively, with 60 pmol siRNA targeting RBBP6. The experiments were done in duplicates and two independent biological repeats (n=2). **p< 0,05, **p<0,01, ***p<0,001, p>0,05 (ns).*

4.3. Telomerase activity in RBBP6-knockdown cells

In this section, detection and quantification of telomerase activity in response to RBBP6 gene silencing was investigated in CaSki and HeLa cell lines using the TRAPeze RT Telomerase detection kit (Merck, USA). HEK293 was used as a telomerase-positive cell line and MRC-5 as a telomerase-negative cell line. Cells were transfected with siRBBP6 for 72 hours prior to obtaining whole cell extracts, which were then subjected to a two-enzyme fluorometric quantitative RT-PCR. A statistically significant reduction in telomerase activity was observed in RBBP6-knockdown CaSki and the positive control HEK293 cells (figure 10 A & C). However, there was a non-significant change in telomerase activity in HeLa cells (Figure 10 D). Telomerase activity in MRC-5 was almost undetectable as expected from a negative control cell line (Figure 10 B).

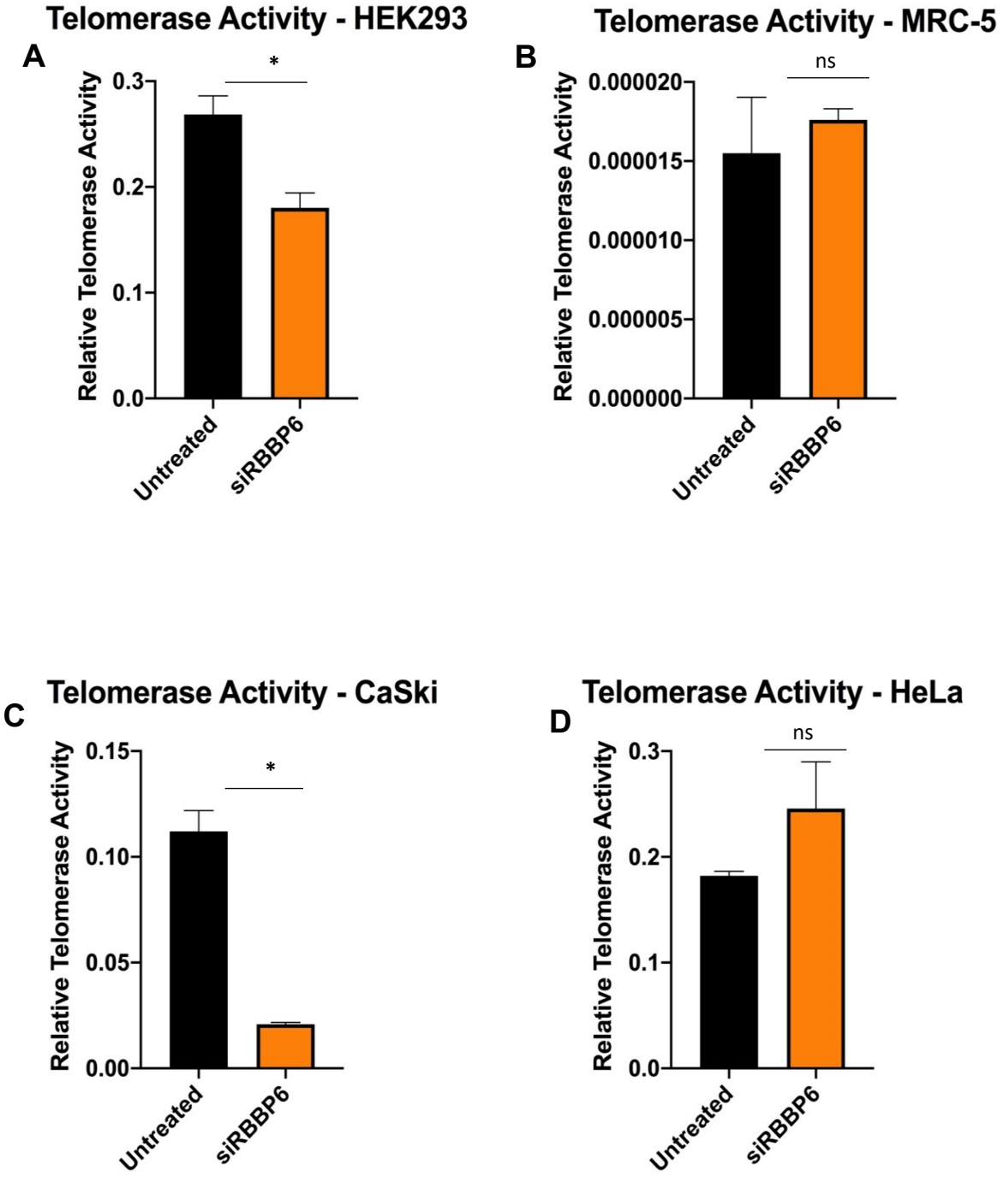

Figure 10: Effects of RBBP6 gene silencing on telomerase activity in HEK293, MRC-5, CaSki and HeLa cell lines Telomerase activity in HEK293 (A), MRC-5 (B), CaSki (C) and HeLa (D) was measured 72 hours post transfection with 60 pmol siRNA targeting RBBP6. The experiments were done in duplicates and two independent biological repeats (n=2), where **p< 0,05, **p<0,01, ***p<0,001, p>0,05 (ns).*

4.4. Cell proliferation in response to RBBP6 and hTERT co-silencing

Previous studies have shown that proliferation of cervical cancer cells is directly proportional to RBBP6 expression, where RBBP6 overexpression results in increased cell proliferation and knockdown reduces cell growth (Moela, Choene et al. 2014). It is also a known fact that hTERT expression strongly associates with advanced cancer. Having observed the consequences of RBBP6 knockdown on hTERT expression and telomerase activity, we were therefore interested in analysing cell proliferation in cells deficient of both RBBP6 and hTERT as this will substantiate our current observations. Cell growth was monitored for a period of ~ 72 hours, with transfection of siRBBP6 and sihTERT after 24 hours cell growth using the xCELLigence system (RTCA S16, ACEA Biosciences, USA). This system uses a non-invasive electrical impedance or cell index (CI) as a measure of cell growth. As shown in figure 11 and 12 below, there was a reduction in cell growth in response to either siRBBP6 or sihTERT in both HeLa and CaSki cells. Co-transfection with sihTERT and siRBBP6 further reduced cell growth in HeLa cells (figure 11) and resulted in the same level of reduction as individual transfections in CaSki cells. Untreated cells and control cells treated with transfection reagent only or negative siRNA showed a steadily increasing growth rate that plateaued at ~ 60 hours in HeLa and 53 hours in CaSki.

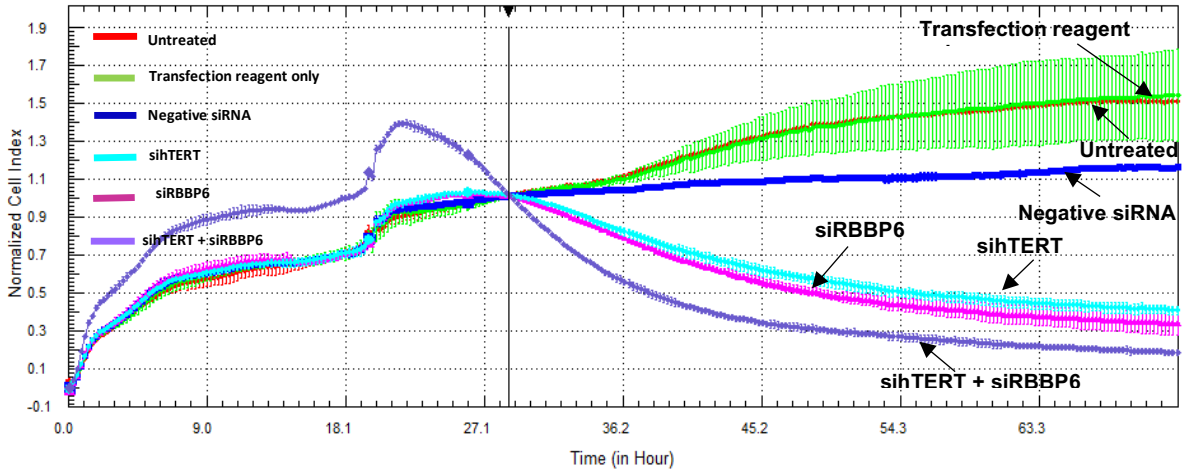

Figure 11: Cell Proliferation of HeLa cells following RBBP6 and hTERT co-silencing. Growth of untreated cells (red), cells treated with transfection reagent only (green), negative siRNA (blue), siRBBP6 (violet), sihTERT (turquoise) and siRBBP6/sihTERT (purple) was monitored in real time over a period of ~72 hours. The experiments were done in duplicates and two independent biological repeats (n=2).

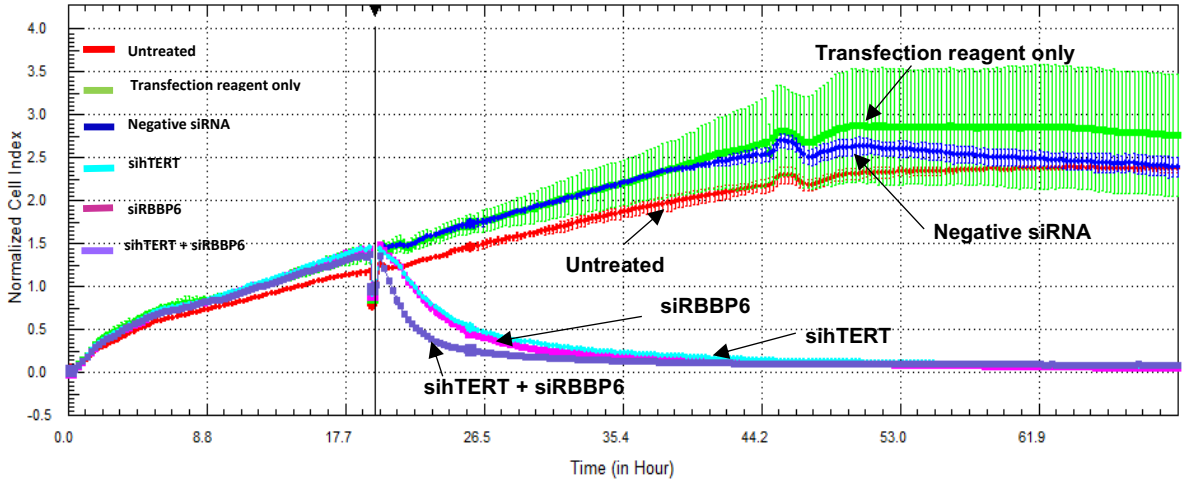

Figure 12: Cell proliferation of CaSki cells following RBBP6 and hTERT co-silencing. Growth of untreated cells (red) and cells treated with transfection reagent only (green), negative siRNA (blue), siRBBP6 (violet), sihTERT (turquoise) and siRBBP6/sihTERT (purple) was monitored in real time over a period of ~72 hours. The experiments were done in duplicates and two independent biological repeats (n=2).

Chapter 5: Discussion and conclusion

Cervical cancer continues to defy medical science, especially in developing countries like South Africa, thus making it a burden to the over stretched health care systems. In addition to this, the high rate of HIV/AIDS infection compromises the immune system of many thus resulting in opportunistic pathogen infections such as HPV to take advantage and cause cervical cancer development. Vaccines against HPV is currently available for young adolescent women, however, those who are already sexually active remain at a risk of developing cervical cancer with no cure insight mostly due to late detection. In this study, we exploited the co-treatment of two genes that might be playing a big role in cancer development in general. One is RBBP6, which according to several researchers, binds to Tp53 and leads to its degradation thereby and a subsequent cancer development and progression (Koivusalo, Mialon et al. 2006, Pugh, Eiso et al. 2006). The other gene of interest in the present study is telomerase, which is highly expressed in several cancers thus resulting in continuous cell proliferation.

Retinoblastoma Binding protein 6 (RBBP6) is a multidomain E3 ubiquitinase protein that is highly expressed in several human cancers (Ntwasa 2008, Motadi, Bhoola et al. 2011, Moela and Motadi 2016). This protein is involved in the proteasomal pathway, which is part of protein modification through ubiquitination. RBBP6 binds to many other proteins to influence their function either by regulation cell cycle or apoptosis (Pugh, Eiso et al. 2006, Cajee, Hull et al. 2012). Cell cycle-dependent phosphorylation regulates the activity of pRB and that of RBBP6. RBBP6 binds to under-phosphorylated but not phosphorylated pRB and regulates its function (Sakai, Saijo et al. 1995). In some studies, they have shown that RBBP6 isoform 3 down regulation resulted in reduced G2/M cell cycle arrest whilst its over-expression results in increased G2/M cell cycle arrest (Mbita, Meyer et al. 2012). The p53 binding domain of the protein bind to TP53 and negatively regulates it, thereby inhibiting apoptosis and cell cycle arrest. The DWNN domain of the isoform 1 is believed to regulate the expression of the whole RBBP6 which lead to its E3 ubiquitinase function (Di Giammartino, Li et al. 2014).

Furthermore, this one study showed that RBBP6 is regulated by two promoters, referred to as P0 and P1 (Pretorius, Kaur et al. 2013). Their findings were that, when apoptosis was induced the activity of the promoter increased. Secondly, both promoter transcription sites are located within CpG islands and they have six-transcription factor binding sites. With regards to HPV E6 oncoprotein, a study showed that the hTERT promoter has CpG islands located within the transcription site (Jiang, Zhao et al. 2012). These studies substantiate our proposed possible link of RBBP6 and telomerase activity. In addition, P0 was associated with apoptosis while P1 was associated with regulation of the cell cycle. Both of these promoters are evidence why RBBP6 has also been implicated in apoptosis and cell cycle regulation. The relationship between the p53, Rb, MDM2 and RBBP6 is continuously being studied and understood. One other important protein that interacts with RBBP6 is MCM10, which is a replication initiator factor that brings together helicase and polymerase and prevents DNA damage (Miotto, Chibi et al. 2014). The interaction of RBBP6 with proteins such as MCM10 and MCM2-7 helicase for the purpose of DNA replication further confirms that the relationship between RBBP6 and telomerase might exist.

In this study, we investigated the mechanism of action of RBBP6 in promoting cancer cell proliferation by analysing a possible relationship between RBBP6 and telomerase activity in cervical cancer cells. Findings reveal early insights into a possible relation between RBBP6 expression and telomerase activity in cervical cancer. We have demonstrated for the first time that successful RBBP6 silencing at mRNA and protein level decreases hTERT expression with a subsequent reduction in telomerase activity at least in the squamous cell carcinoma (SCC)-derived CaSki cell line. This is consistent with findings that hTERT mRNA expression is proportional to telomerase activity (Takakura, Kyo et al. 1998, Kirkpatrick, Clark et al. 2003). In HeLa cells however, this was not the case as seen by the non-significant change in hTERT expression and telomerase activity following knockdown of RBBP6.

One of the experiment performed to establish the relationship between RBBP6 and telomerase activity was to silence RBBP6 and then measure telomerase activity. In brief, Telomerase is the enzyme responsible to maintain the length of the telomeres during cell division (Chong, van Steensel et al. 1995). As reported in many studies,

cancer is characterised by cell proliferation, which subsequently requires increased telomerase activity in order to maintain cell growth (Shay, Zou et al. 2001, Maciejowski and de Lange 2017). Studies have shown that cell lines, immortalized either spontaneously or after transformation by oncogenic viruses, such as simian virus 40 or human papillomavirus types 16 or 18, are usually telomerase-positive (Gutkin, Uziel et al. 2016, Yuan, Larsson et al. 2019). Whereas many normal somatic cells were telomerase-negative. These results were also reflected in our study that involved RBBP6 silencing.

Figure 10 indicates that telomerase activity in untreated CaSki (C) is lower than that of untreated HeLa (D). Therefore, one might argue that the observed differential response of the two cell lines to RBBP6 silencing is as a result of their distinctive telomerase activity. However, similar results were obtained under different siRBBP6 transfection conditions. These observations therefore suggest different mechanisms of RBBP6-mediated telomerase activity in HeLa and CaSki cells. Nonetheless, we cannot rule out the possibility that histological differences between the two cell lines might be an explanation for the differential effects of RBBP6 silencing on telomerase activity. This is in agreement with previous findings where adenocarcinomas in advanced cervical cancer had overall poor response rate to treatment compared to SCC tumours (Katanyoo, Sanguanrungsirikul et al. 2012, Rose, Java et al. 2014). From this preliminary results it was clear that RBBP6 might have an effect on telomerase activity either by reducing cell proliferation or inactivating the enzyme itself.

Over and above, it is important to note that telomerase activity in cervical cancer is greatly influenced by HPV 16/18 E6 oncoprotein. A study by Jiang et al. (2012) on hTERT expression in HPV 16/18-positive cervical cancer cell lines focused mainly on the consequences of HPV16/18 E6 knockdown on hTERT mRNA levels and hTERT promoter DNA methylation in CaSki, HeLa and SiHa cells. Their findings were that hTERT expression in response to HPV 16/18 E6 silencing was highest in HeLa cells with relatively less dense methylation around the transcription start site. On the contrary, hTERT expression was the lowest in E6 knocked-down CaSki cells, with relatively denser methylation around the transcription start site. This differential response to transfection seen in HeLa and CaSki cells is consistent with our findings. Their speculation is that the differential response may be due to the involvement of a

large variety of transcription factors that interact with the hTERT promoter to determine the activity of hTERT (Jiang, Zhao et al. 2012).

Based on the findings, (Koivusalo, Mialon et al. 2006) indicated that E6 oncoprotein through its E3 ligase activity, alters the expression of c-myc transcription factor on the hTERT promoter in cervical cancer cells. These studies substantiate our investigation that RBBP6 is not a DNA binding protein, which means possibilities of it regulating hTERT expression through direct interaction with the hTERT promoter is limited. However, there is evidence showing that E3 ligases have the ability to bind to and alter the expression of transcription factors on the hTERT promoter (James, Lee et al. 2006). Furthermore, (Miotto, Chibi et al. 2014) showed that E3 ubiquitin ligase RBBP6 ubiquitinates and destabilizes the transcriptional repressor called ZBTB38, which is known to negatively regulate transcription. We therefore hypothesize that through its E3 ligase activity, RBBP6 might be indirectly affecting hTERT expression by interacting with certain activators and/or repressors on the hTERT promoter.

This suggestion has merit on the basis that the function of RBBP6 in protein binding and degradation has been strongly elucidated. Chibi et al. revealed that RBBP6 binds to and degrades the transcription factor, Y-box-binding protein 1 (YB-1), an essential regulator of cellular propagation and apoptosis (Chibi, Meyer et al. 2008). Additionally, RBBP6 facilitates MDM2-mediated degradation of p53 through its p53-binding domain and E3 ligase activity (Di Giammartino, Li et al. 2014). The involvement of RBBP6 in p53 degradation has a great potential to result in the inhibition of DNA repair mechanisms and p53-mediated apoptosis, as postulated in previous findings (Motadi, Bhoola et al. 2011, Di Giammartino, Li et al. 2014, Moela, Choene et al. 2014). Loss of DNA repair and evasion of apoptosis are the primary candidates of cancer development and progression. This functioning of RBBP6 in these cellular processes therefore implicates it in cancer cell proliferation.

In cervical cancer, (Motadi, Lekganyane et al. 2018) have shown that RBBP6 is highly expressed in human tissue sections and that cancer cell proliferation is associated with increased expression of RBBP6. Telomerase is also essential for the indefinite proliferation of immortalized cells both in vitro and in vivo (Takakura, Kyo et al. 1998, Kirkpatrick, Clark et al. 2003, Sheng, Tong et al. 2013). As a result, we were interested

in analysing the proliferation of cervical cancer cells that are deficient of both RBBP6 and telomerase activity.

CaSki and HeLa cells were subjected to co-silencing of RBBP6 and hTERT and their growth monitored in real time over a period of ~ 72 hours. As expected, this resulted in a reduced growth rate over time in cells deficient of either RBBP6 or hTERT in both CaSki and HeLa. These observations substantiate the speculation that both RBBP6 and hTERT are strongly associated with cancer cell proliferation. Furthermore, it was interesting to see that co-silencing elicited a much higher cell growth reduction in HeLa cells, highlighting a possible additive effect of RBBP6 and hTERT on cell growth. For the first time, our study has revealed early insights into a possible crosstalk between RBBP6 and telomerase in terms of regulating cell proliferation in cervical cancer cells. Because of the elicited further reduction of cancer cell growth in co-silenced treatment, the results further support the relationship that might exist between RBBP6 and telomerase activity. It would be interesting results that show the relation and the molecular mechanism of the two genes in regulating cancer cell inhibition.

In conclusion, we have shown for the first time that there is a possible relationship between RBBP6 expression and telomerase activity in cervical cancer cells. This was seen by a change in hTERT expression following RBBP6 knockdown. We further indicated that hTERT expression is proportional to telomerase activity in RBBP6-knockdown cells, meaning that RBBP6-mediated change in hTERT expression has consequential effects on telomerase activity. We further illustrated a possible additive effect of both RBBP6 and hTERT on cervical cancer cell proliferation. We, however, draw these conclusions with caution considering the fact that our study was conducted in only two cervical cancer cell lines, which compromises the robustness of our findings. Therefore, a more intensive study is still required in future to deeply explore interrelations between RBBP6 and telomerase activity.

Such future prospects should include investigation on whether there is a possible nuclear co-localization between RBBP6 and hTERT using confocal microscopy. Another similar study we can consider is to determine the interaction between RBBP6 and telomerase by investigating a possible RNA-protein interaction between RBBP6 protein and hTERT mRNA using RNA-Chromatin Immunoprecipitation (ChIP) assay.

This technique is able to determine whether specific proteins (in our case RBBP6) are associated with specific genomic regions such as transcription factors on promoters. This can validate our speculation around the possibility of RBBP6 interacting with hTERT. In addition, a study by (Katzenellenbogen, Egelkrout et al. 2007) identified a novel repressor of hTERT transcription called NFX 1-91, which is targeted for ubiquitin-mediated degradation by HPV type 16 E6/E6AP. In contrast, NFX 1-123, a splice variant of NFX1 increased expression from an hTERT promoter that was activated by E6/E6AP. We propose this may also answer our question of why we saw different outcomes from the cell lines (CaSki and HeLa). Could it be that the decrease in TERT expression in CaSki with HPV type 16 is being repressed by RBBBP6? While on the other hand the expression of hTERT in HeLa cells with HPV type 18 could be being increased via activating transcriptional factors?

In addition to what our findings are on RBBP6 to telomerase activity, a similar prospect can be to study what would happen to RBBP6 expression following silencing of hTERT at RNA and protein level using qPCR and western blotting. This would give us an early indication of whether telomerase can regulate RBBP6 expression before moving to the above-mentioned approaches such as confocal microscopy and ChiP analysis. Furthermore, understanding the mechanism of cell proliferation in hTERT/RBBP6-deficient cells by exploring the cell cycle using is highly important. Lastly, RBBP6 plays a significant role in the apoptosis pathway and it would therefore be interesting to analyze the downstream effects of hTERT/RBBP6 co-silencing on cell death using flow cytometry.

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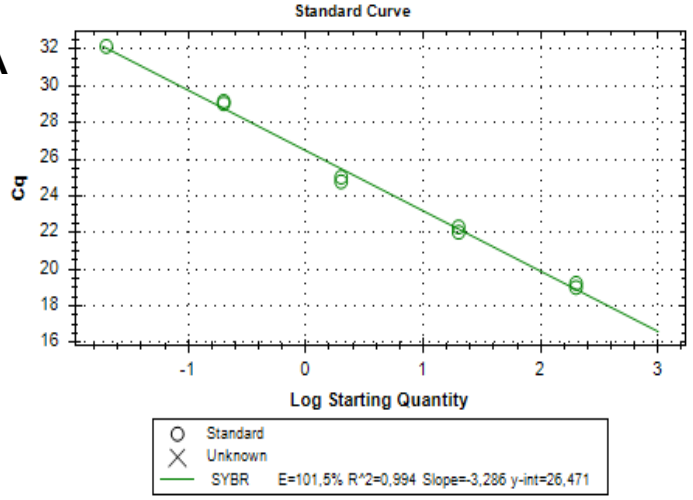
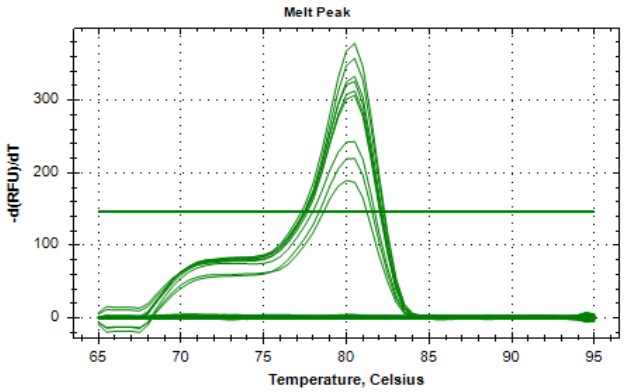
Appendices

Appendix A: Standard curve of target genes qPCR

Standard curves and melting curves of GAPDH, RBBP6 and hTERT. GAPDH(A) was used as positive control for the normalization of gene expression of RBBP6(B) and hTERT(C).

A





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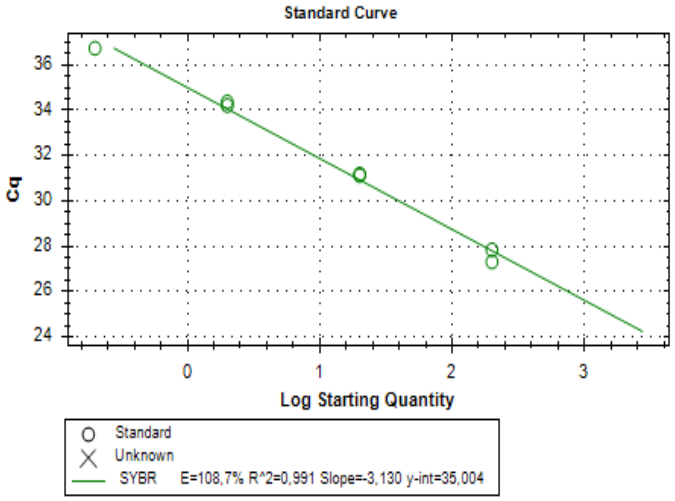
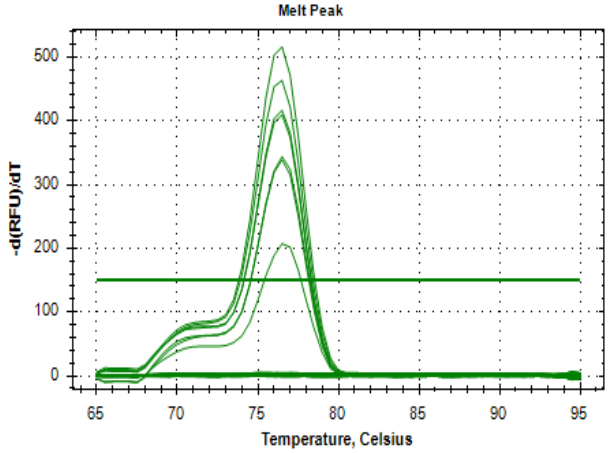
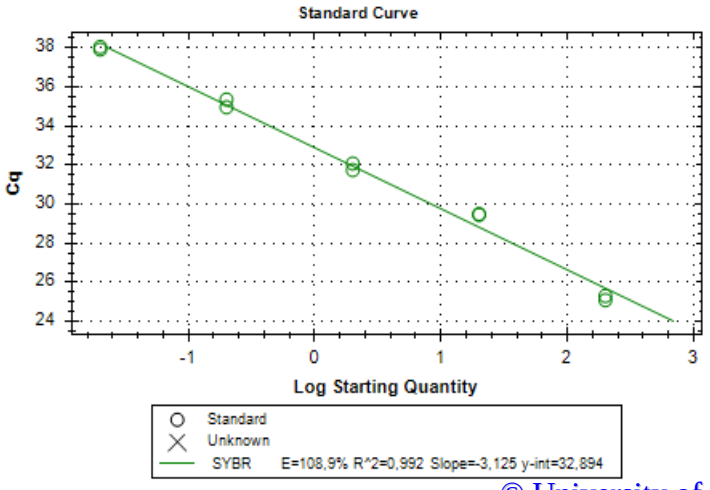
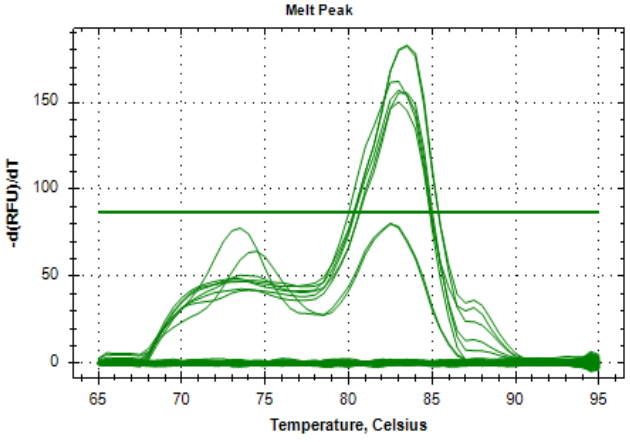




C





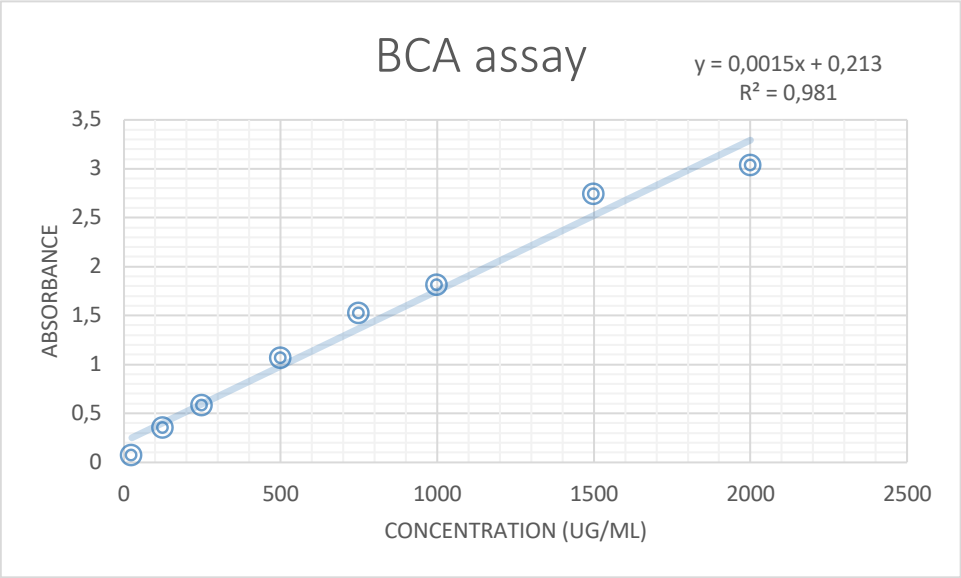
Appendix B1: Albumin standard (BSA) preparation

Table 1. Preparation of Diluted Albumin (BSA) Standards

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20-2,000µg/mL)

Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

Appendix B2: BCA assay standard curve



Protein samples concentration extrapolated from the was as follows:

1. HeLa cells: Untreated cells = Absorbance (A) = 2.7 = 1700 µg/ml
   siRBBP6 cells= A= 3.2 = 1900 µg/ml
2. CaSki cells: untreated cells = A= 2.7 = 1700 µg/ml
   SiRBBP6 cells = A = 1.6 = 900 µg/ml

- Preparation and calculations for protein gel loading involve the following steps:
  The protein assay measures the protein concentration in the assay tube (well) after mixed with working solution. Once the protein concentration is known then the concentration of the original sample can be calculated

Step 1: $C_1V_1 = C_2V_2$

- C_1 = unknown
- V_1 = volume of sample added to assay well
- C_2 = assay well conc.
- V_2 = total assay volume

HeLa cells (untreated)= 1700 µg/ml x 225 µl ÷ 25 µl

C_1 = 15 300 µg/ml

HeLa (siRBBP6) = 1900 µg/ml x 225 µl ÷ 25 µl

C_1 = 17 100 µg/ml

CaSki cells (untreated)= 1700 µg/ml x 225 µl ÷ 25 µl

C_1= 15 300 µg/ml

CaSki (siRBBP6) = 900 µg/ml x 225 µl ÷ 25 µl

C_1= 8100 µg/ml

Step 2: Calculate the protein concentration of gel-ready sample

$C_1V_1 = C_2V_2$

- C_1 = calculated previous step
- V_1 = volume of sample added to gel-ready tube
- C_2 = gel-ready conc. unknown
- V_2 = gel ready volume

HeLa (untreated) = 15 300 µg/ml x 10 µl ÷ 20 µl

C_2= 7650 µg/ml

HeLa (siRBBP6) = 17 100 µg/ml x 10 µl ÷ 20 µl

C_2 = 8550 µg/ml

CaSki cells (untreated)= 15 300 µg/ml x 10 µl ÷ 20 µl

C_2= 7650 µg/ml

CaSki (siRBBP6) = 8100 µg/ml x 10 µl ÷ 20 µl

C_2= 4050 µg/ml

Step 3: Calculate the volume of the gel-ready sample to load into each well

For western blotting an amount of 30 µg protein for each sample was required in order to evaluate the level of protein concentration and this was calculated as follows:

30 µg	1 ml	1000 µl
	7650 µg	1 ml

= 3.92 µl of protein per lane for HeLa and CaSki (untreated cells). To load into a gel a 1:1 volume of protein sample buffer was loaded. For example: 3. 92 µl sample + 3.92 µl buffer = 7.84 ul loaded into the well.

30 µg	1 ml	1000 µl
	8550 µg	1 ml

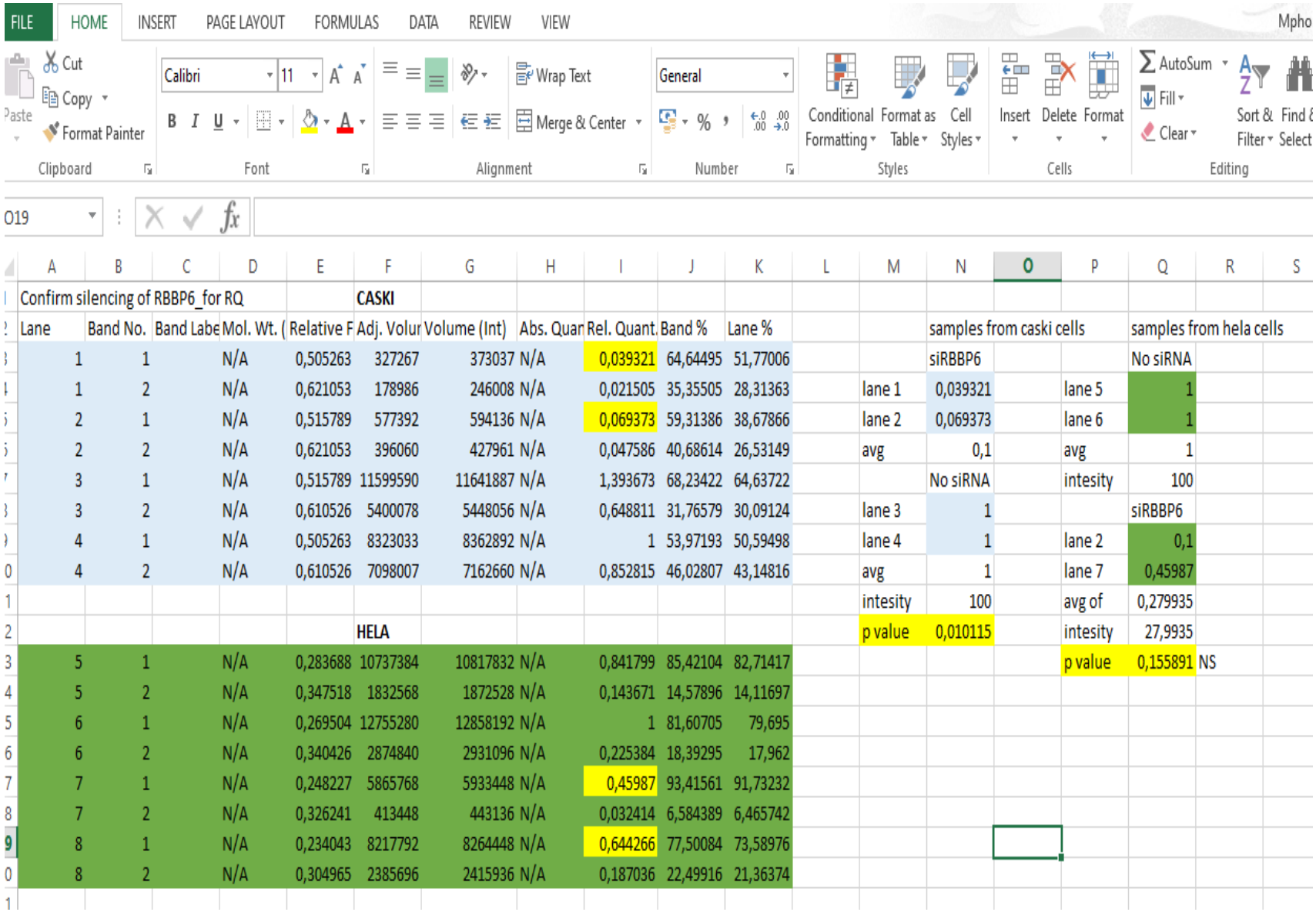
= 3.50 µl HeLa (siRBBP6) + 3.50 µl of buffer = 7.01 µl

30 µg	1 ml	1000 µl
	4050 µg	1 ml

= 7.40 µl CaSki (siRBBP6) + 7.40 µl = 14.81 µl

Appendix B3: Band intensities

- The blots or band intensities were quantified using Image Lab 4.1 software (BioRad, USA). Densitometric analysis evaluating the relative quantity of each band using untreated cell bands as reference. The data was exported to excel in order to calculate the band intensities percentages. Below is an example how that was achieved



FILE HOME INSERT PAGE LAYOUT FORMULAS DATA REVIEW VIEW

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	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1	Confirm silencing of RBBP6_for RQ					CASKI													
2	Lane	Band No.	Band Labe	Mol. Wt. (	Relative F	Adj. Volur	Volume (Int)	Abs. Quan	Rel. Quant.	Band %	Lane %			samples from caski cells			samples from hela cells		
3	1	1		N/A	0,505263	327267	373037	N/A	0,039321	64,64495	51,77006			siRBBP6			No siRNA		
4	1	2		N/A	0,621053	178986	246008	N/A	0,021505	35,35505	28,31363		lane 1	0,039321		lane 5	1		
5	2	1		N/A	0,515789	577392	594136	N/A	0,069373	59,31386	38,67866		lane 2	0,069373		lane 6	1		
6	2	2		N/A	0,621053	396060	427961	N/A	0,047586	40,68614	26,53149		avg	0,1		avg	1		
7	3	1		N/A	0,515789	11599590	11641887	N/A	1,393673	68,23422	64,63722			No siRNA		intesity	100		
8	3	2		N/A	0,610526	5400078	5448056	N/A	0,648811	31,76579	30,09124		lane 3	1			siRBBP6		
9	4	1		N/A	0,505263	8323033	8362892	N/A	1	53,97193	50,59498		lane 4	1		lane 2	0,1		
10	4	2		N/A	0,610526	7098007	7162660	N/A	0,852815	46,02807	43,14816		avg	1		lane 7	0,45987		
11													intesity	100		avg of	0,279935		
12						HELA							p value	0,010115		intesity	27,9935		
13	5	1		N/A	0,283688	10737384	10817832	N/A	0,841799	85,42104	82,71417					p value	0,155891	NS	
14	5	2		N/A	0,347518	1832568	1872528	N/A	0,143671	14,57896	14,11697								
15	6	1		N/A	0,269504	12755280	12858192	N/A	1	81,60705	79,695								
16	6	2		N/A	0,340426	2874840	2931096	N/A	0,225384	18,39295	17,962								
17	7	1		N/A	0,248227	5865768	5933448	N/A	0,45987	93,41561	91,73232								
18	7	2		N/A	0,326241	413448	443136	N/A	0,032414	6,584389	6,465742								
19	8	1		N/A	0,234043	8217792	8264448	N/A	0,644266	77,50084	73,58976								
20	8	2		N/A	0,304965	2385696	2415936	N/A	0,187036	22,49916	21,36374								
21																			

Appendix C1: Telomerase activity assay normalization

- Before loading the telomerase extract into the reaction mix all the samples were calculated to start with 500 ng/µl so we can be able to evaluate the level of telomerase activity in all cell lines equally. Below is an example of how other repeats were calculated:
  - **Conversion:** 1 mg/µl = 1000 ng/µl

- C_1 = measured by NanoDrop
- V_1 = unknown
- C_2 = desired final concentration
- V_2 = desired final volume

Using the formula: **$C_1V_1 = C_2V_2$**

1. Positive extract untreated (HEK293) = 3.92 mg/µl

   $C_1V_1 = C_2V_2$

   3 920 x V_1 = 500 ng/µl x 5 µl

   V_1 = 2500 ÷ 3 920 = 0.63 µl extract (and dilute in 4.36 µl of nuclease free water)

   Positive extract siRBBP6 (HEK293) = 3.0 mg/µl

   $C_1V_1 = C_2V_2$

   3 000 x V_1 = 500 ng/µl x 5 µl

   V_1 = 2500 ÷ 3 000 = 0.83 µl extract (-5 µl= 4.16 µl nuclease free water)

2. Negative extract untreated (MRC-5) = 1.62 mg/µl

   $C_1V_1 = C_2V_2$

   1620 x V_1 = 500 ng/µl x 5 µl

   V_1 = 2500 ÷ 1620 = 1.54 µl telomerase extract

   Negative extract siRBBP6 (MRC-5) = 1.1 mg/µl

   $C_1V_1 = C_2V_2$

   1100 x V_1 = 500 ng/µl x 5 µl

   V_1 = 2500 ÷ 1100 = 2.27 µl

3. HeLa (untreated) = 1.32 mg/µl

   $C_1V_1 = C_2V_2$

   1320 x V_1 = 500 ng/µl x 5 µl

   V_1 = 2500 ÷ 1320 = 1.89 µl extract

   HeLa (siRBBP6) = 1.87 mg/µl

   $C_1V_1 = C_2V_2$

   1870 x V_1 = 500 ng/µl x 5 µl

V_1 = 2500 ÷ 1870 = 1.33 µl extract

4. CaSki (untreated) = 1 mg/µl

$C_1V_1 = C_2V_2$

1000 x V_1 = 500 ng/µl x 5 µl

V_1 = 2500 ÷ 1000 = 2.5 µl extract

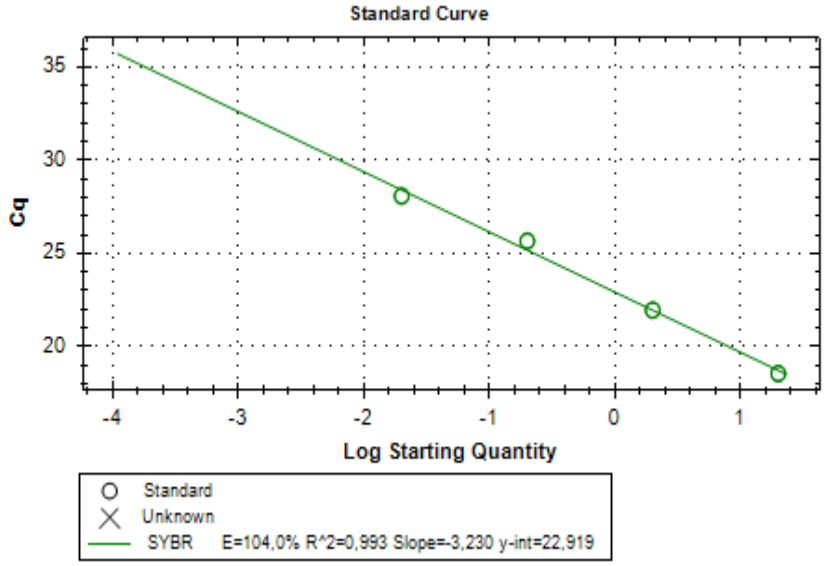
CaSki (siRBBP6) = 0.9 mg/µl

$C_1V_1 = C_2V_2$

900 x V_1 = 500 ng/µl x 5 µl

V_1 = 2500 ÷ 900 = 2.7 µl extract

Appendix C2: Standard curve of TSR8 template for optimization of telomerase activity



Appendix D: Turnitin originality report

Final Dissertation_2

ORIGINALITY REPORT

15%	5%	13%	%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	worldwidescience.org Internet Source	1%
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