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THE CHEMOPREVENTIVE EFFECT OF POLYMETHOXYFLAVONES: TARGETING THE COLORECTAL CANCER STEM CELLS

Fei Xu
University of Massachusetts Amherst

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A Dissertation Presented

by

FEI XU

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Approved as to style and content by:

_____
H. Xiao, Chair

_____
D. Julian McClements, Member

_____
GD. Zhang, Member

_____
ZH. Liu, Member

_____
Eric A. Decker, Department Head

Department of Food Science

DEDICATION

To my dear parents, family members and all the wonderful friends.

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It is almost impossible to fully assess the influence of the graduate study has had on my life. There are so many names poping up in my mind when I am finally handing in this thesis: First, my advisor, Dr. Hang Xiao, deserves the most recognition. It was your positive attitude, intelligence and kindly guidance that opened the scientific world for me. With your trust and encouragement, I have also gained so many valuable experiences in leading and managing. Besides, I was blessed to be able to work with so many talented individuals. Tom, Mingyue, Jingkai, Mon, to you I own the most sincere appreciation; it was your patience and kindness that helped me to learn from almost zero. My fellow lab mates including Christina, Cici, Bonnie, Xiaokun, Zili, Nok, Fang, Yue, Xian, Olivia, Hua, Zhengze, Xiaoqiong, George, Min, Will and Tim, thank you for bearing with me for four years, I know I was such a "demanding" lab manager to deal with. I will never forget the time we have spent together as a family in UMass. Minqi, my "favorite" lab member, without your continuing support I could never overcome all those obstacles and endure through graduate school, I wish I will always be there for you too. I will also acknowledge the friendship with Charmaine, Fang Tian, Fei He, Fran, Debby, Dr. Sun, Dr. Qi, and Dr. Cao. Sincerely I appreciate the time and commitment of Dr. Zhang, Dr. McClements and Dr. Liu for being my committee members. Leigh & Genie Andrews, my dear landlords, it is you who embraced us as family in the US; among all the "life lessons" you have taught me, I love the "living on the edge" one the most. Last, but the most, I want to thank my parents, who have always stood behind me, rain or shine.

ABSTRACT

THE CHEMOPREVENTIVE EFFECT OF POLYMETHOXYFLAVONES: TARGETING THE COLORECTAL CANCER STEM CELLS

SEPTEMBER 2015

FEI XU,

B.Eng., NANJING AGRICULTURAL UNIVERSITY, CHINA

Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Hang Xiao

Among all the cancers, the death rate of colorectal cancer is one of the highest. Evidence from both murine xenograft model and human trials have shown cancer stem cells (CSCs) are responsible for the initiation, metastasis and recurrence of multiple cancers therefore targeting colorectal CSCs would be a promising chemo-preventive/ therapeutic strategy. Polymethoxyflavones including nobiletin (NBT) and 5-demethylatednobiletin (5DN) are exclusively found in citrus peels and have been shown to have anti-cancer effects. Our previous studies in the biotransformation and tissue distribution of NBT and 5DN have shown that in order to fuller evaluate the biological impact of those two PMFs, we also need to take into consideration of their metabolites. In this study, we examined the effects of these NBT and 5DN as well as their metabolites on tumor sphere formation, apoptosis and cell cycle distribution. Plus, we also made the initial attempt to investigate the possible mechanism(s) for the inhibitory effects we have observed. Our results showed that both NBT and its metabolites could inhibit the tumor sphere formation and induced apoptosis, with generally the metabolites having equivalent or stronger effects; On the other hand, M1 exerted the strongest inhibitory effect on colorectal CSCs compared to 5DN and other metabolites. Apoptosis, necroptosis as well as forcing CSCs to reenter the cell cycle from the quiescent states could be accounted to the overall inhibitory effects of NBT, 5DN and their metabolites.

With the achievement of our study, we will be able to better evaluate the overall efficacy of PMFs-based (NBT and 5DN) chemopreventive strategies on colorectal cancer, especially against colorectal CSCs.

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CHAPTER 1

INTRODUCTION

In this industrial world, colorectal cancer is among the most commonly diagnosed cancers. In the United States, it is the third leading cause of death among cancers in both men and women. Multiple barriers obstruct the successful colorectal treatment. Among all the challenges, tumor reoccurrence and metastasis are two major survival-influencing factors of colorectal cancer. For example, up to 40% of colorectal cancer patients who present with stage II or III colorectal cancer will recur after primary treatment [1]. Plus, despite the rapid advances in chemotherapeutic drugs targeting colorectal cancer, 89% patients with metastatic disease cannot survive [2] . This indicates that conventional therapies fail to provide a permanent cure.

There is growing amount of evidence supporting that the cellular heterogeneity found in tumor, is one major factor that hampers the search for new therapies for cancer. Specifically, a small sub-population within the cancers, namely, cancer stem cells (CSCs) are responsible for tumor initiation, metastasis and resistance to conventional therapies such as chemotherapy and radiotherapy. So far, data have been accumulated that several solid tumors, including breast [3], brain [4] and colon [5, 6], follow the CSC model. Thus, with our developing understanding of the mechanism through which CSC direct the tumor growth, targeting CSC has become a very promising preventive/ therapeutic strategy that can ultimately lead to an effective colorectal cancer control.

Compared to other types of tumor, the development of colorectal cancer usually requires a long time span, which provide us with a great opportunity for early detection and prevention [7]. Cancer Chemoprevention, by definition is the inhibition, delay and

reversal of the carcinogenesis by the administration of one or more naturally occurring and/or synthetic agents. Recently, the plausibility of naturally dietary compounds as cancer chemopreventive agents has been discussed extensively mainly for the following reasons [8]: 1. As most of dietary compounds are present in food that people are commonly consuming, they are generally easy accessible and cost-effective. 2. Compared to most drugs used in chemotherapy, they have low or even none toxicity, therefore there will be less concern about the potential side effect. 3. As has been reported by several clinical trials, many of the dietary compounds have already been proved to effect adjunctively with chemotherapy drugs. Epidemiological and dietary interventions studies in both animal and human models have suggested the positive role of many dietary components in inhibiting, reversing tumor development in different type of cancers [9]. So far, several of the dietary agents have also been shown to interfere with the function of CSCs [10]. Generally, this would be achievable by strategies such as inducing of differentiation, inhibiting of self-renewal signaling pathways and sensitizing CSC to go through death pathways such as apoptosis and cell cycle regulation [8]. For example, curcumin was reported to be able to impair the WNT signaling and cell-cell adhesion pathway in human colon cancer cell (HCT-116), which resulted in apoptosis and G2/M cell cycle arrest [11].

Polymethoxyflavones (PMFs) are a group of compounds that are almost exclusively found in the peels of citrus fruit [12]. So far, PMFs have been found to have wide spectrum of health promoting effect including anti-inflammation and anti-carcinogenesis [13]. Previously, as one of the major permethoxylated PMFs, nobiletin (NBT) has been

reported to have various biological properties such as anti-tumorgenesis, anti-inflammation and oxidative stress [14-17].

Among all types of PMFs, 5-demethylated PMFs are a unique subclass, with 5-demethylated nobiletin (5DN) as the most abundantly one that could be found in orange peels (especially in aged orange peels) [18]. The transformation from NBT to 5DN can be happened under the "aging process" of orange peel (e.g. heating) or naturally by auto-hydrolysis. Recent study have shown that 5DN exerted stronger inhibitory effects on the growth of the colon cancer cells compared to their permethoxylated counterparts, NBT, suggesting the critical role of hydroxyl group at 5-position in the function of 5-hydroxy PMFs [18]. Until now, there are multiple reports regarding the bioactivities of 5DN [12, 18, 19].

Biotransformation plays an important role in the biological activities of orally ingested bioactive compounds. In fact, 3′-demethylnobileti (N1) [20], 4′-demethylnobileti (N2) [21], and 3′,4′-didemethynobiletin (N3) [22] were already been identified as major metabolites from NBT and have recently been studied for their biological actions. Evidence have supported that metabolites of NBT, exerted much stronger anti-inflammatory [23] and anti-mutagenic [24] effect compared to the parent compound in the *in vitro* setting. Plus, our unpublished data have shown that, after oral feeding of NBT (1000ppm) to mice, the majority of NBT was transformed into three major metabolites; specifically, in the mice colon mucosa, there were significant higher concentrations of metabolites than NBT. As a result, we need to include the metabolites in our study in order to fully evaluate the biological impact of NBT on colorectal CSCs.

On the other hand, the metabolites of 5DN have been scarcely investigated. Recently, our group successfully isolated and identified three novel urinary metabolites of 5DN, namely, 5,3'-didemethylnobiletin (M1), 5,4'-didemethylnobiletin (M2), and 5,3',4'-tridemethylnobiletin (M3) [25]. Notably, all three metabolites have showed stronger inhibitory effect in human colon cancer cells then 5DN in the in vitro setting [25]. Moreover, according to our unpublished data, after feeding of 5DN (1000PPM) to mice, about half of NBT get transformed into three major metabolites (M1, M2 and M3); specifically, while 5DN was still the most abundant one in the mice colon mucosa, the three metabolites in total was more than the amount of 5DN. This again provide us the rationale that we need to include the metabolites in out study in order to fully evaluate the biological impact of 5DN on colorectal CSCs.

Taken all these together, out long term goal is to improve the current chemo-preventive strategy for colorectal cancer. To achieve this goal, the overall objective of our research will be focused on developing novel chemopreventive -based strategies that target colorectal CSC using multiple PMFs. Our central hypothesis is that NBT, 5DN and their demethylated metabolites will exert their anti-CSC ability by regulating major CSC-associated cellular signaling pathways. Our hypothesis has been formulated on the basis of our and others' preliminary results that NBT, 5DN and their demethylated metabolites have already shown anti-cancer effects both *in vivo* and *in vitro*, with the metabolites showing much more potent effect than their parental compounds.

We plan to test our central hypothesis, and thereby, achieve our objective of this project by pursing these following ***specific aims:***

1. ***Determine the inhibitory effect of NBT and their demethylated metabolites on colorectal CSCs.*** The inhibitory effect of NBT and their metabolites will be analyzed by tumor-sphere formation assay. Flow cytometry analysis will be utilized to determine the effect of NBT and their metabolites on cell cycle distribution and apoptosis. Western blot on various key CSC- related markers, and oncogenic proteins will be conducted. We anticipate seeing that both NBT and their metabolites will have inhibitory effects on colorectal CSCs, with possibility metabolites having stronger effect.

2. ***Determine the inhibitory effect of 5DN and their demethylated metabolites on colorectal CSCs.*** 5DN and their metabolites will be investigated by using the same techniques as described in specific aim 1. We anticipate seeing that both 5DN and their metabolites would exert inhibitory effect on colorectal CSCs, with possibly metabolites having stronger effect.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to colorectal cancer

As part of the gastrointestinal (GI) tract, one of the most regenerative systems in human body, colon and rectum (large intestine) are characterized by unique properties: 1. Large intestine is a 5-feet long muscular tube, which is covered by a sheet of crypts. Crypts are the basic functional unit of the intestine. 2. To maintain the proper function of the larger intestine, billions of specialized intestinal epithelial cells are replaced by new cells in a daily base. All these are regulated by the stem cells harbored in the base of the crypts [26]. These stem cells are able to maintain themselves through self-renewal. Depending on the different location along the crypt axis in colon, intestine cells can be further specialized after receiving signals to differentiate into a variety of cell types [27]. Over all, the highly regenerative and proliferative nature of the intestinal system makes it very vulnerable to multiple genetic mutations followed by the loss of proliferation control, which are thought to lead to colorectal cancer [28].

In this industrial world, colorectal cancer is one of the most commonly diagnosed cancers. In the United States, it is the third leading cause of death among cancers in both men and women (Figure 1). According to American Cancer Society, there would be approximately 26100 and 23600 estimated deaths from colorectal cancer in male and female respectively in 2015. Unlike other cancers such as lung cancer, there is no single risk factor that accounts for the most cases of colorectal cancer. Instead, multiple risk factors (most of which are often interrelated) have been identified through

epidemiological studies, for example, family history of colorectal cancer [29], inflammatory disease [30], smoking [31], and excessive alcohol consumption [32].

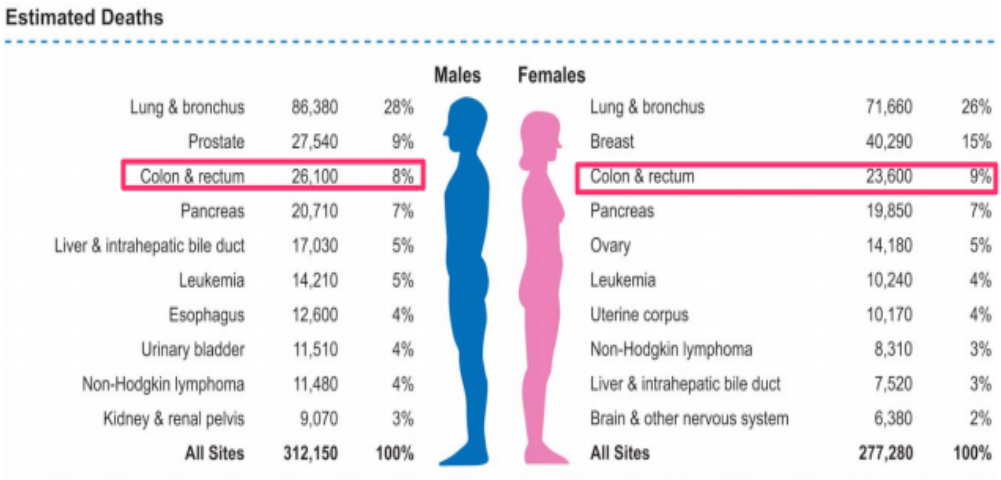

Figure 1. Ten leading cancer types for the estimated deaths by sex in United States, 2015. (Adapted from Siegel et al., 2015) [33]

Traditionally, there is higher incidence of colorectal cancer in developed countries in Europe, North America, and Oceania, while the incidence is much lower in developing countries of East Asia and Africa [34]. Findings from multiple epidemiologic and experimental investigations have shown that the risk of colorectal cancer is linked with the consumption of different foods and nutrients. For example, fiber, milk and whole grains have been associated with a lower risk of colorectal cancer, while the extensive consumption of red meat and other processed meat product are believed to result in a higher risk [35]. There are increasing data supporting that the efficacy of dietary modification and nutrient supplementation for the prevention of colorectal cancer [36].

The development of colorectal cancer usually begins with a growth of tissue or tumor as non-cancerous polyps on the inner layer of the colon or rectum. While most of the polyps are considered to be benign tumors (adenomas), only the certain polyps

(carcinoma) can be further developed into cancer. As most colorectal cancers develop over several years, from adenoma to carcinoma, the disease is most curable if detected at early stages. Multiple observational studies suggest significant reduction in the incidence and mortality of colorectal cancer by screening colonoscopy, which prevents cancer by allowing for the removal of precancerous lesions [37, 38].

For the recent years, compared to the decreasing trend of both the incidence and mortality rate of colorectal cancer in developed countries, there is a rapid increase in the incidence and mortality of colorectal cancer in previous low-risk developing countries such as China. Studies have shown that this trend is related to people's elaboration of the west-life style such as high-fat diet consumption. On the other hand, besides the underdeveloped health-care resources, people in developing countries are more reluctant to take the colorectal cancer screenings such as colonoscopy due to their invasive nature [34, 39].

Multiple barriers obstruct the successful colorectal treatment. Among all the challenges, tumor reoccurrence and metastasis are two major survival-influencing factors of colorectal cancer. For example, up to 40% of colorectal cancer patients who present with stage II or III colorectal cancer will recur after primary treatment [1]. In colorectal cancer, despite the rapid advances in chemotherapeutic drugs, 89% patients with metastatic disease cannot survive [2]. This indicates that conventional therapies fail to provide a permanent cure.

Overall, since the effectiveness of current treatment of colorectal cancer still remains a huge challenge, the long term declines in colorectal cancer incidence rates since the mid-1980s have been attributed to both changes in risk factors and the introduction of

screening [40]. However, the rapid declines in recent years would almost be attributed to the development of colonoscopy [41].

2.2 The evolving concept: cancer stem cells (CSCs) and colorectal cancer

2.2.1 Tumor heterogeneity

Despite the introduction of novel therapies in cancer treatment over the years, there are still many patients suffering from the low survival rate of solid tumors such as colorectal cancer. Traditionally, most of the therapeutic approaches have been focused on targeting the malignant mass, of which the cell growth and survival pathways have been studied for decades. However, rather than archiving the anticipated clinical goal, the tradition treatments usually if not always have result in tumor progression and recurrence.

In response to the therapy failure and disease progression of the conventional cancer therapies, more and more researchers have been focused on identifying new, innovative targets to guide the future drug development as well as chemopreventive strategies. Among all the challenging factors that have been hampering the search for new therapeutic target, it has been widely accepted that the intratumoral heterogeneity is the most important one [42]. In another word, rather than simply as a collection of homogeneous cells, tumor is more likely a complex organization consists of cells with different functions. All these cells, as a whole, influence the development of tumor, and ultimately impact the efficacy of certain therapy [43]. Individual cells within a tumor could be various in multiple aspects such as growth, metabolism and apoptosis [44].

It was more than two decades ago that Vogelstein [45] pointed out that just like most eukaryotic organism begin as single cells (eukaryotic stem cells), a tumor might also

starts from a single cell and then develop into a heterogeneous system. Thus, the understanding of tumor heterogeneity will be of great importance in the flowing two aspects: 1. It will provide insights to the biological base of tumor development and critical events such as initiation, metastasis and resistance to therapy; 2. It will be helpful in guiding the direction of future development of cancer-preventive/ therapeutic interventions.

Firstly, the tumor cell heterogeneity at the genetic level is one major factor that account for the generation of cell diversity within a tumor. With the advance of whole genome sequencing technique [46, 47], there are increasing studies supporting that the variability at the DNA level, or, multiple genetic alterations that accumulated during the progression of cancer, can be account for the phenotypic diversity among tumor cells.

In addition to the genetic difference that contributes to the tumor cell heterogeneity, epigenetic mechanisms can be another factor. Unlike changes at the DNA level, epigenetic modification including methylation, acylation, ubiquitination, phosphorylation and non-coding small RNAs (miRNAs) [48] do not result in alterations in primary DNA sequence while can still alter the transmission of information through cell division [49]. Overall, multiple data support that those epigenetic factors not only play a very important role in normal tissue functioning, they also contribute to the functional heterogeneity in cancers [50, 51].

Given the fact that there are both genetic and non-genetic factors that can lead to the cellular heterogeneity in cancer, it is of great importance to understand that whether all cells within a tumor have the same biological potentials that can be account for the tumor

sustainability, or, is there a particular sub-population of cancer cells that are responsible for the tumor- initiating?

A traditional view of the cellular heterogeneity is known as the stochastic model. It is based on the assumption that since every single cell within a tumor are "equally created"; it is the random variables that decide the fate of cells. In other words, whether or not some cells would possess tumor-initiating ability is simply a stochastic event with relatively low probability [28]. In fact, the stochastic model is in contradiction to the hierarchies in the development of normal organs, where stem cells serve as the most potent cells and have the potentials to differentiate into cells of specific lineage. Plus, with this stochastic model, it basically denies the possibility that cells with tumor-initiating potentials can be separated from bulk tumor.

On the other hand, there is the hierarchical model, also known as the cancer stem cell (CSC) model, which is based on the prediction that bulk tumor cells are consist of intrinsically different subpopulations: tumorigenic and non-tumorigenic. Within the hierarchically system, only a small population of tumorigenic cells, or cancer stem cells (CSCs), are able to give rise to phenotypically and functional diverse non-tumorigenic cells.

According to the hierarchical model, only CSCs have the ability of long-term self-renewal, therefore maintaining the growth and progression of cancer [52]. On the other hand, differentiated non-tumorigenic cells will be consisting the bulk of tumors, though had almost no contribution to the cancer progression. Prospectively, the separation of CSCs from non-CSCs would be achievable based on the hierarchical theory.

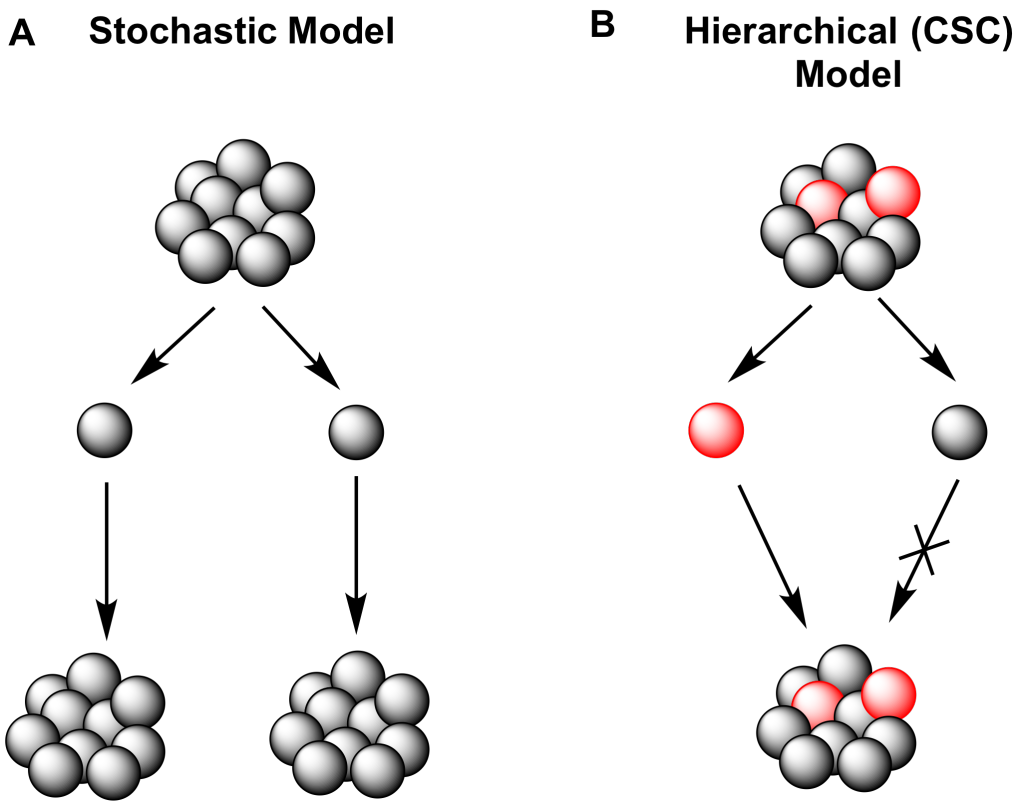

Figure 2. Two models that demonstrate the tumor heterogeneity. **A**. The stochastic model. According to this model, every single cell within the tumor has the tumor-initiating potential. **B**, The hierarchical (CSC) model. According to this model, there is only a small sub-population of cells (CSCs) are responsible for initiating and maintaining tumor growth.

2.2.2 Defining the "Stemness"

As has been reviewed above, there is increasing evidence supporting that rather than a collection of homogeneous malignant cells, tumor is more a complex systems with functionally diverse cell types which influence the overall tumor "fitness" [44]. Historically, principles from normal stem cells (NSC) have been guiding the developing of cancer stem cell (CSC) study. Even though CSC may not necessarily derive from the normal NSCs, they could still possibly share multiple signaling pathways [53]. One of the

key characteristics of stem cells is self-renewal. By definition, self-renewal is the biological process where cells can undergo either symmetric or asymmetric division to form daughter cells with the same biological potential, which ensure the long-term clone ability. Particularly, through asymmetric cell division, one CSC can give rise to two different daughter cells. While one of the daughter cell will maintain the long term self-renewal properties as the mother cell, the other cell can be further specialized according to the different developmental stage [54]. While through symmetric cell division, each CSC can divide symmetrically to generate either two daughter stem cells or two differentiated cells [55]. (Figure 3)

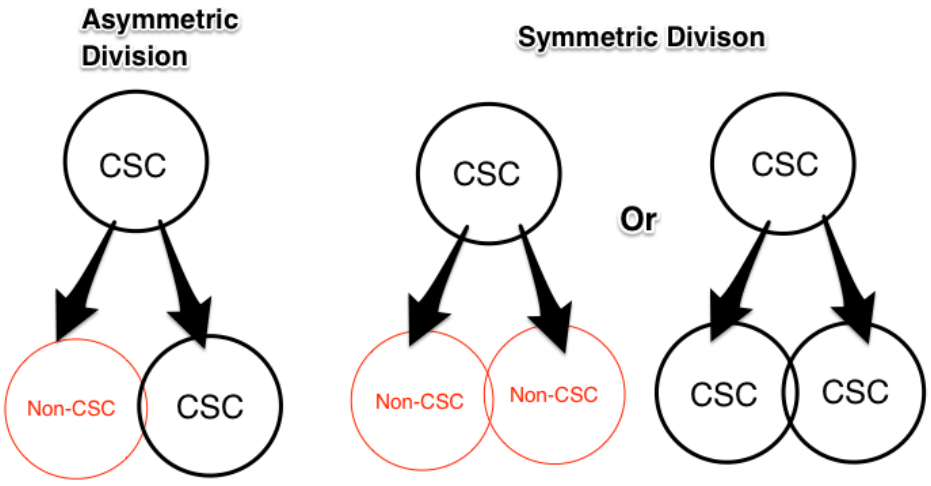

Figure 3. Asymmetric and symmetric division of CSCs.

There is increasing use of the term "stemness" to describe the functioning as well as the serial unique biological properties of CSCs. Generally, "stemness" has been used to characterize the multi-lineage differentiating potential while retaining the ability to self-renewal [56].

The ultimate goal for the study of CSC, as has been pointed out by Kreso et al. is to get a complete view of the underlying mechanisms of the tumor heterogeneity; and how such how they are connected to critical challenges such as therapy resistance and tumor progression and relapse [44]. Recent observations in human studies of leukemia and solid tumor begin to support the central role of the "stemness" in the cancer study [57-60]. In a recent review, the authors for the first time provide us with the notion that three fundamental filed in biology: genetic factors, epigenetic modification (e.g. DNA methylation, histone modification), as well as the non-malignant cells within the microenvironment (niche), could be accounted for the functional heterogeneity in the tumor; more importantly, such factors could either simultaneously and /or independently impact the "stemness" of the CSC [44]. Thus, a more detailed illustration of the "stemness", as well as some of its key regulators will be further reviewed in the following chapters.

2.2.3 Purification of colorectal CSCs

2.2.3.1 CSC markers: popular yet controversial

Generally, the investigation of CSCs have been focused on the use of cancer cell lines, primary cancer cell lines, xenografts from murine model as well as primary human patient tissue sample [61]. Specifically, certain CSC surface markers have been widely used in various CSC models.

During the early studies of leukemia [62, 63], researchers successfully utilized the tools such as fluorescence- activated cell sorting (FACS) and xenograft in immunocompromised mice model to separate CSCs and thereby have them subjected to functional assays. However it took decades of efforts for people to apply the similar

principles from leukemia/ hematopoietic system to solid tumor such as colorectal cancer. Followed by the initial study in breast cancer [3], more and more progress has been made in identifying cancer stem cells in several cancers such as brain [4], pancreas [64, 65], lung [66].

Human colorectal CSCs were first isolated based on the cell surface CD133 in 2007 [5, 6]. CD133 was initially known as a cell surface antigen on both hematopoietic and neural stem cells [67, 68]. Two independent groups utilized CD133 in different murine xenograft models to characterize colorectal CSCs. O'Brien *et al.* [5] demonstrated that the $CD133^+$ cells had much stronger tumor-initiating ability compared to $CD133^-$ cells utilizing non-obese diabetic/severe combined immunodeficiency (NOD/SCID) renal subscapular xenograft model. The similar results from the study of Ricci-Vitiani *et al.* were based on a SCID mouse subcutaneous xenograft model [6]. Both of the two studies have demonstrated that there is enrichment in $CD133^+$ Cells in terms of the tumorigenicity, however that the $CD133^+$ population is still far from a homogeneous system; rather, not every one of CD133 represent a CSC. By *in vivo* limiting dilution assay (LDA), O'Brien *et al.* further revealed compared to $CD133^-$ cells there is 200-fold enrichment in terms of the cancer stem cell activity in the $CD133^+$ subsets [5].

Besides CD133, other markers are also been utilized to identify the tumor-initiating sub-populations in colon cancer. For instance, CD44, an adhesion molecule that once seen as a maker for human breast cancer, got its application expanded to the identification of several of solid tumors such as colon [69] and pancreas [65]. Specifically, Dalerba *et al.* discovered that the combination of CD44 and epithelial cell adhesion molecule (EpCAM) could effectively result in an enrichment of CSCs in a NOD/ SCID

subcutaneous xenograft model [69]. The study was based on eight colorectal cancer specimens, and frequency of the tumor-initiating cells ($EpCAM^+/CD44^+$) ranged from 0.8% to 38%. In addition, the study also focused on the efficacy of CD44 coupled with another marker CD166, a mesenchymal stem cell marker [70]. The previous mentioned publication by Weichert *et al.* had demonstrated the correlation between the increased expression of CD166 in and the poor clinical outcome in CRC [71]. According to their study, the colon cancer cell subpopulation that co-expressing $EpCAM^+/CD44^+/CD166^+$ had high tumor-initiating property.

Aldehyde dehydrogenase 1 (ALDH1) is another popular marker that has been used to separate CSCs from the rest of the bulk cells. As a detoxing enzyme that oxidizes intracellular aldehydes and therefor protects cells from alkylating stress [72, 73], ALDH1 was found to be active in human hematopoietic stem cells (HSCs) [74]. More recent findings have begun to draw people' s attentions to the use of ALDH1 to enrich human colorectal CSC [75, 76]. One of findings showed that as low as 100 $ALDH1^+$ human colon caner cells would be able to give rise to xenografts in NOD/SCID mice (while $ALDH^-$ could not generate any tumor), supporting the high tumorigenicity of $ALDH1^+$ cells [76]. Besides, the authors also explored the combining use of ALDH1 with other stem cell markers such as CD44 and CD133. Interestingly, they found that only the combination of ALDH1 and CD133 was able to generate subpopulations with higher tumor-initiating capacity: while the tumor-initiating rate was 58% when only ALDH1 was used, in comparison, the rate got increased to 89% in the xenografts from $CD133^+$ $ALDH1^+$ cells. Overall, based on their study, the authors concluded that ALDH1 was an ideal candidate with high specificity in identifying and isolating human colorectal CSC.

To date, there has already been a commercially available ALDEFLUOR assay kit, which can be used to detect and isolate subpopulation of cells with high ALDH expression. The use of such kit, as a result, has been applied in various cancer types such as lung [77], breast [78] and prostate [79].

Notably, given the multiple achievements so far in the research of the surface markers as a tool to detect the CSCs, ambiguous results exist. Generally, it is believed that the surface markers are closely related to the binding epitopes, sample size and analyzing methods such as western blot and flow cytometry [80]. Interestingly, some research even stated that the expression of CD133 is not limited to CSC in colorectal, rather that both $CD133^+$ and $CD133^-$ subsets have tumor initiating properties [81]. One possible reason of the opposite observation is that *in vitro* culture of cells before analyzed by flow cytometry and/or FACS, which might have potential influence on the immune-phenotype [81]. On the other hand, even if the cell samples are obtained from solid tumor specimens, it is also critical to have optimized protocols for cell dissociation, where dead cells and debris should be excluded before any tumorigenicity test. The small sample size might be another reason for the controversial results [28]. For example, for the studies of Haraguchi *et al*. [82] and Chu *et al*. [75], neither of them used more than 5 colon cancer samples. These were mostly due to the difficulty in obtaining samples from human patients as well as the complex procedures that would possibly be involved.

Furthermore, it has been considered that the introduction of more than one marker would put another layer of complexity [28]. For example, while multiple independent groups studied the application of high expression of CD133/CD44 as a tool to identify colorectal CSC [69, 82, 83], the study of Chu *et al*. showed quite opposite effect, where

the application of CD44 alone had much more significant efficiency in isolating colorectal CSC, compared to the combination of CD44 and CD133 [75].

In summary, the use of CSC surface markers should be carefully defined for each tissue and tumor sub-type; more importantly, even for a certain tumor type, the solely use of surface maker (s) cannot be account for the "purification" of the CSC population.

Marker(s)	**Marker expression in tumors (%)**	**Reference**
$CD133^+$	1.8 to 24.5	[5]
$CD133^+$	0.7 to 6.1	[6]
$CD133^+$	0.3 to 82	[82]
$CD133^+$	0.3 to 3	[84]
$EpCAM^+CD44^+$	0.03 to 38	[69]
$EpCAM^+CD44^+CD166^+$	3.3 to 35.6	[69]
$CD44^+$	11.5 to 58.4	[82]
$CD133+CD44^+$	0.2 to 50.5	[82]
$ALDH1^+$	2.5 to 4.5	[76]
$ALDH1^+CD44^+$	0.7 to 1.9	[76]
$ALDH1^+CD133^+$	0.7 to 1.1	[76]

Table 1. Isolation of colorectal CSCs by using surface markers.

2.2.3.2 *In vivo* xenograft: the golden standard for CSC study

Questions have been raised regarding whether using solely cell surface makers would be the optimal method in purifying CSCs from the bulk of cancer cells. In fact, as multiple studies have already found, a change in the phenotypic marker expression might not always indicate the unique function of CSCs [85]. Currently, the golden methods of detecting of CSC, as summarized by O' Brien *et al.* [85] and Kreso *et al.* [44], is to use patient tissue samples to generate xenografts in immunocompromised mice in an *in vivo* setting; thereby utilizing robust functional assays such limited dilution assay (LDA), the tumor-initiating capacity can be further tested.

Xenograft, though has been regarded as the golden standard in CSC study, is still facing some important challenges: for example, many researchers would doubt that the use of immune-compromised mice as a reliable model to address the tumorigenicity of human cancer. In other words, would the observation of cells with tumor-initiating potential in murine model be necessarily indicating the "fate" of such cells in human? In response to this critical question, Shackleton [86] suggested that we need to distinguish the different meaning of "potential" and "fate": while "fate" means what are or/and will be happening for sure, "potential" just provide the possibility that certain cells are capable of doing under a particular circumstances. As a result, it is understandable that the interpretation of the above-mentioned "golden standard", are mostly referring to the "potential" of CSCs in human.

In reality, the function of tumorigenic cells might be vastly influenced by the microenvironmental and/ or immunological mechanisms (for instance, immune response in mice might be stronger than in patients). The importance of CSC and its microenvironment (niche) will be discussed in the following chapters. Therefore, high experimental standard should be applied in order to ensure the soundness of the results. Particularly, the conducing of xenograft should be optimized to overcome multiple barriers: for example, depending on which unique type of tumor will be investigated, the xenograft site should be carefully chosen and suitable for the human cancer to grow[28].

In summary, xenografts in mice model are so far the most reliable (yet far from perfect) functional method to assess tumor-initiating potential.

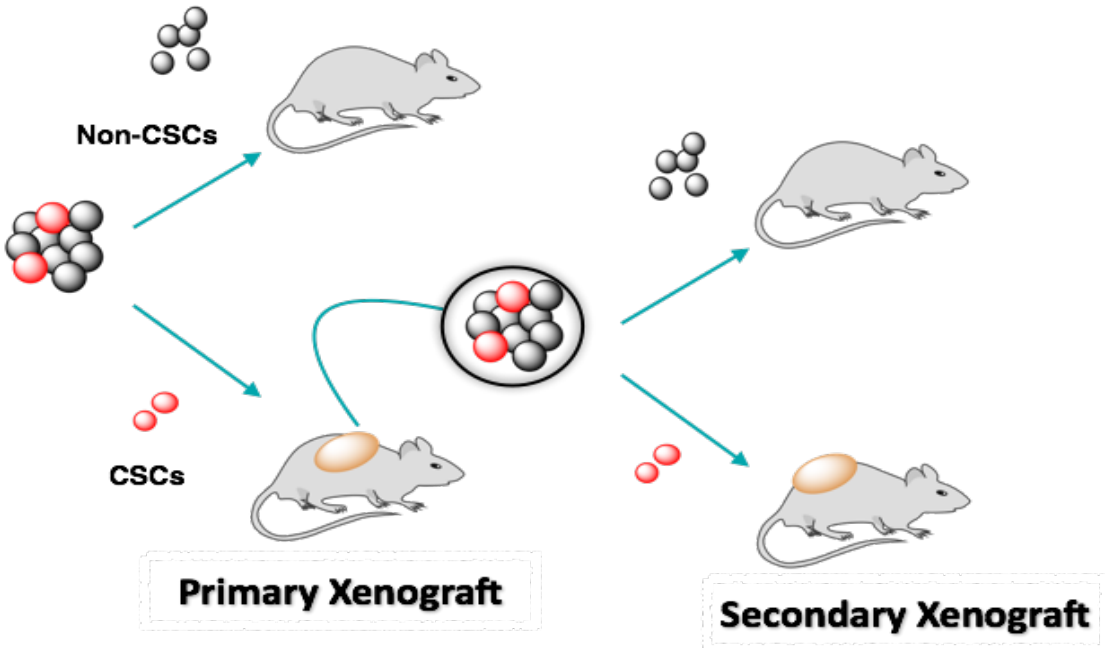

Figure 4. *In vivo* xenograft is the golden standard for CSC study. Theoretically, only CSCs can generate xenografts in the murine model; such xenografts can be further passed to the next generation.

2.2.3.3 *In vitro* sphere culture of CSCs: an effective surrogate for stem cell study

In order to conduct functional assays to investigate the key factors in the CSC-related pathways, large amount of CSCs would be needed. However, given the rarity of CSCs in tumors (sometimes less than 1%), this is hardly achievable. Thus, a simple and effective method is needed for the advance for this field. *In vitro* cell sphere culture has been increasingly used for the enrichment of study of CSCs [87-90]. Specifically, non-adherent, three-dimensional (3D) tumor spheres can be grown under serum-free condition after a period of time (usually 7-14 days). By quantifying the number of spheres in a specific sample, the sphere-forming ability (also as known as the *in vitro* tumorigenicity) of CSCs can be further estimated.

In a mechanism study aimed to elucidate the possible regulators in the maintaining of the "stemness" of colorectal CSCs, O'Brien *et al*. tested the soundness of the *in vitro*

sphere culture assay [85]. Specifically, they compared the CSC- enriching efficacy in both sphere forming (serum-free) medium and colony- forming medium (with 10% FBS). Both of the cells from above two culture mediums were then further compared to cells obtained from in vivo xenograft model. According to their findings, the frequency of sphere forming cells is only 6- fold greater than the frequency of CSCs from the *in vivo* xenograft; while the frequency of colony- forming cells was almost 88- folder higher. This showed that the frequency of the sphere-forming cells was much closer to the "CSCs" derived in the *in vivo* xenograft model. The authors then determined whether the sphere-forming and colony-forming cells still possessed tumor-initiating capacity. By injecting both these group of cells into immunocompromised mice model using LDA, they found that the cells from sphere- forming group showed significant potency in generating new tumors than the colony-forming counterparts. In other words, the results provided solid proof that *in vitro* sphere culture assay can be highly trust to reflect the "real" CSCs frequency as would be generated by the golden standard: *in vivo* xenograft model [85]. So far, multiple independent research groups have been using *in vitro* tumor sphere formation assay as an effective surrogate in the study of CSCs, especially in exploring the molecular mechanisms, where large amount of samples are required [84, 91, 92].

However, given the complexity of CSC, for example, the fact that CSCs are closely related to their microenvironment (see Chapter 2.2.4), there are also some researchers are questioning the soundness of the *in vitro* sphere culture as a valid tool for CSC study [93]. As a result, more progress in the CSC biology would be required for a better evaluation and/or even modification of our current study models; it is also possible that

the rising of novel study models would provide us a better insight of the "stemness" of CSCs.

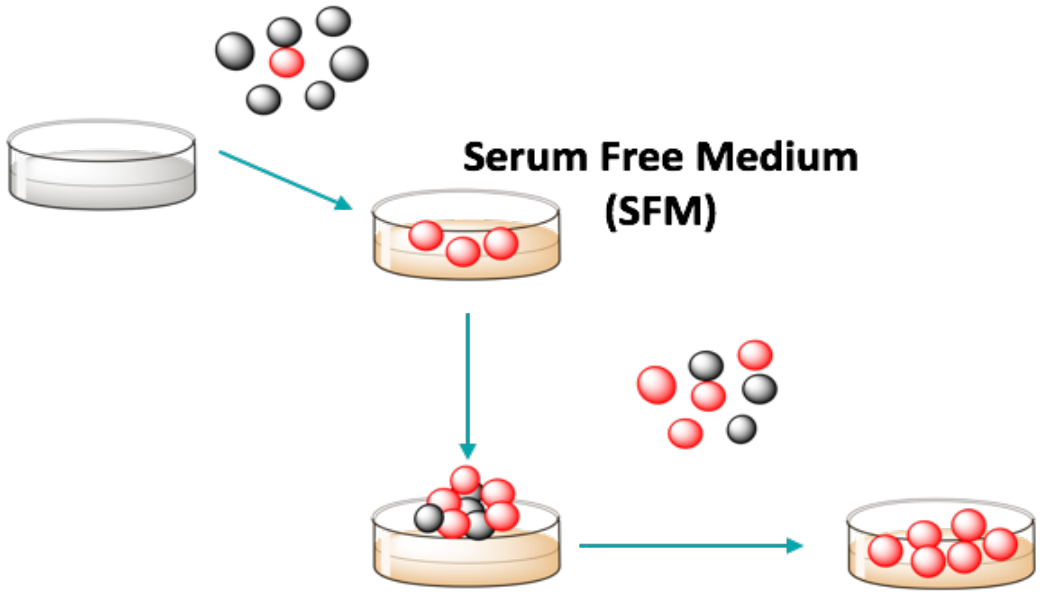

Figure 5. *In vitro* tumor sphere formation is an effective surrogate for CSC study. Theoretically, only CSCs can grow in the SFM and form 3- dimensional unattached spheres. Each tumorsphere are believed to have derived from one or more CSCs.

2.2.4 Multiple layers of complexity

2.2.4.1 CSCs and microenvironment (niche)

In the study of cancer biology, the "seed and soil" hypothesis has been used to explain the close relationship between tumor cells (the seeds) and the microenvironment (the soil) of which they can survive; plus, it also helps us to understand the preferentially metastasis of certain cancer (e.g. colon cancer would preferably metastasize to the liver) [94]. According to this hypothesis, there is a "receptor-ligand" like interaction between the tumor and its local non-malignant cells including inflammatory cells, hematopoietic cells, associated stromal cells and vasculature [95]; such interaction enables the activation

of self-renewal pathways, which will eventually re-initiate the tumor growth in other site [96].

Accumulating evidence are supporting that just like normal adult stem cells, CSCs also exist in an specific microenvironment: the CSC niche. Depending on the individual tumor type, such niche may play either an inductive or selective role in regulating the function of CSC [95]. So far, the brain CSCs and their perivascular niche is one of best-characterized model. Notably CSCs in glioblastoma have been shown to secrete VEGF which help supporting the development of local vascular cells [97]; while on the other hand, endothelial cells would secrete nitric oxide which induces the Notch signaling in glioma cells. Such bi-directional relationship has also been supported by other studies [98-100]. In the study of colorectal cancer, the interaction between CSCs and their niche is also supported by the fact that chronic inflammation which favors the cancer development [101], is closely related to the CSC sub-population [102]. A recent study has shown that the HGF-mediated activation of the Wnt signaling pathway from the colorectal CSC niche play a crucial role in defining the CSC state and thus are responsible for the induction of certain CSC phenotype [103]. In other words, this study suggested that the microenvironment could actually govern the tumor cell "stemness". It has been proposed that targeting of myofibroblasts or the HGF/c-MET pathway would be able to interfere with the maintaining of the CSCs and thus prevent the possible induction of CSCs from the non-CSCs population [95].

Understanding the role of CSC niche will be beneficial in at least following two aspects: 1. It will help us to evaluate of the current study model. As has been pointed out by Kreso *et al.* [28], the xenograft, even though regarded as the golden standard of CSC

study is still facing quite some limitations (see **Chapter 2.2.3**). Basically, xenograft is simply growing human cells in mice (the alien environment), where the microenvironment could be possibly different from that in human setting. Different microenvironment may have completely different impact on CSC behavior. Besides the across-species challenges, studies have also shown that even with one specie, the injection sites should be carefully selected since they may play very crucial role in influencing the frequency of CSCs. For instance, the study by Stewart *et al.*, showed that compared to the ovarian bursa, there is much higher tumor-initiating frequency in the mammary fat pad [104].

Furthermore, the cross talk between the CSCs and the microenvironment, not only has imposed another layer of complexity of the CSC- study, more importantly, findings support the notion that selectively or adjunctively targeting the CSC microenvironment may be used as an alternative for the CSC-targeting therapy [95].

2.2.4.2 Heterogeneous within the CSC pool: Tracking single CSC down

As has been extensively reviewed in chapter 2.2.1, the intratumoral heterogeneity is one of the major factors hampering the current search of new effective therapeutic target against cancer. Still, question arises regarding whether (functional) heterogeneity also exists within the pool of CSCs. If this is true, it will definitely put another layer of complexity to the study of cancer stem cells.

Generally, current CSC-studying models such as surface marker expression and xenografting assay are unable to answer the question above; in other words, “pure” CSCs still cannot be isolated and studied individually. With the advancement of technologies such as gene sequencing, the tracking of CSCs at the single cell level is achievable and

has been utilized in many studies. Tracking CSC down at the single cell level has been believed to further reveal the nature of the potential heterogeneity based on the knowledge from genetic fingerprint of each tumor-initiating cells.

As some of the recent publication have suggested, the overall effect of certain treatment, would rather mask the cellular variation among those CSCs seemly to be homogeneous [44]. A study of the Leukemic stem cells (LSCs) in acute lymphoblastic leukemia showed that such LSCs not only were genetically variable, but also demonstrates different degree of "stemness" (self-renewal and leukemogenicity)[105].

It is until recently that progress has also been made in the study of solid tumors. By the combining use of both lentiviral marking and xenografting techniques in NOD/ SCID mice, Kreso *et al.* showed that rather than being a homogeneous system, the CSC subpopulation within the colorectal cancer is consist of tumor-initiating cells with diverse functionality; moreover, a gradient in proliferation in proliferation and self-renewal kinetics in the colorectal cancer-initiating cell pool was also observed [28, 106]. Interestingly, the authors also found in their study that, as some of the cells within the CSC pool retained the extensive self-renewal ability for as long as a year, there are still some cells preserve their self-renewal potential for a very short period [28, 106]. This pattern is similar to those have been observed in normal stem cell system or leukemic stem cells [105, 107].

In summary, multiple studies have already shown that extensive cellular variability also exists within the CSC population, which was thought to be a uniform system. So far it is not clear whether this heterogeneity would have any implication on the therapeutic outcome of the current anti-CSC strategies. Evidence are still vastly lacked regarding

whether all the cells within the CSC pool will be responded equally to certain therapy aimed to targeting one or more molecular site [44]. As a result, it is of great importance for us to take into consideration that the CSC subpopulation is an actually dynamic heterogonous system and therefore the effects of certain anti-CSC strategy should be carefully interpreted.

2.3 Targeting colorectal CSCs

2.3.1 CSC and the clinical relevance

With the rapid advance of modern technology, researchers have had more "weapons" than ever before to fight against cancer. However, in spite of the consistent efforts in developing novel therapies for the treatment of cancer, we generally fail to improve the patient overall survival [108, 109]. Aggressive tumor growth, which is often linked with metastasis of malignant tumor to remote sites, contributes mostly to the poor prognosis for overall survival [110]. There is growing amount of evidence supporting that the cellular heterogeneity found in tumor, is one major factor that hampers the search for new therapies for cancer. In other words, the "stemness" properties of CSCs enable them to be highly relevant to the clinical outcome.

So far, it is still controversial regarding the soundness of using CSC surface makers as predictors of clinical outcome of cancers. For instance, a meta-analysis of totally 15 studies shown that high expression of CD133 was indeed an independent prognostic marker for over-all survival and disease free survivals; in other words, CD133 over-expression was not significantly related with multiple clinicopathological parameters [111]. However, according to another study, it has been shown that CD133 expression is correlated with poor prognosis in colorectal cancer [112]. Similarly, it was found that

leukemia-initiating cell fractions defined by functional assays from human patients, was highly related to the clinical outcome; however, such correlation was not observed when using phenotypic surface markers to define the L-IC fractions [58]. This study showed the importance of functional assays in the CSC research and also supported the notion that using of surface marker as "clinical predictor" is sometimes insufficient or even unreliable.

Plus, recent gene expression profiling study was able to provide more insights into the prognostic significance of CSCs. In a colorectal cancer study, the receptor tyrosine kinase EphB2, a Wnt target gene, was used to characterize CSCs from colorectal tumors in human patients [60]. Interestingly, the study showed that compared to other current clinical predictors, the high expression of EphB2 is much more efficient in predicting tumor relapse in patients after two years of treatment [60]. According to similar findings reported by Pece *et al.*, the purified stem cells sub-set in the mammary tumor is one of the major factors distinguishing breast cancers of different grades and molecular types from each other; notably, by combining the observations in the expression of surface marker as well the functional assays such as xenograft, they concluded that poorly differentiated tumor sub-types contained higher portion of CSCs population compared to their extensively differentiated counter-part [113].

As a matter of fact, there is so far still very limited number of publications regarding clinical trials incorporated with the study of CSCs. In a breast cancer study, Li *et al.* compared the potential change of the CSC subpopulation after traditional chemotherapy alone and those with the addition treatment of lapitinib [114]. According to their results, only the additional treatment of lapitinib resulted in the change of phenotypic marker

expression, more importantly, a functional change was also observed through the xenograft-forming assay. In spite of the fact there are still no clinical studies on colorectal CSC have been published yet, efforts have been already made in elucidating the clinical relevance between CSC and colorectal cancer. One of the pioneering studies by Kreso *et al.* showed that human colorectal CSC function is highly dependent on BMI-1 [106]. The down-regulation of BMI-1 resulted in a significantly decrease of the self-renewal properties in the colorectal CSC, which in turn lead to the effective control of the *in vivo* tumor growth [106].

In summary, despite the fact that there is still yet an agreement among researchers regarding whether all the tumor types would be following the CSC model; however, the interests on the CSC and their clinical relevance have been accumulated in rapidly in recent years. Particular, data are supporting rather than the changes phenotype changes, it is the functional signature, namely, the "stemness" of CSCs that is closely linked to the clinical outcomes such as prognosis and therapy failure [60, 115]. Such "stemness" nature enables CSCs to make a balance between proliferation, survival and differentiation and thereby render them the resistance against conventional radiotherapies and chemotherapies. One vivid analogy by Winquist *et al.* stated that if the all the cells and signaling events can be seen as an endless train consists of a string of boxcars; targeting cancer stem cells is just like removing the locomotive engine. Even though the extricating of the engine might not impact the size of train immediately, however it would stop the train from further activities such as metastasis and relapse [116]. In order to develop more effective CSC- targeting strategies, it is very importance to gain a better understanding of

the possible molecular mechanisms through which CSCs exert their resistance against conventional therapies.

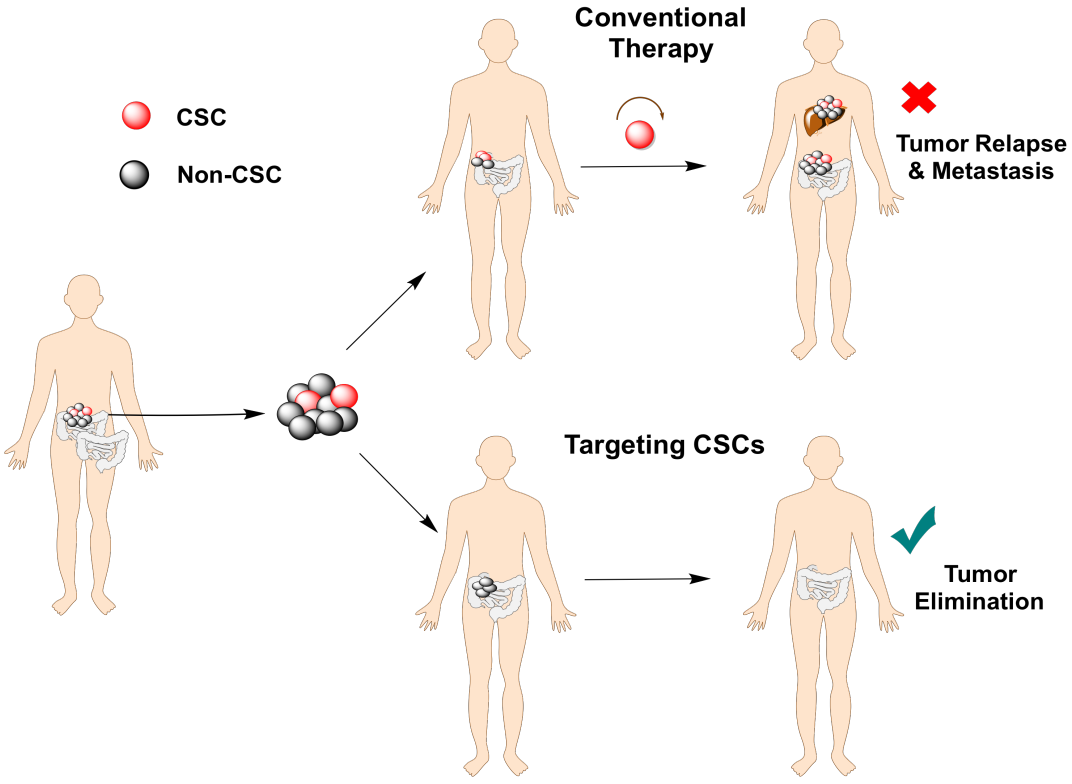

Figure 6. The clinical relevance of CSC. Conventional therapies may be efficient in eliminating the "bulk " of cancer cells (de-bulking), however, with CSCs been left behind, tumor relapse and metastasis will be followed; Only by targeting CSCs, tumor elimination can be achieved.

2.3.2 CSC and its therapeutic resistance: possible mechanisms

To date, there have been increasing number of studies suggesting that the CSC subpopulation demonstrates a stronger capability in resisting both the radiation and chemotherapies than the non-CSC counterpart in many solid tumor types[117, 118]. Plus, Todaro *et al.* observed in their study that CSCs and non-CSCs responded differently when treated with traditional chemotherapeutic agents such as 5- fluorouracil and Oxaliplatin. Multiple mechanisms could be account for the resistance of CSCs.

Firstly, It has generally been accepted that many cancer are resistant to radioactive treatment due to the existence of CSCs [119-121]. In a study of glioblastomas, Bao *et al.* showed the radioactive resistance of $CD133^+$ CSCs could be explained by the up-regulation of the DNA damage response (DDR). The authors further reported that such resistance could be vastly weakened by inhibition of DNA damage checkpoints Chk1 and Chk2. Such Chk1/2 response would therefore have great targeting potential in the development of the CSC-sensitizing agents in the response of the radiation therapy [109, 117].

Conventional anti-cancer therapies (chemotherapies/radiotherapies), may be effective at de-bulking the tumor, where they target proliferating cells and require active cycling for induction of apoptosis [122]; while for CSCs, as cells with the quiescence or slow cycling nature, would survive the conventional therapies and have the potentials to re-enter the cell cycle and re-establish a new tumor in situ (tumor relapse) or at a distant site (metastasis) [122]. Even though the detailed information on the specific signaling pathways through which the slow cycling CSCs exert the death evading behavior is still limited, some of the key regulators of the quiescence of CSCs have been proposed by some groups. For example, in the colorectal cancer cell line HCT116, p21 null cells were shown to have 10-fold lower tumor initiating ability compared to the normal p21 expressing cells [123]. Specifically, the authors found that the p21 null cells were unable to form tumor spheres *in vitro.* The p21- dependence sphere forming was further demonstrated to be related to the lack of expression of E-cadherin and suppression of apoptotic signals. The author further proposed that besides the tradition role of p21 in the cell cycle regulation, it also could be obtaining the “pro-tumorigenic” capacity [123]. As

a result, any agents targeting p21 or its down-stream genes, would possibly be able to break the quiescent status of CSC and thus force them to re-enter cell cycle or undergo apoptosis; once becoming “cycling ”, CSCs would be much more likely to be eliminated [122]. In a word, the quiescent (slow-cycling) nature of the CSC subpopulation, would at least partially account for their resistance against the conventional therapies.

For both normal stem cells and CSCs, the ATP-binding cassette (ABC) transporters serve as drug efflux pumps, which enable cells survive a wide range of xenobiotics [124]. As a result, the ABC transporters have also been known as “guardians of CSCs” [125]. High levels of ABC transporters would mostly, if not always, be a sign of poor prognosis. For example, some researchers have observed reduce in the 4- year survival rate of patients expressing high level of ABCC11 [126]. Other ABC transporters including multidrug resistance transporter (MDR1) and breast cancer resistance protein (BCRP) also have been proved to be CSC related. Specifically, MDR1 has been suggested to remove vinblastine [127] and paclitaxel [128], while BCRP helps avoid the accumulation of imatinib mesylate [129], and methotrexate [130] . So far, evidence have already supported that the over-expression of BCRP could be directly related in multiple human tumor types, such as liver cancer [131] and breast cancer [132].

As mentioned above, targeting DNA damage response (DDR), ABC-transporters, and multiple key regulators in the quiescence nature of CSC could be promising strategies to overcome the drug resistance in CSC. Still, there are many other possible mechanisms can be account for the radioactive/ chemo resistance of CSC. For example, ALDH, (see chapter 2.2.3.1) as an important CSC surface marker, has also been related to the resistance of CSCs: high ALDH1 activity has also been accounted for the

resistance of chemotherapy for multiple tumor types. In a study of the colorectal cancer, Dylla *et al.* found that the tumor-initiating ability was higher in the $ESA^{+}CD44^{+}ALDH1^{+}$ than in the $ESA^{-}CD44^{-}ALDH1^{-}$ cells. Interestingly, the inhibition of the ALDH1 activity by either short –hairpin RNA interference (RNAi) or the specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) was unable to directly induce significant decrease in the xenograft growth; however, such inhibition of ALDH did result in the increased sensitization of cells to the treatment of cyclophosphamide, and led to a significant decrease in the xenograft growth [73]. Similar results were also observed in other studies: for example, sulforaphane (a bioactive compound found in cruciferous vegetables such as broccoli) has been shown to be able to reduce the ALDH activity thus render the $CD44^{+}ALDH^{+}$ cells sensitive to chemotherapy /radiotherapy [133]. In a study of colorectal cancer, Todaro *et al*. found that the colorectal CSCs were chemosensitized by an interleukin-4 blocking antibody. This suggested that autocrine stimulation of IL-4 receptors on CSCs may contribute to their chemo resistance and thereby would render a promising alternative strategy to sensitize CSCs to cytotoxic chemotherapies [84].

With the accumulation of our understanding of the molecular mediators that regulate the therapeutic resistance in CSCs, it would be rational to prioritize the targeting of such mediators. However, it should also be taken into account the complexity of the CSC system: for example, some study indicated that the mechanism of chemotherapy resistance is possibly drug-specific [73]; moreover, it is still not clear that whether certain mechanism of resistance could be applied in different tumor type models. To better decipher such mechanism, deeper insights of the CSC biology will be required.

With the understanding of the stemness as well as its implication on the clinical outcome such as the failure of the conventional therapies, more and more focus have been focused on the development of novel anti-cancer therapies targeting CSCs. So far, the direct targeting of CSC can be achieved by inhibiting self-renewal pathways or by interventions on the cell death pathways such as apoptosis and cycle regulation [134].

2.3.3 Targeting self-renewal signaling pathways in CSCs

The application of CSC surface markers, as many researchers believes, can only take us so far in terms of the studying of the very nature of CSC [135]. As has been discussed in detail above, the fundamental biological property of CSC is self-renewal. Upon cell division, self-renewal enables a stem cell to generate one (asymmetric division) or two (symmetric division) daughter cell, which maintains the long-term tumor growth. The capacity of self-renewal is actually the only property that distinguishes CSCs from the rest of tumor cells. Therefore, targeting the self-renewal has been proposed as a promising therapeutic goal [106, 136-138]. With the advances in understanding signaling pathways in normal intestinal stem cell in recent years, the knowledge of how such pathways are regulated or deregulated in colorectal CSC is till vastly unknown. Several transgenic studies in murine model have already proved the close relationship between activation of the self-renewal pathways and the maintenance of stemness [139, 140]. So far, studies on the human CSC xenografts models are still vastly limited.

Among multiple deregulated self-renewal pathways are functioning to maintain the CSC properties, the WNT pathway, the Notch pathway, and the Hedgehog pathway are generally considered as key players [141].

The aberrant WNT/ β-catenin pathway, first studied by Jamieson *et al*. was shown to be the key driving force of human blast crisis leukemia stem cell (LSC) [142]. Similar results were obtained later in solid tumor study. For example, Vermeulen *et al.* identified WNT activity to be one of the defining features of colorectal CSC. In their study [103], only cells with highest level of WNT pathway activation possess CSC properties. In addition, by genetic knockdown of protein phosphatase and tensin homolog (PTEN), Korkaya *et al.* found the increase in WNT/ β-catenin activity, which indicated that self-renewal of breast cancer stem cell was mediated by β-catenin signaling [143]. 90% of colorectal tumors have a mutation in key regulator (such as *APC*) of the WNT/ β-catenin pathway, which result in a enhancement transcription of WNT target gene [144]. A recent finding found that a *KRAS* mutation could upregulated the WNT/ β-catenin pathway in the genetic background of *APC* loss, which generates cells with CSC phenotype and elevated metastatic potential [145].

Notch pathway is another important player in colorectal CSC growth and differentiation [146]. Evidence have supported that Notch Pathway is required for normal ISCs homeostasis in mouse. Recent study has shown that inhibition of one of the important components of Notch signaling pathway, Delta-like 4 ligand (DLL4), resulted in the growth inhibition of human colon cancer xenografts [147]. Besides, Sikandar *et al.* also found that the elevated level of Notch signaling in colorectal CSC, followed by the prevention of apoptosis and maintenance of an undifferentiated state [148]. The essential roles of Notch pathway activation have also been observed in CSCs from multiple solid tumor types, including breast [147, 149] and glioblastoma [150].

Hedgehog signaling pathway is a known regulator in organ development. It was firstly reported that activated Hedgehog signaling pathway could be observed in pancreatic CSC [65]. Evidence that support its role in the regulating the self-renewal capacity have been accumulated in pancreatic, glioma and leukemic, colon and other solid tumor CSC models [151-154].

The proper function of the self-renewal also depends on one polycomb group protein Bmi-1. Bmi-1 is one of the first regulators, which are closely associated with self-renewal and involved in the maintenance of stem cell in multiple tissues [139, 155, 156]. Evidence have been accumulated supporting that Bmi-1 is highly expressed in head and neck CSCs [157]; genetic knockdown of Bmi-1 has been resulted in the impairment of self-renewal property in both breast [158] and brain [159] xenograft models. As has mentioned above in **Chapter 2.3.1,** a most recent study by Kreso *et al.* showed that the down regulation of Bmi-1 with a small molecule inhibitor had led to the inhibition of the self-renewal capacity in colorectal cancer stem cells, suggesting Bmi-1 as a therapeutic target in the control of colorectal cancer [106].

Self-renewal Pathways	Key regulator	Potential natural inhibitors	Reference
WNT	E-cadherin	Vitamin D	[160]
	β-catenin	EGCG	[161] [162]
Notch	β-catenin	Curcumin	[11, 163]
	DLL4,	Curcumin	[164]
	miR-21, miR-34	Curcumin	[165]
Hedgehog	Gli-mRNA	Curcumin	[166]
	Cyclin D1, cyclin E	Curcumin	[167]
Bmi-1	Bmi-1	Unknown	[106]

Table 2. Current studies on targeting the self-renewal pathways of CSCs.

2.3.4 Targeting cell death pathways in CSC: apoptosis and necroptosis

2.3.4.1 Apoptosis and CSC-regulation

With several research have been focused on the regulation of self-renewal, proliferation and differentiation of CSCs, studies of cell death pathways including apoptosis are still quite limited [168].

Apoptosis, by definition, is the active, strictly regulated programmed cell death, which plays an important "protective" role in the function of normal cells. Apoptosis evasion is one of the hall-markers of cancer [42]. Generally, two major pathways trigger apoptosis: the extrinsic and intrinsic pathways. The extrinsic pathway is active by the binding of extracellular pro-apoptotic ligands to cell surface receptors (death receptor) such as CD95, nerve growth factor receptor (NGFR), and TNF-related apoptosis inducing ligand (TRAIL) receptors [169]. The binding of such death receptors, are then followed by the recruitment of the adaptor molecule Fas-associated Death Domain (FADD) and caspase-8 to form the death inducing signaling complex (DISC). The caspase 8 get activated and initiates apoptosis through cleavage casepase-3 [170].

The intrinsic pathway is also known as mitochondrial pathway. It is initiated by a serial of stresses which generate damages in both cellular and DNA level, such stresses including anticancer drugs, growth factor withdrawal and hypoxia [168]. The stimuli then lead to a mitochondrial permeabilization and the activation of mitochondrial pathway. Thus, the permeability of mitochondrial membrane is a key factor in the apoptosis cascades and mediated by B-cell leukemia/ lymphoma 2 (Bcl-2) family proteins [171]. With the change of the permeability of mitochondrial membrane, apoptogenic factors including cytochrome *c*, apoptosis inducing factor (AIF), second mitochondria-derived

activator of caspase (Smac), get released from inter-membrane space into cytosol [172]. These are then followed by formation of the cytochrome *c* / Apaf-1/casepase-9 complex which will trigger the activation of caspase-3, leading to apoptosis eventually [173]. Smac promotes caspase activation by neutralizing inhibitors of apoptosis protein (IAP), which stands for the last protective measure against both extrinsic and intrinsic apoptosis.

In cancer, multiple key steps with the intrinsic and extrinsic pathways are extensively deregulated. Recently, evidence starts to accumulate supporting the apoptosis pathways are also deregulated in CSCs. For example, the death receptor (DR) 4 mediating the extrinsic pathway has a high expression in chemotherapy resistant colon cancer side population (SP) [174]. Since that many tumors have p53 inactivating mutations, stimulating of DRs has potential application as therapeutic target. The FLICE-like inhibitory proteins (cFLIPs) are a group of negative modulator of DR induced apoptosis [175]. Recently, it has been reported that cFLIPs are upregulated in $CD133^+$ sub-population of glioblastoma [176] and breast cancer [177].

The Bcl-2 family of proteins consists of both anti-apoptotic proteins (Bcl-2, Bcl-X_L, Mcl-1) and pro-apoptotic proteins (Bax, Bik, Noxa and Puma) [178]. Notably, it is the imbalance in the ratio of anti- to pro-apoptotic molecule rather than the expression of any expression of particular molecule that result in cell survival and the sensitivity to apoptotic stimuli [178]. There is emerging evidence supporting that the resistance of stem cell to apoptosis is partly through the regulation of Bcl-2, while non-tumorigenic cells in tumors are more susceptible to the induction of apoptosis or chemotherapy [179-181]. For instance, it was reported that $CD133^+$ gliomas CSCs express a high level of anti-apoptotic proteins Bcl-2 and Bcl-X_L [176]. Plus, the high expression of Mcl-1 is closely

related to the resistance of glioma CSCs to the small molecular inhibitor of Bcl-2, ABT-737 [182]. Additional studies have indicted that downregulation of Bcl-2 or upregulation of Bax induces apoptosis of CSCs [183, 184]. All these data above support the important role of mitochondrial apoptosis pathway in maintaining CSC population thus become a promising target for preventive/ therapeutic interventions [168, 172].

Survivin, a member of the inhibitor of apoptosis (IAP) family, have been shown to regulate cell proliferation, apoptosis and pluripotency [185, 186]. Other publications have also been focused on other upregulated IAP proteins including XIAP, Livin in human CSCs [176, 187].

2.3.4.2 Necroptosis and CSC-regulation

As has been discussed above, apoptosis has been demonstrated as a conserved pathway that regulates programed cell death. However, evidence has been accumulated supporting that apoptosis is not the only mechanisms that could control the cell death. Specifically, necroptosis, also known as the programmed necrosis, has gained more attention in recent years. Similarly as necrosis, necroptosis has been characterized by the loss of membrane integrity, rapid cell swelling and eventually lead to cell lysis; however, besides the necrosis-like morphology changes, necroptosis has been believed to maintains part of the apoptotic features as apoptosis [188, 189]. Compared to apoptosis, necroptosis is mostly independent of the caspase activity. Even that the understanding of the biochemical events mediating necroptosis is still very limited, some recent studies showed that necroptosis and apoptosis share some of the upstream cell signaling pathways. Notably, necroptosis might be activated by tumor necrosis factor α (TNFα), Fas ligand (FasL) and TRAIL, which are also ligands that initiate apoptosis [190]. Such

observation provided the notion that there might be a complex cross-talk between the apoptosis and necroptosis pathways. Interestingly, caspase 8 seems to be playing an opposite role in such cross-talk: TNF activate apoptosis by triggering a series of molecular events which eventually lead to the activation of caspase 8; while in necroptotic pathway, the function of caspase 8 is disrupted or even inhibited [191]. By far, some members of the receptor interacting protein (RIP) family, especially RIP-1 and RIP-3, have been suggested as the key molecular regulators of necroptosis [192, 193].

Recent studies supported that necroptosis is closely related to multiple diseases including inflammatory bowel diseases [194, 195]. Thus, targeting necroptosis-related pathways might as well of important therapeutic value [196]. In light of the significant of necroptosis in the cancer development and control, the role of some anti-cancer drugs has been "rediscovered": for example, curcumin, a well-known pro-apoptotic compound [197], has been found be involved in a complex signaling network, where it might regulate both apoptotic and necroptotic cell death. Particularly, the treatment of curcumin has been shown to induce ERK or p38/ c-jun N-terminal kinase (JNK) phosphorylation at early time points; such induction of JNK phosphorylation would then trigger the formation of Reactive oxygen species (ROS), which activate necroptotic cell death in prostate cancer cells [198].

So far there is no direct studies on the clinical relevance of necroptosis and the CSC-targeting therapeutic/ preventive strategies. However, evidence has shown that certain necroptotic regulators also play important roles in the CSC biology. For example, a recent research has shown that TNFα enhances cancer stem cell-like phenotype via Notch-Hes1 activation in oral squamous cell carcinoma cells; particularly, data showed

that TNFα was closely related to the increase in tumor sphere-forming ability, stem cell-associated genes expression, chemo/radio resistance, as well as tumorigenicity. On the other hand, TNFα also plays a central role in the necroptotic regulation [199].

In summary, apoptosis might not be the only cellular mechanism that would regulate cell deaths. Necroptosis, as well, has been suggested as an alternative cell death pathway especially when apoptosis fails because of the ATP failure (apoptosis is the most energy-consuming process since that it requires the activation of multiple caspases and the formation of apoptosome) [200-202]. The cross talk between the multiple death pathways, therefore need to be further understood in the development of CSC-targeting strategies.

2.3.5 Targeting the cell cycle compartment in CSC

In mammalian cells, multiple surveillance checkpoints monitors the cell cycle, which determines whether cells will continue proliferation or stop diving at certain stages. In cancers including colorectal cancer, genes regulate cell cycle progression are often mutated. Various regulators such as cyclins, cyclin-dependent kinases (e.g. CDK-4, CDK-2) and cyclin-dependent kinase inhibitors (CKIs, including p16, p21, and p27) are playing pivotal roles in controlling cell proliferation. Evidence also supported that disruption of cell cycle inhibition may also contribute to the very nature of CSCs, especially in breaking the balance between the self-renewal and differentiation, which lead to the deregulated self-renewal of CSCs [203].

The DNA damage response (DDR) in response to the chemo- and radiation therapy, is a known mechanism that activates the tumor suppressor p53-induced cell cycle arrest, apoptosis and DNA repair [204] (**see Chapter 2.3.2**). Moreover, there is more and more

evidence supporting that cellular senescence, by assisting DNA repair machinery, promotes tumor progress. A good example will be that Bao *et al.* [117] found that the radio-resistance in gliomas has been associated with the activation of DNA repair in the $CD133^+$ population in both human xenografts and primary patient sample. Notably, the activation of DNA repair can be revered through inhibiting the DNA damage checkpoint kinases, Chk1an Chk2. Interestingly, a recent study in mesenchymal stem cell (MSC) have shown the tumor-promoting role of $p21^{waf1/cip1}$ [205]. Particularly, the lack of $p21^{waf1/cip1}$ in the background of p53 wild type led to accelerated apoptosis and inhibit tumor formation, while its $p21^{waf1/cip1}$ positive counterpart would result in cell senescence. Plus, the author also discovered the important connection between the loss of p53 and the lack of senescence, which is followed by increased tumor growth rate. Another study further demonstrated the role of $p21^{waf1/cip1}$ in maintaining self-renewal in human leukemia stem cell. According to the study, the DNA damage induced by oncogene activated $p21^{waf1/cip1}$ dependent cell cycle arrest [206]. In a study on colorectal CSCs, O'Brien *et al*. have identified multiple inhibitor of DNA binding proteins (IDs), mainly ID1 and ID3, play a critical role in the maintenance of CSC population. Specifically, they have found that ID1/ID knockdown impaired the tumor-initiating capacity of colorectal CSCs and also increased their sensitivity to the treatment of a commonly used chemotherapeutic agent, Oxaliplatin. They further discovered that $p21^{waf1/cip1}$ might function to maintain ID1/ID3-dependent tumor-initiating potential [85]. The dependence of $p21^{waf1/cip1}$ was also observed in colorectal cancer cell line HCT116 under sphere-forming conditions [123]: p21 null cells were not able to form spheres, ceased proliferation, and eventually died. The authors also found that such p21- dependence was related with lack of E-cadherin

expression and suppression of apoptosis signals, which suggested that p21 might play a complex role in tumor cells. It has been proposed that some small molecules targeting p21 or its downstream targets would be promising to force quiescent CSCs to reenter cell cycle or undergo apoptosis. Since that cycling CSCs would be much more susceptible to multiple stresses such as chemotherapy [122].

2.3.6 Targeting other CSC related pathways

A miRNA are small non-coding RNA, which can bind and inhibit the translation of targeted mRNAs [207]. They are generally considered as tumor- suppressors since they often regulate genes which involved in cancer cell differentiation, proliferation and anti-drug resistance [207]. Recently, mir-34 has been reported as suppressor gene in prostate tumor-initiating cells[208]. Similarly, mir-21 was overexpressed in glioblastoma cells; while in breast cancer, it functions as anti- apoptotic factor. The use of miRNA-based tool to target CSCs bears promising therapeutic value [209].

The P13K/AKT/ mTOR signaling pathway has been proved to be closely involved in CSC biology, especially in cell cycle progression and tumor survival [210]. Inhibition of Akt blocks the growth of Brain CSCs [211]. While for tumor-initiating cells in chronic myeloid leukemia, TGF-beta-FOXO signaling pathway is responsible for the maintaining of tumorigenic potential [212]. Targeting the P13K/AKT/mTOR pathway could be another effective strategy in therapeutically sensitizing CSCs as well as decreasing their frequencies in various tumor types [124].

The signal transducer activator of transcription (STAT) and NF-κB Pathways can be activated by several different cytokines and thus have been seen as potential target for cancer treatment [213, 214]. The study of Lin *et al.* showed that compared to the bulk of

cancer cells and $ALDH^-$/ $CD133^-$, $ALDH^+$/ $CD133^+$ cells in colon cancer expressed higher level of STAT3 phosphorylation. They further discovered that by treating with GO-Y030, an analogue of curcumin, the STAT3 phosphorylation was inhibited and thus resulted in reduced tumor sphere formation capacity of $ALDH^+$/$CD133^+$ cells [215]. The transcription factor NF-κB has been linked to various ontogenesis, including the inhibition of apoptosis by inducing the expression of survival factors [216, 217]. One of the examples of targeting the NF-κB pathway is that the use of Eriocalyxin B led to apoptosis in human ovarian CSC [218].

In addition, pluripotency transcription factors such as OCT4, SOX2 and NANOG have gained quite a lot attention because of their possible impaction on the acquisition of stem like state [219]. Plus, evidence has shown that they are also valuable markers of tumorgenesis and will function as molecular regulators that determine the fate of CSC during cancer development [220]. For example, OCT4 has been regarded as a new potential molecular marker in prostate tumors [221]. The overexpression of NANOG, on the other hand, can be used as a predictor of tumor progression and poor prognosis in colorectal cancer [222]. OCT4 and SOX2 are related to distant recurrence after chemo- and radio- therapy in rectal cancer [223].

Collectively, increasing efforts have been made in the development of pre-clinical /clinical interference based on the hypothesis that disrupted cell death pathways in CSCs could be account for the failure of existing therapies for cancers. More and more evidence begin to support that the targeting death pathways and the deregulated cell cycle progression (quiescence) in CSCs is of great importance in the search of novel preventive/ therapeutic solutions [224].

Type of CSC	Target	Potential natural inhibitors	Reference
AML	CD44	Unknown	[225]
			[226]
Brain	MAPK	All-trans retinoic acid	[182, 211,
	Bcl-2, Akt, XIAP	Unknown	227]
Breast	FAS,	Resveratrol	[228]
	P13K/Akt	Unknown	[143]
Colorectal	IL-4	Unknown	[229, 230]
	Akt	Sulforaphane	[231]
	STAT3	Curcumin	[232]
	STAT3	G0-Y030	[215]
Prostate	Akt	Sulforaphane	[233]
Pancreatic	SHH/mTOR	Unknown	[234]
	NF-κB	Genistein	[235]
	NF-κB	Sulforaphane	[236]

Table 3. Current studies on targeting the non-self-renewal pathways of CSCs in different tumor types.

2.4 Chemopreventive and CSC

2.4.1 Introduction to cancer chemoprevention

With the identification of cancer risk factors such as family history, obesity and excessive alcohol consumption and tobacco, researchers are putting more effort in developing preventive strategies to control the cancerogenesis [237]. Cancer chemoprevention, by definition, is the inhibition, delay and reversal of the carcinogenesis by the administration of one or more naturally occurring and/or synthetic agents. Historically, in despite of the huge investment of pharmaceutical companies to develop potent cancer preventive agents, there are so far only 10 of them have been proved by FDA [237]. As one of the most promising group of chemopreventive drug, non-steroidal

anti-inflammatory drugs (NSAIDs), have shown consistently protective effect on cancers especially colorectal cancer; yet the wide use of them are still limited due to the side effects such as a increased risk of bleeding [238]. Recently, the plausibility of naturally dietary compounds as cancer chemopreventive agents has been discussed extensively mainly for the following advantages [8]: 1. As most of them are present in food that people are commonly consumed, they are easily accessible and cost-effective; 2. compared to most drugs used in chemotherapy, they have low or even none toxicity. 3. as has been reported by several clinical trials, many of the dietary compounds have already been proved to effect adjunctively with chemotherapy drugs.

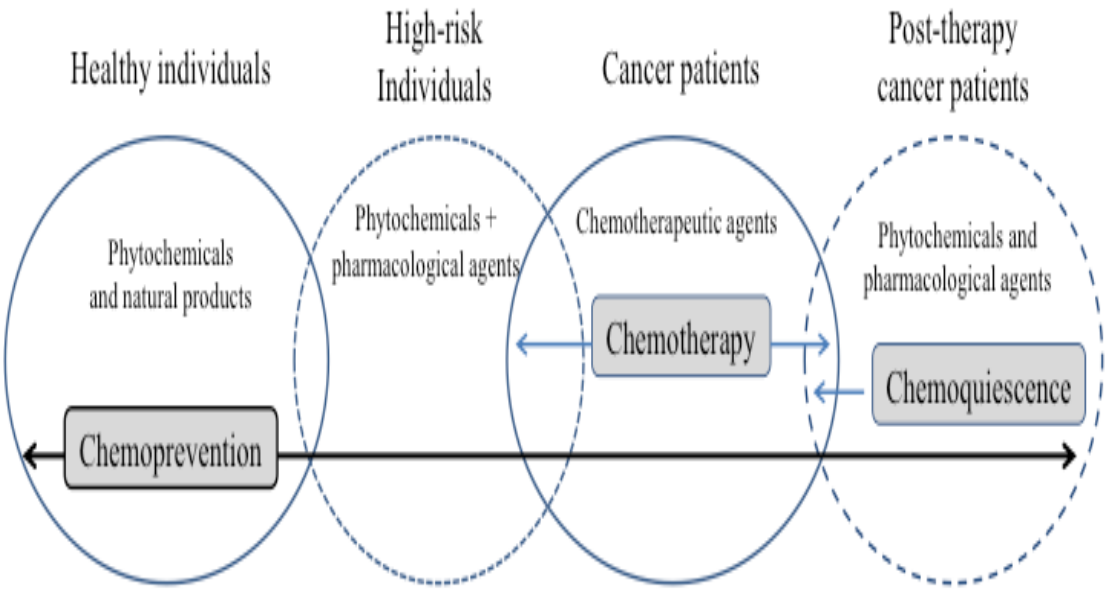

Figure 7. A schematic diagram displaying the selective responsiveness of healthy population as well as cancer patients to chemopreventive agent. Generally, chemopreventive agents should be beneficial for every population. In cancer patients, it is feasible that combination regimens of chemopreventive agents and chemotherapeutic agents should be utilized. Moreover, modification of diet along with pharmological intervention should be considered for retarding and inhibiting cancer recurrence in post-therapy cancer patients (Adapted from Mehta *et al.*, 2010) [239].

As has been reviewed previously, compared to other types of tumor the development of colorectal cancer usually requires a long time span, which provide us great opportunity for early detection and prevention [7]. Some evidence indicated that the recent increase in the survival rate of colorectal cancer was largely due to advances in early detection like colonoscopy and shift in the lifestyle, especially in the dietary pattern [2].

So far, there is a great number of studies in both cultured cell, animal models and human clinical trials that have supported the protective role of dietary compounds. For example, multiple natural polyphenols have been proved to be able to function against different type of cancers [9]. Generally, the compounds with chemopreventive properties can be categorized into several classes, for example, Carotenoid, Flavonoids, Isothiocyanates and omega-3 fatty acids. Pan *et al.* have reviewed the molecular mechanisms of chemopreventive effects of multiple natural dietary compounds on cancer [240].

2.4.2 Natural chemopreventive agents against CSC

According to the CSC model, the lacking of ability to control the CSCs is the very reason for the failure of traditional chemotherapies and cancer relapse. Therefore, targeting the CSC population has become very promising for cancer prevention and therapy [241]. Generally, this would be achievable through strategies such as inducing of differentiation, inhibiting of self-renewal signaling pathways and sensitizing CSCs to chemotherapeutic agents [8].

To make further progress in discovering and developing novel chemopreventive agents for effectively treatment of CSCs, it is of great importance to understand the mechanism through which the CSC can be affected by the dietary compounds. Though

still in the rudimentary stage, new findings in this field have been accumulated in a steady pace.

Curcumin is a well-studied dietary polyphenol abundantly enriched in turmeric, a traditional Indian spice for curry preparation [242]. Multiple bioactive functions of Curcumin including anti-oxidant, anti-inflammatory effect have been studied [243]. Plus, results from pre-clinical trial test has support that preventing and delaying of the progress of cancer is one of the most promising properties of Curcumin [244]. As discussed above, several signaling pathways including WNT/β-catenin, Hedgehog and Notch play a central in self-renewal of CSC. The WNT/β-catenin signaling pathway is closely related to the maintenance of CSC in leukemia, breast, colon and lung cancers [245-247]. Recent finding have shown that curcumin was able to attenuate the WNT/β-catenin signaling in colon cancer cell lines, and also curcumin at 5μM would inhibited 50% of WNT signaling pathway in human breast cancer (MCF-7) [163]. Curcumin also impaired the WNT signaling and cell-cell adhesion pathway in human colon cancer cell (HCT-116), which resulted in apoptosis and G2/M cell cycle arrest [11]. It has also been reported that demethoxycurcumin (DMC) and bisedmethoxycurcumin (BDMC), natural analogues of curcumin, can inhibit the WNT signaling pathway through down regulating of the expression of transcriptional co-activator p300 in several human colon cancer cell lines [163]. Moreover, according to an analysis of gene transcription profile, the expression of Frizzled-1, a WNT receptor, can be suppressed by curcumin [248].

The Hedgehog pathway is another key relator in CSC self-renewal and development, which have been supported by the observation in leukemic, pancreatic and glioma CSC [151-153]. Particularly, by the using of Cyclopamine, a Hedgehog pathway inhibitor, the

self-renewal capacity of CSC derived by tumor sphere formation assay in glioma was significantly inhibited [153]. Curcumin also inhibit the hedgehog pathway in mouse derived TRAM-C2 prostate cancer cell as indicated by the decreased expression of Gli mRNA[166]. Data from another study showed that curcumin encapsulated in nano-particular, can inhibit the expression of Gli mRNA [249]. In addition, hedgehog signaling also regulate cell proliferation by indirectly activation of cell-cycle related gene, such as cyclin D, and cyclin E, proteins that are related in G1-S phase transition [167].

The Notch pathway is related to organ development by regulating cell proliferation and apoptosis [250]. At the level of 5μM and 10μM, curcumin can inhibit the Notch-1 signaling pathway, resulting in apoptosis via the inactivation of transcriptional factor NF-κB in pancreatic cancer cells [164]. Notably, the inhibitory effect of curcumin on the Notch1 pathway was also supported by the observation-reduced expression of Notch-1 specific microRNAs such as miR-21 and miR-34 [165].

In addition, curcumin has also shown to regulate the STAT signaling pathways. As has been reviewed before, phosphorylation of STATs activate their function in regulating critical genes for cell cycle, cell proliferation, invasion and even maintenance of the tumor-initiating properties [232]. The findings of Lin *et al.* showed that the colorectal CSCs, characterized as $ALDH^+/CD133^+$ subpopulation, had higher level of STAT3 phosphorylation [232]. Similarly, curcumin has also been proved to regulate the IL-6 induced STAT3 phosphorylation in myeloma cancer cells [251]. Notably, compared to the curcumin itself, one of its analogues, GO-Y030 had much stronger effect in inhibiting the STAT3 phosphorylation and downstream gene such as cyclin D1, survivin and Bcl-XL in colorectal CSC [215].

Besides the effect on self-renewal pathways related to CSC biology, there are also some publications supporting that curcumin can also inhibit the tumor-sphere formation as well as the expression molecular surface makers [252-255]. The combination use of curcumin and the anti-cancer drug dasatinib, resulted in the decrease level of stem cell specific mRNA as well as the sphere formation ability; such combination of treatment, was in a synergistic manner [254]. Moreover, as resistance to cytotoxic drug is one of the major characters of CSC, it has been proposed that Curcumin can enhance the effect of anti-cancer therapeutics through multiple mechanisms [256, 257].

Sulforaphane, found abundantly in cruciferous vegetables (e.g., broccoli) is another promising dietary compounds that bearing cancer chemopreventive property. A recent study by Li *et al.* showed that a relatively low dose of sulforaphane could inhibit the sphere formation in both MCF-7 and SUM159 breast cancer cells, with an IC50 ranging from 0.5-1μM [133]; more importantly, evidence also support such dose used in their study was physiological achievable by normal dietary consumption [258]. One of the possible mechanisms through which sulforaphane exerts its inhibitory effects on CSCs is by interfering with tumor necrosis factor-related apoptosis inducing ligand (TRAIL) - activated NF-κB signaling [236]. Besides, various studies have reported that sulforaphane could also inhibit the Akt pathway in prostate and colorectal cancer [231, 233]. P13K/Akt pathway also plays a critical role in regulating breast stem cells [143].

Soy isoflavones, especially genistein, have also been reported to have potent anti-cancer effect multiple cancer types [259]. Data have shown that by inhibiting Notch signaling pathway, genistein reduced the NF-κB activity, resulting in a cell growth inhibition and apoptosis in human pancreatic cancer cells [164, 235].

Epidemiological studies have already supported that green tea consumption might have cancer preventive potential [260]. Results from several groups have shown that epigallocatechin-3-gallate (EGCG), the most abundant catechin found in green tea, could be a promising chemopreventive agent [261]. A recent study showed that the Wnt pathway was inhibited by the treatment of EGCG in human breast cancer cell [161]. Specifically, at the dose of 100μM, EGCG treatment lead to a 50% depression of WNT signaling pathway through the down-regulation of HMG box-containing protein 1(HBP1), a well- known WNT suppressor. With the inhibition of WNT, the tumorigenicity and invasiveness of breast cancer cell have been thereby weakened. Similar results were also observed by Zeng *et al*., who concluded that the high expression of WNT signaling pathway in breast cancer stem cell was crucial for the maintenance of their stemness [162]. So far many researchers have begun to connect the chemopreventive effective of EGCG to its function in suppressing WNT pathway in human breast CSC [10, 162].

Vitamin D_3, the active form of vitamin D, has been shown to induce apoptosis and cell cycle arrest in multiple types of cancer [160]. Recent studies have also reported that vitamin D_3 was able to inhibit the cancer stem cell signaling pathways in solid tumor [262]. One the possible mechanisms through which vitamin D_3 inhibit the WNT signaling was by inducing E-cadherin in colon cancer cells [160]. However, it is also worth mentioning that the inhibitory effect of vitamin D_3 also varies on different cell types and specific cell line [262]. In conclusion, vitamin D_3, it could be another therapeutic preventive dietary compound against CSCs.

As been reviewed above, even though there are still limited reports regarding the inhibitory effect of natural dietary compounds against CSCs, many of them have already

shown be actively regulating the CSC related signaling pathways (e.g. self-renewal). Notably, some compounds, such as curcumin and sulforaphane, have shown a promising effect on CSCs at a very low concentration [133, 263]; such observation would provide us with the notion that the chemopreventive effects can be achievable through either normal "healthy" diet or dietary supplements. Overall, the study of the therapeutic/ preventive effect of natural bioactive compounds depends on a better understanding of the CSC biology, especially in the cross talk among multiples mediators in the maintaining of the "stemness". Plus, for each specific agent of interest, a quantity-function study should also be carried to determine the minimal dose required for the health-promoting effects.

2.4.3 Polymethoxyflavones (PMFs) and cancer chemoprevention

Due to their nutritional values and special flavor, citrus fruits are of great commercial importance. World-widely, the estimated number of citrus production in the major producing country was about 72 million metric tons in 2007 to 2008 (about 159 billion lbs.) [15]. In the United States alone, of all the total citrus production (about 10.6 million metric tons), about one third were used for juice production. With such a large amount of citrus used in the juice marker, considerably quantities of by-products such as peels, seeds are also been produced [264].

Traditionally, since citrus peels are enriched with molasses, pectin, they have been used as cattle feed after a serial of processing; one the other hand, citrus peel is also a popular source of bioactive compound such as citrus flavonoids [15]. In fact, citrus peels, as a important traditional medical herb, have been long utilized as traditional medicine for treating diseases like stomach upset and hypertension [265]. Citrus flavonoids,

together with other polyphenolic components found in citrus peels have draw more and more attention due to their underlying anti-inflammation, anti-carcinogenesis health promoting effect [13].

Generally, citrus flavonoids are classified into three different groups: the flavanone glycoside, the flavones glycoside, and polymethoxyflavones (PMFs) [13]. Interestingly, among all the citrus flavonoids, PMFs is the only one that almost exclusively found in the peels of sweet orange (Citrus sinesis) and mandarin oranges (Citrus reticulate) [18].

With more than 20 PMFs have been isolated and identified, Nobiletin (NBT), 3,5,6,7,8,3′, 4′-heptamethoxyflavone (HMF) and tangeretin (TAN) are three major permethoxylated PMFs [264]. In tradition Chinese medicine, orange peels are usually prepared through heating and drying; thereby forms “aged orange peels”. Chemically, under the influence of such “aging process” and auto-hydrolysis during storage, NBT, HMF and TAN can be further converted to 5-hydroxy-6, 7,8,3′,4′-pentamethoxyflavone (5-demethylnobiletin, 5DN), 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (5-HHMF), and 5-hydroxy-6,7,8,4′-tetramethoxyflavone demethyltangeretin (5-DT), respectively. These transformations involve the demethylation at the 5- position in A-ring of the basic tangeretin structure.

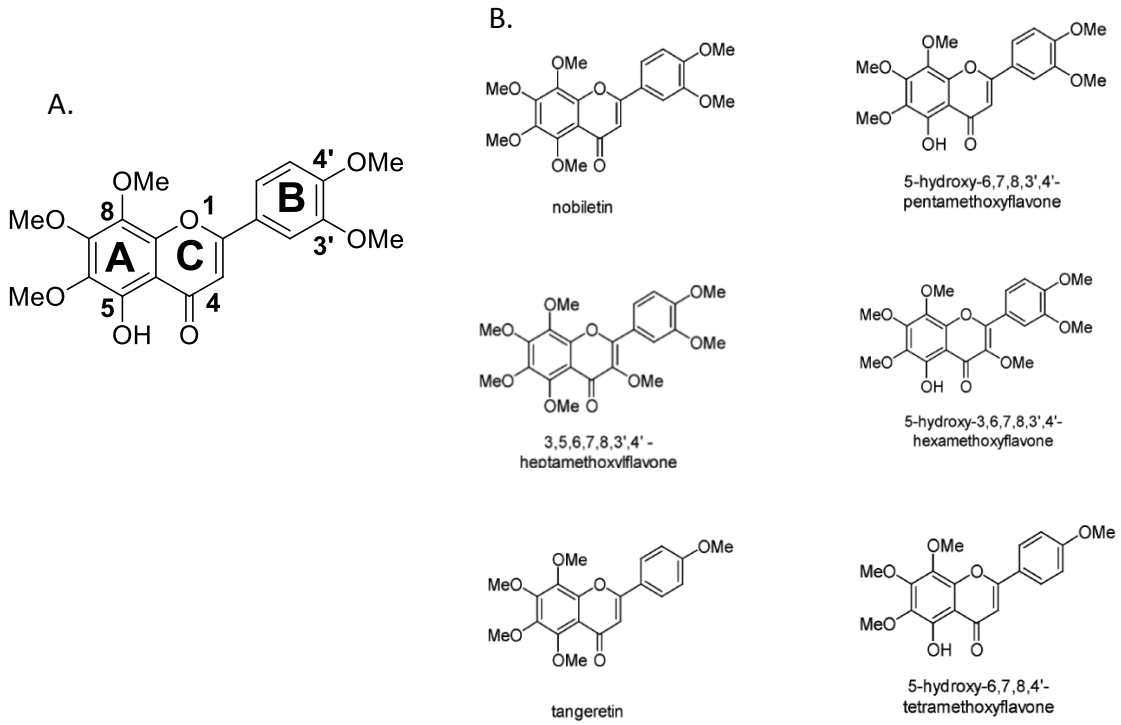

Figure 8. Chemical structures of polymethoxyflavones (PMFs). (A) Chemical structure of nobiletin. (B) Nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone (NBT), and tangeretin are major abundant permethoxylated PMFs found in the peels of sweet oranges. During aging process, 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (5DN), 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (5-HHMF), and 5-hydroxy-6,7,8,4′-tetramethoxyflavone (5-HT) can be converted from nobiletin, HMF, and tangeretin, respectively (Adapted from Qiu *et al*., 2010) [18]

Increasing evidence have been merging to support multiple bioactive properties of PMFs, including anti-inflammatory [17], anti-carcinogenic [12, 18, 266-268], and anti-oxidative [15]. Data from our recent studies showed that compared to their permethoxylated counterparts, demethylated PMFs exerted much more potency in terms of the biological functions as mentioned above [266-268].

Studies have shown that PMFs exhibit their anti-inflammatory function by inhibiting certain pro-inflammation enzymes. For examples, found that nobiletin can suppress the

production of proteases of matrix-metallo-proteinase-9 (MMP-9) family and prostaglandin E2 (PGE2) in human synovial fibroblasts. Specifically, by downregulation of cyclooxygenase-2 (COX-2), which ultimately will lead to the inhibition of the matrix degradation of articular cartilage and pannus formation in osteoarthritis and rheumatoid arthritis [269]. Plus, a recent finding also showed that tangeretin also can suppress COX-2 gene expression, and inhibited the UVB-induced transaction of NF-κB pathway in a dose-dependent manner [270].

Both *in vitro* and *in vivo* studies have that PMFs can be used as potential anti-carcinogenesis agents, and most of the research have been focus on NBT and TAN, as that they abundantly found in citrus peels. A comparative study was conducted by Kandaswami *et al., in* which the growth inhibitory effects of various PMFs were evaluated [271]. Evidence supported that both NBT and TAN could inhibit breast and colon cancer growth by interfering the G1 phase cell cycle [14].

Recent findings of our group also revealed that demethylated PMFs exhibited much stronger anti-cancer activities than their permethoxylated counterparts. Xiao *et al.* found that only demethylated PMFs, 5DN and 5HHMF were able to induce cell cycle arrest and apoptosis in human lung cancer cell [12]. Specifically, 5DN had much stronger inhibitory effect than tangeretin in human non-small cell lung cancer (NSCLC) cells by inducing G2/M cell cycle arrest and apoptosis [268]. In fact, among all the natural PMFs that we have studied, 5DN provided the strongest inhibitory effect [18]. Similar resulted were also obtained from both human breast [19] and leukemia cancer cells [272] studies, where 3′-hydroxy-5, 6,7,4′-tetramethoxyflavone and 5HHMF exhibited much more potent effect than their permethoxylated counterparts, respectively.

When considering the efficacy of certain chemopreventive effect, it is important to take into account the physiological factors such as intracellular accumulation, metabolisms and excretion. Bioavailablity, by definition is the amount of drug or nutrient that can be absorbed to blood circulation and target tissues, is usually characterized by plasma concentration [273]. Four factors should be considered in order to determine the bioavailablity of certain agent: absorption, distribution, metabolism, and excretion (ADME) [274].

As the key factor of bioavailability, absorption is influenced by both solubility and permeability of certain agents. Basically, compounds with high solubility and permeability would have the most absorption. When talking about the permeability, US Food and Drug Administration (FDA) provide us three absolute permeability criteria: 1. mass balance, absolute bioavailablity, or intestinal perfusion studies in human; 2. *In vivo* intestinal perfusion studies in animal model; 3. *In vitro* permeation experiments in passing through monolayer of cultured human intestinal cells such as Caco-2 cells [274]. Generally, our understanding of the PMFs has been based on the assumption that the multiple methoxy groups contribute to their high hydrophobicity. A study showed that after 4 hours of incubation more than 48% of nobiletin permeated to the bilateral side, indicating the high permeability and tendency to be accumulated in the intracellular compartment [275]. Other studies conducted in the SD male rats model by the same group further revealed that nobiletin is tend to accumulate in the mucous membrane and can be detected in significant amount in organs including stomach, intestines and liver [275].

In a recent pharmacokinetic study, Manthey *et al.* compared the bioavailablity between NBT and TAN. According to their study, both NBT and TAN could be detected in rat serum for up to 24 hours after administration. However, compared to NBT, TAN showed much lower plasma concentration after been same amount of gavage [276].

While the bioactivity of multiple PMFs including NBT and TAN have extensively reported as reviewed above, the study of the metabolic fate of PMFs in human body have not been conducted until recently. Basically, biotransformation plays a critical role in the biological activities of dietary compounds [277], and flavonoid compounds are no exceptions [278]. It has been suggested that after oral admiration, the metabolites generated through biotransformation might have even much potent biological activities [25]. A good example will be that Matthies *et al.* have found that after oral consumption, the anti-cancer effect of isoflavones such as daidzein was greatly depended on its biotransformation by phase I/ phase II enzymes as well as the influence of gut microflora [279].

In light of the potential impact of the biotransformation on the health-promoting activities of PMFs, we have expended the current studies to the potential properties of metabolites of both nobiletin (NBT) and its natural demethylated counter-part, 5-demethylated nobiletin (5DN).

Recently, 3′-demethylnobileti (N1) [20], 4′-demethylnobileti (N2) [21], and 3′,4′-didemethynobiletin (N3) [22] were reported as major metabolites from NBT and have recently been studied for their biological actions (Figure 9). Specifically, Li et al. have found that compared to the parent compound NBT, the metabolites including N1, N2 and N3 showed much potent anti-inflammatory effect in mouse macrophage RAW 264.7 [23].

Their study further supported that the anti-inflammatory effect of N2 and N3 was closely associated with the suppressing of the iNOS and COX-2 gene-expression. Other studies also support that the metabolites of NBT have stronger anti-tumorgenesis activity [280]. According to our unpublished data, after oral feeding of NBT (1000 ppm) in mice model for 5 months, only about 2μM of NBT could be detected in mice colon mucosa; while majority of NBT had been transformed in three of its metabolites (N1, N2 and N3). This again indicated that in order to fully evaluate the biological impact of NBT on colorectal cancer, we need to include the metabolites in our study.

Among all types of PMFs, 5-demethylated PMFs are a unique subclass, with 5-demethylated nobiletin (5DN) as the most abundantly one that could be found in orange peel (especially, in aged orange peels) [18]. With the bioactivity of 5DN has already been studied by our group and other groups [12, 18, 19], the metabolites of 5DN, however, has been scarcely investigated. Recently, our group isolated the metabolites of 5DN identified 3 novel urinary metabolites of 5DN, namely, 5,3'-didemethylnobiletin (M1), 5,4'-didemethylnobiletin (M2), and 5,3',4'-tridemethylnobiletin (M3) (Figure 10) [25]. Notably, all three metabolites have showed stronger inhibitory effect in human colon cancer cells [25]. According to our unpublished data, after oral feeding of 5DN (1000 ppm) in mice model for 5 months, while 5DN was still the most abundant that could be detected in the mice colon mucosa (about 15μM), a large portion of 5DN had been transformed into three of its demethylated metabolites (M1, M2 and M3). This indicated that it is also important to include the metabolites in our study in order to fully evaluate the biological impact of 5DN on colorectal cancer.

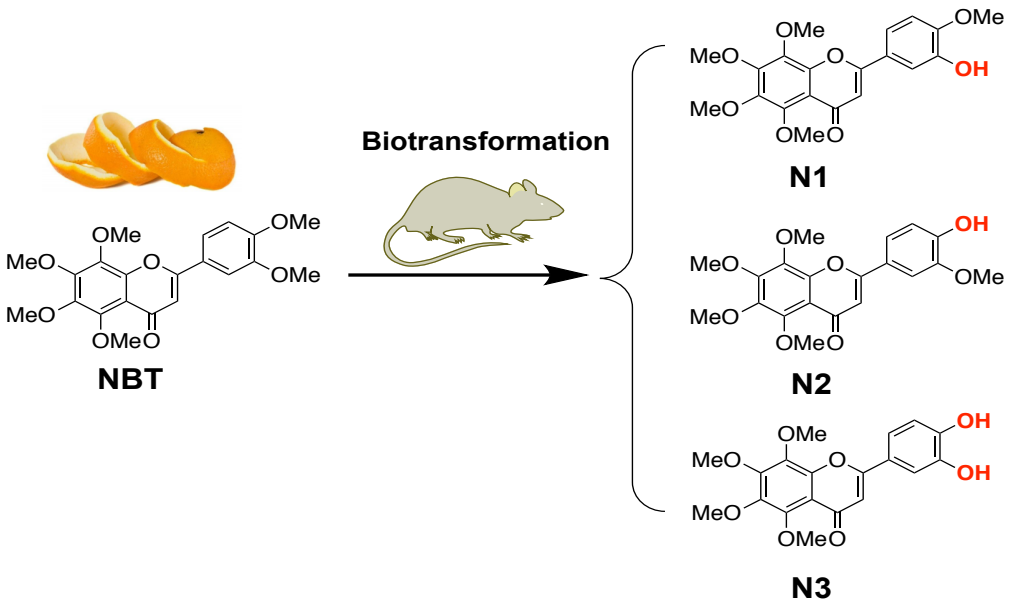

Figure 9. The Biotransformation of NBT. After oral consumption of NBT, under the influence of phase I& II metabolisms, as well as the co-function of microbiota, NBT can be further transferred into three major metabolites: N1, N2 and N3. The only structural difference of each metabolite is demethylation site on the side ring.

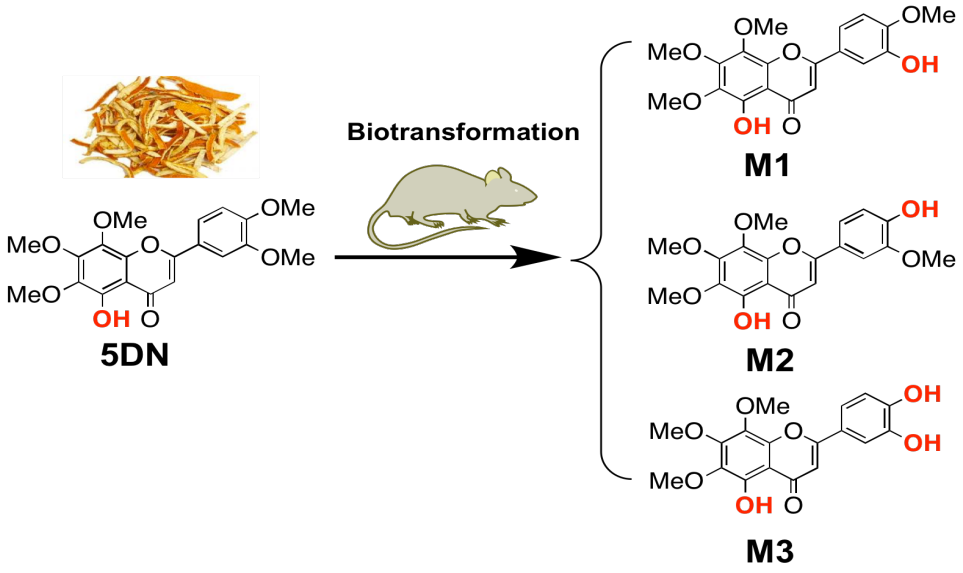

Figure 10. The Biotransformation of 5DN. After oral consumption of 5DN, under the influence of phase I& II metabolisms, as well as the co-function of microbiota, 5DN can be further transferred into three major metabolites: M1, M2 and M3. The only structural difference of each metabolite is demethylation site on the side ring.

CHAPTER 3

INHIBITORY EFFECTS OF NOBILETIN (NBT) AND ITS DEMETHYLETED METABOLITES ON COLON CANCER STEM CELLS

3.1 Introduction

Aggressive tumor growth, which is often linked with metastasis of malignant tumor to remote sites, contributes mostly to the poor prognosis for overall survival [110]. Colorectal cancer is the third leading cause of death in both men and women in the United States. Multiple barriers obstruct the successful colorectal cancer treatment. Among all the challenges, tumor reoccurrence and metastasis are two major survival-influencing factors of colorectal cancer treatment. For example, up to 40% of colorectal cancer patients who present with stage II or III colorectal cancer treatment will recur after primary treatment [1]. In colorectal cancer treatment, despite the rapid advances in chemotherapeutic drugs, 89% patients with metastatic disease cannot survive [2]. All these indicate the failure of conventional interventions including both radio- and chemo-therapies. Thus, it is of great important for us to develop novel and effective strategies for the control of colorectal cancer.

Recently, there is increasing experimental evidence supporting that the existence of a small sup-population of tumorigenic cells, namely, cancer stem cells (CSCs), are responsible for the tumor-initiation, metastasis and recurrence after conventional therapy. Multiple studies have been focused on the relevance between CSCs and colorectal cancer, which support the center role of CSCs in the overall poor prognosis and therapy failure in

colorectal cancer [5, 6, 60, 115]. Targeting CSC compartment, therefore, become a promising solution for both the prevention and /or treatment of colorectal cancer [241].

Epidemiological and dietary interventions studies in both animal and human models have suggested the positive role of many dietary components in inhibiting, reversing tumor development in different type of cancers [9]. So far, several of the dietary agents have also been shown to interfere with the function of CSCs [10]. Generally, this would be achievable by strategies such as inducing of differentiation, inhibiting of self-renewal signaling pathways and sensitizing CSC to chemotherapeutic agents [8]. For example, curcumin was reported to be able to impair the WNT signaling and cell-cell adhesion pathway in human colon cancer cell (HCT-116), which resulted in apoptosis and G2/M cell cycle arrest [11].

Polymethoxyflavones (PMFs) are a group of compounds that are almost exclusively found in the peels of citrus fruit [12]. So far, PMFs have been found to have wide spectrum of health promoting effect including anti-inflammation and anti- carcinogenesis [13]. Previously, as one of the major permethoxylated PMFs, nobiletin (NBT) has been reported to have various biological properties such as anti-tumorgenesis, anti-inflammation and oxidative stress [14-17]. In fact, when considering the efficacy of certain bioactive compound, it is of great importance that one should also take into account of physiological factors such as metabolism; in other word, as a result of biotransformation, the metabolites would deserve more attention since many of them might have even more significant activities than the parent compound [25]. In fact, 3´-demethylnobileti (N1) [20], 4´-demethylnobileti (N2) [21], and 3´,4´-didemethynobiletin (N3) [22] were already been identified as major metabolites from NBT and have recently

been studied for their biological actions. Evidence have supported that metabolites of NBT, exerted much stronger anti-inflammatory [23] and anti-mutagenic [24] effect compared to NBT. Our recent progress in the biotransformation and tissue distribution (unpublished) showed that in order to fully evaluate the biological impact of NBT on colorectal cancer, it is critical to include NBT as well as its metabolites in this study.

Herein, in this study, for the first time we investigated the inhibitory effect of NBT and three of its demethylated metabolites in human colorectal CSCs.

3.2 Materials and methods

3.2.1 Treatment and cell culture

HCT-116 and HT-29 Human colon cancer cell lines were obtained from American Type Cell Collection (ATCC, Manassas, VA, USA), and maintained in RPMI 1640 media (Media tech) supplemented with 5% heat-inactivated fetal bovine serum and 100U/mL of penicillin and 0.1mg/mL of streptomycin at 37°C with 5% CO_2 and 95% air. Cells were maintained in subconfluent culture and media were changed every 3–4 days. All cells used in experiments were between 4 and 20 passages. NBT and their demethylated metabolites were isolated and identified as previously described [12, 264, 281]. DMSO was used as vehicle to deliver NBT and demethylated metabolites to the cells. The final concentration of DMSO in all experiments was 0.1% v/v in cell culture media.

The sphere culture was carried out in serum free DMEM/F12 medium (Life Technologies). Briefly, the serum free medium (SFM) was the supplemented with 1×B27 (Life Technologies), 20ng/ml EGF (Life Technologies), 20ng bFGF (Life Technologies),

5 µg/ml human insulin (Sigma-Aldrich), 100U/ml of penicillin and 0.1mg/ml of streptomycin.

3.2.2 Isolation of colon cancer stem cell and tumor sphere formation assay

Singles cells obtained from HCT-116 cell line were plated in ultra-low attachment 24-well plates (Corning, Lowell, MA, USA) at a density of 12000 viable cells per well in triplicate. Cells were grown in SFM as described above at 37°C with CO_2 and 95% air. Right after seeding, cells were treated with 12.5-50µM of NBT and its demethylated metabolites for 7 days. After 7 days, tumor sphere were observed under microscope. For counting of spheres, cells were collected and transferred onto collagen-coated dishes respectively and cultured in RPMI 1640 medium supplemented with 5% FBS. In approximately 24h, tumor sphere were adhered, then stained with crystal violet and counted.

Secondary tumor sphere formation: for the passage of tumor sphere, single cells suspension was first obtained from primary tumor sphere by brief trypsinization. Then single cells from primary spheres were plated at the density of 2000 cell per well in triplicate. The secondary tumor spheres were grown, treated, harvested and counted as described above.

In order to compare the sphere-forming potential of primary and secondary test, the results were further normalized as number of tumorspheres formed per 1000 seeding cells.

3.2.3 Cell cycle analyses

Cells from HCT-116 and HT-29 cell line were seeded as single cell in 6-well suspension plates in SFM at a density of 12000 cells per well. Right after seeding, cells were treated with different concentrations of NBT and demethylated metabolites. After 7

days, the whole content of each well were collected by brief trypsinization (0.25% trypsin-EDTA; Sigma-Aldrich). Cell pellets were washed with 1mL of ice-cold PBS and then re-suspended in 1mL of 70% ethanol in -20°C overnight. After centrifugation (1600g, 1 min), the supernatant was removed and cells were incubated with 0.3mL of PBS containing 30 µg RNase (Sigma-Aldrich) and 3µg propidium iodine (Sigma-Aldrich) for 30min at room temperature. Single-cell suspension was generated by brief trypsinization. Cell cycle was analyzed using a BD LSR II cell analyzer at the analytical cytometry facility (University of Massachusetts Amherst), and data were processed using Modifit LT software.

3.2.4 Detection of apoptosis

HCT-116, HT-29 cells were seeded and treated exactly the same as described in cell cycle analyses above. After 7 days of incubation, apoptotic cells were quantified by Annexin V/propidium iodide (PI) double staining assay. Annexin V/PI staining was done using apoptotic detection kit (BioVision, Mountain View, CA, USA) following the manufacturer's instruction. Briefly, the content of each well were collected and disrupted by brief trypsinization, and then washed with ice cold PBS. After another wash with binding buffer, cells were suspended in 0.3 mL Annexin V binding buffer containing Annexin V and PI, and incubated for 5min at room temperature before analysis by flow cytometer. Early apoptotic cells were identified as Annexin V-positive/PI-negative cells, while late apoptotic/necrotic cells were identified as Annexin V-positive/PI-positive cells using BD LSRII cell analyzer at the analytical cytometry facility (University of Massachusetts Amherst).

3.2.5 Immunoblotting

After treatment with serial concentration of NBT and its metabolites (namely, N1, N2 and N3) for 7 days, whole cell lysate will be obtained following method as previously described [268]. Briefly, suspended spheroid cells will then be collected and washed with ice- cold PBS. Cells will then be incubated on ice for 10 min in lysis buffer (Cell signaling, Beverly, MA, USA) supplemented with cocktails of protease inhibitor (1:100), phosphatase inhibitor I (1:100), and phosphatase inhibitor II (1:100) (Boston Bio products, Boston, MA, USA). After sonication (4 seconds, 4 times), cells will be incubated for 20 mins on ice and followed by centrifugation at 14,000 rpm using a bench top Eppendorf centrifuge for 20 mins at 4°C. Supernatants will be collected as whole cell lysates. Proteins will be quantified by BCA™ protein assay kit (Pierce Biotechnology, Rockford, IL, USA), and 50-150 μg of proteins will be resolved either by 8%, 12% or 15% SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membrane. The membrane containing the transferred protein will be blocked in blocking buffer (5% nonfat dry milk, 1% Tween-20 in 20 mM Tris-buffered saline, pH 7.4) for 2 h at room temperature, and then incubated with appropriate primary antibodies in blocking buffer overnight at 4°C. After incubation with appropriate secondary antibodies for 2 h at room temperature, the membranes will be washed with Tris buffer containing 0.5% of Tween-20, and then be visualized using enhanced chemiluminescence kit (Boston Bio products, Ashland, MA, USA). Antibodies for Cyclin D1, $p21^{Cip1/Waf1}$, p53, RIP3 and cleaved poly ADP ribose polymerase (PARP) will be obtained from Cell Signaling Technology (Beverly, MA, USA). β-Actin (Sigma-Aldrich) will be used as a loading control.

3.2.6 Statistical analysis

All data were presented as mean ± SD. Student's t-test was used to determine the mean difference between two groups. Analyses of variance (ANOVA) model was used for the comparing the differences among more than two groups.

3.3 Results

3.3.1 NBT and its demethylated metabolites inhibited tumorsphere formation in colon cancer stem cells.

To determine whether the treatment of NBT and their demethylated metabolites would influence the proliferation of colorectal CSCs, we conducted tumor sphere formation assay on spheroid cells derived from both HCT-116 and HT-29 cell lines (Figure.11 and Figure. 12). Specifically, HCT-116 and HT-29 cells were seeded as single cell in Serum-free medium (SFM) and treated with NBT (12.5μM- 50μM) or three demethylated metabolites: N1 (12.5μM-50μM), N2 (12.5μM-50μM) and N3 (12.5μM-50μM). The tumor sphere-forming potential of both primary and secondary CSCs were analyzed. For CSCs derived from HCT-116 cell line (Figure. 11 A), the primary tumorsphere forming frequency in the control group got increase from about 0.4% (4 tumorspheres formed per 1000 seeding cells) to about 1.7% (17 tumorspheres formed per 1000 seeding cells) in the secondary test, this further supported the notion that sphere-culturing in SFM as an effective tool in enriching the CSC subpopulation. Compared to the control group, the sphere-forming ability following each treatment was significantly impaired in a nearly dose-dependent manner. For example, the treatment of NBT at 12.5μM resulted in a nearly 50% percent of inhibition of primary tumorsphere, and at 50μM, the inhibitory effect got increased to almost 66%. Then we compared the

inhibitory effect of three of its demethylated metabolites (N1, N2 and N3) with that of NBT: for the primary tumorsphere test, only N3 (50μM) showed stronger inhibitory effects compared to the parent compound, NBT (50μM); as for the secondary test, the treatment of N1 (12.5μM), N2 (50μM) and N3 (50μM) was showed to be more potent than their NBT-treating counterparts. For example, N3 (50μM) led to a 100% inhibition in secondary tumor sphere formation, while NBT (50μM) only resulted in a 60% reduction. Plus, such inhibition of sphere-forming potential was also observed as the reduction of tumorsphere sizes (Figure 11 B).

Similar pattern was also observed in the tumorsphere-forming ability of CSCs derived from HT-29 cell line (Figure 12 A). Compared to the control group, the treatment of NBT and its demethylated metabolites (N1, N2 and N3) resulted in a significant decrease in the sphere-forming potential in both primary and secondary test. Then we compared the effect of three demethylated metabolites (N1, N2 and N3) with that of NBT: for the primary test, N2 (12.5μM, 25μM), N3 (12.5-50μM) showed significantly stronger effect than those NBT-treating group; for the secondary test, the treatment of N1 (12.5μM, 50μM), N2 (12.5μM) and N3 (12.5μM, 50μM) showed to be more potent than the NBT-treating counter-part. Plus, such inhibitory effects were also observed in the reduction of tumorsphere size (Figure 12 B).

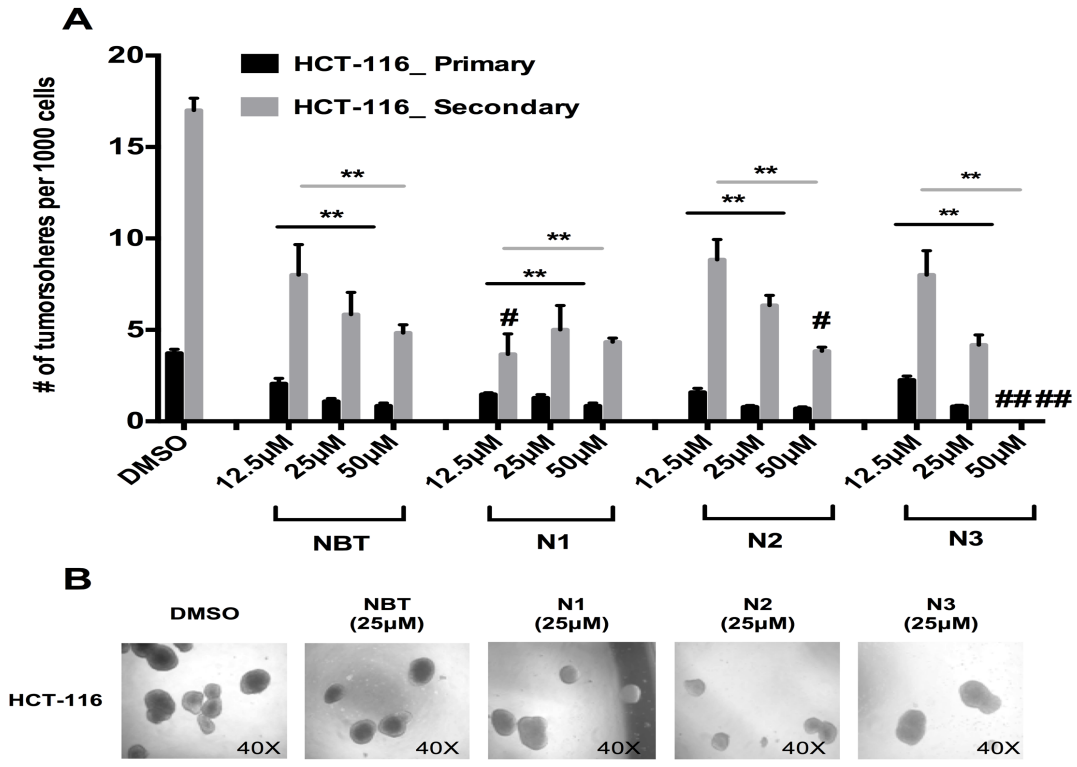

Figure 11. The treatment of NBT and its demethylated metabolites (N1, N2 and N3) inhibit the tumorsphere formation of colorectal CSCs derived from HCT-116 cell line. **(A)** For the primary tumorsphere formation assay, single cells from HCT-116 cell line were seeded in 24-well ultra-low attachment plates (12000 cells per well); for the secondary tumorsphere formation, single cells obtained from the primary tumorspheres as described in Material and Methods were seeded in 24-well ultra-low attachment plates (2000 cells per well,) in triplicate in a serum-free (SFM) medium. Right after seeding, CSCs were treated with NBT (12.5μM to 50μM) and three of its demethylated metabolites: N1, N2 and N3 (12.5μM to 50μM). After 7 days, primary/ secondary tumorspheres were collected and counted. All data represent mean ± SD, * indicated the statistical significance of each treatment in comparison with the control (DMSO) group, as *$p<0.05$, **$p<0.01$, *** $p<0.001$; # indicated the statistical significance of metabolites-treating groups in comparison with the NBT-treating group, as #$p<0.05$, ##$p<0.01$, ###$p<0.001$. **(B)** Tumorsphere culture demonstrating sphere-formation of colorectal CSCs derived from HCT-116 cell line following the treatment with NBT and its demethylated metabolites.

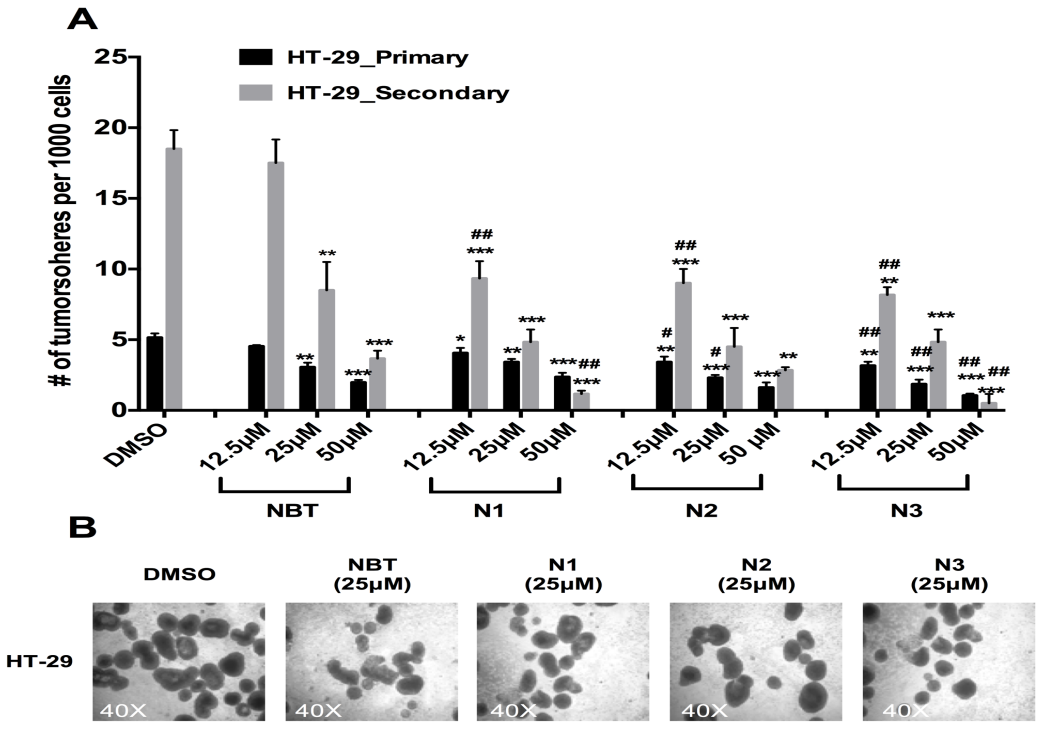

Figure 12. The treatment of NBT and its demethylated metabolites (N1, N2 and N3) inhibit the tumorsphere formation of colorectal CSCs derived from HT-29 cell line. **(A)** For the primary tumorsphere formation assay, single cells from HT-29 cell line were seeded in 24-well ultra-low attachment plates (12000 cells per well); for the secondary tumorsphere formation, single cells obtained from the primary tumorspheres as described in Material and Methods were seeded in 24-well ultra-low attachment plates (2000 cells per well,) in triplicate in a serum-free (SFM) medium. Right after seeding, CSCs were treated with NBT (12.5µM to 50µM) and three of its demethylated metabolites: N1, N2 and N3 (12.5µM to 50µM). After 7 days, primary/ secondary tumorspheres were collected and counted. All data represent mean ± SD, * indicated the statistical significance of each treatment in comparison with the control (DMSO) group, as *$p<0.05$, **$p<0.01$, *** $p<0.001$; # indicated the statistical significance of metabolites-treating groups in comparison with the NBT-treating group, as #$p<0.05$, ##$p<0.01$, ###$p<0.001$. **(B)** Tumorsphere culture demonstrating sphere-formation of colorectal CSCs derived from HT- 29 cell line following the treatment with NBT and its demethylated metabolites.

3.3.2 NBT and its demethylated metabolites induced apoptosis (necroptosis) in colon colorectal stem cells

We next examined the extent to which cell death pathways such as apoptosis and/or necroptosis contributed to the inhibitory effect of NBT and its demethylated metabolites on tumorsphere forming capacity of CSCs as we have demonstrated in **Chapter 3.3.1**. For CSCs derived from HCT-116 cell line (Figure 13), the treatment of NBT (25μM) showed no significant apoptosis (or necroptosis) inducing effect; while for N1 (25μM), N2 (25μM) and N3 (25μM) significantly induced apoptosis (necroptosis) compared to both control and the NBT- treating group. For example, N1 (25μM) resulted in one fold of increase (from 5.8% to 11.4%) in the total apoptotic (or necroptotic) population compared to the control group. Notably, N3 (25μM) increased total apoptotic (or necroptotic) population by 2.6-fold.

Similar pattern were also observed in the CSCs derived from HT-29 cell line (Figure 14). Particularly, compared to the control group, all treatments led to signify of induction of apoptotic (necroptotic) cells. For example, NBT (25μM) increased the total apoptotic (necroptotic) cells by (from 4.4% to 13.7%) by 3.1-fold; N2 (25μM) also increased the early phase apoptotic cells by 7-fold (from 0.4% to 2.8%).

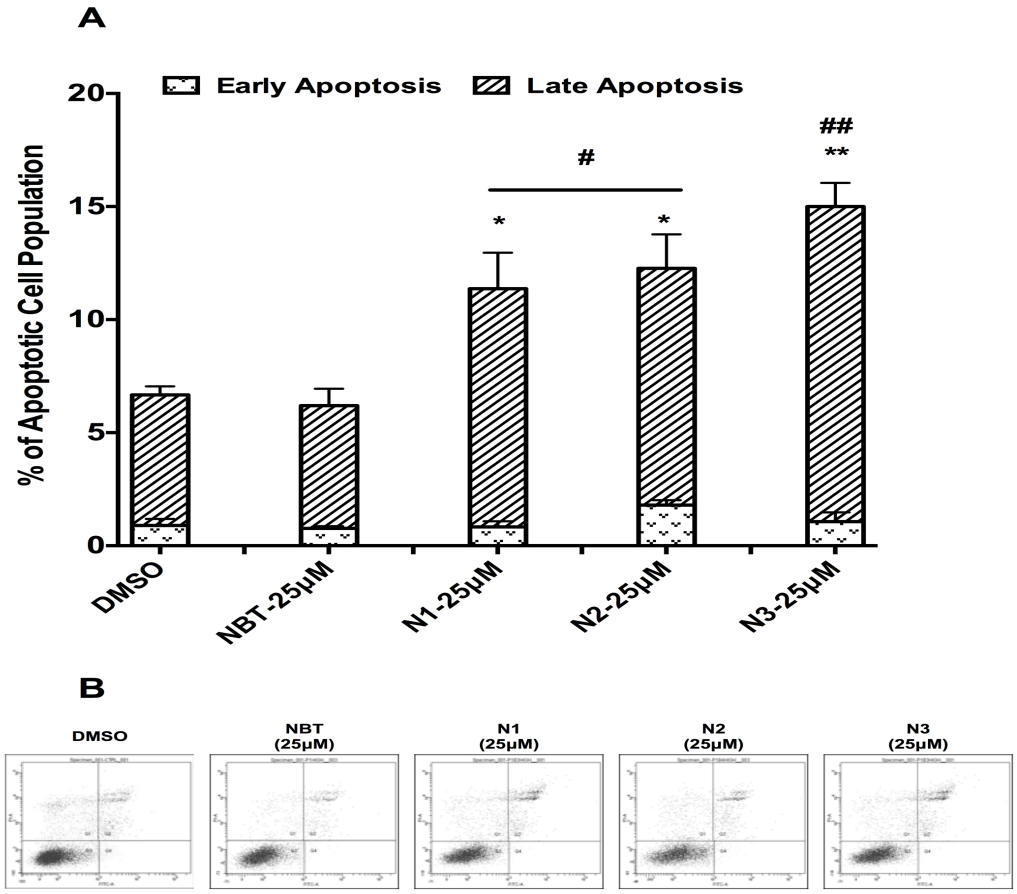

Figure 13. The treatment of NBT and its demethylated metabolites induced apoptosis (or necroptosis) in CSCs derived from HCT-116 cell line. **(A)** HCT-116 cells were seeded as single cell in 6-well suspension plates in SFM at a density of 12000 cells per well. Right after seeding, cells were treated with NBT (25μM) and demethylated metabolites N1 (25μM), N2 (25μM) and N3 (25μM). After 7 days, apoptotic cells were quantified by Annexin V/propidium iodide (PI) double staining assay as described in Materials and Methods. FITC ^{high}/ PI ^{low} cells were defined as early apoptotic cells, FITC ^{high}/ PI ^{high} cells were defined as late apoptotic (or necroptotic) cells. All data represent mean ± SD, * indicated the statistical significance of each treatment in comparison with the control (DMSO) group, as *p<0.05, **p<0.01, ***p<0.001; # indicated the statistical significance of metabolites-treating groups in comparison with the NBT-treating group, as #p<0.05, ##p<0.01, ###p<0.001. (B) The distribution pattern of CSCs (HCT-116) following the treatment of NBT and its demethylated metabolites.

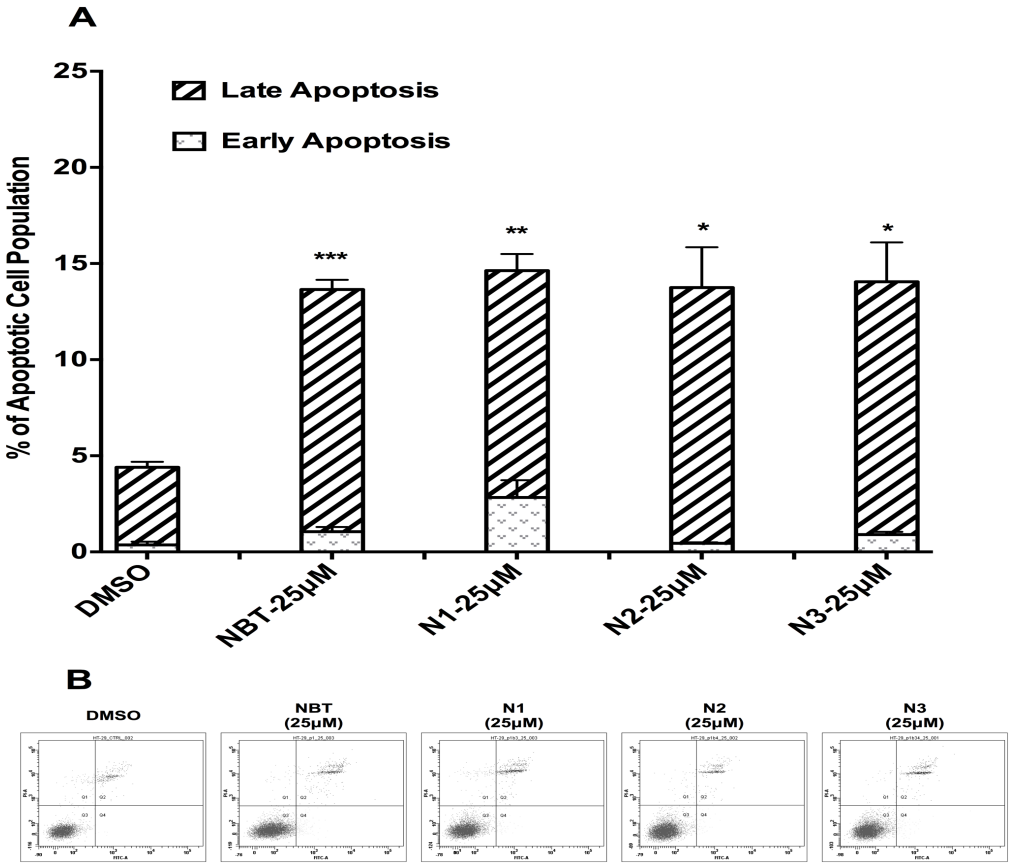

Figure 14. The treatment of NBT and its demethylated metabolites induced apoptosis (or necroptosis) in CSCs derived from HT-29 cell line. **(A)** HT-29 cells were seeded as single cell in 6-well suspension plates in SFM at a density of 12000 cells per well. Right after seeding, cells were treated with NBT (25μM) and demethylated metabolites N1(25μM), N2(25μM) and N3 (25μM). After 7 days, apoptotic cells were quantified by Annexin V/propidium iodide (PI) double staining assay as described in Materials and Methods. FITC ^{high}/ PI ^{low} cells were defined as early apoptotic cells, FITC ^{high}/ PI ^{high} cells were defined as late apoptotic (or necroptotic) cells. All data represent mean ± SD, * indicated the statistical significance of each treatment in comparison with the control (DMSO) group as *p<0.05, **p<0.01, ***p<0.001; # indicated the statistical significance of metabolites-treating groups in comparison with the NBT-treating group, as #p<0.05, ##p<0.01, ###p<0.001. (B) The distribution pattern of CSCs (HT-29) following the treatment of NBT and its demethylated metabolites.

3.3.3 NBT and its demethylated metabolites caused cell-cycle redistribution in colorectal colon cancer cells

We next investigate the possible effects of NBT and its demethylated metabolites on the cell cycle distribution of CSCs. Theoretically, the quiescence (or slow cycling) nature of CSCs would make most of them stay in the G0/G1 phase [122] .

For CSCs derived from HCT-116 cell line (Figure 15), more than 80% of cells in the control group were found in the G1/G0 phase. All the treatment decreased the cell distribution in G1/G0 phase compared to the control group. For example, the treatment of N3 (25μM) decreased the cells distribution in G1/G0 phase from 81% to 72%; Then we compared the effect of three metabolites with that of NBT: compared to the NBT-treating group, N1 (25μM) and N3 (25μM) both significantly decreased the cell population in G1/G0 Phase. Similar patterns were also observed in CSCs followed by the treatment of NBT (25μM) or its metabolites, where the cell cycle distributing was significantly altered. For example, compared to the control group, the treatment of N3 (25μM) decreased the cell distribution in G0/G1 phase from 70% to 63%. However, compared to the NBT-treating group, none of the groups treated with metabolites (N1, N2 and N3) showed higher potency.

Similar pattern was also been observed in CSCs from HT-29 cell line (Figure 16). About 70% cells were in the G0/G1 phase in the control group; the treatment of NBT significantly decreased the number of cells in the G0/G1 phase, while all the three metabolites had almost equivalent effects as NBT.

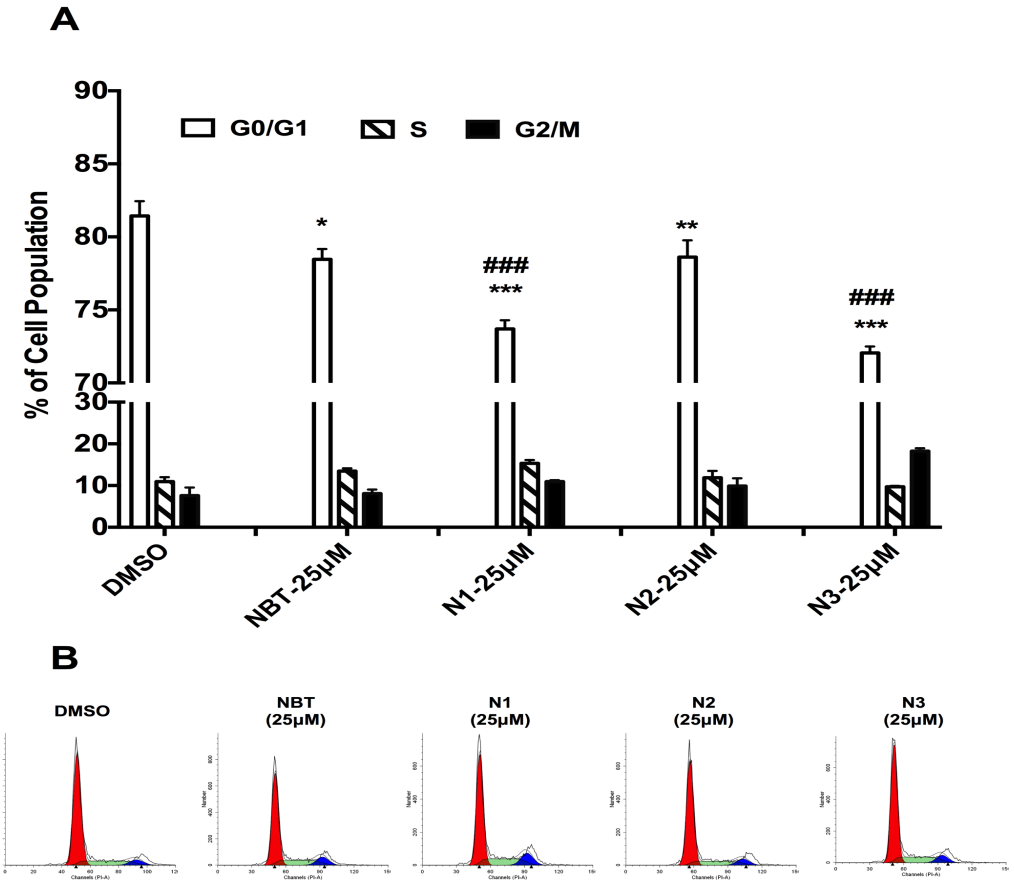

Figure 15. The treatment of NBT and its demethylated metabolites altered the Cell Cycle distribution of CSCs derived from HCT-116 cell line. **(A)** HCT-116 cells were seeded as single cell in 6-well suspension plates in SFM at a density of 12000 cells per well. Right after seeding, cells were treated with NBT (25μM) and demethylated metabolites N1 (25μM), N2 (25μM) and N3 (25μM). After 5 day, cells were collected and subjected to cell cycle analyses as described in Material and Methods. All data represent mean ± SD, * indicated the statistical significance of each treatment in comparison with the control (DMSO) group as *p<0.05, **p<0.01, ***p<0.001; # indicated the statistical significance of metabolites-treating groups in comparison with the NBT-treating group, as #p<0.05, ##p<0.01, ###p<0.001. **(B)** Cell cycle distribution of CSCs (HCT-116) following treatment of NBT and its demethylated metabolites.

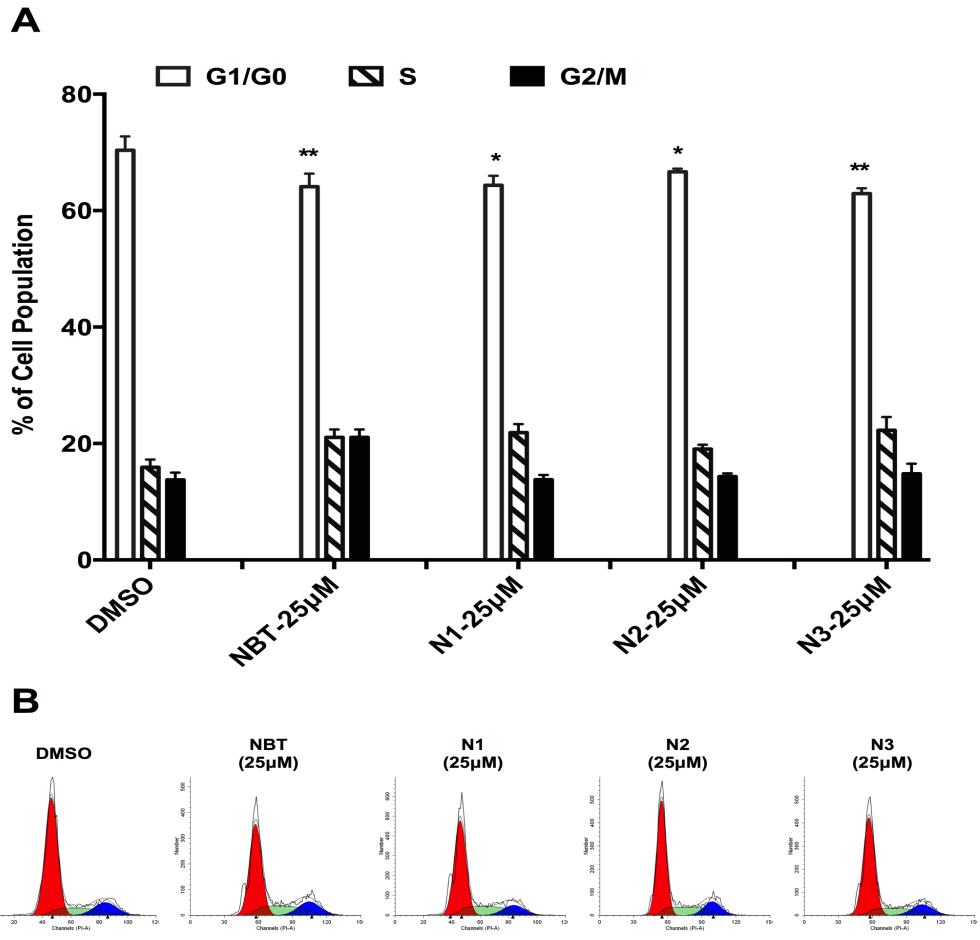

Figure 16. The treatment of NBT and its demethylated metabolites altered the Cell Cycle distribution of CSCs derived from HT-29 cell line. **(A)** HT-29 cells were seeded as single cell in 6-well suspension plates in SFM at a density of 12000 cells per well. Right after seeding, cells were treated with NBT (25μM) and demethylated metabolites N1 (25μM), N2 (25μM) and N3 (25μM). After 5 day, cells were collected and subjected to cell cycle analyses as described in Material and Methods. All data represent mean ± SD, * indicated the statistical significance of each treatment in comparison with the control (DMSO) group as *p<0.05, **p<0.01, ***p<0.001; # indicated the statistical significance of metabolites-treating groups in comparison with the NBT-treating group, as #p<0.05, ##p<0.01, ###p<0.001. **(B)** Cell cycle distribution of CSCs (HT-29) following treatment of NBT and its demethylated metabolites

3.3.4 NBT and its demethylated metabolites inhibited the growth of colorectal CSC by regulating key proteins related to apoptosis, necroptosis and cell cycle.

In order to elucidate the molecular mechanisms governing the inhibitory effects of NBT and its demethylated metabolites (N1, N2 and N3) on CSCs from both HCT-116 and HT-29 cell line, we analyzed the effects of treatments on some key proteins in regulating cell cycle and apoptosis by immunoblotting analysis as described in chapter 3.2.5.

To confirm the apoptosis-inducing effect of our treatment as described in chapter 3.2.3, we examined their effects on poly ADP ribose polymerase (PARP) and p53 (for possible p53-dependent apoptosis). According to our results (Figure 17), for CSCs derived from HCT-116, NBT and all its metabolites increased the level of p53, with N1 (25μM) having the strongest effect. While among all of our treatments, only N1 (25μM) signify increased the expression of cleaved PARP, which is the indicator of apoptosis. For cells derived from HT-29, it was found that only N3 (25μM) resulted in the significant increase of P53; on the other hand, dramatic increasing in the level of cleaved PARP confirmed that occurrence of apoptosis following our treatment.

In cell cycle arrest, P21 is transcriptionally regulated by p53. Plus, p21 has also been found to control entry into quiescence and maintaining of the quiescent state [282]. According to our result (Figure 17), in CSCs derived from HCT-116, there was a significant increase in the level of p21 followed by our treatment; however, as for CSCs derived from HT-29, the opposite trend was observed.

Cyclin D1 plays a central role in promoting the entry into the cell cycle, high level of cyclin D1 is required for the G1/S transition [283]. According to our results (Figure 17)

increased levels of cyclin D1 were detected following the treatments in CSCs derived from both HCT-116 and HT-29 cell line. This could be further used to confirm that our treatments were able to force CSCs to re-enter cell cycle.

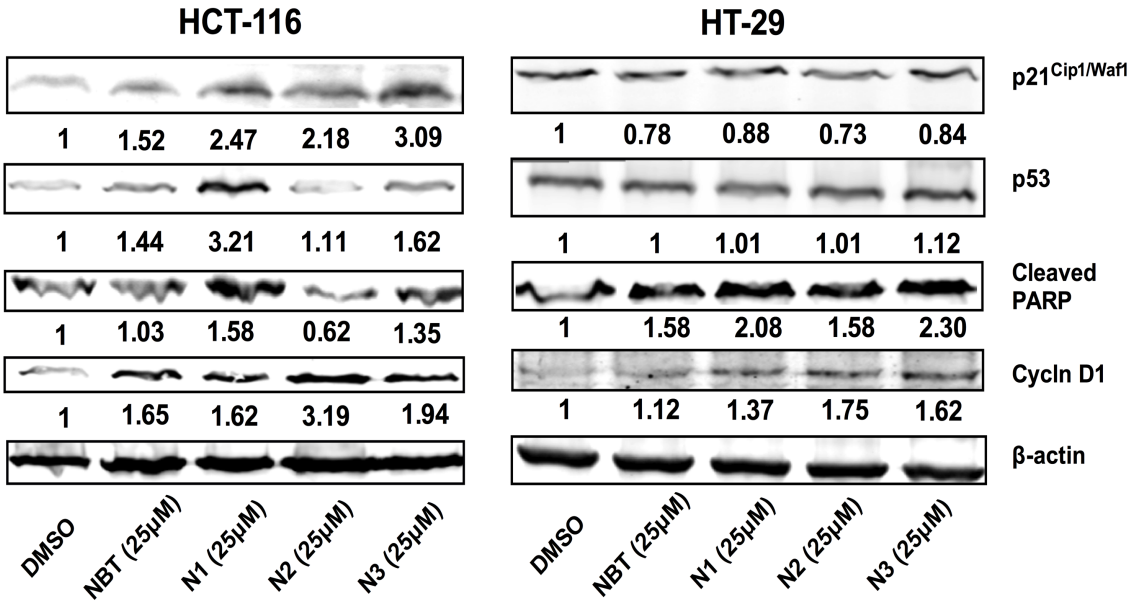

Figure 17. The treatments of NBT and its demethylated metabolites (N1, N2 and N3) in modulating key proteins regulating apoptosis and cell cycle. Immunoblots displayed the treatment of NBT (25μM) and three of its metabolites: N1 (25μM), N2 (25μM) and (25μM) on $p21^{cip1/Waf1}$, p53, cleaved PARP and Cyclin D1 on CSCs derived from HCT-116 and HT-29. The numbers under the blots represent band intensity (normalized to β-actin, means of three independent experiments). The standard deviations (all within ± 15% of the means) were not shown. β- actin served as an equal loading control.

Besides apoptosis, necroptosis is also regarded as an important programmed pathway way, especially when apoptosis fails for multiple reasons. Therefore, in our study, we also test that whether our treatments could result in the level change of the RIP3, one of the markers of the necroptosis pathway. As shown in Figure 18, for CSCs derived from HCT-116, the treatment of NBT (25μM), N1 (25μM) and N3 (25μM)

increased the level of RIP3; while for CSCs derived from HT-29, only NBT (25μM) and N2 (25μM) induced the increase in RIP3.

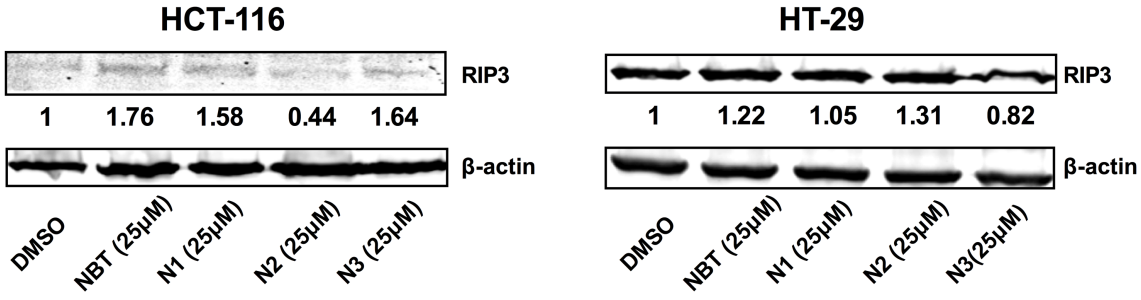

Figure 18. Effects of NBT and its three demethylated metabolites (N1, N2 and N3) on the expression of RIP3, one of the key regulators of necroptosis. Immunoblots displayed the treatment of NBT (25μM) and three of its metabolites: N1 (25μM), N2 (25μM) and (25μM) on one of the key molecular marker of necroptosis, RIP3. The numbers under the blots represent band intensity (normalized to β-actin, means of three independent experiments). The standard deviations (all within ± 15% of the means) were not shown.β-actin served as an equal loading control.

3.4 Discussion

Currently, NBT, as one of the major permethoxylated PMFs has been reported to have various biological properties such as anti-tumorgenesis, anti-inflammation and oxidative stress [14-17]. Plus, in light of the importance of biotransformation in the understanding the overall function of certain bioactive compound, attention have also been focused on three of the demethylated metabolites of NBT, namely, N1, N2 and N3. Recently, the metabolites of NBT have been studied for their biological properties. Evidence have supported that metabolites of NBT, exerted much stronger anti-inflammatory [23] and anti-mutagenic [24] effect compared to parent compound . So far, there has been no publish on NBT and its metabolites targeting the CSCs, which is

believed to be the fundamental factors that the hinters the effectiveness of current preventive/ therapeutic strategies for cancer control.

In vitro tumor sphere formation has already been proved to be an effective tool in the study of CSCs [85]. In this study, we determined the effects of NBT and their metabolites in both primary and secondary tumor formation. According to our results we found that the frequency of sphere-forming cells in the secondary culture was almost 3-folds as high as the frequency in the primary culture, which further confirmed the "CSCs-enriching" function of the suspension sphere culture. There are evidence supporting that such spheroid- forming cells has significant higher "tumor-initiating" ability in the transgenic mouse model than cells cultured with conventional methods [84, 91, 92]. According to the result from the tumorsphere formation study, we found that for both CSCs derived from HCT-116 and HT-29, the treatment of NBT or their demethylated metabolites could significantly impair the *in vitro* sphere-forming ability. Furthermore, prolonged treatment of NBT or their metabolites also resulted in the induction of apoptotic cells as well as a shift the cell cycle redistribution in CSCs derived from both cell lines. Interestingly, we have observed that for each cell line, the level of responses differs when cells were subjected to different treatment (NBT or metabolites), generally, it was observed that metabolites had stronger effects on the cells than the parent compound; on the hand, results also showed that CSCs derived from the two cell line, responded differently when subjected to the same treatment. This may indicate that the phenotype difference may contribute to the different cellular-sensitivity to our treatments.

So far, with several research have been focused on the regulation of self-renewal, proliferation and differentiation of CSCs, studies of cell death pathways including

apoptosis and necroptosis are still quite limited [168]. In this study, we examined whether NBT and their demethylated metabolites exert the inhibitory effect on CSCs by regulating some of the key modulators in apoptosis and cell cycle distribution (quiescence maintaining).

The cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis [284]. Our results in immunoblotting showed that within each cell line we have test, the treatment of NBT or metabolites resulted in the increased level of cleaved -PARP compared to the control group. Such increase in cleaved PARP can be used to further support our observation in the apoptosis test by flow cytometry.

The tumor suppressor, p53, has been implicated in a variety of cellular processes and regarded as the "guardian of the genome'' in cells. It exerts its function by inducing apoptosis and growth arrest. According to our results, treatment of NBT and metabolites resulted in the up-regulation of p53 in CSCs derived from HCT-116. This may indicate that the inhibitory effect of our treatment was p53 -dependent in CSCs derived from HCT-116 cell line. To our surprise, for CSCs derived from HT-29 cell line, the level of total p53 protein almost kept unchanged following our treatment. A possible explanation would be that rather than a elevation in the total p53 level, the treatment could lead to the increase in the serine phosphorylation level of p53. In a study using curcumin to treat HT-29 cells, such high level of the serine phosphorylation of p53 (total p53 remains unchanged) was shown to be able to down-regulate the anti-apoptotic factor, Bcl-2 and up-regulating the pro-apoptotic factor Bax, thereby decreasing the Bcl-2/Bax ratio and disposing to apoptosis [285].

Recently, the role of p53 in cancer stem cell has been discussed in detail by Aloni-Grinstein et al.[286]. Particular, it has been proposed that p53 can also function as a differentiation inducer in various cell types, which place it as an attractive candidate for differentiation therapy in control of CSCs [286]. According to our result in this study, increased level p53 was observed in CSCs derived from HT-116 cell line. Though evidence is still vastly lacked, this might also be novel for our understanding of the mechanism behind the inhibitory effect of NBT and metabolites on colorectal CSCs.

The CDK inhibitor $p21^{CIP1/WAF1}$, is a member of Cip/Kip family and responsible for both p53 dependent and p53-independent cell cycle arrest [287]. During the p53-dependent cell cycle arrest, p21 served as a down stream transcriptional targes of p53; increased levels of p53 would also result in increased level of $p21^{CIP1/WAF1,}$ which will in turn contributes to cell cycle arrest. As for our study, such pattern (increased $p21^{CIP1/WAF1}$, plus increased p53 protein level) was observed in CSCs derived from HCT-116 followed by the treatment of NBT and metabolites. This further supported that NBT and metabolites might probably exert their function through p53- dependent p21 mediated pathway.

Evidence supported that disruption of cell cycle inhibition may also contribute to the very nature of CSCs, especially in breaking the balance between the self-renewal and differentiation, which lead to the deregulated self-renewal of CSCs [203]. Theoretically, the quiescence (or slow cycling) nature of CSCs would make most of CSCs stay in the G0/G1 phase. As has been discussed in chapter 2.3.2, quiescence is one of the major mechanisms contributing the therapy resistance of CSCs. Particularly, G0 phase is viewed as either an extended G1 phase, where the cell is neither dividing nor preparing to

divide, or a distinct quiescent stage that occurs outside of the cell cycle [122]. It has also been proposed that, targeting p21 or its down stream target may be an effective means to force quiescent CSCs to cycle and thereby be more susceptible to chemotherapies and undergo apoptosis. As for our cell cycle analysis, we found that for both control and test group, more than 80% of total population of CSCs was in the G0/G1 phase, which is not usual in our study of the bulk of cancer cells (data not shown). This could be an indication that majority of cells in the suspension culture are CSCs. According to our observation in the cell cycle detection and immunoblotting, a decrease in G0/ G1 phase cell population for both cell lines as well as a reduced levels of p21 in HT-29 were observed; Cyclin D1 plays a central role in the entry of cell-cycle, particularly, high level of cyclin D1 expression can be an indicator G1/S transition [283]. According to our results, in CSCs derived from both HCT-116 and HT-29 cell line, all our treatments (NBT and metabolites) led to increased expression cyclin D1, which were in line with our observation in the cell cycle distribution. Still, further evidence are still needed to connect the inhibitory of our treatment on CSCs and the "re-enter" into cell cycle.

Besides apoptosis, evidence has been accumulated supporting that apoptosis is not the only mechanisms that could control the cell death. Specifically, necroptosis, also known as the programmed necrosis, has gained more attention in recent years. Besides the necrosis-like morphology changes, necroptosis are also believed to maintains part of the apoptotic features as apoptosis [188, 189]. In our research, we also hypothesize that necroptosis could be another factor contributed to the inhibitory effects of NBT and metabolites on colorectal CSCs. Specifically, our hypothesis was based on the following reasons: 1. Possible limitation exists for our methods in "apoptotic" cell detection. Time-

lapse imaging, which allows monitoring of morphological changes in individual cells in a dynamic and comparative manner, is so far the most effect way to reveal the differences between apoptosis and necrosis (necroptosis) [288]. However, during our research, cells were analyzed by flow fluorocytometry for exposure of phosphatidylserine (PS, characterized by the binding of FITC) and changes in permeability (uptake of propidium iodide, characterized by binding of PI). Notably, in apoptosis, membrane changes will lead to a rapid PS exposure, while the permeability change will be lagged behind [288] . Such phenomena result in the steady shift from early phase apoptosis (PI^{low} / $FITC^{high}$ cells) to late phase apoptosis (PI^{high} / $FITC^{high}$ cells); for necroptosis (necrosis), PS exposure overlaps with the changes with permeability changes, which result in the rapid shift of cell population from lower left corner (PI^{low} / $FITC^{low}$ cells) to the upper right corner (PI^{high} / $FITC^{high}$ cells). Therefore, in our research, since we only tested the cell distribution pattern at the end of 7- day incubation, the upper right cells preciously defined as “late phase apoptosis” cells, could also be cells went through “necrosis (necroptosis)”; 2. It has been reported that necroptosis is an alternative cell death pathway especially when apoptosis cannot be archived because of the ATP failure (apoptosis is the most energy-consuming process since that it requires the activation of multiple caspases and the formation of apoptosome) [200-202]. As in our study, CSCs were derived in serum free medium (SFM) supplemented with growth factors (see Material and Methods) and cultured extensively for 7 days. Such condition would result in the possible “energy starvation” thus necroptosis could be accounted as an alternative death pathway involved. 3. One of the key characters of CSC is higher apoptosis-resistance compared to the non-

CSC counterpart [289], this could be another reason besides ATP failure that can make necroptosis an alternative explanation for the inhibitory effect of our treatment.

To test hypothesis, we test the expression of the RIP3, one of the key molecular regulators of necroptosis [192, 193] by immunoblotting. It was shown that our treatment caused increased RIP3 level in CSCs from both cell lines: for HCT-116 derive CSCs, NBT, N1 and N3 result in the increasing in RIP3 while for CSCs derived from HT-29, it was the treatments of NBT, N1 and N3 resulted in the elevated level of RIP3 expression. Evidence have been supporting that caspase 8 plays opposite roles in cross-talk between apoptosis and necroptosis: TNF activate apoptosis by triggering a series of molecular events which eventually lead to the activation of caspase 8; while in necroptotic pathway, the function of caspase 8 is disrupted or even inhibited [191]. Therefore, we will further confirm our observation by assessing the level of caspase 8 in order to elucidate the possible relationship between the inhibitory effect of our compounds and the necroptosis pathways.

In our current study, we conducted the initial investigation of the possible mechanisms of which NBT and its metabolites exerted their inhibitory effects on colorectal CSCs. However, we believed that NBT and each of its metabolite should be studied independently in order to elucidate the mechanisms. There are multiple factors exist and make it hard to make the assumption that there is only one universal explanation for all our observation. Among all the factors, the uncertainty of the – structure-function relationship was the most critical one that require us to be extremely careful and avoid over-simplifying the interpretation of certain mechanism. For example, it has been proposed that the elevated anti-cancer effects 5-demethylatedtangeretin (5DT)

was closely related to the demethylation at the 5- position in their A- ring of Tangeretin (TAN) [19, 267]. However, the exactly mechanism of how the structural change in the functional group are related to the possible change of biological activity is still unknown. In our case, the only structure differences between NBT and their metabolites are the different demethylation site at the B- ring structure. So far, there is very limited study have been conducted regarding such structure-functional relation (e.g. different demethylation site Versus different anti-cancer properties). Therefore, we suspect that for each compound we are studying, it could exert their functions, interacting with even completely different signaling pathways, based on their characterized structure.

In conclusion, our current study for the first time demonstrated the inhibitory effects of NBT and its demethylated metabolites on colorectal CSCs derived from HCT116 and HT-29 cell line. Generally we found that compared to NBT, its metabolites were shown to have equivalent or even stronger effects. Our initial attempt to elucidate the mechanism behind such inhibitory effects indicated that apoptosis, necroptosis and forcing CSCs reentering cell cycle could be potential explanations for the inhibitory effects. We will put our future effort to further elucidation of the possible mechanism (s) for anti-CSC property of NBT and their metabolites, which is crucial for the overall assessment for their potential preventive/ therapeutic application. Most importantly, our previous study (unpublished) in the biotransformation of NBT and the tissue distribution of NBT and their metabolites in the colon mucosa provided with strong rationale to include the metabolites in our study; in this way, as we believe, we have made significant progress in evaluating the overall biological impact of NBT on colorectal cancer, especially on colorectal CSCs

CHAPTER 4

INHIBITORY EFFECT OF 5-DEMETHYLNOBILETIN (5DN) AND ITS DEMETHYLATED METABOLITES ON COLORECTAL CSCS

4.1 Introduction

Colorectal cancer is the third leading cause of death in both men and women in the United States. Multiple barriers obstruct the successful colorectal cancer treatment. Among all the challenges, tumor reoccurrence and metastasis are two major survival-influencing factors of colorectal cancer treatment. For example, up to 40% of colorectal cancer patients who present with stage II or III colorectal cancer treatment will recur after primary treatment [1]. In colorectal cancer treatment, despite the rapid advances in chemotherapeutic drugs, 89% patients with metastatic disease cannot survive [2]. All these indicate the failure of conventional interventions including both radio- and chemo-therapies. Thus, it is of great important for us to develop novel and effective strategies for the control of colorectal cancer.

Increasing experimental evidence begin to support that the existence of a small sup-population of tumorigenic cells, namely, cancer stem cells (CSCs), are responsible for the tumor-initiation, metastasis and recurrence after conventional therapy. Multiple studies have suggested that the development of colorectal cancer is also CSC-dependent, which explains the poor prognosis and therapy failure in colorectal cancer [5, 6, 60, 115]. Targeting CSC compartment, therefore, become a promising solution for both the prevention and /or treatment of colorectal cancer [241].

Epidemiological and dietary interventions studies in both animal and human models have suggested the positive role of many dietary components in inhibiting, reversing

tumor development in different type of cancers [9]. So far, several of the dietary agents have also been shown to interfere with the function of CSCs [10]. Generally, this would be achievable by strategies such as inducing of differentiation, inhibiting of self-renewal signaling pathways and sensitizing CSC to chemotherapeutic agents [8]. For example, curcumin was reported to be able to impair the WNT signaling and cell-cell adhesion pathway in human colon cancer cell (HCT-116), which resulted in apoptosis and G2/M cell cycle arrest [11].

Polymethoxyflavones (PMFs) are a group of compounds that are almost exclusively found in the peels of citrus fruit [12]. So far, PMFs have been found to have wide spectrum of health promoting effect including anti-inflammation and anti-carcinogenesis [13]. Among all types of PMFs, 5-demethylated PMFs are a unique subclass, with 5-demethylated nobiletin (5DN) as the most abundantly one that could be found in orange peel (especially, in aged orange peels)[18]. Even though that multiple bioactivities of 5DN have already been studied by our group and other groups [12, 18, 19], the metabolites of 5DN have been scarcely investigated. Recently, our group isolated the metabolites of 5DN identified three novel urinary metabolites of 5DN, namely, 5,3'-didemethylnobiletin (M1), 5,4'-didemethylnobiletin (M2), and 5,3',4'-tridemethylnobiletin (M3) [25]. Notably, all three metabolites have showed stronger inhibitory effect in human colon cancer cells (SW480, SW620) [25]. Interestingly, according to our recent study (unpublished), after feeding the mice with 5DN (1000 ppm) for 5 months, we found an interesting tissue distribution of 5DN and its three metabolites: even 5DN was still the most abundant one that detected in the colon mucosa, quite a lot of 5DN had also been transformed into M1, M2 and M3. Therefore, in order to fully

evaluate the biological impact of 5DN on colorectal cancer (or colorectal CSCs), we need to all the major metabolites of 5DN as well in this study.

Herein, in this study, for the first time we investigated the inhibitory effect of 5DN and three of its recent identified demethylated metabolites in human colorectal CSCs.

4.2 Materials and methods

4.2.1 Treatment and cell culture

HCT-116 and HT-29 Human colon cancer cell lines were obtained from American Type Cell Collection (ATCC, Manassas, VA, USA), and maintained in RPMI 1640 media (Media tech) supplemented with 5% heat-inactivated fetal bovine serum and 100U/mL of penicillin and 0.1mg/mL of streptomycin at 37°C with 5% CO_2 and 95% air. Cells were kept subconfluent and media were changed every 3- 4 days. All cells used in experiments were between 4 and 20 passages. 5DN and their demethylated metabolites (M1, M2 and M3) were isolated and identified as previously described [25]. DMSO was used as vehicle to deliver 5DN and three of its metabolites to the cells. The final concentration of DMSO in all experiments was 0.1% v/v in cell culture media.

The sphere culture was carried out in serum free DMEM/F12 medium (Life Technologies). Briefly, the serum free medium (SFM) was the supplemented with 1×B27 (Life Technologies), 20ng/ml EGF (Life Technologies), 20ng bFGF (Life Technologies), 5 µg/ml human insulin (Sigma-Aldrich), 100U/ml of penicillin and 0.1mg/ml of streptomycin.

4.2.2 Isolation of colon cancer stem cell and tumor sphere formation assay

Singles cells obtained from HCT-116 and HT-29 cell line were plated in ultra-low attachment 24-well plates (Corning, Lowell, MA, USA) at a density of 12000 viable cells

per well in triplicate. Cells were grown in SFM as described above at 37°C with CO_2 and 95% air. Right after seeding, cells were treated with 5DN (6.25μM, 12.5μm) and its demethylated metabolites (for HCT-116 M1: 0.15 μM, 0.3 μM; M2: 6.25 μM, 12.5 μM; M3: 6.25 μM, 12.5 μM; for HT-29 M1: 1.25 μM and 2.5μM; M2: 6.25 μM, 12.5 μM; M3: 6.25 μM, 12.5 μM) for 7 days. After 7 days, tumor sphere were observed under microscope. For counting of spheres, cells were collected and transferred onto collagen-coated dishes respectively and cultured in RPMI 1640 medium supplemented with 5% FBS. In approximately 24h, tumor sphere were adhered, then stained with crystal violet and counted.

Secondary tumor sphere formation: For the passage of tumor sphere, single cells suspension was first obtained from primary tumor sphere by brief trypsinization. Then single cells from primary spheres were plated at the density of 2000 cell per well in triplicate. The secondary tumor spheres were grown, treated and counted as described above.

In order to compare the sphere-forming potential of primary and secondary test, the results were further normalized as number of tumorspheres formed per 1000 seeding cells.

4.2.3 Detection of apoptosis

HCT-116 and HT-29 cells were seeded and treated exactly the same as described in cell cycle analyses above. After 7 days of incubation, apoptotic cells were quantified by Annexin V/propidium iodide (PI) double staining assay. Annexin V/PI staining was done using apoptotic detection kit (BioVision, Mountain View, CA, USA) following the manufacturer's instruction. Briefly, the content of each well were collected and disrupted by brief trypsinization, and then washed with ice cold PBS. After another wash with

binding buffer, cells were suspended in 0.3 mL Annexin V binding buffer containing Annexin V and PI, and incubated for 5min at room temperature before analysis by flow cytometer. Early apoptotic cells were identified as Annexin V-positive/PI-negative cells, while late apoptotic/necrotic cells were identified as Annexin V-positive/PI-positive cells using BD LSRII cell analyzer at the analytical cytometry facility (University of Massachusetts Amherst).

4.2.4 Cell cycle analyses

HCT-116 and HT-29 cells were seeded as single cell in 6-well suspension plates in SFM at a density of 12000 cells per well. Right after seeding, cells were treated with different concentrations of NBT and demethylated metabolites. After about 7 days, the whole content of each well were collected by brief trypsinization (0.25% trypsin-EDTA; Sigma-Aldrich). Cell pellets were washed with 1mL of ice-cold PBS and then re-suspended in 1mL of 70% ethanol in -20°C overnight. After centrifugation (1600g, 1 min), the supernatant was removed and cells were incubated with 0.3mL of PBS containing 30 µg RNase (Sigma-Aldrich) and 3µg propidium iodine (Sigma-Aldrich) for 30min at room temperature. Single-cell suspension was generated by brief trypsinization. Cell cycle was analyzed using a BD LSR II cell analyzer at the analytical cytometry facility (University of Massachusetts Amherst), and data were processed using Modifit LT software.

4.2.5 Immunoblotting

After treatment with serial concentration of 5DN and their metabolites for 7 days, whole cell lysate will be obtained following method as previously described [268]. Briefly, suspended spheroid cells will then be collected and washed with ice- cold PBS.

Cells will then be incubated on ice for 10 min in lysis buffer (Cell signaling, Beverly, MA, USA) supplemented with cocktails of protease inhibitor (1:100), phosphatase inhibitor I (1:100), and phosphatase inhibitor II (1:100) (Boston Bio products, Boston, MA, USA). After sonication (4 seconds, 4 times), cells will be incubated for 20 mins on ice and followed by centrifugation at 14,000 rpm using a bench top Eppendorf centrifuge for 20 mins at 4°C. Supernatants will be collected as whole cell lysates. Proteins will be quantified by BCA™ protein assay kit (Pierce Biotechnology, Rockford, IL, USA), and 50-150 μg of proteins will be resolved either by 8%, 12% or 15% SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membrane. The membrane containing the transferred protein will be blocked in blocking buffer (5% nonfat dry milk, 1% Tween-20 in 20 mM Tris-buffered saline, pH 7.4) for 2 h at room temperature, and then incubated with appropriate primary antibodies in blocking buffer overnight at 4°C. After incubation with appropriate secondary antibodies for 2 h at room temperature, the membranes will be washed with Tris buffer containing 0.5% of Tween-20, and then be visualized using enhanced chemiluminescence kit (Boston Bio products, Ashland, MA, USA). Antibodies for $p21^{Cip1/Waf1}$, p53, RIP3, poly ADP ribose polymerase (PARP) and cyclin D1 will be obtained from Cell Signaling Technology (Beverly, MA, USA). β-Actin (Sigma-Aldrich) will be used as a loading control.

4.2.6 Statistical analysis

All data were presented as mean ± SD. Student's t-test was used to determine the mean difference between two groups. Analyses of variance (ANOVA) model was used for the comparing the differences among more than two groups. A1% significant level was used for all tests.

4.3 Results

4.3.1 5DN and its demethylated metabolites inhibited tumor-sphere formation in colorectal CSCs

To determine whether the treatment of 5DN and their demethylated metabolites would influence the proliferation of colorectal CSCs, we conducted tumor sphere formation assay on spheroid cells derived from both HCT-116 and HT-29 cell lines (Figure.19 and Figure. 20). Specifically, HCT-116 and HT-29 cells were seeded as single cell in Serum-free medium (SFM) and treated with NBT (6.25μM- 12.5μM) or three demethylated metabolites: M1 (0.15μM-0.3μM), M2 (6.25μM-12.5μM) and M3 (6.25μM-12.5μM). The tumor sphere-forming potential of both primary and secondary CSCs were analyzed. For CSCs derived from HCT-116 cell line (Figure 19 A and B), the primary tumorsphere forming frequency in the control group got increase from about 0.4% (4 tumorspheres formed per 1000 seeding cells) to about 1.7% (17 tumorspheres formed per 1000 seeding cells) in the secondary test, this further supported the notion that sphere-culturing in SFM as an effective tool in enriching the CSC subpopulation. Compared to the control group, the sphere-forming ability following each treatment was significantly impaired in a nearly dose-dependent manner. As shown in Figure 19 the treatment of 5DN at 6.25μM resulted in a nearly 65% percent of inhibition of primary tumorsphere, and at 12.5μM, the inhibitory effect got increased to almost 100%. Then we compared the inhibitory effect of three of its demethylated metabolites (M1, M2 and M3) with that of 5DN (Figure 19 A): for both primary and secondary tumorsphere formation test, the treatment of 5DN (12.5μM) showed stronger inhibitory effects compared to the

two of metabolites, M2 (12.5μM) and M3 (12.5μM); For example, while 5DN (12.5μM) led to a 100% inhibition in secondary tumor sphere formation, M2 (12.5μM) only resulted in a 75% reduction. Among all the demethylated metabolites, M1 showed to have the strongest effect (Figure19 B). For example, in both primary and secondary tumorsphere formation test, M1 at the dose as low as 0.15μM, showed almost the same inhibitory effect as that exerted by 5DN at 6.25μM, which indicated that the M1 was more than 40 times potency than 5DN. Plus, such inhibition of sphere-forming potential was also observed as the reduction of both the numbers and the sizes of tumorspheres (Figure 19 C).

Similar pattern was also observed in the tumorsphere-forming ability of CSCs derived from HT-29 cell line compared to the control group, the treatment of 5DN and its demethylated metabolites (M1, M2 and M3) resulted in a significant decrease in the sphere-forming potential in both primary and secondary test (Figure 20 A and B). For example, M2 (12.5μM) resulted in the inhibition of tumorsphere formation by 67% and 82% in primary and secondary test, respectively. Then we compared the effect of three demethylated metabolites (M1, M2 and M3) with that of 5DN: as shown in Figure 20 A, for both primary and secondary test, at the dose of 12.5μM, 5DN showed significantly stronger inhibitory effect than those groups treated with M2 (12.5μM) or M3 (12.5μM). Among all the demethylated metabolites of 5DN, M1 showed the highest potency (Figure 20 B). For example, in the primary test, M1 at the does as low as 1.25μM, led to almost 80% inhibition in tumorsphere formation, while 5DN at 12.5μM only resulted in 68% inhibition. Plus, such inhibitory effects were also observed in the reduction of both the numbers and sizes of tumorspheres (Figure 20 C).

Overall, we found that the effects of metabolites were comparable to that of 5DN.

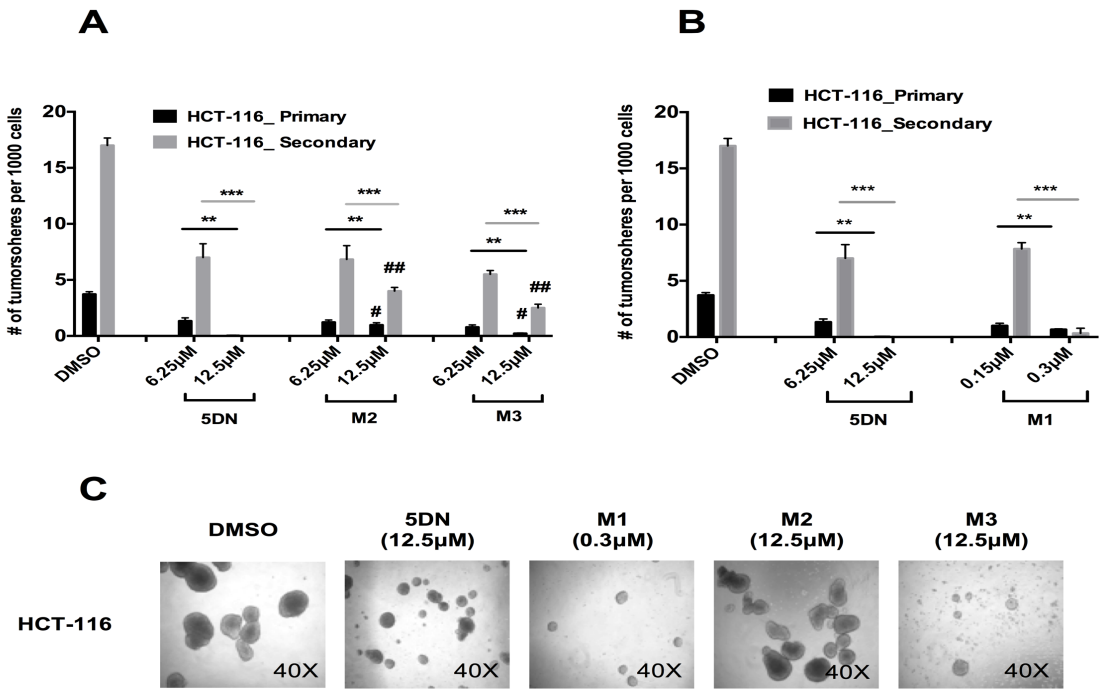

Figure 19. The treatment of 5DN and its demethylated metabolites (M1, M2 and M3) inhibit the tumorsphere formation of colorectal CSCs derived from HCT-116 cell line. **(A) and (B)** For the primary tumorsphere formation assay, single cells from HCT-116 cell line were seeded in 24-well ultra-low attachment plates (12000 cells per well); for the secondary tumorsphere formation, single cells obtained from the primary tumorspheres as described in Material and Methods were seeded in 24-well ultra-low attachment plates (2000 cells per well,) in triplicate in a serum-free (SFM) medium. Right after seeding, CSCs were treated with 5DN (6.25µM to 12.5µM) and three of its demethylated metabolites: M1 (0.15µM to 0.3µM), N2 and N3 (6.25µM to 12.5µM). After 7 days, primary/ secondary tumorspheres were collected and counted. All data represent mean ± SD, * indicated the statistical significance of each treatment in comparison with the control (DMSO) group, as *p<0.05, **p<0.01, ***p<0.001; # indicated the statistical significance of metabolites-treating groups in comparison with the 5DN-treating group, as #p<0.05, ##p<0.01, ###p<0.001. **(C)** Tumorsphere culture demonstrating sphere-formation of colorectal CSCs derived from HCT-116 cell line following the treatment with 5DN and its demethylated metabolites.

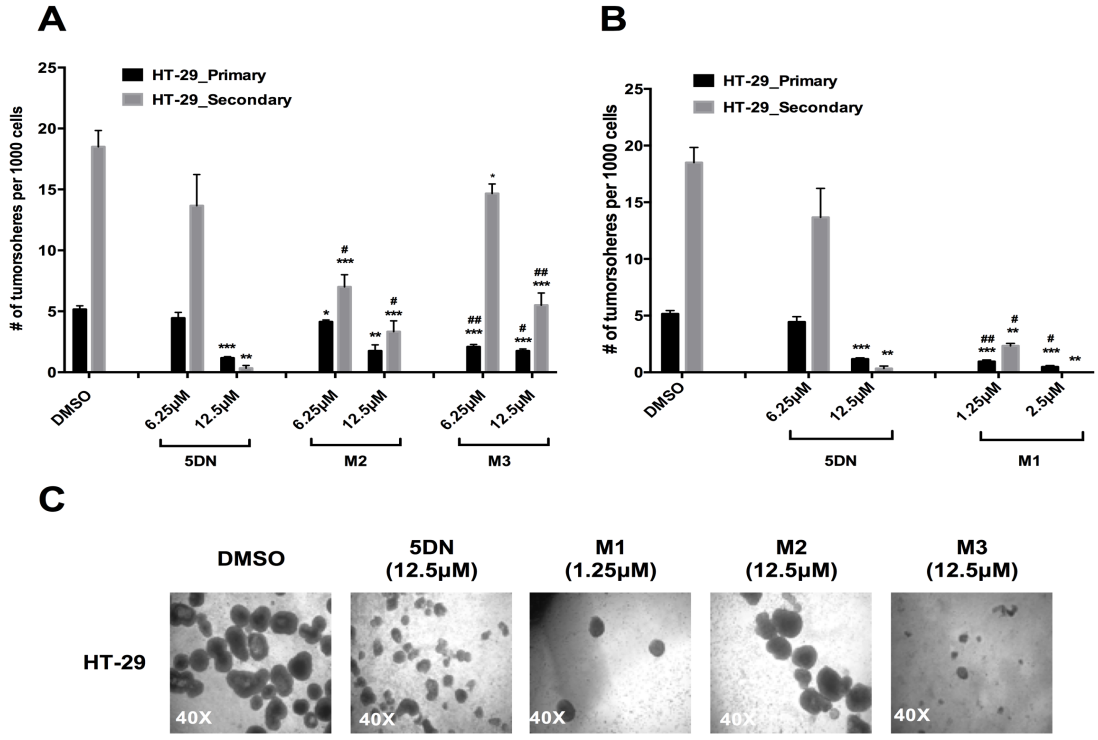

Figure 20. The treatment of 5DN and its demethylated metabolites (M1, M2 and M3) inhibit the tumorsphere formation of colorectal CSCs derived from HT-29 cell line. **(A) and (B)** For the primary tumorsphere formation assay, single cells from HT-29 cell line were seeded in 24-well ultra-low attachment plates (12000 cells per well); for the secondary tumorsphere formation, single cells obtained from the primary tumorspheres as described in Material and Methods were seeded in 24-well ultra-low attachment plates (2000 cells per well,) in triplicate in a serum-free (SFM) medium. Right after seeding, CSCs were treated with 5DN (6.25μM to 12.5μM) and three of its demethylated metabolites: M1 (1.25μM to 2.5μM), N2 and N3 (6.25μM to 12.5μM). After 7 days, primary/ secondary tumorspheres were collected and counted. All data represent mean ± SD, * indicated the statistical significance of each treatment in comparison with the control (DMSO) group, as *$p<0.05$, **$p<0.01$, ***$p<0.001$; # indicated the statistical significance of metabolites-treating groups in comparison with the 5DN-treating group, as #$p<0.05$, ##$p<0.01$, ###$p<0.001$. **(C)** Tumorsphere culture demonstrating sphere-formation of colorectal CSCs derived from HT-29 cell line following the treatment with 5DN and its demethylated metabolites.

4.3.2 5DN and its demethylated metabolites induced apoptosis (necroptosis) in colorectal CSCs.

We next examined the extent to which cell death pathways such as apoptosis and/or necroptosis contributed to the inhibitory effect of 5DN and its demethylated metabolites on tumorsphere forming capacity of CSCs as we have demonstrated in Chapter 4.3.1. For CSCs derived from HCT-116 cell line (Figure. 21), all the treatments including 5DN (12.5μM), M1 (0.3μM), M2 (12.5μM) and M3 (12.5μM) significantly induced apoptosis (necroptosis) compared to the control group. As shown in Figure 21 A, 5DN (12.5μM) resulted in a 2.5 -fold increase (from 5.5% to 13.9%) in the late phase apoptotic (or necroptotic) population compared to the control group. Notably, M3 (12.5μM) increased the total apoptotic (or necroptotic) population by 5-fold, compared to the control group (from 6.7% to 30.7%). Among all the metabolites of 5DN, M1 was the most potent one (Figure 21 B). For example, the treatment of M1 at the concentration as low as 0.3μM resulted in the total apoptotic population as 24%, while 5DN (12.5μM) only resulted in the total apoptotic (or necroptotic) population of 16%.

Similar pattern were also observed in the CSCs derived from HT-29 cell line (Figure 22). Particularly, compared to the control group, all treatments led to signify of induction of apoptotic (necroptotic) cells. As shown in Figure 22 A, 5DN (12.5μM) increased the total apoptotic (necroptotic) cells population from 4.4% to 13.7%; then we compared the apoptosis (necroptosis) inducing ability of 5DN (12.5μM) with those of M2 (12.5μM) and M3 (12.5μM). The results showed that 5DN(12.5μM) induced more apoptotic (necroptotic) cell population than M2 (12.5μM); no significant difference was observed between 5DN(12.5μM) and M3 (12.5μM). As shown in Figure 22 B, M1 (1.25μM)

induced nearly 10% of total apoptotic (or necroptotic) cell population; even though 5DN (12.5μM) induced more apoptosis cells than M1, when considering that the concentration for M1 was 10-time less than that of 5DN, it might still indicate that M1 was much more potent than 5DN.

Overall, we found that the effects of metabolites were comparable with that of the parent compound, 5DN. Especially, we found that M1 was the most potent one among all the metabolites and 5DN.

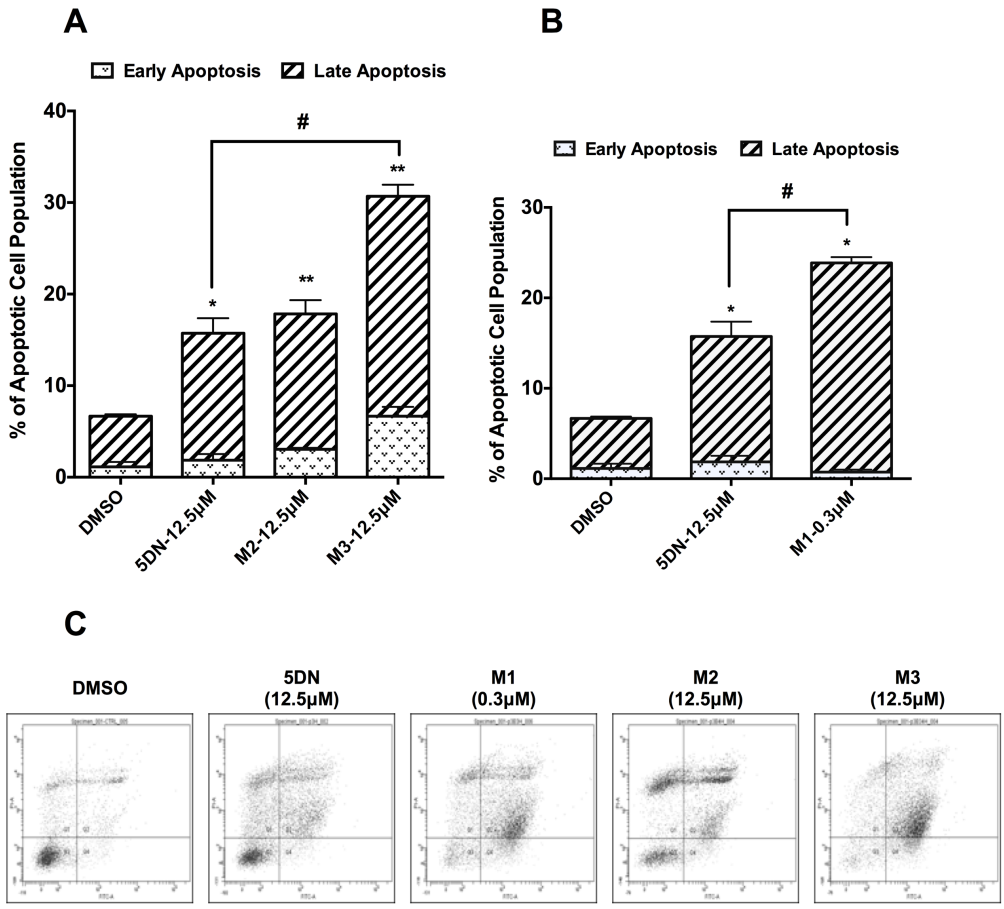

Figure 21. The treatment of 5DN and its demethylated metabolites induced apoptosis (or necroptosis) in CSCs derived from HCT-116 cell line. **(A)** and **(B)** HCT-116 cells were seeded as single cell in 6-well suspension plates in SFM at a density of 12000 cells per well. Right after seeding, cells were treated with 5DN (12.5µM) and demethylated metabolites M1(0.3µM), M2(12.5µM) and M3 (12.5µM). After 7 days, apoptotic cells were quantified by Annexin V/propidium iodide (PI) double staining assay as described in Materials and Methods. FITC ^{high}/ PI ^{low} cells were defined as early apoptotic cells, FITC ^{high}/ PI ^{high} cells were defined as late apoptotic (or necroptotic) cells. All data represent mean ± SD, * indicated the statistical significance of each treatment in comparison with the control (DMSO) group, as *p<0.05, **p<0.01, ***p<0.001; # indicated the statistical significance of metabolites-treating groups in comparison with the 5DN-treating group, as #p<0.05, ##p<0.01, ###p<0.001. **(C)** The distribution pattern of CSCs (HCT-116) following the treatment of 5DN and its demethylated metabolites.

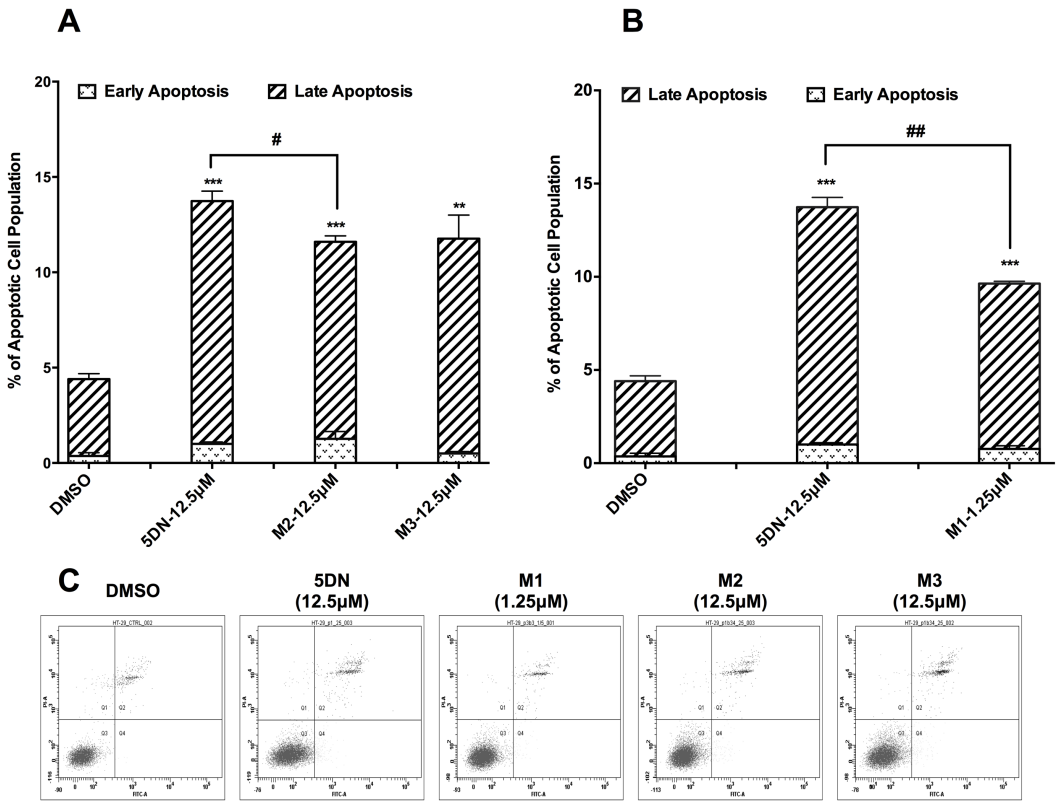

Figure 22. The treatment of 5DN and its demethylated metabolites induced apoptosis (or necroptosis) in CSCs derived from HT-29 cell line. **(A)** and **(B)** HT-29 cells were seeded as single cell in 6-well suspension plates in SFM at a density of 12000 cells per well. Right after seeding, cells were treated with 5DN (12.5μM) and demethylated metabolites M1(1.25μM), M2(12.5μM) and M3 (12.5μM). After 7 days, apoptotic cells were quantified by Annexin V/propidium iodide (PI) double staining assay as described in Materials and Methods. FITC ^{high}/ PI ^{low} cells were defined as early apoptotic cells, FITC ^{high}/ PI ^{high} cells were defined as late apoptotic (or necroptotic) cells. All data represent mean ± SD, * indicated the statistical significance of each treatment in comparison with the control (DMSO) group, as *p<0.05, **p<0.01, ***p<0.001; # indicated the statistical significance of metabolites-treating groups in comparison with the 5DN-treating group, as #p<0.05, ##p<0.01, ###p<0.001. **(C)** The distribution pattern of CSCs (HCT-116) following the treatment of 5DN and its demethylated metabolites.

4.3.3 5DN and its demethylated metabolites caused cell-cycle redistribution in colorectal colon cancer cells

We next investigate the possible effects of 5DN and its demethylated metabolites on the cell cycle distribution of CSCs. Theoretically, the quiescence (or slow cycling) nature of CSCs would make most of CSCs stay in the G0/G1 phase [122] .

For CSCs derived from HCT-116 cells (Figure 23), about 80% of cells in the control group were found in the G1/G0 phase. Plus, All the treatment decreased the cell distribution in G1/G0 phase compared to the control group. For example, the treatment of M3 (12.5μM) decreased the cells distribution in G1/G0 phase from 80% to 74%; Then we compared the effect of three metabolites with that of 5DN. As shown in Figure 23 A and B, compared to M1 (0.3μM), M2 (12.5μM) and M3 (12.5μM), the treatment of 5DN (12.5μM) showed stronger capacity in reducing the cell population in G1/G0 Phase.

Similar pattern was also observed in CSCs derived from HT-29 cell line (Figure 24). The treatment of 5DN (12.5μM), M1 (1.25μM), M2 (12.5μM) and M3 (12.5μM) all decreased the cell cycle population in G0/G1 phase.

Generally, the effects of metabolites were comparable with that of 5DN, with M1 having the strongest effects.

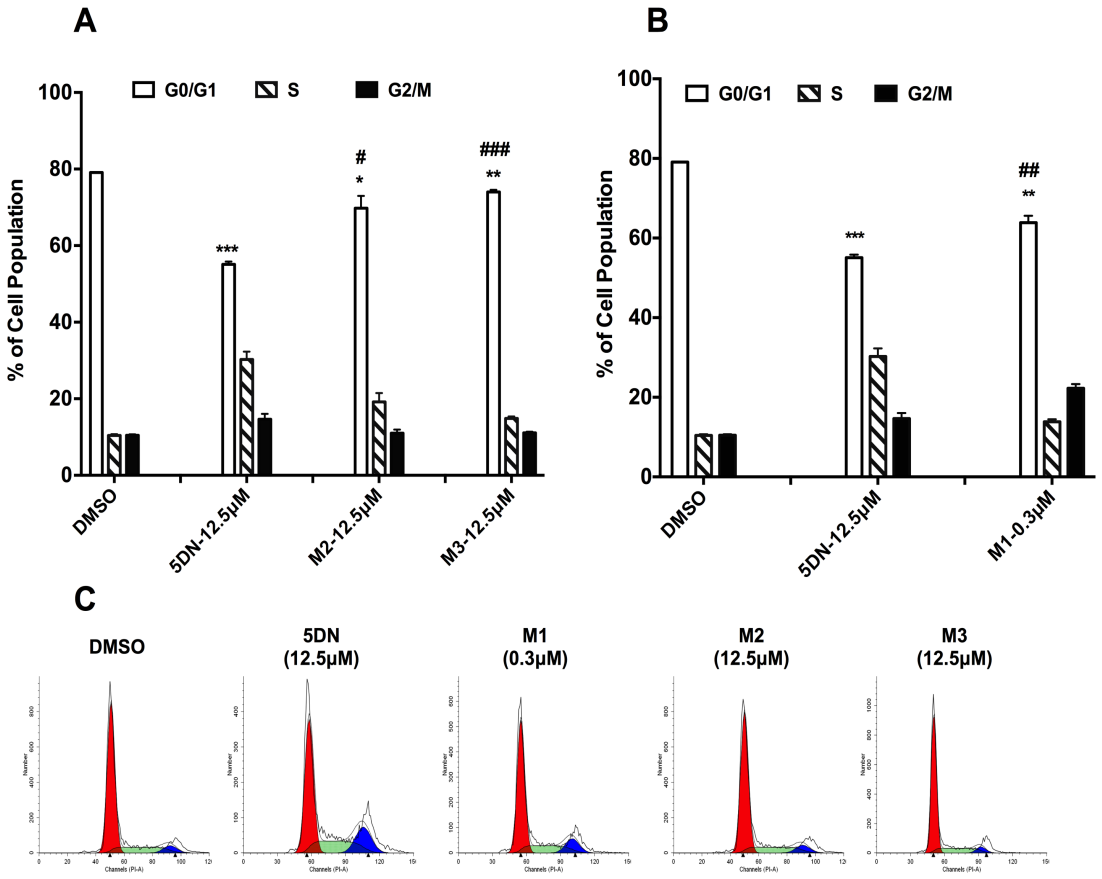

Figure 23. The treatment of 5DN and its demethylated metabolites altered the Cell Cycle distribution of CSCs derived from HCT-116 cell line. **(A)** and **(B)** HCT-116 cells were seeded as single cell in 6-well suspension plates in SFM at a density of 12000 cells per well. Right after seeding, cells were treated with 5DN (12.5µM) and demethylated metabolites M1 (0.3µM), M2 (12.5µM) and M3 (12.5µM). After 5 day, cells were collected and subjected to cell cycle analyses as described in Material and Methods. All data represent mean ± SD, * indicated the statistical significance of each treatment in comparison with the control (DMSO) group as *p<0.05, **p<0.01, ***p<0.001; # indicated the statistical significance of metabolites-treating groups in comparison with the NBT-treating group, as #p<0.05, ##p<0.01, ###p<0.001. **(B)** Cell cycle distribution of CSCs (HCT-116) following treatment of 5DN and its demethylated metabolites.

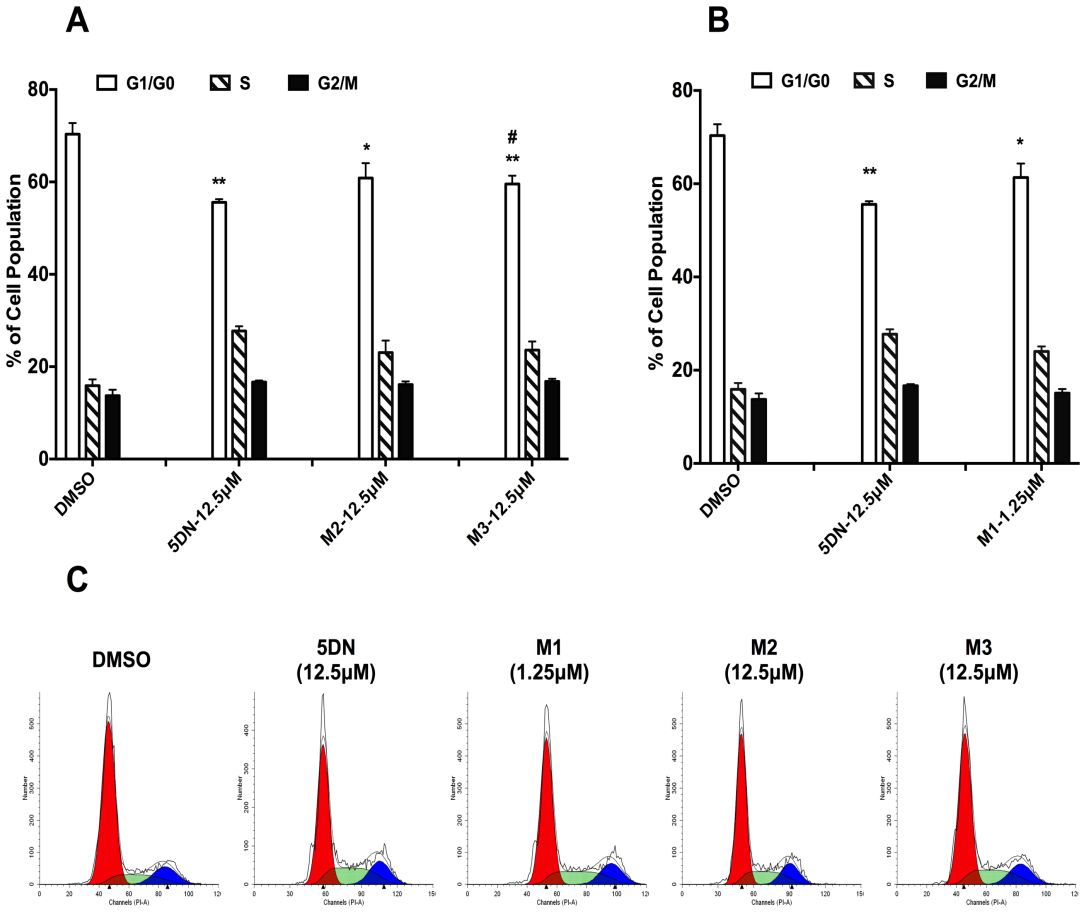

Figure 24. The treatment of 5DN and its demethylated metabolites altered the Cell Cycle distribution of CSCs derived from HT-29 cell line. **(A)** and **(B)** HT-29 cells were seeded as single cell in 6-well suspension plates in SFM at a density of 12000 cells per well. Right after seeding, cells were treated with 5DN (12.5µM) and demethylated metabolites M1 (1.25µM), M2 (12.5µM) and M3 (12.5µM). After 5 day, cells were collected and subjected to cell cycle analyses as described in Material and Methods. All data represent mean ± SD, * indicated the statistical significance of each treatment in comparison with the control (DMSO) group as *p<0.05, **p<0.01, ***p<0.001; # indicated the statistical significance of metabolites-treating groups in comparison with the NBT-treating group, as #p<0.05, ##p<0.01, ###p<0.001. **(B)** Cell cycle distribution of CSCs (HT-29) following treatment of 5DN and its demethylated metabolites.

4.3.4 5DN and its demethylated metabolites inhibited the growth of colorectal CSCs by regulating key proteins related to apoptosis, necroptosis and cell cycle.

In order to elucidate the molecular mechanisms governing the inhibitory effects of 5DN and its demethylated metabolites (M1, M2 and M3) on CSCs from both HCT-116 and HT-29 cell line, we analyzed the effects of treatments on some key proteins in regulating cell cycle and apoptosis by immunoblotting analysis as described in chapter 3.2.5.

To confirm the apoptosis-inducing effect of our treatment, we examined their effects on poly ADP ribose polymerase (PARP) and p53 (for possible p53-dependent apoptosis). In Figure 25, we compared the effects of M2 and M3 with that of 5DN. According to the results, for CSCs derived from HCT-116, 5DN and all its metabolites increased the level of p53; plus, all of our treatments resulted in increased the expression of cleaved PARP, which is the indicator of apoptosis. For CSCs derived from HT-29, it was found that both the level of p53 and cleaved PARP got increased following our treatment. In Figure 26, we compared the effects of M1 and 5DN. According to the results, both 5DN and M1 resulted in the increased expression of p53 and cleaved-PARP, which could be used to support our observation in the apoptosis detection.

In cell cycle arrest, p21 is transcriptionally regulated by p53. Plus, p21 has also been found to control entry into quiescence and maintaining of the quiescent state [282]. According to our result (Figure.25 and Fig. 26), in CSCs derived from HCT-116, there was a significant increase in the level of p21 followed by the treatment of 5DN (12.5μM), M2 (12.5μM) and M3(12.5μM); however, an opposite trend was found following the treatment of M1 (0.3μM), where the level of p21 get dramatically decreased. As for CSCs

derived from HT-29, the level of p21 got decreased after the treatments of 5DN(μM) and M2 (12.5μM), while M1 (1.25μM) and M3 (12.5μM) led to the increased level of p21. Cyclin D1 Plays a central role in the entry of cell cycle from G0/G1 phase, especially, in the G1/S transition [283]. According to our results (Figure 25 and 26), all the treatments led to the increased expression of cyclin D1 in CSCs derived from both cell lines.

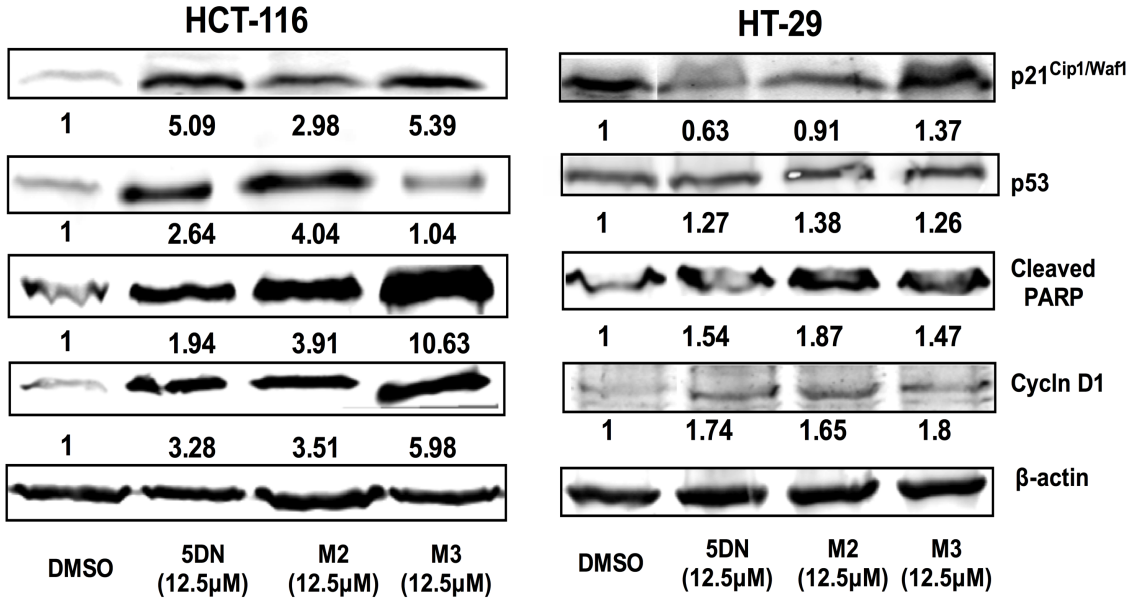

Figure 25. Effects of 5DN and its demethylated metabolites (M2 and M3) in modulating key proteins regulating apoptosis and cell cycle. Immunoblots displayed the treatment of NBT (12.5μM) and three of its metabolites: M2 (12.5μM), M3 (12.5μM) on $p21^{cip1/Waf1}$, p53, cleaved PARP and cyclin D1 on CSCs derived from HCT-116 and HT-29. The numbers under the blots represent band intensity (normalized to β-actin, means of three independent experiments). The standard deviations (all within ± 15% of the means) were not shown.β-actin served as an equal loading control.

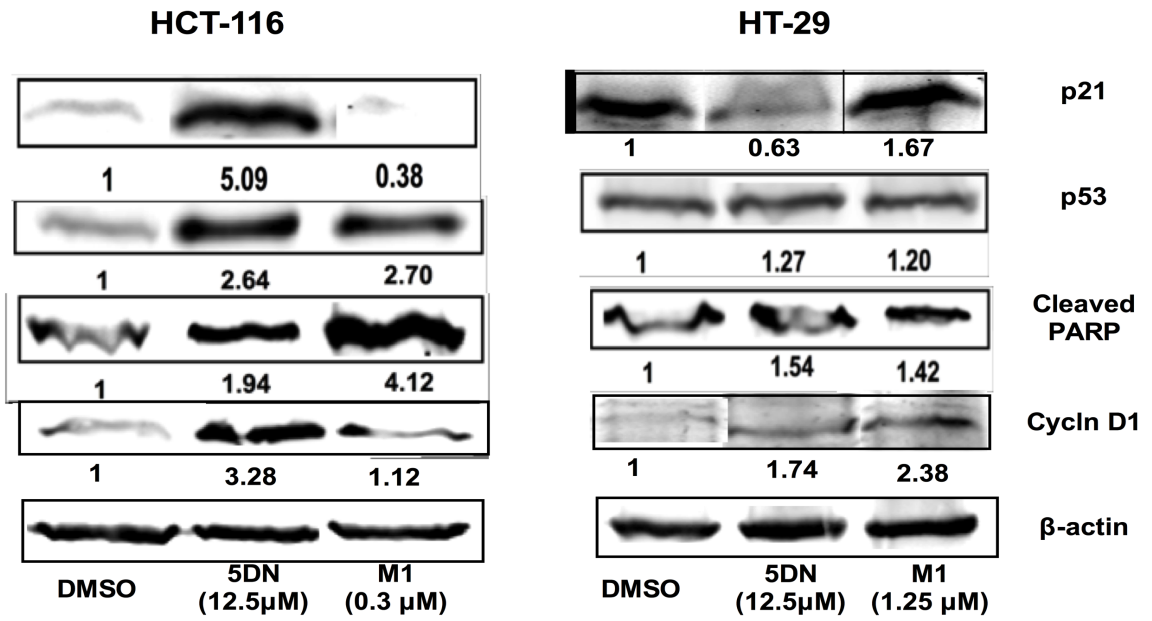

Figure 26. Effects of 5DN and its demethylated metabolites M1 in modulating key proteins regulating apoptosis and cell cycle. Immunoblots displayed the treatment of M1 (0.3μM for HCT-116 and 1.25μM for HT-29) on $p21^{cip1/Waf1}$, p53, cleaved PARP and cyclin D1 on CSCs derived from HCT-116 and HT-29. The numbers under the blots represent band intensity (normalized to β-actin, means of three independent experiments). The standard deviations (all within ± 15% of the means) were not shown. β-actin served as an equal loading control.

Besides apoptosis, necroptosis is also regarded as an important programmed pathway way, especially when apoptosis fails for multiple reasons. Therefore, in our study, we also test that whether our treatments could result in the level change of the RIP3, one of the markers of the necroptosis pathway. As shown in Figure 27, for CSCs derived from HCT-116, all the treatments increased the expression of RIP3; while for CSCs derived from HT-29, only M2 (12.5μM) induced the increase in the RIP3 expression.

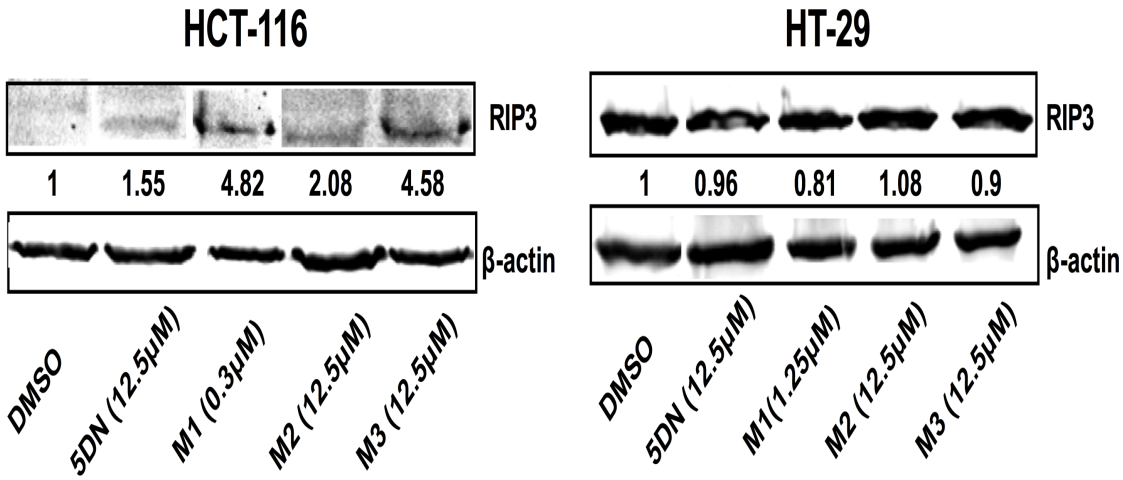

Figure 27. Effects of 5DN and its demethylated metabolites (M1, M2 and M3) in modulating one of key molecular marker of necroptosis, RIP3. Immunoblots displayed the treatment of 5DN (12.5μM), M1 (0.3μM for HCT-116 and 1.25μM for HT-29), M3 (12.5μM) and M3 (12.5μM) on the protein expression of RIP3 in CSCs derived from HCT-116 and HT-29. The numbers under the blots represent band intensity (normalized to β-actin, means of three independent experiments). The standard deviations (all within ± 15% of the means) were not shown.β-actin served as an equal loading control.

4.4 Discussion

Among all types of PMFs, 5-demethylated PMFs are a unique subclass, with 5-demethylated nobiletin (5DN) as the most abundantly one that could be found in orange peel (especially, in aged orange peels) [18]. The bioactivities of 5DN have already been studied by our group and other groups [12, 18, 19]. In order to understand the overall function of certain bioactive compound, we also need to take the biotransformation into account. So far, the metabolites of 5DN have been scarcely investigated. Recently, our group isolated and identified 3 novel urinary metabolites of 5DN, namely, 5,3'-didemethylnobiletin (M1), 5,4'-didemethylnobiletin (M2), and 5,3',4'-

tridemethylnobiletin (M3) [25]. Notably, all three metabolites have shown stronger inhibitory effect in human colon cancer cells (SW480, SW620) [25]. However, all these studies mentioned above were only focused on their effects on the bulk of cancer cells, no reports has yet been published on possible effects of 5DN and its metabolites targeting the CSCs.

In vitro tumor sphere formation has already been proved to be an effective tool in the study of CSCs [85]. In our research, we determined the effects of 5DN and their metabolites in both primary and secondary tumor formation. According to our results, it was found that the frequency of sphere-forming cells in the secondary culture was almost 3 folds as high as that in the primary culture, which further confirmed the "CSCs-enriching" function of the suspension sphere culture. There are evidence supporting that such spheroid- forming cells has significant higher "tumor-initiating" ability in the transgenic mouse model than cells cultured with conventional methods [84, 91, 92]. Since we have found in our previous study in the study of bulk cancer that M1, one of the major metabolites of 5DN, showed significantly potency compared to 5DN and metabolites, thus in this study, the effects of M1 will be separated from other two metabolites and then compared with the effects of 5DN. According to the result from the tumorsphere formation study, we found that for both CSCs derived from HCT-116 and HT-29 cell line, the treatment of 5DN or their demethylated metabolites could significantly impair the *in vitro* sphere-forming ability. Notably, M1 at much lower concentration, showed nearly the same level of effects compared to both 5DN and other metabolites. For example, according to our results, it was shown that M1 was 40 and 10

times as potency as the parent compound 5DN, in treating CSCs from HCT-116 and HT-29, respectively.

Furthermore, prolonged treatment of 5DN or their metabolites resulted in the induction of apoptotic cells as well as a shift in the cell cycle redistribution in CSCs derived from both cell lines. Interestingly, we have observed that for each cell line, the level of responses differs when cells were subjected to different treatment (5DN or metabolites), generally, it was observed that the effects of metabolites were comparable with those of the parent compound, 5DN; particularly, we have found that M1 exerts the strongest effects among all the metabolites as well 5DN. On the hand, results also showed that CSCs derived from the two cell line, responded differently when subjected to the same treatment. This may indicate that the phenotype difference may contribute to the different cellular- sensitivity.

So far, with several research have been focused on the regulation of self-renewal, proliferation and differentiation of CSCs, studies of cell death pathways including apoptosis and necroptosis are still quite limited [168]. In this study, we examined whether 5DN and their demethylated metabolites exert the inhibitory effect on CSCs by regulating some of the key modulators in apoptosis, necroptosis, cell cycle distribution (re-entering the cell cycle).

The cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis [284]. Our results in immunoblotting showed that within each cell line we have test, the treatment of NBT or metabolites resulted in the increased level of cleaved -PARP compared to the control group. For example, M3 (12.5μM) resulted in more 10-fold increase in the level of the cleaved -PARP compared to the control group.

Such increases in cleaved PARP were in line with our observation in the apoptosis test by flow cytometry.

The tumor suppressor, p53, has been implicated in a variety of cellular processes and regarded as the "guardian of the genome'' in cells. It exerts its function by inducing apoptosis and growth arrest. According to our results, treatment of NBT and metabolites resulted in the up-regulation of p53 in CSCs derived from HCT-116, especially significant after the treatment of 5DN (12.5μM), M1 (0.3μM) and M2 (12.5μM). This may indicate that the inhibitory effect of our treatment was p53 -dependent in CSCs derived from HCT-116 cell line. On the hand, for CSCs derived from HT-29 cell line, the level of total p53 protein only got slightly increased following our treatment. A possible explanation would be that rather than an elevation in the total p53 level; the treatment could lead to the more significant increase in the serine phosphorylation level of p53. In a study using curcumin to treat HT-29 cells, high level of the serine phosphorylation of p53 (while total p53 remains unchanged) was shown to be able to down-regulate the anti-apoptotic factor, Bcl-2 and up-regulating the pro-apoptotic factor Bax, thereby decreasing the Bcl-2/Bax ratio and disposing to apoptosis [285].

Recently, the role of p53 in cancer stem cell has been discussed in detail by Aloni-Grinstein et al.[286]. Particular, it has been proposed that p53 can also function as a differentiation inducer in various cell types, which place it as an attractive candidate for differentiation therapy in control of CSCs [286]. According to our result in this study, increased level p53 was observed in CSCs derived from HT-116 and HT-29. Though evidence is still vastly lacked, this might also be novel for our understanding of the mechanism behind the inhibitory effect of NBT and metabolites on colorectal CSCs.

The CDK inhibitor $p21^{CIP1/WAF1}$, is a member of Cip/Kip family and responsible for both p53 dependent and p53-independent cell cycle arrest [287]. During the p53-dependent cell cycle arrest, p21 served as a down stream transcriptional targets of p53; increased levels of p53 would also result in increased level of $p21^{CIP1/WAF1,}$ which will in turn contributes to cell cycle arrest. As for our study, such pattern (increased $p21^{CIP1/WAF1}$, plus increased p53 protein level) was observed in CSCs derived from HCT-116 followed by the treatment of 5DN (12.5μM), M2 (12.5μM) and M3 (12.5μM). A similar trend was also found in HT-29 derived CSCs after the treatment M1 (1.25μM). This could indicate that above mentioned treatments might probably exert their function through p53-dependent p21 mediated pathway.

Evidence supported that disruption of cell cycle inhibition may also contribute to the very nature of CSCs, especially in breaking the balance between the self-renewal and differentiation, which lead to the deregulated self-renewal of CSCs [203]. Theoretically, the quiescence (or slow cycling) nature would make most of CSCs stay in the G0/G1 phase. As has been discussed in chapter 2.3.2, quiescence is one of the major mechanisms contributing the therapy resistance of CSCs. Particularly, G0 phase is viewed as either an extended G1 phase, where the cell is neither dividing nor preparing to divide, or a distinct quiescent stage that occurs outside of the cell cycle [122]. It has also been proposed that, targeting p21 or its down stream target may be an effective means to force quiescent CSCs to cell cycle and thereby cells will be more susceptible to chemotherapies and undergo apoptosis. As for our cell cycle analysis, we found that for control group from each cell line, 70% to 80% of total population of CSCs was in the G0/G1 phase, which is not usual in our study of the bulk of cancer cells (data not shown). This could be an

indication that majority of cells in the suspension culture are CSCs. According to our observation in the cell cycle detection and immunoblotting, a decrease in G0/ G1 phase cell population for both cell lines; In addition, reduced levels of p21 was found in HCT-116 after the treatment of M1 (0.3μM) and in HT-29 after treatment of 5DN (12.5μM), M2 (12.5). Cyclin D1 plays a central role in the entry of cell-cycle, particularly, high level of cyclin D1 expression can be an indicator G1/S transition [283]. According to our results, in CSCs derived from both HCT-116 and HT-29 cell line, all our treatments (NBT and metabolites) led to increased expression cyclin D1, which were in line with our observation in the cell cycle distribution (CSCs re-entered the cell cycle). Still, further evidence is needed to connect the inhibition of above-mentioned treatments on CSCs and the possible inducing of CSCs to "re-enter" the cell cycle.

Besides apoptosis, evidence has been accumulated supporting that apoptosis is not the only mechanisms that could control the cell death. Specifically, necroptosis, also known as the programmed necrosis, has gained more attention in recent years. Besides the necrosis-like morphology changes, necroptosis are also believed to maintains part of the apoptotic features as apoptosis [188, 189]. In our research, we also hypothesize that necroptosis could be another factor contributed to the inhibitory effects of 5DN and metabolites on colorectal CSCs. Specifically, our hypothesis was based on the following reasons: 1. Possible limitation exists for our methods in "apoptotic" cell detection. Time-lapse imaging, which allows monitoring of morphological changes in individual cells in a dynamic and comparative manner, is so far the most effect way to reveal the differences between apoptosis and necrosis (necroptosis) [288]. However, during our research, cells were analyzed by flow fluorocytometry for exposure of phosphatidylserine (PS,

characterized by the binding of FITC) and changes in permeability (uptake of propidium iodide, characterized by binding of PI). Notably, in apoptosis, membrane changes will lead to a rapid PS exposure, while the permeability change will be lagged behind [288] . Such phenomena result in the steady shift from early phase apoptosis (PI ^{low} / FITC ^{high} cells) to late phase apoptosis (PI ^{high} / FITC ^{high} cells); for necroptosis (necrosis), PS exposure overlaps with the changes with permeability changes, which result in the rapid shift of cell population from lower left corner (PI ^{low} / FITC ^{low} cells) to the upper right corner (PI ^{high} / FITC ^{high} cells). Therefore, in our research, since we only tested the cell distribution pattern at the end of 7- day incubation, the upper right cells preciously defined as "late phase apoptosis" cells, could also be cells went through "necrosis (necroptosis)"; 2. It has been reported that necroptosis is an alternative cell death pathway especially when apoptosis cannot be achieved because of the ATP failure (apoptosis is the most energy-consuming process since that it requires the activation of multiple caspases and the formation of apoptosome) [200-202]. As in our study, CSCs were derived in serum free medium (SFM) supplemented with growth factors (see Material and Methods) and cultured extensively for 7 days. Such condition would result in the possible "energy starvation" thus necroptosis could be accounted as an alternative death pathway involved. 3. One of the key characters of CSC is higher apoptosis-resistance compared to the non-CSC counterpart [289], this could be another reason besides ATP failure that can make necroptosis an alternative explanation for the inhibitory effect of our treatment.

To test hypothesis, we test the expression of the RIP3, one of the key molecular regulators of necroptosis [192, 193] by immunoblotting. It was shown that our treatment

caused increased RIP3 level in CSCs derived from HCT-116, with M1 (0.3μM) and M3 (12.5μM) having the strongest effect. While for CSCs derived from HT-29, only the treatment of M1 (1.25μM) showed RIP increasing potential. Evidence have been supporting that caspase 8 plays opposite roles in cross-talk between apoptosis and necroptosis: TNF activate apoptosis by triggering a series of molecular events which eventually lead to the activation of caspase 8; while in necroptotic pathway, the function of caspase 8 is disrupted or even inhibited [191]. Therefore, we will further confirm our observation by assessing the level of caspase 8 in order to elucidate the possible relationship between the inhibitory effect of our compounds and the necroptosis pathways.

In our current study, we conducted the initial investigation of the possible mechanisms of which 5DN and its metabolites exerted their inhibitory effects on colorectal CSCs. However, we believed that 5DN and each of its metabolite should be studied independently in order to elucidate the mechanisms. There are multiple factors exist and make it hard to make the assumption that there is only one universal explanation for all our observation. Among all the factors, the uncertainty of the – structure-function relationship was the most critical one that require us to be extremely careful and avoid over-simplifying the interpretation of certain mechanism. For example, it has been proposed that the elevated anti-cancer effects 5-demethylatedtangeretin (5DT) was closely related to the demethylation at the 5- position in their A- ring of Tangeretin (TAN) [19, 267]. However, the exactly mechanism of how the structural change in the functional group are related to the possible change of biological activity is still unknown. In our case, the only structure differences between 5DN and their metabolites are the

different demethylation site at the B- ring structure. So far, there is very limited study have been conducted regarding such structure-functional relation (e.g. different demethylation site Versus different anti-cancer properties). Therefore, we suspect that each compound we are studying could exert their functions by interacting with completely different signaling pathways, based on their characterized structure.

In conclusion, our current study for the first time demonstrated the inhibitory effects of 5DN and its demethylated metabolites on colorectal CSCs derived from HCT116 and HT-29 cell line. Generally, we have found that the effects of metabolites were comparable to those of 5DN, with M1 having the strongest effects among all our treatments. Our initial attempt to decipher the mechanism behind such inhibitory effects indicated that apoptosis, cell cycle pathways, necroptosis as well as the "quiescence" nature of CSCs could be potential targets of our compounds of interests. We will put our future effort to further elucidation of the possible mechanism (s) for anti-CSC property of 5DN and their metabolites, which is crucial for the overall assessment for their potential preventive/ therapeutic application. Most importantly, our previous study (unpublished) in the biotransformation of 5DN and the tissue distribution of 5DN as well as their metabolites in the colon mucosa provided with strong rationale to include the metabolites in our study; in this way, as we believe, we have made significant progress in evaluating the overall biological impact of 5DN on colorectal cancer, especially in colorectal CSCs.

CHAPTER 5

GENERAL DISCUSSION AND THE ROAD AHEAD

5.1 Challenges in the cancer stem cell study

Though regarded as one of the hottest topic in the research of cancer, the field of CSC-study it still at the nascent stage; as a result, multiple challenges exist with the field.

One of the most debated areas is the isolation and quantification of CSCs. As we have discussed in previous chapters, the use of surface of markers, xenograft in murine model and *in vitro* spheroid cultures have been the major tools used to study the colorectal CSCs. The use of surface makers, as many researchers believes may not be the optimal method to purify CSCs from the tumor bulk. One argument is that the significant amount of genetic variability exist in the colorectal caner may not necessarily be reflected as change in the phenotype [44]. For example, due to the absence of mRNA downregulation after colorectal CSC differentiation, CD133, generally regarded as "solid" marker, has been criticized recently [290]. In a study of lung CSCs, it was shown that the cell surface marker differs among tumors initiated by oncogenic K-ras, EGFR, suggesting that the genotype of the tumor must be identified for the proper characterization of the CSCs , in other world, the use of CSC surface markers should be carefully defined for each tissue and tumor sub-type.

The xenograft assay, though regarded as the "golden standard" in the preclinical CSC research, is still facing criticisms. One of the major concerns is that since xenograft is basically identifying CSC fraction in transgenic murine model, the microenvironment of the mouse might have completely different impact on CSC behavior relative to that of human. Plus, evidence has shown that the CSC frequency varies among different

transgenic models used [291]. Given the clinical relevance of both the frequency and properties of CSCs, it is of great important to choose the best available xenograft model for every individual tumor subtype.

The spheroid culture has been seen as the most convenient tool in isolating and expanding colorectal CSCs [6, 92, 103]. Utilizing such tool, a large number of CSCs can be prepared for multiple experimental purposes such as *in vivo* xenografts. However, some scientists have criticized the use of convention cell lines as the source of the spheroid culturing. The multiple passages that cell lines undergo in the *in vitro* setting may result in huge behavioral change compared to the cells in human patients. Rather, it has been suggested that using primary samples from human patients may render a better credibility.

The other challenge in the study of CSC has been to answer the question the whether the CSCs are static or plastic. It has been found that normal stem cells can re-enter the stem cell [292], this shed light on the idea that is could also be possible that CSCs can be generated from non-CSCs under certain situation [293]. Such reversibility of tumorigenicity has been supported by some studies using *in vitro* models [294, 295]. Besides, the microenvironment, which CSCs reside, might be able to induce the "stemness" in non-CSC component. For example, in an intestinal tumor initiation mouse model, epithelial non-CSC can reexpress stem cell markers followed by Wnt activation, and thereby "dedifferentiate" to CSCs [296]. A better understanding of the possible plasticity nature of CSC would help us to gain deeper insights into the cancer hierarchies.

5.2 Friend or Foe: the multiple roles of intestinal microbiota in the cancer development and diet-based chemoprevention.

Traditionally, our perception of the function of the large intestine has been limited to the reabsorption of water, salt as well as removing of the "useless" food debris; such understanding almost completely ignores the presence of the microbiota. The fact is, in human body, more than 100 trillion microbiota are found in the intestines, which are necessary for the maintenance of physiologic homeostasis. Notably, significant variability exists in terms of the density as well as the complexity of the microbiota: for example, the gut flora consists of 300 to 1000 different species of bacteria, which exert multiple metabolic activities [297]. Evidence have shown that gut microbiota have about 100 times more genes compared to those in human genome [298]. In recent years, advances in technology such as sequencing has fueled the microbiota research, especially, it has been widely accepted that microbiota can be seen as the "forgotten" organ [299]. Interestingly, it has been proposed that the higher microbial density in the colorectal compared to that in small intestine, may contribute to the much higher incidence of cancer in the colorectal relative to the small intestine [300].

As has been discussed in chapter 2.2.4, the tumor microenvironment has been recognized as one of the "hallmarks" epithelial cancers including colorectal cancer. As one of the key compartment of the microenvironment, microbiota also contribute to the carcinogenesis. According to studies in germ-free animals, a number of microbiota species are able to initiate and promote intestinal tumorigenesis [301, 302]. So far, it is still unknown that whether such tumor-promoting effects of the microbiota are due to one particular species or whether a small community of species is responsible [303]. Even

though that there is so far very limited reports concerning the implication of microbiota on the development of colorectal cancer under the CSC hypothesis; however, the future study of the host-microbiota interaction may shed light on the understanding of the CSC biology.

Besides the tumor-promoting potential of certain specifies within the intestinal microbiota, there might also be some species that are playing important, if not central roles in the metabolism of bioactive compound found in the food matrix. Generally, with so many efforts have been put in search of the diet-base chemopreventive strategies, our understanding of the possible influence of diet on cancer occurrence is still poorly characterized [28]; this may at least partial due to our previous ignorance of the microbiota in the large intestine. Studies begin to accumulating showing that while in most cases, bacterial metabolism reduces the activity of dietary compounds; however, sometimes a specific product of bacterial transformation exhibits enhanced or more beneficial properties [304]. In other words, the bioactive properties of metabolites can be completely different from the parent compounds. For example, while the majority of dietary anthocyanins cannot be absorbed in the upper gastrointestinal tract, they would therefore be further metabolized by intestinal microbiota and become “absorbable” in the colon; such transformation may render anthocyanins with stronger impact on the microbiota as well as the host [305].

Nevertheless, due to the huge diversity of intestinal microbial exist within different individuals, the metabolic profile as well the final impact of certain bioactive compounds would also be highly variable. For example, in many situations, only the “products” of certain bacterial metabolism can be absorbed and exert the beneficial impact on human

health; for people who is lacking of certain microbiota which are necessary for such "desirable" biotransformation, the beneficial effect the consumption of the bioactive compounds would hardly be achievable [304].

In conclusion, it will be crucial to elucidate the multiple roles that microbiota plays in the development of colorectal cancer as well as the metabolism of certain bioactive components. In order to achieve better chemopreventive effect, it will be crucial to make dietary suggestion according to the unique "signature" of intestinal microbiota for each individual; plus, implantation of new or even genetically altered microbiota while excluding carcinogenic species might also be used to maximize the health-promoting benefit of the consumption of certain bioactive compounds while minimizing the possible tumor-promoting risk such as inflammation.

5.3 Future Direction

With the completion of this project, we have gathered important data, which will provide rationale for the future pre-clinical and clinical application of PMFs and their metabolites in the control of colorectal cancer. Notably, we invested the possible effect of PMFs and their metabolites in targeting the CSC subpopulation. The revolutional theory of CSC changed our previous view of cancer development. However, the current understand of CSCs is still at the infant stage and many issues still haven' t be solved with the complexity of the hierarchical organization of some tumor types. With the development of technologies such as gene sequencing, we will be able to gain better insights of the stemness nature of CSCs within the tumor bulk. Plus, more attentions should also be focused on the interaction between the microenvironment and the CSC subpopulation, since it may be playing an important role in maintaining the proper

behavior of CSCs. More efforts should be put on the improvement of the purification of CSCs, as well as the better understanding of the cross- talk among multiple molecular events in the CSC biology.

The interaction between PMFs and the intestinal microbiota are crucial for understanding the role of these compounds and their effects on human health. The knowledge obtained in pre-clinical models has to be further verified in human trials. The microbiota that would be identified as the key players in the biotransformation of PMFs, could thereby be used as biomarkers to anticipate the possible difference responses among individuals followed by the consumption of PMFs; while it might also be promising that certain PMFs can be chemically modified before consumption to achieve the maximum biological potential.

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