Targeting mTORC2 inhibits colon cancer cell proliferation in vitro and tumor formation in vivo

Didier Roulin†, Yannick Cerantola†, Anne Dormond-Meuwly, Nicolas Demartines and Olivier Dormond*

Abstract
The mammalian target of rapamycin (mTOR), which exists in two functionally distinct complexes, mTORC1 and mTORC2, plays an important role in tumor growth. Whereas the role of mTORC1 has been well characterized in this process, little is known about the functions of mTORC2 in cancer progression. In this study, we explored the specific role of mTORC2 in colon cancer using a short hairpin RNA expression system to silence the mTORC2-associated protein rictor. We found that downregulation of rictor in HT29 and LS174T colon cancer cells significantly reduced cell proliferation. Knockdown of rictor also resulted in a G1 arrest as observed by cell cycle analysis. We further observed that LS174T cells deficient for rictor failed to form tumors in a nude mice xenograft model. Taken together, these results show that the inhibition of mTORC2 reduces colon cancer cell proliferation in vitro and tumor xenograft formation in vivo. They also suggest that specifically targeting mTORC2 may provide a novel treatment strategy for colorectal cancer.

Findings
The mammalian target of rapamycin (mTOR) is a serine-threonine kinase that regulates cell growth and proliferation in response to the availability of growth factors and nutrients [1,2]. mTOR exists in two functionally distinct complexes: mTORC1 and mTORC2, which have different functions and are differentially sensitive to rapamycin [3]. mTORC1 is composed of mTOR, mLST8, PRAS40, depitor and raptor and is sensitive to rapamycin. mTORC1 enhances cell growth and proliferation through various mechanisms including synthesis of proteins and lipids as well as reduction of autophagy. Part of the functions of mTORC1 are mediated by the phosphorylation and activation of S6K1 and 4E-BP1, two well-characterized downstream effectors of mTORC1 [1]. mTORC2 contains mTOR, mLST8, mSIN1, dector, protor-1 and rictor. Although mTORC2 is classically insensitive to rapamycin, prolonged exposure to rapamycin may also inhibit mTORC2 activity by blocking its assembly in certain cell types [4]. In contrast to mTORC1, relatively little is known about the functions of mTORC2. It has been described that mTORC2 regulates cell survival and cytoskeletal organization through the regulation of Akt and PKCα respectively [5,6].

Since mTOR plays a major role in cell growth and proliferation, targeting mTOR in cancer therapy has been viewed as a promising approach [3,7]. Indeed, alterations of mTOR signaling pathway are commonly observed in solid tumors. Whereas the role of mTORC1 in cancer progression has been extensively characterized, the role of mTORC2 is much less documented. So far it has been shown that targeting mTORC2 might be beneficial in tumors harboring high levels of activated Akt, such as gliomas or tumors caused by PTEN deletion [8,9]. Since the activation of Akt is frequently observed in colorectal cancer [10], we wish here to analyse the role of mTORC2 in colon cancer.

To investigate the role of mTORC2 in colon cancer, we used a lentiviral short hairpin RNA (shRNA) expression system that suppresses the expression of rictor to block the activity of mTORC2 [5]. In addition we also suppressed the expression of raptor to block mTORC1 or the expression of mTOR to block both complexes. HT29 and LS174T colon cancer cells were infected with lentiviruses expressing either a scramble shRNA, raptor shRNA, rictor shRNA or mTOR shRNA. The gene knockdown efficiency following infection was analysed by Western Blot. Using this technique, we observed that raptor, rictor and mTOR expression was significantly reduced in both
addition, we also found that mTOR and rictor shRNA
phosphorylation of S6K1, a downstream target of mTORC1. In
that mTOR and raptor downregulation reduced the phos-
HT29 and LS174T cells (Figure 1A). We further observed
mTOR or rictor knockdown in LS174T cells
resulted in a G1 arrest as observed by a marked increase
inhibitor of mTORC1. Similarly to the inhibition of
mTORC1 by raptor shRNA, we found that, while block-
ing S6K1 phosphorylation, rapamycin increased Akt
phosphorylation. These results show that rapamycin
inhibits mTORC1 but not mTORC2 in HT29 and LS174T
cells (Figure 1B).

After selection by puromycin for 48 hours, we also
found that knockdown of mTOR and rictor significantly
changed the morphology of HT29 and LS174T cells. While
HT29 and LS174T cells had a small distinct cyto-
plasm and grew in cell clusters, mTOR or rictor deficient
HT29 and LS174T cells were small and round with absent
cytoplasm, and which failed to grow (Figure 2A). In con-
trast, raptor knockdown did not alter the morphology of
HT29 and LS174T cells. To exclude that the morphologi-
changes observed in mTOR and rictor knockdown
cells was indeed due to an increased cell death, we moni-
tored apoptosis by quantifying DNA fragmentation. We
did not find a significant change in cell apoptosis between
cells expressing scramble, raptor, mTOR or rictor shRNA
(Figure 2B).

To further characterize the role of mTORC2 in colon
cancer progression, we analyzed the effect of rictor
knockdown on colon cancer cell proliferation. We found
that the proliferation of HT29 and LS174T cells deficient
for rictor or mTOR was markedly reduced (Figure 3A).
Knockdown of raptor also significantly reduced the pro-
liferation of HT29 and LS174T cells compared to control
cells, however to a lesser extend than rictor or mTOR
knockdown. Finally, rapamycin treatment also reduced
cell proliferation to a similar extend than raptor knock-
down. To gain insight into the mechanism by which rictor
and mTOR knockdown reduced colon cancer cell prolif-
eration, we analyzed cell cycle progression using propid-
ium iodide staining and flow cytometry analysis. We
found that mTOR or rictor knockdown in LS174T cells
resulted in a G1 arrest as observed by a marked increase
in the G1 population. Similar results were observed in
HT29 cells (Figure 3B).

We next tested the effect of mTOR and rictor knock-
down on tumor growth in vivo. LS174T cells deficient for
mTOR, raptor and rictor as well as LS174T expressing a
scramble shRNA as a control were injected subcutane-
ously into nude mice and tumor growth was monitored.
We found that mTOR or rictor deficient LS174T cells failed to form a tumor xenograft even after 60 days of observations (Figure 4). In contrast, LS174T cells deficient for raptor formed tumor xenografts which grew however slower than control xenografts.

Several studies have demonstrated that the activity of mTOR is increased in tumors [7]. Particularly in colorectal cancer, mTOR signaling components were highly activated in tumor specimen compared to non-cancerous mucosa [11]. Furthermore, targeting mTOR with a specific siRNA to mTOR also reduced SW480 and HCT116 colon cancer cell proliferation and survival in vitro and injection of small interfering RNA to mTOR into HCT116 tumor xenografts also blocked tumor growth in vivo [11]. In addition, the inhibition of mTORC1 suppressed the formation of colonic adenomas and cancers in a mouse model of familial adenomatous polyposis [12].

Therefore, targeting mTOR in colorectal cancer might be a successful strategy. mTOR exists in two distinct complexes, however, no study so far has analyzed the specific role of mTORC2 in colorectal cancer development. Here, we found that mTORC2 plays an important role in colon cancer cell proliferation. Downregulation of mTORC2, by either blocking the expression of mTOR or rictor, reduced the proliferation of HT29 and LS174T colon cancer cells in vitro and inhibited the formation of tumor xenografts in vivo. We therefore propose that targeting mTORC2 in colon cancer could be a promising therapeutic strategy.

Targeting mTOR with rapamycin or its analogs in clinical studies has been less successful than expected [7]. However, as mTORC2 and part of mTORC1 functionality are resistant to rapamycin, one may speculate that therapies that target both complexes will be much more efficient in cancer therapy. Consistent with this hypothesis, we found that the inhibition of mTORC1 reduced colon cancer cell proliferation, however to a lesser extent than the inhibition of both complexes, or mTORC2 alone.
Recently, several groups have developed ATP-competitive and selective mTOR inhibitors that target simultaneously both complexes [13-15]. Initial experiments have shown that the antiproliferative efficacy of these inhibitors is superior to rapamycin [15]. Future studies will reveal the efficacy of such inhibitors in cancer therapy; one major concern being that these inhibitors might have considerable toxicity in vivo. However, our results suggest that targeting only mTORC2 might be sufficient to prevent colon cancer progression and thus give a rationale for the development of drugs that specifically target mTORC2.

Abbreviations
mTOR: mammalian target of rapamycin; shRNA: small hairpin RNA.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
DR, YC, ADM performed the experiments and interpreted the experimental findings. YC and OD conceived the study. DR drafted the manuscript. ND and OD wrote the final version of the manuscript. All authors read and approved the final manuscript.

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Author Details
Department of Visceral Surgery, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Pavillon 3, Av de Beumont, 1011 Lausanne, Switzerland

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References
1. Wullschleger S, Loewith R, Hall MN: Defining the role of mTOR in cancer.
2. Hay N, Sonenberg N: Upstream and downstream of mTOR.
3. Guertin DA, Sabatini DM: The mTORC2 complex: Novel targets for mTOR inhibitors.
4. Roulin et al.: Defining the role of mTOR in cancer.
5. Guertin DA, Sabatini DM: Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex.
6. Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, Hall A, Hall MN: Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive.
7. Favier S, Kromer C, Raymond E: Current development of mTOR inhibitors as anticancer agents.
8. Mair J, Bermuth A, Martin J, Jo-OD, Vartanian R, Funk A, Gena J: mTORC2 activity is elevated in gliomas and promotes growth and cell motility via overexpression of rictor.
9. Guertin DA, Stevens DM, Saitoh M, Kinkel S, Crosby K, Sheen JH, Mullholland DJ, Magnuson MA, Wu H, Sabatini DM: mTOR complex 2 is required for the development of prostate cancer induced by Pten loss in mice.
10. Rychahou PG, Kang J, Gulhati P, Deon HO, Chen LA, Xiao SY, Chung DH, Evers BM: Akt2 overexpression plays a critical role in the establishment of colorectal cancer metastasis.
11. Zhang YJ, Dai Q, Sun DF, Xiong H, Tian XQ, Gao FH, Xu MH, Chen GQ, Han ZG, Fang JY: mTOR signaling pathway is a target for the treatment of colorectal cancer.
12. Fujishita T, Aoki K, Lane HA, Aoki M, Taketo MM: Inhibition of the mTORC1 pathway suppresses intestinal polyp formation and reduces mortality in ApcDelta716 mice.
13. Thoreen CC, Kang SA, Chang JW, Liu Q, Zhang J, Gao Y, Reichling LJ, Sim T, Sabatini DM, Gray NS: An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1.
14. Feldman ME, Apel S, Uottia A, Loewith R, Knight ZA, Ruggero D, Shokat KM: Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2.
15. Yu K, Toral-Barza L, Shi C, Zhang WG, Lucas J, Shor B, Kim J, Verheijen J, Curr J, Malwitz DJ, et al.: Biochemical, cellular, and in vivo activity of novel ATP-competitive and selective inhibitors of the mammalian target of rapamycin.

Figure 4 Colon cancer cells require mTOR or rictor to form tumors as xenografts. LS174T cells were infected with lentiviral particles containing scramble, raptor, or mTOR shRNA. Stable transfectants were selected for resistance to puromycin for 48 hours and subsequently cultured in DMEM 10% FBS for 2 days. An equal amount (1 × 10⁶) of LS174T cells deficient for raptor, rictor, or mTOR, or expressing a scramble shRNA were harvested and injected subcutaneously into immunodeficient mice (n = 5 in each group). Tumor volumes were evaluated using caliper measurements and calculated with the formula V = π/6 × a² × b where a is the short axis and b the long axis of the tumor. Mice bearing the scramble or the raptor shRNA xenografts were sacrificed after 20 days. Mice bearing the mTOR or the rictor shRNA xenografts were observed during 60 days. Points, average value of tumor volume. Bars, SD. Animal experiments were in accordance with the Swiss federal animal regulations and approved by the local veterinary office. *p < 0.001 comparing with cells expressing the Scramble shRNA (one-way ANOVA).

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