

AMERICAN UNIVERSITY OF BEIRUT

THE PLANT-DERIVED BIOACTIVE MOLECULE
THYMOQUINONE NEGATIVELY REGULATES
COLORECTAL CANCER STEM CELLS' SELF-RENEWAL
CAPACITY

by
CARLA HENRY HANKACHE

A thesis
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for the degree of Master of Science
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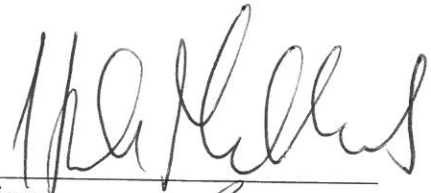
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by
CARLA HENRY HANKACHE

Approved by:

Dr. Hala Muhtasib, Professor
Department of Biology

Advisor



Dr. Wassim Abou-Kheir, Assistant Professor
Department of Anatomy, Cell Biology and
Physiological Sciences

Co-Advisor



Dr. Noel Ghanem, Assistant Professor
Department of Biology

Member of Committee



Date of thesis defense: July 7, 2015

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I hope you enjoy reading my thesis ☺

AN ABSTRACT OF THE THESIS OF

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Title: The Plant-Derived Bioactive Molecule Thymoquinone Negatively Regulates Colorectal Cancer Stem Cells' Self-Renewal Capacity

Cancer relapse following therapy remains a leading cause of death among humans worldwide. Colorectal cancer, after a potentially curative surgery, has the tendency to relapse after 5 years on average. It is believed that cancer stem cells (CSCs), which are a subpopulation of cancer cells that retain the ability of self-renewal and differentiation into different mature cells, are the main factor in cancer relapse. Although CSCs pose a significant problem for cancer relapse, they provide a target for drug therapy. Thymoquinone (TQ) is a promising anticancer molecule shown to inhibit cancer cell growth and progression in numerous cancer systems both *in vitro* and *in vivo*. We hypothesize that TQ targets colon cancer cells with stem-like properties by inhibiting their self-renewal property. Here, we investigated the effect of TQ on colon cancer cells with stem-like properties using two isogenic HCT116 human colon cancer cell lines that differ in their p53 status. We also investigated the relevance of stem cell markers profiling in 2-dimensional (2-D) monolayer cultured cells versus 3-dimensional (3-D) colonospheres. Sphere-formation and propagation assays were used to assess TQ effect on self-renewal potential of enriched CSC populations. Inhibition of self-renewal required 10-fold lower concentrations of TQ in 3-D HCT116 colonospheres when compared to those required to inhibit cell growth in two-dimensional 2-D monolayers. Colonospheres which survived TQ treatment at 1 and 3 μ M TQ were propagated from generation 1 to 5 and showed, at every generation, similar dose-dependent decrease in sphere viability upon TQ treatment. Colonospheres treated with 1 μ M TQ showed a consistent decrease in sphere viability over serial passages from generation 1 to 5 regardless of their p53 status. We also showed that a pure CSC population with wild type p53 is less responsive to TQ treatment when compared to a CSC population with p53 deletion. TQ significantly decreased HCT116 p53^{+/+} sphere size at generation 1 but not in HCT116 p53^{-/-} cells. However, no decrease in sphere size was noted in subsequent generations in both cell lines. We also showed that TQ treatment decreased expression levels of colorectal stem cell markers EpCAM and CD44 in monolayer HCT116 p53^{+/+} and p53^{-/-} cells and HCT116 p53^{-/-} spheres, highlighting the difference between 2D and 3D culture systems. TQ also induced higher apoptosis in HCT116 p53^{-/-} colonospheres than those with wild type p53. Altogether, our findings document for the first time TQ's effect on colon cancer stem cells and provide insight on the underlying mechanisms of TQ inhibition of colon cancer relapse, information which is essential for clinical translation of this natural anticancer molecule.

CONTENTS

ACKNOWLEDGMENTS.....	viii
ABSTRACT.....	ix
LIST OF ILLUSTRATIONS.....	xiii
LIST OF TABLES.....	xiv
LIST OF ABBREVIATIONS.....	xv
Chapter	
I. INTRODUCTION	1
A. Colorectal Cancer.....	1
1. Epidemiology.....	1
2. Development and Causes.....	3
3. Stages and Treatment.....	5
4. Genetic Alterations: p53 Tumor Suppressor Gene.....	6
B. Cancer Stem Cells.....	9
1. Concept.....	9
2. Normal Stem Cells vs. Cancer Stem Cells.....	12
a. Cancer Stem Cell Signaling and Markers.....	12
b. Metastasis Determinants in Stem Cell Niche.....	13
C. Colorectal Cancer Stem Cells.....	14
1. Overview.....	14
2. Enrichment of Colorectal Cancer Stem Cells.....	16
3. Targeting Cancer Stem Cells.....	17

D. Thymoquinone.....	19
1. Chemical Characteristics.....	20
2. TQ and Cancer.....	21
E. Aim of Study.....	23
II. MATERIALS & METHODS.....	25
A. Cell Culture Conditions.....	25
B. Drug Preparation and Treatment.....	25
C. MTT Cell Viability Assay.....	26
D. Sphere Formation Assay.....	26
E. Propagation Assay.....	27
F. RNA Extraction and Quantitative Real Time PCR (qRT-PCR).....	27
G. Immunofluorescence and Microscopic Imaging.....	28
H. Statistical Analysis.....	29
III. RESULTS.....	30
A. Effect of TQ on Viability of Human Colorectal Cancer Cell Lines HCT116 p53 ^{+/+} and p53 ^{-/-} in 2D Culture.....	30
B. Optimization of Three - Dimensional Sphere Culture Serum Conditions.....	32
C. Comparison of TQ Concentration-Dependent Effect on Two- Dimensional vs. Three-Dimensional HCT116 Cultures.....	33
D. TQ Effect in Targeting an Enriched Population of Human Colorectal Cancer Stem-like Cells.....	36
1. TQ Effect on HCT116 Sphere Counts.....	36

2. TQ Effect on HCT116 Sphere Size.....	41
E. Stem Cell Markers Gene Expression in 2-D HCT116 Cells vs. 3-D HCT116 Spheres.....	43
F. TQ-Induced Apoptosis in HCT116 p53-/- Colonospheres.....	45
IV. DISCUSSION	48
REFERENCES.....	53

ILLUSTRATIONS

Figure	Page
1. Gender specific number of colorectal cancer cases per year	1
2. Colorectal cancer as a multistep process	4
3. Colorectal cancer stages and treatment	6
4. p53 functions as a homotetrameric transcription factor	8
5. p53 activating signals and downstream effects	9
6. Origin of cancer stem cells.	11
7. Location of normal or cancer colorectal stem cells within a crypt	15
8. Propagation of progenitor/stem-like cell population	17
9. Strategies sensitizing CSCs and inducing complete tumor regression	19
10. TQ chemical structure	20
11. MTT viability assay on HCT116 cells in response to TQ treatment.	31
12. Optimizing serum conditions in HCT116 sphere culture	33
13. Sensitivity of HCT116 p53 ^{+/+} and p53 ^{-/-} cells to TQ concentrations	34
14. Effect of TQ on generation 1 HCT116 p53 ^{+/+} and p53 ^{-/-} spheres.....	35
15. TQ decreases self-renewal in enriched HCT116 progenitor/stem-like cells.	38
16. Effect of TQ on a pure CSC population at generation 5 in HCT116 p53 ^{+/+} and p53 ^{-/-} cell lines.	40
17. Effect of TQ on size of colonospheres at G1.....	42
18. EpCAM and Lgr5 gene expression levels in HCT116 2-D monolayer Cells and G1 spheres	44
19. TQ induces apoptosis in HCT116 colonospheres	46

TABLES

Table	Page
1. Risk factors involved in colorectal cancer	3
2. Normal and cancer stem cell markers in different solid tumors	13

ABBREVIATIONS

μ M: Micromolar

2D: Two-dimension

3D: Three-dimension

ABCR5: ATP Binding Cassette Receptor 5

ACF: Aberrant Crypt Foci

ALDH1: Aldehyde dehydrogenase 1

AML: Acute myeloid leukemia

APC: Adenomatous polyposis coli

AUBMC: American University of Beirut Medical Center

Bcl-2: B-cell lymphoma 2

Bcl-xL: B-cell lymphoma-extra large

CDF: Difluorinated-curcumin

cDNA: complementary desoxyribonucleic acid

CHEK1: Checkpoint kinase 1

COX2: Cyclo-oxygenase 2

CRC: Colorectal cancer

CRCSC: Colorectal cancer cancer stem cell

CSC: Cancer stem cell

Ctl: Control

DNA: Deoxyribonucleic acid

EpCAM: Epithelial cell adhesion marker

FBS: Fetal bovine serum

G1: Generation 1

G2: Generation 2

G3: Generation 3

G4: Generation 4

G5: Generation 5

GSH: Glutathione

GSK-3 β : Glycogen synthase kinase 3 β

HCT116: Human colorectal cell line

Hrs: Hours

IC₅₀: Half maximal inhibitory concentration

ISEMF: Intestinal subepithelial myofibroblast

Lgr5: Leucin rich G-protein coupled receptor 5

Mdm2: Mouse double minute 2 homolog

MLH1: Mult homolog 1

MMP: Matrix metalloproteinase

MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

NSG: NOD SCID Gamma

OD: Optical density

P19ARF: p19 alternate reading frame

PBS: Phosphate-buffered saline

PC: Progenitor cell

PPAR: Peroxisome proliferator-activated receptor

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RT-PCR: Reverse transcription polymerase chain reaction

SD: Standard deviation

SEM: Standard error mean

SFU: Sphere formation unit

Shh: Sonic hedgehog

TAC: Transit-amplifying cell

TCF: T-cell factor

TQ: Thymoquinone

WT: Wild type

CHAPTER I

INTRODUCTION

A. Colorectal Cancer

1. *Epidemiology*

Colorectal cancer (CRC) is the third commonly diagnosed cancer in men and women and one of the leading cancer killers' worldwide (Jemal *et al.*, 2011). According to the World Health Organization, in the United States, advances in surgical techniques, radiotherapy, use of combinatorial chemotherapy and improved prevention have resulted in increased survival rate in patients diagnosed with CRC (Jemal *et al.*, 2011). In Lebanon, prostate and colorectal cancers occupy the top three ranks in the cancer occurrences chart (Shamseddine *et al.*, 2014). The American University of Beirut Medical Center (AUBMC) tumor registry annual report 2010 highlighted the progressive increase in colorectal cancer incidence within the past two decades (Figure 1).

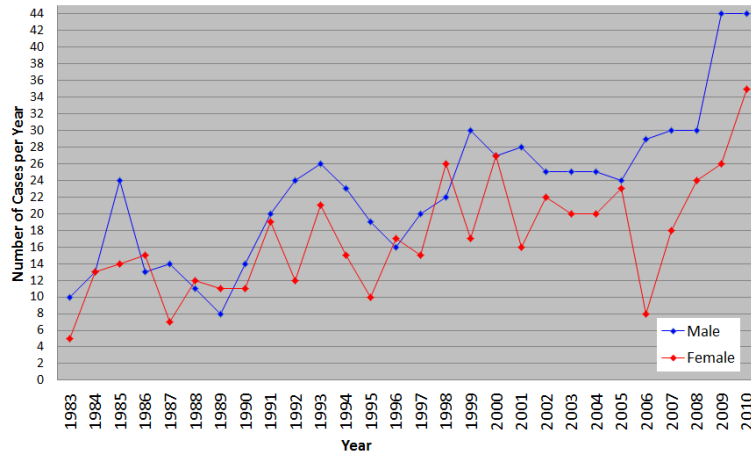


Figure 1. Gender specific number of colorectal cancer cases per year. (From the AUBMC tumor registry annual report 2010).

Due to the rapid metastasis of this cancer, patients are not diagnosed until late and approximately 50% of them encounter metastatic progression (Arvelo *et al.*, 2015). Colorectal cancer incidence is highly influenced by environmental, nutritional habits and variations in the genetic profile of different ethnicities and different populations which constitute 20% of CRC (Arvelo *et al.*, 2015; Terzic *et al.*, 2010). Therefore, diet and sedentary lifestyle are the most important risk factors for high CRC incidence in Europe, North America and Australia whereas Western Africa possess the lowest occurrence rate (Durko, 2014). The following table 1 summarizes factors which increase colorectal cancer occurrence.

Factors increasing the risk	Factors decreasing the risk
Heredity and Medical History	Fiber rich foods
Family History (HNPCC, FAP)	Plant extracts
Inflammatory bowel disease Crohn disease Ulcerative colitis	Milk consumption
Diabetes	Vitamin B and E
Other factors	Regular and daily amount of sleep
Obesity	Moderate daily physical activities
Red meat consumption	
Tobacco	
Alcohol consumption	
Lack of physical activity	
Sleep deprivation	

Table 1. Risk factors involved in colorectal cancer. [Modified and adapted from Durko 2014].

2. Development and Causes

The development of colorectal cancer is a multistep process in which accumulation of mutations and epigenetic changes lead to uncontrolled proliferation and switch from normal to malignant cells (Figure 2). Alterations in cell growth, differentiation and apoptotic cell death result from deregulated expression of oncogenes and tumor suppressor genes, most prominently KRAS, APC and p53 (Fearon, 2011). Often referred to the ‘Vogelstein model’, colorectal epithelial cells initiate their development towards malignancy by acquiring early mutations in their tumorigenic pathway, precisely adenomatous polyposis coli APC tumor suppressor gene. Cells with APC mutations are shed in the intestinal lumen where they accumulate second and third mutations necessary for their malignant behavior. Loss of

function of the tumor suppressor APC gene and aberrant mutations in other components of the pathway are crucial for adenoma formation (Terzic *et al.*, 2010). Aberrant activation of K-Ras oncogene, B-Raf and cyclo-oxygenase 2 (COX2) and inactivation of TGF- β receptor II, p53 and the proapoptotic protein Bax are altogether responsible for adenoma-to-carcinoma transition (Terzic *et al.*, 2010).

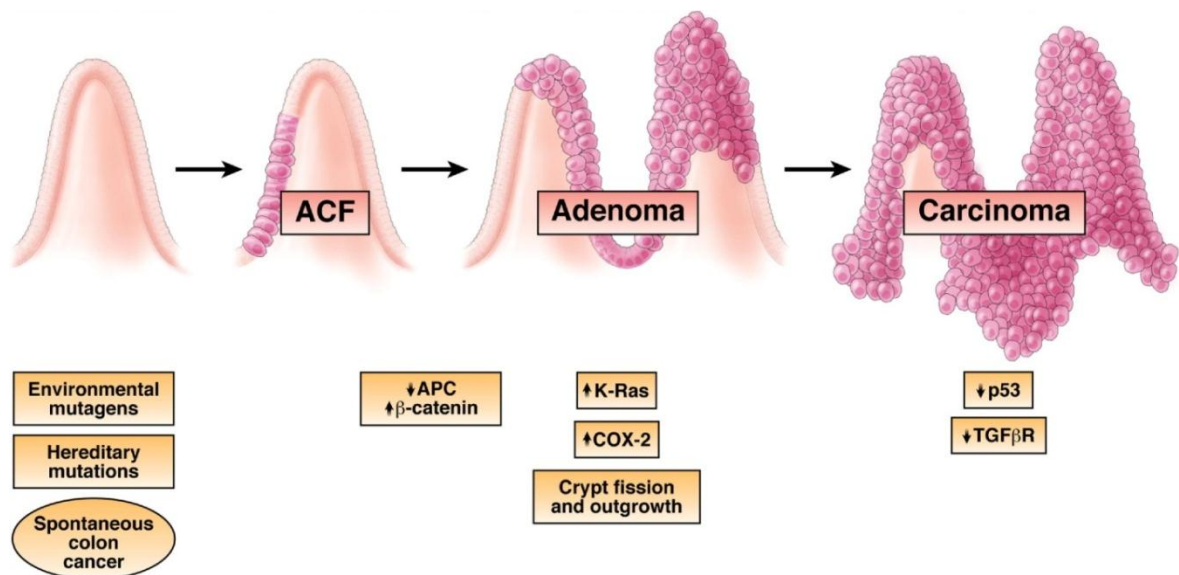


Figure 2. Colorectal cancer as a multistep process. Colorectal cancer is characterized by accumulation of mutation in tumor suppressor genes (APC: adenomatous polyposis coli, β -catenin, p53) and oncogenes (K-Ras and COX-2) which lead to progression from pre-neoplastic cells to Aberrant Crypt Foci (ACF), followed by adenoma and carcinoma stages. [Modified and adapted from Terzic *et al.*, 2010].

Additional evidence suggested that CRC is also dictated by genetic instability and epigenetic modifications. Colorectal malignancies sharing microsatellite instabilities are considered 15% of colorectal cancer cases whereas 80% of the cases are due to epigenetics

(Grady and Carethers, 2008). Genetic alterations favor uncontrolled cell growth, chemoresistance and apoptosis evasion by the aberrant activation of oncogenes, loss of function of tumor suppressor genes and inactivation of DNA repair genes, such as *MLH1* and *MYH* (Grady and Carethers, 2008). Loss of DNA methylation and CpG islands was significantly observed in early stages of colorectal cancer formation whereby minimal contribution was held in later stages of adenoma-to-carcinoma transition. Recent studies show variations in epigenetic mechanisms among normal intestinal cells, precancerous and malignant cells, such as inactivation of the mismatch repair system through DNA methylation of repair genes (Wang *et al.*, 2004), abnormal centrosome number regulation and microsatellite instability (Eshleman *et al.*, 1998), thereby promising early cancer detection techniques (Grady and Carethers, 2008).

3. Stages and Treatment

Colorectal cancer treatment is driven by the tumor stage at the time of diagnosis. Stage I is defined by the appearance of small tumorigenic nodules progressing to Stage II when neighboring tissues are attained. Stage III is characterized by the tumor mass reaching lymph nodes whereas Stage IV is metastasis to distant organs, mainly to liver and lung (Curtin, 2013). Surgery and/or radiation and chemotherapy are conventional in treating colorectal cancer (Figure 3). 5-fluorouracil, irinotecan and oxaliplatin are commonly used chemotherapy drugs which succeeded in increasing colorectal patients' life expectancy (Rodriguez, 2007). Nevertheless, radiotherapy and standard chemotherapy have shown side effects ranging from mild to severe ones, most importantly cardiotoxicity (de Brujine *et al.*,

1999) and taxanes-induced neuropathy (Miltenburg *et al.*, 2014) which have led researchers to explore the efficacy of novel ‘safer’ anticancer agents.

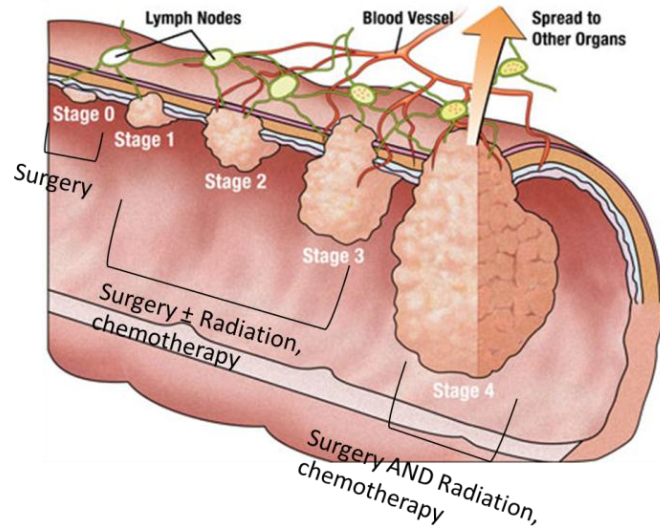


Figure 3. Colorectal cancer stages and treatment. Stage 0-2 are characterized by progressive growth of small tumorigenic nodules at the lining of the colon. Stage 3 tumors attain neighboring lymph nodes. Stage 4 is the metastatic tumor spreading to distant organs. Treatment is applied based on the stage advancement [Modified and adapted from Hollister Inc 2014].

4. Genetic Alterations: p53 Tumor Suppressor Gene

Half of cancer types are associated with mutations or inactivation of the tumor suppressor gene p53. In the context of colorectal cancer, studies have identified a role of p53 in cancer progression and prognosis. Adenomas rarely show inactivation of p53 whereas later in carcinogenesis, carcinoma majorly have p53 mutations followed by complete loss of both alleles (Sarasqueta *et al.*, 2013). p53 is essential in preventing malignant transformation and progression. Almost all cancers show loss of p53 function either by p53 mutations or by

deregulation of upstream mediators that activate p53 (Gurpinar, 2015). Evidence showed that p53 is not a typical tumor suppressor gene. Usually, homozygously inactivated tumor suppressor genes disrupt normal development and are consequently considered as negative regulators of cell proliferation in several cell types (Weinberg, 2013). On the other hand, when both p53 alleles are deleted cell development remained intact but animal models had a short life span. Effects of the homozygous inactivation of tumor suppressor genes are also seen in heterozygous mutation of p53. Consequently, p53 function does not obey Knudson's hypothesis in which inactivation of a tumor suppressor gene in neoplastic cells is restricted to loss of both normal alleles. Heterozygous p53 mutation is explained by the domination of the mutant allele on the normal functioning of the intact gene copy, called *dominant-negative* alleles (Brachmann *et al.*, 1996). Another concept explaining the originality of p53 function is its homotetrameric structure, meaning the assembly of four identical subunits. The tetramerization α -helix domain is not mutated in mutant p53 proteins, which allows diversity of p53 homotetramers formation between wild type and mutant p53 proteins (Figure 4). The presence of a single mutant allele may influence p53 tetramer cell function (DiGiammarino *et al.*, 2002).

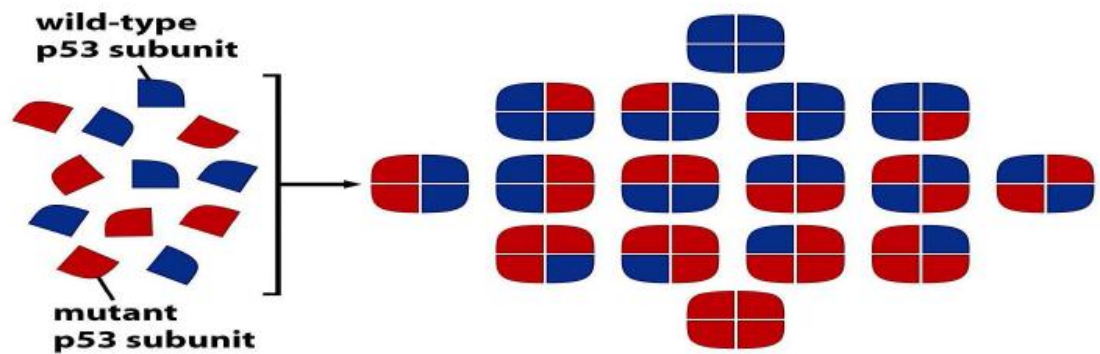


Figure 4. p53 functions as a homotetrameric transcription factor. Different proportions of wild type p53 (blue) and mutant p53 (red) may interact to give mixed tetramers where one mutant monomer may influence the entire function of the protein. [Adopted from the Biology of Cancer © Garland Science 2013].

p53 does not regulate proliferation signals but rather is the watchman of the genome by avoiding the occurrence of cell abnormalities capable of tumor initiation (Weinberg, 2013). It acts by arresting damaged or defective cells in the cell cycle or by eliminating them through apoptosis or senescence (Goh *et al.*, 2011; Ashcroft *et al.*, 2000; Weinberg, 2013) (Figure 5).

In the context of colorectal cancer, p53 is constantly produced and cellular steady levels of the protein are maintained by proteasome degradation. In a normal cell, Mdm2 protein recognizes ubiquitinated p53 and mediates its degradation. p53 levels are controlled by a p53-Mdm2 negative feedback loop: an increase in p53 levels leads to increased transcription of target genes. Mdm2 is one of the highly transcribed target genes. Thus, Mdm2 protein binds to excessive p53 monomers and induces their ubiquitination, export to the cytoplasm and proteasomal degradation (Weinberg, 2013; Lavin *et al.*, 2006). Mdm2

antagonist p19ARF inhibit mdm2-induced p53 degradation and stabilizes p53 levels regardless of Mdm2 binding to p53 (Gallagher, 2005).

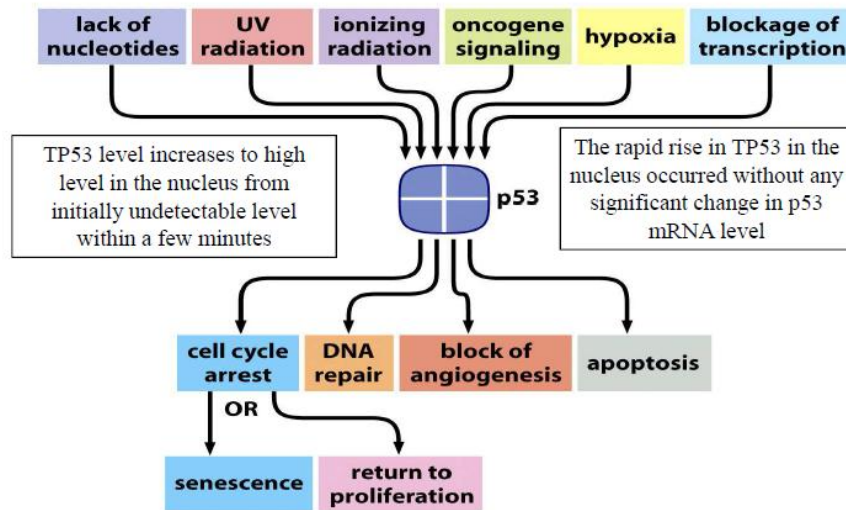


Figure 5. p53 activating signals and downstream effects. [Adopted from The Biology of Cancer © Garland Science 2013].

B. Cancer Stem Cells

1. Concept

Aggressiveness, invasiveness, metastasis and uncontrolled proliferation of tumor cells are associated with accumulation of genetic abnormalities. In different tumors, cells show heterogeneous potentials in terms of their function, proliferative and differentiation states. Two cancer models have been established to account for tumor heterogeneity: the stochastic and hierarchical models (O'Brien *et al.*, 2009). The stochastic model suggests

that all tumor-forming cells are biologically homogeneous, thus equally contributing to regeneration and maintenance of tumor growth. On the contrary, the hierarchical model, also known as the cancer stem cell model, proposes that only a small subpopulation of cells within a tumor have the capacity to regenerate it. The fraction of cells that are capable of tumor initiation are referred to as cancer stem cells (CSCs) and defined by their self-renewal, pluripotency and tumor expansion potentials (Jordan *et al.*, 2006). It is true that CSCs share similarities with normal stem cells, but they do not necessarily originate from them. However, stem cells have a greater probability of accumulating genetic mutations compared to differentiated cells, mainly due to a longer life span. Since a normal differentiating cell requires six genetic mutations to dedifferentiate and acquire stemness properties, it is most likely that normal stem cells give rise to cancer stem cells after one or two mutations (Figure 6), such as growth factor self-sufficiency and insensitivity to inhibitory growth signals (Li & Neaves, 2006). Not all tumors arise from self-renewing CSCs (Al-Hajj & Clarke, 2004), they can also come from progenitor cells (Jamieson *et al.*, 2004). Mutated progenitor cells have therefore acquired self-renewal capacity leading to CSC production. CSCs were first identified in acute myeloid leukemia (AML) by Dick *et al.* in the 1990s, where only a fraction of primary human AML cells was able to initiate leukemia in immunodeficient mice and could be serially transplanted (Bonnet & Dick, 1997). One decade after the identification of leukemic stem cells in hematological malignancies, studies started to show the presence of CSCs within solid tumors, ruling out application of the stochastic model in favor of the hierarchical model (O'Brien *et al.*, 2009). Cancers of the brain (Singh *et al.*, 2003), breast (Al-Hajj *et al.*, 2003), colon (O'Brien *et al.*, 2007), ovary (Zhang *et al.*, 2008), pancreas (Li *et al.*, 2007), prostate (Maitland & Collins,

2008), melanoma (Schatton *et al.*, 2008; Schmidt *et al.*, 2011) and multiple myelomas (Matsui *et al.*, 2008) have shown to follow the CSC model. Nevertheless, Quintana *et al.* have highlighted controversies regarding hierarchical organization of melanoma cells where transplantation of all melanoma cells, melanoma ABCR5⁺ stem cells and ABCR5⁻ cancer cells, can both initiate tumors in NSG mice and show unlimited proliferation during serial passages (Quintana *et al.*, 2010). Understanding stem cell biology and its relation with oncogenesis hold insight for defeating and understanding certain types of cancer, therefore ultimately seeking efficient cancer cures.

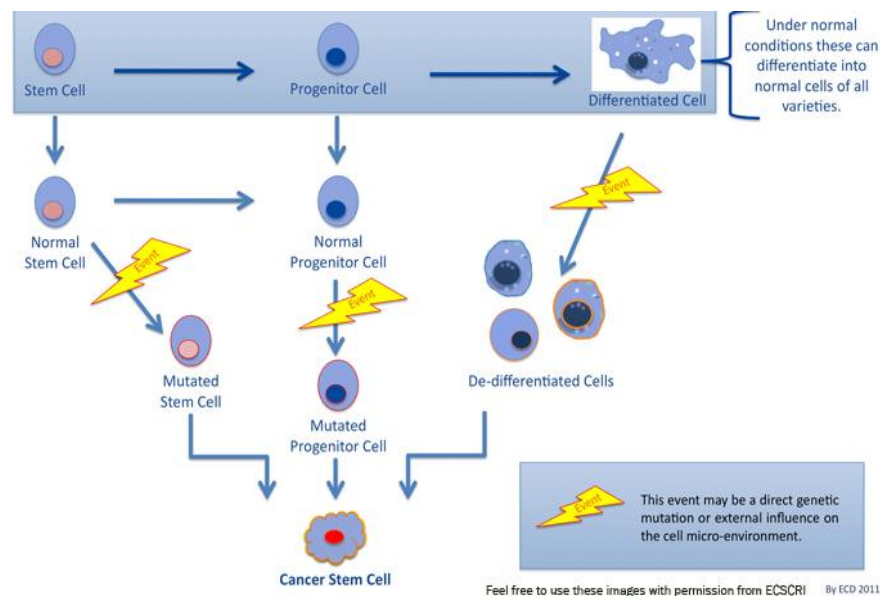


Figure 6. Origin of cancer stem cells. A normal stem cell divides to a new stem cell and a progenitor cell. The normal progenitor cell then matures into differentiated cell required by the body. Direct genetic mutations or the effect from external factors lead to cell mutations or de-differentiation such as losing their specialized function at any stage. These affected cells could then produce a cancer stem cell. [Adopted from The European Cancer Stem Cell research Institute].

2. Normal Stem Cells vs. Cancer Stem Cells

Cancer stem cells are often compared with normal stem cells. Both types of stem cells share their undifferentiated quiescent state and their self-renewal and proliferation capacity.

Normal stem cells produce functional differentiated progeny and cancer stem cells have the capacity to initiate a heterogeneous population of a tumor (Visvader and Lindeman, 2012).

Furthermore, differences in signaling pathways, degree of dependence on the stem cell niche and differential expression of stem cell markers are detailed below.

a. Cancer Stem Cell Signaling and Markers

Evidence suggests that it is the fine tuning between pathways involved in self-renewal that switch a normal stem cell into a malignant stem cell (Reya *et al.*, 2001). Three major signaling pathways contribute to stem cell development and oncogenesis: the Notch, Sonic hedgehog (Shh) and Wnt signaling (Taipale *et al.*, 2001); Bhardwaj *et al.*, 2001; Reya *et al.*, 2001), with Wnt being the most relevant for colon cancer development. *In vivo* studies on transgenic mice suggest that activated Wnt signaling in epidermal stem cells contribute to epithelial tumors (Gat *et al.*, 1998), while mice lacking the transcription factor TCF-4 of the Wnt pathway exhibit decreased numbers of undifferentiated progenitors in the intestinal crypts, suggesting that regulation of this pathway is also required for epithelial stem cell maintenance (Korinek *et al.*, 1998). The three key Wnt signaling molecules which have been found to be modulated by drugs (including thymoquinone) are β -catenin, GSK-3 β , and c-myc. Epithelial cell adhesion marker EpCAM and leucin-rich-repeat-containing G-protein coupled receptor 5 Lgr5 are putative colorectal CSC markers also involved in Wnt signaling (Langan *et al.*, 2013; Barker *et al.*, 2007). After extensive CSC research in the

hematopoietic system, focus has been translated to investigate the presence of CSC subpopulations in solid tumors. Unique sets of cancer stem cell markers were specifically identified for major solid tumors arising from cancer stem cells (Table 2). However, CSC populations among a specific cancer type are not universally characterized by their CSC markers but need to be further supported by functional assays.

Tumor model	Normal stem cell markers	Cancer stem cell markers
Breast	CD44, CD29, CD49f, EpCAM, CD24 (Petersen & Polyak, 2010)	CD44; CD133; ALDH; c-kit; ESA; ABCG2 (Bao et al., 2013)
Prostate	CD49f, Trop2, CD166, Sca-1, CD44, CD133, CD117 (Kwon & Xin, 2014)	CD44; CD133; stem cell antigen 1 (Sca-1); collagen receptor $\alpha 2\beta 1^{hi}$; CK5/14; CK8/18; CD49f; ABCG2 (Bao et al., 2013)
Colon	Msi-1; CD29; Lgr5; DCAMKL-1 (Lindeman and Visvader, 2012)	CD133; CD44; EpCAM; CD24; ALDH1; CD16; Lgr5 (Todaro et al., 2010)
Brain	GFAP, Sox2, Nestin, Pax6, Tbr2 (Bergstrom and Forsberg-Nilsson, 2012)	CD133, ALDH1A3 (Mao et al., 2013; O'Brien et al., 2009)
Pancreas	CD133; CD44; EpCAM; CD24 (Bao et al., 2013)	CD133; CXCR4; CD44; EpCAM; CD24; ESA; enhancer of zeste homolog 2 (EZH2) (Bao et al., 2013)

Table 2. Normal and cancer stem cell markers in different solid tumors.

b. Metastasis Determinants in Stem Cell Niche

The microenvironment where stem cells reside, creates the balance between stem cell self-renewal and inhibition of tumorigenesis (Li & Neaves, 2006). The stem cell niche is a group of cells which favor an anchoring site for stem cells along with adhesion molecules between stem cells and neighboring cells, and the extracellular matrix. Extrinsic regulatory signal molecules produced within the niche determine cell fate. Maintaining a balance

between signals of proliferation and differentiation prevents tumorigenesis and ensures homeostasis in the self-renewing stem cell pool during tissue regeneration. The molecules known to play a role in homing and mobilizing cells are mainly integrins, cadherin, β -catenin and matrix metalloproteinases (MMPs) (Li & Neaves, 2006). β -catenin/ α -catenin form a heterodimer that binds to cadherin while the β -catenin monomer binds to transcriptional mediators TCFs in the nucleus to activate cell cycle target genes. Consequently, β -catenin is a key molecule in determining stem cell fate (Song *et al.*, 2002; Lowry *et al.*, 2005).

C. Colorectal Cancer Stem Cells

1. Overview

Colon cancer has shown to follow the stem cell model (Todaro *et al.*, 2010) in which a small subpopulation of CSCs has the ability to initiate the heterogenous population of the tumor. To date, the location and origin of colorectal cancer cancer stem cells (CRCSCs) are not fully clear. The colon wall is characterized by four layers: the inner most layer is the mucosa followed by the submucosa, muscularis externa and the serosa. The external mucosal surface is lined by an absorptive and secretory columnar epithelium forming invaginations embedded in the connective tissue. The aforementioned test-tube structures constitute the functional unit of the colon called the crypts of Lieberkühn (Todaro *et al.*, 2010). Each crypt is formed of columnar cells or colonocytes, mucin-secreting cells and the endocrine cells. In normal conditions, regeneration of these cells arises from a population of colorectal adult stem cells and occurs every 2-7 days (Leedham, 2014). In the colon,

stem cells were located in the midcrypt of the ascending colon and at crypt base of the descending colon (Clevers, 2013) (Figure 7). Normal stem cells undergo symmetrical cell division that result in another stem cell and a progenitor cell restricted to a cell lineage. After genetic and epigenetic aberrations, colorectal cancer stem cells divide both symmetrically and asymmetrically giving rise to a heterogeneous cell pool containing tumorigenic and metastatic cancer stem cells (Todaro *et al.*, 2010).

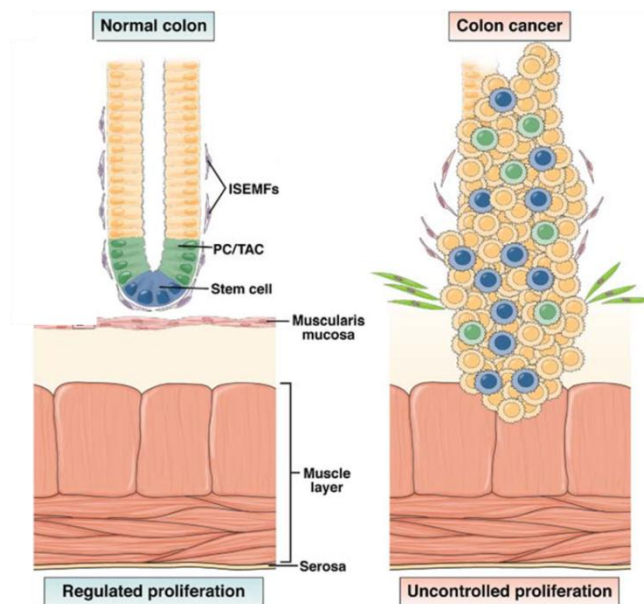


Figure 7. Location of normal or cancer colorectal stem cells within a crypt.

(Left) Stem cells commonly located at the crypt base are surrounded by intestinal subepithelial myofibroblast (ISEMFs). Upon paracrine secretion of growth factors and cytokines, ISEMFs regulate stem cell function along with Wnt signaling pathway. (Right) Deregulation of stem cell function and aberrant activation of signaling pathways develops malignancy within the digestive tract. PC: Progenitor cell; TAC: Transit-amplifying cell. [Adopted and modified from Todaro *et al.*, 2010].

2. Enrichment of Colorectal Cancer Stem Cells

Three-dimensional (3D) culture systems provide a mean to evaluate the self-renewal capacity and the differentiation potential of CSCs (Bao *et al.*, 2012). Sphere formation assays, also called tumorspheres, grow spheres in 3D in two different ways: in a suspension or on a reconstituted basement membrane (BD Matrigel matrix) (Kosovsky, 2012). Sphere formation assays follow a similar concept of colony formation with the exception of the possibility to propagate growing spheres to assess self-renewal (Figure 8). Floating sphere formation assays plate cells in serum-free medium whereas basement membrane sphere formation mixes single cell suspension in serum-free medium with matrigel in a 1:1 ratio. The media and treatment conditions are replenished every 2-3 days until images, counts and size of spheres are evaluated at day 12-15 (Abou-Kheir *et al.*, 2010). BD Matrigel™ matrix is a semi-solid basement membrane majorly composed of laminin, collagen IV, heparin sulfate proteoglycans, entactin, nidogen and growth factors (Kosovsky, 2012). 3D sphere culture in matrigel mimics the interaction between CSCs and the ECM, major factor in the CSC microenvironment *in vivo* (Kosovsky, 2012). The presence of a matrix *in vitro* relatively demonstrates the dependence of CSCs on their surrounding (Li & Neaves, 2006). Nevertheless, there is existing debate about whether to use in suspension or 3D basement membrane sphere formation assays. The first sphere formation assay was originally performed on floating neural stem cells capable of formation of floating neurospheres (Reynolds & Weiss, 1992). In suspension, spheres may encounter the problem of merging of spheres and aggregation of cells, consequently leading to underestimation of the number of stem cells and over estimation of stem cell activity. Using a semi-solid matrix embeds

cells and spheres within the matrigel, preventing in suspension aggregation (Reynolds & Rietze, 2005).

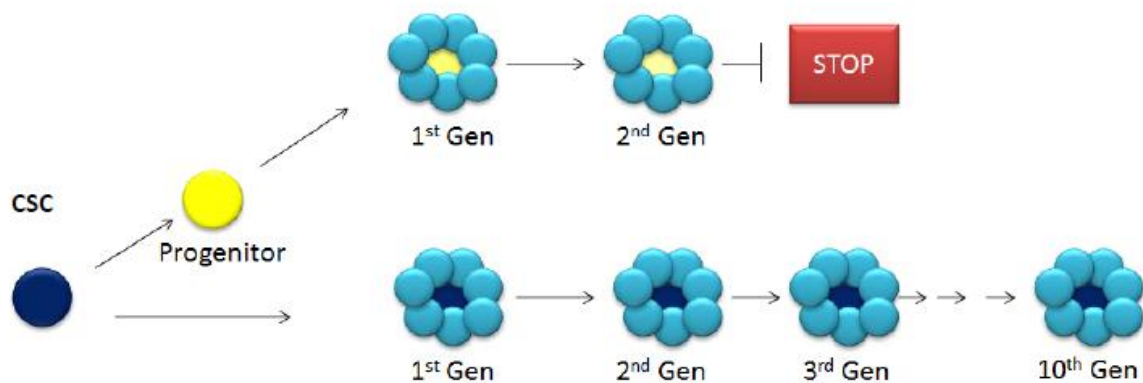


Figure 8. Propagation of progenitor/stem-like cell population. Spheres from progenitor cells are able to form spheres for one or two generations, while CSC are able to form spheres indefinitely. [Adopted from Rabih Bassam El-Merehbi MS Thesis, 2013].

To date, sphere formation and propagation assay is used as a functional assay to enrich for progenitor/stem-like cells within cell lines or primary tumor tissues. Upon propagation of spheres, progenitors are capable of self-renewal up to 2-3 generations due to their limited self-renewal potential. However, the indefinite self-renewal property of stem cells is assessed by propagation of spheres for up to 10 generations.

3. Targeting Cancer Stem Cells

Combination therapies have been recently adopted for metastatic cancers leading to improved overall survival of cancer patients (Todaro *et al.*, 2010). Despite clinical progress,

tumor metastasis remains the leading cause of death in patients. Cancer stem cells, which are spared by many chemotherapeutics, are believed to be the reason behind cancer relapse. Different strategies aim to sensitize CSCs including the use of differentiation-inducing agents (Gupta *et al.*, 2009; Todaro *et al.*, 2010), inhibitors of survival pathways (Van *et al.*, 2005; Kismet *et al.*, 2009; Mueller *et al.*, 2009; Vazquez-Martin *et al.*, 2011; Bao *et al.*, 2012), immune therapy (Jin *et al.*, 2006; Todaro *et al.*, 2007; Chan *et al.*, 2009; Todaro *et al.*, 2009; Jin *et al.*, 2009; Majeti *et al.*, 2009; Bao *et al.*, 2013), conventional chemotherapy (Todaro *et al.*, 2010) as well as by triple-target therapeutic strategies (Willett *et al.*, 2004; Yi *et al.*, 2013; Liao *et al.*, 2014; Ye *et al.*, 2014) (Figure 9). So far, there have been no effective drugs targeting CSCs and CSC metastasis while sparing normal stem cells. One way to target CSCs therapeutically is to identify and purify CSC subpopulations from the heterogeneous cancer population by sphere formation assay and immunostaining for specific markers on cell surfaces and on spheres (Kong *et al.*, 2009; Abou-Kheir *et al.*, 2010).

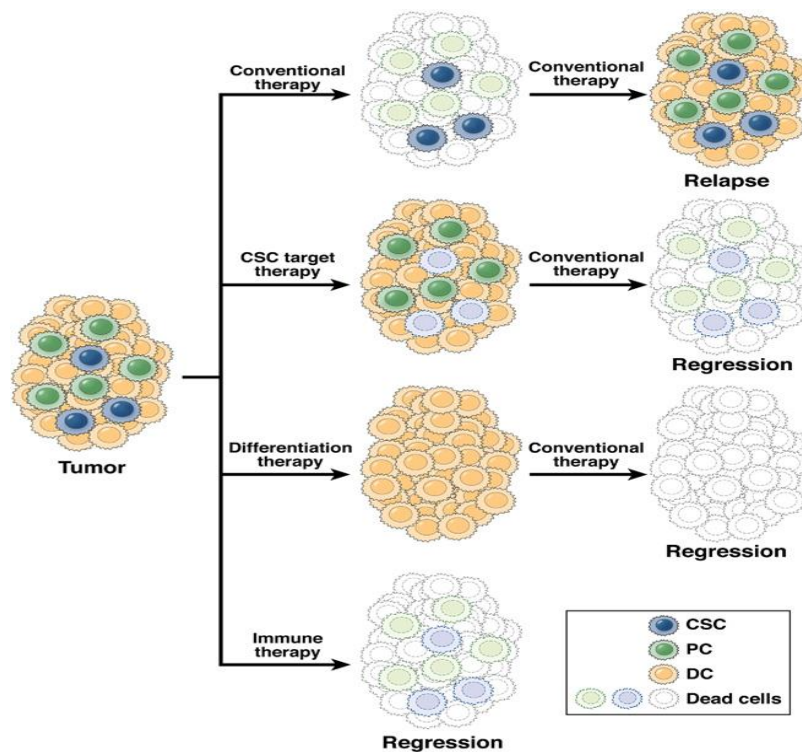


Figure 9. Strategies sensitizing CSCs and inducing complete tumor regression.

Different therapeutic strategies sensitize both CSCs and highly proliferating cells. Agents inducing differentiation of CSCs, immune cells, chemotherapy and inhibitors of survival pathways are strategically combined to eradicate CSCs and achieve complete tumor regression [Adopted from Todaro *et al.*, 2010].

D. Thymoquinone

Thymoquinone, 2-isopropyl-5-methylbenzo-1,4-quinone (TQ) is isolated from the essential oil of the *Nigella sativa* L. black seed (Aboul-Enein *et al.*, 1995). Also, it has been extracted from other plants, as from *Eupatorium ayapana* (Trang *et al.*, 1993), the leaves of several *Origanum* species and the oils of different *Satureja* species (Schneider-Stock *et al.*, 2014). TQ is chemically synthesized by oxidation of thymol with hydrogen peroxide (Schneider-Stock *et al.*, 2014).

1. Chemical Characteristics

Thymoquinone has a basic quinone structure consisting of a para substituted dione conjugated to a benzene ring to which a methyl and an isopropyl side chain groups are added in positions 2 and 5, respectively (Gad *et al.*, 1963; Schneider-Stock *et al.*, 2014) (Figure 10).

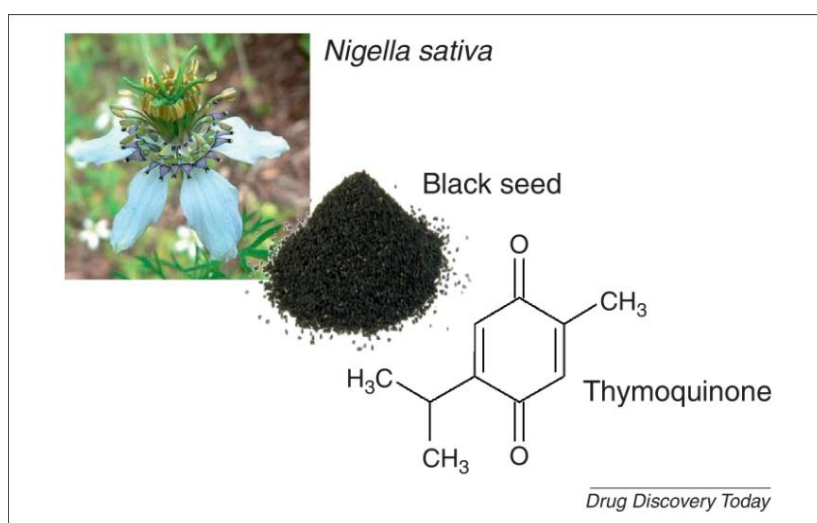


Figure 10. TQ chemical structure. [Adopted from Schneider-Stock *et al.*, 2014].

TQ quinone structure is involved in reactions with amino or thio groups of amino acids (Cremer *et al.*, 1987) and in redox cycles leading to reactive oxygen species (ROS) generation (Gali-Muhtasib *et al.*, 2008; Schneider-Stock *et al.*, 2014). In normal tissues, TQ acts as a strong antioxidant by inhibition of superoxide radicals or an increase in the antioxidant enzymes activity, namely superoxide dismutase (SOD), catalase, glutathione

(GSH) and quinone reductases (Banarjee *et al.*, 2010; Woo *et al.*, 2012; Mansour, 2000; Badary *et al.*, 2000). In cancer, TQ may act as an anti-oxidant or as pro-oxidant by decreasing GSH levels or inducing ROS generation (Dergarabetian *et al.*, 2013; Rooney *et al.*, 2005).

2. TQ and Cancer

In the development and discovery of new potential anticancer agents, growing interest is heading towards 'safe' and widely available molecules, prominently from plant extracts. Excellent reviews reported chemoprotective effects of TQ in the colon, kidneys, liver and brain (Darakhshan *et al.*, 2015) and positive effects against diabetes (AbuKhader, 2012), circulatory and reproductive disorders (Darakhshan *et al.*, 2015). TQ has shown promising effects against cancer (Schneider-Stock *et al.*, 2014). For more than 10 years, studies investigated the potential anticancer effect of TQ and its efficacy and selectivity against cancer cells and lack of toxicity to normal tissues (Schneider-Stock *et al.*, 2014). Interesting evidence has reported the anti-cancer effects of TQ to target nine out of ten traits of cancer first cited by Hanahan and Weinberg in 2000 (Hanahan and Weinberg, 2011). It has also been proven to be effective against the classical hallmark of apoptosis by several ways such as chromatin condensation, flipping of phosphatidyl-serine on the plasma membrane and DNA fragmentation (Banerjee *et al.*, 2010). Furthermore, TQ induced apoptosis in breast MCF7 cell line by activation of PPAR- γ , activation of caspases and down-regulation of PPAR- γ related genes, namely Bcl-2, Bcl-xL and survivin (Abu Khader, 2013). In human colorectal HCT116 p53^{+/+} and p53^{-/-} cells, TQ treatment caused apoptosis through

inactivation of the stress response pathway sensor CHEK1 (Gali-Muhtasib *et al.*, 2008b). In the context of 2D monolayer culture model, HCT116 with wild type (WT) p53 status showed higher sensitivity to TQ translated by higher apoptosis when compared to TQ-treated HCT116 p53^{-/-}-cells. Initially, TQ treatment in HCT116 cells increased apoptosis levels due to elevated p53 protein levels silencing the survival gene CHEK1 transcription. In the absence of p53, HCT116 p53^{-/-} cells demonstrate high levels of CHEK1, making them more resistant to TQ treatment (Gali-Muhtasib *et al.*, 2008b).

Thymoquinone's ability to inhibit colon cancer growth and invasion, and induce cell cycle arrest and apoptosis in colon cancer cell culture and animal models has been documented by us and others (Gali-Muhtasib *et al.*, 2004; Gali-Muhtasib *et al.*, 2008a; Gali-Muhtasib *et al.*, 2008b; Wirries *et al.*, 2010; Alenzi *et al.*, 2010; Jrah-Harzallaha *et al.*, 2013; Schneider-Stock *et al.*, 2014). Interestingly, TQ has been shown to modulate Wnt signaling through GSK-3 β activation, β -catenin translocation and reduction of nuclear c-myc (Lang *et al.*, 2013), knowing that Wnt signaling pathway is aberrantly active in stem cells (Visvader and Lindeman, 2012). Up to this date, TQ effect has not yet been tested in a cancer stem cell context. The only published reports showed the neuroprotective effect of TQ on synapse damage in human induced pluripotent stem cells-induced neurons (Alhebshi *et al.*, 2014).

E. Aim of Study

No efficient drug treatment was launched clinically as to target CSCs, but putative and promising studies developed immune therapies such as monoclonal antibodies against specific CSC markers, differentiation-induced drugs that stimulate differentiation pathways and inhibit self-renewal signaling (Wnt, Hedgehog and Notch pathways) as well as affecting hypoxia, angiogenesis and other microenvironmental factors. However, cancer and CSC eradication use strategic therapeutics which combines several targeted factors within the heterogeneous population of a tumor. Conventional chemotherapy shrinks the tumor by killing highly proliferating cells whereas CSC-targeted immune and differentiation-inducing drugs target the quiescent population of CSCs for the ultimate goal of tumor regression and avoiding cancer recurrence. There is growing interest in the ability of plant-derived chemicals to regulate cell death mechanisms, inhibit cell growth and interfere with several specific stages of tumorigenesis. Since healthcare costs are a key problem for cancer treatment, it would be cost-effective to focus on developing inexpensive cancer treatments, namely compounds derived from nature.

Considering that colon cancer is the second leading cause of death in patients, it is essential to adopt new approaches for the treatment of this disease. Most colon cancer patients die of metastasis due to the resistance of their disease to standard therapies. Recent literature attributes cancer deaths to a small population of cancer stem cells that are capable of self-renewal and are resistant to therapy (Todaro *et al.*, 2010). My work aims to investigate for the first time the ability of the natural compound TQ to target colon cancer cells with stem-like properties. To date, no published data reported TQ effect on CSCs nor on colorectal

CSCs. By investigating the *in vitro* self-renewal capacity of an enriched progenitor/stem-like cell population in human colon cancer HCT116 cell lines and their response to TQ, this project will also offer an understanding of colon cancer stem cell biology. It also provides insights on resistance mechanisms, thus ultimately finding ways to effectively combat the disease, either by TQ alone or in combination treatment. *In vitro* identification of colorectal cancer cells with stem cell properties is through the assessment of their ability to form spheroids under low-serum conditions. In order to trigger *in vitro* expansion of the putative colorectal CSCs, human colorectal cell lines were grown in 3D sphere-forming assays to study them as reported for mammospheres, neurospheres and colonospheres (Kanwar *et al.*, 2010).

CHAPTER II

MATERIALS & METHODS

A. Cell Culture Conditions

Experimentally, human colorectal HCT116 p53^{+/+} and p53^{-/-} cell lines are cultured in their respective media either on matrigel or in 2D monolayer conditions. HCT116 p53^{+/+} cells are cultured in RPMI 1640 (Sigma-Aldrich, UK) with 20mM HEPES and L-Glutamine, while HCT116 p53^{-/-} are grown in Dulbecco's Modified Eagle medium (DMEM; 4.5 g/L D-glucose) supplemented with sodium pyruvate (Sigma-Aldrich). All cells are maintained in an incubator at 37 °C in a humidified atmosphere of 5% CO₂ and 95 % air. Media of all cell lines are supplemented with antibiotics [1% Penicillin-Streptomycin (100 U/ml)] and 10% heat-inactivated FBS (Sigma-Aldrich, Germany).

B. Drug Preparation and Treatment

Directly before use, fresh stock of the purified synthetic compound thymoquinone (Sigma-Aldrich: CAS: 490-91-5; 99.5% purity) 16.4mg/mL in 1mL methanol is prepared.

Intermediate concentrations and combination of TQ are prepared by serial dilutions from stock every 2 days during sphere formation assay. Cells were cultured in different concentrations. We assessed the sphere formation unit variation in response to different treatment conditions. Spheres are exposed to the treatment for 13 days.

C. MTT Cell Viability Assay

HCT116 p53^{+/+} and p53^{-/-} cells were plated in 100 μ L complete medium in 96-well culture plates at a density of 18 000 cells/well. Cells were incubated overnight then treated with various TQ concentrations for 24, 48 and 72 hours. Each condition was reproduced in triplicate within one experiment. Cytotoxic effect of TQ was assessed by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] that measures the ability of metabolically active cells to convert tetrazolium salt into a violet formazan crystals. At each time point, MTT reagent was added to each well and incubated at 37° for 4 hours. 100 μ L isopropanol was used as a solubilizing solution to dissolve violet crystals. Consequently, MTT optical density (OD) is measured at a wavelength of 595 nm using ELISA reader (Multiskan Ex). The percentage cell viability is expressed as the percentage of cell growth at treated conditions relative to untreated cells.

D. Sphere Formation Assay

HCT116 p53^{+/+} and p53^{-/-} cells were able to generate spheres in non-adherent cultures. Cells were passaged through different syringes for single cell suspension and then counted by trypan blue exclusion method. A density of 1000 cells/well is suspended in free medium, respectively for each cell line. Therefore, 50 μ L cold mix (25 μ L cold Growth Factor Reduced Matrigel™/ 25 μ L suspended cells) is prepared for each well. Note that each experimental condition was performed in duplicate. Mastermix of cells with matrigel were circularly plated at the rim of the well of a 24-well plate and allowed to solidify in the incubator at 37°C for 45 minutes. We gently added 1mL media with 5% FBS (with or

without treatment) at the center of the well. Media or treatments were replenished every 2 days. Sphere counts and imaging were performed at day 13 of sphere culture.

E. Propagation Assay

In order to enrich the stem-like population of cells, the media is aspirated from the well and the matrigel-containing spheres are digested by 500 μ L dispase solution (Invitrogen, Carlsbad, CA, 1 mg dissolved in 1 mL RPMI-1640 incomplete medium) for 1 hour at 37°C. Spheres were collected and incubated in 1mL Trypsin/EDTA at 37°C for 3minutes, passed through 20, 27 and 30 gauge syringes 4 times each. Single cells resulting from the dissociation of spheres were re-plated at the same density of 1000 cells/well in 24-well plate dishes. We assume the requirement of 5 generations of colonospheres to enrich the subpopulation of progenitor/stem-like colon cancer cells.

F. RNA Extraction and Quantitative Real Time PCR (qRT-PCR)

Total RNA was isolated from untreated and TQ-treated HCT116 p53^{+/+} and p53^{-/-} monolayer cells, according to manufacturers' instructions using the RNeasy Mini Kit (Qiagen). Colonospheres were dissociated by dispase solution for 1 hour before proceeding with RNA extraction as mentioned above. cDNA was generated from total RNA derived from HCT116 monolayer or sphere derived cells using the Super Script III First Strand Synthesis System for RT-PCR (Invitrogen). PCR was performed using platinum taq polymerase (Invitrogen). SYBR Green Mastermix or Taqman Gene Expression Master Mix

was used for quantitative RT-PCR on the BioRad RT PCR System. Gene expression levels were normalized to GAPDH. All reactions were performed in duplicate using primers (IDT) listed below:

GAPDH: Forward: 5' - CCACTTTGTCAAGCTCATTTTCCT-3'

Reverse: 5' -TCTCTTCCTCTTGTGCTCTTGCT-3'

CD44: Forward: 5' -TTTGCATTGCAGTCAACAGTC-3'

Reverse: 5' - GTTACACCCCAATCTTCATGTCCA-3'

EpCAM: Forward: 5' - CCATGTGCTGGTGTGTGAAC-3';

Reverse: 5' - ACGCGTTGTGATCTCCTTCT-3'

G. Immunofluorescence and Microscopic Imaging

Immunofluorescence is a technique used to detect the presence of cell surface and intracellular proteins by the use of specific antibodies. Spheres are grown until day 13 with or without treatment. Matrigel was digested by 500 μ L dispase solution (Invitrogen, Carlsbad, CA, 1 mg dissolved in 1 mL RPMI-1640 incomplete medium) for 45 minutes. Spheres were then collected and centrifuged to washout all Matrigel debris. After centrifugation, spheres were fixed by formalin for 20 minutes. After washing with PBS 3 times, cells were permeabilized with 0.5% Triton X-100 for 30 minutes, and blocked with sphere blocking buffer (0.1% BSA, 0.2% Triton X-100, 0.05% Tween-20, and 10% normal goat serum in PBS) for 2 hours at room temperature. Cells were washed and then incubated overnight at 4°C with conjugate Annexin V-FITC at a concentration 1:100 with blocking solution. Cells were then washed with PBS and finally washed and mounted using the 5-7

μ L anti-fade reagent Fluoro-gel II with DAPI. Fluorescent signals were captured using Zeiss LSM 710 confocal microscope and images were acquired and analyzed using the Zeiss LSM image software.

H. Statistical analysis

Statistical analysis was performed using Microsoft Excel 2013. Data presented are the average \pm SEM of at least two independent assays as noted in the figure legends. Student's t-test was performed between control and treated conditions and statistical significance was reported when the P-value was < 0.05 (*): $P < 0.05$; (**): $P < 0.01$.

CHAPTER III

RESULTS

A. Effect of TQ on Viability of Human Colorectal Cancer Cell Lines HCT116 p53^{+/+} and p53^{-/-} in 2D Culture

First, we investigated the *in vitro* anti-proliferative effect of different doses of TQ on HCT116 cell lines using the MTT assay. HCT116 p53^{+/+} and p53^{-/-} cells were treated with different concentrations of TQ (1-100 μ M) for 24, 48 and 72 hours. As shown in Figure 10, 72 hour exposure to 100 μ M TQ inhibited cell viability by 60% in both cell lines. Our previous work showed the IC₅₀ of TQ to be 40 μ M on HCT116 cell lines (Gali-Muhtasib *et al.*, 2004). In accordance with our previous results, at 72 hours, cell viability in response to 40 μ M of TQ was 65% in HCT116 p53^{+/+} and 45% in p53^{-/-} (Figure 11). HCT116 p53^{-/-} cell line was more sensitive to TQ than p53^{+/+} cells, while low concentrations of 1 μ M and 3 μ M of TQ do not show any effect on cell viability in traditional monolayer cell cultures. These results were comparable to TQ IC₅₀ on HCT116 cells to be 40 μ M as published earlier (Gali-Muhtaseb *et al.*, 2004) and thus, serve as a baseline for further comparison of TQ effect in 3D environment.

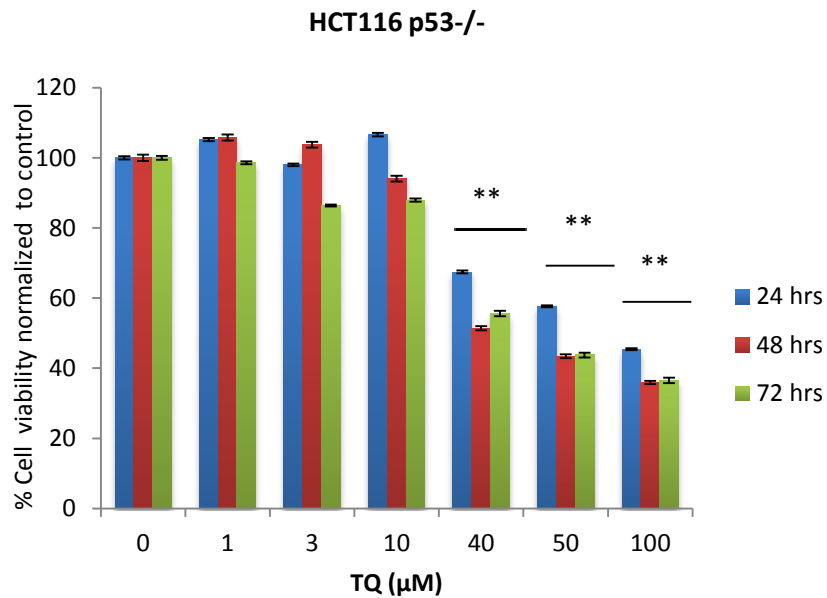
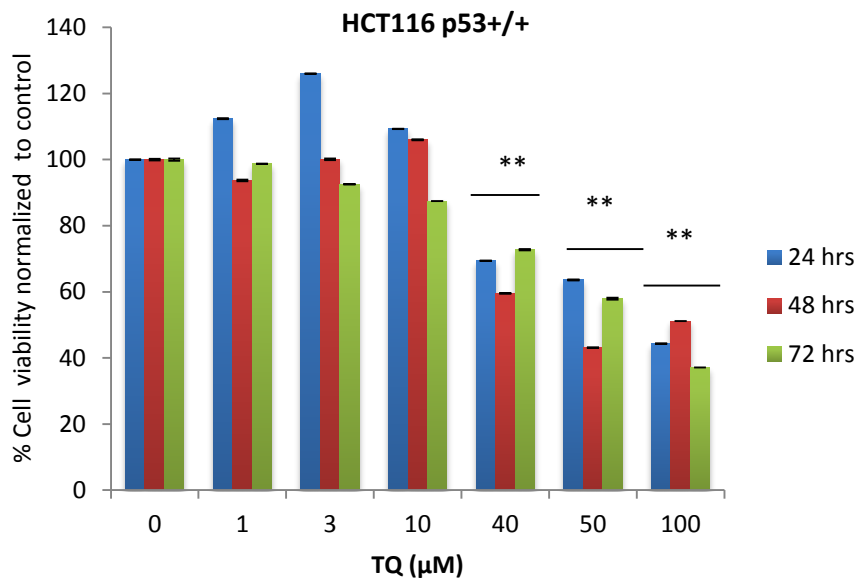


Figure 11. MTT viability assay on HCT116 cells in response to TQ treatment.

Bar graphs show MTT viability tests on HCT116 wild type and p53^{-/-} cells respectively in the presence or absence of TQ treatment at 24, 48 and 72 hours. Percentage of cell viability was normalized to control (untreated) cells. Cell viability was assayed in triplicates for each condition. Values represent the average of three independent experiments \pm SEM. Asterisks indicate a significant difference between untreated and treated cells using Student's t-test: (*) represents $p < 0.05$ and (**) represents $p < 0.01$.

B. Optimization of Three - Dimensional Sphere Culture Serum Conditions

Next, we attempted to optimize the serum conditions that would result in the optimal growth of colonospheres. Sphere formation is a functional assay to determine self-renewal property of cells at low cell density and low serum concentration. In conventional 2D monolayer cultures, HCT116 cell lines were grown in media supplemented with 10% fetal bovine serum (FBS). In 3D sphere culture, we tested different concentrations of FBS ranging from 0 to 10% and counted the number of sphere formation units (SFU) after 1 generation of growth in both HCT116 (p53^{+/+}) and (p53^{-/-}) cell lines. No spheres were formed in 0% FBS. HCT116 p53^{+/+} cells formed spheres in 1%, 2%, 5% and 10% FBS whereas HCT116 p53^{-/-} started forming spheres starting at 5% FBS concentration (Figure 12). In order to choose an adequate FBS concentration for both cell lines, we adopted 5% as the optimal FBS concentration to be used in all subsequent experiments for both p53^{-/-} and p53^{+/+} HCT116 isogenics. Unexpectedly, in 5% FBS conditions, HCT116 p53^{+/+} attained 15% SFU whereas HCT116 p53^{-/-} cells had 10% SFU only (Figure 12). Therefore, %SFU was higher in cells with intact p53, and, p53^{-/-} cells did not form spheres at 1% and 2% FBS. These results are somehow counter- intuitive since loss of the tumor suppressor gene p53 would ultimately result in increased proliferation reflected by a higher number of spheres as seen with other cell types (Gil-Perotin *et al.*, 2011).

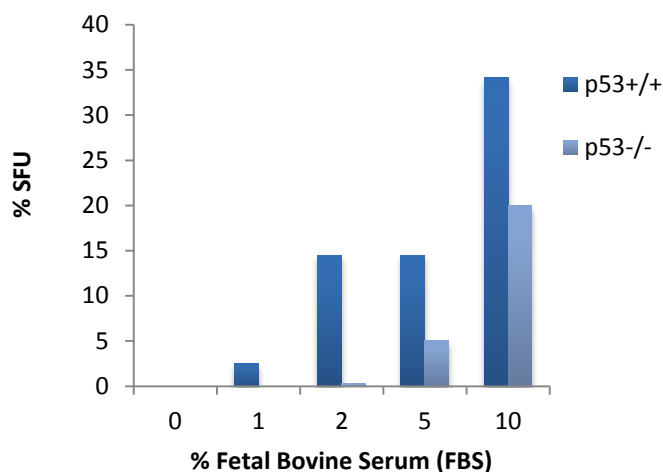


Figure12. Optimizing serum conditions in HCT116 sphere culture. The percentage of sphere forming units without FBS and with 1, 2, 5 and 10% FBS in a 13 day sphere culture of HCT116 p53+/+ and p53-/- cells. Cells were plated in Matrigel™ at a density of 2000 cells/well. Spheres were counted by phase-contrast microscopy. Sphere formation unit (SFU) is the ratio of counted spheres to the number of plated cells.

C. Comparison of TQ Concentration-Dependent Effect on Two-Dimensional vs. Three-Dimensional HCT116 Cultures

Next, we tested the effect of concentrations of TQ ranging from 1 to 20μM on sphere formation in the two HCT116 cell lines (Figure 13). Interestingly, at least 10-fold lower concentrations of TQ were required to inhibit viability of HCT116 in 3D culture in comparison to 2-dimensional (2D) culture conditions, raising hope that TQ could be used effectively to inhibit the self-renewal of colon cancer cells.

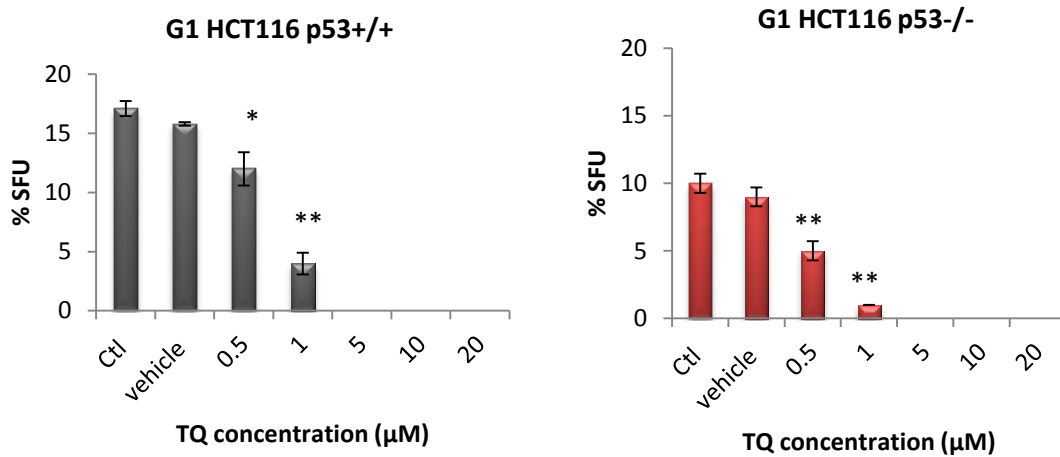


Figure 13. Sensitivity of HCT116 p53+/+ and p53-/- cells to TQ concentrations. Spheres grown in 5% FBS were counted after 13 days of exposure to TQ concentrations. Cells were plated in Matrigel at a density of 1000 cells/well. Methanol was the vehicle used to dissolve TQ and was found not to significantly affect sphere formation at concentrations used. SFU is the ratio of counted spheres to the number of plated cells. Data is the average count of first generation (G1) spheres from two independent experiments \pm SD. Asterisks indicate a significant difference between untreated cells and 0.5 μ M TQ or 1 μ M TQ treated cells using Student's t-test, (*) represents $p < 0.05$ and (**) represents $p < 0.01$.

Knowing that concentrations of TQ above 5 μ M result in death of spheres, we next examined the effects of lower concentrations of TQ ranging from 1 to 5 μ M on the percentage of sphere formation units in HCT116 p53+/+ and p53-/- cells (Figure 14). We also noted the higher ability of HCT116 p53+/+ cells in forming spheres when compared to HCT116 p53-/- cells. However, HCT116 p53+/+ and p53-/- cells demonstrated a similar dose-dependent decrease in sphere viability in response to low concentrations (1 μ M and 3 μ M) of TQ treatment (Figure 13; Figure 14).

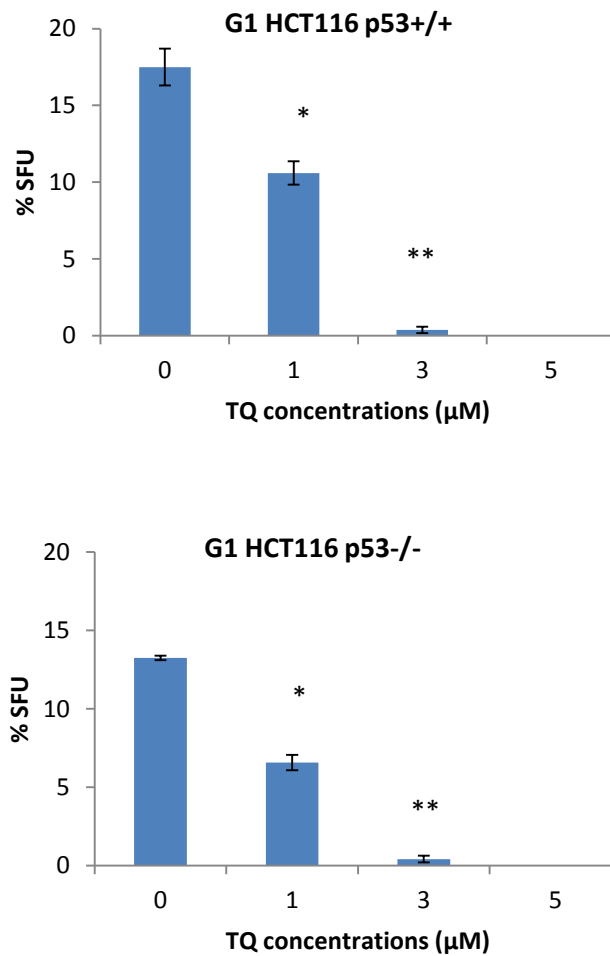


Figure 14. Effect of TQ on generation 1 HCT116 p53+/+ and p53-/- spheres.

Spheres grown in 5% FBS were counted after 13 days of exposure to TQ concentrations. HCT 116 p53+/+ and p53-/- cells were plated in Matrigel at a density of 1000cells/well. SFU is the ratio of counted spheres to the number of plated cells. Data is the average of three independent experiments \pm SEM. Asterisks indicate a significant difference between untreated cells and 1 μM TQ or 3 μM TQ treated cells using Student's t-test, (*) represents $p < 0.05$ and (**) represents $p < 0.01$.

D. TQ Effect in Targeting an Enriched Population of Human Colorectal Cancer Stem-like Cells

1. TQ Effect on HCT116 Sphere Counts

In order to determine the presence of colorectal CSC pool within HCT116 cell lines, we performed sphere formation assay in a matrigel semi-solid matrix to mimic the *in vivo* microenvironment. Self-renewal capacity of the progenitor/stem-like population was studied upon propagation of colonospheres from generation 1 (G1) to generation 5 (G5). In p53^{+/+} cells, control spheres that were consistently untreated upon propagation from G1 to G5 showed a high % SFU ranging from 17.5 % SFU at G1 to 11.1 % SFU at G5 (Figure 15). Similarly, in p53^{-/-} cells, untreated spheres formed 13.2 % SFU at G1 and 7.2 % SFU at G5. Therefore, a relatively high % SFU was maintained upon propagation assay, suggesting the presence of selection against short-term progenitors and in favor of long-term cancer stem-like pool with self-renewal property. Comparing sphere formation capacity between HCT116 p53^{+/+} and p53^{-/-} unexpectedly showed a higher sphere forming ability in the presence of p53 as obtained previously.

After G1, spheres that survived 1 μ M and 3 μ M TQ were divided in two halves at generation 2 (G2): the first half was untreated and the second was treated with the same TQ concentration (1 μ M or 3 μ M) that was applied in G1. The same protocol was performed during subsequent passages up to G5 (Figure 15). For the sake of simplicity, % SFU for both treated and untreated conditions are shown for G2 only (Figure 15). In HCT116 p53^{+/+} cells, spheres treated with 1 μ M TQ showed 10.6 % SFU at G1. At G2, these spheres showed 8% and 3% SFU when they were untreated and treated, respectively.

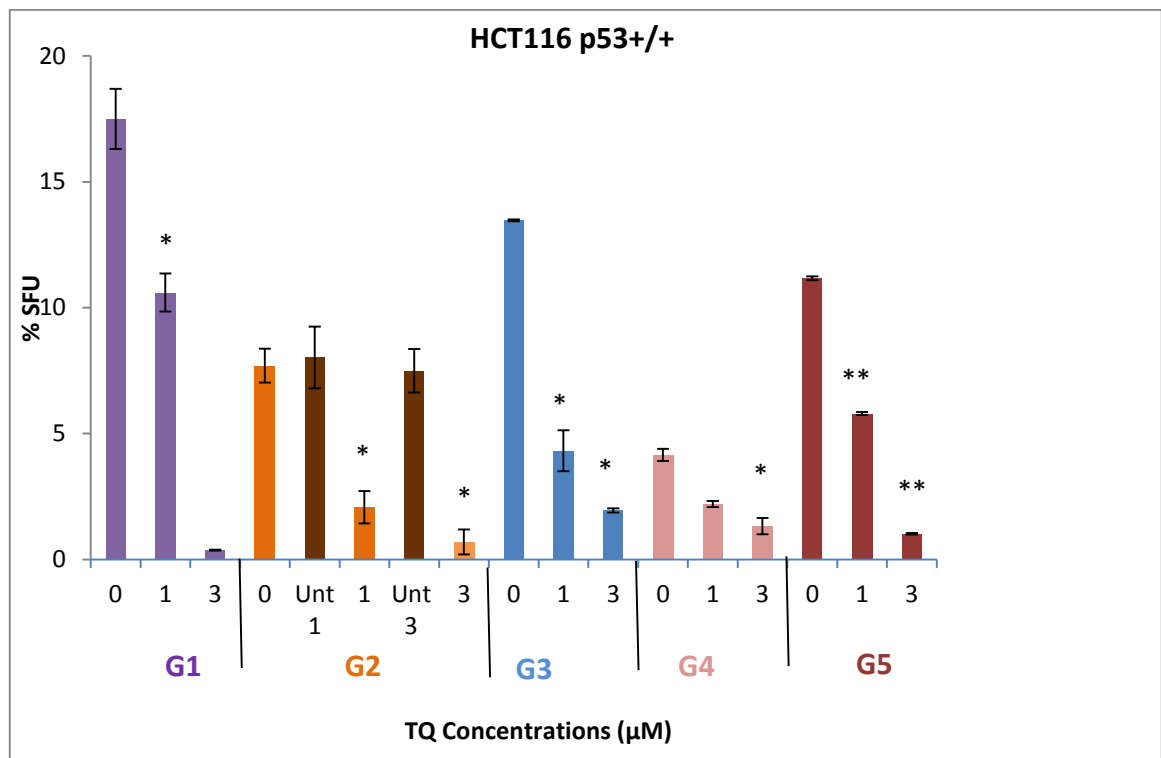
HCT116 p53^{-/-} cells revealed 6 % SFU at 1 μ M TQ treatment at G1 also showed around 7% SFU when treated again at G2 and 7.5% SFU when untreated at G2 (Figure 15). These results highlight the reversibility of TQ treatment on both HCT116 p53^{+/+} and p53^{-/-} cells.

We next compared %SFU after propagation of untreated spheres and those treated with 1 μ M and 3 μ M between G1 to G5 in order to assess TQ effect on self-renewal capacity of HCT116 cells. Treatments with 1 μ M and 3 μ M TQ resulted in a consistent effect of TQ over passages and a similar dose-dependent trend of decrease in sphere viability every generation in both cell lines (Figure 15). Hence, in 1 μ M TQ treatment of HCT116 p53^{+/+}, there was a consistent decrease in sphere viability from 10.6% SFU at G1 to 5.8% SFU at G5. Similarly, HCT116 p53^{-/-} spheres treated with 1 μ M TQ showed 6% SFU at G1 and 3% SFU at G5 (Figure 15). These findings highlight the negative effect of TQ on the self-renewal capacity of HCT116 cells irrespective of the p53 status. Of note, SFU levels followed similar rates of proliferation in both untreated versus treated spheres over 5 generations (Figure 15).

Next, we assessed the acute effect of TQ on the stem-cell like population that persisted after G5 and was not treated previously, thus characterized by its self-renewal property.

Untreated HCT116 p53^{+/+} spheres obtained at G5 formed 11.1 % SFU in comparison to 10.3% SFU after one-time exposure to 3 μ M TQ (Figure 16). However, in untreated HCT116 p53^{-/-} spheres, one-time exposure to 3 μ M TQ at G5, % SFU decreased from 7.3% to 2.2 % SFU (Figure 16). Altogether, these data indicate that TQ treatment negatively affects viability and/or self-renewal of the pure HCT116 p53^{-/-} stem cell-like

population obtained at G5 but did exhibit similar effect on the HCT116 p53+/+ population, indicating that the absence of p53 may increase CSC sensitivity to TQ.



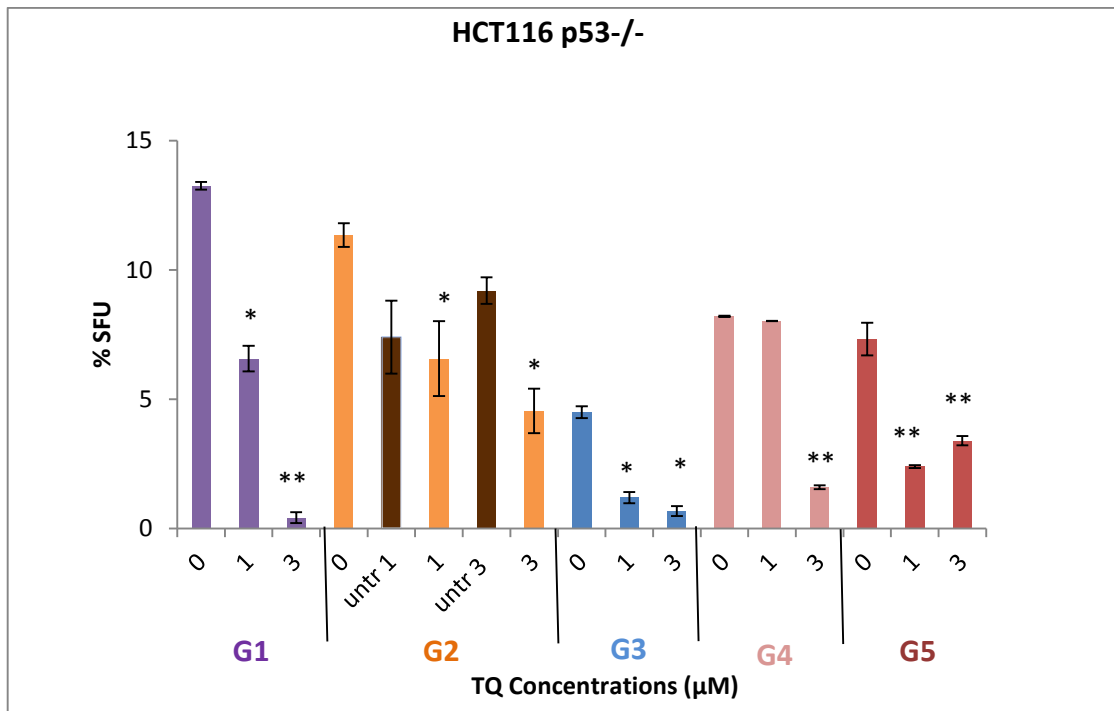


Figure 15. TQ decreases self-renewal in enriched HCT116 progenitor/stem-like cells.

Graphical representation of % SFU of sphere culture and propagation assay in generations 1 (G1), 2 (G2), 3 (G3), 4 (G4) and 5 (G5) of HCT116 p53^{+/+} cells and HCT116 p53^{-/-} cells. Cells were plated in matrigel at a density of 1000 cells/well. Fresh media and TQ treatment were replenished every 2 days. Sphere propagation was performed every 12 days. Each graph represents %SFU obtained in untreated versus treated cultures with 1µM and 3µM TQ over 5 generations. Untreated versus treated spheres are shown at G2 only in 1µM and 3µM conditions and omitted from other passages for the sake of simplicity. SFU represents the number of counted spheres over the number of plated cells. Data represent the average of two independent experiments ±SEM. Asterisks indicate a significant difference between untreated cells and 1 µM TQ or 3 µM TQ treated cells using Student's t-test, (*) represents p<0.05, (**) represents p<0.01.

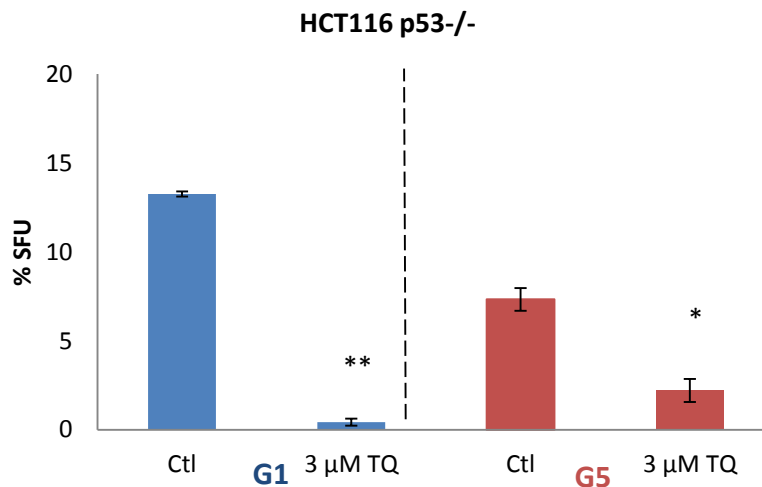
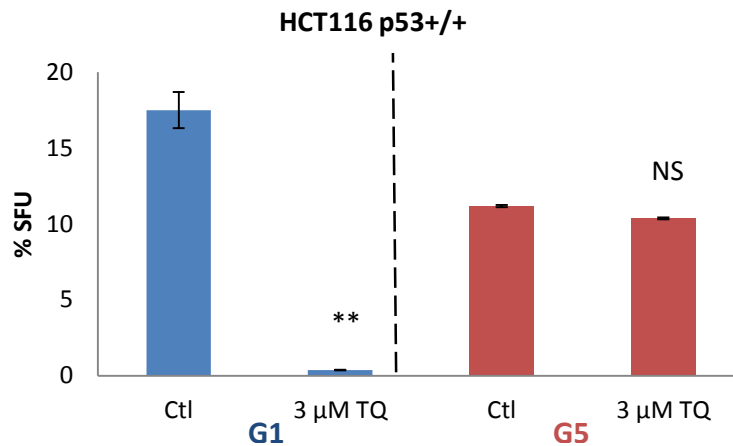
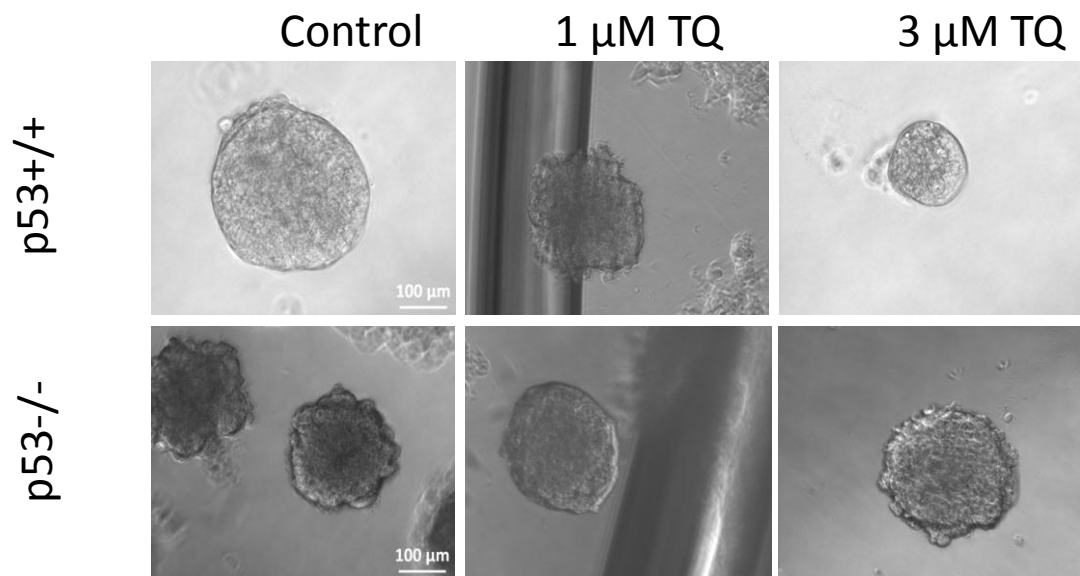


Figure 16. Effect of TQ on a pure CSC population at generation 5 in HCT116 p53+/+ and p53-/- cell lines. Graphical representation of % SFU in untreated cells and cells treated for once with 3 μM TQ at generation 1 (G1) and 5 (G5) in HCT116 p53+/+ and HCT116 p53-/- cells. Cells were plated in matrigel at a density of 1000 cells/well. SFU is calculated as the number of counted spheres over the number of plated cells. Values represent the average of two independent experiments ±SEM. Asterisks indicate a significant difference between control (Ctl) and 3 μM TQ treated cells using Student's t-test, (*) represents p<0.05, (**) represents p<0.01 and (NS) non-significant.

2. TQ Effect on HCT116 Sphere Size

To assess the proliferation potential of the enriched progenitors/stem-like population in the presence or absence of p53 within HCT116 cell lines, we measured sphere diameter at day 13 of sphere culture and determined the average diameter for the majority of spheres within each condition. Our results showed a decrease in HCT116 p53^{+/+} average sphere size at G1 (Figure 17) from 137 μ m diameter in the control to 75 μ m and 68 μ m in 1 μ M and 3 μ M TQ treated spheres, respectively. TQ treatment did not however affect sphere size in subsequent generations in HCT116 p53^{+/+} cells suggesting that its effect was reversible thereafter. In comparison, no decrease in sphere size was noted in all generations of HCT116 p53^{-/-} cells (Figure 17). We also noted the formation of heterogeneous population of spheres with diversified sizes in both cell lines. Treated spheres at G5 showed larger spheres than those at G1 regardless of their p53 status (Figure 17).

A



B

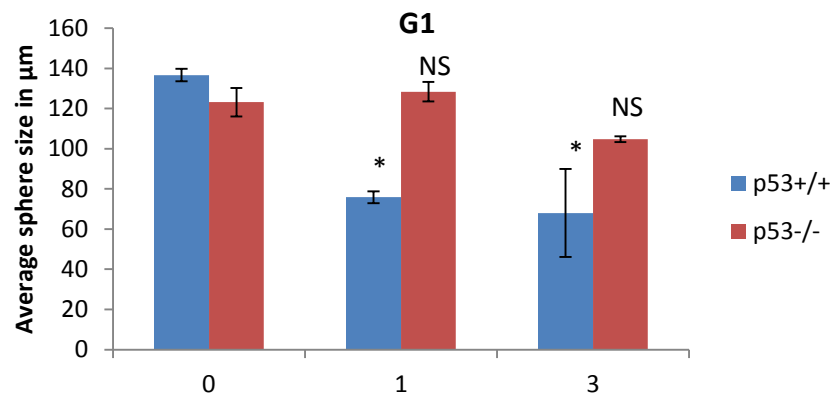


Figure 17. Effect of TQ on size of colonospheres at G1.

A) Representative images of HCT116 p53^{+/+} spheres were taken at generation 1, at different conditions: Ctl untreated spheres; 1 μ M TQ treated spheres; 3 μ M TQ treated spheres. Images were visualized by Carl Zeiss Microscope at 10x magnification and sphere diameters were measured by Carl Zeiss Zen 2012 image software. B) Quantification of the average diameter of spheres with or without treatment (1 μ M TQ and 3 μ M TQ) at generation 1. Diameters spheres were quantified in each condition. Error bars represent SEM of sphere size in each condition. Asterisks indicate a significant difference between control and 1 μ M and 3 μ M TQ treated cells using Student's t-test, (*) represents $p < 0.05$ and (NS) represents non-significant.

E. Stem Cell Markers Gene Expression in 2-D HCT116 Cells vs. 3-D HCT116 Spheres

We further investigated the presence of colon cancer stem cells markers EpCAM and CD44 (Kanwar *et al.*, 2010; Langan *et al.*, 2013) in 2D mixed population of human colorectal HCT116 cells and 3-D enriched progenitor/stem-like population at G1. In 2D culture of both HCT116 p53^{+/+} and p53^{-/-} cells, preliminary data of quantitative RT-PCR showed a decrease in EpCAM and CD44 gene expression upon 40 μ M TQ treatment (Figure 18 A, C). RT-PCR performed on G1 spheres revealed that EpCAM and CD44 gene expression was not affected by TQ treatment in HCT116 p53^{+/+} G1 spheres whereas 1 μ M and 3 μ M TQ treatment decreased EpCAM and CD44 gene expression in HCT116 p53^{-/-} G1 spheres (Figure 18 B, D). EpCAM RNA expression decreased in a dose dependent manner from an average expression of 1.75 in untreated G1 p53^{-/-} spheres to 1.4 when treated with 1 μ M TQ and 0.15 when 3 μ M TQ was applied. A similar pattern was observed for CD44 average RNA expression where the average decreased from 0.8 in untreated G1 p53^{-/-} spheres to 0.7 and 0.1 when treated with 1 μ M and 3 μ M TQ, respectively. These results suggest that the effect of TQ is dependent on p53 absence in the enriched progenitor/stem-like HCT116 population. Further experiments investigating CSC markers expression in a pure CSC population at G5 are required to validate the aforementioned results.

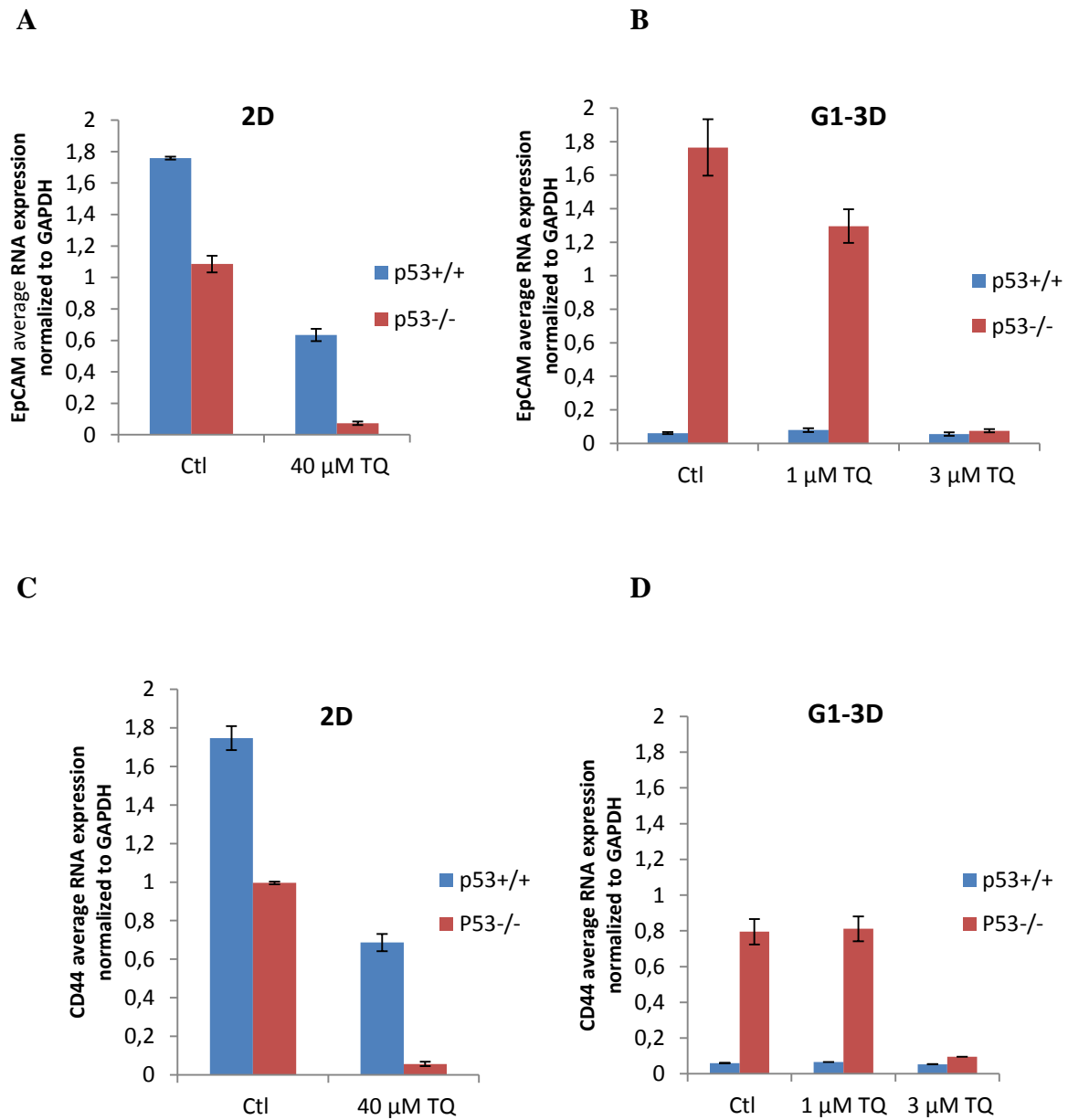


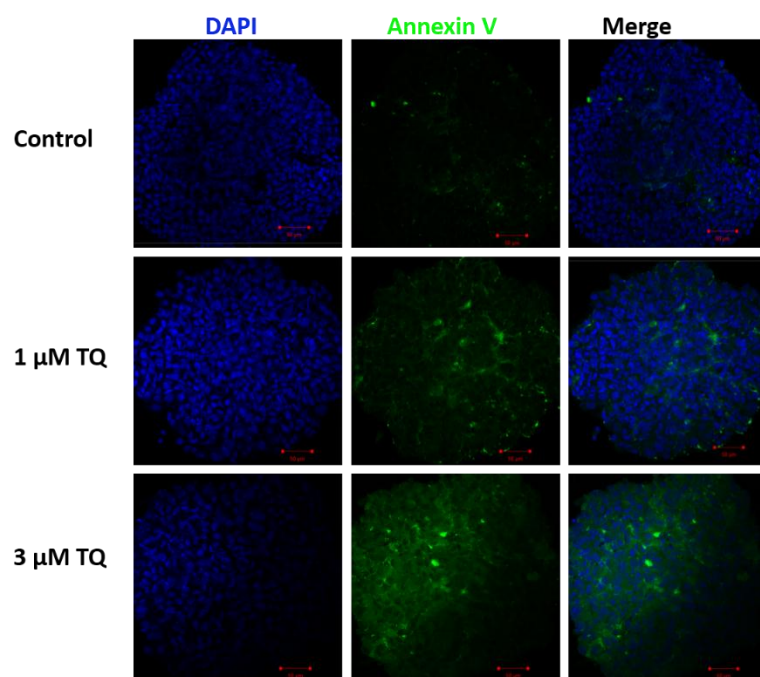
Figure 18. EpCAM and Lgr5 gene expression levels in HCT116 2-D monolayer cells and G1 spheres.

EpCAM and CD44 expression levels were determined using quantitative RT-PCR analysis and values were normalized to GAPDH (N=1). A) and C) represent, respectively, EpCAM and CD44 expression levels in HCT116 p53+/+ and p53-/- monolayer cells untreated (Ctl) and treated with 40 μ M TQ. B) and D) represent, respectively, EpCAM and CD44 expression levels in G1 HCT116 p53+/+ and p53-/- spheres collected at day 12 of sphere culture. Spheres were dissociated from matrigel after 1 hour incubation in dispase solution.

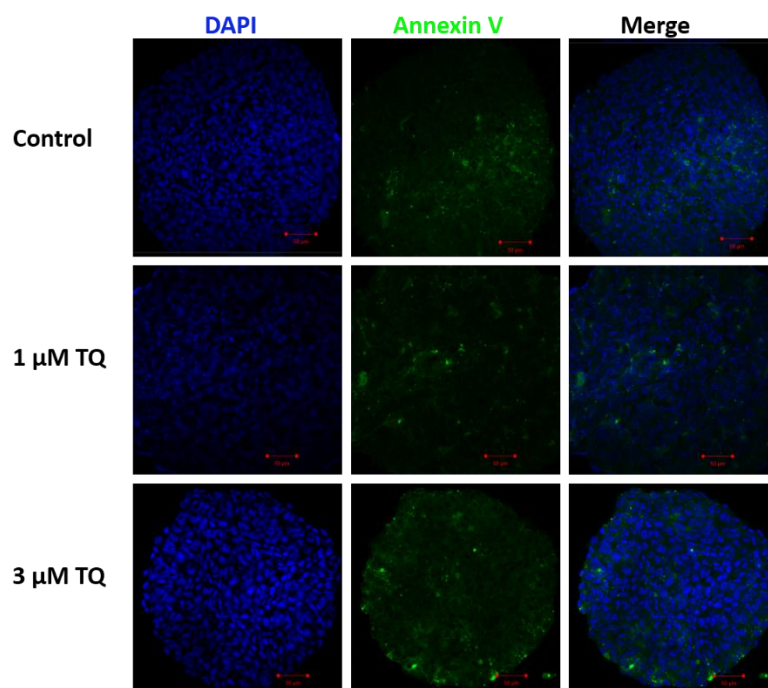
F. TQ-Induced Apoptosis in HCT116 p53^{-/-} Colonospheres

Since evading apoptosis is one mechanism that can be triggered by CSC in response to chemotherapy, we next aimed to study whether TQ induces apoptosis in HCT116 colonospheres. Annexin V immunostaining assay on G1 colonospheres revealed that TQ treated HCT116 p53^{-/-} spheres contained higher amount of apoptotic cells when compared to control spheres. Furthermore, Annexin V expression increased in a dose dependent manner in HCT116 p53^{-/-} colonospheres treated with TQ (Figure 19A). Hence, at G1, HCT116 p53^{-/-} Annexin V positive spheres increased from 60% in control to 90% and 100% Annexin V positive spheres in cultures treated with 1 μ M and 3 μ M TQ, respectively (Figure 19C). In parallel, in G1 HCT116 p53^{+/+} spheres, TQ treatment increased Annexin V expression from 60% in control spheres to 80% in spheres treated with 3 μ M TQ (Figure 19 B; C). Interestingly, these data suggest that TQ induces higher apoptosis in both HCT116 p53^{-/-} spheres and p53^{+/+} cells with a slightly higher effect on the former type.

A



B



C

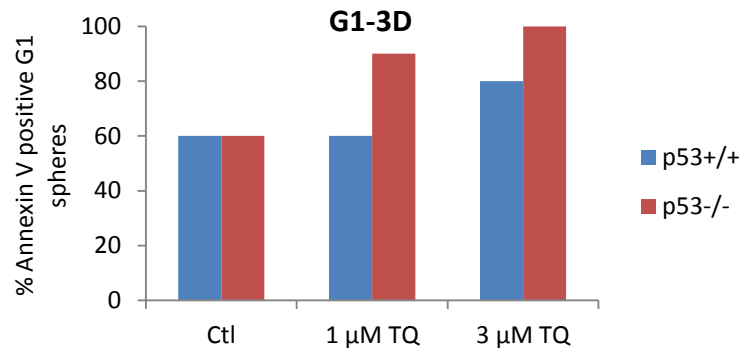


Figure 19. TQ induces apoptosis in HCT116 colonospheres.

AnnexinV colonospheres immunostaining at day13 of sphere culture. Cell nuclei are labeled by DAPI staining (blue) and flipped phosphatidyl-serine are labeled by annexin V-conjugated FITC (green) in HCT116 G1 p53 -/- spheres (A) and HCT116 G1 p53+/+ spheres (B). Images were taken by confocal microscope at 63x magnification with oil. C) Semi-quantitative analysis of immunofluorescence: Annexin V positive spheres were counted out of 10 spheres in each condition. Values represent the % of Annexin V positive spheres over total counted spheres.

CHAPTER IV

DISCUSSION

Local recurrence and distant metastasis cause the majority of cancer mortality (Vaiopoulos *et al.*, 2012; Anderson *et al.*, 2011). Colorectal cancer after a potentially curative surgical resection has the tendency to relapse in the liver and lung after 5 years on average (Young *et al.*, 2014). It is believed that a major determinant for post-therapeutic cancer recurrence in colorectal cancer patients is the presence of self-renewing and maintained population of CSCs. Evidence suggests that epithelial cancer cells possess a small sub-population of these self-renewing multipotent CSCs (Kanwar *et al.*, 2010). Although conventional therapies inhibit highly proliferative cells, remaining slow cycling CSCs lead to local and distant tumor initiation. In an attempt to completely eradicate cancer cells, extensive research is focused on CSC-targeted therapy. Here we showed that TQ targets the enriched CSC population in the human colorectal cancer cell line HCT116, which we used as an *in vitro* model for colorectal cancer, having a high number of CSCs when compared to other human colorectal cell lines (Chen *et al.*, 2011; Yeung *et al.*, 2010). We showed that 10-fold lower concentrations of TQ IC₅₀ resulted in inhibition of HCT116 self-renewal capacity using 3D sphere formation assay. Since CSC are capable of chemotherapy resistance by their unique property to initiate the heterogeneous population of a tumor, we were interested in determining the effect of TQ on targeting the progenitor/stem-like population of cells within HCT116 p53^{+/+} and p53^{-/-} cell lines. Sphere formation and propagation results of

untreated versus TQ treated spheres revealed that low concentrations of 1 μM and 3 μM TQ were able to inhibit sphere formation in HCT116 cells regardless of their p53 status. This suggested that TQ negatively regulates self-renewal capacity of progenitor/stem-like pool via p53-independent mechanisms, namely the aberrantly activated β -catenin/Wnt pathway in colorectal CSCs (Todaro *et al.*, 2010) as well as through extrinsic apoptosis and p53-independent DNA damage pathways as shown in leukemic Jurkat cells (Alhosin *et al.*, 2010). In our 3D culture system, we unexpectedly found that cells lacking the tumor suppressor gene p53 did not show an increase in sphere number compared to p53^{+/+} cells which is counter-intuitive and requires additional experiments to understand the role of p53 in sphere formation of HCT116 human colorectal cancer cell line.

In HCT116 p53^{+/+} spheres, TQ decreased sphere size at G1 but not in HCT116 p53^{-/-} cells. However, sphere size and proliferation rate were not significantly affected by TQ treatment upon propagation in both cell lines. Adopting a different counting methodology in the future might reveal any size difference that we might have missed by calculating the average sphere size in each condition overall. It is thus better to count and compare the average sphere size in distinct sphere categories ranging in size from small or < 70 μm to medium 70-100 μm and large >100 μm spheres. It might also be beneficial to measure sphere diameters at an earlier time-point of culture *i.e.* day 6 in order to capture the logarithmic cell growth of HCT116 cells.

Furthermore, our propagation data showed that TQ concentrations as low as 1 μM and 3 μM inhibited sphere formation by 50% from G1 up to G5, suggesting the consistent

inhibitory effect of TQ on CSCs. In every generation, treated spheres propagated and treated again showed more reduced % SFU than that of the previous generation.

A wide diversity of stem cell markers help identify the CSC population in colorectal cancer and no specific combination of colon cancer stem cell markers was identified to date (Stuellten *et al.*, 2010). To provide insights on the mechanisms involved in colorectal stemness properties and to decipher pathways that might be targeted by TQ, we studied gene expression levels of EpCAM and CD44 known to be putative colorectal CSC markers. EpCAM is an epithelial cell adhesion molecule in the Catenin pathway and Wnt pathway (Langan *et al.*, 2013). Activated EpCAM provides Wnt-like signals that enforce stem cell properties in normal cells and cancer stem cells. Furthermore, enhanced EpCAM expression enhances Oct 4 expression (Munz *et al.*, 2009). CD44 is a transmembrane glycoprotein crucial in tumor initiation and colonosphere propagation *in vitro* (Du *et al.*, 2009). CD44 acts as downstream target of the Wnt/ β -catenin pathway (Subramaniam *et al.*, 2010). Our preliminary data showed that TQ treatment decreased CD44 and EpCAM RNA expression levels in enriched human colorectal progenitor/stem-like pool with p53 deletion in 3D model. The presence of p53 in HCT116 p53^{+/+} spheres seems to prevent TQ effect on CD44 and EpCAM gene expression. These results are in line with the fact that CSCs resist therapy by proliferation and initiation of the tumorigenic heterogeneous population. TQ treatment reduced CSC markers gene expression in G1 p53^{-/-} spheres but not in G1 p53^{+/+} spheres, suggesting a possible involvement of p53 in progenitor/stem-like pool response to TQ. We also assessed TQ effect on the enriched sub-population at G5 to the presence or absence of p53; CSCs with p53 deletion were more sensitive to 3 μ M TQ one-

time treatment at G5 as opposed to CSCs wild type for p53. Obtained G5 spheres are assumed to be formed from pure CSC population. Therefore, up to G5, TQ treatment was able to reduce the stem cell pool in human colorectal HCT116 cell lines. Moreover, chronic TQ treatment from G1 to G5 might have weakened sphere-forming cells making them more susceptible to TQ response compared to one-time TQ treatment at G5 showing the stem-like cells pure response to the drug.

p53 $+/+$ and p53 $-/-$ HCT116 cell lines were equally sensitive to TQ treatment at G1. However, EpCAM and CD44 expression levels only decreased in HCT116 p53 $-/-$ G1 spheres. These findings suggest the involvement of other stem cell markers associated with TQ response, namely Lgr5 and ALDH1. Another mechanism of action of TQ on HCT116 sphere formation is apoptosis induction. However, HCT116 p53 $+/+$ G1 spheres did not show high apoptotic levels upon TQ treatment opposite to the high decrease in sphere counts, suggesting that apoptosis is not the only mechanism responsible for TQ response.

Studies on the effect of plant-derived molecules on a cancer stem cell pool have been recently reported. Another plant-derived extract, LH4 from *Berberis Libanotica* reduced the stem cell pool in human prostate cancer DU145 cell line (Rabih El-Merehbi, 2014). Recent evidence reported the use of the dietary ingredient curcumin along with novel analogs such as Difluorinated-curcumin (CDF) to target cancer stem-like population in colorectal cancer, breast, pancreatic, brain cancer and head and neck cancer (Zang *et al.*, 2014; Kanwar *et al.*, 2011). The next step after finding a potential anti-cancer effect of a plant-derived drug on CSCs is to develop combinatorial treatments with conventional chemotherapy. CDF in combination with the commonly used chemotherapy for colon cancer, 5-fluorouracil and

Oxaliplatin showed efficiency in elimination of colorectal CSCs (Kanwar *et al.*, 2011). Due to the inhibitory effect of TQ on self-renewal property of CSCs, it would be of interest to conduct further investigations on TQ in combination with chemotherapeutic drugs against CSCs.

Our study demonstrated that low concentrations of TQ can target enriched CSCs from colorectal cancer HCT116 cell lines, suggesting promising effect of TQ on resistant cells. Testing TQ with commonly used chemotherapy for colorectal patients, namely 5-fluorouracil would investigate synergism and future *in vitro* work is required to uncover the mechanism of action of TQ underlying its inhibitory effect on CSCs.

Further investigations should be conducted to determine TQ effect on protein levels of CSC markers, EpCAM, CD44 and others such as Lgr5 and ALDH1 activity (Todaro *et al.*, 2010). To further address TQ effect on proliferation of the progenitor/stem-like pool, immunostaining with antibodies against proliferation marker e.g. Ki67 can be performed on colonospheres and primary gut tissue. Furthermore, experiments are required to validate our results and determine the effect of TQ on stem cell protein expression levels by western blot analysis and whether TQ effect is similar in G5 enriched CSCs. *In vivo* xenotransplantation experiments should be performed to address the effect of TQ alone or in combination on the tumor initiation ability of HCT116-derived CSCs and thus its potential for clinical translation.

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