CRISPR screens identify cholesterol biosynthesis as a therapeutic target on stemness and drug resistance of colon cancer

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INTRODUCTION
Colon cancer is one of the most commonly diagnosed malignancy worldwide. The current standard of care for patients with colon cancer is maximal surgical removal of tumor, followed by adjuvant chemotherapy or targeted therapy [1]. However, drug resistance and tumor recurrence represent major clinical challenges of colon cancer. CSCs, or so-called cancer-initiating cells, comprise a tiny fraction of a tumor, but they are highly tumorigenic cells essential for tumor maintenance and a root cause of therapy resistance and tumor relapse [2–5]. Therefore, targeting CSCs is an attractive approach to eradicate tumors and prevent tumor recurrence, in contrast to conventional therapies that mostly target bulk tumor cells [6, 7]. For colon cancer, CSCs have been isolated with non-uniform surface markers, including CD133 [2, 3], Lgr5 [8], EpCAM, CD44, CD166 [9], ALDH [10], and EphB2 [5]. CSCs from primary colon tumors can be enriched and maintained by the well-established three-dimensional (3D) spheroid culture system, which preserves the capacity to re-establish cellular hierarchy recapitulating that of parental tumor and patient-specific genotypic and epigenetic signatures [2, 9, 11, 12]. These in vitro CSC-enriched spheroid models are clinically relevant in terms of their intrinsic resistance to conventional drugs [13]. In this study, we aim to uncover genetic vulnerabilities of colon CSC-enriched spheroids with a novel high-throughput CRISPR dropout screen.

CRISPR/Cas9-based dropout screening has emerged as a powerful tool for systematic identification of essential/fitness genes governing cell proliferation and survival [14–17]. In this study, we constructed an Epi-Drug single-guide RNA (sgRNA) library comprising of focused druggable genome targeted by FDA-approved drugs, together with numerous epigenetic regulators. Our Epi-Drug library enables the rapid prioritization of existing drug targets and drug repositioning for novel indications. In two colon cancer patient-derived CSC-enriched spheroid models, we performed Epi-Drug CRISPR dropout screens and identified potential druggable targets that could synergize with conventional chemotherapy. Our data provide a rationale for the novel druggable targets in colon cancer, especially for cell subpopulations harboring stemness and drug resistance characteristics.
RESULTS
Epi-Drug CRISPR dropout screens identify genetic vulnerabilities of colon CSC-enriched spheroids
In this study, we utilized two well-characterized, low-passage, patient-derived colon CSC-enriched spheroid lines, POP92 and POP66 (Fig. 1A) [11, 13]. Flow cytometry analysis confirmed >50% positivity in the stemness markers CD133 or CD44 in POP66/POP92 spheroids (Supplementary Fig. 1A). In addition, these stemness markers are functionally critical for maintenance of CSC-enriched spheroids, as CD133 knockout in spheroids inhibited cell proliferation, impaired sphere-formation ability, and induced cell differentiation (Supplementary Fig. 1B–D). To unravel druggable essential genes in these characterized CSC-enriched spheroids, we performed CRISPR/Cas9 dropout screening using our in-house Epi-Drug library comprising of over 12,500 sgRNAs targeting 657 Drugbank-based targets and 317 epigenetic regulators (Fig. 1B). We first generated stable Cas9-expressing spheroid models and confirmed the highly effective endonuclease activity with two sgRNAs targeting an individual gene METTL3 (Fig. 1C). Lentiviral Epi-Drug library was transduced into Cas9-expressing POP92 and POP66 spheroids at a low multiplicity of infection (MOI) of 0.3, and the abundance of individual sgRNAs at days 8 and 16 was quantified by next-generation sequencing compare to baseline pool. As shown in Fig. 1D, we observed the gradual depletion of sgRNAs over time in culture. Using the MAGEK algorithm, we identified 121 and 59 essential genes depleted in POP92 and POP66 at a P value threshold of 0.01, respectively (Fig. 1E and Supplementary Tables S1, S2). We compared our list of essential genes to CSciDB, a database that includes 74 CSC markers and 1769 functional regulatory genes [18], and identified a significant overlap between the two datasets (Fig. 1E), implying that our screen successfully validated some well-known CSC regulators (e.g., mTOR, IGF1R, METTL3, HDAC3) [19–23].

Cholesterol biosynthesis pathway is essential for colon CSC-enriched spheroids
There were 44 common dropout essential genes in POP92 and POP66 (Fig. 2A, Table 1, and Supplementary Table S3), and pathway enrichment analysis revealed several significantly enriched terms, including cholesterol biosynthesis (Fig. 2B). Within the cholesterol biosynthesis pathway, HMGCR, FDPS, and GPPS1 were top-ranked essential genes for both POP92 and POP66, while SQLE and CYP51A1 were also important for POP92 (Fig. 2C). We next determined the “druggability” of the 44 common dropout genes using Drug Gene Interaction database and identified 23 genes that could be classified as “druggable” [24], including HMGCR and FDPS (Fig. 2D). HMGCR, known as HMG-CoA reductase, is the first rate-limiting enzyme for the mevalonate pathway that produces endogenous cholesterol (Fig. 2E). On the other hand, FDPS catalyzes the generation of farnesyl pyrophosphate (FPP) and GGPP, intermediates of cholesterol biosynthesis that serve as substrates for protein post-translational modification (Fig. 2E) [25]. Taken together, our findings indicate that cholesterol biosynthesis pathway is essential for the growth of colon CSC-enriched spheroids.

Cholesterol biosynthesis genes are overexpressed in human colon cancer and CSC-enriched spheroids
In light of our findings, we next investigated the potential role of cholesterol biosynthesis pathway in human colon cancer. We examined the expression of several key cholesterol biosynthetic enzymes (HMGCR, FDPS, and SQLE) in paired colon tumor and non-tumor tissues performed by western blot (Fig. 3A) and immunohistochemistry analysis (Supplementary Fig. 2A, B). Consistent with our hypothesis, all three genes are significantly upregulated in colon tumors compared to paired non-tumor tissues.

To investigate if cholesterol biosynthesis pathway is selectively upregulated in colon CSCs, we induced differentiation of CSC-enriched spheroids by culturing them in 2-dimensional adherent culture flasks with 10% FBS-containing medium for 10 days (Fig. 3B). Differentiation was validated by the reduced expression of stemness markers including CD133, CD44, Lgr5, ALDH1A1, and EphB2 (Fig. 3C, D). We next compared the expression of cholesterol biosynthetic enzymes in 3D CSC-enriched spheroids and their differentiated counterparts. Increased protein expression of several key genes including HMGCS1, HMGCR, SQLE, and FDPS was found in 3D CSC-enriched spheroids (Fig. 3D). In addition, marked and coordinated upregulation of most cholesterol biosynthesis enzymes were validated in 3D CSC-enriched spheroids by real-time PCR (Fig. 3E). Consistent with the augmented expression of cholesterol biosynthesis genes, total intracellular cholesterol content was significantly increased in both POP92 and POP66 spheroids as compared to their differentiated counterparts (Fig. 3F). These results indicate that cholesterol biosynthesis is markedly upregulated in colon CSC-enriched spheroids.

We then analyzed the correlation between cholesterol biosynthesis pathway and CSC markers in human colon cancer samples. We analyzed the correlation between stemness markers and cholesterol biosynthesis genes in TCGA colorectal cancer (COADREAD) cohort and revealed positive correlations between CSC markers (EphB2 and CD44) and cholesterol biosynthesis genes (HMGCR, HMGCS1, FDPS, and FDT1) (Supplementary Fig. 3A). As EphB2 is highly correlated with cholesterol biosynthesis genes at mRNA level, we thus determined the correlation of cholesterol biosynthesis genes with EphB2 in a cohort of 21 human colon tumors by western blot and demonstrated that HMGCS1, HMGCR, FDPS, and SQLE positively correlated with EphB2 protein expression (Fig. 3G). In agreement with our data, analysis of the TCGA reverse phase protein array dataset revealed positive associations between several cholesterol biosynthetic enzymes and EphB2 (Supplementary Fig. 3B). Previous work has indicated EphB2 as a surface marker for stem-like tumor cells with robust tumor-initiating capacity and long-term self-renewal potential in human colorectal cancer [5]. Together, cholesterol biosynthesis activation is associated with cancer stemness traits in colon cancer.

Genetic or pharmacological blockade of cholesterol biosynthesis impairs self-renewal and tumorigenic potential of CSC-enriched spheroids
To validate the functional importance of the cholesterol biosynthesis pathway in colon CSC-enriched spheroids, we evaluated the effect of HMGCR or FDPS knockout in POP92, POP66, and two additional 3D spheroid models CSC28 and LS174T-S. Two specific sgRNAs were designed to target HMGCR and FDPS, respectively, and both sgRNAs ablated protein expression of their target genes in four colon CSC-enriched spheroid models (Fig. 4A). Depletion of HMGCR or FDPS significantly inhibited CSC-enriched spheroid growth, as determined by cell viability assay (Fig. 4B). Apoptosis assay demonstrated that HMGCR or FDPS knockout both induced a significant increase of early and late apoptotic cells (Fig. 4C). In addition, we found that loss of HMGCR or FDPS significantly impaired sphere formation (Fig. 4D, E) and self-renewal capacity (Fig. 4F) as determined by sphere formation and limiting dilution assays (LDAs). Corroborating our in vitro findings, HMGCR or FDPS deletion significantly inhibited tumor growth in subcutaneous xenograft assay in nude mice (Fig. 4G). To pinpoint the metabolites involved in self-renewal capacity of colon CSCs, we performed rescue assays with downstream metabolites FPP, GGPP, and cholesterol in HMGCR-depleted CSC-enriched spheroids. GGPP and cholesterol, but not FPP, rescued growth inhibitory effect of HMGCR knockout in colon spheroids, implying the
Targeted CRISPR-Cas9 dropout screens identify unique genetic vulnerabilities in patient-derived colon CSC-enriched spheroids.

A. Experimental workflow of the targeted CRISPR-Cas9 dropout screens. B. Composition of the Epi-Drug sgRNA library. C. Validation of the endonuclease activity of Cas9 protein with two sgRNAs targeting an individual gene METTL3 in two Cas9-expressing spheroids. D. Distribution of sgRNA reads per gene at indicated time points post-transfection. E. Volcano plots showing the genes significantly depleted in POP92 and POP66. Genes with P value < 0.01 were considered as essential for colon CSC-enriched spheroids propagation in vitro (threshold indicated by dashed line). Depleted genes were overlapped with a public CSC database, with matching genes highlighted in red. Source data of all genes are provided in Supplementary Tables S1 and S2.
requirement of cholesterol and protein prenylation for the growth of CSC-enriched spheroids in vitro (Fig. 4H, I).

Given the important role of HMGCR and FDPS in the maintenance of colon CSC-enriched spheroids, we next determined if pharmacological targeting of these two genes could suppress their survival. As expected, both lovastatin (HMGCR inhibitor) and zoledronate acid (FDPS inhibitor) significantly inhibited colon spheroid growth in a time- and dose-dependent manner (Fig. 5A). Lovastatin or zoledronate acid also induced cell apoptosis (Fig. 5B), and impaired sphere-formation of colon CSC-enriched spheroids in vitro (Fig. 5C). In addition, both lovastatin and zoledronate acid exerted antitumor growth effect in vivo, with little effect on body weight of mice (Fig. 5D). Consistently, either HMGCR or FDPS depletion reduced intracellular cholesterol levels in POP92 and POP66 spheroids, and POP92-derived xenografts in vivo (Supplementary Fig. 4A, B). Supplementation with cholesterol or GGPP rescued impaired growth of colon CSC-enriched spheroids treated with lovastatin (Fig. 5E, F), confirming the role of cholesterol biosynthesis pathway-derived metabolites in the survival of CSC-enriched spheroids. NCM460, a normal colon epithelial cell line, was less sensitive to lovastatin or zoledronate acid treatment (Supplementary Fig. 5). And lovastatin or zoledronate acid treatment had no significant effect on mice body weight, indicating a potential therapeutic window. Collectively, these results demonstrate the role of cholesterol biosynthesis pathway in contributing to metabolites required for survival and pluripotency of colon CSC-enriched spheroids.

Fig. 2 Analysis of essential genes for colon CSC-enriched spheroids expansion in vitro. A Venn diagram showing common essential genes in both CSC-enriched spheroid models, as listed in Table 1 and Supplementary Table S3. Genes are ranked by false discovery rate (FDR) in both models. B Gene set enrichment analysis of 44 common essential genes for both spheroid models. Pathways with FDR < 0.05 are shown. C Frequency histograms indicating distribution of CRISPR scores for all sgRNAs. Bottom panel shows distribution of individual sgRNAs (red lines) for genes involved in cholesterol biosynthesis pathway. D Drug Gene Interaction database (DGIdb) [24] categorization of the essential genes based on potential druggability defined by the DGIdb. Three categories are depicted. E Schematic diagram of the cholesterol biosynthesis pathway, also known as mevalonate pathway.
Cholesterol biosynthesis pathway inhibition deregulates metabolic- and TGF-β-associated signaling cascades in colon CSC-enriched spheroids

To probe the molecular basis underlying cholesterol biosynthesis is important for colon CSC-enriched spheroids, we evaluated the effect of TGF-β on the self-renewal capacity. CSC28 and POP66 were treated with TGF-β1 or a TβR inhibitor to induce or suppress TGF-β signaling, respectively. TGF-β1 attenuated cell viability and sphere formation in both models, while TβR inhibitor exerted an opposite effect (Fig. 6F). Moreover, the blockade of HMGCR or FDPS-knockout-induced TGF-β signaling by TβR inhibitor (Fig. 6G) rescued cell viability and sphere-forming capacity (Fig. 6H, I) of CSC28 and POP66, indicating that TGF-β signaling functions downstream of cholesterol biosynthesis pathway to modulate CSC self-renewal. Overall, our data indicate that cholesterol biosynthesis pathway represses TGF-β signaling to induce self-renewal capacity of colon CSC-enriched spheroids.

Cholesterol biosynthesis inhibitors synergize with chemotherapeutics to suppress colon cancer growth

Cancer stemness key drivers of therapeutic resistance in various cancers, including colon cancer [4, 30]. Concordantly, we found that POP92 and POP66 spheroids were considerably more resistant to conventional chemotherapeutic drugs, such as 5-FU and oxaliplatin, as compared to HCT116, HT29, and SW480 cell lines (Supplementary Fig. 7A, B). Given that cholesterol biosynthesis inhibitors effectively suppressed POP92 and POP66 spheroid growth and self-renewal as shown in Fig. 5A–F, we thus hypothesized that lovastatin and zolendronate acid treatment could sensitize them to conventional therapy. We therefore treated POP92 and POP66 spheroids with lovastatin or zolendronate alone or in combination with 5-FU, a commonly employed chemotherapeutic drug in colon cancer (Fig. 7A). Both lovastatin and zolendronate acid co-operatively inhibited POP92 and POP66 viability in conjunction with 5-FU, as evidenced by combination indices of <1 for all drug combinations (Fig. 7A). The co-treatment of lovastatin or zolendronate acid with 5-FU significantly reduced the sphere-formation capacity of POP92 and POP66 spheroids compared to single drug treatment (Fig. 7B), implying synergy between cholesterol biosynthesis inhibition and 5-FU. To determine whether cholesterol biosynthesis inhibitors confer a therapeutic benefit in combination with 5-FU in colon cancer, we determined the effect of the aforementioned drug combination(s) in three colon cancer patient-derived organoids (PDOs). Consistently, cholesterol biosynthesis inhibitors enhanced the anticancer effect of 5-FU in all the PDOs (Fig. 7C). Microscopic images and H&E staining showed decreased organoid-forming cells and impaired organoid structure in PDO

Table 1. Essential genes for both colon CSC-enriched spheroids propagation in vitro.

| Rank | Gene symbol | Rank | Gene symbol | Rank | Gene symbol |
|------|-------------|------|-------------|------|-------------|
| 1    | ATP6V1B2    | 16   | WDR5        | 31   | HUWE1       |
| 2    | WDR75       | 17   | DR1         | 32   | RTF1        |
| 3    | ACTL6A      | 18   | NOP2        | 33   | POLA1       |
| 4    | FDPS        | 19   | DDB1        | 34   | CHEK1       |
| 5    | METTL3      | 20   | RPL3        | 35   | FBL         |
| 6    | GGP51       | 21   | WDR74       | 36   | TAF1        |
| 7    | HDAC3       | 22   | RUVBL2      | 37   | RRM1        |
| 8    | FTSJ3       | 23   | RPL8        | 38   | RUVBL1      |
| 9    | TUG81       | 24   | PHB         | 39   | TRMT112     |
| 10   | HCFC1       | 25   | HMGCR       | 40   | U2AF1       |
| 11   | WDR82       | 26   | SUSD3       | 41   | RBBP5       |
| 12   | PRMT5       | 27   | NAA50       | 42   | TUBB3       |
| 13   | METAP2      | 28   | RPL4        | 43   | PSM2D2      |
| 14   | POLR1C      | 29   | DMAP1       | 44   | BRAF        |
| 15   | RPL19       | 30   | RNF20       |      |             |

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828 after treatment with 5-FU plus lovastatin or zoledronate acid (Fig. 7D, E). To corroborate our in vitro data, we determined the efficacy of 5-FU, lovastatin or their combination in low dosage using POP92-derived xenografts in nude mice. As shown in Fig. 7F, 5-FU or lovastatin treatment alone slightly inhibited tumor growth, whereas their combination significantly suppressed xenograft growth compared to other groups. Collectively, these data indicate targeting cholesterol biosynthesis pathway sensitizes CSC-enriched spheroids to chemotherapy.

Finally, we determined whether targeting cholesterol biosynthesis pathway is a viable approach in drug-resistant cells. To this end, we evaluated the growth inhibitory effect of cholesterol biosynthesis inhibitors in two drug-resistant cell lines, HCT15/FU and SW620/FU, which were resistant to 5-FU. Cell viability assays
showed that lovastatin or zoledronate both significantly impaired cell viability of HCT15/FU and SW620/FU (Supplementary Fig. 8). Hence, cholesterol biosynthesis represents a druggable target for colon cancer cells exhibiting stemness or drug resistance characteristics.

**DISCUSSION**

Cancer stemness are considered the basis of drug resistance, tumor relapse, and metastasis. Hence, it is critical to unravel novel molecular targets to eradicate cells with stemness characteristics. Here, we utilized a CRISPR/Cas9 Epi-Drug library to systematically...
screen genetic vulnerabilities in colon CSC-enriched spheroid models and identified that cholesterol biosynthesis pathway is essential for spheroid survival. We validated functional role of cholesterol biosynthetic genes HMGCR and FDPS for colon CSC-enriched spheroid viability and self-renewal; and established druggability of the two targets with FDA-approved drugs. Finally, we demonstrated that combining cholesterol biosynthesis inhibitor with conventional chemotherapy was synergistic in suppressing CSC-enriched spheroids and overcomes drug resistance in colon cancer cells, implying that cholesterol biosynthesis is a druggable pathway for the chemosensitization of colon cancer.

Our targeted CRISPR screens identified cholesterol biosynthesis as essential for survival and pluripotency in colon cancer patient-derived CSC-enriched spheroids. Concordantly, we found the upregulation of cholesterol biosynthesis genes in CSC-enriched spheroids and primary colon tumors. Genetic ablation or pharmacological inhibition of two critical molecular targets, HMGCR and FDPS, confirmed functional importance of this pathway in colon CSC-enriched spheroids. Cholesterol biosynthesis pathway, also known as the mevalonate pathway, is a multistep process that produces cholesterol and other intermediates such as GGPP, FPP, and sterols. We found that supplementation with cholesterol or GGPP rescued growth and pluripotency of colon CSC-enriched spheroids with knockout or blockade of HMGCR, thus substantiating that cholesterol biosynthesis-derived intermediate metabolites are directly involved in the maintenance of colon cancer stemness.
Our results indicate that cholesterol biosynthesis-mediated cancer stemness might involve two distinct mechanisms. The first one is directly associated with cholesterol, while an alternative mechanism involves GGPP-dependent protein prenylation. In agreement with our findings, multiple lines of evidence suggest the role of cholesterol in intestinal CSCs regulation. For example, cholesterol was found to promote intestinal stem cell growth and tumorigenesis in intestinal-specific Sreb2 transgenic mice; and biosynthesis of cholesterol played a determinant role for tumor formation in Apcmin/+ mice model [31]. Apart from intestinal CSCs, cholesterol biosynthesis was shown to enhance self-renewal and tumorigenic capacity of mammosphere and neurosphere [32–34], implying an important role of cholesterol in pluripotency in multiple malignancies. GGPP, on the other hand, serves as a substrate for prenylation of small GTPases, such as Ras, Rho, and Rac [25], as a prerequisite step necessary for membrane localization and activation. GTPases activation also endows cancer stemness properties, as reported by others [32]. Taken together, cholesterol biosynthesis pathway is activated in colon CSC-enriched spheroids to sustain pluripotency, with cholesterol and GGPP as the major effectors.

The underlying mechanism linking cholesterol biosynthesis pathway to colon CSC-enriched spheroids survival is unclear. We identified TGF-β signaling as a mechanistic link between upregulated cholesterol biosynthesis and colon CSC-enriched spheroids survival by RNA sequencing analysis. Cholesterol deprivation by genetic inhibition or pharmacological blockade of HMGCR or FDPS activated TGF-β signaling to repress the expression of ID proteins, which has an established role in driving stemness [11, 35, 36]. Consistently, TGF-β impaired self-renewal capacity, while the inhibition of TβR promoted spheroid growth and rescued the inhibitory effect of cholesterol deprivation.

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However, in the context of colon CSC-enriched spheroids, cholesterol appears to be the active metabolite, as direct cholesterol addition overcomes the functional effects of HMGCR/FDPS knockout. In agreement with our data, cholesterol itself has been shown to promote intestinal stem cell proliferation and tumorigenesis in Apc<sup>min</sup> mice [31]. Several studies have implied a potential role of cholesterol in the regulation of TGF-β signaling. For instance, cholesterol could induce the rapid degradation of TGF-β receptors.

Fig. 7  Cholesterol biosynthesis inhibitors synergize with 5-FU to suppress colon cancer in vitro and in vivo. A The dose–response curve of indicated drug or drug combination(s) was plotted and half maximal inhibitory concentration (IC50) was determined. Combination index (CI) for 5-FU and cholesterol biosynthesis inhibitors (lovastatin and zoledronate acid) were calculated according to Chou–Talalay’s method at 72-h timepoint. CI < 1, CI = 1, and CI > 1 indicate synergistic, additive, and antagonistic effects, respectively. B Sphere-formation frequency of colon CSC-enriched spheroids after treatment with 5-FU and lovastatin/zoledronate acid, alone or in combination. C Cell viability of colon cancer PDOs after treatment with 5-FU and lovastatin/zoledronate acid, alone or in combination. D Microscopic images and E H&E staining of colon cancer PDO 828 treated with 5-FU and lovastatin/zoledronate acid, alone or in combination at indicated concentrations for 6 days. F In vivo xenograft assay in nude mice bearing colon CSC-enriched spheroids-derived tumors. Mice were treated with vehicle, 5-FU, lovastatin, or 5-FU plus lovastatin at low dosages as indicated. Representative images, tumor volume, and tumor weight were shown. Error bar, mean ± SD. For F, error bar, mean ± SEM. *P < 0.05 (one-way ANOVA).
In agreement with our observations, others showed that cholesterol biosynthesis of cholesterol biosynthesis appeared safe for mice in our study. We systematically identify and formulate the inhibitory effect of meta-analyses of clinical trials have shown that statins are targeting of colon cancer. Indeed, epidemiological studies and strategy for treating cancer [6], as both CSCs and non-CSCs are therapy and conventional chemotherapy represent a promising CSC-enriched spheroids were highly resistant to 5-FU and oxaliplatin in vitro and blocking this cholesterol response was further shown to reported in acute myelocytic leukemia (AML). Cholesterol levels are impaired self-renewal and augmented cell differentiation by cholesterol biosynthesis inhibitors. Such phenomenon was also observed in pancreatic cancer cells [43]. Cholesterol thus negatively modulates TGF-β response holds promise for colon cancer treatment. Our work suggests a novel therapeutic approach for colon cancer spheroids self-renewal in vitro and tumorigenic potential in vivo. Others have also proposed statins or bisphosphonates as potential antineoplastic agents in a variety of cancers [45–47]. Nevertheless, we systematically identify and formulate the inhibitory effect of these drugs on colon CSC-enriched spheroids. Importantly, targeting of cholesterol biosynthesis appeared safe for mice in our study. In agreement with our observations, others showed that cholesterol biosynthesis inhibition did not impair normal intestinal crypt proliferation [31], implying a therapeutic window for selective targeting of colon cancer. Indeed, epidemiological studies and meta-analyses of clinical trials have shown that statins are chemopreventive and protective against colon cancer [48–50]. Our results thus advocate for the strategy of utilizing statins to target colon cancer cells with stemness traits.

Numerous studies have reported that traditional chemotherapy preferentially targets fast-proliferating differentiated cancer cells, whilst sparing CSCs [13]. Concordantly, we demonstrated that colon CSC-enriched spheroids were highly resistant to 5-FU and oxaliplatin compared to colon cancer cell lines. Combinatorial CSC-targeting therapy and conventional chemotherapy represent a promising strategy for treating cancer [6], as both CSCs and non-CSCs are simultaneously targeted. We found a synergistic anticancer effect between cholesterol biosynthesis inhibitors (lovastatin/zoledronate acid) and chemotherapy in colon CSC-enriched spheroids, primary colon tumor organoids, and spheroid-derived xenografts, all of which models the heterogenous nature of CSCs and non-CSCs population in human tumors. Such synergism is likely the consequence of impaired self-renewal and augmented cell differentiation by cholesterol biosynthesis inhibitors. Such phenomenon was also reported in acute myelocytic leukemia (AML). Cholesterol levels are abnormally induced in AML cells after exposure to chemotherapy in vitro and blocking this cholesterol response was further shown to sensitize AML cells to standard therapeutic drugs [51]. Collectively, our work suggests a novel therapeutic approach for colon cancer treatment.

In conclusion, our work highlighted a crucial role of cholesterol biosynthesis pathway in the maintenance of colon CSC-enriched spheroids and established this pathway as an attractive therapeutic target for the eradication of colon cancer cells with stemness and drug resistance traits. The combination of cholesterol biosynthesis inhibitors plus conventional chemother- apy holds promise for colon cancer treatment.

TfRs localized in lipid rafts and repress TGF-β responsiveness. Conversely, the inhibition of cholesterol biosynthesis pathway markedly increased the expression of TfRs, TGF-β1, and TGF-β in different cell types [39–42]. Disruption of cholesterol biosynthesis by Nsdhl knockout or statins treatment also induced SREBP1-dependent Tgfbr1 expression and autocrine TGF-β1-SMAD2/3 signal- ing in pancreatic cancer cells [43]. Cholesterol thus negatively modulates TGF-β signaling via diverse mechanisms, which in turn, can promote the survival of CSC-enriched spheroids.

Cholesterol biosynthesis pathway represents an attractive therapeutic target, as it has well-defined molecular targets and FDA-approved drugs that enables potential drug repurposing. HMGCR is a primary target of statins, a large class of cholesterol-lowering drugs commonly prescribed to individuals with hypercholesterolemia, whereas FDPS is targeted by bisphosphonates, a class of drugs used for treating bone diseases [44]. Here, we demonstrated that therapeutic targeting of HMGCR or FDPS with lovastatin or zolendronic acid effectively suppressed colon cancer spheroids self-renewal in vitro and tumorigenic potential in vivo. Others have also proposed statins or bisphosphonates as potential antineoplastic agents in a variety of cancers [45–47]. Nevertheless, we systematically identify and formulate the inhibitory effect of these drugs on colon CSC-enriched spheroids. Importantly, targeting of cholesterol biosynthesis appeared safe for mice in our study. In agreement with our observations, others showed that cholesterol biosynthesis inhibition did not impair normal intestinal crypt proliferation [31], implying a therapeutic window for selective targeting of colon cancer. Indeed, epidemiological studies and meta-analyses of clinical trials have shown that statins are chemopreventive and protective against colon cancer [48–50]. Our results thus advocate for the strategy of utilizing statins to target colon cancer cells with stemness traits.

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**Materials and Methods**

**Cell culture and reagents**

Human colon CSC-enriched spheroid models (POP92, POP66, CSC28, LS174T-S) and colon cancer PDOs (PDO 828, 1482, 816) were kindly provided by Dr CAOB in Princess Margaret Cancer Center, University of Toronto. POP92, POP66, and CSC28 were derived from a primary colon tumor specimen and two liver metastases of colon adenocarcinoma, respectively, and cultured in 3D suspension state. Sphere number and diameter were evaluated after 5–7 days of culture. For in vitro LDAs, viable spheroid cells were sorted into 96-well plates at densities of 10, 5, or 1 cell per well, using FACS cell sorters, followed by drug treatments. About 2 weeks later, wells containing spheres were counted and the results were analyzed using a web-based tool (ELDA, http://bioinf.wehi.edu.au/software/elda/index.html).

**CRISPR dropout screens**

Pooled Epi-Drug sgRNA library was designed and cloned into lentivirus plasmid by He lab. Colon CSC-enriched spheroids were transduced with the predetermined volume of the pooled sgRNA lentiviral supernatant. About 1 day after transduction, the cells were selected with puromycin for 2 days and further cultured in stem cell primitive medium without puromycin. Approximately 15 million cells were harvested for DNA extraction, next-generation sequencing, and data analysis on “Day 0,” “Day 8,” and “Day 16.” See also Supplementary Materials and Methods.

**Sphere-formation assays and in vitro LDAs**

Colon CSC-enriched spheroids were digested into single cell suspension, counted, and seeded in 6-well plates at 3000 cells per well in suspension state. Sphere number and diameter were evaluated after 5–7 days of culture. For in vitro LDAs, viable spheroid cells were sorted into 96-well plates at densities of 10, 5, or 1 cell per well, using FACS cell sorters, followed by drug treatments. About 2 weeks later, wells containing spheres were counted and the results were analyzed using a web-based tool (ELDA, http://bioinf.wehi.edu.au/software/elda/index.html).

**Xenograft assays**

The xenograft animal experiments were carried out under the approval of Animal Experimentation Ethics Committee in the Chinese University of Hong Kong (Ref no. 19-098-MIS). Colon CSC-enriched spheroids (1 × 10^6 cells) for tumor formation (POP92 and LS174T-S, 5 × 10^5 cells for CSC28) were subcutaneously injected into right flanks of 4–6-week-old male nude mice. To investigate the tumorigenesis ability after cholesterol biosynthesis genes deletion, cells with/without HMGCR/FDSPs were injected subcutane- ously (n = 6 or 7), and tumors were measured and recorded every 2–3 days until the end point. For drug treatment assays, tumor-bearing mice were randomized into control group and treatment group when the average tumor volume reached 30–60 mm^3 (4–8 mice per group). To test the anticancer effect of single drug treatment, lovastatin (50 mg/kg per day, oral gavage), or zoledronate acid (100 µg/kg per day, subcutane- ously) was given to mice in treatment groups (n = 4). To investigate combinatorial effect of 5-FU and lovastatin, four groups (n = 7) were included: control (olive oil per day + saline, 5 times), 5-FU (30 mg/kg/day, 5 times, intraperitoneal, i.p.), lovastatin (30 mg/kg/day) or their combination (5-FU 30 mg/kg/day, 5 times, i.p. and lovastatin 30 mg/kg/day). Body weight and tumor volume were measured every 2–3 days until the end point.

**RNA sequencing data analysis**

Total RNA from POP92 cells were sequenced on HiSeq2500 (Novogene, Beijing, China) after the indicated treatments. Reads were aligned with HISAT2 v2.1.0 to hg38 human reference genome, and raw count of each sample was evaluated by StringTie v0.11.2. R DESeq2 package was employed for the normalization and transformation of gene expression. GSEA was also performed using gene sets from KEGG pathways in the Molecular Signatures Database [52].
Statistics
All experiments were done at least twice independently. Statistical analyses were justified as appropriate in terms of assumptions of tests and variation estimate, performed on the software Prism 7 (GraphPad). Two-tailed unpaired t-tests or paired t-tests were performed for comparisons of two groups. One-way ANOVA (for more than 2 group comparisons) was performed followed by the Bonferroni multiple comparison post test. For all comparisons, a P value of <0.05 was considered statistically significant. Data are presented as mean ± SD (ns not significant, *P < 0.05).

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COMPETING INTERESTS
The authors declare no competing interests.

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