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Malignancies of ISCs and their niche emerge as frontiers in precision therapy for colorectal cancer

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Highlights:

- ISCs and their niche drive colorectal cancer initiation and progression.
- CSC plasticity and dedifferentiation fuel tumor heterogeneity and relapse.
- Targeting the ISC-niche ecosystem enables precision colorectal cancer therapy.

Abstract: Colorectal cancer (CRC) ranks among the leading malignancies globally in both incidence and mortality. Treatment failure and disease recurrence are largely attributable to significant heterogeneity and acquired resistance to current therapies. Recent studies have extensively demonstrated that the initiation, progression, and recurrence of CRC are closely associated with the malignancy of intestinal stem cells (ISCs) and their derived cancer stem cells (CSCs). CSCs possess the characteristics of sustained self-renewal and multi-potent differentiation, constituting a key cellular population that sustains tumor growth, metastasis and drug resistance. Concurrently, CSCs evade the host immune system by reducing tumor antigen presentation, secreting immunosuppressive factors, and remodeling tumor microenvironment, thereby significantly limiting the clinical efficacy of immunotherapy. Consequently, immunotherapeutic strategies targeting ISCs and CSCs have emerged as a pivotal research direction for precision treatment of CRC. This paper systematically reviews recent advances in this field, discussing vaccine strategies based on CSC-specific antigens, bispecific antibodies (BsAbs) and antibody-drug conjugates (ADCs), CAR-T cells, and multimodal therapeutic approaches. Further, this paper summarizes the application of multi-omics technologies, spatial biology, and organoid models in elucidating the plasticity and drug resistance mechanisms of CSCs. We also discuss the potential role of gut microbial regulation in enhancing immunotherapy response. In summary, comprehensive immunotherapy strategies targeting ISCs and their ecological niches hold promise for overcoming current treatment bottlenecks in CRC, providing new theoretical foundations and practical pathways towards achieving long-term disease control.

Keywords: colorectal cancer; ISCs; CSCs; immunotherapy; niche; precision medicine

1. Introduction

1.1. Transformation of ISCs to CSCs during colorectal tumorigenesis

Colorectal cancer (CRC) ranks among the most prevalent and lethal gastrointestinal malignancies globally, with a substantial proportion of patients ultimately succumbing to tumor recurrence, metastasis, and chemotherapy resistance. Annually, approximately 1.85 million new cases are diagnosed, resulting in 850,000 deaths [1]. The initiation and, at least in part, the progression of CRC widely thought to be intrinsically linked to abnormalities in intestinal stem cells (ISCs). Under normal conditions, ISCs reside at the base of intestinal crypts, possessing the capacity for self-renewal and differentiation into epithelial cells [2]. Accumulating experimental evidence indicates that, when these stem cells accumulate oncogenic mutations (such as adenomatous polyposis coli (APC) gene inactivation leading to uncontrolled Wingless-related integration site (Wnt) signaling), they may initiate tumorigenesis in defined experimental contexts. Consistent with this notion, genetically engineered mouse models have demonstrated that specific induction of APC deficiency in $Lgr5^+$ ISCs efficiently results in intestinal adenoma formation [3]. Consequently, CRC is widely considered, based largely on lineage-tracing and mouse genetic studies, to frequently originate from ISC, with tumor microenvironments harboring distinct cancer stem cell (CSC) subpopulations that exhibit phenotypic and functional similarities to normal ISCs [4]. CSCs display sustained self-renewal capacity and multipotent differentiation potential, and are commonly regarded as a key cellular population that contributes to, rather than exclusively determines, tumor initiation, maintenance, metastasis, and recurrence [5]. In recent years, substantial evidence has supported the CSC hypothesis, suggesting that most tumors, including CRC, harbor a hierarchical structure with varying degrees of differentiation. Within this framework, a minority of CSCs occupy central positions within the tumor niche and have been proposed to act as primary drivers of tumor growth and metastatic dissemination in multiple experimental tumor models [6].

However, current CRC treatment modalities remain limited in their capacity to effectively address CSC-associated tumor heterogeneity and therapeutic resistance. Clinically, standard therapies for CRC encompass radical surgical resection combined with radiotherapy and chemotherapy, molecularly targeted drugs, and immune checkpoint inhibitors. Whilst these approaches have improved outcomes for patients with early-stage CRC, therapeutic efficacy often remains suboptimal in advanced or metastatic disease, with persistently high rates of recurrence and metastasis [7]. This limitation is thought to arise, at least in part, from the pronounced heterogeneity of CRC tumors, in which distinct cellular subpopulations display variable sensitivities to treatment, thereby limiting the ability of conventional therapies to eradicate all tumor cells comprehensively [8]. CSCs are widely regarded as a major contributor to, rather than the sole determinant of, tumor heterogeneity and therapeutic resistance [9]. In multiple experimental settings, CSCs have been reported to preferentially adopt relatively quiescent cellular states and to express elevated levels of drug resistance–associated molecules, features that may confer intrinsic resistance to radiotherapy and chemotherapy. As a result, these cells can survive therapeutic pressure and are proposed to contribute to tumor recurrence and regrowth.

Although cancer stem cells (CSCs) have been described across multiple malignancies, colorectal cancer represents a biologically distinctive context in which CSC biology can be directly linked to a well-defined physiological stem cell system. Unlike many solid tumors in which CSCs are inferred primarily through functional assays, CRC-CSCs arise within an epithelium sustained by a hierarchically

organized and rapidly cycling intestinal stem cell (ISC) compartment, characterized by Lgr5$^+$ active stem cells and injury-responsive reserve stem cells. The exceptionally high turnover rate of the intestinal epithelium, together with the spatially ordered crypt–niche architecture governed by Wnt, Notch, bone morphogenetic protein (BMP), and Hippo gradients, provides a unique framework in which stem cell plasticity is not an aberration but an intrinsic biological property.

1.2. ISC subpopulations and tumor-associated stem-like states in CRC

In intestinal biology, the term "intestinal stem cells" refers to multiple functionally distinct populations. Under physiological conditions, actively cycling Lgr5$^+$ ISCs at the crypt base sustain rapid epithelial turnover and homeostasis. In parallel, quiescent or reserve stem cells can be activated upon tissue injury to support epithelial regeneration and ensure functional resilience.

In contrast, tumor-associated stem-like cells in colorectal cancer are not equivalent to normal ISCs or reserve stem cells. Accumulating evidence indicates that colorectal cancer stem cells (CRCSCs) represent aberrant stem-like states arising through oncogenic transformation and cellular plasticity. These CSC-like ISCs retain partial stem cell programs but display dysregulated self-renewal, impaired differentiation, and enhanced resistance to therapy and environmental stress. Lineage-tracing and functional studies further suggest that such CSC-like populations can originate both from transformed Lgr5$^+$ ISCs and from differentiated tumor cells that reacquire stemness under therapeutic or microenvironmental pressure.

This distinction is particularly important in CRC, where the intestinal epithelium exhibits rapid turnover and a well-defined crypt–niche architecture. Unlike many other solid tumors, CRC offers a unique framework in which the continuum from normal ISCs to tumor-initiating and CSC-like states can be directly interrogated *in vivo*. Understanding how physiological stem cell programs are co-opted or reactivated during colorectal tumorigenesis is therefore central to the development of precision therapies that selectively target malignant stem-like populations while preserving normal tissue regeneration.

1.3. CSC plasticity and metastatic organotropism in CRC

Beyond tumor initiation and therapy resistance, accumulating evidence indicates that CSC populations also play a central role in metastatic dissemination and organ-specific colonization in CRC. Metastasis is a multistep process requiring invasion, survival in circulation, and successful colonization, all of which rely on the high cellular plasticity characteristic of CSC-enriched tumor fractions. CRC exhibits a characteristic metastatic pattern, with the liver and lung as the most frequent target organs. This organotropism is not solely dictated by anatomical blood flow but is increasingly attributed to selective interactions between CSC-like tumor cells and permissive metastatic niches. CSC-enriched populations, particularly those displaying Lgr5$^+$ or regenerative stem-like states, have been shown to possess enhanced metastatic seeding and outgrowth capacity compared with more differentiated tumor cells. The liver microenvironment provides a supportive niche for disseminated CRC-CSCs through paracrine signals derived from hepatic stellate cells, Kupffer cells, and sinusoidal endothelial cells, including Wnt ligands, hepatocyte growth factor (HGF), and inflammatory cytokines such as Interleukin (IL)-6. Single-cell and lineage-tracing studies further suggest that CSC states in liver metastases differ from those in primary tumors, exhibiting increased stress tolerance and immune-evasive features. CSC-mediated metastasis in CRC is highly dynamic and involves reversible epithelial–mesenchymal transition (EMT)

and mesenchymal–epithelial transition (MET), enabling invasion, dissemination, and re-establishment of proliferative growth at distant sites. Together, these findings support a model in which CSC plasticity and niche compatibility jointly determine organ-specific metastatic behavior in CRC, underscoring the necessity of targeting metastatic CSC populations and their niches to achieve durable therapeutic control.

1.4. Heterogeneity and drug resistance as limitations of CRC therapy

Despite significant advances over recent decades in surgical resection techniques, adjuvant chemotherapy (such as the combination of fluorouracil and oxaliplatin) [10], radiotherapy, and molecularly targeted therapies (including anti–epidermal growth factor receptor (anti-EGFR) and anti-Vascular endothelial growth factor (VEGF) antibodies) [11,12] and immune checkpoint inhibitors (ICIs) [13] have significantly improved overall survival rates for CRC patients. However, outcomes for advanced or metastatic CRC often remain suboptimal, with persistently high rates of tumor recurrence and metastasis [7]. This therapeutic limitation is widely attributed to, rather than exclusively determined by, two major biological challenges: pronounced tumor heterogeneity and complex mechanisms of treatment resistance. CRC tumors display substantial internal heterogeneity, whereby distinct cellular subpopulations respond differentially to therapeutic interventions, thereby limiting the capacity of conventional treatments to eradicate all tumor cells comprehensively [8]. Within this context, CSCs are widely regarded as a critical contributor to, but not the sole driver of, tumor heterogeneity and therapeutic resistance [9]. In multiple experimental and preclinical settings, CSCs have been reported to preferentially adopt relatively quiescent states and to overexpress resistance-associated molecules (such as drug efflux transporters), features that may confer reduced sensitivity to radiotherapy and chemotherapy. As a result, CSC-enriched populations can persist under therapeutic pressure and are proposed to contribute to tumor recurrence and disease progression [14].

1.4.1. Tumor heterogeneity, a key factor in precision medicine

Tumor heterogeneity refers to the emergence of molecular, genetic, and phenotypic diversity among tumor cells during successive rounds of proliferation, resulting in variability in tumor growth dynamics, invasive potential, drug sensitivity, and clinical prognosis [15]. This heterogeneity exists not only between different patients (inter-patient heterogeneity) but more significantly within tumors from the same patient (intratumoral heterogeneity (ITH)) and between primary and metastatic sites [16]. Such variability is widely considered to pose a major challenge to therapeutic strategies, undermining treatment efficacy primarily through two dimensions: molecular subtyping differences between patients [17] and clonal diversity within individual patients [18].

First, at the population level, substantial variation in tumor molecular characteristics across patients limits the efficacy of universal therapies. Whilst transcriptomic-based ‘consensus molecular subtypes’ (CMS) categorise CRC into four groups (CMS1–4), aiming to provide a theoretical framework for clinical stratification. However, clinical and translational studies have shown that even patients assigned to the same CMS category can display markedly different responses to standard treatments. For example, although CMS2 tumors—characterized by Wnt/MYC pathway activation—are generally considered responsive to EGFR-targeted therapies, a subset of CMS2 patients develops primary or acquired resistance, potentially due to low-frequency subclonal alterations (such as minor KRAS proto-oncogene (KRAS) or B-Raf proto-oncogene, serine/threonine kinase (BRAF) mutations) that may

escape detection by routine diagnostic assays. These observations indicate that CMS classification alone is often insufficient to accurately predict therapeutic responses across all patients.

Second, ITH is increasingly recognized as a critical factor contributing to treatment failure and disease recurrence. Single-cell sequencing studies reveal that CRC tumors are not homogeneous cellular masses but complex communities composed of multiple subclones that are genetically and functionally distinct [19]. Under the selective pressure of chemotherapy or targeted therapies, tumor evolution can resemble Darwinian selection processes [20]: initially disadvantaged resistant subclones (such as cells harboring minimal KRAS or MET amplification) [21,22] may gain a growth advantage, gradually supplanting sensitive clones to become dominant, ultimately leading to disease recurrence. The presence of ITH further induces spatial heterogeneity within tumors, posing significant challenges for clinical diagnosis. Given that drug-resistant clones may exhibit patchy distribution within the tumor, examining only a small tissue sample fails to reflect the tumor's full landscape. As a result, treatment decisions informed by single-site biopsies may be suboptimal, highlighting a major practical limitation that currently constrains the efficacy of precision medicine approaches.

1.4.2. Drug resistance, from genetic mutations to cellular plasticity

Whilst mutations in key genes such as KRAS, NRAS proto-oncogene, GTPase (NRAS), or BRAF do indeed confer intrinsic resistance to EGFR therapies, mounting preclinical and clinical evidence suggests that non-genetic mechanisms—namely cellular plasticity—play a more subtle yet pivotal role in acquired resistance [23]. Traditional views posited that tumor tissues adhere to a strict hierarchical structure: CSCs at the apex govern tumor growth and maintenance, while differentiated daughter cells are destined for death and lack oncogenic potential. However, recent advances in single-cell lineage tracing and functional studies have challenged the rigidity of this classical view. Under strong selective pressure imposed by chemotherapy or targeted agents, differentiated cancer cells have been reported, in specific experimental and preclinical models, to undergo phenotypic reprogramming or dedifferentiation, thereby reacquiring stem cell-like features, including self-renewal capacity. This process may partially replenish stem-like cell pools depleted by therapy. Importantly, these newly generated "stem-like" cells often display distinct metabolic programs and altered cell-cycle dynamics compared with their parental populations, features that may reduce their sensitivity to conventional therapies primarily targeting rapidly proliferating cells. As a result, therapeutic strategies exclusively targeting proliferating tumor cells may be insufficient in certain contexts, as the tumor cell pool can be replenished through the dedifferentiation of non–stem-like cells.

A critical intermediate state exists before genetically resistant clones fully dominate the tumor: drug-tolerant persistent cells (DTPs) [24]. This concept originated in microbiology, describing a minute fraction of genetically identical bacterial populations that evade antibiotic killing by entering a dormant state. In oncology, DTPs are recognized as a major component of minimal residual disease (MRD) and an important contributor to tumor recurrence. These cells evade chemotherapy-induced cell death by hijacking embryonic developmental "dormancy" mechanisms, actively suppressing mechanistic target of rapamycin (mTOR) signaling, reducing metabolic activity, and entering a state of slow proliferation.

Notably, this plasticity driven by epigenetic reprogramming is not an endpoint but a bridge to permanent resistance. Cells in the DTP state exhibit "adaptive mutation" characteristics, temporarily downregulating DNA repair capacity under stress, thereby increasing the probability of acquiring permanent genetic resistance mutations (such as KRAS amplification) [25]. These observations highlight

the limitations of therapeutic strategies focused solely on static genotypic alterations and underscore the need to consider dynamic cell-state transitions in the design of effective treatment regimens.

1.5. A new paradigm targeting ISC transformation and their niche

Given the accumulating evidence implicating CSCs in treatment resistance and disease recurrence in CRC, an emerging therapeutic framework has been proposed that centers on targeting ISCs and their niche. Rather than focusing solely on reducing tumor burden, this approach aims to disrupt the cellular populations and regulatory programs that sustain persistent tumor growth and evolutionary adaptation, thereby providing a conceptual framework for the development of more effective CRC therapies.

The conceptual basis of this framework encompasses multiple therapeutic dimensions. First, targeting CSC-associated maintenance pathways has been proposed as a potential intervention point. CSC stemness is supported by key signaling networks, including Wnt and Notch, and modulation of these pathways may impair CSC self-renewal capacity, promote differentiation or cell death, and thereby limit tumor regenerative potential. Second, disrupting supportive signals within the stem cell niche is increasingly recognized as a complementary strategy for constraining CSC survival. Components of the tumor microenvironment, including fibroblasts, immune cells, and the extracellular matrix, secrete paracrine factors such as HGF and Wnt ligands, which provide survival and adaptive cues for CSCs. Interfering with these paracrine support axes may reduce CSC tolerance to hostile microenvironmental conditions and therapeutic pressure.

Building on these concepts, synthetic lethality strategies have attracted increasing interest as a means of preferentially targeting CSCs in defined molecular contexts. By identifying metabolic dependencies or DNA repair vulnerabilities enriched in CSC populations harboring specific driver alterations (such as APC loss), it may be possible to achieve selective targeting while minimizing damage to normal ISCs. In parallel, remodeling the immune microenvironment has been proposed as an important component of this therapeutic framework. Strategies aimed at alleviating CSC-associated immunosuppression, enhancing effector immune cell infiltration, and improving antigen presentation may facilitate the conversion of immunologically “cold” tumors into more immunoreactive states, thereby potentially enhancing immune-mediated recognition and clearance of CSCs.

Collectively, these integrated strategies targeting ISCs and their niche reflect an evolving shift in perspective within CRC precision medicine—from a tumor-burden–centric approach toward greater consideration of stem cell–driven tumor maintenance and adaptation. While this framework offers valuable conceptual guidance and emerging therapeutic opportunities, its clinical translation remains constrained by tumor heterogeneity, cellular plasticity, and safety considerations, underscoring the need for continued experimental and clinical validation.

2. Molecular mechanisms of ISC transformation

2.1. Driver mutations hijack stem cell regulatory networks

2.1.1. APC mutation

The APC gene is among the most frequently inactivated tumor suppressor genes in gastrointestinal malignancies, particularly CRC, and its functional loss is widely regarded as an early and critical event

in tumor initiation [26]. Under physiological conditions, APC plays a central role in maintaining the balance between ISC proliferation and differentiation through regulation of the Wnt signaling pathway. Loss-of-function mutations in APC disrupt this homeostatic control and are thought to reprogram stem cell regulatory networks through coordinated alterations in signaling activity, epigenetic state, and microenvironmental interactions, thereby promoting malignant transformation and tumorigenesis [27].

Based on recent experimental evidence, this section summarizes how APC mutations reshape ISC regulatory networks across three interconnected dimensions: molecular signaling mechanisms, stem cell behavior, and niche-level regulation.

(a) Disrupted Balance between ISC Proliferation and Differentiation

The core function of the APC protein is to act as a "molecular brake" in the Wnt signaling pathway. By forming complexes with β-catenin, Glycogen synthase kinase 3 beta (GSK3β), and other molecules, it promotes the phosphorylation and degradation of β-catenin, preventing its accumulation within the cell nucleus [28]. Once APC inactivating mutations (such as truncating or frameshift mutations), this regulatory mechanism fails, triggering persistent overactivation of the Wnt signaling pathway. Through this mechanism, APC loss profoundly alters stem cell regulatory dynamics, contributing to dysregulated ISC proliferation and impaired differentiation programs [29].

At the base of intestinal crypts, $Lgr5^+$ ISCs constitute the principal population sustaining epithelial renewal, a process critically dependent on tightly regulated Wnt signaling [30]. APC inactivation promotes nuclear accumulation of β-catenin in $Lgr5^+$ ISCs, where it associates with T-cell factor (TCF)/lymphoid enhancer-binding factor (LEF) transcription factors and induces aberrant activation of downstream target genes [31]. This transcriptional program includes proliferation-associated genes such as c-MYC and Cyclin D1, facilitating escape from normal cell-cycle constraints and promoting sustained proliferative capacity.

In genetically engineered mouse models and organoid-based systems, APC loss in $Lgr5^+$ ISCs has been reported to result in an approximately 2–3-fold increase in proliferative indices relative to wild-type controls, as assessed by proliferation and cell-cycle–associated readouts (including EdU incorporation, Ki67 labeling, and flow-cytometric cell-cycle profiling) at defined time points following APC inactivation [31]. This enhanced proliferative activity is frequently accompanied by G1-phase shortening and an increased proportion of cells in S phase. Importantly, the magnitude and kinetics of these effects appear to be model- and context-dependent, underscoring the need for cautious interpretation.

(b) The clonal "Supercompetition" mechanism

The intestinal stem cell niche is constrained by limited spatial and trophic resources. Within this context, APC-mutant cells may acquire a competitive advantage not only through enhanced proliferative capacity but also by modulating the local niche environment in ways that disadvantage neighboring wild-type stem cells. This phenomenon has been described as a form of clonal "supercompetition," reflecting a relative fitness advantage rather than an absolute exclusion mechanism [32].

One mechanism underlying this competitive behavior involves the secretion of Wnt pathway antagonists by APC-mutant cells. Notably, expression of the Notum gene is significantly upregulated following APC loss. The secreted Notum protein inhibits Wnt ligand–Frizzled receptor interactions by enzymatically removing the palmitoleate modification of Wnt ligands, thereby attenuating Wnt signaling in neighboring wild-type ISCs [33]. Because Wnt pathway activation in APC-mutant cells occurs downstream at the level of β-catenin stabilization, Notum-mediated inhibition exerts minimal effects on mutant cells themselves, creating a context-dependent selective disadvantage for surrounding wild-type

stem cells. In addition to Notum, APC-mutant cells have been reported to secrete other Wnt antagonists, including Dkk1, Sfrp5, and Wif1, forming a redundant inhibitory network that further constrains Wnt responsiveness in non-mutant ISCs [33].

Beyond paracrine signaling effects, APC inactivation also influences the epigenetic landscape of ISCs. Experimental studies indicate that APC loss is associated with early and dynamic changes in DNA methylation profiles within $Lgr5^+$ stem cells, including aberrant methylation of genes involved in differentiation and lineage commitment [33]. These epigenetic alterations may further destabilize normal differentiation trajectories and reinforce stem-like transcriptional programs, thereby cooperating with Wnt signaling dysregulation to promote ISC transformation.

(c) Niche remodeling

Stem cell function is critically dependent on tightly regulated signaling interactions within the niche. In addition to intrinsic molecular alterations, APC-mutant cells have been shown in experimental models to modulate niche components, creating a microenvironment that preferentially supports mutant stem cell maintenance while constraining the function of neighboring wild-type stem cells. Through these niche-level alterations, APC mutations may further reinforce aberrant stem cell regulatory programs and clonal expansion [34].

Alterations in crypt architecture and cellular composition have been proposed to contribute to niche expansion for mutant stem cells. Under physiological conditions, intestinal crypts display an inverted cone–like organization, with $Lgr5^+$ stem cells and Paneth cells localized at the crypt base and differentiated cells occupying the upper regions. In contrast, APC inactivation is associated with aberrant crypt proliferation and structural remodeling, characterized by crypt enlargement and increased crypt density.

In murine models of APC deficiency, histological analyses have reported an approximately 1.8-fold increase in crypt number or density relative to wild-type controls, based on quantitative metrics defined within individual studies [35]. Notably, the extent of crypt expansion and its temporal dynamics appear to vary depending on experimental context, underscoring the importance of model-specific interpretation.

2.1.2. KRAS mutation

KRAS primarily hijacks stem cell regulatory networks through multiple mechanisms, including sustained activation of downstream pathways, reshaping cellular identity, maintaining tumor stem cell characteristics, and manipulating the microenvironment. Collectively, these alterations have been implicated in tumor initiation, progression, and therapeutic resistance, with supporting evidence derived from diverse experimental systems.

(a) Disrupted ISC Proliferation and Differentiation

Under normal physiological conditions, KRAS cycles between an active guanosine triphosphate (GTP)-bound state and an inactive guanosine diphosphate (GDP)-bound state, thereby participating in the regulation of proliferation and differentiation in intestinal epithelial cells, including stem cell populations. Common oncogenic KRAS mutations, such as G12D or G12C, impair this regulatory cycling and result in sustained activation of downstream signaling pathways, most notably the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase–protein kinase B (PI3K–AKT) cascades.

In experimental models, persistent activation of the rapidly accelerated fibrosarcoma–mitogen-activated extracellular signal-regulated kinase–extracellular signal-regulated kinase (RAF–MEK–ERK) axis has been associated with disruption of the balance between stem cell proliferation and differentiation,

favoring enhanced proliferative capacity. In parallel, activation of PI3K–AKT signaling has been reported to attenuate apoptosis and promote cell survival. Together, these pathway alterations may facilitate the accumulation of aberrant stem-like cell populations, thereby creating conditions permissive for tumor initiation and early progression [36].

(b) Differentiation of ISCs toward a Regenerative Phenotype

In mouse intestinal tissues harboring KRAS-G12D or KRAS-G12C mutations, sustained activation of the MAPK signaling pathway significantly elevates expression of regenerative stem cell markers Anxa1 and Ly6a while reducing expression of the classical stem cell marker Lgr5. This pushes cells toward a more plastic and adaptive regenerative stem cell state.

Notably, even in the context of APC deficiency—which constitutively activates Wnt signaling and typically supports a classical ISC identity—oncogenic KRAS signaling appears to bias cellular states toward a regenerative phenotype during early tumor formation. Conversely, pharmacological inhibition of MAPK signaling has been shown, in certain experimental settings, to promote a reversion toward Wnt-dependent classical stem cell programs. These observations highlight the dynamic interplay between KRAS-driven MAPK signaling and canonical stem cell regulatory pathways, underscoring the plasticity of stem-like states in KRAS-mutant tumors [37].

(c) CSC Reprogramming

KRAS mutations maintain cancer stem cell properties through multiple mechanisms, firmly controlling stem cell regulatory networks to drive tumor progression. On one hand, KRAS mutations activate cyclin-dependent kinase 1 (CDK1), promote N-acetylglucosamine glycosylation modifications, and upregulate the cancer stem cell marker SRY-related transcription factor SOX2 (SOX2), thereby enhancing tumor stem cells' self-renewal capacity and resistance to treatment. On the other hand, studies in lung cancer reveal that KRAS (G12D) can slowly and asynchronously reprogram differentiated quiescent AT1 cells into AT2 stem cells. These reprogrammed stem cells persistently generate inert tumors. In this process, KRAS disrupts normal stem cell differentiation regulation, hijacking the stem cell population through reverse reprogramming to drive tumorigenesis [37]. While these findings highlight the capacity of oncogenic KRAS to drive cellular reprogramming, their direct applicability to CRC requires careful validation, given tissue-specific differences in stem cell hierarchies and differentiation programs.

(d) Niche regulation

Beyond cell-intrinsic effects, KRAS mutations can also influence stem cell regulatory networks indirectly through modulation of the tumor microenvironment. For instance, it activates the transcription factor transcription factor CP2 (TFCP2), inducing cancer-associated fibroblasts to differentiate into lipid-enriched cells. These cells provide nutritional support and a favorable microenvironment for stem cell-like tumor cells, promoting their proliferation.

In parallel, KRAS-mutant tumor cells have been reported to secrete cytokines, including Transforming growth factor beta (TGF-β) and IL-6, that facilitate the recruitment of regulatory T cells and myeloid-derived suppressor cells, contributing to an immunosuppressive microenvironment. Through these stromal and immune-modulatory effects, KRAS signaling may create conditions that favor the persistence and expansion of stem-like tumor cells, thereby indirectly reinforcing dysregulated stem cell regulatory programs [38].

2.1.3. TP53 mutation

The tumor suppressor p53 plays a central role in regulating stem cell proliferation, differentiation, and genomic stability. Mutations in tumor protein p53 (TP53) are among the most frequent genetic alterations across human cancers and have been shown to affect stem cell regulatory networks through multiple mechanisms, including perturbation of differentiation programs, dysregulation of cell division patterns, and modulation of the tissue microenvironment. Through loss of tumor-suppressive functions and, in some contexts, gain-of-function activities, p53 mutations have been implicated in promoting aberrant stem cell behavior and facilitating tumor initiation and progression, as supported by extensive experimental evidence.

(a) Altering stem cell differentiation pathways

Under physiological conditions, p53 promotes appropriate stem cell differentiation by regulating the expression of downstream target genes and microRNAs. For example, in human embryonic stem cells, p53 activation induces microRNAs such as miR-34a and miR-145, which suppress core pluripotency factors including octamer-binding transcription factor 4 (OCT4) and SOX2, thereby limiting excessive self-renewal and uncontrolled proliferation.

Loss or mutation of p53 has been shown to interfere with these differentiation programs. In studies of Barrett's esophagus–associated neoplasia, single-cell RNA sequencing analyses revealed that TP53-mutant $Cck2r^{+}$ stem-like cells in the gastroesophageal junction, particularly under chronic inflammatory conditions, display impaired differentiation trajectories and instead preferentially progress toward dysplastic states [39]. These cells exhibit activation of stemness-associated signaling pathways, including Notch and PI3K–AKT, and demonstrate enhanced proliferative capacity and tolerance to DNA damage. Consistently, organoids derived from such mutant stem-like populations display increased growth potential and accelerated dysplastic transformation following transplantation. In addition, p53 deficiency has been reported to influence differentiation programs in mesenchymal stem cell populations in other experimental systems [40].

(b) Disrupting stem cell division patterns

Normal p53 exerts strict control over stem cell division patterns. For instance, in Drosophila neural stem cells, p53 activates core regulatory factors such as Numb and Brat to maintain asymmetric division. This ensures one daughter cell retains stemness while the other differentiates into a mature functional cell, preventing disorderly accumulation of stem cells. Mutations in p53 disrupt this balance of asymmetric division. For example, studies in mammary stem cell models indicate that intact p53 signaling suppresses symmetric self-renewing divisions, whereas p53 loss shifts division patterns toward symmetric expansion of stem-like cells. Such alterations in division behavior may lead to an increased abundance of aberrant stem-like populations, thereby creating conditions that are permissive for tumor initiation and progression [41].

(c) Regulating niche-related pathways

p53 can also indirectly regulate stem cell control by modulating the microenvironment. Its mutations disrupt this regulation, creating a favorable environment for abnormal stem cells. For instance, in intestinal tissue, normal p53 is temporarily activated after radiation damage, prompting damaged epithelial cells to dedifferentiate into regenerative stem cells (revSCs) and other cell types to aid intestinal repair. This process is regulated by the p53-Mdm2 negative feedback loop to prevent excessive regeneration. However,

p53 dysfunction disrupts this repair balance. Conversely, when p53 mutations cause persistent activation or abnormal loss, it either excessively induces regenerative stem cell formation or fails to initiate regeneration, allowing abnormal stem cells to occupy intestinal crypt niches. Furthermore, in a prostate cancer bone metastasis model, p53 deficiency elevates osteoprotegerin (OPG) secretion by mesenchymal stem cells, inhibiting osteoclast differentiation and increasing bone density. These findings illustrate how p53 loss can influence stem cell–supportive microenvironments in a context-dependent manner.

2.2. Dysregulation of stemness pathways in ISCs

ISCs play a central role in maintaining epithelial homeostasis and regenerative capacity in the intestine, and their dysregulation has been closely associated with CRC development. Under physiological conditions, signaling pathways such as Wnt, Notch, and Hippo coordinately regulate the balance between ISC self-renewal and differentiation. In the context of CRC, these pathways are frequently dysregulated and reorganized, giving rise to aberrant signaling networks that are associated with altered stem cell behavior and expansion of CSC populations.

Based on current literature, this section reviews the activation patterns, context-dependent remodeling, and functional interactions of these core stemness pathways in ISCs under CRC conditions, with an emphasis on their contributions to tumor initiation and progression as well as their potential therapeutic relevance.

For clarity, the major cellular components of the ISC niche, their associated signaling pathways, and functional impacts on ISCs/CSCs in colorectal cancer are summarized in table (Table 1).

Table 1. Key components of the ISC niche and associated signaling pathways in colorectal cancer.

ISC niche component	Major cell types	Key signals/ligands	Dominant pathways	Functional impact on ISCs/CSCs	Therapeutic relevance
Epithelial niche	Paneth cells, niche epithelial cells	Wnt3, Epidermal growth factor (EGF), Notch ligands (DLL1/4)	Wnt/β-catenin, Notch	Maintain ISC self-renewal and crypt homeostasis; support CSC maintenance under oncogenic activation	Wnt and Notch pathway inhibitors; differentiation-inducing strategies
Stromal fibroblasts	Cancer-associated fibroblasts (CAFs), myofibroblasts	Wnt ligands, R-spondins, HGF, TGF-β	Wnt, TGF-β, Hippo/Yes-associated protein (YAP)	Enhance ISC/CSC stemness, promote plasticity and therapy resistance	Targeting CAF–CSC interactions; TGF-β and YAP inhibition
Immune niche	Tumor-associated macrophages, T cells, dendritic cells	IL-6, IL-1β, TNF-α, TGF-β	Janus kinase (JAK)/ Signal transducer and activator of transcription 3 (STAT3), NF-κB	Induce CSC stemness, immune evasion, and inflammatory niche remodeling	Combination of immunotherapy with CSC-targeted or niche-modulating agents
Endothelial niche	Endothelial cells, pericytes	VEGF, angiocrine factors	VEGF, Notch	Support ISC proliferation and tumor angiogenesis; facilitate CSC survival	Anti-angiogenic therapy combined with ISC/CSC targeting
Extracellular matrix (ECM)	Collagen, laminin, fibronectin	Integrin ligands, mechanical cues	FAK, Hippo/YAP	Regulate ISC anchorage, mechanotransduction, and CSC invasion	ECM remodeling inhibitors; mechanotransduction-targeted strategies
Microbial niche	Gut microbiota	Microbial metabolites, short-chain fatty acids (SCFAs), pathogen-associated molecular patterns (PAMPs)	toll-like receptors/myeloid differentiation primary response protein 88 (TLR/MyD88), epigenetic regulation	Modulate ISC epigenetic state, stemness, and drug response	Microbiota modulation to enhance therapeutic sensitivity

2.2.1. Physiological regulation of ISCs by stemness pathways

Under physiological conditions, the Wnt, Notch, and Hippo signaling pathways precisely regulate the fate determination of ISCs through synergistic effects. The Wnt signaling pathway is the core pathway for maintaining the stemness of ISCs. It regulates the expression of downstream stemness-related genes (such as Lgr5) through the β-catenin/TCF complex, ensuring the self-renewal capacity of ISCs [42,43]. As a specific marker of ISCs, the expression of Lgr5 is directly regulated by the Wnt signal, and Lgr5+ ISCs are the main source of intestinal epithelial regeneration [44,45].

The Notch signaling pathway mainly regulates the differentiation direction of ISCs. By inhibiting their differentiation into secretory cells (such as goblet cells), it maintains the proliferative state of absorptive epithelial cells [46,47]. The Hippo signaling pathway regulates the nuclear localization and activity of YAP/transcriptional coactivator with PDZ-binding motif (TAZ) transcriptional co-activators through phosphorylation, thereby regulating the proliferation and regeneration of ISCs and playing an important role in intestinal injury repair [48,49].

Importantly, these pathways do not function in isolation. Multiple studies have demonstrated extensive crosstalk among Wnt, Notch, and Hippo signaling, which collectively contributes to ISC pool homeostasis [50,51]. For example, Hippo pathway activity can modulate Wnt signaling output through YAP/TAZ-dependent mechanisms, while Wnt signaling has been shown to influence Notch pathway activation status [52].

2.2.2. Hyperactivation of stemness pathways in CRC

(a) Wnt Signaling Pathway

Abnormal activation of the Wnt signaling pathway is an early key event in the occurrence of CRC and the most typical feature of signal reconstruction in ISCs under colorectal cancer conditions [53]. Under physiological conditions, Wnt signaling is initiated by ligand–receptor interactions that inhibit β-catenin degradation, allowing β-catenin to translocate to the nucleus and associate with TCF/LEF transcription factors to regulate downstream gene expression [54] (Figure 1A).

In CRC, sustained Wnt pathway activation can arise through multiple mechanisms. Genetic alterations affecting core regulatory components, particularly loss-of-function mutations in APC, represent a major route by which β-catenin degradation is impaired, leading to persistent activation of Wnt target genes involved in stemness and proliferation [55,56]. In addition, activating mutations in catenin beta 1 (CTNNB1) (encoding β-catenin) can further stabilize β-catenin in a ligand-independent manner [56].

Beyond core pathway mutations, dysregulation of the R-spondin/Lgr5/Rnf43 axis also contributes to enhanced Wnt signaling output. R-spondins act as potent Wnt pathway amplifiers by binding Lgr5 and inhibiting the E3 ubiquitin ligase Rnf43, thereby stabilizing Frizzled receptors at the cell surface [57]. Gene fusions involving RSPO family members and loss-of-function mutations in RNF43 have been identified in subsets of CRC, collectively promoting heightened Wnt responsiveness and expansion of ISC/CSC populations [58,59] (Figure 1B).

In addition to cell-intrinsic alterations, the CRC microenvironment can further potentiate Wnt signaling through increased ligand availability. Tumor-associated fibroblasts and immune cells have been reported to secrete Wnt ligands, creating localized niches with elevated Wnt activity that may

reinforce stem-like properties in ISCs and CSCs [60]. Collectively, these mechanisms highlight how sustained Wnt signaling is associated with maintenance of stemness and tumor persistence, rather than serving as a singular determinant of CRC progression [60,61].

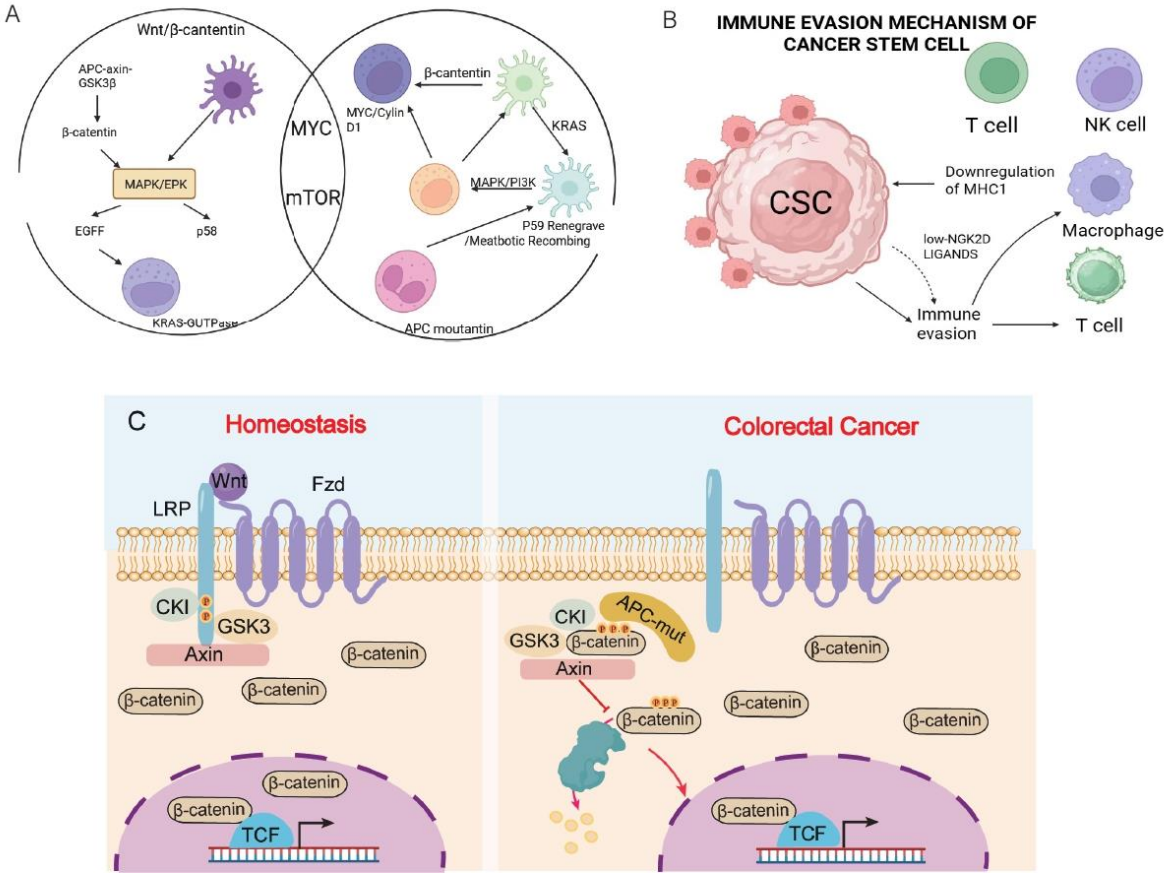

Figure 1. Dysregulation of intestinal stem cell signaling and immune evasion during colorectal cancer progression. **(a)** Integrated oncogenic signaling pathways driving the transformation of ISCs into colorectal cancer stem cell. Aberrant activation of Wnt/β-catenin signaling, commonly caused by APC mutation, cooperates with KRAS-dependent MAPK/ERK and PI3K–AKT–mTOR pathways to promote sustained proliferation, metabolic reprogramming, and stemness maintenance. MYC and Cyclin D1 function as key downstream effectors coordinating these oncogenic signals. **(b)** Immune evasion strategies employed by colorectal cancer stem cell within the tumor immune microenvironment. CSCs escape immune surveillance through downregulation of Major histocompatibility complex (MHC) class I molecules and reduced expression of natural killer (NK) cell–activating ligands, thereby impairing cytotoxic T cell and NK cell–mediated killing while fostering immunosuppressive interactions with macrophages and other immune cells. **(c)** Canonical Wnt/β-catenin signaling under intestinal homeostasis and colorectal cancer conditions. In normal epithelium, β-catenin is tightly controlled by the destruction complex (casein kinase 1 (CK1), GSK3, Axin, and APC). In colorectal cancer, APC mutation disrupts β-catenin degradation, leading to its nuclear accumulation and persistent activation of TCF-dependent transcriptional programs that drive ISC expansion and CSC maintenance. Created with BioRender.com.

(b) Notch Signaling Pathway

Under physiological conditions, Notch signaling primarily regulates ISC lineage commitment. Activation of Notch receptors through ligands such as Jagged or Delta-like proteins induces downstream transcriptional programs, including Hes1 expression, which suppress secretory lineage differentiation and favor maintenance of proliferative absorptive progenitors [62,63].

In CRC, Notch signaling activity is frequently elevated and appears to undergo functional reprogramming. Increased expression of Notch receptors and ligands, particularly Jagged1 and Notch1, has been observed in CRC tissues and is associated with adverse clinical outcomes in multiple studies [64]. Notably, Jagged1 expression is transcriptionally regulated by Wnt signaling, and in APC-mutant CRC, enhanced Wnt activity can promote Jagged1 upregulation, thereby facilitating coordinated activation of Wnt and Notch pathways [52].

Pharmacological inhibition of Notch signaling, including the use of γ-secretase inhibitors, has been shown in experimental models to suppress CRC cell proliferation and promote differentiation toward secretory lineages, supporting a role for Notch signaling in maintaining stem-like tumor cell states [52]. In addition, Notch pathway activation has been implicated in CRC invasion, metastasis, and angiogenesis through regulation of epithelial–mesenchymal transition–associated genes and endothelial cell function [65,66]. Importantly, Notch signaling activity appears to be cell-type– and context-dependent, reflecting heterogeneity within the CRC microenvironment [65,67].

(c) Interaction Network and Synergistic Regulation

Under CRC conditions, Wnt, Notch, and Hippo signaling pathways do not operate independently but instead form an interconnected regulatory network that shapes ISC and CSC behavior. Among these pathways, Wnt signaling frequently occupies an upstream or coordinating position by influencing the activity of key components within the Notch and Hippo pathways.

Wnt–Notch cooperation represents an important regulatory axis during early tumor development. Wnt-driven upregulation of Jagged1 can activate Notch signaling, and together these pathways promote ISC proliferation while limiting differentiation [51]. Experimental studies in APC-mutant CRC mouse models have demonstrated that Notch pathway inhibition can reduce tumor burden, highlighting the functional relevance of this cooperative interaction [68]. Conversely, Notch signaling can modulate Wnt pathway output by enhancing β-catenin nuclear localization, thereby reinforcing oncogenic signaling loops [65].

Crosstalk between Wnt and Hippo pathways is mediated in part through interactions between β-catenin and YAP/TAZ. YAP/TAZ can act as transcriptional co-activators that enhance Wnt target gene expression, while Wnt signaling can promote YAP/TAZ nuclear localization by suppressing Hippo kinase activity [65,69]. During tissue repair, such interactions support ISC regeneration; under sustained oncogenic conditions, however, they may contribute to malignant transformation and tumor progression [69,70].

Interactions between Notch and Hippo signaling further add to this complexity. Hippo pathway inactivation has been reported to upregulate Notch signaling, while Notch activation can influence YAP/TAZ activity, collectively promoting CRC cell proliferation and invasion in experimental models [51,71]. Together, these multilayered interactions illustrate how dysregulated stemness pathways form a context-dependent signaling network rather than a linear oncogenic cascade [61,72].

In summary, Wnt, Notch, and Hippo signaling pathways undergo context-specific dysregulation and functional remodeling in ISCs under CRC conditions, giving rise to interconnected regulatory networks

that influence stem cell maintenance, plasticity, and tumor progression. While aberrant Wnt activation frequently represents an early and prominent feature, its oncogenic effects are shaped by dynamic interactions with Notch and Hippo signaling. A deeper understanding of these signaling networks and their context-dependent regulation may inform the development of therapeutic strategies aimed at targeting ISC/CSC-associated vulnerabilities in CRC.

2.3. Immune evasion properties of CSCs

Studies have reported associations between CSC marker expression in human tumors and the abundance and composition of tumor-infiltrating immune cells, suggesting a close relationship between CSC states and the tumor immune microenvironment (TIME). Accumulating evidence indicates that immune responses against CSC-enriched populations can be impaired through multiple, and often context-dependent, immune evasion mechanisms. Importantly, the TIME evolves dynamically across stages of tumor progression, and the dominant immunosuppressive mechanisms may differ by disease stage, anatomical site, and tumor type. Therefore, in this section we summarize CSC-associated immune evasion mechanisms with emphasis on CRC evidence when available, and we explicitly distinguish findings derived from non-CRC tumor models.

2.3.1. Impaired antigen presentation

The interaction between the T-cell receptor (TCR) on the surface of cytotoxic T lymphocytes (CTLs) and major histocompatibility complex (MHC) class I–associated antigens constitutes an essential prerequisite for effective T-cell–mediated immune responses against tumor cells. Tumor antigens must be appropriately degraded by the proteasome, transported into the endoplasmic reticulum via dedicated transporter proteins, and subsequently loaded onto MHC class I molecules for surface presentation. In the absence of efficient antigen processing and presentation, transformed cells fail to be recognized and eliminated by cytotoxic T cells.

Accumulating studies suggest that key steps of antigen processing and presentation can be disrupted in CSC-enriched populations, representing an important immune evasion mechanism. In CRC, CSC-like tumor fractions have been reported to exhibit reduced MHC class I–associated antigen presentation, which may decrease susceptibility to CTL-mediated immune surveillance and facilitate persistence under immune pressure, thereby contributing to immune escape and disease progression [73].

2.3.2. Downregulation of tumor-associated antigens

In addition to defects in antigen processing and presentation, cancer stem cells may further evade immune surveillance by reducing the expression or immunogenicity of tumor-associated antigens. In colorectal cancer, this antigenic invisibility limits effective recognition by antigen-specific T cells, thereby weakening antitumor immune responses [74].

Notably, while CSCs may exhibit reduced visibility to the immune system, they continue to express a set of stemness-associated proteins that are essential for maintaining self-renewal and tumor-initiating capacity. These CSC-associated molecules represent a distinct class of tumor-associated antigens and have attracted increasing interest as potential immunotherapeutic targets. However, the immune tolerance surrounding such antigens in colorectal cancer poses a significant challenge, underscoring the

need for strategies that simultaneously enhance antigen immunogenicity and overcome CSC-mediated immune evasion.

2.3.3. CSC–NK cell interactions and NK-resistance phenotypes

NK cells preferentially target "missing-self" cells with reduced MHC-I expression, as MHC-I engagement of inhibitory NK receptors suppresses NK activation [75]. This framework suggests that CSCs with low MHC-I expression might be susceptible to NK-mediated killing. However, the sensitivity of CSC-enriched populations to NK cytotoxicity remains context-dependent and sometimes controversial, and may be influenced by both tumor-intrinsic factors and immunosuppressive components of the microenvironment [76].

Several studies have reported enhanced NK cytotoxic activity against CSC-enriched tumor cells in co-culture systems [77], and preferential killing of CSC-like cell populations characterized by markers such as cluster of differentiation 24/cluster of differentiation 44 (CD24/CD44), cluster of differentiation 133 (CD133), or aldehyde dehydrogenase (ALDH) *in vitro* [78]. NK cells can execute cytotoxicity through perforin/granzyme release as well as death receptor pathways (Fas cell surface death receptor/Fas ligand (FAS/FASL) and death receptor 4/death receptor 5/tumor necrosis factor-related apoptosis-inducing ligand (DR4/DR5/TRAIL)) [79]. For example, CSC-like fractions derived from colon cancer cell lines have been reported to exhibit cMYC-associated DR4 expression and sensitivity to TRAIL-induced apoptosis. In other tumor models, including breast cancer, CSC-enriched populations have displayed variable sensitivity to FASL- and TRAIL-mediated apoptosis compared with non-CSCs [80].

Conversely, CSCs may develop NK resistance by downregulating ligands for NK activating receptors (e.g., MHC class I polypeptide-related sequence A/MHC class I polypeptide-related sequence B, UL16-binding proteins (MICA/MICB, ULBPs)) or by upregulating MHC-I expression, as reported in multiple non-CRC tumor contexts including adult T-cell leukemia, glioblastoma, ovarian cancer ascites–derived CSC-like cells, and renal carcinoma spheroids [81–83]. In CRC, STAT3 activation has been linked to reduced expression of NK-activating ligands, and STAT3 inhibition has been reported to induce MICA expression and enhance NK activation, suggesting that CSC-associated transcriptional programs may contribute to NK resistance [82].

2.3.4. Resistance to cytolytic granule–mediated killing

Upon recognition of target cells, CTLs and NK cells release cytolytic granules containing perforin and granzymes, which induce apoptosis through caspase activation and cleavage of nuclear and mitochondrial substrates [84]. CSC-enriched populations have been reported to employ multiple strategies to attenuate granzyme-mediated killing in certain tumor contexts [85].

Serglycin (SG) is a proteoglycan with high affinity for granzyme B and has been implicated in regulating granzyme stability and delivery. In glioma models, mast cell infiltration has been reported to induce SG expression alongside CSC- and EMT-associated markers such as CD44 and zinc finger E-box binding homeobox 1 (ZEB1), and SG expression correlates with poor prognosis [86]. These findings suggest that SG-associated programs may facilitate immune evasion of CSC-like populations, although additional mechanisms are likely involved given the multifunctional roles of SG.

2.3.5. Immune factors

TGF-β is a multifunctional cytokine implicated in CSC state induction, immune suppression, and EMT. CSC-enriched populations in several tumor models, including breast cancer and glioblastoma, have been reported to exhibit elevated TGF-β signaling activity, which may enhance stemness programs while dampening antitumor immune responses [87–89]. Pharmacological interventions (e.g., metformin in trastuzumab-resistant breast CSC models) have been reported to attenuate TGF-β–associated EMT and self-renewal phenotypes. Although much of this evidence derives from non-CRC contexts, TGF-β signaling is also relevant to CRC immune suppression and may intersect with CSC-associated programs, warranting further CRC-focused validation [90].

IL-6 is another multifunctional cytokine that promotes inflammatory signaling and has been implicated in CSC induction and maintenance through the JAK/STAT3 pathway across multiple tumor types [91]. STAT3 is also a key transcription factor associated with CSC-like programs and immune evasion, and epigenetic regulators (e.g., histone-lysine N-methyltransferase 1A (KMT1A)) have been reported to influence CSC properties through STAT3-dependent transcriptional circuits in certain cancers [92]. In CRC, STAT3 activation has been linked to CSC-associated phenotypes and may contribute to immune escape, including reduced NK-activating ligand expression as noted above.

Chemokine signaling can further support CSC-associated immune evasion. For example, the C–C motif chemokine ligand 20–C–C motif chemokine receptor 6 (CCL20–CCR6) axis has been reported to recruit regulatory T cells and promote immunosuppressive niches across several tumor types, thereby facilitating tumor progression, metastasis, and therapeutic resistance [93]. Whether and how these cytokine/chemokine circuits preferentially sustain CSC-enriched states in CRC remains an active area of investigation.

Collectively, CSC-associated immune evasion can arise through impaired antigen visibility to CTLs, context-dependent resistance to NK-mediated cytotoxicity, and cytokine/chemokine-driven immunosuppressive circuits within the TIME. Importantly, many mechanistic insights derive from non-CRC tumor models, and their direct relevance to CRC requires careful validation in appropriate clinical and experimental contexts. These considerations are essential when designing immunotherapies intended to eradicate CSC-enriched populations.

2.4. CSCs from animal models to human organoids

The CSC hypothesis represents an influential framework for explaining intratumoral heterogeneity, therapeutic resistance, and recurrence/metastasis in solid tumors. In CRC, this concept has progressed from early histopathological observations to a model supported by genetic lineage tracing, functional perturbation studies, and patient-derived platforms, including organoids and xenografts. In this section, we summarize key experimental milestones linking physiological ISCs to CSC-like tumor-propagating populations and highlight how these models have shaped current views of CSC plasticity and therapeutic vulnerabilities.

2.4.1. Animal models

(a) Pioneering Breakthroughs in Lineage Tracing

Two seminal lineage-tracing studies reported around 2009 provided foundational *in vivo* evidence supporting the CRC-CSC hypothesis. Using Lgr5-CreERT2 mouse models, the groups of Hans Clevers and Doug Winton performed inducible lineage tracing in APC-deficient intestinal adenomas. They showed that, following tumor initiation, labeled $Lgr5^+$ cells not only persisted but also generated progeny that contributed broadly to adenoma cell populations, whereas concurrently labeled differentiated cells were progressively lost over time [94]. These findings provided direct evidence in a spontaneous tumor context that specific stem-like subpopulations possess long-term clonal tumor-propagating capacity, supporting a functional link between physiological ISCs and CSC-like cells.

(b) Functional Validation and Preliminary Attempts in Clinic

To functionally test the requirement of $Lgr5^+$ CSC-like cells for tumor maintenance, multiple studies have employed inducible genetic ablation approaches to selectively eliminate $Lgr5^+$ cells in established intestinal tumors. In these experimental settings, depletion of $Lgr5^+$ cells was associated with reduced tumor growth and, in some cases, tumor regression as assessed by standard tumor-burden readouts [95].

Importantly, such experiments also highlighted a key translational challenge: tumors were often not completely eradicated. Residual $Lgr5^-$ tumor cells—including populations marked by alternative stem-like features (e.g., Dclk1) or cells undergoing dedifferentiation—have been reported to regain stem-like properties through activation of compensatory programs (including Hippo/YAP-associated regeneration pathways), thereby enabling tumor regrowth [96]. These observations support the concept of CSC plasticity, in which stem-like states are dynamically regulated rather than permanently fixed, and are influenced by microenvironmental cues.

2.4.2. Human organoids and PDX models

(a) Functional Validation

Patient-derived organoids (PDOs) established from fresh CRC tumor specimens and cultured in three-dimensional matrices can preserve key genomic alterations and aspects of tumor architecture over extended culture, making them valuable platforms for functional interrogation [97]. Using fluorescence-activated cell sorting (FACS)-based enrichment and reporter strategies, multiple studies have reported that CSC-associated markers—including CD44, CD133, ALDH1, and Lgr5—can enrich for tumor cell subpopulations with increased spheroid-forming capacity, enhanced chemotherapy tolerance, and elevated tumor-initiating potential *in vivo*.

For instance, in limiting-dilution transplantation assays in immunodeficient mice, $CD44^+$ fractions derived from CRC PDOs have been reported to exhibit markedly higher tumor-initiating frequencies than unsorted or $CD44^-$ populations across graded inoculation cell numbers [98]. Together, these PDO- and xenograft-based studies support the existence of functional heterogeneity and CSC-like hierarchies in human CRC specimens.

(b) Disease Modeling for Therapeutic Response

Beyond mechanistic validation, human PDOs and patient-derived xenograft (PDX) models provide tractable systems for modeling therapeutic responses and exploring resistance trajectories under controlled perturbations. These platforms have been used to examine how CSC-enriched fractions

respond to cytotoxic agents, targeted therapies, and immune-modulatory interventions, and to identify adaptive state transitions that may underlie residual disease and relapse [99].

Importantly, while conventional therapies primarily eliminate rapidly proliferating tumor populations, CSC-enriched or stem-like states may persist under treatment pressure in certain contexts, thereby contributing to recurrence. This evidence motivates therapeutic strategies that combine tumor debulking with approaches aimed at limiting CSC maintenance programs and plasticity, a theme that is discussed in detail in subsequent sections.

3. The potential of ISC transformation as CRC therapeutic targets

CRC remains a major cause of cancer-related mortality worldwide, largely due to therapeutic resistance, recurrence, and metastatic progression [100]. From a precision medicine perspective, ISC- and niche-targeted therapies offer multiple layers of selectivity, including genetic context (e.g., APC or KRAS mutations), stem cell state (e.g., $Lgr5^+$ versus regenerative CSC-like states), and microenvironmental dependencies. Preclinical and emerging clinical studies increasingly demonstrate that exploiting these layers of vulnerability can enhance therapeutic efficacy while limiting toxicity to normal intestinal epithelium. Increasing evidence suggests that aberrant activation of ISC programs and the emergence of CSC-like states are associated with disease persistence and relapse. CSC-enriched populations display key features such as self-renewal capacity, tumor-propagating potential, and relative resistance to conventional therapies [101]. These observations have motivated therapeutic strategies aimed at targeting ISC/CSC-associated regulatory mechanisms, with the goal of complementing existing cytotoxic and targeted treatments.

3.1. Suppressing CRC by regulating ISCs self-renewal

The balance between self-renewal and differentiation in ISCs is governed by interconnected signaling networks, disruption of which has been linked to CRC development [102]. Among these pathways, Wnt/β-catenin signaling plays a prominent role in maintaining ISC identity and proliferative capacity, and is frequently dysregulated in CRC. Loss-of-function mutations in APC impair β-catenin degradation, allowing nuclear β-catenin accumulation and transcriptional activation of downstream targets such as MYC and cyclin D1 (CCND1), thereby promoting sustained proliferative signaling [103,104].

Experimental studies have further suggested that enhanced self-renewal activity in $Lgr5^+$ ISCs—the stem cell population located at the crypt base—is associated with early tumorigenic processes, and that this phenotype is modulated by cooperative signaling inputs from pathways including Notch and Hedgehog [105]. However, the relative contribution of these pathways may vary across tumor stages and genetic backgrounds, highlighting the need for context-specific therapeutic strategies.

Interventions targeting ISC self-renewal pathways have demonstrated antitumor activity in preclinical models. For example, salinomycin, a reported inhibitor of Wnt signaling, has been shown to disrupt the β-catenin/TCF complex, reduce ISC self-renewal, and promote differentiation, resulting in decreased tumor burden in CRC animal models [106]. In parallel, γ-secretase inhibitors targeting the Notch pathway suppress Notch receptor activation and have been reported to attenuate stemness programs and enhance chemotherapy sensitivity in experimental settings [107].

Despite these promising results, pathway-targeted approaches face challenges including on-target toxicity, pathway redundancy, and adaptive resistance, underscoring the importance of careful patient selection and combination strategies in future clinical applications.

3.2. CSC targeting strategies with small molecules

Building on the recognition of ISC/CSC-associated vulnerabilities, multiple therapeutic approaches have been explored, including small-molecule inhibitors, antibody-based therapies, and gene-editing technologies [108]. Each modality offers distinct advantages and limitations, providing complementary options for precision treatment of CRC.

Small-molecule inhibitors, characterized by favorable tissue penetration and oral bioavailability, have been widely investigated for targeting ISC/CSC-associated signaling pathways and epigenetic regulators. For instance, alkB homolog 5, RNA demethylase (ALKBH5), an m^6A demethylase reported to be upregulated in colorectal cancer stem cell, has been implicated in maintaining stemness and chemotherapy resistance via regulation of the family with sequence similarity 84 member A (FAM84A)–β-catenin axis. Genetic and organoid-based studies suggest that ALKBH5 inhibition may enhance chemotherapy sensitivity in specific contexts [109].

Additional strategies include synthetic vulnerabilities identified in APC-mutant CRC, where combined modulation of Wnt signaling and apoptotic pathways has been reported to enhance tumor cell killing in preclinical systems [110]. As well as inhibition of MyD88 signaling to suppress growth of intestinal tumor–derived organoids [111]. While these findings highlight potential therapeutic avenues, their clinical relevance requires validation in well-defined patient subsets.

Antibody-based therapies aim to selectively target ISC/CSC surface markers such as CD44, CD133, epithelial cell adhesion molecule (EpCAM), and Lgr5 [112]. Preclinical studies have shown that antibodies against CD44 or EpCAM can reduce stem-like properties and induce immune-mediated cytotoxicity through mechanisms including antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [113]. However, marker heterogeneity and overlap with normal ISCs raise concerns regarding specificity and potential toxicity, which remain major barriers to clinical translation.

Gene-editing technologies, particularly clustered regularly interspaced short palindromic repeats–CRISPR-associated protein 9 (CRISPR–Cas9), have enabled precise interrogation of ISC-associated oncogenic drivers in experimental CRC models [114]. Conditional knockout studies targeting genes such as ALKBH5 in ISC compartments have provided mechanistic insights into tumor initiation and progression [115]. Nevertheless, challenges related to *in vivo* delivery, off-target effects, and ethical considerations currently limit direct clinical application.

3.3. Synthetic lethality and combined therapies

Given the genetic and signaling complexity of CRC, single-agent therapies often result in adaptive resistance. Synthetic lethality and rational combination therapies aim to exploit context-specific vulnerabilities created by oncogenic mutations, thereby enhancing tumor selectivity while minimizing toxicity [116].

In CRC characterized by aberrant Wnt/β-catenin activation, particularly in the setting of APC mutations present in approximately 80% of cases [117], synthetic lethal strategies have been explored.

One example involves combined inhibition of glycogen synthase kinase-3 (GSK-3) and B-cell lymphoma-extra large (BCL-XL), where further activation of Wnt signaling in APC-mutant cells is proposed to exceed cellular tolerance thresholds, sensitizing cells to apoptosis induced by low-dose BCL-XL inhibition. This approach has been validated in organoid-based competition assays and quantitative synergy analyses, and may mitigate dose-limiting toxicities associated with BCL-XL monotherapy [110]. Whether such strategies can be safely translated to clinical settings remains an open question.

Combination therapies targeting additional mutational contexts have shown encouraging early clinical signals. In patients with KRAS^G12C-mutant metastatic CRC, combinations such as divarasib plus cetuximab or adagrasib plus cetuximab have demonstrated objective response rates ranging from approximately 46% to 62.5% in early-phase trials, with median progression-free survival of 6.9–8.1 months as assessed by Response Evaluation Criteria in Solid Tumors (RECIST) v1.1 criteria [118,119].

Similarly, in BRAF-mutant CRC, triple therapy combining BRAF, EGFR, and MEK inhibitors has been reported to overcome feedback activation mechanisms and reduce expression of stemness-associated markers in preclinical models [120]. Additional combinatorial approaches, including inhibition of JAK/STAT signaling alongside immune checkpoint blockade, have been proposed to enhance antitumor immunity by attenuating CSC-associated immune evasion programs [121].

3.4. Differentiation-targeting sequential therapy

Tumor cell heterogeneity represents a major barrier to durable responses in colorectal cancer. Conventional targeted therapies may preferentially eliminate therapy-sensitive tumor fractions, whereas differentiated tumor cells can, in some contexts, reacquire stem-like properties through dedifferentiation after treatment, thereby contributing to relapse [122]. On this basis, an emerging sequential paradigm—often described as a “prime-then-kill” strategy—has been proposed: first inducing a transient stem-like or ISC-like state in differentiated tumor cells, followed by administration of ISC/CSC-targeted agents to broaden therapeutic vulnerability and potentially improve tumor control [123].

Dedifferentiation is a complex and context-dependent process regulated by multiple signaling pathways, with reactivation of stemness-associated transcriptional programs representing a common feature. Evidence suggests that both cytotoxic stressors and epigenetic modulators can promote stem-like state transitions in differentiated tumor cells [124]. For example, low-dose chemotherapy has been reported to activate EMT-associated programs and induce expression of stemness markers such as Lgr5 and CD44 in colorectal cancer cells in specific experimental settings [125].

Importantly, inducing dedifferentiation is a double-edged sword: while it may increase the fraction of cells susceptible to CSC/ISC-targeted agents, it may also transiently expand stem-like populations if the subsequent “kill” phase is suboptimal. Thus, the therapeutic window, induction strength, timing, and dosing sequence are likely to be critical determinants of efficacy.

Sequential therapy paradigms have demonstrated promising potential in preclinical studies. For instance, in colorectal cancer xenograft models, pretreatment with the MEK inhibitor trametinib for 7 days induced dedifferentiation of differentiated tumor cells toward an ISC-like state; subsequent administration of a Wnt pathway inhibitor or anti-Lgr5 monoclonal antibody resulted in a tumor growth inhibition (TGI) of 92% relative to vehicle or single-agent treatment, as calculated over the defined treatment window [126]. In colorectal cancer organoid models, histone deacetylase (HDAC) inhibitors were first used to induce dedifferentiation of differentiated tumor cells, significantly increasing the

proportion of ISCs-like cells. Subsequent application of ALKBH5 inhibitors or anti-Lgr5 monoclonal antibodies markedly enhanced organoid killing efficacy [109]. Furthermore, mathematical modeling confirmed that rationally designed sequential treatment regimens can effectively overcome therapeutic resistance caused by tumor heterogeneity, achieving more stable tumor control [127]. The core advantage of this therapeutic paradigm lies in proactively expanding the pool of sensitive cells, addressing the issue of incomplete treatment caused by tumor heterogeneity in traditional targeted therapies. This provides a novel strategy for improving colorectal cancer cure rates.

Nevertheless, this paradigm remains predominantly preclinical. Key translational challenges include identifying clinically actionable and safe "priming" agents, optimizing sequencing and dosing to avoid inadvertent expansion of stem-like populations, and validating efficacy across genetically and immunologically diverse CRC contexts.

3.5. Immunotherapy targeting ISCs/CSCs and their niche

Accumulating evidence indicates that immune resistance in colorectal cancer is tightly linked to the immune-evasive properties of intestinal stem cells and their niche. Accordingly, effective immunotherapy must simultaneously target CSCs and remodel the tumor immune microenvironment to enable durable antitumor immunity.

3.5.1. Niche-driven immune evasion of intestinal CSCs

CSC-enriched tumor fractions can shape an immunosuppressive microenvironment through multiple mechanisms, including reduced antigen visibility, inhibitory cytokine signaling, and bidirectional crosstalk with myeloid and dendritic compartments. In CRC, CSC-like populations have been reported to exhibit reduced MHC-I–associated antigen presentation, potentially limiting $CD8^+$ T-cell recognition. In addition, CSC-associated cytokine outputs—including IL-4 in some experimental contexts—have been implicated in suppressing local T-cell activity [128]. CSCs may also impair dendritic cell (DC) function. For example, studies have reported that $CD133^+$ CSC-enriched populations can reduce activated DC numbers and promote tolerogenic DC phenotypes, thereby facilitating regulatory T cell differentiation and reinforcing immunosuppression [129]. Conversely, reciprocal regulation between tumor-associated macrophages (TAMs) and CSC-like states has been described: M2-like TAMs secrete cytokines (e.g., IL-6, IL-1β, TNF-α) that activate STAT3/NF-κB signaling and enhance stemness-associated programs, whereas CSC-associated factors can promote immunosuppressive macrophage polarization [130].

Together, these observations support a niche-level feedback structure that can sustain CSC persistence and immune escape, although the dominant mechanisms may vary by tumor stage and patient-specific TIME composition.

3.5.2. Immunotherapeutic strategies directly targeting ISCs/CSCs

Several immunotherapeutic approaches aim to directly target CSC-associated antigens or stem-like states. Vaccine strategies based on CSC-associated antigens seek to enhance antigen presentation by loading tumor antigens into dendritic cell vaccines or nanoparticle platforms, thereby eliciting CSC-directed T-cell responses and potentially overcoming immune tolerance [131]. Antibody engineering approaches,

including bispecific antibodies and antibody–drug conjugates (ADCs), have been explored to block oncogenic signaling and engage immune effector functions. For example, the EGFR × Lgr5 bispecific antibody petosemtamab has demonstrated clinical activity in head and neck squamous cell carcinoma, and its potential relevance to CRC CSC targeting has attracted interest [132]. However, translation to CRC will require careful evaluation of target expression heterogeneity, on-target/off-tumor risk in normal intestinal stem compartments, and CRC-specific clinical efficacy.

Concurrently, chimeric antigen receptor (CAR) cell therapies offer more aggressive approaches for direct CSC elimination. CAR-T cell therapies targeting CSC markers have progressively advanced into clinical phases. Among these, an autologous CAR-T product targeting Lgr5 has advanced into early-phase clinical evaluation for patients with recurrent or metastatic colorectal cancer. Preclinical studies have demonstrated that Lgr5-directed CAR-T cells can effectively eliminate CSC-enriched tumor fractions and suppress tumor growth, supporting the feasibility of targeting intestinal CSCs using adoptive cell therapy strategies. However, the clinical translation of Lgr5-targeted CAR-T therapy in CRC requires careful evaluation of on-target toxicity, antigen heterogeneity, and intestinal stem cell safety [133]. Furthermore, CAR-T therapies targeting CSC-associated markers such as CD133 and EpCAM have demonstrated manageable safety profiles and preliminary antitumor activity in early studies, offering novel therapeutic possibilities for refractory colorectal cancer [134].

Beyond these approaches, cell therapies leveraging innate immune responses have also garnered attention. Engineering NK cells or employing allogeneic cytokine-induced killer (CIK) cells leverages their innate ability to recognize and eliminate MHC-I-low CSCs, partially circumventing the immune evasion achieved by CSCs through downregulation of antigen presentation [135]. Such approaches offer novel tools for combining or augmenting adaptive immunotherapy.

3.5.3. Converting MSS/pMMR CRC from “cold” to “hot” tumors

Despite these advances, approximately 85%–95% of advanced CRC exhibit microsatellite stability (MSS) or intact proficient mismatch repair (pMMR). Such tumors typically exhibit a classic immunological “cold” phenotype, characterized by insufficient intratumoral effector T-cell infiltration, impaired antigen presentation, and a markedly immunosuppressive microenvironment, leading to primary resistance to ICIs. Consequently, single-agent immunotherapies struggle to achieve durable responses in these patients. Current research priorities are shifting towards multidimensional, combined interventions aimed at systematically remodeling the tumor microenvironment to convert “cold” tumors into “hot” tumors [136].

3.5.4. Synergistic remodeling of the CSC niche and tumor immunity

Against this backdrop, multiple immunostimulation strategies have been proposed and refined. Oncolytic viral therapy preferentially infects tumor cells, inducing immunogenic cell death (ICD) to promote tumor antigen release and enhance $CD8^+$ T cell recruitment [137]. Although monotherapy with oncolytic viruses remains limited in clinical efficacy, viral engineering or combination with extracellular matrix degradation strategies significantly improves viral and immune cell penetration within dense tumor stroma, thereby amplifying antitumor immune responses.

Concurrently, the combination of immune checkpoint inhibitors with conventional therapies has demonstrated promising outcomes. Chemotherapy, radiotherapy, or anti-angiogenic treatments not only directly suppress tumor growth but also enhance tumor immunogenicity by inducing immunogenic cell death, disrupting immune barriers, and improving antigen presentation. Extensive research indicates that such multi-modal combination strategies represent a key pathway to overcoming immune tolerance in "cold" tumors, with chemotherapy- or radiotherapy-induced ICD being recognized as a crucial molecular event triggering tumor immune ignition [138].

Furthermore, the application of immune-activating factors provides novel tools for enhancing anti-tumor immunity. For instance, TLR or stimulator of interferon genes (STING) agonists, CD40 agonist antibodies, and pro-inflammatory cytokines (such as IL-12) significantly enhance dendritic cell maturation and antigen presentation functions, promoting effector T cell activation. This synergizes with PD-1/PD-L1 inhibitors to breach tumor immune tolerance [139]. Conversely, targeting immunosuppressive cell populations represents another crucial strategy for remodeling the tumor microenvironment. Reprogramming TAMs and myeloid-derived suppressor cells (MDSCs) effectively alleviates tumor-induced immunosuppression. Strategies targeting gut-specific or tissue-resident macrophages are also emerging as promising new directions in colorectal cancer immunotherapy [140].

Furthermore, it is noteworthy that gut microbial regulation is emerging as a key factor influencing the efficacy of CRC immunotherapy. Recent studies indicate that the composition and functional state of a patient's gut microbiota can significantly modulate anti-tumor immune responses. Transplantation of immunostimulatory microbiota can reprogramme immunologically "cold" tumors towards a more inflamed "hot" immune phenotype, thereby enhancing the therapeutic efficacy of PD-1 inhibitors. This finding suggests that modulating the gut microbiota and its metabolites may offer novel, precision-targeted interventions for colorectal cancer immunotherapy [141].

Collectively, these TIME-remodeling strategies intersect with CSC biology by altering the immunosuppressive niche that supports CSC persistence, enhancing antigen exposure, and restoring effector immune infiltration, thereby potentially increasing CSC vulnerability to immune-mediated clearance.

To strengthen the translational relevance of ISC/CSC- and niche-targeted concepts, we summarized representative ongoing or recent clinical efforts and late-preclinical exemplars in CRC, highlighting patient populations, biomarker-based selection, endpoints, and common causes of limited efficacy (Table 2).

Figure 2 summarizes ISC-centered therapeutic strategies aimed at eliminating CRC at its cellular root.

At the crypt base, aberrant activation of ISCs drives CSC formation and tumor initiation. Therapeutic interventions can be broadly classified into six interconnected strategies: (1) Direct targeting of ISCs self-renewal, inhibiting core stemness pathways to suppress tumor initiation and maintenance; (2) Stem cell–targeted therapies, including small-molecule inhibitors, monoclonal antibodies, and gene-editing approaches selectively acting on ISCs/CSCs; (3) ISC-based immunotherapy and tumor microenvironment modulation, enhancing immune-mediated clearance of stem-like tumor cells; (4) Synthetic lethality and rational combination therapy, selectively eliminating mutation-defined ISCs (e.g., APC-deficient cells) while sparing normal epithelium; (5) Sequential therapy paradigms, in which transient pathway inhibition (e.g., MEK inhibition) induces an ISC-like state, followed by ISC-targeted killing; (6) Immunotherapy synergy, converting immunologically "cold" tumors into "hot" tumors through combined ISC targeting and immune activation. Created with BioRender.com.

Table 2. Selected clinical/late-preclinical examples of ISC/CSC- and niche-targeted strategies in CRC.

Strategy	Target	Trial/Evidence	Subjects	Disease setting /Precursor lesions	Biomarker	Surrogate endpoint	Efficacy	Safety	Key limitations
Targeted therapy combination	KRAS G12C + EGFR	Adagrasib + cetuximab (KRYSTAL-1; Food and Drug Administration (FDA) accelerated approval)	Previously treated KRAS G12C mCRC	Metastatic CRC	KRAS G12C mutation	Objective response rate (ORR), Progression-free survival (PFS) (RECIST)	Clinically meaningful responses	Manageable class-related toxicities	Adaptive resistance; tumor heterogeneity
Targeted therapy combination	KRAS G12C + EGFR	Divarasib + cetuximab (phase Ib)	KRAS G12C mCRC	Metastatic CRC	KRAS G12C mutation	ORR, PFS	High early antitumor activity	Manageable toxicity profile	Clonal evolution; pathway bypass
CAR-T cell therapy	Lgr5	CNA3103 (NCT05759728)	Advanced or metastatic CRC	Metastatic CRC	Tumor Lgr5 expression	Safety, dose escalation	Ongoing clinical evaluation	Potential on-target/off-tumor toxicity	Antigen heterogeneity; intestinal toxicity risk
CAR-T cell therapy	CD133	CD133-targeted chimeric antigen receptor T cells (CART-133, early-phase trials)	CD133-positive advanced tumors	Advanced disease	CD133 expression	Safety, tumor response	Preliminary antitumor activity	Cytokine-related adverse events	Marker overlap with normal stem cells
CAR-T cell therapy	EpCAM	NCT05028933; NCT03013712	Advanced epithelial cancers	Advanced CRC and others	EpCAM expression	ORR, safety	Ongoing	Risk of epithelial toxicity	Broad expression in normal tissues
Bispecific antibody	EGFR × Lgr5	Petosemtamab (clinical activity in Head and neck squamous cell carcinoma (HNSCC))	Solid tumors; CRC of interest	Translational relevance to CRC	EGFR/Lgr5 co-expression	ORR, PFS	CRC efficacy not yet established	Expected EGFR-related toxicities	Non-CRC extrapolation
Pathway inhibition	Wnt/ Notch	Multiple preclinical and early clinical agents	CRC; FAP (exploratory)	Adenoma and carcinoma	APC loss; Wnt activation	Gastrointestinal (GI) toxicity; biomarker modulation	Limited clinical window	Significant intestinal toxicity	Dependence of normal ISCs on pathway
Stemness pathway inhibition	STAT3-associated programs	Napabucasin (phase III negative)	Metastatic CRC	Advanced CRC	STAT3 activation signatures	OS, PFS	No survival benefit	Generally tolerable	Lack of biomarker stratification

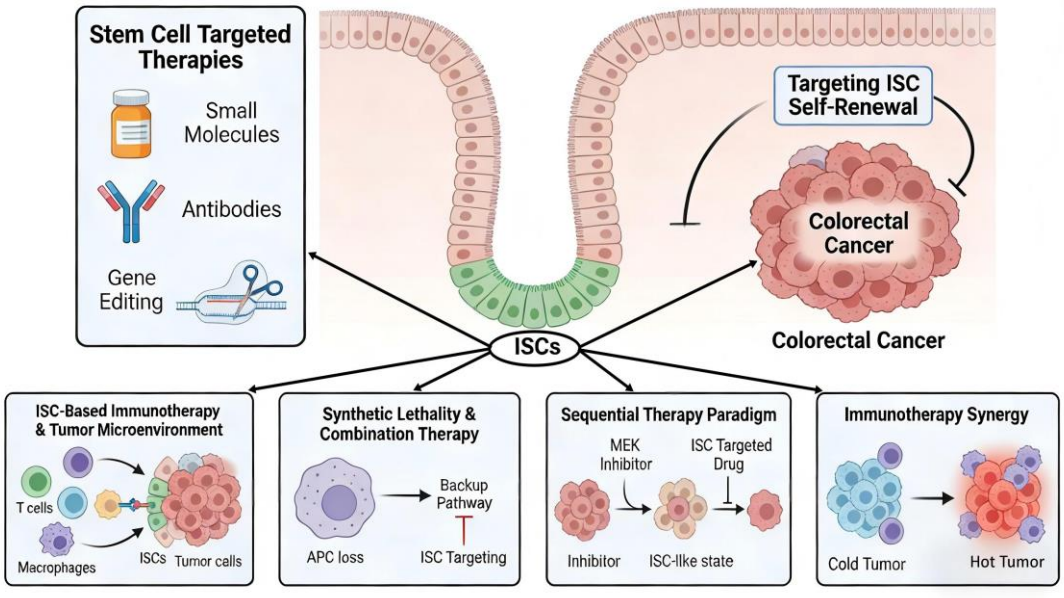

Figure 2. Schematic overview of six therapeutic strategies targeting ISC transformation for colorectal cancer elimination.

4. Frontier advances

Long-term *in vitro* culture of intestinal organoids depends on recreating key niche cues that support ISC survival, self-renewal, and differentiation. Many widely used protocols build upon the embedded 3D culture framework originally established by Sato and colleagues, with subsequent refinements aimed at improving robustness, scalability, and physiological relevance [142].

4.1. Advances in in vitro culture technology of ISCs and organoids

4.1.1. Core culture system

Cell Sources and Matrix Support: The starting cells for culture can be adult stem cells (ASCs) derived from biopsy tissues or induced pluripotent stem cells (iPSCs) obtained through directed differentiation. These cells are encapsulated in Matrigel or other matrices rich in laminin and collagen, which not only provide physical support but also mimic key biological signals of the basement membrane [143].

Key Signaling Pathways and Growth Factors: The medium formulation is crucial for maintaining the self-renewal and lineage differentiation of stem cells. Standard formulations must include components that activate the Wnt signaling pathway, such as Wnt3a and the potent enhancer R-spondin-1 [144]. Meanwhile, inhibitors of the bone morphogenetic protein (BMP) signaling pathway (e.g., Noggin) need to be added to prevent stem cell differentiation [145]. Epidermal growth factor (EGF) is essential for the formation of crypt-villus structures. Additionally, regulation of the Notch signaling pathway and YAP signaling plays important roles in maintaining stem cell characteristics and promoting proliferation [146].

4.1.2. Recent technical optimizations and innovations

To improve reproducibility and support potential clinical translation, efforts have increasingly focused on chemically defined and xeno-free media formulations [147]. Commercial kits (e.g., STEMdiff™ (stem cell differentiation) and IntestiCult™-SF (intestinal culture – serum-free)) facilitate serum-free culture and may reduce batch-to-batch variability. Recent work has further explored fully defined formulations by controlling the concentrations of growth factors and small-molecule modulators (e.g., CHIR99021, Y-27632), enabling more standardized regulation of growth and differentiation states [148]. Comparative evaluations of commercial and in-house formulations have also been reported, providing useful evidence for medium standardization and benchmarking [149].

Suspension Culture and Bioreactors: Traditional Matrigel-embedded culture methods are labor-intensive and costly, making large-scale production difficult. To address this issue, researchers have developed suspension culture systems based on bioreactors. These systems allow organoids to grow without Matrigel embedding and improve nutrient and oxygen exchange by controlling medium flow, paving the way for automation and large-scale production [150].

Microfluidics and “Organ-on-a-Chip” Technology: To better simulate the complex physiological environment of the intestine, such as intestinal peristalsis, fluid flow, and microbial symbiosis, researchers have combined organoid culture with microfluidic technology to construct “gut-on-a-chip” models. These models can introduce mechanical stress and immune cells, thereby more realistically simulating the human intestine at the functional level, particularly showing great potential in drug absorption and toxicity testing [151].

4.2. Intestinal organoid transplantation technology

Intestinal organoid transplantation aims to promote mucosal repair and restore barrier and absorptive functions *in vivo*. Most evidence currently derives from animal studies, while early-stage clinical exploration remains limited and primarily focuses on feasibility and safety endpoints.

4.2.1. Transplantation strategies and evaluation in animal models

Transplantation Methods and Sites: Successful transplantation of human intestinal organoids has been achieved in various animal models (mainly immunodeficient mice). Transplantation methods include endoscopic injection of organoid single-cell suspensions or organoids themselves into the submucosa of damaged colon, or direct transplantation into ectopic sites such as the small intestinal mesentery and renal capsule. Studies have found that transplantation into the physiologically relevant small intestinal mesentery better supports the differentiation and maturation of organoids and the formation of intestinal epithelium with crypt-villus structures [152].

Evidence of Engraftment and Functional Recovery: After successful transplantation, organoid-derived cells can integrate into the host's intestinal epithelium to form chimeric tissue and exhibit functions such as nutrient absorption. Studies have shown that in colitis mouse models, transplanted human intestinal organoids can effectively repair mucosal damage and reduce inflammatory responses [153]. Notably, some studies indicate that successful engraftment of mouse intestinal organoids often requires pre-induced inflammatory damage, while organoids derived from human embryonic stem cells may achieve engraftment in the absence of inflammation [154].

Immunodeficient recipients are commonly used to reduce rejection in xenotransplantation studies [155]. For future allogeneic transplantation in immunocompetent hosts, immunomodulatory regimens may be required, raising additional safety considerations (e.g., infection risk and long-term immune perturbation) [156]. Autologous approaches using patient-derived ASCs or iPSC-derived organoids may mitigate rejection risk, but introduce other translational challenges, including manufacturing time, cost, and release testing for safety and genetic stability [157].

4.2.2. Preliminary attempts toward clinical application

Encouraging animal data have motivated limited early-stage clinical exploration. For example, a group in Japan reported a clinical study investigating autologous colonic organoid–based transplantation for refractory ulcerative colitis, in which stem cells from non-inflamed colonic regions were expanded into organoid-derived epithelial sheets and transplanted endoscopically to ulcerated sites. Early observations primarily supported feasibility and short-term safety, with preliminary indications of mucosal repair that warrant confirmation in larger, controlled studies [158]. Organoid-based approaches have also been explored for repair of other epithelial tissues (e.g., salivary glands), providing additional translational insights into delivery and safety considerations [159].

4.3. Core challenges and countermeasures in clinical translation

Despite significant progress in intestinal organoid technology, the path from the laboratory to clinical application is fraught with obstacles. The main current challenges are as follows:

4.3.1. Vascularization dilemma: limiting organoid size and function

In vitro cultured organoids generally lack a vascular network, leading to necrosis in their inner core due to insufficient oxygen and nutrient supply. This greatly limits the size (usually between several hundred micrometers and one millimeter) and long-term survival capacity of organoids [160]. The absence of blood vessels also prevents them from fully simulating complex cellular interactions and physiological functions *in vivo* [161].

Countermeasures: Addressing the vascularization issue is a current research focus. Strategies include: (1) Co-culture: Co-culturing intestinal organoids with vascular endothelial cells (e.g., human umbilical vein endothelial cells (HUVECs)) and mesenchymal cells to induce spontaneous formation of vascular networks around or within organoids [162]. (2) Organ-on-a-Chip Technology: Using microfluidic chip perfusion culture to simulate blood flow, promoting angiogenesis and stabilization [163]. (3) Genetic Engineering: Overexpressing pro-angiogenic factors (e.g., VEGF) through gene editing technology to promote vascularization [164]. (4) 3D Bioprinting: Using bioinks to print complex structures containing cells and vascular channels [165].

Recent studies have used quantitative image analysis software (e.g., ImageJ, AngioTool) to accurately evaluate indicators such as total length and branch point density of vascular networks, providing quantitative basis for optimizing vascularization protocols [166].

4.3.2. Standardization of large-scale production and quality control

Clinical applications (especially for the treatment of short bowel syndrome) require a large number of high-quality organoids, which current culture methods are difficult to meet.

Large-Scale Production: As mentioned earlier, the development of automated bioreactors and suspension culture technology is the key path to achieving large-scale production. This not only increases yield but also reduces costs and human operation errors [167].

Quality Control (QC) and Release Standards: As a living “cell therapy product”, the safety and efficacy of organoid products are crucial [168]. Currently, there are no unified global standards for the production and quality control of clinical-grade organoids. The challenges include: (1) Lack of Clear Quality Control Parameters: How to define a “qualified” organoid? Critical Quality Attributes (CQAs) need to be clarified, such as morphological uniformity, proportion of specific cell types (e.g., $Lgr5^+$ stem cells), stability of gene expression profiles, barrier function (e.g., transepithelial electrical resistance), as well as sterility, endotoxin-free status, and non-tumorigenicity [169]. (2) Batch-to-Batch Variations: Due to changes in cell sources, culture conditions, and other factors, there are significant batch-to-batch variations in organoid production. Ensuring product consistency is a major challenge [170]. Establishing standardized operating procedures (SOPs) and strict release testing standards is an inevitable path.

4.3.3. Regulatory approval pathways and challenges

Regulatory science for organoid-based products is still evolving. In the United States, the FDA has increasingly emphasized the value of alternative methods and human-relevant models for preclinical evaluation, including organoid-based platforms, while guidance for organoid-derived therapeutic products continues to develop [171]. In China, regulatory authorities and professional organizations have

also begun to discuss quality standards and technical guidance relevant to organoid applications, although unified, product-specific regulatory pathways remain under active refinement.

Good Manufacturing Practice (GMP) Production Requirements: Any cell product used in humans must be produced in facilities that comply with GMP. This means that the entire process from cell acquisition, culture, and cryopreservation to final product preparation requires strict quality management and documentation, posing significant technical and financial barriers for most research laboratories.

Ethical Considerations: Particularly for iPSC-derived organoids, ethical issues related to embryonic stem cell research are involved. Establishing a sound ethical review and informed consent mechanism is an indispensable part of clinical translation.

Figure 3 illustrates the translational pipeline of ISC and organoid technologies from laboratory research to clinical application.

Stage 1: Laboratory research (*in vitro* culture). ISCs derived from patient biopsies or pluripotent stem cells are expanded in three-dimensional culture systems supplemented with defined growth factors to recapitulate the intestinal stem cell niche. Organoids undergo serial passaging, expansion, and phenotypic characterization, supported by microscopy-based analyses to assess morphology, differentiation status, and growth dynamics.

Stage 2: *In vivo* transplantation. Expanded organoids are prepared for delivery using hydrogels or scaffolds and transplanted into animal models. Following intestinal engraftment, organoid-derived cells integrate into host tissue, where vascularization, epithelial differentiation, and functional integration are evaluated to assess regenerative potential and tissue repair capacity.

Stage 3: Clinical application and key challenges. ISC- and organoid-based platforms enable applications in drug screening, disease modeling, and regenerative medicine, including personalized therapeutic testing. Major translational challenges include the lack of unified standardization, difficulties in large-scale production, and rigorous quality control requirements, which collectively limit widespread clinical implementation. Created with BioRender.com.

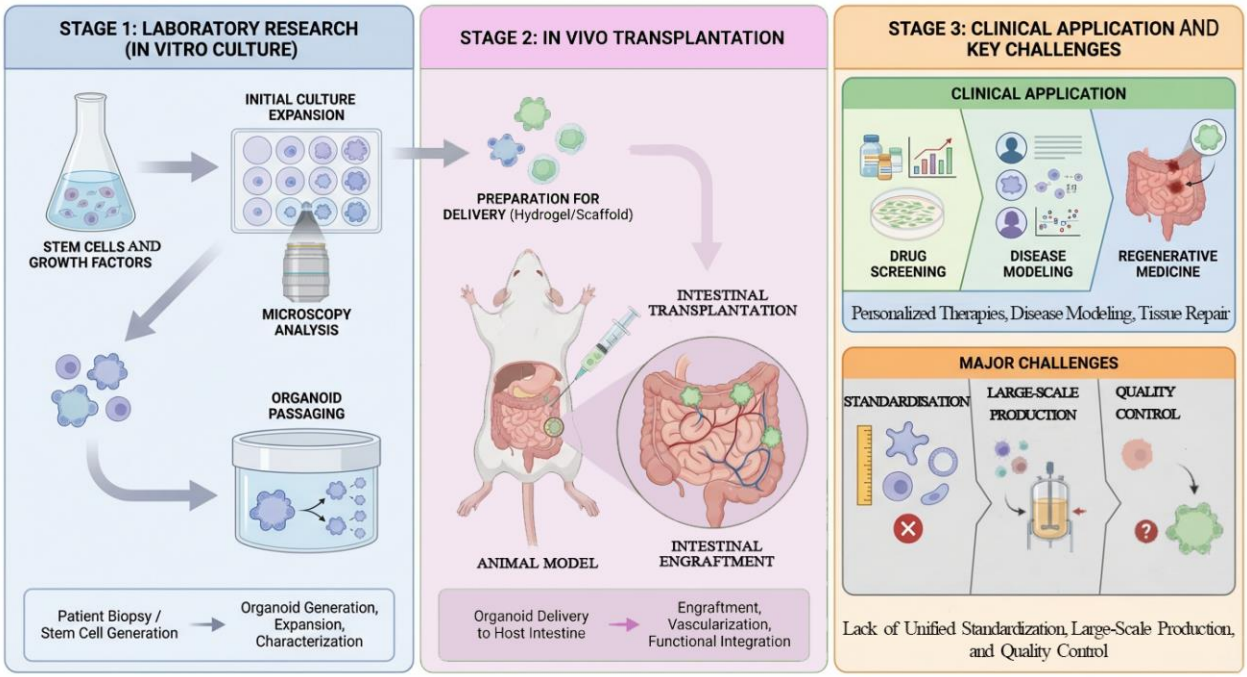

Figure 3. From *in vitro* culture to clinical application of ISCs and organoids.

5. Challenges and future directions in ISC therapy

Whilst ISC-targeted therapies represent a promising direction in CRC treatment, bridging the gap between fundamental discoveries and reproducible clinical benefit remains challenging. This involves not only scientific and technological bottlenecks, but also complex biological heterogeneity and ethical boundaries.

5.1. Individual variability for precision medicine: an ecosystem perspective beyond genomics

Precision medicine aims to match therapies to patient- and tumor-specific features rather than applying uniform regimens to all patients with the same diagnosis. However, patient stratification based solely on genomic sequencing (e.g., KRAS/BRAF mutation status) has proven inadequate, necessitating a broader perspective encompassing the entire tumor ecosystem.

Increasing evidence suggests that the gut microbiota can modulate treatment response and resistance in CRC, rather than acting solely as a bystander. Fusobacterium nucleatum activates CSC autophagy via the TLR4/MyD88 pathway in preclinical CRC models, thereby inducing chemoresistance to fluorouracil and oxaliplatin [172]. Furthermore, microbial metabolites (e.g., short-chain fatty acids) directly modulate the epigenetic state of ISCs, influencing their therapeutic sensitivity [173]. Future precision treatment regimens must incorporate microbiome assessment and may require adjunctive probiotics or faecal microbial transplantation to normalize the intestinal microenvironment, thereby disrupting the microbial basis of resistance.

Furthermore, while the aforementioned CMS classification provides macro-level guidance, it overlooks intra-tumoral spatial heterogeneity (ITH). Clinically, distinct clonal populations may exist between primary and metastatic sites in the same patient, or even within different regions of a single tumor. Consequently, future diagnostics may increasingly rely on longitudinal monitoring (e.g., liquid biopsy) rather than a single time-point assessment to detect MRD and adaptive evolution under treatment pressure [174].

5.2. Precision implementation: optimal target population and timing of intervention

Precision implementation: who to treat and when to intervene. The clinical value of CSC/ISC-directed strategies will likely depend on selecting (i) the right disease setting and (ii) the right biological state. First, the most actionable target populations include: (1) molecularly defined subgroups with tractable dependencies (e.g., KRAS^G12C CRC for KRAS+EGFR combinations, or APC/Wnt-addicted tumors for synthetic-lethal windows); (2) patients at high risk of relapse where CSC-like reservoirs are most consequential, such as ctDNA-defined MRD after surgery or poor-risk MSS/pMMR tumors with stemness/EMT/YAP–STAT3 programs; and (3) metastatic lesions with niche support (e.g., liver metastases) where organ-specific microenvironments reinforce stem-like states. Second, timing matters: CSC/ISC-targeted interventions may be most effective (a) early in the treatment course (neoadjuvant/adjuvant) before resistant clones dominate, (b) at MRD detection to prevent regrowth from minimal stem-like reservoirs, and (c) as rational combinations/sequences (e.g., “prime-then-kill” to transiently expose stem-like vulnerabilities followed by CSC-directed killing), guided by longitudinal biomarkers (ctDNA dynamics, stemness signatures, spatial immune context). This framework also provides testable explanations for prior disappointing results—namely insufficient biomarker

enrichment, antigen/state heterogeneity, and persistent niche-mediated rescue—suggesting that future trials should incorporate state-aware stratification and adaptive scheduling.

5.3. Ethical concerns and safety considerations in stem cell therapy

The clinical application of organoids and stem cell technologies presents unprecedented ethical and safety challenges, necessitating rigorous regulatory frameworks alongside technological advancement.

PDOs, serving as *in vitro* surrogates of patients, raise controversies regarding informed consent rights when stored long-term or used commercially. Do patients retain ownership over research outcomes derived from their cells? Should patients share in profits if organoids are used to develop new drugs? Furthermore, as organoid complexity increases, their bioethical status sparks debates concerning the rights of "quasi-living entities".

Beyond this, the foremost obstacle for drugs targeting stem cell-critical pathways like Wnt and Notch is "on-target toxicity." Since normal intestinal epithelial renewal similarly relies on these pathways, systemic administration frequently induces severe gastrointestinal side effects (e.g., crypt atrophy, mucosal ulceration) [175]. Future drug development must identify suitable therapeutic windows, such as employing synthetic lethal strategies (targeting specific vulnerabilities in APC-deficient cells) or developing smart delivery systems (e.g., pH-responsive nanoparticles) to achieve precise targeting with drug release confined to the tumor microenvironment [176,177].

5.4. Future research directions: integration of multi-omics

Over the past two to three years, increasing evidence has refined the understanding of ISC plasticity and its contribution to therapy resistance in colorectal cancer. Recent single-cell and spatial transcriptomic studies have revealed that stem-like states in CRC are highly dynamic and can be reversibly acquired in response to therapeutic stress, inflammatory cues, or niche-derived signals. Rather than representing a fixed cell population, CSC-like ISCs appear to occupy a spectrum of transitional states shaped by microenvironmental interactions.

Notably, emerging studies have demonstrated that chemotherapy and targeted therapies can actively induce dedifferentiation programs in non-stem tumor cells through activation of YAP/TAZ, STAT3, and NF-κB signaling, thereby generating regenerative stem-like populations that drive relapse. Parallel work has highlighted the role of niche components—including tumor-associated macrophages, cancer-associated fibroblasts, and endothelial cells—in sustaining these plastic states by providing cytokines (e.g., IL-6, TGF-β) and metabolic support that buffer therapeutic pressure.

Importantly, emerging data from patient-derived organoids, spatial multi-omics, and *in vivo* lineage-tracing models indicate that niche-driven resistance mechanisms are context-dependent and may vary across tumor stages and genetic backgrounds. These recent findings underscore the necessity of therapeutic strategies that simultaneously target intrinsic ISC plasticity programs and extrinsic niche-derived resistance signals, reinforcing the rationale for combination and sequential therapies in precision treatment of colorectal cancer.

Precisely due to the aforementioned challenges, future breakthroughs will no longer rely on advances in individual technologies, but rather on the deep integration of multidisciplinary techniques. This aims to construct a "holographic map" of CRC, thereby systematically deciphering its dynamic

evolution and therapeutic response mechanisms, laying the foundation for developing precision treatment strategies.

Whilst traditional single-cell sequencing (scRNA-seq) has revealed cellular diversity, it sacrifices crucial spatial information. Emerging spatial transcriptomics technologies now enable in situ observation of physical interactions between CSCs and their microenvironment (fibroblasts, immune cells, vasculature), elucidating how the spatial architecture of the "stem cell niche" determines CSC fate [178].

Moreover, single-dimensional data cannot elucidate complex resistance mechanisms. Future approaches must integrate genomic, transcriptomic, epigenomic (particularly chromatin accessibility via assay for transposase-accessible chromatin using sequencing (ATAC-seq)), and proteomic data. Analysing these vast datasets through AI and machine learning algorithms will identify key transcription factor networks driving CSC plasticity, predict cellular evolutionary trajectories under drug stress, and enable the design of combination therapies that block "cell state transitions".

6. Conclusion

6.1. ISCs and CSCs reshape the landscape of colorectal cancer therapy

Looking back over the past two decades, the research paradigm for CRC has undergone a profound transformation: our understanding of tumors has leapt from a singular "accumulation of gene mutations model" to a dynamic "stem cell ecosystem model". ISCs and their malignant counterparts—colorectal cancer stem cell (CRC-SCs)—have been increasingly recognized as key contributors to tumor initiation, progression, metastatic competence, and therapeutic resistance. The existing body of evidence clearly demonstrates that CRC is not a homogeneous mass of proliferating cells, but rather a complex organ driven by a hierarchically organized and highly plastic stem cell community [179].

Consequently, targeting ISC/CSC-associated programs is increasingly viewed as an important complement to existing modalities, with the potential to improve durability of response in selected CRC contexts. While conventional radiotherapy and chemotherapy effectively eliminate rapidly proliferating differentiated cancer cells, they often fail to reach quiescent or highly plastic stem cell subpopulations. As explored in this review, strategies such as employing "synthetic lethality" approaches to precisely target stem cells harboring APC or KRAS mutations, utilising the novel "sequential therapy" paradigm to block cellular dedifferentiation escape routes, and reshaping the immune microenvironment to transform stem cell "cold" barriers into "hot" targets for immune attack, are progressively establishing the framework for next-generation precision medicine. As a pivotal technology in this field, organoid technology not only bridges fundamental biology with clinical practice but also offers the potential for "surrogate drug testing" for each patient, bringing the vision of personalized medicine closer to reality than ever before.

6.2. Current limitations and future breakthroughs

Despite the encouraging prospects, we must remain acutely aware that the journey from laboratory to clinical application remains fraught with challenges. The foremost bottleneck in current research lies in the delicate balance between specificity and safety. Given that CRC-SCs largely retain the signaling dependencies of normal ISCs (such as the Wnt and Notch pathways), a critical challenge in drug

development remains how to eradicate tumor stem cells while preserving the regenerative capacity of normal intestinal epithelium and avoiding severe gastrointestinal toxicity. Moreover, tumor heterogeneity and plasticity far exceed expectations. Complex subclonal structures revealed by single-cell sequencing, coupled with the dedifferentiation of differentiated cells back into stem-like states under chemotherapy stress, indicate we face an evolving, moving target where monotherapy is inherently inadequate [180].

Looking ahead, breakthroughs will hinge on multidimensional, systematic technological convergence. Firstly, higher-precision targeting strategies must be developed. This encompasses not only identifying surface antigens or metabolic vulnerabilities unique to CRC-SCs—distinct from normal stem cells (such as specific lipid metabolism or ferroptosis susceptibility)—but also leveraging antibody-drug conjugates (ADCs) or bispecific antibodies for precise delivery. Secondly, dynamic monitoring and adaptive therapy will become standard practice. By integrating liquid biopsy (ctDNA) with spatial transcriptomics, we must track tumor clonal evolution under therapeutic pressure in real time, shifting from reactive response to proactive prediction to block escape pathways before tumor cells exploit plasticity. Finally, systemic combination therapy represents the central strategy. Future CRC treatment will no longer be a monotherapy solo act, but rather combined therapies integrating stem cell differentiation inducers, microenvironment modulators (targeting fibroblasts or the microbiome), and immune checkpoint inhibitors. Particularly by deciphering the complex interactions between the gut microbiota-neuro-immune axis and stem cells, we may uncover novel intervention targets.

In summary, precision therapy research centred on ISCs stands at the forefront of the convergence between fundamental science and clinical medicine. Although challenges remain formidable, the deep integration of multi-omics technologies and translational medicine supports the view that CRC management may become increasingly durable and personalized as biomarker-guided combinations, multi-omics integration, and translational platforms continue to mature.

Declaration of generative AI and AI-assisted technologies

During the preparation of this manuscript, the authors used generative AI tools only to improve language and readability. The authors take full responsibility for the content of the manuscript.

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Authors' contribution

Conceptualization, Pingping Zhu; methodology, Pingping Zhu; software, Ziliang Li; validation, Ziliang Li, Xi Li, Guo Qing, Jiao Lu, Yingjie Zhu, Jinhai Xu and Xinmeng Li; formal analysis, Ziliang Li; investigation, Ziliang Li, Xi Li, Guo Qing, Jiao Lu, Yingjie Zhu, Jinhai Xu and Xinmeng Li; resources, Pingping Zhu; data curation, Ziliang Li; writing—original draft preparation, Ziliang Li; writing—review and editing, Pingping Zhu; visualization, Xi Li and Guo Qing; supervision, Pingping Zhu; project

administration, Pingping Zhu; funding acquisition, Pingping Zhu. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

Pingping Zhu holds the position of Editorial Board Member for *Advanced Cancer Research* and has not peer reviewed or made any editorial decisions for this paper. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

Abbreviation	Full term
ASC	Adult stem cell
ATAC-seq	Assay for transposase-accessible chromatin using sequencing
BMP	Bone morphogenetic protein
CDC	Complement-dependent cytotoxicity
CRC	Colorectal cancer
CRC-SC	Colorectal cancer stem cell
CSC	Cancer stem cell
CTL	Cytotoxic T lymphocyte
EGF	Epidermal growth factor
EMT	Epithelial–mesenchymal transition
FDA	Food and Drug Administration
GMP	Good Manufacturing Practice
HNSCC	Head and neck squamous cell carcinoma
IFN	Interferon
IL	Interleukin
iPSC	Induced pluripotent stem cell
ISC	Intestinal stem cell
ITH	Intratumoral heterogeneity
JAK	Janus kinase
MEK	Mitogen-activated protein kinase kinase
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MRD	Minimal residual disease
NKG2D	Natural killer group 2D
NK	Natural killer
NMPA	National Medical Products Administration
ORR	Objective response rate
PDO	Patient-derived organoid
PDX	Patient-derived xenograft
PFS	Progression-free survival
QC	Quality control
RECIST	Response Evaluation Criteria in Solid Tumors
scRNA-seq	Single-cell RNA sequencing
STAT3	Signal transducer and activator of transcription 3
TAM	Tumor-associated macrophage
TCF	T-cell factor
TGF-β	Transforming growth factor beta
TGI	Tumor growth inhibition
TIME	Tumor immune microenvironment
TME	Tumor microenvironment
uPAR	Urokinase plasminogen activator receptor
VEGF	Vascular endothelial growth factor
Wnt	Wingless-related integration site
YAP	Yes-associated protein

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