Institut für Tumorbiologie, Universitätsklinikum Hamburg-Eppendorf

Prof. Dr. Klaus Pantel
Institutsdirektor

Institut für Tumorbiologie

Martinistraße 52
20246 Hamburg

Dr. med. Dr. rer. biol. hum. Daniel J. Smit
Arzt / Forschungsgruppenleiter

Institut für Tumorbiologie

Gebäude N27 4. OG
Mobil: +49 (0) 152 22815034
Intern: (9) 65385
d.smit@uke.de
www.uke.de

Universitätsklinikum Hamburg-Eppendorf | Martinistraße 52 | 20246 Hamburg
Dr. Dr. Daniel J. Smit |Institut für Tumorbiologie

Hamburger Krebsgesellschaft
Butenfeld 18
22529 Hamburg

Abschlussbericht zum Projekt „Funktionelle Untersuchungen zur Rolle von AKT und NCKAP1 für die hepatische Metastasierung von kolorektalen Karzinom-Zellen“

Hamburg, 23.12.2024

Sehr geehrte Frau Holz, sehr geehrter Vorstand der Hamburger Krebsgesellschaft,

zunächst möchten wir uns nochmals recht herzlich für die großzügige Forschungsförderung der Hamburger Krebsgesellschaft unseres Projektes bedanken. Wie mit Frau Holz besprochen, haben wir in den letzten Monaten das Projekt aus Eigenmitteln fortgeführt und einige u.a. zusätzliche Experimente durchgeführt.

Derzeit verfassen wir die Publikation, die aus dem Projekt entstanden ist und die synergistische Wirkung von Inhibitoren gegen AKT/mTOR und gegen Regulatoren des Aktin-Zytoskeletts (Fascin) in primären und metastatischen CRC-Zelllinien beschreibt. Weiterhin konnten wir durch Knockdown-Experimente zeigen, dass die von AKT2 vermittelte Steigerung des Wachstums von CRC-Zellen über NCKAP1 vermittelt wird. Wir bitten daher den Abschlussbericht lediglich für interne Zwecke zu nutzen.

Im Anhang erhalten Sie den ausführlichen wissenschaftlichen Abschlussbericht, der Ihnen einen Überblick über die durchgeführten Arbeiten gibt. Der rechnerische Verwendungsnachweis sollte Ihnen bereits von unserer hiesigen Drittmittelabteilung zugegangen sein. Bei Fragen stehen wir Ihnen selbstverständlich jederzeit zur Verfügung.

Wir bedanken uns recht herzlich für die Förderung und das entgegengebrachte Vertrauen und verbleiben mit den besten Grüßen,

Dr. med. Dr. rer. biol. hum. Daniel J. Smit

Prof. Dr. rer. nat. Manfred Jücker

Final report on the research project 'Functional analysis of AKT and NCKAP1 for liver metastasis of colorectal cancer cells' funded by the Hamburger Krebsgesellschaft

1. Effect of combined treatment of colorectal cancer cells with AKT, mTOR and fascin inhibitors

Sustained proliferative signalling and activated invasion are two of several hallmarks and drivers of cancer (Hanahan, 2022). The PI3K/AKT/mTOR signaling pathway is a crucial driver of cancer development and progression. Remodeling of the actin cytoskeleton with the F-actin bundling protein fascin playing a pivotal role, propels invasive behavior. Fascin upregulation in metastatic cancers associates with poor clinical outcomes, highlighting its clinical significance (Puppa et al., 2007; Lin et al., 2021). To simultaneously target both proliferation and dissemination, we used a combination of AKT inhibitor MK2206, mTOR inhibitor RAD001, and fascin inhibitor NP-G2-044.

Four colorectal cancer cell lines were used in our experiments. SW480 and SW620 cell lines were derived from the same patient. SW480 was isolated from the primary adenocarcinoma arising in the colon and SW620 was isolated from a lymph node one year later when cancer recurred with widespread metastasis (Leibovitz et al., 1976). The other two colorectal carcinoma cell lines HROC147 T0M1 and HROC147 Met1 are also originated from one patient. HROC147 T0 M1 was established from a xenograft from a sigmoid colon tumor of the patient. The HROC147 Met1 cell line originates directly from the liver metastasis of the same patient (Wagner et al., 2021).

We treated the cells with increasing concentrations of MK2206, RAD001 and NP-G2-044 and analyzed the proliferation. **Figure 1** demonstrates that the effect of the treatment is concentration-dependent, and the highest inhibition of proliferation was reached by the combination of all three inhibitors in the highest concentration. The effectiveness of the combined treatment was observed for all four cell lines tested (i.e. SW480, SW4620, HROC147T0M1 and HROC147Met1).

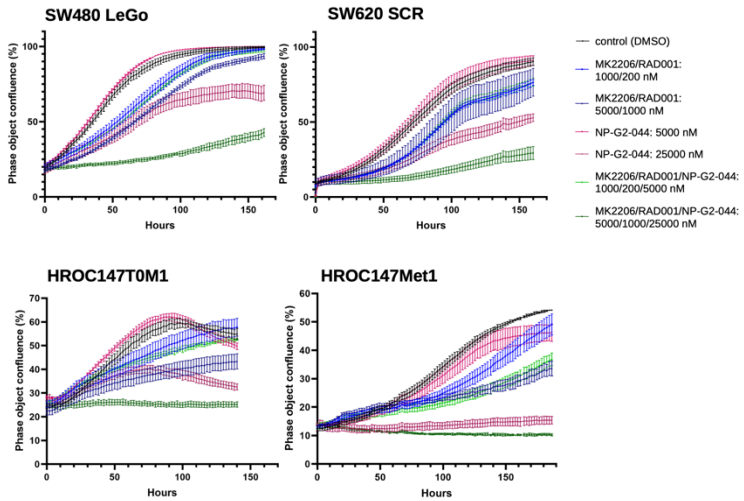


Figure 1. Titration of the AKT/mTOR and fascin inhibitors alone or in combination

Growth curves of colorectal cancer cell lines under AKT (MK2206)/mTOR (RAD001) or fascin (NP-G2-044) inhibitor treatment from the IncuCyte Zoom live cell imaging system.

Then, we determined the combination indexes (CI) using CompuSyn software (Chou, 2010). For each drug combination, CI<1, =1, and >1 indicates synergism, additive effect and antagonism, respectively. The combination of all three inhibitors demonstrated the synergistic effect in all four cell lines tested at the highest concentration (MK2206/RAD001/NP-G2-044: 5000/1000/25000 nM) and for three out of four cell lines also for the lower concentrations of MK2206/RAD001/NP-G2-044 (1000/200/5000 nM) **(Table 1)** indicating that the combination of anti-proliferative drugs (i.e. AKT and mTOR inhibitors) with anti-metastatic drugs (i.e. Fascin inhibitors) can improve the anti-tumor effect *in vitro*.

Table 1. Combination indexes for the MK2206/RAD001/NP-G2-044 treatment (mean values ± standard deviation) by 3 experiments.

Concentation	SW480 LeGo	SW620 SCR	HROC147T0M1	HROC147Met1
MK2206/RAD001/ NP-G2-044: 40/8/200 nM	3.11 ± 1.58	7.51 ± 10.03	1,713 ± 2,952	31.75 ± 35.31
MK2206/RAD001/ NP-G2-044: 200/40/1000 nM	8.52 ± 12.01	25.32 ± 29.47	6.00 ± 6.02	17.59 ± 27.15
MK2206/RAD001/ NP-G2-044: 1000/200/5000 nM	3.46 ± 2.73	**0.85 ± 0.38**	**0.47 ± 0.33**	**0.64 ± 0.11**
MK2206/RAD001/ NP-G2-044: 5000/1000/25000 nM	**0.02 ± 0.02**	**0.31 ± 0.41**	**0.01 ± 0.01**	**0.12 ± 0.03**

Solid tumors have a three-dimensional structure in which not all cells are equally exposed to therapeutic agents. Poor penetration and heterogeneity of the drug distribution in solid tumors are also one of the possible causes of the limited efficacy of chemotherapy and so-called “pseudo-resistance” (Davoli et al., 2022).

In addition to our previous experiments in 2D culture conditions, we also tested the combination of three inhibitors MK2206/RAD001/NP-G2-044 in a three-dimensional tumor spheroid model. We formed spheroids from HROC147 T0M1 and HROC147 Met1 and then treated the spheroids with the MK2206/RAD001 and NP-G2-044 inhibitors alone or in combination. Effectiveness of the treatment was estimated by the size reduction of the spheroids **(Figure 2, Figure 3)**. We also stained the spheroids with Calcein to test the cell viability after the treatment. Calcein-positive staining of the spheroids after FOLFOX treatment indicates that, despite the strong reduction of the spheroid size, the cells forming the spheroid are still alive **(Figure 4)**.

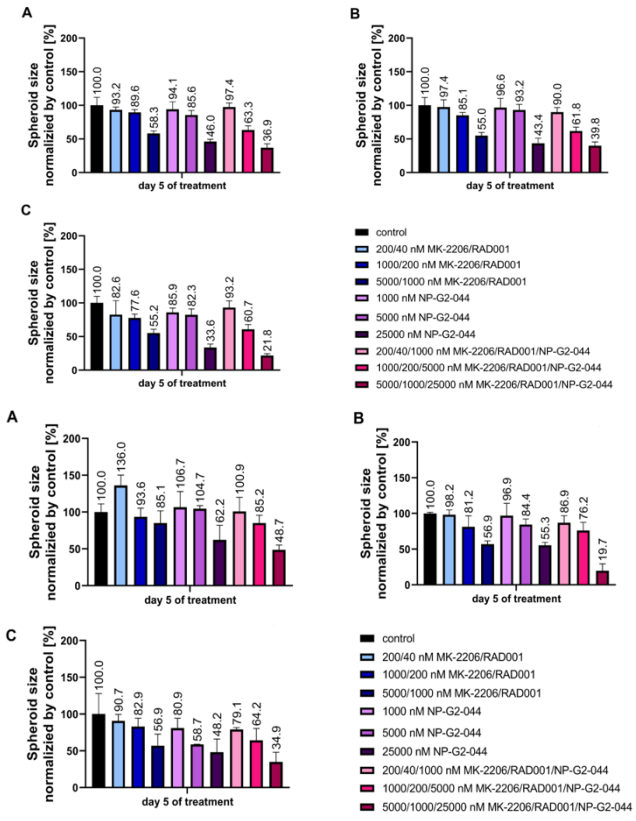


Figure 2. *Titration of the AKT/mTOR and fascin inhibitors alone or in combination in 3D cultures*

Relative size of the spheroids formed by HROC147 T0M1 after AKT (MK2206)/mTOR (RAD001) or fascin (NP-G2-044) inhibitor treatment. Results of three independent experiments are presented.

Figure 3. Titration of the AKT/mTOR and fascin inhibitors along or in combination in 3D cultures

Relative size of the spheroids formed by HROC147 Met1 after AKT (MK2206)/mTOR (RAD001) or fascin (NP-G2-044) inhibitor treatment. Results of three independent experiments are presented.

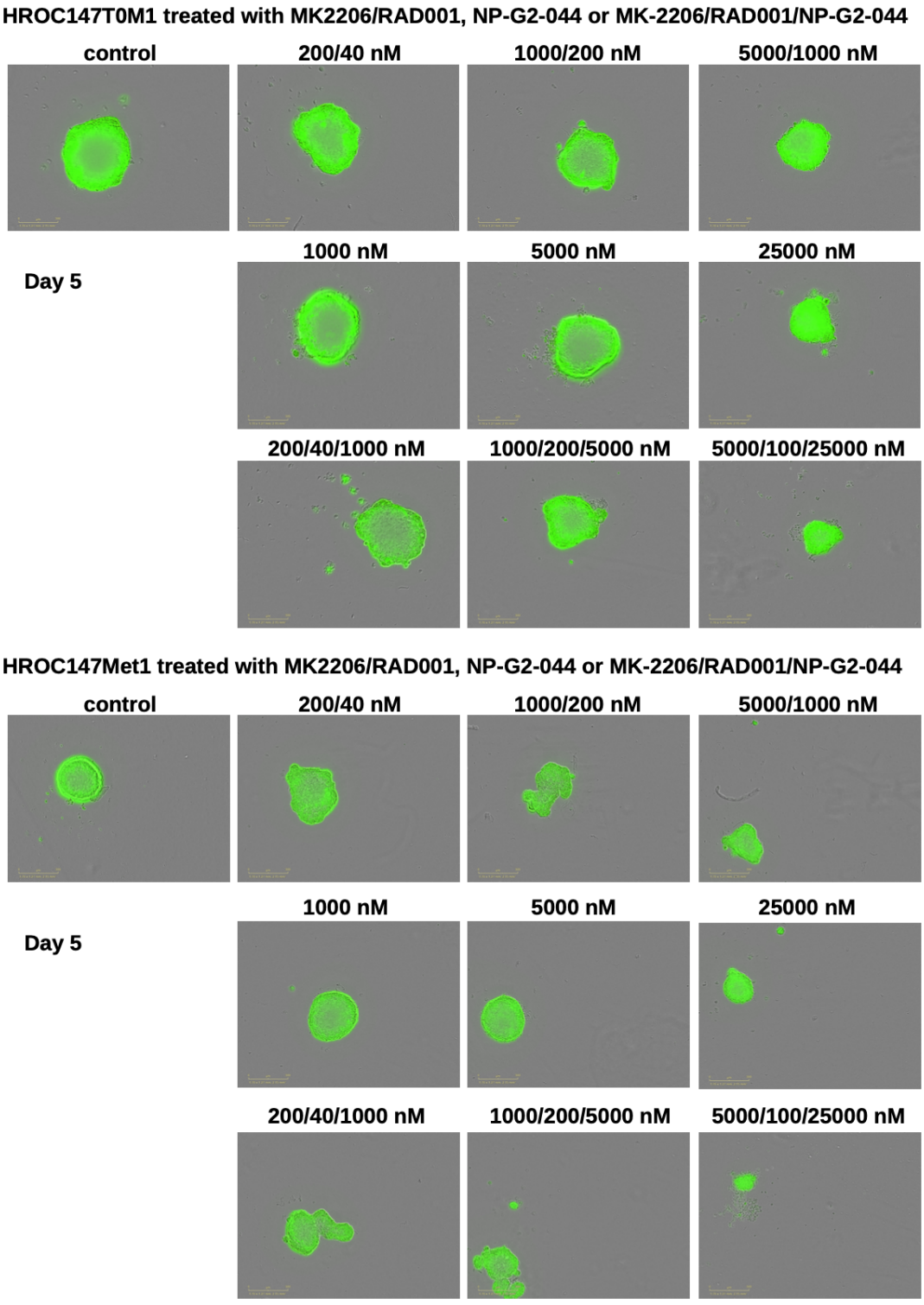


Figure 4. Calcein AM staining of HROC147 T0M1 and HROC147 Met1 spheroids

Spheroids formed by HROC147 T0M1 or HROC147Met1 cells were stained with Calcein AM (green) after 5 days of the treatment with AKT (MK2206)/mTOR (RAD001) or fascin (NP-G2-044) inhibitors alone or in combination

Combination indexes (CI) were also analyzed using CompuSyn software and are listed in Table 2. Treatment of spheroids with the combination of MK2206/RAD001/NP-G2-044 inhibitors also demonstrated synergism

for the two highest concentrations, although to a lesser extent **(Table 2)** compared to the results from the 2D model.

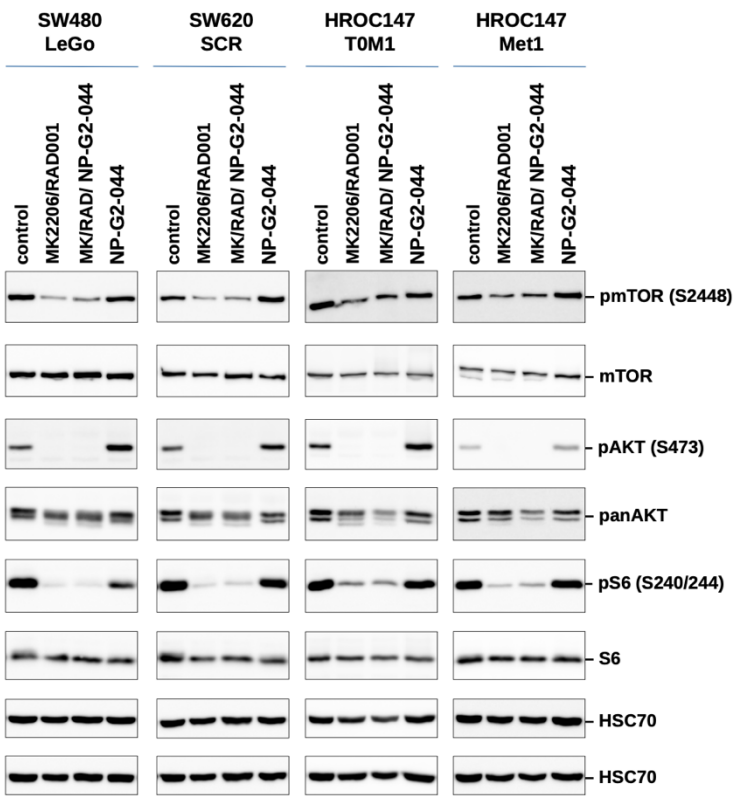
Concentration	HROC147T0M1	HROC147Met1
MK2206/RAD001/ NP-G2-044: 200/40/1000 nM	4.94 ± 5.57	1.17 ± 0.91
MK2206/RAD001/ NP-G2-044: 1000/200/5000 nM	**0.66 ± 0.13**	**0.83 ± 0.42**
MK2206/RAD001/ NP-G2-044: 5000/1000/25000 nM	**0.96 ± 0.66**	**0.88 ± 0.29**

Table 2. Combination indexes for the MK2206/RAD001/NP-G2-044 treatment of spheroids formed by HROC147 T0M1 and HROC147 Met1 (mean values ± standard deviation) by 3 experiments.

2. Molecular mechanisms of MK2206/RAD001/NP-G2-044-mediated inhibition

We characterized the molecular effects underlying MK2206/RAD001 and fascin inhibitor-mediated inhibition of colon cancer cell proliferation. We analyzed the phosphorylation levels of AKT, mTOR and ribosomal protein S6 by Western blot with the appropriate antibodies.

High levels of phosphorylated mTOR, AKT and S6 can be observed in all four untreated CRC cell lines **(Figure 5)**. Treatment with the combination of MK2206/RAD001 or MK2206/RAD001/NP-G2-044 resulted in the complete inhibition of AKT and S6 phosphorylation and strong reduction of mTOR phosphorylation in all four cell lines tested (Figure 5 and 6). Treatment with NP-G2-044 (fascin inhibitor) alone has no influence on the level of mTOR and S6 phosphorylation and also increased the phosphorylation of AKT (Fig. 5 and 6). As fascin inhibitors are aiming to block the fascin–actin interaction, the lack of effect on phosphorylation of the PI3K/AKT/mTOR pathway was expected. However, the increase in AKT phosphorylation following NP-G2-044 treatment was a novel finding that has not been reported before.



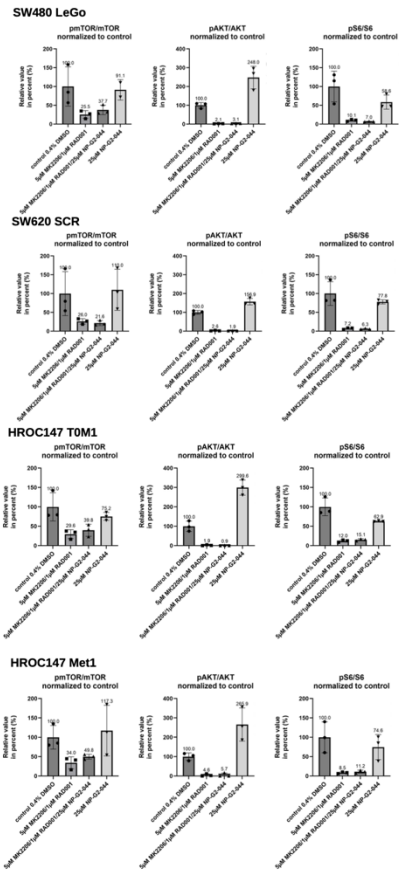


Figure 5. Western blot of CRC cell lines after treatment with AKT, mTOR and fascin inhibitors. *Cells were treated with the 5 µM MK-2206/1µM RAD001 and 25 µM fascin inhibitor (NP-G2-044) alone or in combination for 24 hours. Proteins of cell lysates were separated by SDS-PAGE, transferred onto the nitrocellulose membrane that was incubated with the indicated antibodies. The intensity of the bands was quantified, normalized to the unphosphorylated protein first and then to the loading control.*

3. Imaging of the actin cytoskeleton in CRC-cells with constantly activated or knock-down of AKT-isoforms

Cancer cells form actin-based structures that drive the proteolytic invasion by degrading the extracellular matrix (Linder et al., 2023). We tested the influence of constantly activated AKT-isoforms in the CRC cell line SW480 or stable knockdowns of the AKT isoforms in the CRC cell line SW620 on the actin cytoskeleton **(Figure 6)**.

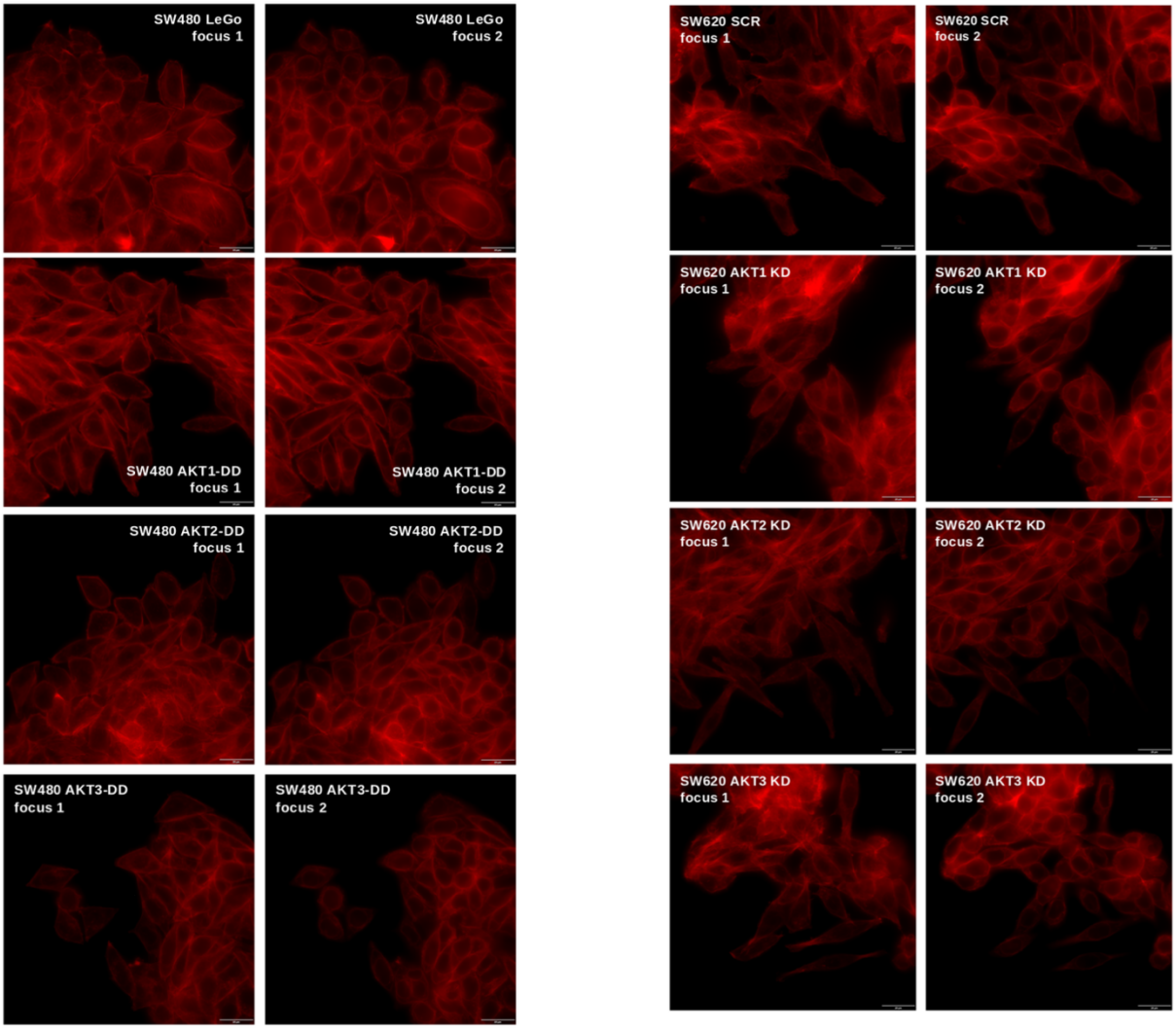


Figure 6. Staining of actin in SW480 cells expressing activated AKT isoforms and SW620 cells with reduced expression of AKT isoforms. *Left panel: SW480 AKT1-DD, AKT2-DD, AKT3-DD and control cell line SW480 LeGo; Right panel: SW620 AKT1 KD, AKT2 KD, AKT3 KD and control cell line SW620 SCR were stained with phalloidin-rhodamine.*

In a next step, we have treated the SW480 AKT-DD as well as SW620 AKT-KD cell lines with the 25 µM of fascin inhibitor to evaluate the effect on the actin cytoskeleton. Surprisingly, despite the strong synergistic effects observed in 2D and 3D proliferation experiments and the absence of PI3K/AKT/mTOR regulation, very weak changes could be observed after incubation with the fascin inhibitor (Figure 7).

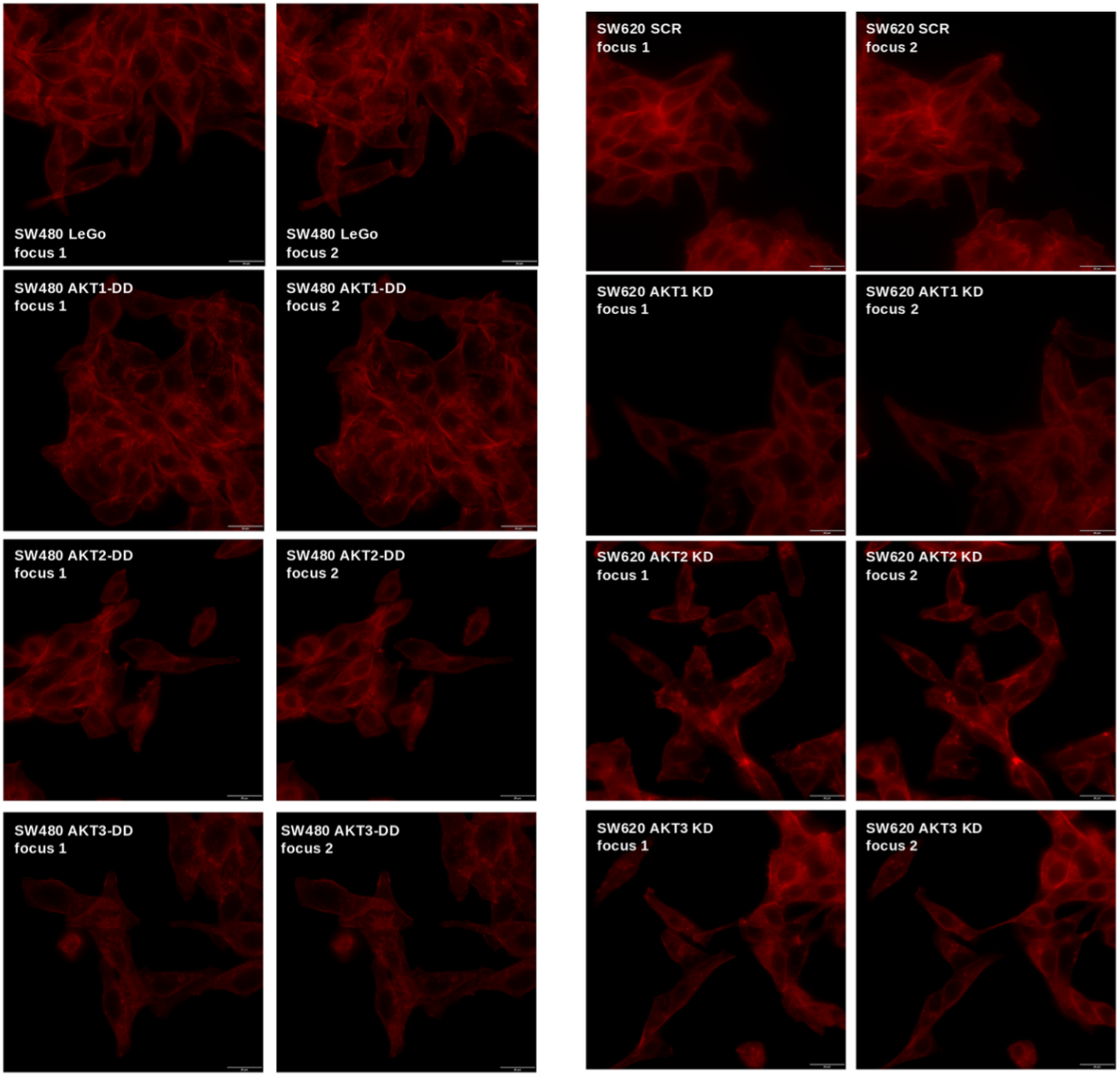


Figure 7. Staining of actin in SW480 cells with constantly activated AKT isoforms and in SW620 cells with AKT isoform knockdowns after treatment with fascin inhibitor NP-G2-044.

Colorectal cancer cells SW480 AKT1-DD, AKT2-DD, AKT3-DD and control cell line SW480 LeGo Left panel: SW480 AKT1-DD, AKT2-DD, AKT3-DD and control cell line SW480 LeGo; Right panel: SW620 AKT1 KD, AKT2 KD, AKT3 KD and control cell line SW620 SCR were treated with 25µM NP-G2-044 for 3 days and stained with phalloidin-rhodamine.

4. Cooperative effect of AKT1-DD, AKT2-DD and NCKAP1

In a next step, we have established SW480 cells with NCKAP1 KD alone as well as SW480 cells with AKT1-DD/NCKAP1-KD. The establishment of the knockdown was successfully confirmed by western blot analysis **(Figure 8A)**. Although multiple unspecific bands are present, the quantification of the western blot in the expected molecular range thus confirmed the successful NCKAP1 knockdown to a relative expression of 20% **(Figure 8B)**. For the cells with AKT1-DD, a NCKAP1 KD efficacy of 90% relative to the LeGO control was also observed.

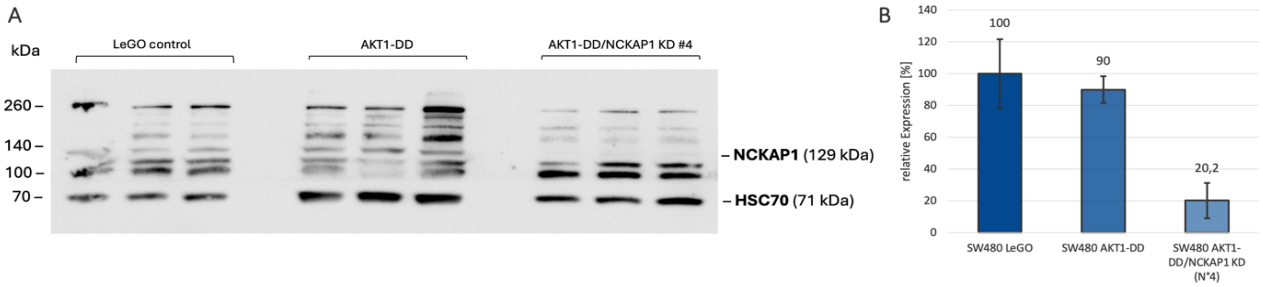


Figure 8. Western blot analysis of the cell line SW480 with an NCKAP1 antibody. (A) Image of the chemiluminescence with the corresponding HSC70 loading control. Triplicates with 20 µg total protein each were prepared. The membrane was sectioned between the 70 and 100 kDa bands. From the left: SW480 LeGO_puromycin; SW480_SCR_neomycin; SW480 AKT1-DD_puro/SCR_neomycin, AKT1-DD_puro/NCKAP KD_neomycin. NP40 buffer was applied between the respective triplicates. (B) Graphical representation of the relative expression of NCKAP1 normalized to HSC70.

In a next step, we analyzed the proliferation of the SW480 AKT1-DD/NCKAP1 KD cell line compared to the AKT1-DD and SW480 LeGO cell line. Surprisingly, we could not see an activating effect of AKT1-DD as reported previously, however, NCKAKP1 KD in the SW480-AKT1-DD cells (AKT1-DD/NCKAP1 KD) could diminish the proliferation capacity of the SW480-AKT1-DD cells by approximately 23% **(Figure 9)**.

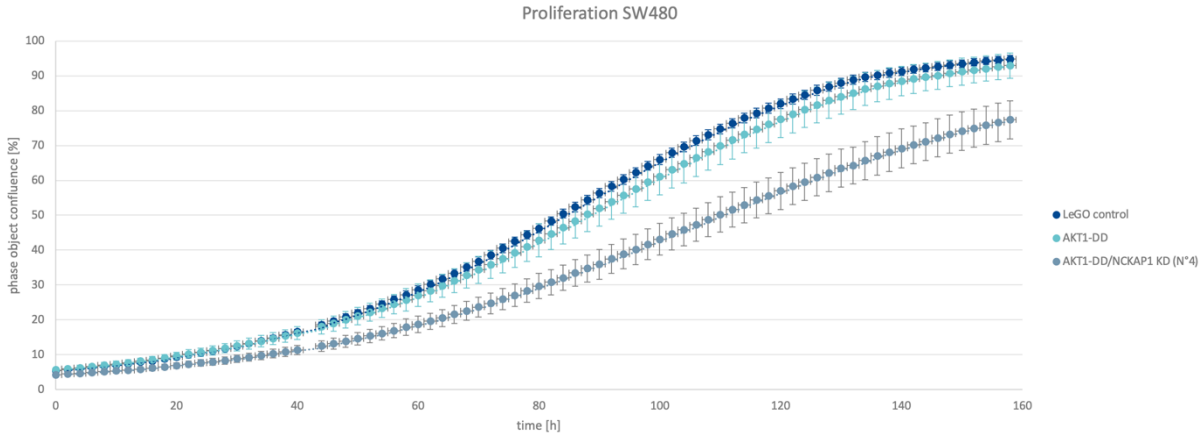


Figure 9. Growth curves of colorectal carcinoma cells of the SW480 cell line. The growth curves of the cells SW480 LeGO/puro - SCR/neo, SW480 AKT1-DD/puro - SCR/neo and SW480 AKT1-DD/puro - NCKAP1 KD (N°4)/neo are shown. The proliferation assay was terminated after 158h, and the confluence was visualized as a function of time.

We have also successfully generated the SW480 AKT2-DD/NCKAP1 KD line that shows a strong KD of NCKAP1 **(Figure 10)**.

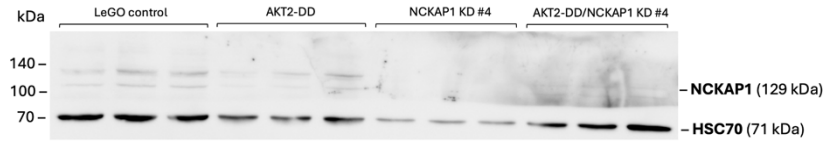


Figure 10. Western blot analysis of the cell line SW480 with an NCKAP1 antibody. (A) Image of the chemiluminescence with the corresponding HSC70 loading control. Triplicates with 40 µg total protein each were prepared. The membrane was sectioned between the 70 and 100 kDa bands. From the left: SW480 LeGO/puro - SCR/neo, SW480 AKT2-DD/puro - SCR/neo, SW480 LeGO/puro - NCKAP1 KD (N°4)/neo, SW480 AKT1-DD/puro - NCKAP1 KD (N°4)/neo.

In the proliferation assays, AKT2-DD alone, as well as NCKAP1 KD alone increase the proliferation rate of SW480 compared to LeGO control. Strikingly, AKT2-DD/NCKAP1 KD reverses the observed increase in proliferation **(Figure 11)**.

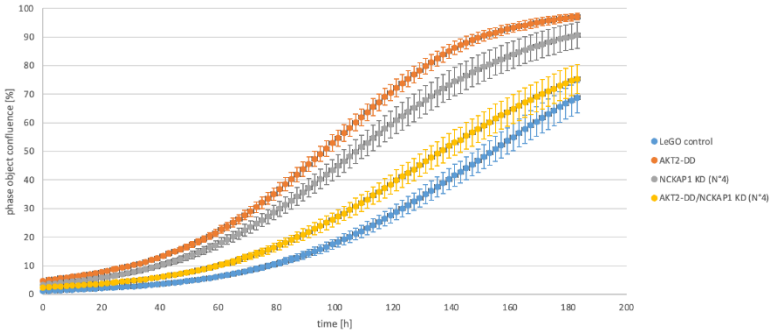


Figure 11. Growth curves of colorectal carcinoma cells of the cell lines SW480. Shown are the growth curves of the cells SW480 LeGO/puro - SCR/neo, SW480 AKT2-DD/puro - SCR/neo, SW480 LeGO/puro - NCKAP1 KD (N°4)/neo, SW480 AKT2-DD/puro - NCKAP1 KD (N°4)/neo. The proliferation assay was terminated after 183 hours and the confluence was visualized as a function of time.

5. Invasion assay of SW620 and AKT isoform-specific knockdowns in 3D culture conditions

To investigate the influence of AKT-isoforms on the ability of cancer cells to invade, we used 3D-invasion assay. Spheroids, formed by SW620 cells with knock-down of one AKT-isoform, were embedded in Matrigel to mimic extracellular matrix. In this assay, the “growth” of spheroids indicates that cells are able to degrade the extracellular matrix (Matrigel) and invade **(Figure 12)**.

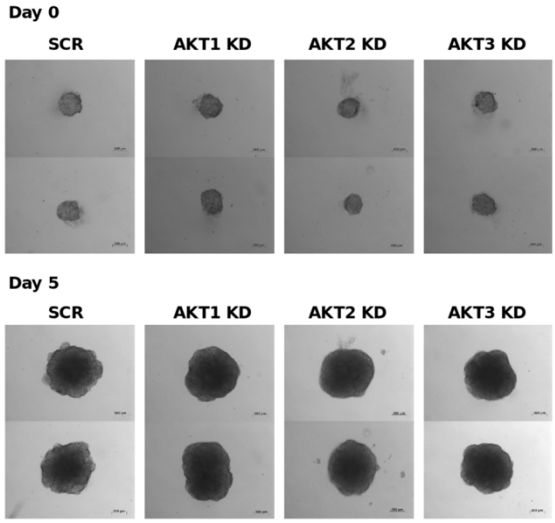


Figure 12. 3D invasion assay of SW620 cells with knock-down of AKT isoforms and control cell line SW620

SW620 AKT1 KD, AKT2 KD, AKT3 KD and control cell line SW620 SCR were seeded into ultra-low attachment plate to form spheroids (Day -3). After three days, spheroids were embedded in Matrigel (Day 0) and were incubated for 5 days.

We measured the size of the spheroids on Day 0 and after 5 days of incubation (Day 5) and found no significant differences between the cell lines with the knock-down of different AKT-isoforms (Figure 13).

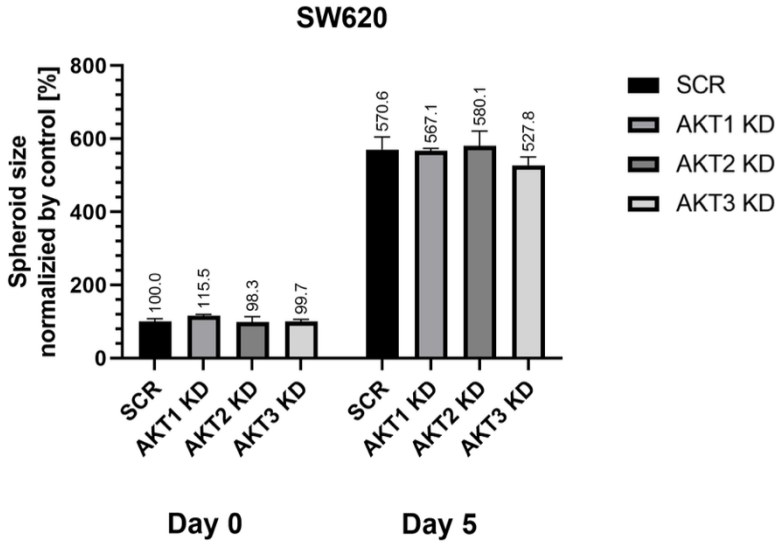


Figure 13. 3D invasion assay of SW620 cells with knock-down of AKT isoforms and control cell line SW620

Spheroids formed by SW620 AKT1 KD, AKT2 KD, AKT3 KD and control cell line SW620 SCR were embedded in Matrigel (Day 0) and were incubated for 5 days. The areas of the spheroids were estimated and normilized to the size of the control cell line SW620 SCR on Day 0.

Although we did not find any influence of different AKT-isoforms on the invasion potential of the SW620 cells, we received encouraging preliminary results when the spheroids were treated with the combination of AKT/mTOR/fascin inhibitors (5 µM MK-2206, 1 µM RAD001, 25 µM NP-G2-044). The inhibitors almost did not suppress the growth of spheroids formed by cancer cells SW620 AKT1 KD, AKT2 KD and SCR but significantly slowed down the growth of cells with reduced expression of the AKT3 isoform after 5 days of treatment (Figure 14).

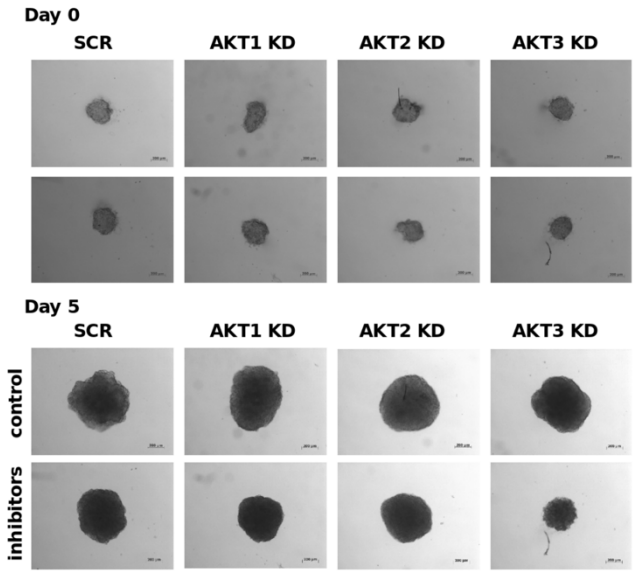


Figure 14. 3D invasion and treatment of SW620 cells with knock-down of AKT isoforms and control cell line SW620

SW620 AKT1 KD, AKT2 KD, AKT3 KD and control cell line SW620 SCR were seeded into ultra-low attachment plate to form spheroids (Day -3). After three days, the inhibitors were added, and spheroids were embedded in Matrigel (Day 0) and incubated for 5 days.

6. Conclusion

In conclusion, we have demonstrated here, for the first time that combined targeting of anti-proliferative (i.e. AKT inhibitors /mTOR inhibitors), as well as anti-metastatic drugs (i.e. fascin inhibitors) can synergistically reduce the proliferative effects of a primary CRC and a metastatic CRC cell line in 2D cell cultures and partly with additive effect in 3D spheroid culture. Moreover, we have revealed that fascin inhibitors can upregulate pAKT S473, highlighting that addition of AKT and mTOR inhibitors can be beneficial and that the mechanism of action which is currently not known yet is presumably mediated by AKT/mTOR signalling. Regarding the AKT isoforms, NCKAP1 KD was able to revert the AKT2-DD dependent increase in proliferation, indicating AKT isoform-specific effects. Our work suggests a complex network which goes beyond the PI3K/AKT/mTOR signalling that contributes to the molecular mechanism of action. Nevertheless, NCKAP1 inhibition and shRNA-mediated KD consistently reduces proliferation in the CRC cell line, indicating a noteworthy novel drug target.

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