**Marquette University**

**e-Publications@Marquette**

***Dental Faculty Research and Publications/School of Dentistry***

***This paper is NOT THE PUBLISHED VERSION*.**

Access the published version via the link in the citation below.

*Medicinal Research Reviews*, Vol. 41, No. 1 (January 2021): 395-434. [DOI](https://doi.org/10.1002/med.21735). This article is © Wiley and permission has been granted for this version to appear in [e-Publications@Marquette](http://epublications.marquette.edu/). Wiley does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from Wiley.

Recent Developments in Targeting Genes and Pathways by RNAi-based Approaches in Colorectal Cancer

Asiyeh Jebelli

Higher Education Institute of Rab-Rashid

Behzad Baradaran

Tabriz University of Medical Sciences

Jafar Mosafer

Torbat Heydariyeh University of Medical Sciences

Amir Baghbanzadeh

Tabriz University of Medical Sciences

Ahad Mokhtarzadeh

Tabriz University of Medical Sciences

Lobat Tayebi

Marquette University

# Abstract

A wide spectrum of genetic and epigenetic variations together with environmental factors has made colorectal cancer (CRC), which involves the colon and rectum, a challenging and heterogeneous cancer. CRC cannot be effectively overcomed by common conventional therapies including surgery, chemotherapy, targeted therapy, and hormone replacement which highlights the need for a rational design of novel anticancer therapy. Accumulating evidence indicates that RNA interference (RNAi) could be an important avenue to generate great therapeutic efficacy for CRC by targeting genes that are responsible for the viability, cell cycle, proliferation, apoptosis, differentiation, metastasis, and invasion of CRC cells. In this review, we underline the documented benefits of small interfering RNAs and short hairpin RNAs to target genes and signaling pathways related to CRC tumorigenesis. We address the synergistic effects of RNAi-mediated gene knockdown and inhibitors/chemotherapy agents to increase the sensitivity of CRC cells to common therapies. Finally, this review points new delivery systems/materials for improving the cellular uptake efficiency and reducing off-target effects of RNAi.

# Abbreviations

**5-FU =** 5-fluorouracil

**APC =** anaphase promoting complex/cyclosome

**Asp =** aspirin

**BORIS =** brother of the regulator of imprinted sites

**bPEI =** branched polyethyleneimine

**CDK =** cyclin-dependent kinase

**CIN =** chromosomal instability

**CNT =** convallatoxin

**CRC =** colorectal cancer

**DOX =** doxorubicin

**dsRNA =** double strand RNA

**EMT =** epithelial-mesenchymal transition

**FZD =** frizzled

**HRT =** hormone replacement therapy

**IAP =** inhibitor of apoptosis protein

**JAK =** Janus kinase

**LS =** left-sided

**mAB =** monoclonal antibody

**mCRC =** metastatic CRC

**MIN =** microsatellite instability

**MMP =** matrix metallopeptidase

**MMR =** mismatch repair

**NP =** nanoparticle

**NSAID =** nonsteroidal anti-inflammatory drug

**PDX =** patient-derived xenograft

**PLK =** polo-like kinase

**RISC =** RNA-induced silencing complex

**RNAi =** RNA interference

**RRM2 =** M2 subunit of ribonucleotide reductase

**RS =** right-sided

**shRNA =** short hairpin RNA

**siRNA =** small interfering RNA

**SNALP =** stable nucleic acid lipid particles

**STAT3 =** signal transducer and activator of transcription

**STK33 =** serine/threonine kinase 33

**TTR =** transthyretin

**uPA = u**rokinase-type plasminogen activator

**VEGF =** vascular endothelial growth factor

**WT =** wild type

# 1 INTRODUCTION

The third most common cancer diagnosed in either men or women is known as colorectal cancer (CRC).1, 2 CRC occurs in the colon and rectum and develops slowly from adenomatous polyps.3 The overgrowth of the colonic mucosa creates these polyps with either a pedunculated or sessile morphology.4

Different pathways for CRC development are considered; among them, the serrated pathway and classical adenoma-carcinoma sequence are more notable.5 The serrated pathway involves serrated lesions, which are detected in one-third of all CRCs. These serrated lesions are defined by saw-toothed appearance of the crypt epithelium and occur mainly in the proximal colon. Finally, they have variable potential to arise CRC and are characterized by the distinct molecular profiles.6

Regarding the adenoma-carcinoma sequence, it is proposed that the progression from small adenomas to large adenomas or advanced adenocarcinomas is mediated by multiple molecular pathways, including the microsatellite instability (MIN), the chromosomal instability (CIN) and the epigenetic pathway. MIN comes from a germline mutation in the DNA mismatch repair (MMR) genes like *PMS2, MLH1, MSH2*, or *MSH6* that causes the loss of MMR activity and accounts for about 15% of all CRCs.7 CIN is based on the acquisition of mutational events in oncogenes and tumor suppressor genes, such as *APC* and *KRAS*, respectively.8 The last pathway arises through hyper-methylation of genes in their promoters rather than mutational occurrence that leads to silencing of tumor suppressor genes like *MLH-1*, one of the MMR compartments.8

Based on location, CRC tumors are classified into two groups: right-sided (RS) tumors in the proximal colon and left-sided (LS) tumors in the distal colon. LS CRC tumors occur more frequently than RS ones, generally 60% versus 40%, respectively. LS and RS tumors have differences in clinical and molecular aspects. Inflammatory reaction, poor differentiation, and high TNM are common characteristics observed in RS tumors, which mostly occur in females and/or the elderly.9 While RS tumors represent a higher frequency of abdominal viscera and lymph nodes metastases, LS tumors disseminate often to the liver and chest. RS tumors are associated with MIN, diploid DNA content and more mutations in signaling pathways like *MAPK, ErbB, TGFβ* compared to LS tumors.10 In return, CIN and amplification/deletion (in 20q and 18q, respectively) are more common in LS carcinomas. Some *HOX* genes such as *HOXC6, HOXB6*, and *HOXB13* are also differentially expressed in LS and RS carcinomas.11

It is possible for interventional strategies to prevent cancer because it estimated that it takes at least 10 years for a polyp to transform to a cancer cell.4 Early diagnosis (through colonoscopy, sigmoidoscopy and, newer modalities like stool DNA-based screening) and improvements of treatment methods have contributed to prolonged survival in the curable phase of CRC. Nonetheless, metastasis is present in around 25% of CRC patients during the diagnosis, and, in total, 50% of patients with CRC will develop metastasis.12 A substantial prognostic factor in metastatic CRC (mCRC) is the location of primary tumor.9 Due to the heterogeneous and multifactorial nature of CRC, studies for finding clinical and molecular predictive and prognostic factors have considerably increased over the last decade and have the potential to improve patient management.3, 9

This review article first presents the various traditional therapies of CRC, after providing overall information about epidemiology and risk factors related to colon carcinogenesis. Then, the review focuses on the new nucleic acid-based medicines, defined as RNA interference (RNAi) therapy, with promising prospects for CRC therapy.

# 2 EPIDEMIOLOGY

CRC mortality and incidence rates notably vary based on the location in the world. The highest incidence rates are estimated in North America, Australia, New Zealand, and Europe (especially in Slovakia, Hungary, and Czech Republic). In contrast, the lowest rates of CRC are reported in South-Central Asia and Africa.1, 13 CRC incidence is approximately 20% higher in African Americans than in whites. These differences seem to be associated to diet differences, environmental exposures, socioeconomic status, race, and ethnicity.1, 4 However, it seems that the true prevalence of colorectal polyps is unknown in many developing countries because of less accessibility to endoscopy in these areas.4 The rates of CRC incidence and mortality are approximately 25% higher in males than females, presumably due to the protective role of female hormones.14

At least 600,000 CRC-related deaths occur, along with one million new cases of CRC diagnosed every year worldwide.2, 15 In the United States, there were about 97,220 new cases and 50,630 deaths in 2018, according to the American Cancer Society's estimates. Overall, there is a slightly higher risk of developing CRC in men than women, with a lifetime risk of about 1 in 22 (4.49%) for men and 1 in 24 (4.15%) for women.16 The management of CRC is associated with notable health care costs, with national expenditures being more than $14 billion annually in the United States.17

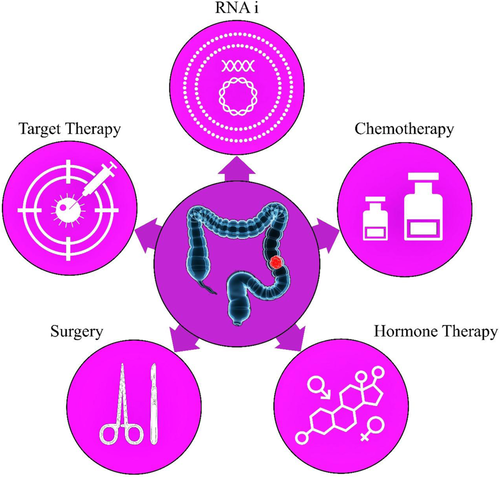
In total, the rates of CRC incidence and mortality have been dropping for men and women from 1970 to now due to early detection.13, 18 For example, CRC incidence rates over the last 15 years in the United States have decreased approximately 2.5%–4% per year. However, during this period, these rates have rapidly grown in several regions historically at low risk, including Spain and a number of countries within Eastern Europe and Eastern Asia, while have remained unchanged in most other western countries.1 These findings demonstrate differences in lifestyle modifications and risk of CRC, but the majority of CRC occurs in individuals without known risk factors.4 Table **1** summarizes some known positive and negative factors associated with the risk of CRC.

**Table 1.**The factors with positive and negative effects on the risk of CRC4, 17

|  |  |
| --- | --- |
| **Factors associated with a decreased risk** | **Factors associated with an increased risk** |
| Healthy diet (high in fruits, vegetables, calcium, fiber, folate, and vitamin D) | Unhealthy diet (high in red and processed meat) |
| Healthy body weight | Obesity |
| Minimum alcohol intake | High alcohol consumption |
| No smoking | Smoking |
| Regular physical activity | Physical inactivity |
| Nonsteroidal anti-inflammatory drugs, like aspirin and NSAIDs | Family history of colorectal polyps |
|  | Family history of colon or rectal cancer |
|  | Inflammatory bowel disease |
|  | Type 2 diabetes mellitus |
|  | Older age |
|  | Male sex |

# 3 CONVENTIONAL TREATMENT

Although a range of therapies for advanced mCRC are available, the results remain suboptimal19 due to the fact that heterogeneity, prognosis, molecular characteristics, response to therapy, and clinical presentation of CRC greatly vary among patients.20 Common treatments of CRC are based on surgery, chemotherapy, targeted therapy, and hormone replacement therapy (HRT).13 Figure **1** represents possible therapeutic strategies for CRC therapy.

[](https://onlinelibrary.wiley.com/cms/asset/e1eef97a-6dad-4005-b61b-43e6a3e368bd/med21735-fig-0001-m.jpg)

**Figure 1** A schematic image of possible therapeutic strategies for the management of CRC.

At least 50% of patients with CRC will relapse after surgical resection and ultimately die of metastatic disease. Despite adjuvant postoperative chemotherapy uses in patients to decrease the risk of recurrence by eliminating any latent viable tumor cells that may be stable after surgery,15 adjuvant treatment gives no survival benefit for patients, especially those diagnosed with stage I CRC.3

For improvement of survival in CRC patients, antimetabolite 5-fluorouracil (or simply 5-FU) is commonly used as a chemotherapeutic agent.21 In most cases, the overall response rate of 5-FU monotherapy in advanced CRC is restricted to 10%–15%. A potential way to improve the response rates and reduce adverse effects is combination therapy.13 However, drug resistance, toxicity and nonspecific action of chemotherapy agents are non-negligible adverse effects of conventional cytotoxic chemotherapy. In addition to tumor cells, nonmalignant cells—such as follicle cells, erythrocytes, leukocytes, and mucous membranes of the oral cavity and gastrointestinal tract are also affected by chemotherapeutics. These side effects impair life quality, physical health, and emotional state of patients.22-26

Targeted therapy is based on monoclonal antibodies and small molecule inhibitors, which selectively block the cancer cell proliferation through intervention with certain molecules and overexpressed proteins required for tumor expansion and growth.27 Targeted therapy is often less toxic and better tolerated than traditional chemotherapy. However, large alterations between individual treatment responses exist.20, 27 This treatment tool is usually effective in cancers with a specific molecular target. Hence, absence of such a target could markedly influence the treatment effectiveness.This distinction may be influenced by patient sex, ethnicity, and tumor histology.27 For instance, targeted therapy has shown no benefit of anti-EGFR agents in mCRC tumors with mutations in exon 2, 3, or 4 of *KRAS* that are negative predictive factors for anti-EGFR therapy. For these reasons, some anti-EGFR agents—such as cetuximab and panitumumab—have been used only in mCRC patients with wild-type *KRAS*.9, 12, 20 On the other hand, it must be emphasized that a main challenge in using antibodies is allergic reactions. Panitumumab is a fully humanized antibody, and the occurrence of allergic reactions in this antibody is lower than cetuximab. The cost of targeted therapy agents, determining optimal dosing, and effectiveness of targeted therapy are other critical barriers that must be overcome.

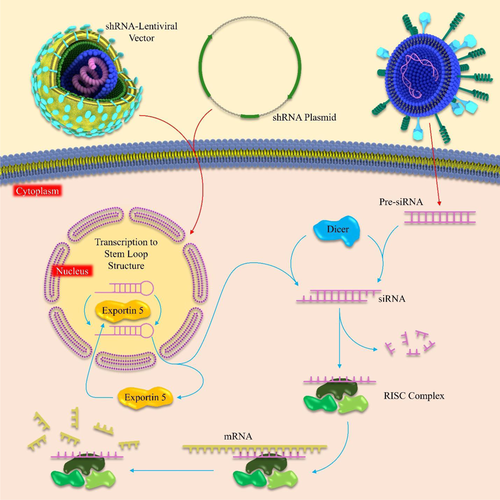
It is known that HRT can have a protective role in CRC. The estrogen-alone or combined with progestin therapy is associated with the inferior cancer development risk.28 However, since numerous studies have yielded inconsistent results related to the effect of estrogen on the increase of proliferation of CRC cell lines, it seems that HRT plays a dual role in CRC pathogenesis and clinical management.29, 30 In addition, serious side effects of HRT, including cardiovascular complications and increased breast cancer risk, have limited the use of this method for control and treatment of CRC.28

Despite the advances in disease treatment tools discussed in this section, the outcomes in CRC therapy are far from satisfactory. However, the novel nucleic acid medicines can be more encouraging as an anticancer approach for this purpose.

# 4 RNA INTERFERENCE

RNAi is an endogenous biological process that can control vital progressions such as cell growth/proliferation, heterochromatin formation and tissue differentiation through highly specific and selective knockdown of target genes.31, 32 Accordingly, RNAi dysfunction is connected to neurological disorders, cardiovascular diseases and several types of cancer.31 Furthermore, to ameliorate the efficacy of cancer treatment, RNAi therapy could also be combined with immune modulating agents.33 Short hairpin RNA (shRNAs) and small interfering RNA (siRNA) are two classes of noncoding RNAs which regulate gene expression via RNAi phenomenon.31, 34

SiRNAs are processed from a double strand RNA (dsRNA), either transcribed or artificially introduced into the target cells. In the first step, siRNAs are excised from long, fully complementary dsRNAs by an endo-ribonuclease protein called Dicer to create a RNA with 20–30 nucleotides, commonly bearing a two nucleotide (usually TT and UU) overhang at the 3′ end of each strand. These nucleotides are important for recognition by the RNAi machinery. Then, siRNA is loaded into a multiprotein component complex denoted as the RNA-induced silencing complex (RISC). Within the RISC complex, siRNA is unwound to form two single strands. The strand with stable 5′ end typically remains part of the RISC complex, guiding and aligning the RISC complex on the target messenger RNA (mRNA). Finally, through the action of catalytic RISC proteins (i.e., Argonaute), mRNA is cleaved (Figure **2**).35, 36 Dicer knockout embryonic stem cells can efficiently burden processed siRNA onto RISC to carry out RNAi as effectively as Dicer wild type (WT) cells. This result indicates that the load of absolutely processed exogenous siRNA onto RISC for RNAi is independent of Dicer and its auxiliary proteins, TRBP, and PACT.37

[](https://onlinelibrary.wiley.com/cms/asset/ac6003af-74a0-482a-94ce-12bdff774de8/med21735-fig-0002-m.jpg)

**Figure 2** The schematic of RNAi. RNAi can be initiated by introducing of synthetic double strand siRNAs delivered using liposomes or lentiviral/plasmid driven shRNA into cells. Pre-siRNA is processed into a 22–30 bp dsRNA by Dicer. DsRNA is then recognized by RISC complex. One strand of RNA is degraded and another strand remains a part of RISC complex. Specific cellular mRNAs are bound to siRNA-RISC complex due to complementary sequences and then cleaved. ShRNA is transcribed in the nucleus and processed by Drosha as a stem-loop structure. Then, it is exported to the cytoplasm where is cut by Dicer into siRNA. The following steps are similar to those mentioned for siRNA. dsRNA, double strand RNA; mRNA, messenger RNA; RISC, RNA-induced silencing complex; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, small interfering RNA

Expression of shRNA in cells is typically directed through viral/bacterial vectors or delivery of plasmids. The RNA polymerase II or III, depending on the promoter choice, generate the primary transcript that contains a hairpin like stem-loop structure with a two nucleotide 3′ overhang. Exportin 5 transports this stem-loop structure to the cytoplasm, where it is processed to two strand RNA structure by Dicer and bound to RISC. The mRNA cleavage is induced by binding the antisense strand to mRNA via a complementary sequence, while the sense strand is degraded (Figure **2**).38-40

## 4.1 Comparison between siRNA and shRNA

Although siRNA and shRNA provide similar functional outcomes, they are intrinsically distinct molecules. Thereby, the RNAi pathways, the molecular mechanism of action, the off-target effects and their applications can also show differences.33 The exogenously introduced siRNA containing appropriate length and two nucleotide 3′ overhang could be loaded onto RISC without interaction with Dicer, PACT, or TRBP. Nevertheless, the loading process is 10 times less effective than shRNA. Increasing siRNA length may appear to enhance the process efficacy.41 In addition, exogenously introduced siRNA presents high turnover and degradation. Around 1% of the introduced siRNA duplex persists in the cell until 48 h following administration. In contrast, shRNA can be constantly synthesized in the host cell, highlighting much more durable of its effect. The effective knockdown for most siRNAs is reported in concentrations less than nM, while fewer than five copies of shRNA integrated in the cell genome is adequate to provide frequent gene knockdown effect. The higher dose for siRNA-mediated knockdown can contribute to the further off-target effects. n comparison, low copy numbers of shRNA lead to less off-target effects.33 To improve the stability and efficacy of RNAi agents, various modifications can be applied on siRNA. While it is easier to modify the chemically synthesized siRNA, manufacturing of modified siRNA is more expensive. In return, the dependency of vector-based shRNA to the expression machine of host cell makes difficult its modifications. These modifications are limited to the manipulating expression methods and promoter regulation.33, 42

The comparison of the potency of shRNA versus siRNA mediated knockdown was achieved by several studies in vivo. The potency of siRNA and shRNA for luciferase knockdown was equivalent at 10 μg dose. However, the shRNA, on a molar basis, was 250-fold more potent than the siRNA.43 To investigate the possibility of RNAi as a therapeutic strategy for hepatitis C virus, 19- and 25-bp shRNAs were compared with same length of siRNA directed against gene coding for protein 5B viral polymerase and IRES of hepatitis C. In both cases, the shRNAs were more effective than siRNAs.44, 45

## 4.2 SiRNA/shRNA delivery strategies

A wide range of gene delivery approaches including viral and nonviral carriers have been surveyed for the delivery of shRNA and siRNA into target cells and tissues.46 Viruses can be used as gene vehicles by removing unnecessary or pathogenic genes from virus genome and then replacing them with a therapeutic nucleic acid. These recombinant viral vectors are nonpathogenic and they are able to infect and then replicate in special target cells.47, 48 Viral vectors are favorite strategy for laboratory delivery of shRNA due to their great transfection efficiency and effectual integration of exogenous nucleic acid.49 Lentiviral vectors have ability to infect a wide variation of dividing and nondividing cells and integrate permanently into the host genome, leading to long-term expression of the transgene. HIV-1, the best specified of the lentiviruses, has been developed as a common gene delivery vehicle. A silencing lentiviral vector typically contains both an antibiotic resistance gene or a marker gene like green fluorescent protein (GFP) and the shRNA silencing cassette with ~350 bp.50 Despite the greater efficiency of viral vectors as effective carriers for nucleic acids, potential oncogenicity, immunogenicity, safety, storage difficulties, size limitations, inflammatory potential, limited cell-targeting, and random integration in host genome are main concerns over viral vectors that lead to losing support of using them in recent years.49, 51

Nonviral delivery strategies provide much further safety profiles compared to their viral counterparts. Nonviral delivery systems are broadly classified into physical and chemical subgroups.46 In physical strategies (such as hydrodynamic injection, jet injection, biolistics, ultrasound, magnetic field, iontophoresis, and electroporation), nucleic acid molecules can penetrate directly into the cytosol of cells. Iontophoresis and electroporation are the most commonly used physical approach for local siRNA delivery in the eye, skin, lung, nervous system, and digestive system. The repeated administration of nucleic acids and no limitation in the length of carried nucleic acids are the main advantages of this strategy. However, the biggest challenge for physical delivery assay is its low efficiency compared with other methods.52

Chemical delivery strategies are predominantly based on nanoparticles (NPs) especially cationic lipids and cationic nanopolymers.46, 47, 53 The positive charge of these materials facilitates the loading of negatively charged nucleic acids into them and also their binding to the negatively charged glycocalyx on outer cell membranes boosting endocytosis. The encapsulation of RNAi agents with suitable NPs helps their protection from nuclease-mediated degradation, facilitates their cellular uptake via endocytosis pathway, enhances their escape from endosomes, supports efficient biodistribution and solubility, declines immune responses, and promotes specific delivery of RNAi agents to target cells.46, 54 Importantly, three major groups of nonviral delivery systems including lipids, biodegradable/natural polymers and synthetic polymers provide potential vehicles for siRNA delivery. Many of the promising systems for clinical trials are actually hybrids of these groups.48, 55

Lipid-based NPs like liposomes and stable nucleic acid lipid particles (SNALP) obtained more interest for delivery of siRNA in RNAi research.56 SNALP, for example, is an efficient systemic delivery carrier for targeting siRNAs, consisting of synthetic cholesterol, 1,2-dilinoleyloxy-3-(*N*,*N*-dimethyl) aminopropane, 1,2-distearoyl-sn-glycero-3-phosphocholine and 3-*N*-(-methoxy poly(ethylene glycol)2000)carbamoyl-1,2-dimyristyloxy-propylamine.57 The popular commercially available lipid-based NPs that commonly used in studies include DharmaFECT, Lipofectamine, Oligofectamine, HiPerFect, and RNAiMAXTM.

NPs based on poly (lactic coglycolic acid) have been applied as a biodegradable, biocompatible, safe, and nontoxic nonviral vehicle for shRNA delivery for in vivo studies. Moreover, nonviral delivery carriers provide the potential for shRNA-based therapy in humans.58, 59

## 4.3 Clinical applications of RNAi

The discovery of RNAi has introduced it as an innovative therapeutic approach to treat irremediable illnesses. RNAi-based therapies in a number of Phase I clinical trials considering patients with solid tumors have now been accomplished.31, 32 For example, a cyclodextrin-based cationic polymer was designed to successfully deliver siRNA against to ribonucleotide reductase (RRM2) for various in vivo cancer-related models.60, 61 This preparation is the first therapy on the basis of siRNA that has entered in Phase I clinical trials (clinical version recognized as CALAA-01).62 Onpattro, the commercially available siRNA-based medicine, was introduced for the treatment of hereditary transthyretin (TTR) polyneuropathy by Alnylam Pharmaceuticals in August 2018. This drug is consisted a siRNA against TTR in complex with a cationic lipid, cholesterol, phospholipid, and a conjugate of lipid and polyethylene glycol.63 More than 20 RNAi-based drugs are presently in early phase clinical trials against different targets including RRM2,61 tenascin-C,64 TTR,65 apolipoprotein B,66, 67 and so forth, for solid tumors and other diseases. ShRNA for HIV and hepatitis B treatment was also approved by Food and Drug Administration for clinical trial.33

This is the first article that focuses on the current debate over the use of shRNA and siRNA molecules for CRC therapy through targeting genes and pathways playing a critical function in tumorigenesis. This review elaborates the role of genes and signaling pathways in cells and the effect of their knockdown to control tumorigenesis of colon cells. Besides, this article addresses the synergistic effects of RNAi-mediated gene knockdown and inhibitors/chemotherapy agents to increase the sensitivity of tumor cells to common therapy approaches. Finally, this updated review points new delivery systems/materials that used to improve the cellular uptake efficiency and lessen off-target effects of shRNA and siRNA. The outcomes of RNAi in CRC cells together with target genes have been summarized in Table **2**.

**Table 2.**Summary of some therapeutic siRNA and shRNA targets in CRC

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Silencing outcomes** |  |  |
| **Targets** | **Decrease** | **Increase** | **References** |
| Apoptosis |  |  |  |
| *BORIS* | Colony formation ability  Proliferation and cell viability  Expression of *FerT, MYC*, and *BRCA1* genes  5-FU treatment dose | ROS production  Apoptosis  Expression of *EIF3E, PTPRK* and fusion transcripts *TADA2A-MEF2B* and *MED13L-CD4* | 68–71 |
| *Bcl-xL* | mRNA and protein levels of *Bcl-xL*  Protein levels of uPA  Ability of CDK10 to inhibit apoptosis  Cell colony growth  Invasion and migration | Sensitivity to apoptosis induced by TAK-901, Oxaliplatin, Selumetinib, and Bcl-xL inhibitors  Caspase 3/7 activity | 72–78 |
| *BCL2* | *BCL2* expression  Ability of CDK10 to inhibit apoptosis  Invasion and migration  occurrence of multidrug resistance | Sensitivity to Oxaliplatin-induced cell death  Caspase 3/7 activity  Apoptosis  cell cycle arrest | 74, 75, 79–81 |
| *MCL1* | Cell viability  Migration and invasion | Sensitivity to ABT-263, A-1155463, and oxaliplatin  Trametinib/TRAIL-mediated cell death | 73, 75, 77, 82–84 |
| *Livin* | mRNA and protein levels of *Livin* and *Survivin*  Migration and invasion  Cyclin and CDKs  Phosphorylation levels of MAPK signaling proteins  Expression levels of *P62, BCL2*, and *AKT*  Cell number and proliferation  Rates of tumor growth | Apoptosis  Cleaved caspases-3/7 and PARP  Cell cycle arrest  *CDKI* p*27* expression  Sensitivity to 5-FU, cisplatin, vincristine, and etoposide  Number of autophagosomes  Ratio of LC3-II to LC3-I  Caspase*-3* and *SMAC* expression | 85–90 |
| Cell cycle |  |  |  |
| *CDK10* | CDK10 expression in vitro and in vivo  Cell survival  BCL2 and Bcl-xL expression  In vivo tumor growth | Apoptotic response  Caspase 3/7 activity | 74 |
| *CDK8* | CDK8, β-catenin, E-cadherin and MMP-7 expression  Cell cycle progression  Viability and cell proliferation  Migration and invasion | Apoptotic response  *E2F1* expression | 91, 92 |
| *CDC6* | Number of cells in S phase  Sub-G1 cells | Number of cells in G1 phase  Cell accumulation in G1/S–S phases  Expression levels of *p53, γH2AX*, the cyclin-dependent kinase inhibitor and *p21WAF1/CIP1*  Apoptosis | 93, 94 |
| *PLKs* | Expression of *PLKs, BCL2* and *MCL1*  Cell viability and proliferation | Cleavage of caspase-3/9 and PARP  Fbxw7 levels  Apoptosis, especially in *KRAS* Mut cancer cells | 93, 95–97 |
| Signal transduction pathway |  |  |  |
| *KRAS* | Expression of *KRAS, MCL1*, and *MYC in vitro* and in vivo  Level of pMEK, pERK, pAKT, and p4EBP1 in *vitro* and in vivo  Tumor growth  Cell viability and proliferation  Cell migration  Accumulation of LC3-II  Levels of Atg5-Atg12 conjugate and Beclin1  Lysosomal degradation by Baf. A1  Aggregate liver metastasis | Levels of p21 and caspase-3 in vivo  *PARP* cleavage  Apoptosis  STAT3 phosphorylation  Sensitivity to Cetuximab or Panitumumab | 83, 93, 98–107 |
| *RAF* | Level of pERK  Expression of *RAF* isoforms  Cell viability and proliferation  Invasion | Accumulation of BIM protein  Apoptotic response  Sensitivity to cetuximab or panitumumab | 99, 103, 105, 108, 109 |
| *PIK3CA* | Cell viability and proliferation  Colony growth in vitro  Tumor growth in vivo  Phosphorylation of 4E-BP1, AKT, and ERK | Apoptosis  Sensitivity to cetuximab or panitumumab | 98, 99, 103, 105 |
| *MEK1/2* | Autophagic flux  MEK1/2 protein  Viability and cell growth | Sensitivity to cetuximab or panitumumab | 92, 100, 105 |
| *AKT* |  | Sensitivity to cetuximab or panitumumab  Apoptosis | 105, 108 |
| *STAT3* | Cell viability  Expression of *STAT3*  Phosphorylated STAT3, BCL2, IL-6R, SNAIL, N-cadherin, vimentin, and ZEB1  Cell proliferation, migration, and invasion  Metastasis in NOD/SCID mice  Number of metastatic tumor nodules in the lungs of mice  Number of cancer cell colonies  Secretion of MMP2 and VEGF  IL-6-mediated increased invasiveness  Core stem cell gene expression  IL-22-induced H3K79 methylation on the stem cell core gene promoters of *NANOG, SOX2*, and *POU5F1*  Repression of the microRNA *MIR34A* after IL-6 treatment  Recruitment to HK2 promoter and reduced its expression | Sensitivity to MEK inhibitor AZD6244 and5-FU, specifically in the *KRAS* mutant cells  Chemosensitivity to fluorouracil  Apoptosis  Expression of *p16, p21, p27, E-cadherin*, and *miR-34a* | 99, 104, 110–113 |
| *JAK1/2* | p-JAK2, p-mTOR, p-STAT3 (S727), and p-STAT3 (T705)  Cell viability  STAT3 phosphorylation  AZD6244-induced STAT3 activation  Basal and MEKi-induced STAT3 activity | PARP cleavage  MEK inhibitor-induced apoptosis | 104, 114 |
| *MET* | MEK inhibition-induced activation of STAT3  MACC1-induced cell motility and proliferation  HGF-induced scattered phenotype  Liver metastatic in vivo | Apoptosis | 104, 115 |
| Ca2+/calmodulin-dependent protein kinase mediated pathway |  |  |  |
| *STK33* | Proliferation**\*** in vitro and in vivo  HIF-1α accumulation  VEGF promoter activity  Levels of extracellular VEGF  Colony formation in semisolid medium and tumor formation in vivo  Tumor size  Tumor-driven vasculature in vivo | Proliferation**\***  Apoptosis  Cleaved PARP and caspase 3 | 116–118 |
| Wnt pathway |  |  |  |
| *β-catenin* | mRNA and protein levels of *β-catenin, ITF2, Sp1, AXIN2, c- MYC, EPHB2, BMP4*, and *LGR5*  Level of T4-enhanced cyclin D1, *β-catenin*, and CCND1  Cell viability and proliferation  Invasion  Tumor growth | Expression of *p21, CA2, MUC2*, and *TM4SF4*  Levels of caspase-3 activity and LC3 protein  Apoptosis | 119–123 |
| *FZD7* | Expression of FZD7, c-JUN, p-JNK, p-c-JUN, TCF, CD44v8-9, MT1-MMP, β-catenin, MYC, survivin, and E-cadherin  Percentage of cells at G2/M phase  RhoA activation  Cell viability and proliferation  Invasion  Liver metastases in vivo models | SNAI2 | 109, 124–126 |

Abbreviations: 5-FU, 5-fluorouracil; BORIS, brother of the regulator of imprinted sites; CDK, cyclin-dependent kinase; FZD, frizzled; JAK, Janus kinase; MMP, matrix metallopeptidase; mRNA, messenger RNA; PLK, polo-like kinase; STAT3, signal transducer and activator of transcription 3; STK33, serine/threonine kinase 33; uPA, urokinase-type plasminogen activator; VEGF, vascular endothelial growth factor.

\* Inconsistent data. See Section 7.2 for more information.

# 5 APOPTOSIS

Programmed cell death, or apoptosis, is an extremely regulated process that happens normally during development to maintain homeostasis. This phenomenon is mediated by three main pathways, including granzyme B, extrinsic (death receptor), and intrinsic (mitochondrial) pathways. Since these pathways are linked with each other, a molecule in one pathway can affect the others. Abnormalities in cell death regulation are known to be one of the essential hallmarks of cancers. Disabled apoptosis in tumor cells renders them resistant to DNA-damaging anticancer agents.127, 128 In this section, we highlight evidence for the potential applications of siRNA and shRNA to induce apoptosis in treatment of CRC.

## 5.1 BCL2 family

BCL2 family proteins regulate apoptosis through control of caspase activation and the intrinsic mitochondrial apoptosis pathway, including the release of cytochrome C into the cytoplasm. The members of this family are classified in two groups: (1) antiapoptotic group, including A1 and MCL1 regulators, Bcl-w, BCL2 and Bcl-xL (encoded by *BCL2L1* gene); and (2) proapoptotic group, including two BAX and BH3-only family. The BAX family consists of BAX, BAK, and BOK proteins, while BIM, BIK, BAD, BMF, HRK, Noxa, and Puma belong to the BH3-only family. Since apoptosis is often impaired in cancer due to deregulation of BCL2 family members, targeting of these proteins could result in more effective therapeutic approaches.129, 130

TAK-901 is a potent inhibitor of aurora B (a member of the chromosomal passenger complex that extremely expresses in CRC) and makes HCT116 cells sensitive to apoptosis induced by p53-mediated BAX. In contrast, Bcl-xL binding to BAX and suppressing apoptosis renders cancer cells resistant to TAK-901-induced cell killing. The combined use of TAK-901 or Bcl-xL inhibitors and *Bcl-xL* knockdown with siRNA employing Lipofectamine RNAiMAX can cause synergistic cell-growth inhibition in CRC cell lines, which may suggest an effective strategy in clinical treatment of CRC.72, 73 *BCL2* and *Bcl-xL* knockdown in CRC RKO cells transfected by siRNA using DharmaFECT transfection reagent considerably increased caspase 3/7 activity and omitted the ability of CDK10 to inhibit apoptosis, even in cells overexpressing *CDK10*.74 Upregulation of *CDK10* in CRC, promoting tumor growth and suppressing apoptosis, is prognostic of poor overall survival.131

Inhibition of Bcl-xL by its inhibitor, ABT-263 alone, not only did not seem to be efficiently cytotoxic to SW620 (colon *KRAS*/*BRAF*-mutated, *BCL2L1*-amplified cell line), but also led to *MCL1* upregulation, which in turn causes resistance to *Bcl-xL*-targeted treatments. This observation shows that Bcl-xL and MCL1 play compensatory roles in apoptosis regulation. For this reason, the binary inhibition of Bcl-xL (by ABT-263) and MCL1 (by chemical inhibitor YM-155 or siRNA transfected employing Lipofectamine 2000 reagent) has been shown to have a synergistic influence in vitro and in vivo patient-derived xenograft (PDX) models. Besides, siRNA *MCL1* dramatically decreased the relative cell viability in SW620 cells.82

These outcomes are in agreement with the obtained results by Faber et al.83 that indicate the targeting of *MCL1* with siRNA transfected by HiPerFect reagent sensitized mutant SW620 cells, but not WT CRC cells, to ABT-263. Furthermore, *MCL1* knockdown restored the sensitivity of resistant CRC cell lines with lower expression of *Bcl-xL* and higher expression of *MCL1* to the Bcl-xL inhibitor.73 These results propose that the combined suppression of *BCL2, Bcl-xL*, and *MCL1* should be an applicable therapeutic strategy in mutant CRC cells.

Additionally, siRNA experiments (with Lipofectamine 2000) against *BCL2L1* and *MCL1* were performed in two A-1155463-resistant (*RKO* and *GEO*) and two sensitive (*LS1034* and *SW1417*) CRC cell lines.77 A-1155463 is a highly potent and selective BCL2 and Bcl-xL inhibitor. *BCL2L1* siRNA efficiently silenced *Bcl-xL* expression completely in three cell lines and around 80% in SW1417 cells. Nevertheless, the viability of only the A-1155463-sensitive CRC cells were largely reduced following *BCL2L1* knockdown (more than 50% reduction), confirming a critical function of Bcl-xL in the survival of a subgroup of CRC cells. On the other hand, while *MCL1* targeting alone had no notable effect, its silencing in the presence of A-1155463 rendered complete apoptosis.77 These observations highlight the role of MCL1 as a resistance factor for the Bcl-xL inhibitors A-1155463 and ABT-263. While A-1155463 and ABT-263 induce the apoptotic pathway through releasing BIM from Bcl-xL, MCL1 sequesters BIM released by Bcl-xL inhibitor and thereby suppresses apoptosis in resistant cells.132

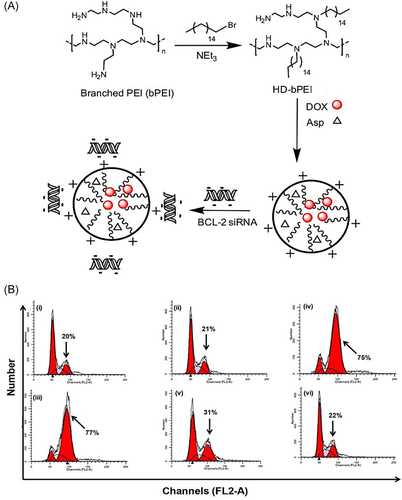
In experiment performed by Liu et al.,78 CRC HT29 cells were transfected by *Bcl-xL* siRNA entrapped in liposomes. The outcomes of this experiment represented that *Bcl-xL* siRNA could successfully inhibit the mRNA and protein levels of Bcl-xL in a concentration- and time-dependent manner. The cell colony growth as well as invasion ability of HT29 cells were decreased.78 However, despite massive impairment of invasion and migration, the efficient downregulation of *Bcl-xL* and *BCL2* by siRNAs transfected via RNAiMAXTM did not alter cell death, viability, proliferation and morphology in SW480 and HT29 over a period of 72 h.75 A very slight increase in cleaved PARP levels (a target of activated caspase 3) was detected after *BCL2* and *Bcl-xL* knockdown. In addition, HT29 and SW480 cells lacking MCL1 indicated a slightly higher viability and proliferation, likely due to antiproliferative functions of MCL1. This data supports the claim that a single knockdown of *BCL2, MCL1*, or *Bcl-xL* is quite well compensated.75

Importantly, the level of the urokinase-type plasminogen activator (uPA) protein of *Bcl-xL* siRNA transfection group was obviously declined in a concentration- and time-dependent way in HT29 cells transfected by siRNA-liposomes.78 Since the increase of uPA activity in CRC is closely associated with invasion and metastasis,133 it seems that RNAi silencing of *Bcl-xL* gene inhibits the invasion of CRC cells in a way that might be related to uPA downregulation. However, the exact mechanism by which *Bcl-xL* regulates the uPA expression in cancer cells is not known.78 Moreover, *BCL2* and *Bcl-xL* knockdown induced great apoptosis in BAX+/− cells but not BAX−/− HCT116 cells. In addition, apoptosis mediated by *BCL2* or *Bcl-xL* silencing was blocked following silencing of *caspase 2*, which shows cell death pathways regulated by *BCL2* and *Bcl-xL* share commonalities in requirements for *BAX* and *caspase 2*.79

A profound sensitization to cell death induced by chemotherapy drug oxaliplatin, an inhibitor of DNA synthesis, was observed after silencing of *BCL2, Bcl-xL*, and *MCL1* in HT29 and SW480 cells.75 Presumably, impaired DNA repair following targeting of BCL2 proteins caused the synergistic effects of oxaliplatin. Conversely, this synergistic interaction was not found for knockdown of these antiapoptotic proteins and 5-FU or irinotecan chemotherapy drugs.75 A similar result was observed for MEK/PI3K inhibitor selumetinib in *KRAS* mutant sensitive HCT116 and insensitive SW620. About 5000 shRNA constructs in the pLKO.1 lentiviral vector targeting around 1200 druggable genes infected HCT116 and SW620 cells at the presence or absence of selumetinib. Among silencing of these genes, *Bcl-xL* knockdown afforded profound inhibition induced by selumetinib.76

*BCL2* siRNA formulated into liposomes caused barely detectable levels of *BCL2* in the clones of HCT116 *p53*+/+ and *p53*−/− cells.79 In comparison with the cells transfected by control siRNA and *BCL2* antisense RNA, extreme apoptosis is only observed in the *p53*+/+ cells and also found in siRNA treated cells. Similar outcomes were observed in other human CRC cell lines defective for DNA MMR, including HT29, SW48, DLD1, and LS174T (all p53 mutant), along with RKO and LoVo (p*53* WT). These findings notice that enormous apoptosis of CRC cells is induced by selective knockdown of *BCL2*.79 This effect is selectively dependent upon p*53*, likely through the rescue of cytochrome *C* from the mitochondria. The proapoptotic task of p*53* keeps cells away from treatment by chemotherapeutic agents, such as 5-FU. However, *Bcl-xL* silencing induced apoptotic pathway in both the *p53*−/− and *p53*+/*+* HCT116 cells, indicating p*53* activity is not a selective requirement for Bcl-xL induced apoptosis.79

Doxorubicin (DOX) antibiotic is one of the broadly used antitumor drugs for the treatment of a wide spectrum of cancers like CRC.134 However, the toxicity of DOX limits its clinical application. The NP DOX formulations could avoid its nonspecific distribution.135 In addition, nonsteroidal anti-inflammatory drugs (NSAIDs) can successfully avoid cancer-related inflammation, critical reason of tumor progression to the metastatic phase.136 Aspirin (Asp), a type of NSAIDs, decreases cancer cell progression.137 Ray et al.81 used branched polyethyleneimine (bPEI), a well-known gene carrier, for encapsulation of anticancer drugs DOX and Asp along with *BCL-2* siRNA to generate DOX-Asp-*BCL2*-bPEI NP (Figure **3A**). The codelivery of two anticancer drug and *BCL-2* siRNA in HCT-116 cells reduced the occurrence of multidrug resistance. The synergistic effect of this system also activated apoptosis by ROS generation in HCT-116 cells as compared to free DOX-treated cells. As shown in Figure **3B**, the more cell cycle arrest at G2/M phase observed following transfection of DOX-Asp-*BCL2*-bPEI NP is due to enhancement of mitochondrial BCL-2 and intranuclear DOX levels to above the threshold level essential to induce apoptosis compared to free DOX. In addition, the expression of *BCL-2* using treatment of NP was remarkably decreased rather than naked *BCL-2* siRNA, indicating more efficiency of NP in the transfection of siRNA.81

[](https://onlinelibrary.wiley.com/cms/asset/bdd2c20f-4927-4cf5-9c18-f21333180d4e/med21735-fig-0003-m.jpg)

**Figure 3** (A) Schematic diagram for the production of DOX-Asp-*BCL2*-bPEI NP consisting of dual drugs (Asp and DOX) and siRNA against *BCL2*. (B) Flow cytometry analysis of HCT-116 cells following transfection of DOX-Asp-*BCL2*-bPEI NPs. These NPs were used in two different concentrations: 1 μg/ml (including 0.8 μg/ml of DOX, 0.4 μg/ml of Asp, and 20 ng of siRNA) and 10 μg/ml (including 8 μg/ml of DOX, 4.4 μg/ml of Asp, and 200 ng of siRNA). G2/M DNA content in (i) Control, (ii) bPEI (1), (iii) NP (1 μg/ml), (iv) NP (10 μg/ml), (v) free DOX, (vi) free Asp.81 Asp, aspirin; bPEI, branched polyethyleneimine; DOX, doxorubicin; NP, nanoparticle; siRNA, small interfering RNA

## 5.2 Inhibitor of apoptosis protein (IAP) family

IAP family comprises of a various group of signaling molecules that suppress apoptosis through the inhibition of caspases and the degradation of apoptotic proteins by ubiquitination. In addition, the members of this family are involved in other cellular pathways, including signal transduction, cell cycle control and gene regulation. The XIAP, NAIP, ILP-2, c-IAP1, c-IAP2, survivin, BRUCE and livin are eight IAP relatives that have been discovered in humans so far.138, 139

Livin inhibits apoptosis via two pathways: first, the suppression of *caspase 9, 7*, and *3*, and second, E3 ubiquitin-ligase-like activity. The latter induces degradation of *DIABLO*/*SMAC*. Livin is overexpressed in cancer cells and makes them resistant to apoptotic stimuli.140 *Livin* siRNA was transfected using LipofectamineTM RNAiMAX and LipofectamineTM 2000 in SW480 and DKO1. The targeting of *Livin* in these cells had several overriding consequences85:

1) Large decline in the mRNA and protein levels of livin and survivin

2) Substantial rise in apoptosis of SW480, but not DKO1 cells, by elevation of *p27* expression

3) Downregulation of phosphorylation levels of MAPK signaling proteins involving JNK, p38, and ERK1/2, but not AKT, NF-κB, and p65

4) Upregulation of cleaved caspases 3/7 and PARP

5) Withholding of tumor cell invasion and migration

6) Supporting apoptosis

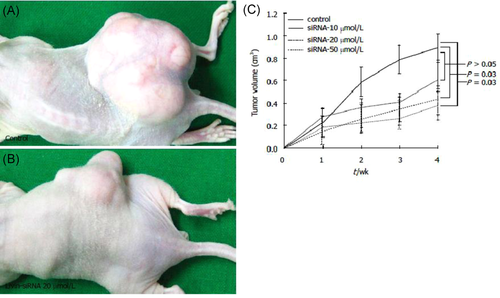
7) Inducing cell cycle captured in the S phase of DKO1 and the G0/G1 phase of SW480 by declining cyclin and cyclin-dependent kinases (CDKs) such as CDK4, CDK6, cyclin D1, and cyclin D3

Liu et al.86 used *Livin* shRNA lentiviral vector pGCL-GFP to infect HCT116 and SW620 cells. *Livin* gene was markedly downregulated and the chemotherapeutic sensitivity of these cells to 5-FU was improved. The expression of *SMAC* and *caspase 3* (apoptosis executioners), the ratio of *LC3-II* to *LC3-I* (an autophagy indicator) and the number of autophagosomes were notably escalated after *Livin* silencing. Additionally, the knockdown of *Livin* decreased the expression levels of *p62, BCL2*, and *AKT*. This data reveals that apoptosis and autophagy contribute to *Livin* silencing-induced 5-FU sensitivity restoration in mentioned cells. In line with these observations, pretreatments of HCT116 and SW620 cells using autophagy and apoptosis inhibitors 3-MA and Z-VAD-FMK significantly damaged *Livin* knockdown-induced cell death.86

*Livin* expression was efficiently knocked down in vincristine-resistance HCT-8/V cells using shRNA subcloned into pGCsi-H1 vector.87 The down-expression of *Livin* promoted apoptosis of HCT-8/V in presence of anticancer agents such as vincristine, etoposide and 5-FU. In addition, the chemosensitivity of HCT-8/V to these agents was enhanced.87 Similarly, the considerable downregulation of *Livin* mRNA and protein was found in *Livin* shRNA-transfected HCT116 cells mediated by Lipofectamine 2000.88

The inhibitory effect of *Livin* knockdown on gene expression was not dose-dependent and attenuated over time in siRNA transfection HCT116 cells that suggests duration of *Livin* siRNA is short in these cells, and minimal dosage of siRNA is sufficient for *Livin* suppression.90 Other critical effects of *Livin* silencing in HCT116 cells was significant reduction in cell proliferation, metastasis, and invasiveness, as well as induction of apoptosis.88, 90

Similar results were observed in *Livin* shRNA-transfected LoVo cells.89 The silencing of *Livin* decreased cell number and proliferation and increased apoptosis and cell sensitivity to Cisplatin, a chemotherapy drug commonly used for cancer treatment, about 50% in these cells.89 With higher than 20 μmol/L concentration of *Livin* siRNA, a dramatic decline in tumor growth rates was observed in a xenograft mice model of CRC (Figure **4**). No difference in body weight and no remarkable toxic reaction in brain, liver, and kidney of siRNA-exposed mice were observed as toxic side effects of siRNA silencing.90

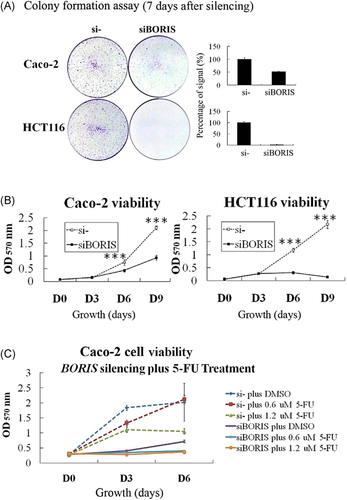
[](https://onlinelibrary.wiley.com/cms/asset/4974b41d-e7e0-4eea-a9ad-fee4c257f730/med21735-fig-0004-m.jpg)

**Figure 4** The effect of Livin siRNA on the tumor growth and volume in xenograft mice. (A,B) The inoculated mice were divided into two groups—control and siRNA-20 μmol/L treated—then, tumor volume was measured. The tumor volume is considerably reduced as compared with control. (A) Control group; (B) siRNA-20 μmol/L treated group. (C) The inoculated mice were divided into four groups (one control and three treated) and treated with 10, 20, and 50 μmol/L doses of siRNA weekly for 4 weeks. Tumor volumes were checked every week. In higher than 20 μmol/L siRNA, the tumor volumes are meaningfully diminished as compared with control group from 2 weeks after transfection (*p* = .03).90

## 5.3 Brother of the regulator of imprinted sites

Brother of the regulator of imprinted sites, or simply *BORIS*, is known as an inimitable tumor-promoting and epigenetically acting transcription factor.141 While *BORIS* transcript is not detected or expressed in a very low amount in normal tissues, it is overexpressed in cancers, including CRC.142, 143 The aberrant expression of *BORIS* in CRC inhibits apoptosis via recruiting antiapoptosis proteins, like BCL2, and changes the expression of downstream genes, such as *c-MYC* and *BRCA1*, by affecting the methylation status of the promoters of these genes. These results highlight the potential clinical applications of *BORIS* knockdown for the treatment of CRC.68, 144

Zhang et al.69 used Lipofectamine to deliver *BORIS* siRNA in the two CRC cell lines, Caco-2 and HCT116. *BORIS* silencing considerably inhibited the colony formation ability and decreased the cell viability (Figure **5A,B**). In addition, the proliferation of CRC cells was suppressed following *BORIS* silencing due to increasing the ROS production and inducing apoptosis. The effects of *BORIS* downregulation in HCT116 were stronger than Caco-2, likely because of the heterogeneous nature of CRC in different cell backgrounds. The suppression of *BORIS* resulted in an intense growth inhibition, even higher than that observed in treatment with 5-FU alone in Caco-2 cells. The synergistic influence of combined treatment of *BORIS* knockdown and lower dose of 5-FU to arrest cell growth could allow the 5-FU dose to be reduced in CRC treatment (Figure **5C**).69

[](https://onlinelibrary.wiley.com/cms/asset/9a3121f6-a853-468b-a800-06d0b7aee22e/med21735-fig-0005-m.jpg)

**Figure 5** The effect of *BORIS* silencing on the colony formation, cell viability, and sensitivity to 5-FU in HCT116 and Caco-2 CRC cells. (A) The colony formation ability of HCT116 and Caco-2 cells was evaluated by crystal violet staining 7 days following siRNA transfection. The right panel displays the percentage of signal for each well compared to that in the control siRNA-treatment cells. (B) HCT116 and Caco-2 cells viability were determined by the MTT assay. The viability of cells was suppressed after *BORIS* siRNA transfection. Statistical differences between the control and treatments were analyzed by two-tailed Student's *t* test. \*\*\**p* < .001. (C) Caco-2 cell lines were treated with 5-FU together with *BORIS* siRNA. The viability of the cells was assessed. The combination of 5-FU and *BORIS* siRNA significantly decreased cell viability. DMSO and negative control siRNA-treated cells were used as controls.69 5-FU, 5-fluorouracil; BORIS, brother of the regulator of imprinted sites; CRC, colorectal cance; siRNA, small interfering RNA

The knockdown of *BORIS* by shRNA transfected using vector pCMV6 in HCT116 cells prevented the assembly of BAT3, SET1A, and ASH2 (transcriptional regulators for chromatin remodeling by H3K4 dimethylation) at the promoters of *MYC* and *BRCA1* genes, and declined the expression of these genes. Conversely, *BORIS* targeting had no effect on the expression of two tumor suppressor genes: *p21* and *p14*.68

The transfection of *BORIS* siRNA by Lipofectamine RNAiMAX and 2000 in *HCT116* cells caused a remarkable reduction in the *FerT* expression (a meiosis and postmeiosis specific tyrosine kinase) through altering the methylation profile of the *FerT* promoter70 and upregulation of *EIF3E* and *PTPRK* genes.69 In addition, the expression of fusion transcripts *TADA2A*-*MEF2B* and *MED13L*-*CD4* was promoted via upregulating of the parent genes, *TADA2A* and *CD4*. These fusion transcripts were undetected in the genome of control *HCT116* cells.69 Since all these genes are involved in gene expression and signaling pathways, they are a double-edged sword with both oncogenic and tumor suppressive abilities.145, 146 However, the association between *BORIS* and chimeric fusion transcripts in tumorigenesis requires further determination.

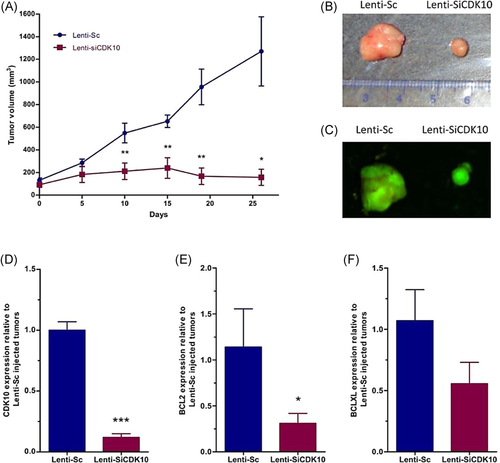
# 6 CELL CYCLE

The CDKs are the central machines that drive cell cycle progression. They are serine/threonine kinases (STKs) whose activity is associated with a regulatory subunit called cyclin. CDKs act as a main eukaryotic protein kinase family involved in the regulation of transcription and cell division. In addition to CDKs, there are other diverse compounds that regulate the cell cycle. Given that deregulation of CDKs and cell-cycle related pathways are a hallmark of cancer, they can be considered as interesting targets for cancer therapy.147

## 6.1 Cyclin-dependent kinases

CDK10 has a main role in cellular proliferation during cell cycle G2-M phase and is constantly upregulated in CRC.74 The transfection of HCT116, RKO, and MIP101 cells by *CDK10* siRNA using DharmaFECT1 transfection reagent led to diminution of cell survival and >70% knockdown in *CDK10* expression. The increased apoptotic response was evidenced as a great number of TUNEL-positive cells, downregulation of *BCL2* and *Bcl-xL* especially in RKO cells—as well as elevation of caspase 3/7 activity by 1.8-fold in HCT116 and 2.3-fold in RKO and MIP101. Nevertheless, proapoptotic proteins, BAD and BAX, remained unaffected. Also, the level of cleaved caspase 8 that only was detectable in HCT116 and RKO cells failed to change, suggesting that there is not an association between the extrinsic apoptosis pathway and CDK10-mediated inhibition of apoptosis in CRC. It should be emphasized that a notable reduction in the siRNA-induced expression of *CDK10* in RKO was affected by relatively lower*CDK10* expression in RKO compared to MIP101 and HCT116 cells.74

For in vivo experiments, the direct intratumoral injection of PDX in mice was performed using vectors expressing *CDK10* siRNA. In line with in vitro observations, effective suppression in *CDK10* expression and concomitant inhibition of *BCL2* and *Bcl-xL* was observed following *CDK10* knockdown in vivo. Moreover, a dramatic decline in tumor growth was reported within 10 days of siRNA treatment (Figure **6**). Despite increase in the average volume of tumors receiving scramble siRNA, those tumors exposed to *CDK10* siRNA remained principally unaltered by day 26 of treatment.74

[](https://onlinelibrary.wiley.com/cms/asset/efd08484-89ba-4b6f-8b77-8eded229cada/med21735-fig-0006-m.jpg)

**Figure 6** CDK10 suppression by siRNA inhibited PDX of CRC tumors in vivo. (A) CRC PDX tumors implanted in C.B-17/SCID mice (volume: 50–100 mm3) were injected using lentivirus vectors expressing CDK10 siRNA (Lenti-siCDK10) or scramble siRNA (Lenti-Sc). Tumor volumes were measured every 5 days after implantation of PDX, when tumors reached volume 50–100 mm3 (results based on *n* = 6–7/group, mean ± SEM; \**p* < .05; \*\**p* < .01). (B) PDX tumors harvested on day 10 of intratumoral injection, indicating the expression of: (C) GFP and by qRT-PCR: (D) CDK10, (E) BCL2, and (F) BCL-XL. Fold changes were calculated relative to tumors treated with Lenti-Sc and statistical significance analyzed by Student t test (\**p* < .05; \*\*\**p* < .001).74 GFP, green fluorescent protein; qRT-PCR, quantitative reverse transcription polymerase chain reaction; siRNA, small interfering RNA

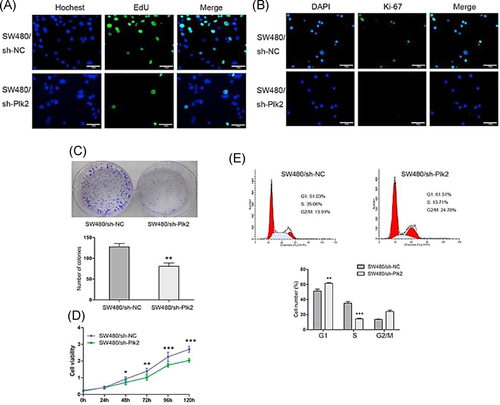
Similar results were obtained for CDK8, a component of the mediator transcriptional regulatory complex, which has been reported to inhibit E2F1-induced suppression of Wnt/β-catenin pathway and is expressed in a great fraction in CRC.148 HCT116 cell lines were transfected with *CDK8*-siRNA using Lipofectin2000 or lentiviral vectors encoding shRNA targeting *CDK8*.91, 92 The maximal transfection efficacy of *CDK8*-siRNA was gained when the ratio of Lipofectin to siRNA is 1:1.92 *CDK8* expression was evidently reduced after 48 and 72 h of transfection at the mRNA and protein level, respectively. Moreover, *CDK8* silencing significantly suppressed invasion and migration, facilitated apoptosis of cancer cells in vitro, reduced cell proliferation and viability, and arrested cancer cells in the G0/G1 phase. It has been revealed that the RNAi effectively inhibited the transcription action of the β-catenin signaling pathway, because following the *CDK8*-siRNA transfection, the mRNA and protein levels of β-catenin were extremely decreased.91, 92

After injection of the CRC cells into mice, reduced migration and invasion ability were observed following transfection with *shCDK8*. The western blot analysis also indicated the lower protein expression of cell metastasis regulators matrix metallopeptidases-7 (MMP-7), E-cadherin, β-catenin, and CDK8 in metastatic liver lesions of shCDK-8 groups compared to the control group, however, protein expression of *E2F1* was increased.91 These observations indicate that the prometastasis function of *CDK8* is mediated within the Wnt/β-catenin pathway and could be considered as an oncogene in CRC. Thereby, regulating colon cancer by controlling the *CDK8* is theoretically possible.91, 92

## 6.2 Polo-like kinases (PLKs)

A member of serine/threonine protein kinases that mediate normal cell division are PLKs. In early G1 phase, PLK2 is activated and directly targets Fbxw7 which regulates the ubiquitin-mediated degradation of cyclin E.149 Although *PLK2* is silenced in B-cell lymphomas, it has also a potential of protumor function, because in comparison to normal tissues, *PLK2* is more upregulated in the more aggressive CRC tumor tissues.95

In study directed by Ou et al.,95 A notable elevation in cleavage of caspase 3 and 9, as well as PARP, was detected when *PLK2* expression was downregulated in SW480 cells through lentivirus-mediated shRNA targeting *PLK2* mRNA in the presence of polybrene. The knockdown of *PLK2* also triggered the decline of *BCL2* and *MCL1*. These findings suggest that depletion of *PLK2* could provoke apoptosis through both intrinsic and extrinsic apoptotic pathways. Cell cycle analysis revealed that *PLK2* silencing inhibited proliferation of the SW480 cells, as represented in Figure **7**. As PLK2 negatively participates in the regulating Fbxw7 levels and leads to converse changes of cyclin E protein, *PLK2* silencing could improve *Fbxw7* levels in SW480 cells. Cyclin E silencing in SW480 cells using siRNA specific for cyclin E transfected by Lipofectamine 2000 could reverse the protumor effects directed by *PLK2* upregulation that confirm positive correlation between cyclin E and *PLK2* expression in cancer cells. This data shows that *PLK2*, as an oncogene in CRC, targets Fbxw7/cyclin E pathway and could be considered as a new diagnostic biomarker and a therapeutic target for CRC therapy.95

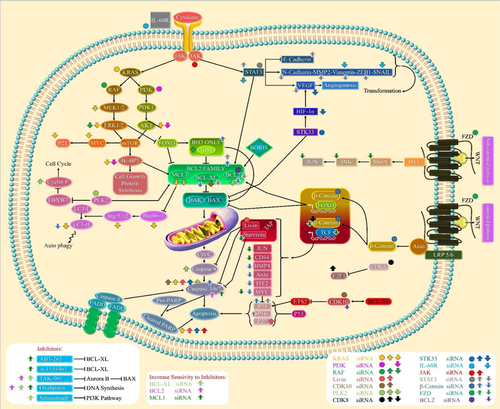
[](https://onlinelibrary.wiley.com/cms/asset/1d454dfd-e2f5-44f8-8405-02927ce1e307/med21735-fig-0007-m.jpg)

**Figure 7** PLK2 contributes in SW480 cells proliferation. (A) Cell proliferation is inhibited by PLK2 knockdown in SW480 cells as measured by 5-ethynyl-2ʹ-deoxyuridine incorporation. Scare bars = 200 μm. (B) Representative images of Ki-67 staining in tumor cells. Scare bars = 200 μm. (C) Colony formation numbers are less in PLK2 low-expression group (SW480/sh-Plk2) in comparision with controls. (D) As measured by Cell Counting Kit-8 assay, PLK2 depletion is found to lower the SW480 cells viability. (E) Cell cycle analysis shows that the rate of G1 phase is more in SW480/sh-Plk2 cells than the control group. Data represents the mean ± SD and are representative of three independent examinations. \**p* < .05, \*\**p* < .01, \*\*\**p* < .00195

The targeting *PLK1*—a mitosis regulator whose dysfunction is directly associated with cancerous transformation—with shRNA yields enhanced toxicity toward *RAS* Mut parental *DLD-1* and *HCT116* cells compared to *RAS* WT isogenic cells in which the mutant *RAS* allele has been removed.96 Additionally, *PLK1*-siRNAs were formulated into SNALP with a final lipid/siRNA mass ratio of 9:1 to silence *PLK1* in HT29 and LS174T cells. The transfection data shows an induction of apoptosis in a dose-dependent manner 48 h after transfection of siRNA in LS174T and a dose-dependent decline in cell viability related to the degree of *PLK1* mRNA downregulation in LS174T and HT29.97 This data led to design of the lipid-siRNA NPs named TKM-080301 (*TKM-PLK1*) against *PLK1* that is being appraised in a first-in-human phase I study in advanced solid tumors involving CRC.150-152

## 6.3 CDC6

CDC6 is an unstable protein synthesized at the end of mitosis. It is a regulator of DNA replication initiation.153 A large-scale siRNA differential apoptosis screen employed a library with more than 7000 siRNA pools directed against the human genome in HCT116 cells and the isogenic derivative HKE-3 with removal in activated mutant *KRAS* allele.93 HCT116 cells displayed a more degree of dependency on CDC6, along with PSMD14, PSMB6, and PSMA1 (components of the proteasome) than HKE-3 cells. *CDC6* depletion in both cells caused a notable decay in cell numbers in S and a rise of G1 phase cells. Whereas S phase-labeled HKE-3 cells were stably arrested following *CDC6* depletion, S phase-labeled HCT116 cells endured to cycle, even without *CDC6* expression. The levels of p*53* and p*21* expression are considerably upregulated in only HCT116 cells with depletion in *CDC6* (Figure **8**).93

[](https://onlinelibrary.wiley.com/cms/asset/acdd1e67-af93-4fdb-b005-90bd4b4e3fe8/med21735-fig-0008-m.jpg)

**Figure 8** An overview of major signal transduction and apoptosis pathways that dysregulate in CRC. siRNAs or shRNAs targeting potential genes involved in theses pathways could suppress aberrant gene perturbations. Up/down arrows indicate increase/reduction in the expression of target mRNAs or proteins. CRC, colorectal cancer; mRNA, messenger RNA; siRNA, small interfering RNA; shRNA, short hairpin RNA

In accordance with the robust p*53* reaction to *CDC6* knockdown, the expression of γH2AX (a C-terminally phosphorylated histone isoform confined in places of DNA damage) was strongly induced in HCT116 cells, while HKE-3 cells treated by *CDC6*-siRNA exhibit a profoundly diminished response.93 This data suggests that while *p21* levels were upregulated in both HKE-3 and HCT116 cells following *CDC6* depletion, DNA damage reaction in the *KRAS* mutant cells was much stronger, presumably due to overruling cell cycle checkpoint control. Moreover, the combined treatment of HKE-3 and HCT116 cells with the small-molecule proteasome inhibitor Bortezomib and *CDC6* siRNA exhibited a synergic effect in apoptosis induction compared to each treatment alone. This result was also seen for the combination treatment of *GATA2* siRNA and bortezomib in both cells. However, a striking growth in apoptosis was accompanied by rising the drug dosage in HCT116, representing a degree of synergy for this arrangement in *KRAS* mutant cells.93 Moreover, transfection of *CDC6*-targeted siRNA using Oligofectamine in HCT116 cells triggered apoptosis after 72 h and perturbed cell cycle progression, evidenced by decline of subG1 cells together with cell accumulation in G1/S–S phases.94

# 7 SIGNAL TRANSDUCTION PATHWAYS

Signal transduction is the process in which a physical or chemical signal is transmitted into a cell through cell surface receptors, resulting in a cellular response via a series of molecular events, most commonly protein phosphorylation. Many of the genes commonly mutated in cancer encode components of the distinct signaling pathways, including the PI3K-AKT, RAS/RAF/MAPK, JAK/STAT3, Wnt, and Ca2+/calmodulin dependent kinase mediated pathways. Targeting of these pathways through systemically delivered siRNAs or shRNAs could be utilized as a novel therapeutic potential for further drug development of mutant CRC cells.106

## 7.1 The RAS/RAF/MAPK pathway and PI3K/AKT/mTOR pathway

There are at least six distinguished *MAPK* signaling pathways that can be named according to the terminal kinases: ERK7/8, ERK5, ERK3/4, ERK1/2, SAPKs or JNK1/2/3 and the p38 MAPK. The best-studied MAPK signaling pathway is RAS/RAF/MAPK, which integrates extracellular signals into the nucleus through ERK1/2.154 On the other hand, the starting point of PI3K/AKT/mTOR pathway is PI3K activation, followed by phosphorylation and activation of AKT. The active AKT is then translocated to the nucleus where many of its substrates are localized.155 The multiple cross-talk points between these two pathways coordinate actions and determine the cell fate. Recent studies have shown that components of MAPK and PI3K/AKT/mTOR signaling pathway are often changed in human cancers,154, 155 suggesting that targeting them may provide new therapeutic strategies.

Yuan et al.99 employed a functional “sensor” assay to establish RNAi libraries targeting several human RAS pathway genes as well as their mouse orthologs. Nine human CRC cell lines, including *KRAS*-WT (Caco-2 and SW48), *KRAS*-mutant (SW620 and SW1116), *BRAF*-mutant (LS411N and RKO), SW403, LoVo, and LS123, were transfected with low dose of sensor siRNAs using Lipofectamine RNAiMAX. The low dose of siRNAs not only could inhibit aberrant gene perturbations, but also decreased both general off-target and sequence-specific effects. The ability of combination gene silencing was another important outcome of using low dose of siRNAs. Among CRC cells, *KRAS* WT cell lines are resistant to *KRAS* silencing, whereas *KRAS* mutant cells are sensitive, highlighting an intense relationship between *KRAS* mutational status and *KRAS-*siRNA-induced cell death. Importantly, effective *KRAS* depletion resulted in reduction of phospho-MEK (pMEK) and phospho-ERK (pERK) in SW1116 cells. The results of *RAF* isoform silencing were reported as the comparative resistance of SW48 and Caco-2 cells to individual and combinations of *RAF* isoform depletion, complete sensitivity of RKO and LS411N cell lines to *BRAF* knockdown and partial sensitivity of SW1116 and SW620 cells to the suppression of either *CRAF* or *BRAF*.99

The combined knockdown of all three *RAF* isoforms in SW620 and SW1116 led to the progressive decrease in pERK levels and cell viability, higher accumulation of BIM protein and strong apoptotic response, representing that silencing of *RAF* isoforms is essential for inhibiting ERK activation and promoting apoptosis in the *KRAS*-mutant context.99 In contrast, these findings were not observed in the treatment of cells by small molecule BRAF inhibitor, PLX4720, reflecting distinct biological effects of siRNA and inhibitor molecules. Furthermore, ablation of all three *PI3K* variants declined cell viability in all cell lines. To provide in vivo proof-of-principle evidence, mice bearing SW620 xenografts were generated and cyclodextrin-polymer NPs were used to deliver sensor siRNAs to tumors. Main consequences of *KRAS* depletion in xenografted mice includes slowed tumor growth, increased levels of *p21* and *caspase-3*, reduced levels of KRAS protein and concomitant decline in pCRAF and pERK, specifically in regions proximal to blood vessels (Figure **8**). In line with in vitro experiments, *A/B/C-RAF-*siRNA and *KRAS-*siRNA treatment equally suppressed tumor growth, but did not induce regression in vivo. Cotargeting of *KRAS* together with *PIK3CA/B* provided remarkably better suppression of tumor growth, especially in later stages of treatment, surpassing the efficacy of the high dose *KRAS-*siRNA treatment. Taken together, these results display combined inhibition of effector nodes, which could confer a therapeutic benefit, likely owing to the redundancy function of RAS pathway compartments. The ability to validate such combination targets *in vivo* with siRNA has important effects, particularly in the absence of small molecules against putative cancer targets.99 Similarly, shRNA-mediated suppression of *NRAS* also inhibited cell growth in DLD-1 cells.91

Similar results were reported in the transfection of HCT116 and DLD-1 cell lines (with coexistent *PIK3CA* and *KRAS* mutations) by siRNAs guided against components of the RAS/RAF/MAPK (*ERK1* or *ERK2, MEK1, MEK2, BRAF*, and *KRAS*) or PI3K/AKT/mTOR (*RAPTOR* or *RICTOR, AKT*1, *AKT*2, *PIK3R1*, and *PIK3CA*) pathways using Lipofectamine RNAiMAX.103 The extreme reduction in proliferation was noted for *KRAS* or *PIK3CA* siRNA in the DLD-1 cell line and almost all of siRNAs in the HCT116 cells, introducing *KRAS* and *PIK3CA* as optimum siRNA treatments. Furthermore, combination *KRAS* + *PIK3CA* siRNA treatments evidently diminished proliferation over either siRNA alone. Additionally, in comparison to the cells exposed to 5-FU and single siRNAs, the combination *KRAS*+*PIK3CA* siRNA and 5-FU treatments led to the highest overall decline in proliferation. While no noteworthy elevation in apoptosis was observed for DLD-1, combination treatment of *KRAS*+ *PIK3CA* siRNAs significantly increased apoptosis compared to *KRAS* siRNA treatments alone. *PIK3CA* siRNA treatment showed negligible apoptotic outcomes in both cell lines. In accordance with apoptosis data, reduced phosphorylation of 4E-BP1, a downstream target of RAS/RAF/MAPK and PI3K/AKT/mTOR pathways, was observed for *KRAS* + *PIK3CA* siRNA combination and *KRAS* siRNA treatments in HCT116 cells. The reduced phosphorylation of ERK and AKT was seen in cells treated via *KRAS* or *PIK3CA* siRNA, respectively. These results indicate the effect of *PIK3CA* and *KRAS* mutational status and differences in genetic profiles of cell lines on responsiveness of CRC to siRNA treatments.103

In an individual treatment, *KRAS*-mutant SW620 and *KRAS-*WT CW-2 cells were transfected by doxycycline-inducible *KRAS* shRNA lentiviral vectors generated using a three plasmid system.83 In comparison to the CW-2 cells, 4E-BP1 phosphorylation in SW620 cells was sufficiently suppressed through the knockdown of *KRAS*. The loss of 4E-BP1 phosphorylation caused a downregulation of *MCL1* via mTOR complex 1 inhibition.83 The patient-derived CRC cell line with a *KRAS* G12D mutation, HCP1, was administrated subcutaneously or intrasplenic in mice to establish a CRC model. *In vivo* siRNA delivery was performed by the intraperitoneal injection of nanoliposomes/siRNA mixture at a ratio of 10:1. The obvious reduction in pERK and tumor growth were observed following siRNA treatment. After 3 weeks of treatment, *KRAS* siRNA caused a 73% reduction in aggregate liver metastasis, a major site of distant metastasis in CRC patients. No clear signs of toxicity from *KRAS* siRNA treatment were exhibited in mice.102

*RAF1* and *AKT1* depletion using pLKO.1 lentiviral shRNA vector also increased apoptosis in HCT116 cells. In contrast, while the silencing of *RAF1* inhibited CRC proliferation and invasion, it did not have significant effect on apoptosis in the SW480 cells.108 Moreover, inhibition of *MEK1/2* using siRNAs transfected by HiPerFect reagent significantly reduced MEK1/2 protein 72 h posttransfection in HCT15 and HCT116 cells. More importantly, the reduced cell growth and restored sensitivity to Cetuximab were reported following RNAi-silencing of *MEK1/2*.92 In a large scale experiment, siRNAs directed against *AKT*1, *PIK3CA, HER2, HER3, MEK1/2, CRAF, BRAF, HRAS, NRAS*, and *KRAS*, effectively knocked down the designated target gene and provided sensitivity to panitumumab or cetuximab in a panel of resistant CRC cell lines. Concomitant suppression of *MEK1/2* and *EGFR* decreased the survival fraction below 50% in all cells.105

Emerging evidence using isogenic derivative cell lines have indicated that mutational status of cancer cells has a strong effect on predicting the outcome of siRNA treatments in CRC.103 For example, isogenic DLD-1 cells with disrupted *KRAS*G13D allele displayed decreased proliferation on adherent surfaces, reduced MAP kinase signaling and impaired ability to sustain anchorage-independent development *in vitro* or tumor growth *in vivo*.96 In isogenic derivative cell lines, the mutant or WT specific alleles have been removed using targeted homologous recombination.101 In DLD-1 and HCT116 isogenic cells with deleted mutant *PIK3CA, PIK3CA* siRNA showed less effective as an individual treatment. In contrast, *KRAS* siRNA treatment alone was considerably more effective in these cells. Although *PIK3CA* siRNA was substantially more effective as a single treatment of deleted mutant *KRAS* cells, *KRAS* siRNA treatments reduced proliferation compared to control.103

Additionally, a genome-wide screen96 using pool-based shRNA platform packaged into retroviruses in isogenic *KRAS* WT/− (*RAS* WT) HCT116 and DLD-1 cells and parental *KRAS* WT/G13D (*RAS* Mut) HCT116 and DLD-1 cells recognized candidate *RAS* synthetic lethal (RSL) genes whose depletion constitute synthetic lethality in combination with the *KRAS* oncogene. For example, the growth and fitness of *RAS* Mut cells, compared to the *RAS* WT cells, were impaired by knockdown of either *COPS3* or *COPS4*. These RSL genes have diverse functions, like ribosomal biogenesis and translation control, RNA splicing, protein neddylation and sumoylation. Therefore, to retain the oncogenicity, *RAS* depends on additional support from numerous genes. The recognition of various mitotic genes as RSL candidates proposes that *RAS* Mut cells likely experience enhanced mitotic stress. This observation was confirmed by different responsiveness of *RAS* WT and Mut cells to inhibitors of mitotic spindle function like paclitaxel and nocodazole. Furthermore, siRNA knockdown of anaphase promoting complex/cyclosome (APC) strongly showed the same phenotype achieved using BI-2536, an inhibitor of PLK1. These results confirm that many genes in RLS network could be used as potential therapeutic targets. On the other hand, although the knockdown of *KRAS* (both WT and Mut protein) in HCT116 and DLD-1 cells led to only a modest reduction in growth on cohesive surfaces, profoundly damaged colony formation in soft-agarose was observed. This result supports the fact that *KRAS* silencing is adequate to suppress the malignant phenotype of these cells.96

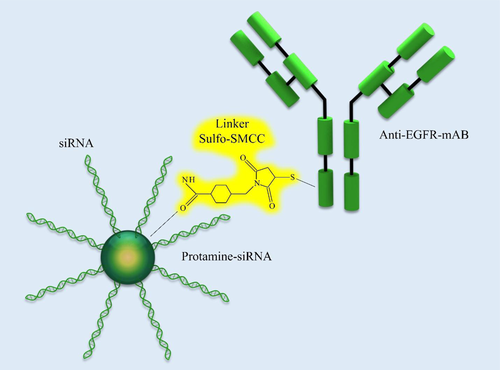
Steckel et al.93 transfected HCT116 cells and the isogenic derivative, HKE-3, by *KRAS* and *PLK1* targeting siRNAs using Dharmafect reagent. In HKE-3 cells, activated but not the normal *KRAS* allele was removed. SiRNA-mediated suppression of *KRAS* in HCT116 led to a robust loss of cell viability, marked decrease in levels of pAKT and pERK and profound induction of apoptosis. In contrast, a much attenuated apoptotic response was reported in HKE-3 following knockdown of *KRAS*. However, apoptosis induction mediated by *PLK1* silencing and reduction in *CDC6* expression following *KRAS* siRNA treatment were in comparable levels of both cells, reflecting alteration in the distribution of cell cycles once *KRAS* function is ablated.93

Despite the protumorigenic effects of mutant *RAS* alleles, a WT copy of *RAS* plays a tumor-suppressive role.101 HCT116 and DLD-1 cell line derivatives with a single copy of mutant *KRAS* (*G13D* mutations) were transfected with *KRAS* siRNAs using RNAiMax. Knockdown data reveals that while mutant *KRAS* had strong consequences on cellular RAS-GTP levels, it had surprisingly moderate influences on downstream signaling through RAF and PI3K pathways. The modest effects of *KRAS* knockdown on AKT phosphorylation were observed in the basal and starved conditions. Despite incomplete knockdown efficiency in HCT116, *KRAS* depletion increased epidermal growth factor receptor (EGFR) phosphorylation. Suppression of *KRAS* showed low effect on migration and proliferation. However, *KRAS* siRNA effectively reduced *KRAS* expression and decreased pERK in both cell lines. The mutant *KRAS*-specific genes, such as *DUSP6, DUSP5, NT5E, ETV1, IER3, UPP1*, and *ETV5*, were generally downregulated following *KRAS* knockdown in the mutant *KRAS*-expressing cells. However, no significant proliferation inhibition was detected following knockdown of *NT5E* or *ETV1* in mutant *KRAS* cells. It is presumable that *KRAS* employs multiple target genes to entirely elicit transformation properties.101

Increasing evidence presents that targeting PI3K/AKT and RAS/RAF/MAPK pathway can regulate autophagy in cancer cells.100 SW480 (*KRAS*G12V) and HCT116 (*KRAS*G13D) cells were transiently transfected by siRNAs targeting *KRAS, ATG5, BECN1, MAP2K1, MAP2K2*, and *PIK3CA* using Lipofectamine 2000. The KRAS depletion dramatically harmed the autophagic flux, evidenced by the reduced cumulation of LC3-II and levels of Atg5-Atg12 conjugate and beclin 1 (components of autophagosome formation) upon lysosomal degradation inhibition by Baf.A1 in both cells. Under nitrogen starvation conditions that induce autophagy, suppression of *KRAS* raised AKT phosphorylation in SW480 and prevented ERK phosphorylation in SW480 and HCT116, representing that the *KRAS*-induced autophagy is regulated via downregulation of the PI3K/AKT pathway and upregulation of the MEK/ERK pathway. Because of an activating *PIK3CAH1047R* mutation, *KRAS* knockdown did not affect AKT activation in the HCT116. Additionally, knockdown of *PI3KCA* had no effect on autophagy induction, whereas *MEK1/2* suppression decreases the autophagic flux of SW480. The depletion of *BECN1, ATG5*, and *KRAS* in SW480 cells increased cell death in whole medium and higher extent in starvation situations. This data supports the notion that increased capacity of autophagy induction in *KRAS* mutant cells is efficiently countered by declining mutant KRAS protein levels beneath a certain threshold.100

The emerging evidence107 indicates that *KRAS* is a potential target gene of microRNA called miR-204-3p. This miRNA binding to *KRAS* mRNA inhibits its expression. On the other hand, long noncoding RNA BCYRN1 deteriorates the occurrence and progression of CRC via competitively binding to miR-204-3p and its inhibiting as well as promoting KRAS expression. These results confirm the role of BCYRN1/miR-204-3p/KRAS axis in the proliferation, apoptosis, invasion, and migration of CRC cells. The transfection of shRNA-KRAS through Lipofectamine 3000 reagent in LoVo and SW480 cells efficiently reversed the increased proliferation cells treated with sh-BCYRN1 and induced the apoptosis of sh-BCYRN1-treated CRC cells. Additionally, sh-KRAS reversed the elevated migration of sh-BCYRN1-treated CRC cells.107

The mutations in *KRAS* commonly lead to resistance for anti-EGFR monoclonal antibody (mAB).98 To overcome this struggle in patients harboring *KRAS* mutations, at least eight molecules of siRNA against *KRAS* were bound to one molecule anti-EGFR-mAB-protamine by electrostatic interactions (Figure **9**) and were introduced into *BRAF* V600E mutation HT29 cells and *KRAS* mutations (in codon 12 or 13*) HCT116, SW620, and LoVo* cell lines. The cells effectively took up anti-EGFR-mAB-protamine-*KRAS*-siRNA complexes. While *KRAS* siRNA bound to anti-EGFR-mAB-protamine sufficiently suppressed KRAS protein expression in LoVo and HCT116 cell lines by more than 80%, control siRNA did not change KRAS expression. The cell viability, ERK1/2 phosphorylation and c-MYC protein expression (target molecule of the ERK signaling) were substantially diminished in cells treated with anti-EGFR-mAB-*KRAS*-siRNA. The anti-EGFR-mAB-protamine-*KRAS*-siRNA complex exhibited signs of apoptosis and inhibited clonogenic growth of HCT116 and LoVo cell lines. The *BRAF*-mutant HT29 cells remained resistant to *EGFR*-targeting siRNA treatment. Since the SW620 (an EGFR-negative cell line) did not represent reaction upon anti-EGFR-mAB-control or anti-EGFR-mAB-*KRAS*-siRNA treatment, it seems a threshold expression of EGFR is essential to regulate the efficacy of anti-EGFR-mAB-*KRAS*-siRNA complex.98

[](https://onlinelibrary.wiley.com/cms/asset/696b16d7-efd8-41a8-85ff-a44f39a64107/med21735-fig-0009-m.jpg)

**Figure 9** The schematic image of EGFR-targeting siRNA carrier system. One anti-EGFR-mAB couples to protamine by bivalent cross-linker sulfo-SMCC. At least, eight siRNAs could bind to one molecule protamine through electrostatic interactions.98 EGFR, epidermal growth factor receptor; mAB, monoclonal antibody; siRNA, small interfering RNA

Subsequently, mice were transplanted with HCT116, DLD1, HT29, or SW480 cells harboring different cetuximab resistance mutations and treated with cetuximab-protamine coupled to *KRAS* siRNA. Anti-EGFR-mAB-protamine-*KRAS*-siRNA complexes notably suppressed *KRAS* mRNA levels, reduced Ki-67 staining (proliferation marker) and inhibited tumor growth, indicated as reduction in tumor volumes and weights. As the most cetuximab-resistant HCT116 cells contain a *H1047R* mutation activating *PIK3CA* besides the *KRAS* mutation, anti-EGFR-mAB carrying combined *KRAS* and *PIK3CA* siRNAs cooperatively reduced colony growth. In line with *in vitro* experiments, *in vivo* observations indicate that *BRAF*-mutated HT29 tumors did not react to this complex. Accumulating evidence proposes that antibody-siRNA conjugates could have a contribution in personalized anticancer therapeutic method against specific mutations.98

## 7.2 Ca2+/calmodulin dependent kinase mediated pathway

STK33, belonging to the Ca2+/calmodulin dependent kinases family, plays a key role in numerous cellular functions in the range of the regulation of DNA replication, signal transduction, cell proliferation, cell differentiation, apoptosis, and tumor development.156 STK33 has been upregulated in many types of cancers.157 However, the mechanism by which STK33 results in neoplasm transformation is not well understood.156 It is suggested that STK33 expression alone likely will not be sufficient for tumor development and instead depend on genes likes *KRAS*.117

RNAi experiments using pLKO.1 lentiviral shRNA vectors were performed to knockdown *STK33* in HCT116 cell lines.116 The lentiviral mediated transduction of cells was used with psPAX2 and pMD2.G packaging vectors in high-titer virus-containing supernatants obtained from HEK293FT cells. The targeting of *STK33* led to an impaired proliferation and elevated apoptosis, as represented by completed cleaved caspase 3 and PARP in HCT116 (Figure **8**). Abrogation of *STK33* resulted in a decreased hypoxia-inducible factor 1α (HIF-1α) accumulation that, in turn, led to the reduced levels of extracellular vascular endothelial growth factor (VEGF) via a considerably decreased VEGF promoter activity. HIF-1α is a transcription factor that responds to hypoxia and VEGF is a signaling protein that enhances the growth of new blood vessels. As corroborated with *in vitro* findings, tumor xenograft experiments on chicken using the chorionallantoic membrane exhibited a strong relationship between reduced proliferation index, as revealed by damaged tumor-driven vasculature and the number of Ki67 positive tumor cells *in vivo*.116

In addition, the infection of mutant *KRAS*-dependent cell lines, such as HCT116, SW480, and *KRAS* WT COLO-320 HSR cell line with lentivirus-mediated shRNA library against *STK33* destroyed colony formation in semisolid medium by SW480 and HCT116 as well as tumor formation ability of SW480 cells in immunocompromised mice.117 This effect was not observed in COLO-320 HSR cells. The sensitivity to *STK33* inhibition was reported to DLD-1, another mutant *KRAS*-dependent cell line. In contrast, *KRAS* dependency was not presented by HCT-15, a sister cell line of DLD-1 and it was resistant to *STK33* knockdown. Therefore, a selective sensitivity to *STK33* silencing could be observed in cells with dependency on mutant *KRAS*.117

Surprisingly, the obtained results reported by Yin et al.118 have been shown to not be in agreement with previous studies. They observed that *STK33* is hypermethylated and thereby downregulated in DLD-1 and HCT116 cells relative to NCM460, normal human cell line deriving from colon mucosal epithelial cells. The rate of cell proliferation of CRC cells was raised compared with normal cells. Next, *STK33* depletion using pSilencer4.1-CMV plasmid with *Hin*dIII and *Bam*HI enzyme sites transfected by Lipofectamine 2000 significantly increased the cell proliferation rate of cells more than siRNA-untransfected cells. It seems that the limitation of this study was the lack of scramble or negative control siRNA transfected cells.118 In addition, whereas overexpression of *STK33* was reported in hypopharyngeal squamous cell carcinoma, hepatocellular carcinoma and lung cancer cells,158, 159 the expression level of this protein has been demonstrated to downregulate in CRC cell lines through hypermethylation.160 Thereby, the mechanisms underlying the exact effect of *STK33* in CRC require further study.

## 7.3 JAK/STAT3 signaling

Janus kinase/signal transducer and activator of transcription, or JAK/STAT3, signaling pathway plays a critical role in several physiological procedures, comprising cell growth, differentiation, hematopoiesis and immune function. The abnormalities in the JAK/STAT3 pathway have exhibited the crucial importance in the tumorigenesis and development of several cancers, including CRC.161 Since main components of this pathway, including JAK1 and JAK2 (tyrosine kinases), MET (receptor tyrosine kinase), and STAT3 (transcription factor), are involved in invasion, migration, survival, and cell growth of tumor cells, intervention with their expression renders an encouraging anticancer method in the CRC treatment.110, 162

In a primary screen,104 siRNAs against 160 targets were evaluated in the *KRAS* mutant (MT) HCT116 and *KRAS* WT HKH-2 isogenic paired cell line model. *KRAS* siRNA did not change the cell viability in the HKH-2 cells, but significantly reduced it in the HCT116 cell line, approving the *KRAS* reliance of the HCT116 cells. RNAi against *STAT3, INPP4B, BIRC5, EFNA1, EFNA2*, and *EPHA1* considerably influenced the cell viability, enhanced sensitivity to MEK inhibitor (AZD6244) and sensitized to 5-FU treatment, particularly in the *KRAS* MT cell line in comparison with a *KRAS* WT daughter cell line. In a secondary RNAi screen employing three extra mutant *KRAS*-dependent cells DLD-1 (G13D), SW620 (G12V), and LoVo (G13D)—siRNAs against STAT3 signaling (*STAT3, JAK1, JAK2*, and *OSM*) showed a considerable inhibitory effect on the survival of these cell lines. In addition, *ERK1/2* or *KRAS* siRNA promoted STAT3 phosphorylation in *KRAS* MT cells, representing that this is an ordinary influence of inhibiting the MEK/ERK pathway in *KRAS* MT cells.104

Cotreatment of oxaliplatin, SN-38, 5-FU or AZD6244 and *STAT3* siRNA induced apoptosis in the *KRAS* MT cell lines, but not in the *KRAS* WT daughter cell line. This data introduces STAT3 as a significant survival compartment in the oncogenic KRAS-MEK signaling pathway.104 Interestingly, the potent suppression of constitutive STAT3 phosphorylation together with a growth in PARP cleavage, elevation MEK inhibitor-induced apoptosis and abrogation of AZD6244-induced STAT3 activation were important results following silencing of *STAT3*-upstream kinases JAK1 and JAK2 in the HCT116, but not in the *KRAS* WT isogenic cells. Whereas *JAK2* knockdown had no effect on the phosphorylation levels of JAK1, it reduced both basal and MEK inhibitor-induced STAT3 activity. Nevertheless, siRNA directed against *JAK1* decreased MEK inhibition-induced elevation in pJAK2 levels and triggered strong inhibition of basal and MEK inhibition-induced STAT3 activity. These observations support cooperative function of JAK1 and 2 in STAT3 survival reaction to MEK inhibition. Taken together, these RNAi data introduced *S*TAT3, JAK2 and JAK1 as critical mediators of resistance to MEK inhibition and chemotherapy in *KRAS* MT cells that play a main role in preserving the viability of *KRAS* MT, but not *KRAS* WT, cells.104

The JAK/STAT3 signaling pathway was also blocked in SW1116 and HT29 using *STAT3* siRNA transfected by DharmaFECT1 transfection reagent.110 The obtained results of RNAi have been revealed to be very comparable to that from AG490, a JAK2 inhibitor. Both phosphorylated STAT3 and unphosphorylated STAT3 were suppressed by 80.3% and 59.8% in *STAT3*-siRNA transfected HT29 cells, respectively. Besides, *STAT3* knockdown resulted in downregulation of *BCL2* and upregulation of *p16, p21*, and *p27* in both cells at 72 h posttransfection. The other worthwhile outcomes achieved following RNAi-induced *STAT3* deficiency in SW1116 and HT29 included as the E-cadherin upregulation, decreased secretion of VEGF and MMP2, apoptosis induction, cell migration, and invasion suppression, as well as the cell cycle arrest in the G1 phase. These results support that STAT3 is a critical mediator in JAK/STAT-dependent angiogenesis and cell invasion. However, no detectable alterations in MMP9 secretion and FAK (a tyrosine kinase upregulated in cancer cells) expression were seen in response to *STAT3* knockdown, which indicate MMP9 and FAK are not presumably required for STAT3-mediated regulation.110

The treatment of DLD-1 cells with *STAT3* siRNA using HiPerfect transfection reagent prevented IL-6-mediated increased invasiveness. *STAT3* depletion, on the other hand, blocked IL-6-induced repression of the microRNA, MIR34A, representing the effect of *STAT3* in the IL-6-induced suppression of MIR34A.111 The suppression of the *MIR34A* was critically associated with epithelial-mesenchymal transition (EMT) in CRC cells.163 In SW480 cells with high levels of IL-6R and pSTAT3, silencing of either *IL-6R* or *STAT3* through siRNAs led to the reduction of *IL-6R, STAT3* and phosphorylated STAT3 levels, *MIR34A* expression induction, and diminution in the expression of *MIR34A* target *SNAIL* together with other mesenchymal marker proteins, such as Vimentin and ZEB1 (E-cadherin repressor). The decreased invasion was also observed for SW620-luc2 and SW480 cells following knockdown of *IL-6R* or *STAT3*. The targeting of *IL-6R* or *STAT3* by siRNAs in SW620-luc2 cells blocked metastasis formation in NOD/SCID mice and decreased the quantity of metastatic tumor nodules in the site of mice lungs. These results depict the necessary function of MIR34A/STAT3/IL-6R feedback loop in metastasis and invasion of CRC cells.111

PGIPZ lentiviral vectors encoding *STAT3*-shRNAs were used for transfection of DLD1 and HT29 cells.99 Sh-*STAT3* decreased the expression of core stem cells, reduced colon cancer sphere numbers and abrogated IL-22-induced H3K79 methylation on the promoters of stem cell core gene, such as *POU5F1, SOX2*, and *NANOG*. These results reveal a relationship between STAT3 and IL-22-mediated H3K79 in regulating cancer stemness.99

Similarly, *STAT3* abrogation by siRNA transfected using DharmaFECT 1 led to the following results: a significant decline in STAT3 phosphorylation and expression, a reduction in the quantity of cancer cell colonies by nearly 80%, an inhibition of cell survival, a notable increase in E-cadherin and a reduction in the expression of N-cadherin, vimentin, and ZEB1 in highly invasive LoVo cells. These results propose that the STAT3 may contribute to the EMT development and invasion in LoVo cells. More importantly, the knockdown of *STAT3* substantially improved the chemosensitivity of LoVo cells to fluorouracil.112

Du et al.113 investigated the effect of *STAT3* silencing on hexokinase 2 (HK2) expression. HK2 enzyme catalyzes the conversion of glucose to 6-phosphate-glucose, the irreversible first stage of glycolysis. The overexpression of HK2 occurs in a several cancers like CRC and affiliates with poor prognosis in patients.164, 165 STAT3 binding to the promoter region of HK2 regulates its expression in transcriptional level. The STAT3 knockdown using lipofectamine 2000 in SW480 cells decreased its recruitment to HK2 promoter and thereby, reduced HK2 expression.113

In a study conducted by Ueno et al.,115 SW620 cells were transfected with *MET* siRNA using Oligofectamine. The results showed a reduction in MACC1 (trigger of metastasis) induced cell motility and proliferation as a result of *MET* knockdown. However, *MACC1* expression was unaltered in response to *MET* siRNA. Furthermore, silencing *MET* mediated by siRNA blocked the *MET* ligand HGF-induced proliferation and scattered phenotype. Importantly, intrasplenic transplantation of SW620 cell lines carrying *MET* shRNA-harboring plasmids by lipofectin diminished liver metastatic rates by 50%.115 In addition, *MET* siRNA in HCT116 cells could abrogate *MEK* inhibition-induced activation of *STAT3* and increase apoptosis.104

The crosstalk between PI3K/AKT/mTOR and JAK/STAT3 signaling pathways is mediated by STAT3.166 Convallatoxin (CNT), triterpenoid ingredient, possesses anticancer effects. CNT inhibits the tyrosine-705 phosphorylation of STAT3 (T705) via JAK2 pathway and serine-727 phosphorylation of STAT3 (S727) via mTOR pathway in CRC cells. The treatment of HCT116 cells with *JAK2*-siRNA using Lipofectamine 2000 reagent resulted in the downregulation of p-JAK2, p-mTOR, p-STAT3 (S727), and p-STAT3 (T705). Cotreatment of *JAK2*-siRNA and CNT exhibited same results for p-JAK2, but downregulation was further augmented for others. Similarly, *mTOR* silencing in HCT116 cells downregulated p-mTOR, p-JAK2, p-STAT3 (S727), and p-STAT3 (T705). While co-treatment of *mTOR*-siRNA and CNT downregulated p-mTOR as same as single *mTOR*-siRNA treatment, the phosphorylation levels of JAK2, STAT3 (S727), and STAT3 (T705) were more inhibited. These observations confirm the involvement of both mTOR and JAK2 in CNT-mediated prevention of STAT3 activation.114

## 7.4 Wnt pathway

The Wnt signaling pathway contains components that positively and negatively regulate it. The genes involved in this pathway are strongly conserved through evolution. The Wnt pathway is divided into two distinct signaling ways: canonical or comparatively well understood β-catenin dependent and noncanonical or less well-characterized β-catenin independent. The balance between both these pathways is mediated by mechanisms that are different for various tissue types and derivative tumors. It has been indicated that the aberrant Wnt signaling, especially canonical pathway, clearly contributes to different types of cancer. In addition, a direct connection between Wnt signaling and other pathways helps maintain growth and survival of tumor cells. The preclinical experiments propose that suppression of Wnt pathway can counteract cancer cell growth and survival. This evidence highly encourages development of therapies interfering with Wnt pathway in cancer cells.167, 168

### 7.4.1 β-Catenin

β-Catenin is a major multitasking effector in canonical Wnt signaling pathway that is responsible for the transmission of signal to the nucleus. In the nucleus, β-catenin together with some transcription factors from the TCF/Lef family induces the transcription of Wnt-specific genes.169 According to the genetics of Wnt signaling in cancer, in which interfering with β-catenin provides antitumorigenic effects, it is suggested that the β-catenin would hold greatly promising potential for cancer therapy.168

The following *β-catenin*-siRNA transfection using Lipofectamine 2000 in SW480 cells, the relative mRNA, along with protein levels of β-catenin and also the SW480 proliferation were effectively reduced. In addition, the apoptosis rate of SW480 cells and the levels of caspase-3 activity were dramatically elevated in siRNA-treatment groups compared to control cells. SiRNA targeting *β-catenin* inhibited the invasion ability of cells 24, 48, and 72 h after transfection.119

In another study,120 HCT116 and Ls174T cells harboring a *β-catenin* stabilizing mutation were transfected by *β-catenin* shRNA cloned into pTER plasmid. The downregulation of β-catenin protein and ITF2 (β-catenin/TCF target), along with proliferation suppression were achieved following *β-catenin* inhibition. The expression of Sp1, an inducer of surviving transcription, was also decreased in combined *β-catenin*/*ITF2* or *β-catenin*/*KRAS* shRNA cells, as well as in *β-catenin* shRNA cells. The double knockdown of *β-catenin*/*ITF2* or *β-catenin*/*KRAS* induced apoptosis in approximately 45% of treated cells and suppressed cell growth by around 99% and 97%, respectively. This apoptosis effect was correlated with strong downregulation of *BCL2* and moderate upregulation of *FAS*. However, one difference in the apoptotic response reported for *β-catenin*/*ITF2* or *β-catenin*/*KRAS* double shRNAs was downregulation of antiapoptotic Bcl-xL protein following *β-catenin*/*KRAS* shRNA treatment and upregulation of proapoptotic BIM by *β-catenin*/*ITF2* silencing. Similar results were also obtained from *in vivo* studies where the double-shRNA *β-catenin*/ITF2 cells or *β-catenin*/*KRAS* cells were injected into nude mice. These mentioned studies reported significant inhibition of tumor growth compared with single shRNA cells.120

In accordance with the former study, Scholer et al.123 reported similar results in their experiments in which both SW403 and LS411N cells (*APC* mutant) were transfected by *β-catenin* shRNA using doxycycline inducible pLKO Tet on lentiviral vectors and then transplanted in mice. A concomitant and considerable decrease in the mRNA and protein levels of β-catenin and its target genes *c-MYC* and *AXIN2* was observed *in vivo* through the silencing of *β-catenin*. Interestingly, a prominent inhibition of tumor growth and nuclear *β-catenin* depletion were reported in tumors treated by *β-catenin* shRNA. While *p21* induction arose in both SW403 and LS411N *β-catenin* shRNA xenografts, it was stronger in SW403-derivated tumors where the most profound effect of *β-catenin* shRNAs was observed on c-MYC expression. These results confirm the effect of *β-catenin* knockdown on cell cycle arrest. Additionally, the cell cycle arrest was not coupled to apoptotic response because the level of cleaved caspase 3 was unaltered. As the level of total LC3 protein was increased at day 3 after *β-catenin* silencing, cell cycle probably cross talked with autophagy.123

Importantly, three crypt progenitor/stem cell markers *LGR5, BMP4*, and *EPHB2* were substantially reduced in LS411N *β-catenin* shRNA tumors.123 Concomitantly, differentiation markers *TM4SF4, MUC2*, and *CA2* were dramatically upregulated. These observations suggest that the *β-catenin* inflicts a crypt progenitor/stem cell phenotype and induces dedifferentiation on colorectal cells. Interestingly, *β-catenin* suppression caused morphological changes of cancer cells similar to that of normal intestinal epithelial cells. Besides, no EMT was observed in *β-catenin* depletion tumors. In keeping with *in vivo* data, shRNA targeting *β-catenin in vitro* downregulated *β-catenin, AXIN2, c-MYC, LGR5, BMP4*, and *EPHB2* together with upregulation of *CA2, MUC2* and *TM4SF4* in LS411N cells. The cell viability and proliferation were only diminished in SW403 and LS411N cell lines, but not in WT *β-catenin* and *APC* RKO cells *in vitro*. Taken together, this data represents that the *β-catenin* shRNA could effectively and specifically suppress the Wnt/β-catenin pathway, and the activation of this pathway is needed for the maintenance of *APC*-mutant CRC cells.123

Interestingly, in addition to proteins implicated in Wnt/β-catenin pathway, patients’ thyroid hormone status might play a notable role in the therapeutic strategies of CRC. l-Thyroxine (T4) enhances the expression of proliferation markers, including c-MYC and cyclin D1, involved in cancer risk and tumor development. Human CRC cell line HCT116 was transfected by SignalSilence *β-Catenin* siRNA using Lipofectamine 3000. Forty-eight hours after transfection, T4 was applied to cells. The silencing of *β-catenin* lowered the T4-enhanced *cyclin D1, β-catenin* and *CCND1* (downstream target of Wnt/β-catenin pathway) expression compared to the T4-treated control cells.121

### 7.4.2 FZD7

Human frizzled (FZD) family of Wnt receptors consists of 10 well studied members that are involved with Wnt proteins to transduce downstream Wnt signals. Some investigations highlight the key roles of FZDs in the progress of cancers.168, 170

In 2009, Ueno et al.124 reported a greater expression level of *FZD7* mRNA in the stage II, III, or IV of primary CRC compared to normal tissues that was related to the shorter survival and poor prognosis in these patients. To determine the significance of FZD7 as a potential therapeutic target in CRC, *FZD7* was downregulated through RNAi in HCT116 cell line. Thirteen different siRNAs against *FZD7* were created in the piGENE hU6 Vector and transiently delivered into HCT116 cells by FuGENE HD transfection reagent. The *FZD7* siRNA number 8 reduced *FZD7* expression more efficiently to 30% and it was used for the following experiments. *FZD7* silencing led to a decline in the fraction of cells at G2/M phase, the expression level of p-c-JUN, p-JNK, and c-JUN (involved in MAP kinases pathway), invasion and cell viability activity. Since small GTPase RhoA involves in the migration of CRC cells and regulates JNK/c-JUN implicated in the noncanonical Wnt pathway, it proposes that FZD7 could be a receptor for the noncanonical signaling pathway and Wnt-stimulated cell migration might be independent of canonical Wnt signaling.124

On the other hand, *FZD7* knockdown reduced the expression levels of *TCF, CD44v8-9*, and matrix metalloproteinase *MT1-MMP*.124 Given that the canonical Wnt pathway associates with TCF and includes several extracellular proteinases likes CD44 and MT1-MMP/MMP-14, FZD7 could regulate migration and invasion via canonical Wnt pathway as well. The antimetastatic activity of *FZD7* siRNA was examined in an *in vivo* liver metastasis model by transplantation of *FZD7* siRNA transfectants into the mice spleen. The results showed that the liver metastases in these mice were significantly decreased. However, no apoptotic cells were detected in this study. One possibility is that Wnt signaling via other FZDs might preserve *FZD7* siRNA transfectants from apoptosis.124

Similar results were observed when *FZD7* siRNAs were created in the piGENE-hU6 vector in HCT116 (with *β-catenin* mutation), SW480 (with *APC* mutations), DLD-1, LoVo, HT-29, and Caco-*2*.109 *FZD7* siRNA decreased TCF activity in these cells by 20%–80% and suppressed the expression level of Wnt canonical targets, such as β-catenin, MYC, survivin, and JUN, supporting the role of FZD7 in the activation of this pathway even in the presence of *APC* or *β-catenin* mutation. The viable cell number was reduced to 20% or 80% and the cell invasion number reduced to 40% or 60% in HCT116 and HT-29, respectively. However, *FZD7* siRNA could not induce *OAS1* expression as an indicator of interferon response as well as trigger cleavage of caspase-3 and release of cytochrome *C*, as apoptotic responses in HCT116 cells. Although the molecular mechanism by which *FZD7* activates the cell invasion remains unknown, accumulating evidence suggests that *FZD7* affects metastasis and invasion of CRC cells via expression of Wnt target genes.109, 124

In an experiment designed by Vincan et al.,125 shRNA oligoes were cloned into the *Hind*III and *Bgl*II sites of retrovirus based pRETROSUPER vector and introduced into monolayer colon carcinoma cell line LIM1863 Mph and two other colon cell lines SW480 and SW620. The *FZD7* expression was notably reduced in the LIM1863-Mph cells transfected with *FZD7* shRNA. Moreover, these cells did not undergo mesenchymal epithelial transition and subsequent carcinoid assembly. While *FZD7* shRNA transduced LIM1863-Mph experienced a reduction in E-cadherin expression (EMT characteristics) and a concomitant slight elevation in *SNAI2* that displaced permanence of the mesenchymal phenotype in these cells, the expression level of other EMT inducers (such as SNAI1, SIP-1, ZEB1, and laminin-5 α3 and γ2) was not increased in these cells. This data could explain why the LIM1863-Mph-*FZD7* siRNA cells did not migrate on the tissue culture plastic. Resembling results were observed with the transfection of *FZD7* shRNA in *SW480* cells. In addition, the proliferation of *FZD7* shRNA transfected SW480 and LIM1863-Mph cells appeared to be decreased, but unaffected in *FZD7* shRNA transfected SW620 cells. These findings highlight a crucial function for *FZD7* in both de-differentiated and differentiated carcinoma cells.125

Additionally, transfection of pcDNA3.1-Wnt11 into HCT116 established two clones—WC-L and WC-H—with relatively lower and higher expression of Wnt11, respectively. Then, *FZD7* siRNA was transiently transferred into WC-L and WC-H cells by the nucleofector system. The *FZD7* expression, but not Wnt11, was clearly depleted after transfection of *FZD7* siRNA. Although *FZD7* siRNA did not change the invasion ability in both WC-L and WC-H cells, it substantially decreased the migration ability of WC-H cells, but not WC-L cells. These findings suggest that while *FZD7* might contribute to Wnt11-induced migration ability of HCT116 cells, the Wnt11-induced invasion activity is presumably mediated through other receptors, like FZD4 and FZD5.126

# 8 CLINICAL APPLICATIONS OF RNAI IN CRC

Despite the promising results of the RNAi usage in the suppression of tumor progression for *in vitro* and *in vivo* models of CRC, its application in clinical therapy is still pending mostly because of the lack of effective delivery systems. Cationic polymers and liposomes are the gold standard carriers for delivery of RNAi agents to mCRC cells.171 Although both vehicles are efficient on gene silencing, they can enforce toxicity and do not carry out the incessant release of siRNA. The last issue is very important to retain the therapeutic effects.172 The encapsulation of siRNA into less toxic and biodegradable matrices like poly (lactic-co-glycolic acid) (PLGA) polymers provides the long-term release of the cargo.173, 174 However, hydrophobic PLGA polymers are not able to effectively entrap the highly hydrophilic and anionic siRNAs.175 It makes difficult the siRNA delivery into CRC metastases in therapeutic amounts.

At present, only a few clinical trials are being accomplished with RNAi strategy in CRC (ClinicalTrials.gov). One clinical trial has been studying the administration of siRNA in patients with stage IV CRC, mCRC and recurrent colorectal carcinoma that cannot be submitted to resectable surgery. In this phase I trial with 11 participants, patients received peripheral blood mononuclear cells transfected *ex vivo* with siRNA targeting the E3 ubiquitin ligase casitas B-lineage lymphoma-b gene (Cbl-b). Cbl-b acts as a major intracellular checkpoint restricting lymphocyte activation. Inhibiting Cbl-b enhances natural killer cells and T cells that inhibit the growth of tumor cells. siRNA-transfected autologous peripheral blood mononuclear cell, APN401, was administrated intravenously over 30 min on days 1, 29, and 57 in the absence of unacceptable toxicity or disease progression. As intravenous infusion of 50 × 105/kg of APN401 into patients was safe and feasible, the injections were proceeding for Phase II clinical trials.176, 177

In another important clinical trial, TKM-080301 (TKM-PLK1), a lipid-siRNA NP against *PLK1*, was appraised in a first-in-human phase I and II study in 68 patients with advanced solid tumors involving CRC. Single administration of TKM-080301 resulted in silencing of *PLK1* for up to 7–10 days. The patients have been treated with TKM-080301 at concentrations ranging from 0.15 to 0.9 mg/kg body weight/week. The data showed the most common drug-related adverse events including mild to moderate levels of chills, fever, vomiting, nausea, and fatigue led to selection of maximum tolerated dose of 0.75 mg/kg body weight/week. One patient with stable CRC received TKM-080301 for more than six months with no cumulative toxicity.150, 152, 178, 179 Other clinical trials using siRNA vehicles have been performed for investigation of RNAi application in the treatment of more developed stages of digestive tract cancers, involving mCRC.171 For instance, Atu027 is an RNAi therapeutic that silences the gene expression of protein kinase N3 in the vascular endothelium. Atu027 consists of chemically stabilized siRNA and liposomal NPs. A phase 1 survey of Atu027 indicated that it was well tolerated in patients with advanced solid tumors including CRC.180 CALAA-01, a cyclodextrin-based polymeric NP including a siRNA targeting RRM2, was well tolerated by patients with different cancers in a phase I study.181

# 9 DISCUSSION

RNAi therapeutics has recently emerged as a promising therapeutic perspective for cancer and several other diseases.46 This approach has various benefits over traditional pharmaceutical drugs. Almost all genes can be strongly silenced by siRNA and shRNA. Moreover, the recognition of very selective and inhibitory sequences is speedier than the finding of new chemicals, and the synthesis of siRNA and shRNA on a large scale is relatively simple.31 However, there are barriers for the development of effective and safe clinical RNAi therapy. To overcome nondesirable outcomes of gene silencing by RNAi systems, various strategies including combination of RNAi-based therapy by traditional chemotherapy or simultaneous delivery of multiple siRNAs to inhibit different pathways in the target cells have been applied for the rising of therapeutic effects.46 Remaining challenges for siRNA and shRNA include selective and efficient delivery systems and off-target effects. New delivery systems/materials are being explored to improve the cellular uptake efficiency. Less off-target effects are also achieved by usage of optimized shRNA constructs to permit good potential and stable influences employing low copy numbers.33 Although shRNA requires the use of an expression vector, which can pose safety concerns, it has a low rate of degradation and turnover.31

# 10 CONCLUSION AND OUTLOOK

*In vitro* and *in vivo* experiments underline the potent role of siRNA and shRNA in controlling CRC through induction of apoptosis and inhibition of the CRC growth. In addition, the synergistic effects of inhibitors and RNAi-mediated gene knockdown could develop novel treatment strategies in oncology.

RNAi-based approaches offer a wide range of potential applications with a high degree of flexibility, providing valuable opportunity to manipulate formerly “undruggable” targets. However, the delivery of RNAi agents is major barrier to be overcome in clinical trials. Moreover, less clinical trials have been managed for colon cancer due to the hardness to reach tissues in comparison to other tumors like melanoma. Although new delivery systems and materials are being investigated for promotion of the delivery efficiency, it is necessary to design randomized controlled trials before implementation of RNAi in clinical guidelines. The development of safe biodegradable and biocompatible NP delivery systems is still obligatory. More importantly, it is necessary to develop reproducible and simple protocols for the manufacture of batches for clinical trials and regulatory assessments. As unmodified siRNAs suffer low and inefficient cellular uptake, they need to be conjugated or complexed with a suitable carrier systems. In addition, the short half-life of siRNAs due to their fast degradation in plasma and cellular cytoplasm may be modulate with the combination of siRNAs with versatile and biocompatible nonviral carriers. Future research needs to expand clinical trials with improved strategies for increasing of the encapsulation of RNAi agents in lipid based NPs such as liposomes or biodegradable polymers such as PLGA, their cellular uptake and also endosomal escape in mCRC cells. Advances in nanotechnology and medicine chemistry may help to solve these issues and RNAi-based therapies will become more broadly adopted.

The recognition of suitable targets is another important issue for applying RNAi-based therapeutics. In addition of understanding of target genes and pathways for the progress of CRC, it is important to know modifying genes that compensate to the effect of loss function of target genes and the degree of gene silencing needed.

# ACKNOWLEDGMENT

The authors are grateful for financial support from the Immunology Research Center, Tabriz University of Medical Sciences (Grant No: 61733).

# CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

# References

1 Macrae F. A. Colorectal cancer: epidemiology, risk factors, and protective factors. 2020. http://www.uptodate.com/contents/colorectal-cancer-epidemiology-risk-factors-and-protective-factors

2 Seeber A, Gastl G. Targeted therapy of colorectal cancer. *Oncol Res Treatment*. 2016; **39**(12): 796- 802.

3 Aran V, Victorino AP, Thuler LC, Ferreira CG. Colorectal cancer: epidemiology, disease mechanisms and interventions to reduce onset and mortality. *Clin Colorectal Cancer*. 2016; **15**(3): 195- 203.

4 Øines M, Helsingen LM, Bretthauer M, Emilsson L. Epidemiology and risk factors of colorectal polyps. *Best Pract Res*. 2017; **31**(4): 419- 424.

5 Witold K, Anna K, Maciej T, Jakub J. Adenomas—genetic factors in colorectal cancer prevention. *Rep Pract Oncol Radiother*. 2018; **23**(2): 75- 83.

6 Rex DK, Ahnen DJ, Baron JA, et al. Serrated lesions of the colorectum: review and recommendations from an expert panel. *Am J Gastroenterol*. 2012; **107**(9): 1315- 1329.

7 Vilar E, Gruber SB. Microsatellite instability in colorectal cancer—the stable evidence. *Nat Rev Clin Oncol*. 2010; **7**(3): 153- 162.

8 Ahnen DJ. The American college of gastroenterology Emily couric lecture—the adenoma–carcinoma sequence revisited: has the Era of genetic tailoring finally arrived? *Am J Gastroenterol*. 2011; **106**(2): 190- 198.

9 Gallois C, Pernot S, Zaanan A, Taieb J. Colorectal cancer: why does side matter? *Drugs*. 2018; **78**: 1- 10.

10 Sinicrope FA, Rego RL, Foster N, et al. Microsatellite instability accounts for tumor site-related differences in clinicopathologic variables and prognosis in human colon cancers. *Am J Gastroenterol*. 2006; **101**(12): 2818- 2825.

11 Missiaglia E, Jacobs B, D'ario G, et al. Distal and proximal colon cancers differ in terms of molecular, pathological, and clinical features. *Ann Oncol*. 2014; **25**(10): 1995- 2001.

12 Ohhara Y, Fukuda N, Takeuchi S, et al. Role of targeted therapy in metastatic colorectal cancer. *World J Gastrointest Oncol*. 2016; **8**(9): 642- 655.

13 Rejhová A, Opattová A, Čumová A, Slív D, Vodička P. Natural compounds and combination therapy in colorectal cancer treatment. *Eur J Med Chem*. 2017; **144**: 582- 594.

14 Jianguang J, Jan S, Kristina S. Use of hormone replacement therapy improves the prognosis in patients with colorectal cancer: a population-based study in Sweden. *Int J Cancer*. 2018; **142**(10): 2003- 2010.

15 Bastos DA, Ribeiro SC, de Freitas D, Hoff PM. Combination therapy in high-risk stage II or stage III colon cancer: current practice and future prospects. *Ther Adv Med Oncol*. 2010; **2**(4): 261- 272.

16 Key statistics for colorectal cancer. http://www.cancer.org/cancer/colon-rectal-cancer/about/key-statistics.html

17 Ansa BE, Coughlin SS, Alema-Mensah E, Smith SA. Evaluation of colorectal cancer incidence trends in the United States (2000–2014). *J Clin Med*. 2018; **7**(2): 22.

18 Siegel R. L., Mill K. D., Ahmedin J. Cancer statistics, 2018. *CA Cancer J Clin*. 2018; **68**(1): 7- 30.

19 Smith KM, Desai J. Nivolumab for the treatment of colorectal cancer. *Expert Rev Anticancer Ther*. 2018; **18**: 611- 618.

20 Linnekamp JF, Wang X, Medema JP, Vermeulen L. Colorectal cancer heterogeneity and targeted therapy: a case for molecular disease subtypes. *Cancer Res*. 2015; **75**(2): 245- 249.

21 Rachel MM, Vanesa S, Joel CB, Kulmira N. Colorectal cancer chemotherapy: the evolution of treatment and new approaches. *Curr Med Chem*. 2017; **24**(15): 1537- 1557.

22 McLeod HL, Sargent DJ, Marsh S, et al. Pharmacogenetic predictors of adverse events and response to chemotherapy in metastatic colorectal cancer: results From North American Gastrointestinal Intergroup Trial N9741. *J Clin Oncol*. 2010; **28**(20): 3227- 3233.

23 Pearce A, Haas M, Viney R, et al. Incidence and severity of self-reported chemotherapy side effects in routine care: a prospective cohort study. *PLOS One*. 2017; **12**(10):e0184360.

24 Nadége C, Estelle P, Agnés B, Jerome G, Alain T, David K. Changing patient perceptions of the side effects of cancer chemotherapy. *Cancer*. 2002; **95**(1): 155- 163.

25 Ghasabi M, Mansoori B, Mohammadi A, et al. MicroRNAs in cancer drug resistance: basic evidence and clinical applications. *J Cell Physiol*. 2019; **234**(3): 2152- 2168.

26 Asadzadeh Z, Mansoori B, Mohammadi A, et al. microRNAs in cancer stem cells: biology, pathways, and therapeutic opportunities. *J Cell Physiol*. 2019; **234**(7): 10002- 10017.

27 Gerber DE. Targeted therapies: a new generation of cancer treatments. *Am Fam Physician*. 2008; **77**: 3.

28 Witold K, Anna K, Maciej T, Jakub J. Adenomas—genetic factors in colorectal cancer prevention. *Rep Pract Oncol Radiother*. 2018; **23**(2): 75- 83.

29 Gilligan LC, Gondal A, Tang V, et al. Estrone sulfate transport and steroid sulfatase activity in colorectal cancer: implications for hormone replacement therapy. *Front Pharmacol*. 2017; **8**: 103.

30 Symer MM, Wong NZ, Abelson JS, Milsom JW, Yeo HL. Hormone replacement therapy and colorectal cancer incidence and mortality in the prostate, lung, colorectal, and ovarian cancer screening trial. *Clin Colorectal Cancer*. 2018; **17**(2): e281- e288.

31 Dana H, Chalbatani GM, Mahmoodzadeh H, et al. Molecular mechanisms and biological functions of siRNA. *Int J Biomed Sci*. 2017; **13**(2): 48- 57.

32 Sledz CA, Williams BRG. RNA interference in biology and disease. *Blood*. 2005; **106**(3): 787- 794.

33 Rao DD, Vorhies JS, Senzer N, Nemunaitis J. siRNA vs. shRNA: similarities and differences. *Adv Drug Deliv Rev*. 2009; **61**(9): 746- 759.

34 Ebrahimian M, Taghavi S, Mokhtarzadeh A, Ramezani M, Hashemi M. Co-delivery of doxorubicin encapsulated PLGA nanoparticles and Bcl-xL shRNA using alkyl-modified PEI into breast cancer cells. *Appl Biochem Biotechnol*. 2017; **183**(1): 126- 136.

35 Carthew RW, Sontheimer EJ. Origins and mechanisms of miRNAs and siRNAs. *Cell*. 2009; **136**(4): 642- 655.

36 Lam JKW, Chow MYT, Zhang Y, Leung SWS. siRNA versus miRNA as therapeutics for gene silencing. *Mol Ther Nucleic Acids*. 2015; **4**: 4.

37 Murchison EP, Partridge JF, Tam OH, Cheloufi S, Hannon GJ. Characterization of Dicer-deficient murine embryonic stem cells. *Proc Natl Acad Sci USA*. 2005; **102**(34): 12135- 12140.

38 Herrera-Carrillo, E, Gao, et al. B. The influence of the 5΄-terminal nucleotide on AgoshRNA activity and biogenesis: importance of the polymerase III transcription initiation site. *Nucleic Acids Res*. 2017; **45**(7): 4036- 4050.

39 Moore CB, Guthrie EH, Huang MT-H, Taxman DJ. Short hairpin RNA (shRNA): design, delivery, and assessment of gene knockdown. *Methods Mol Biol*. 2010; **629**: 141- 158.

40 Mokhtarzadeh A, Parhiz H, Hashemi M, Abnous K, Ramezani M. P53-derived peptides conjugation to PEI: an approach to producing versatile and highly efficient targeted gene delivery carriers into cancer cells. *Expert Opin Drug Delivery*. 2016; **13**(4): 477- 491.

41 Kim D-H, Behlke MA, Rose SD, Chang M-S, Choi S, Rossi JJ. Synthetic dsRNA dicer substrates enhance RNAi potency and efficacy. *Nature Biotechnol*. 2005; **23**(2): 222- 226.

42 Gupta S, Schoer RA, Egan JE, Hannon GJ, Mittal V. Inducible, reversible, and stable RNA interference in mammalian cells. *Proc Natl Acad Sci USA*. 2004; **101**(7): 1927- 1932.

43 McAnuff MA, Rettig GR, Rice KG. Potency of siRNA versus shRNA mediated knockdown in vivo. *J Pharm Sci*. 2007; **96**(11): 2922- 2930.

44 McCaffrey AP, Meuse L, Pham T-TT, Conklin DS, Hannon GJ, Kay MA. RNA interference in adult mice. *Nature*. 2002; **418**(6893): 38- 39.

45 Vlassov AV, Korba B, Farrar K, et al. shRNAs targeting hepatitis C: effects of sequence and structural features, and comparision with siRNA. *Oligonucleotides*. 2007; **17**(2): 223- 236.

46 Mokhtarzadeh A, Alibakhshi A, Hashemi M, et al. Biodegradable nano-polymers as delivery vehicles for therapeutic small non-coding ribonucleic acids. *J Controlled Release*. 2017; **245**: 116- 126.

47 Mokhtarzadeh A, Alibakhshi A, Yaghoobi H, Hashemi M, Hejazi M, Ramezani M. Recent advances on biocompatible and biodegradable nanoparticles as gene carriers. *Expert Opin Biol Ther*. 2016; **16**(6): 771- 785.

48 Soltani F, Parhiz H, Mokhtarzadeh A, Ramezani M. Synthetic and biological vesicular nano-carriers designed for gene delivery. *Curr Pharm Des*. 2015; **21**(42): 6214- 6235.

49 Nguyen T, Menocal EM, Harborth J, Fruehauf JH. RNAi therapeutics: an update on delivery. *Curr Opin Mol Ther*. 2008; **10**(2): 158- 167.

50 Singer O, Verma IM. Applications of lentiviral vectors for shRNA delivery and transgenesis. *Curr Gene Ther*. 2008; **8**(6): 483- 488.

51 Yin H, Kanasty RL, Eltoukhy AA, Vegas AJ, Dorkin JR, Anderson DG. Non-viral vectors for gene-based therapy. *Nat Rev Genet*. 2014; **15**(8): 541- 555.

52 Villemejane J, Mir LM. Physical methods of nucleic acid transfer: general concepts and applications. *Br J Pharmacol*. 2009; **157**(2): 207- 219.

53 Ayatollahi S, Hashemi M, Kazemi Oskuee R, et al. Synthesis of efficient gene delivery systems by grafting pegylated alkylcarboxylate chains to PAMAM dendrimers: evaluation of transfection efficiency and cytotoxicity in cancerous and mesenchymal stem cells. *J Biomater Appl*. 2015; **30**(5): 632- 648.

54 Afsharzadeh M, Hashemi M, Mokhtarzadeh A, Abnous K, Ramezani M. Recent advances in co-delivery systems based on polymeric nanoparticle for cancer treatment. *Artif Cells, Nanomed, Biotechnol*. 2018; **46**(6): 1095- 1110.

55 Khansarizadeh M, Mokhtarzadeh A, Rashedinia M, et al. Identification of possible cytotoxicity mechanism of polyethylenimine by proteomics analysis. *Hum Exp Toxicol*. 2016; **35**(4): 377- 387.

56 Vorhies JS, Nemunaitis J. Nonviral delivery vehicles for use in short hairpin RNA-based cancer therapies. *Expert Rev Anticancer Ther*. 2007; **7**(3): 373- 382.

57 Zimmermann TS, Lee ACH, Akinc A, et al. RNAi-mediated gene silencing in non-human primates. *Nature*. 2006; **441**(7089): 111- 114.

58 Zou L, Song X, Yi T, et al. Administration of PLGA nanoparticles carrying shRNA against focal adhesion kinase and CD44 results in enhanced antitumor effects against ovarian cancer. *Cancer Gene Ther*. 2013; **20**(4): 242- 250.

59 Qazi Y, Stagg B, Singh N, et al. Nanoparticle-mediated delivery of shRNA. VEGF-a plasmids regresses corneal neovascularization. *Invest Ophthalmol Vis Sci*. 2012; **53**(6): 2837- 2844.

60 Heidel JD. Linear cyclodextrin-containing polymers and their use as delivery agents. *Expert Opin Drug Delivery*. 2006; **3**(5): 641- 646.

61 Heidel JD, Liu JY-C, Yen Y, et al. Potent siRNA inhibitors of ribonucleotide reductase subunit RRM2 reduce cell proliferation in vitro and in vivo. *Clin Cancer Res*. 2007; **13**(7): 2207- 2215.

62 Davis ME, Zuckerman JE, Choi CHJ, et al. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature*. 2010; **464**(7291): 1067- 1070.

63 Chernikov IV, Vlassov VV, Chernolovskaya EL. Current development of siRNA bioconjugates: from research to the clinic. *Front Pharmacol*. 2019; **10**: 444.

64 Zukiel R, Nowak S. Suppression of human brain tumor with interference RNA specific for tenascin-C. *Cancer Biol Ther*. 2006; **5**(8): 1002- 1007.

65 Alnylam Pharmaceuticals. Trial to evaluate safety and tolerability of ALN-TTR01 in transthyretin (TTR) amyloidosis. ClinicalTrials.gov. Bethesda MUNLo. MD: US National Library of Medicine; 2012 [updated 2012 May 23]. http://clinicaltrials.gov/ct2/show

66 Tekmirapharm.com. [homepage on the Internet]. Tekmira Pharmaceuticals completes ApoB SNALP Phase 1 clinical trial. British Columbia: Tekmira Pharmaceuticals Corp.; c 2012 [updated 2010 January 7]. http://files.shareholder.com/downloads/ABEA-50QJTB

67 TheFreeLibrary.com [homepage on the Internet]. Santaris Pharma A/S Advances a Second Drug From Its Cardiometabolic Program. SPC4955, Inhibiting apoB, into Phase 1 Clinical Trials for the Treatment of High Cholesterol. Pennsylvania: Farlex, Inc.; c 2012 [updated 2011 May 11]. http://www.thefreeli-brary.com

68 Nguyen P, Bar-Sela G, Sun L, et al. BAT3 and SET1A form a complex with CTCFL/BORIS to modulate H3K4 histone dimethylation and gene expression. *Mol Cell Biol*. 2008; **28**(21): 6720- 6729.

69 Zhang Y, Fang M, Song Y, Ren J, Fang J, Wang X. Brother of regulator of imprinted sites (BORIS) suppresses apoptosis in colorectal cancer. *Sci Rep*. 2017; **7**: 40786.

70 Makovski A, Yaffe E, Shpungin S, Nir U. Intronic promoter drives the BORIS-regulated expression of FerT in colon carcinoma cells. *J Biol Chem*. 2012; **287**(9): 6100- 6112.

71 Zhang Y, Ren J, Fang M, Wang X. Investigation of fusion gene expression in HCT116 cells. *Oncol Lett*. 2017; **14**(6): 6962- 6968.

72 Murai S, Matuszkiewicz J, Okuzono Y, Miya H, De Jong R. Aurora B inhibitor TAK-901 synergizes with BCL-xL inhibition by inducing active BAX in cancer cells. *Anticancer Res*. 2017; **37**(2): 437- 444.

73 Lam LT, Zhang H, Xue J, et al. Abstract 2759: colorectal cancer cell lines with high BCL-XL and low MCL-1 expression are sensitive to a potent and selective BCL-XL inhibitor. *Cancer Res*. 2014; **74**(19 Suppl): 2759.

74 Weiswald L-B, Hasan MR, Wong JCT, et al. Inactivation of the kinase domain of CDK10 prevents tumor growth in a preclinical model of colorectal cancer, and is accompanied by downregulation of Bcl-2. *Mol Cancer Ther*. 2017; **16**(10): 2292- 2303.

75 Koehler BC, Scherr A-L, Lorenz S, et al. Beyond cell death–antiapoptotic Bcl-2 proteins regulate migration and invasion of colorectal cancer cells in vitro. *PLOS One*. 2013; **8**(10):e76446.

76 Corcoran RB, Cheng KA, Hata AN, et al. Synthetic lethal interaction of combined BCL-XL and MEK inhibition promotes tumor regressions in KRAS mutant cancer models. *Cancer Cell*. 2013; **23**(1): 121- 128.

77 Zhang H, Xue J, Hessler P, et al. Genomic analysis and selective small molecule inhibition identifies BCL-X L as a critical survival factor in a subset of colorectal cancer. *Mol Cancer*. 2015; **14**(1): 126.

78 Liu W-D, Bo Y-Z, Fan Y, et al. Effect of Bcl-xL gene expression silenced by RNA interference on invasion of human colorectal cancer cells. *J BUON*. 2014; **19**(4): 925- 929.

79 Jiang M, Milner J. Bcl-2 constitutively suppresses p53-dependent apoptosis in colorectal cancer cells. *Genes Dev*. 2003; **17**(7): 832- 837.

80 Kim HJ, Kang S, Kim DY, et al. Diallyl disulfide (DADS) boosts TRAIL-mediated apoptosis in colorectal cancer cells by inhibiting Bcl-2. *Food Chem Toxicol*. 2019; **125**: 354- 360.

81 Ray L. Synergistic anticancer activity by co-delivered nanosized dual therapeutic agents and siRNA in colon cancer. *J Drug Delivery Sci Technol*. 2020; **55**: 101351.

82 Cho S-Y, Han JY, Na D, et al. A novel combination treatment targeting BCL-XL and MCL1 for KRAS/BRAF-mutated and BCL2L1-amplified colorectal cancers. *Mol Cancer Ther*. 2017; **16**(10): 2178- 2190.

83 Faber AC, Coffee EM, Costa C, et al. mTOR inhibition specifically sensitizes colorectal cancers with KRAS or BRAF mutations to BCL-2/BCL-XL inhibition by suppressing MCL-1. *Cancer Discovery*. 2014; **4**(1): 42- 52.

84 Lin L, Ding D, Xiao X, Li B, Cao P, Li S. Trametinib potentiates TRAIL-induced apoptosis via FBW7-dependent Mcl-1 degradation in colorectal cancer cells. *J Cell Mol Med*. 2020; **24**(12): 6822- 6832.

85 Myung D-S, Park Y-L, Chung C-Y, et al. Expression of Livin in colorectal cancer and its relationship to tumor cell behavior and prognosis. *PLOS One*. 2013; **8**(9):e73262.

86 Liu S, Li X, Li Q, et al. Silencing Livin improved the sensitivity of colon cancer cells to 5-fluorouracil by regulating crosstalk between apoptosis and autophagy. *Oncol Lett*. 2018; **15**(5): 7707- 7715.

87 Wang X, Xu J, Ju S, Ni H, Zhu J, Wang H. Livin gene plays a role in drug resistance of colon cancer cells. *Clin Biochem*. 2010; **43**(7-8): 655- 660.

88 Liu B, Ge Y, Cao X, et al. Overexpression of Livin promotes migration and invasion of colorectal cancer cells by induction of epithelial–mesenchymal transition via NF-κB activation. *Onco Targets Ther*. 2016; **9**: 1011- 1021.

89 Zou A, Wang H, Zhu W, Wang F, Shen J. Effect of RNAi-mediated silencing of Livin gene on biological properties of colon cancer cell line LoVo. *Genet Mol Res*. 2014; **13**(2): 3832- 3841.

90 Oh B-Y, Lee R-A, Kim KH. siRNA targeting Livin decreases tumor in a xenograft model for colon cancer. *World J Gastroenterol*. 2011; **17**(20): 2563- 2571.

91 Cai W, Shen F, Feng Z, et al. Downregulation of CDK-8 inhibits colon cancer hepatic metastasis by regulating Wnt/β-catenin pathway. *Biomed Pharmacother*. 2015; **74**: 153- 157.

92 He S-B, Yuan Y, Wang L, Yu M-J, Zhu Y-B, Zhu X-G. Effects of cyclin-dependent kinase 8 specific siRNA on the proliferation and apoptosis of colon cancer cells. *J Exp Clin Cancer Res*. 2011; **30**(1): 109.

93 Steckel M, Molina-Arcas M, Weigelt B, et al. Determination of synthetic lethal interactions in KRAS oncogene-dependent cancer cells reveals novel therapeutic targeting strategies. *Cell Res*. 2012; **22**(8): 1227- 1245.

94 Lau E, Zhu C, Abraham RT, Jiang W. The functional role of Cdc6 in S–G2/M in mammalian cells. *EMBO Rep*. 2006; **7**(4): 425- 430.

95 Ou B, Zhao J, Guan S, et al. Plk2 promotes tumor growth and inhibits apoptosis by targeting Fbxw7/Cyclin E in colorectal cancer. *Cancer Lett*. 2016; **380**(2): 457- 466.

96 Luo J, Emanuele MJ, Li D, et al. A genome-wide RNAi screen identifies multiple synthetic lethal interactions with the Ras oncogene. *Cell*. 2009; **137**(5): 835- 848.

97 Judge AD, Robbins M, Tavakoli I, et al. Confirming the RNAi-mediated mechanism of action of siRNA-based cancer therapeutics in mice. *J Clin Invest*. 2009; **119**(3): 661- 673.

98 Baumer S, Baumer N, Appel N, et al. Antibody-mediated delivery of anti-KRAS-siRNA in vivo overcomes therapy resistance in colon cancer. *Clin Cancer Res*. 2015; **21**: 1383- 1394.

99 Kryczek I, Lin Y, Nagarsheth N, et al. IL-22+CD4+ T cells promote colorectal cancer stemness via STAT3 transcription factor activation and induction of the methyltransferase DOT1L. *Immunity*. 2014; **40**(5): 772- 784.

100 Alves S, Castro L, Fernandes MS, et al. Colorectal cancer-related mutant KRAS alleles function as positive regulators of autophagy. *Oncotarget*. 2015; **6**(31): 30787- 30802.

101 Vartanian S, Bentley C, Brauer MJ, et al. Identification of mutant K-Ras-dependent phenotypes using a panel of isogenic cell lines. *J Biol Chem*. 2013; **288**(4): 2403- 2413.

102 Pecot CV, Wu SY, Bellister S, et al. Therapeutic silencing of KRAS using systemically delivered siRNAs. *Mol Cancer Ther*. 2014; **13**(12): 2876- 2885.

103 Valentino JD, Elliott VA, Zaytseva YY, et al. Novel siRNA co-targeting strategy as treatment for colorectal cancer. *Surgery*. 2012; **152**(2): 277- 285.

104 Van Schaeybroeck S, Kalimutho M, Dunne PD, et al. ADAM17-dependent c-MET-STAT3 signaling mediates resistance to MEK inhibitors in KRAS mutant colorectal cancer. *Cell Rep*. 2014; **7**(6): 1940- 1955.

105 Thoms HC, Dunlop MG, Stark LA. p38-mediated inactivation of cyclin D1/cyclin-dependent kinase 4 stimulates nucleolar translocation of RelA and apoptosis in colorectal cancer cells. *Cancer Res*. 2007; **67**(4): 1660- 1669.

106 Aghamiri S, Jafarpour A, Malekshahi ZV, Mahmoudi Gomari M, Negahdari B. Targeting siRNA in colorectal cancer therapy: nanotechnology comes into view. *J Cell Physiol*. 2019; **234**: 14818- 14827.

107 Yang L, Zhang Y, Bao J, Feng J-F. Long non-coding RNA BCYRN1 exerts an oncogenic rrole in colorectal cancer through regulating miR-204-3p/KRAS axis. *Cancer Cell Int*. 2020; **20**: 453.

108 Azoitei N, Hoffmann CM, Ellegast JM, et al. Targeting of KRAS mutant tumors by HSP90 inhibitors involves degradation of STK33. *J Exp Med*. 2012; **209**(4): 697- 711.

109 Ueno K, Hiura M, Suehiro Y, et al. Frizzled-7 as a potential therapeutic target in colorectal cancer. *Neoplasia*. 2008; **10**(7): 697- 705.

110 Xiong H, Zhang Z-G, Tian X-Q, et al. Inhibition of JAK1, 2/STAT3 signaling induces apoptosis, cell cycle arrest, and reduces tumor cell invasion in colorectal cancer cells. *Neoplasia*. 2008; **10**(3): 287- 297.

111 Rokavec M, Öner MG, Li H, et al. IL-6R/STAT3/miR-34a feedback loop promotes EMT-mediated colorectal cancer invasion and metastasis. *J Clin Invest*. 2014; **124**(4): 1853- 1867.

112 Xiong H, Hong J, Du W, et al. Roles of STAT3 and ZEB1 proteins in E-cadherin down-regulation and human colorectal cancer epithelial-mesenchymal transition. *J Biol Chem*. 2012; **287**(8): 5819- 5832.

113 Du W, Liu N, Zhang Y, et al. PLOD2 promotes aerobic glycolysis and cell progression in colorectal cancer by upregulating HK2. *Biochem Cell Biol*. 2020; **98**(3): 386- 395.

114 Zhang ZH, Li MY, Wang Z, et al. Convallatoxin promotes apoptosis and inhibits proliferation and angiogenesis through crosstalk between JAK2/STAT3 (T705) and mTOR/STAT3 (S727) signaling pathways in colorectal cancer. *Phytomedicine*. 2020; **68**: 153172.

115 Stein U, Walther W, Arlt F, et al. MACC1, a newly identified key regulator of HGF-MET signaling, predicts colon cancer metastasis. *Nature Med*. 2009; **15**(1): 59- 67.

116 Liu Y, Steinestel K, Rouhi A, et al. STK33 participates to HSP90-supported angiogenic program in hypoxic tumors by regulating HIF-1α/VEGF signaling pathway. *Oncotarget*. 2017; **8**(44): 77474- 77488.

117 Scholl C, Fröhling S, Dunn IF, et al. Synthetic lethal interaction between oncogenic KRAS dependency and STK33 suppression in human cancer cells. *Cell*. 2009; **137**(5): 821- 834.

118 Yin MD, Ma SP, Liu F, Chen YZ. Role of serine/threonine kinase 33 methylation in colorectal cancer and its clinical significance. *Oncol Lett*. 2018; **15**(2): 2153- 2160.

119 Li KUI, Zhou Z-Y, Ji P-P, Luo H-S. Knockdown of β-catenin by siRNA influences proliferation, apoptosis and invasion of the colon cancer cell line SW480. *Oncol Lett*. 2016; **11**(6): 3896- 3900.

120 Mologni L, Dekhil H, Ceccon M, et al. Colorectal tumors are effectively eradicated by combined inhibition of β-catenin, KRAS, and the oncogenic transcription factor ITF2. *Cancer Res*. 2010; **70**(18): 7253- 7263.

121 Lee Y-S, Chin Y-T, Shih Y-J, et al. Thyroid hormone promotes β-catenin activation and cell proliferation in colorectal cancer. *Hormones Cancer*. 2018; **9**(3): 156- 165.

122 Ohsugi T, Yamaguchi K, Zhu C, et al. Anti-apoptotic effect by the suppression of IRF1 as a downstream of Wnt/β-catenin signaling in colorectal cancer cells. *Oncogene*. 2019; **38**(32): 6051- 6064.

123 Scholer-Dahirel A, Schlabach MR, Loo A, et al. Maintenance of adenomatous polyposis coli (APC)-mutant colorectal cancer is dependent on Wnt/β-catenin signaling. *Proc Natl Acad Sci USA*. 2011; **108**(41): 17135- 17140.

124 Ueno K, Hazama S, Mitomori S, et al. Down-regulation of frizzled-7 expression decreases survival, invasion and metastatic capabilities of colon cancer cells. *Br J Cancer*. 2009; **101**(8): 1374- 1381.

125 Vincan E, Darcy PK, Farrelly CA, Faux MC, Brabletz T, Ramsay RG. Frizzled-7 dictates three-dimensional organization of colorectal cancer cell carcinoids. *Oncogene*. 2006; **26**: 2340- 2352.

126 Nishioka M, Ueno K, Hazama S, et al. Possible involvement of Wnt11 in colorectal cancer progression. *Mol Carcinog*. 2013; **52**(3): 207- 217.

127 Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol*. 2007; **35**(4): 495- 516.

128 Brown JM, Attardi LD. The role of apoptosis in cancer development and treatment response. *Nat Rev Cancer*. 2005; **5**: 231- 237.

129 Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer*. 2002; **2**: 647- 656.

130 Frenzel A, Grespi F, Chmelewskij W, Villunger A. Bcl2 family proteins in carcinogenesis and the treatment of cancer. *Apoptosis*. 2009; **14**(4): 584- 596.

131 Chan SK, Griffith OL, Tai IT, Jones SJ. Meta-analysis of colorectal cancer gene expression profiling studies identifies consistently reported candidate biomarkers. *Cancer Epidemiol Biomarkers Prevent*. 2008; **17**(3): 543- 552.

132 Lam LT, Zhang H, Chyla B. Biomarkers of therapeutic response to BCL2 antagonists in cancer. *Mol Diagn Ther*. 2012; **16**(6): 347- 356.

133 Zhang J, Sud S, Mizutani K, Gyetko MR, Pienta KJ. Activation of urokinase plasminogen activator and its receptor axis is essential for macrophage infiltration in a prostate cancer mouse model. *Neoplasia*. 2011; **13**(1): 23- 30.

134 Seymour LW. Phase II studies of polymer-doxorubicin (PK1, FCE28068) in the treatment of breast, lung and colorectal cancer. *Int J Oncol*. 2009; **34**(6): 1629- 1636.

135 Qi J, Yao P, He F, Yu C, Huang C. Nanoparticles with dextran/chitosan shell and BSA/chitosan core—doxorubicin loading and delivery. *Int J Pharm*. 2010; **393**(1-2): 177- 185.

136 Yin M-J, Yamamoto Y, Gaynor RB. The anti-inflammatory agents aspirin and salicylate inhibit the activity of IκB kinase-β. *Nature*. 1998; **396**(6706): 77- 80.

137 Pathak RK, Marrache S, Choi JH, Berding TB, Dhar S. The prodrug platin-A: Simultaneous release of cisplatin and aspirin. *Angew Chem*. 2014; **126**(7): 1994- 1998.

138 Deveraux QL, Reed JC. IAP family proteins—suppressors of apoptosis. *Genes Dev*. 1999; **13**(3): 239- 252.

139 Rumble JM, Duckett CS. Diverse functions within the IAP family. *J Cell Sci*. 2008; **121**(21): 3505- 3507.

140 Yan B. Research progress on Livin protein: an inhibitor of apoptosis. *Mol Cell Biochem*. 2011; **357**(1-2): 39- 45.

141 Chen K, Huang W, Huang B, et al. BORIS, brother of the regulator of imprinted sites, is aberrantly expressed in hepatocellular carcinoma. *Genet Test Mol Biomarkers*. 2013; **17**(2): 160- 165.

142 Martin-Kleiner I. BORIS in human cancers—a review. *Eur J Cancer*. 2012; **48**(6): 929- 935.

143 Vatolin S, Abdullaev Z, Pack SD, et al. Conditional expression of the CTCF-paralogous transcriptional factor BORIS in normal cells results in demethylation and derepression of MAGE-A1 and reactivation of other cancer-testis genes. *Cancer Res*. 2005; **65**(17): 7751- 7762.

144 Renaud S, Loukinov D, Alberti L, et al. BORIS/CTCFL-mediated transcriptional regulation of the hTERT telomerase gene in testicular and ovarian tumor cells. *Nucleic Acids Res*. 2010; **39**(3): 862- 873.

145 Sesen J, Casaos J, Scotland SJ, Seva C, Eisinger-Mathason T, Skuli N. The Bad, the Good and eIF3e/INT6. *Front Biosci*. 2017; **22**: 1- 20.

146 Agarwal S. Protein tyrosine phosphatase receptor type kappa is a glioma tumor suppressor that predicts survival and response to therapy. Iowa City, IA: University of Iowa. 2013

147 Malumbres M. Cyclin-dependent kinases. *Genome Biol*. 2014; **15**(6): 122.

148 Firestein R, Hahn WC. Revving the throttle on an oncogene: CDK8 takes the driver seat. *Cancer Res*. 2009; **69**(20): 7899- 7901.

149 Cizmecioglu O, Krause A, Bahtz R, Ehret L, Malek N, Hoffmann I. Plk2 regulates centriole duplication through phosphorylation-mediated degradation of Fbxw7 (human Cdc4). *J Cell Sci*. 2012; **125**(4): 981- 992.

150 Liu X. Targeting polo-like kinases: a promising therapeutic approach for cancer treatment. *Transl Oncol*. 2015; **8**(3): 185- 195.

151 Northfelt DW, Hamburg SI, Borad MJ, et al. A phase I dose-escalation study of TKM-080301, a RNAi therapeutic directed against polo-like kinase 1 (PLK1), in patients with advanced solid tumors: expansion cohort evaluation of biopsy samples for evidence of pharmacodynamic effects of PLK1 inhibition. *J Clin Oncol*. 2013; **31**(15\_suppl): TPS2621.

152 Semple SC, Judge AD, Robbins, et al. Preclinical characterization of TKM-080301, a lipid nanoparticle formulation of a small interfering RNA directed against polo-like kinase 1. *Cancer Res*. 2011; **71**(8 Supplement): 2829.

153 Piatti S, Lengauer C, Nasmyth K. Cdc6 is an unstable protein whose de novo synthesis in G1 is important for the onset of S phase and for preventing a 'reductional' anaphase in the budding yeast *Saccharomyces cerevisiae*. *EMBO J*. 1995; **14**(15): 3788- 3799.

154 Aksamitiene E, Kiyatkin A, Kholodenko BN. Cross-talk between mitogenic Ras/MAPK and survival PI3K/Akt pathways: a fine balance. *Biochem Soc Trans*. 2012; **40**: 139- 146.

155 Fresno Vara JA, Casado E, de Castro J, Cejas P, Belda-Iniesta C, González-Barón M. PI3K/Akt signalling pathway and cancer. *Cancer Treat Rev*. 2004; **30**(2): 193- 204.

156 Lu Y, Tang J, Zhang W, Shen C, Xu L, Yang D. Correlation between STK33 and the pathology and prognosis of lung cancer. *Oncol Lett*. 2017; **14**(4): 4800- 4804.

157 Mujica AO, Brauksiepe B, Saaler-Reinhardt S, Reuss S, Schmidt ER. Differential expression pattern of the novel serine/threonine kinase, STK33, in mice and men. *FEBS J*. 2005; **272**(19): 4884- 4898.

158 Huang L, Chen C, Zhang G, et al. STK33 overexpression in hypopharyngeal squamous cell carcinoma: possible role in tumorigenesis. *BMC Cancer*. 2015; **15**: 13.

159 Yang T, Song B, Zhang J, et al. STK33 promotes hepatocellular carcinoma through binding to c-Myc. *Gut*. 2016; **65**(1): 124- 133.

160 Moon J, Lee S, Lee J, et al. Identification of novel hypermethylated genes and demethylating effect of vincristine in colorectal cancer. *J Exp Clin Cancer Res*. 2014; **33**(1): 4.

161 Wang S-W, Sun Y-M. The IL-6/JAK/STAT3 pathway: potential therapeutic strategies in treating colorectal cancer. *Int J Oncol*. 2014; **44**(4): 1032- 1040.

162 Lee H, Herrmann A, Deng J-H, et al. Persistently activated Stat3 maintains constitutive NF-κB activity in tumors. *Cancer Cell*. 2009; **15**(4): 283- 293.

163 Siemens H, Jackstadt R, Hünten S, et al. miR-34 and SNAIL form a double-negative feedback loop to regulate epithelial-mesenchymal transitions. *Cell Cycle*. 2011; **10**(24): 4256- 4271.

164 Krasnov GS, Dmitriev AA, Lakunina VA, Kirpiy AA, Kudryavtseva AV. Targeting VDAC-bound hexokinase II: a promising approach for concomitant anti-cancer therapy. *Expert Opin Ther Targets*. 2013; **17**(10): 1221- 1233.

165 Liu S, Chen Q, Wang Y. MiR-125b-5p suppresses the bladder cancer progression via targeting HK2 and suppressing PI3K/AKT pathway. *Hum Cell*. 2020; **33**(1): 185- 194.

166 Britschgi A, Radimerski T, Bentires-Alj M. Targeting PI3K, HER2 and the IL-8/JAK2 axis in metastatic breast cancer: which combination makes the whole greater than the sum of its parts? *Drug Resist Updates*. 2013; **16**(3-5): 68- 72.

167 Zhan T, Rindtorff N, Boutros M. Wnt signaling in cancer. *Oncogene*. 2016; **36**: 1461- 1473.

168 Polakis P. Wnt signaling in cancer. *Cold Spring Harbor Perspect Biol*. 2012; **4**(5): a008052.

169 Valenta T, Hausmann G, Basler K. The many faces and functions of β-catenin. *EMBO J*. 2012; **31**(12): 2714- 2736.

170 Cao T-T, Xiang D, Liu B-L, et al. FZD7 is a novel prognostic marker and promotes tumor metastasis via WNT and EMT signaling pathways in esophageal squamous cell carcinoma. *Oncotarget*. 2017; **8**(39): 65957- 65968.

171 Sousa AR, Oliveira AV, Oliveira MJ, Sarmento B. Nanotechnology-based siRNA delivery strategies for metastatic colorectal cancer therapy. *Int J Pharm*. 2019; **568**: 118530.

172 Zhou J, Patel TR, Fu M, Bertram JP, Saltzman WM. Octa-functional PLGA nanoparticles for targeted and efficient siRNA delivery to tumors. *Biomaterials*. 2012; **33**(2): 583- 591.

173 Martins C, Sousa F, Araújo F, Sarmento B. Functionalizing PLGA and PLGA derivatives for drug delivery and tissue regeneration applications. *Adv Healthc Mater*. 2018; **7**(1): 1701035.

174 Hafezi Ghahestani Z, Alebooye Langroodi F, Mokhtarzadeh A, Ramezani M, Hashemi M. Evaluation of anti-cancer activity of PLGA nanoparticles containing crocetin. *Artif Cells Nanomed Biotechnol*. 2017; **45**(5): 955- 960.

175 Woodrow KA, Cu Y, Booth CJ, Saucier-Sawyer JK, Wood MJ, Saltzman WM. Intravaginal gene silencing using biodegradable polymer nanoparticles densely loaded with small-interfering RNA. *Nat Mater*. 2009; **8**(6): 526- 533.

176 Triozzi P, Kooshki M, Alistar A, et al. Phase I clinical trial of adoptive cellular immunotherapy with APN401 in patients with solid tumors. *J Immunother Cancer*. 2015; **3**(S2): P175.

177 Triozzi P. APN401 in treating patients with recurrent or metastatic pancreatic cancer, colorectal cancer, or other solid tumors that cannot be removed by surgery. NCT03087591 NIH: USA. 2017.

178 Northfelt DW, Hamburg SI, Borad MJ, et al. A phase I dose-escalation study of TKM-080301, a RNAi therapeutic directed against polo-like kinase 1 (PLK1), in patients with advanced solid tumors: expansion cohort evaluation of biopsy samples for evidence of pharmacodynamic effects of PLK1 inhibition. *J Clin Oncol*. 2013; **31**(15\_suppl.

179 Wan C, Allen T, Cullis P. Lipid nanoparticle delivery systems for siRNA-based therapeutics. *Drug Delivery Transl Res*. 2014; **4**(1): 74- 83.

180 Schultheis B, Strumberg D, Santel A, et al. First-in-human phase I study of the liposomal RNA interference therapeutic Atu027 in patients with advanced solid tumors. *J Clin Oncol*. 2014; **32**(36): 4141- 4148.

181 Zuckerman JE, Gritli I, Tolcher A, et al. Correlating animal and human phase Ia/Ib clinical data with CALAA-01, a targeted, polymer-based nanoparticle containing siRNA. *Proc Natl Acad Sci USA*. 2014; **111**(31): 11449- 11454.