Selective targeting of human colon cancer stem-like cells by the mTOR inhibitor Torin-1

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ABSTRACT:

Metastatic colorectal cancer (CRC) is incurable for most patients. Since mammalian target of rapamycin (mTOR) has been suggested as a crucial modulator of tumor biology, we aimed at evaluating the effectiveness of mTOR targeting for CRC therapy. To this purpose, we analyzed mTOR expression and the effect of mTOR inhibition in cancer stem-like cells isolated from three human metastatic CRCs (CoCSCs).

CoCSCs exhibited a strong mTOR complex 2 (mTORC2) expression, and a rare expression of mTOR complex 1 (mTORC1). This latter correlated with differentiation, being expressed in CoCSC-derived xenografts. We indicate Serum/glucocorticoid-regulated kinase 1 (SGK1) as the possible main mTORC2 effector in CoCSCs, as highlighted by the negative effect on cancer properties following its knockdown. mTOR inhibitors affected CoCSCs differently, resulting in proliferation, autophagy as well as apoptosis induction. The apoptosis-inducing mTOR inhibitor Torin-1 hindered growth, motility, invasion, and survival of CoCSCs in vitro, and suppressed tumor growth in vivo with a concomitant reduction in vessel formation. Torin-1 also affected the expression of markers for cell proliferation, angio-/lympho-genesis, and stemness in vivo, including Ki67, DLL1, DLL4, Notch, Lgr5, and CD44. Importantly, Torin-1 did not affect the survival of normal colon stem cells in vivo, suggesting its selectivity towards cancer cells. Thus, we propose Torin-1 as a powerful drug candidate for metastatic CRC therapy.

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer death in the United States [1]. Despite new treatment options developed in the last decade, the prognosis for patients with advanced or recurrent CRC remains poor.

The serine/threonine kinase mammalian target of rapamycin (mTOR) has been suggested as a crucial modulator of tumor cell growth and proliferation, and therefore a potential target for anticancer therapy [2]. Unfortunately, drug development against mTOR started when knowledge of its function was very preliminary, resulting in contradictory data and unsuccessful clinical trials. Several breakthroughs have recently changed the course of mTOR-oriented drug discovery. First, the fact that mTOR exists in two distinct complexes: mTORC1 containing Raptor, and mTORC2 containing Rictor [3]. Second, the discovery that Rapamycin (the first mTOR inhibitor identified) exerts an incomplete inhibition of mTORC1 and is inactive against mTORC2 under short-term conditions [4]. Third, the existence of negative feedback loops linking mTOR to other pathways. Akt can phosphorylate mTOR in mTORC1, consequently leading to phosphorylation of ribosomal S6 protein kinase 1 (S6K1) and eIF4E-binding protein 1 (4E-BP1), mediators of protein translation and cell growth [5]. mTORC1-activated S6K1 phosphorylates Rictor and/or Insulin receptor substrate (IRS)-1, thus inhibiting mTORC2 and phosphatidyl inositol 3-kinase (PI3K)/Akt signaling,
分别 [6, 7]。最近的发现表明，mTORC1也磷酸化生长因子受体结合蛋白10（Grb10），导致Glb10和负反馈抑制PI3K和微管相关的蛋白激酶/细胞外信号调节激酶（MAPK/ERK）通路 [8]。mTORC2在细胞生存中起着重要的作用，包括代谢、分化、细胞骨架组织。它磷酸化蛋白质激酶Cα（PKCα）、SGK1，以及Akt，允许其完全激活 [3, 9-11]。文献中描述了生存率而不是抗肿瘤效果的依赖于Rapamycin的效果，从mTORC1依赖的负反馈通路中导致mTORC2和IRS-1/PI3K。这种意识推动了新代抑制剂的发展，这些抑制剂与ATP在催化位点竞争mTOR激酶，抑制两个复合体mTORC1, mTORC2是CRC中过度表达 [12]。不幸的是，被称为mTOR激酶的抑制剂（mTorKIs）在CRC细胞中几乎没有被完全研究 [2]。ApcΔ716小鼠，一种家族性腺瘤性息肉病的动物模型，表现为mTORC1靶向药物的开发和CRC癌和息肉的预防 [13, 14]。然而，抑制一种特异性途径可以扰乱信号通路之间以及信号通路的信号，并增强肿瘤信号。在这一点上，与Rapamycin的细胞静止效应有关，Rapamycin显著增加了MAPK激酶（MEK)/ERK活性，导致一种纺锤体的形态改变。这与K-Ras转变的肠上皮细胞（IECs） [15]。这表明对新的药物研发的需要，这些药物能够克服抑制剂的负反馈抑制通路。如上所述，mTORC1, mTORC2是CRC中过表达的两个复合体，并且在癌症生物学中起着重要作用 [16]。最近的研究已经证明了两个激酶抑制剂NVP-BEZ235和pp242在CRC细胞系中与CRC细胞系（CoCSCs）中磷酸化p70S6K1的mTORC1效应器（Ser2481）增加以及mTORC2激活 [2]。有趣的是，mTOR Ser2448、mTOR Ser2481、SGK1 Ser422、PKCα Ser657，在三个来自人类转移的CoCSCs中具有低Akt信号，但mTORC2信号明显升高。此外，mTOR Ser2448（mTORC2激活的标志）和Thr389在三个细胞系中磷酸化 [2]。Akt信号的异常导致mTORC2激活（Figure 1A） [2]。罕见的Akt信号（mTORC1激活状态 [2]）和Thr389的磷酸化表明没有非人类（stroma）细胞。可比较的结果在所有三个细胞系中被获得。所有CoCSCs都具有强Akt信号，但mTORC2激活，如磷酸化p70S6K1的mTORC1效应器（data not shown）在所有细胞中被发现，表明mTORC2信号在CRC中的重要性。另一方面，mTOR Ser2481和Rictor共定位，进一步表明mTORC2激活在CRC中的重要性。有趣的是，mTOR Ser2481共定位与γ-转录质在中心粒，这表明mTORC2在CRC的CoCSC增殖（Figure 1B）中可能存在的潜在作用。mTORC2可能调节CRC细胞癌干细胞和成熟细胞的生理学。尽管mTOR途径的各个成分都已研究过，但尚未理解其在CRC中的效应。mTOR激酶抑制剂（mTorKIs）被研究用于CRC治疗。我们研究了不同mTOR抑制剂的效果，单独使用或与其他标准化疗一起使用。文献中描述了生存率而不是抗肿瘤效果的依赖于Rapamycin的效果，从mTORC1依赖的负反馈通路中导致mTORC2和IRS-1/PI3K。这种意识推动了新代抑制剂的发展，这些抑制剂与ATP在催化位点竞争mTOR激酶，抑制两个复合体mTORC1, mTORC2是CRC中过度表达 [12]。不幸的是，被称为mTOR激酶的抑制剂（mTorKIs）在CRC细胞中几乎没有被完全研究 [2]。ApcΔ716小鼠，一种家族性腺瘤性息肉病的动物模型，表现为mTORC1靶向药物的开发和CRC癌和息肉的预防 [13, 14]。然而，抑制一种特异性途径可以扰乱信号通路之间以及信号通路的信号，并增强肿瘤信号。在这一点上，与Rapamycin的细胞静止效应有关，Rapamycin显著增加了MAPK激酶（MEK)/ERK活性，导致一种纺锤体的形态改变。这与K-Ras转变的肠上皮细胞（IECs） [15]。这表明对新的药物研发的需要，这些药物能够克服抑制剂的负反馈抑制通路。如上所述，mTORC1, mTORC2是CRC中过表达的两个复合体，并且在癌症生物学中起着重要作用 [16]。最近的研究已经证明了两个激酶抑制剂NVP-BEZ235和pp242在CRC细胞系中与CRC细胞系（CoCSCs）中磷酸化p70S6K1的mTORC1效应器（Ser2481）增加以及mTORC2激活 [2]。有趣的是，mTOR Ser2448、mTOR Ser2481、SGK1 Ser422、PKCα Ser657，在三个来自人类转移的CRCs (Tu12, Tu21, and Tu22 cells) [23]。这些细胞生长在一种特定细胞系（CoCSCs）上，我们的研究证明了CoCSCs作为CRC癌干细胞的潜在标记物。多个细胞表面蛋白已经被提出作为CRC癌干细胞候选标记物，包括CoCSCs和我们的CoCSCs。CoCSCs的形成需要使用替代方法来识别和鉴定CRC癌干细胞。
Ser2448 and p70S6K1 Thr389 were barely detectable in vitro, they were clearly expressed in vivo, together with mTOR Ser2481 and SGK1 Ser422 (Figure 1C). Because CoCSC differentiation has been previously observed after transplantation in immunodeficient mice [23], our immunostaining suggests a possible regulatory role of mTORC1 in CoCSC differentiation and of mTORC2 in the physiology of both stem/progenitor and mature tumor cells.

**SGK1 facilitates CoCSC growth and drug resistance.**

The finding that CoCSCs expressed low levels of activated Akt might be explained in light of the recent observation that activated Akt in CRC correlates with low stage and good prognosis, [25] and this was not our case, since we were analyzing cells isolated from CRC metastases to the liver [23]. The fact that despite low Akt activation, CoCSCs showed mTORC2 activation, led us to hypothesize that Akt could not have been a major mTORC2 effector in our system. SGK1 is the main mTORC2 effector in yeast and worms [26]; thus, we hypothesized that this could also have been the case with CoCSCs. To prove this hypothesis, we investigated whether SGK1 knockdown could affect cancer properties. Tu12, Tu21 and Tu22 cells were purified from contaminating feeder cells, grown on plastic, and infected with copGFP control, control shRNA or SGK1 shRNA lentiviral particles (Figure 2A). RT-PCR analysis revealed a significant decrease in SGK1 mRNA levels 72h after transfection (Figure 2B, left), while immunofluorescence analysis highlighted a strong decrease of SGK1 phosphorylation following puromycin selection (Figure 2B, right). Decreased clonogenicity (Figure 2C), invasive ability (p=0.0006) (Figure 2D), and a 2-5fold increased Oxaliplatin-induced apoptosis (Figure 2E-F), were also observed following SGK1 knockdown. Thus, rather than Akt, SGK1 could be the main mTORC2 effector in CoCSCs.

**mTOR inhibitors differently affect CoCSC viability.**

Since mTORC2 activation characterized CoCSCs in vitro, we compared the effects of various mTorKIs...
(Ku-0063794, WYE-354, pp242, and Torin-1) to first-generation mTOR inhibitors (Rapamycin and Temsirolimus) on the three CoCSC lines previously examined. Feeder cell-depleted CoCSCs were treated with vehicle or serial dilution (0.001-10µM) of the above-mentioned compounds for 72h. Even high drug concentrations were often insufficient to affect cell viability (Figure 3A, left). Interestingly, mTOR inhibitors sometimes induced cell proliferation. Only Torin-1 decreased cell viability in a dose-dependent fashion.

1µM decreased Tu12, Tu21, and Tu22 cell viability to 65% ± 2.3, 60% ± 5.2, and 51% ± 4.3%, respectively, while 10µM killed all of the cells. 10µM pp242 caused the accumulation of large cytoplasmic vacuoles inside the cells (Figure 3A, right). Additional studies revealed that pp242 induced autophagy, as indicated by acidic vesicular organelle (AVO) formation, changes in the localization of the autophagosome marker LC3 from diffuse cytosolic to a punctate distribution (Supplementary Figure 1A), and increased Beclin-1 mRNA levels.

Figure 2: SGK1 knockdown reduces CoCSC growth, invasive ability and chemoresistance. Tu12, Tu21 and Tu22 were infected with copGFP control, control shRNA or SGK1 shRNA lentiviral particles (MOI=6.5). 72h post-transfection, cells were subjected to (A) light microscopy, and (B, left) RT-PCR analysis for SGK1 or 18S housekeeping gene as internal control. Scale bars, 100μm. After additional 5 days of puromycin selection (1.5-2µg/mL), cells were analyzed by immunofluorescence for SGK1 Ser422 (B, right). Scale bar, 100μm. (C) Decreased CoCSC clonogenicity following SGK1 knockdown. One hundred thousand cells infected with control or SGK1 shRNA lentiviral particles were plated in each well of a 6-well plate. 2 weeks later, colonies were fixed and stained with 0.2% crystal violet (CV) in 10% ethanol. (D) Bar graph showing mean percentage (±SD) of apoptotic cells calculated considering three different microscopic fields of TUNEL assay and CC3 staining (**p<0.01, ***p<0.001).
(Supplementary Figure 1B). A late-stage inhibitor of autophagolysosome formation, Bafilomycin A1, effectively blocked the accumulation of pp242-induced AVOs, but did not further sensitize cells to the effects of mTOR inhibition (Supplementary Figure 1C). In contrast, autophagy inhibition alleviated pp242 cytostatic effects, as proliferative rates of treated cells returned to basal levels (data not shown). Moreover, a transient and weak S phase cell cycle arrest accompanied pp242-induced autophagy (Supplementary Figure 1D), further indicating that autophagy was not delaying apoptotic cell death, but effectively rescuing cells from death.

As expected, both Rapamycin and Temsirolimus did not affect mTOR Ser2481 phosphorylation (Supplementary Figure 2A) and low Rapamycin doses (10nM) induced Akt feedback activation (Supplementary Figure 2B). p70S6K1 Thr389 levels were very low in CoCSCs and not altered by Rapamycin exposure up to 1µM. Conversely, Rapamycin markedly decreased Grb10 protein abundance (Supplementary Figure 2C), suggesting that the relief of Grb10-mediated negative feedback inhibition of PI3K may have sometimes induced CoCSCs to activate Akt and proliferate.

We then aimed at testing mTOR inhibitors in combination with standard chemotherapy. Tu12, Tu21 and Tu22 cells were treated with 5µM Oxaliplatin or 100µM 5-Fluorouracil alone or in combination with 10µM Rapamycin, Temsirolimus or WYE-354 for 72h. The mTOR inhibitors tested did not show additive or synergistic effects in combination with chemotherapeutics (Figure 3B). Rather, Temsirolimus rescued Oxaliplatin-treated Tu22 cells (p=0.0075), while WYE-354 rescued Oxaliplatin-treated Tu12 (p=0.04) and Tu22 cells (p=0.008), as well as 5-Fluorouracil-treated Tu12 cells (p=0.03). Thus, mTOR inhibitors may have a beneficial role by protecting cells against pro-apoptotic insults. There was no observed benefit of combining Ku-0063794, Torin-1 or pp242 with chemotherapeutics (data not shown).

Figure 3: Differential effects of mTOR inhibitors on CoCSC viability. (A) Line graphs (left) and optical imaging (right) showing viability of Tu12, Tu21 and Tu22 cells treated with vehicle (V) or a serial dilution (0.001-10µM) of Rapamycin (R), WYE-354 (W), Temsirolimus (T), pp242 (P), Ku-0063794 (K) or Torin-1 (T1). Scale bars, 200µm. The number of viable cells was measured by MTS assay after 72h of drug exposure. Data are expressed as mean percentage (±SD) of viable cells as compared to respective control cultures, obtained from triplicate absorbance readings from at least three independent experiments. Optical images were taken from Tu22 cells exposed to 10µM of each drug for 72h. (B) Bar graph showing mean percentage (±SD) of viable cells following treatment with Oxaliplatin (Ox, 5µM) or 5-Fluorouracil (5-FU, 100µM) alone or in combination with 10µM Rapamycin (Rap), Temsirolimus (Tem) or WYE-354 (WYE) for 72h. The number of viable cells was obtained as in (A) (*p<0.05, **p<0.01).
shown). Taken together, Torin-1 as a single agent, is the most powerful inhibitor among those examined for CRC therapy, as it efficiently suppresses tumor growth in vitro.

**Torin-1 triggers CoCSC apoptosis.**

Various techniques were used to determine whether Torin-1 induced apoptosis. 5µM Torin-1 treatment for 15h suppressed mTOR Ser2481 phosphorylation (Figure 4A) and induced apoptosis, as indicated by caspase-3 activation, monitored by both immunofluorescence and flow cytometry analysis (Figure 4B), and appearance of TUNEL-positive half moon shaped and fragmented nuclei (Figure 4C). Following 72h of treatment, all the cells were apoptotic (Figure 4D). Importantly, 5µM Torin-1 treatment for 15h resulted in sub-G1 cell accumulation (Figure 4E), and appearance of single Annexin V− cells (Figure 4F), both indicative of apoptosis induction.

Emergence of resistant clones represents a main problem of current therapies. To understand whether CoCSCs could develop Torin-1 resistance, Torin-1 resistant Tu12, Tu21, and Tu22 cells were generated, as depicted in Supplementary Figure 3A. Cells could now survive 5µM Torin-1, but they were no longer able to extensively proliferate in vitro (Supplementary Figure 3B). S.c. injection of Torin-1 resistant cells into mice (n=7) did not generate palpable tumors during a 7-wk observation.

**Figure 4: Torin-1 triggers CoCSC apoptosis.** Immunofluorescence pictures of control or Torin-1-treated (5µM, 15 h) Tu12 cells showing expression of (A) mTOR Ser2481 and (B, left) cleaved caspase-3 (CC3) Asp175. Scale bars, 100µm. (B, right) Flow cytometry histogram showing CC3 Asp175 staining on PFA- and methanol-fixed Tu12 cells treated as in (A). (C) Optical imaging of TUNEL assay on Tu12 cells treated as in (A) (DAB, brown color). Scale bar, 100µm. (D) Hoechst 33342 staining and merged pictures of Hoechst staining and optical imaging of Tu12 cells treated with 5µM Torin-1 or vehicle as control for 72h. Scale bar, 100µm. Condensed chromatin, fragmented nuclei, and apoptotic bodies can be observed following treatment. (E) DNA content histogram (left) and table (right) showing sub-G1 cell accumulation after Torin-1 treatment (5µM, 15h). Tu12 cells were fixed with 70% ethanol, RNase treated (0.2mg/ml), and stained with PI (40µg/ml) before being analyzed with a flow cytometer. Quantitative analysis is based on the Dean-Jett-Fox curve-fitting model. (F, left) Stacked bar graph showing mean percentage (±SD) of viable, early apoptotic or late apoptotic/necrotic Tu12, Tu21 or Tu22 cells treated as in (A), determined using FITC Annexin V/PI staining. Results are representative of at least two experiments. (F, right) Flow cytometry contour plots of FITC Annexin V/PI-stained Tu12, Tu21 and Tu22 cells treated as in (A).
period (Supplementary Figure 3C). Nevertheless, examination of skinned mice revealed two mice had formed very small tumors. Thus, CoCSC cultures that have been subjected to a prolonged, continuous, multistep selection with Torin-1 contain a strikingly reduced tumor-initiating cell population, thus encouraging Torin-1 potential use for CRC therapy.

**Torin-1 hinders growth, motility, invasion, and survival of distinct CoCSC subpopulations.**

Despite the first wave of enthusiasm surrounding the CSC field, no consensus has emerged so far about cell surface marker profiles that define CoCSCs, Initially

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**Figure 5: Rapamycin, WYE-354 and Torin-1 differently affect cancer properties of distinct CoCSC subpopulations.**

(A) Flow cytometry dot plots (left) showing gates used for sorting Tu12 cells. Cells were stained with CD326 PerCP-Cy5.5, CD24 FITC, CD49f PE, CD29 APC, and Sytox Blue or CD326 PerCP-Cy5.5, CD166 PE, CD44 SAv-APC, and Sytox Blue. CD326+/CD24+/CD49f+ and CD326+/CD44+/CD166+ subpopulations were sorted directly into 96-well plates in medium containing vehicle, 1 µM Rapamycin, WYE-354 or Torin-1 (see the table for cell densities, middle panel). Medium was replaced every 72h up to 12 days, when wells containing colonies were scored and CFU frequencies (±SE) estimated using the LCalc™ software (StemCell Technologies) (upper right panel). Optical imaging of CV-stained wells (lower right panel) showing confluence rates of CD326+/CD44+/CD166+ cells exposed to different drugs (pictures are from wells originally plated with the highest number of cells; white arrows indicate colonies). Scale bar, 200 µm. (B) Optical imaging of unstained or CV-stained CD326+/CD44+/CD166+ cells treated as in (A) and migrating across the gap up to 72h. Scale bar, 200 µm. Thirty thousand cells were seeded into each well of a culture insert and grown overnight. After removal of the insert, a 500 µm cell-free gap was created. Cells were therefore treated, and migration monitored at different time points. (C) Optical imaging of CV-stained transwells (left) showing invasive potential of CD326+/CD24+/CD49f+/CD29+ and CD326+/CD44+/CD166+ Tu12 cells treated as in (A). Scale bar, 100 µm. Fifteen thousand cells were sorted directly into growth factor reduced Matrigel (2 mg/ml)-coated transwells and allowed to invade for 72h. Drugs were added to bottom chambers. Bar graph (middle) shows the mean (±SD) invasion area (pixels) from one representative experiment of two in triplicate, with similar results (statistical analysis was performed with respect to control samples; *p<0.05, **p<0.01). Optical imaging (right) of a bottom chamber showing CD326+/CD44+/CD166+ cells crossing the porous membrane under Rapamycin exposure (white arrows indicate cells). Scale bar, 100 µm. (D) Bar graph (left) showing relative caspase 3/7 activity levels (fold increase over control) of CD326+/CD24+/CD49f+/CD29+ and CD326+/CD44+/CD166+ Tu12 cells following treatment with vehicle or Torin-1 (5 µM, 15h), and optical imaging (right) showing morphological changes in treated versus control cells. Scale bars, 200 µm. Data of caspase 3/7 activities are presented as mean (±SD) of the luminescence values obtained in triplicate determination from at least three independent experiments.
described as a unique marker for immature intestinal cells, CD133 was later subject of huge controversy [27]. Conversely, the combined expression of CD326+/CD44+/CD166+ was suggested as being more robust for CoCSC isolation [28]. Both CD24+/CD29+ and CD24+/CD44+ signature have been suggested to characterize putative mammary stem/progenitor cells [29]. Interestingly, we found colony-forming unit (CFU) frequencies of CD326+/CD24+/CD44+/CD29+ and CD326+/CD44+/CD166+ CRC subpopulations to be very similar. For this reason, we chose these two subpopulations within Tu12 cells to further confirm Torin-1 anti-CoCSC activity. Particularly, we performed limiting dilution analysis, migration, and invasion assays, in the presence or absence of 1µM Rapamycin, WYE-354, or Torin-1. While CFU frequencies among Control, Rapamycin-, and WYE-354-treated cells were similar, CFU frequencies following Torin-1 treatment were significantly decreased (Figure 5A, upper right panel). However, cells exposed to Rapamycin or WYE-354 were less confluent than control cells, and Torin-1-treated cells generated very small colonies (Figure 5A, lower right panel). Compared to control cells, more cells migrated through the gap at 48h and invaded the Matrigel layer at 72h under Rapamycin exposure (p=0.035 for CD326+/CD24+/CD44+/CD29+ and 0.0056 for CD326+/CD44+/CD166+ cells) (Figure 5B-C). Moreover, Rapamycin caused some cells to cross the porous membrane and adhere to the bottom chamber (Figure 5C, right). In contrast, control cell were completely unable to cross the membrane under experimental conditions. Although 72h WYE-354 treatment did not affect Tu12 cell viability (Figure 3A, left), it slightly reduced migration, but not invasion of both subpopulations (p=0.37 and p=0.36, respectively) (Figure 5B-C). Conversely, Torin-1 strongly inhibited both migration and invasion of the selected subpopulations (p=0.024 and 0.009, respectively), and activated apoptosis as evidenced by increased caspase 3/7 activity and morphological changes (Figure 5B-D), confirming its anti-CSC activity.

![Figure 6](image-url)
Torin-1 reduces tumor growth and vessel formation in vivo.

To test whether Torin-1 could suppress CRC progression in vivo, we established Tu12 and Tu22 xenografts in nude mice. Then, mice received 20mg/Kg Torin-1 or vehicle every day up to 12 days. Torin-1-treated tumors grew slower than control tumors (Figure 6A). Drug was well tolerated as no significant differences in body weight between the two groups were observed (Figure 6B). The mean weights of the excised tumors were approximately 50% less in mice treated with Torin-1 with respect to control mice (Tu12, p=0.025; Tu22, p=0.0009) (Figure 6C). Importantly, tumor vasculature was less developed in treated tumors (Figure 6D), suggesting Torin-1 anti-angiogenic activity.

Torin-1 decreases the expression of proliferative, angio-/lympho-genic, and stem cell markers, and activates apoptosis in vivo.

Different markers were evaluated by real-time qPCR analysis in both untreated and treated tumors (Figure 7A). With respect to control tumors, the proliferation
marker Ki67, the Notch pathway members involved in angiogenesis DLL4 and Notch1 [30], and the CSC surface marker CD44 were significantly reduced in both Tu12 and Tu22 treated tumors. No changes in the mRNA levels of the goblet cell differentiation marker Muc2, the Notch effector Hes1, and the Polycomb member Bmi1 were observed. The enterocyte differentiation marker Villin, the Notch pathway component DLL1, and the crypt stem cell marker Lgr5 were decreased in Tu22, but not in Tu12 tumors, following treatment. Anti-CSC therapy is expected to promote differentiation; the reduction in Villin levels may therefore seem paradoxical. However, a previously unrecognized function of Villin in IEC survival was reported [31], indicating that Torin-1 might favor apoptosis by reducing Villin levels. A decrease in mTOR Ser2481, and an increase in CC3 and fibrotic tissue (Azan Mallory, A.M.) were observed in treated versus control tumors (Figure 7B). In accordance with molecular analysis, no changes in goblet cell numbers were found, as investigated by Muc2 and Alcian Blue (A.B.) stainings (Figure 7B). Importantly, treated tumors contained fewer blood vessels, as examined through CD31 staining (Figure 7B). Interestingly, Podoplanin expression characterized both lymphatic vessels and tumor cells at the invasive front of control tumors, while no positivity was observed in treated tumors (Figure 7C). Podoplanin+ vessels were CD31+. Podoplanin+ cells located outside vessels were human in origin, although HLA expression was dispersed throughout their cytoplasm. This is not surprising since tumor cells often down-regulate HLA antigens surface expression to escape immunological attack. Podoplanin+ cells exhibited round morphology typical of amoeboid motility and were CD44+. Loss of CD44 expression in invaded area is a good indicator of lymph-node metastasis in CRC [32]. Thus, while control tumors comprised cells with high metastatic potential, cells in treated tumors were less prone to migrate to distant sites.

Since in vitro cultured tumor cells might not maintain the exact properties of the primary tumor, by acquisition of additional gene alterations, we aimed at assessing Torin-1 effects on xenografts obtained from injection of freshly isolated metastatic CRC cells. After 5 months from injection, 6 out of 10 tumors had formed. Mice were therefore divided in two groups, and treated with 20mg/Kg Torin-1 or vehicle every day up to 12 days (parental tumor was confirmed for mTOR pathway activity).

Figure 8: Torin-1 does not affect the survival of normal colon stem cells in vivo. (upper panel) H&E or immunofluorescence staining of PFA-fixed, paraffin-embedded sections of a mouse lymph node showing engraftment of human fetal intestinal epithelial cells as revealed by Muc2 positivity. Scale bar, 100μm. (lower panel) Representative Muc-2 reactive-colon crypts repopulating lymph nodes of mice (Mo) receiving injection of human fetal colon #54 or 15 (Co#54 and Co#15, respectively), and subjected to vehicle or Torin-1 treatment (Ve and To, respectively). Scale bar, 100μm. Enlargements of boxed regions for images Co#15 Ve_Mo#1 and Co#54 Ve_Mo#2 are shown.
activation before beginning Torin-1 treatment, data not shown). Torin-1-treated tumors grew slower than control tumors (data not shown). Importantly, CD326 expression was reduced after treatment, as assessed by flow cytometry analysis on freshly isolated cells (data not shown). CD326 is one of the first and prominent immunotherapeutic targets in cancer therapy due to its frequent and high-level expression on most carcinomas of various origins. It can abrogate E-cadherin mediated cell-cell adhesion thereby promoting metastasis [33], and can support proliferation by enhancing Wnt signaling [34]. CD326+ cells from both groups were sorted and plated on stroma feeder layer. CD326+ cells isolated from vehicle-treated xenografts generated a higher number of colonies than CD326+ cells isolated from Torin-1-treated xenografts, and colonies were significantly bigger (Figure 7D). All these data support the potential of further development of Torin-1 as therapeutic for the treatment of CRC.

Torin-1 does not affect the survival of normal colon stem cells in vivo.

Because normal stem cells and CSCs share many traits, it seems reasonable to think that any therapy targeting CSCs may also destroy healthy tissues [35]. Given our recent finding that the mouse lymph node can be used as an in vivo organ factory to build up complex organ structures [36], we aimed at assessing the in vivo toxicity of Torin-1 against normal colon stem cells by recapitulating colon organ inside the mouse lymph node. To this purpose, we used human fetal intestinal epithelial cells, thought to be enriched in normal stem cells (Figure 8, upper panel). Following 3 weeks from cell transplantation, mice were treated with vehicle or 20mg/Kg Torin-1 once a day for 12 days. Tridimensional crypts could be observed in lymph nodes of both untreated and treated mice, to the same extent (Figure 8, lower panel). Thus, Torin-1 did not affect the survival of normal colon stem cells in vivo, suggesting its selectivity towards cancer cells.

DISCUSSION

Although during the last decades in vivo preclinical studies revealed the antitumor effect of several drugs against CRC, most patients experience a tumor recurrence. The main explanation for such a dismal prognosis is that common drugs leave behind CoCSCs, whose drug-induced positive selection renders tumors more aggressive. As cancer’s Achilles’ heel, CSCs have been intensively studied to develop more effective therapies. Because it plays such a crucial role in cancer biology, mTOR has emerged as a potential target for drug development. Several mTOR inhibitors, including Rapamycin, have already gone through clinical trials as single agents for treating various cancers without great success. Of the huge list of mTOR inhibitors developed, only Temsirolimus and Everolimus have been approved by the FDA for the treatment of advanced renal cell carcinoma [37]. Nevertheless, the role of mTOR inhibitors in cancer therapy continues to evolve, as new compounds are synthetized. Despite this, data comparing the effects of distinct mTOR inhibitors on CSCs are incomplete.

Here, we first analyzed metastatic CRC cells with the properties of intestinal stem cells as well as tumor-initiating cells for mTOR activation. We found mTOR complexes to be differently expressed in these cells, concluding that mTORC2 might be involved in the maintenance of the cancer stem-like phenotype, while mTORC1 might be involved in CoCSC maturation. This is in agreement with previous studies showing a role for mTORC1 in driving the differentiation of normal and cancer stem/progenitor cells [38-41].

CoCSCs expressed low levels of Akt Ser473, indicating that this kinase could not have been a major mTORC2 effector in our system. We indicated SGK1 as the possible main mTORC2 effector in CoCSCs, as highlighted by the negative effect on cancer properties following its knockdown. Akt hypophosphorylation and dependence of SGK family members for viability are known to occur most frequently in the context of wild-type Phosphatase and tensin homolog (PTEN), and helical PI3K alpha catalytic subunit kinase domain (PIK3CA) mutations [42]. Future studies will be conducted to confirm whether this genetic signature can predict resistance or sensitivity of CoCSCs to the different mTorKIs here studied. Unfortunately, the prognostic and predictive value of common mutations in patients with colon cancer is controversial, due to bias in research settings [43]. In our opinion, mTorKI resistance might also occur through less well-studied but equally important epigenetic mechanisms [44].

We therefore analyzed the effects of six mTOR inhibitors, including Torin-1, a highly potent and selective mTorKI [45]. Of the examined compounds, Torin-1 proved the most powerful inhibitor, suppressing CoCSC growth both in vitro and in vivo.

mTOR inhibitors can relieve negative feedbacks, resulting in strengthened oncogenic signals. This happened under Rapamycin exposure, which drove cells towards a pro-metastatic phenotype, confirming the notion that Rapamycin can have pro-tumor effects. Nevertheless, despite its role in activating oncogenic pathways, Rapamycin has also been considered a tumor-preventive agent since it delays carcinogenesis in tumor-prone p53-/- and p53+/- mice, most likely by slowing down the process of aging [46, 47]. mTOR inhibitors can have a double-edged sword by activating autophagy, and autophagy itself has a double-edged sword in cancer by the promotion of metabolically stressed tumor cell survival. Indeed, pp242 protected CoCSCs from the effects of mTOR inhibition.
through autophagy activation, warranting considerable attention on newly developed inhibitors. The goal of any therapeutic strategy is to trigger CSC apoptosis, and Torin-1 successfully achieved this aim both in vitro and in vivo. It is possible that higher Torin-1 concentrations could increase antitumor response in vivo. However, 20mg/Kg Torin-1 was sufficient to hinder CoCSC expansion, angio-/lympho-genesis, and invasion in vivo. Importantly, Torin-1-resistant CoCSCs exhibited much reduced tumorigenicity or were even non-tumorigenic. Moreover, Torin-1 did not affect the survival of normal colon stem/progenitor cells in vivo, thus encouraging its potential use for metastatic CRC therapy. Recently, Torin-2, which has better pharmacokinetic properties and an improved synthetic route than Torin-1 has been synthetized [48]. Although to our knowledge no studies have been reported so far to elucidate its effectiveness in cancer, we do not exclude it could also be used to successfully treat CRC. Inappropriate activation of PI3K signaling is one of the most common features of CRC. Several studies have shown that inhibition at multiple levels of the PI3K pathway results in FOXO-dependent feedback reactivation of several receptor tyrosine kinases (RTKs), which, in turn, limit the sustained inhibition of this pathway [49]. This suggests that if used as single agents, PI3K pathway inhibitors may have limited clinical activity. Conversely, dual inhibition of PI3K and mTORC1/2 signaling induced tumor regression in several in vivo human tumor xenograft models, and might show greater efficacy than mTorKIs at depleting CoCSCs [50].

METHODS

Cells.

CoCSCs were cultured as previously described [23].

Reagents, plastics, and instruments:

Antibodies used are indicated in Supplementary Table 1. CopGFP control (sc-108084), control shRNA (sc-108080), and SGK1 shRNA (sc-38913-V) lentiviral particles, and SGK1 primers (sc-38913-PR) were purchased from Santa Cruz. Sequences of other primers used are indicated in Supplementary Table 2. Drugs were purchased as follows: Rapamycin (R-5000) from LC Laboratories; WYE-354 (CD0270), pp242 (CD0258), and Ku-0063794 (CD0274) from Chemdea; Tensirolimus (PZ0020), 5-Fluorouracil (F6627), and Oxaliplatin (O9512) from Sigma; Torin-1 (1222998-36-8) from Tocris Biosciences. All drugs except Oxaliplatin were dissolved in DMSO as stock solutions, and then diluted with culture medium. Oxaliplatin was dissolved in deionized water. Kits were purchased as follows: RNeasy Mini Kit (74104) from Qiagen; iScript™ Reverse Transcription Supermix for RT-qPCR (170-8841), iTaq DNA Polymerase kit (170-8870), and SsoAdvanced™ SYBR® Green supermix (172-5261) from Bio-Rad; CellTiter 96® AQueous Assay (G3580), DeadEnd™ Colorimetric TUNEL System (G7360), and Caspase-Glo® 3/7 Assay (G8091) from Promega. Transwells (07-200-150) were purchased from Corning. Ibidi culture inserts (80209) were used for migration assay. Flow analysis was performed on a Miltenyi MACSQuant Analyzer. Sorting was performed on a BectonDickenson FACSAria II SORP cell sorter. Final data analysis was done using FlowJo software (TreeStar). A C1000 thermal cycler (Biorad) and a StepOnePlus™ Real-Time PCR System (Applied Biosystems) were used for gene amplification.

Colon cancer xenografts and treatment.

Tumor tissues were minced into small fragments with scalpels and then digested using a two-step collagenase method. Freshly isolated cells from primary tumors or CoCSCs (5 x 10⁶) were suspended in HBSS:Matrigel (1:1) and injected s.c. into both flanks of 5-week-old BALB/c nude mice (Charles River, n=5 per group). 20mg/Kg Torin-1, as a suspension in 20% N-methyl-2-pyrrolidone/40% PEG400/40% water [45], or vehicle was delivered by i.p. injection once daily for 12 days after the tumor was established. Tumors were measured every day using a caliper and tumor volumes calculated according to the formula $V=\frac{\pi}{6}r^2(small\ diameter)\times(small\ diameter)^2$. At the end of the treatment mice were killed by CO₂ euthanasia. Xenografted tumors were excised, weighed, photographed, fixed in buffered formalin, and embedded in paraffin for histological and immunohistochemical examination, or stored in RNA later (Qiagen) prior to RNA isolation, or subjected to flow-cytometric analysis.

Fetal intestinal epithelial cell transplantation and treatment.

Normal colon was obtained from 2 fetuses (#15 and #54, 21 and 23 weeks, respectively) from elective abortions performed at Magee Women’s Hospital, Pittsburgh, PA. The colon was cut longitudinally in HBSS, contents rinsed, cut into 1-inch pieces, transferred to EBSS/10mM EGTA/1% HEPES (Life Technologies/Sigma-Aldrich/Mediatech) and minced. Tissue was then transferred to a tube and incubated for 5 min at room temperature. After an EBSS wash, the tissue was treated three times with a cocktail containing 1mg/mL collagenase II (Life Technologies), 1mg/mL hyaluronidase (Sigma-Aldrich), and 20µg/mL DNase I (Roche) in HBSS/1% HEPES for 20 min. Tissue/cell suspensions were passed through a 100m cell strainer to isolate single cells from...
undigested tissue.

For lymph node transplantation, recipient mice (BALB/c nude, n=12) were anesthetized with 1–3% isoflurane. A small incision was made in the abdomen to expose jejunal lymph nodes. A 25μL gas-tight removable needle syringe (Hamilton, 7656-01) with a removable needle (gauge 27) (Hamilton, 7803-01) was used to slowly inject the cell suspension (2X10^6 cells/mouse) into a single lymph node. Light cauterization was used to seal the opening. The wound was then closed with surgical sutures. Ketoprofen (2 mg/kg, IM) treatment for postoperative pain relief was initiated right after surgery and continued for 2 additional consecutive days. Three weeks later, mice were divided in two groups; one group received i.p. injection of 20mg/Kg Torin-1 once a day for 12 days (n=7); the other group received vehicle (n=5). At the end of Torin-1 treatment, all mice were euthanized, their lymph nodes were collected, fixed 2 hours in 4% PFA, and embedded in paraffin for analysis. Sections were stained with H&E or Muc2 antibody (antibody used is indicated in Supplementary Table 1).

Statistical Analysis.

Data are presented as means ±SD. Statistical analysis was performed using Student’s t test (p<0.05 was considered significant).

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