A Study of interaction between KRAS and SMARCA4 (Brg-1) protein in Human colorectal carcinoma cell lines

1. Aim

To determine if the oncogene KRAS (Kirsten rat sarcoma viral oncogene homolog) binds to and regulates SMARCA4 (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4) function, which if proven could help create a SMARCA4 targeted therapy for colorectal cancers expressing KRAS.

2. Introduction

Colorectal cancer is an important health issue in the United States. The American Society of Clinical Oncology estimates that 52,580 people will die of colorectal cancer in the United States in 2022 [1]. An important gene being studied for the treatment of colorectal cancer is KRAS. KRAS mutations occurs early in colorectal cancer [4]. KRAS is a GTPase that is the main signaling intermediary in the EGFR pathway, and influences cell survival via the PI3K pathway, and cell proliferation via the MAPK pathway [4]. Furthermore, the RAS family of proteins are part of the ERK1/2 pathway, which plays a role in the development of some cancers [5]. Thus, mutations in KRAS cause excessive proliferation of cells, such as in the colon, and thus leads to cancer tumor formation.

The protein Brahma-related gene 1 (BRG1), encoded by the gene SMARCA4, is part of the SWI/SNF chromatin remodeling complex, and regulates gene promoters and enhancers, [2] and SMARCA4 is also a tumor suppressor [3]. BRG1 is overexpressed in prostate cancer but can also be down regulated [2]. Function of BRG-1 in colon cancer, however, remain largely unknown. In this study we will find out the interaction of KRAS protein and BRG-1 in two human colorectal cancer cell lines (wild type, KRAS mutant cell lines) which could be a potential therapeutic approach to cure colon cancer.

3. Experimental Plan

3.1 Methods and Materials

We used the human colorectal cancer cell lines HKE3 and HCT116. HCT116 is a KRAS mutant cell line with a G13D KRAS mutation, and the HKE3 cell line is a KRAS wt. (wild type) cell line [8]. These two cell lines were used by Shifteh et al. to show that Protein Arginine Methyltransferase 5 (PRMT5) is overexpressed in all KRAS cells, and more so in KRAS mutant cells than KRAS wt, at both the transcriptional and translational levels [8]. It was also shown, with these cell lines, that PRMT5 inhibitor caused G_2 phase cell cycle arrest in KRAS mutant cells [8]. It should be noted that HCT116 usually proliferates faster than HKE3. Therefore, HCT116 and HKE3 cell lines are excellent cell lines for researching KRAS.

We used Brg-1 shRNA (short hairpin RNA) (sc-29827-SH) (Santa Cruz Biotechnology, Inc.) to knock down SMARCA4. This shRNA is a combination of 3 target-specific lentiviral vector plasmids and contains a puromycin resistance gene to allow for puromycin-based selection of successfully transfected cells [6]. Lentiviral vectors, which are retroviruses, are derived from the Human Immunodeficiency Virus (HIV) and are used to introduce genes into target cells [5]. shRNA works by entering the nucleus of the target cell, where it is transcribed into the host cell's genome [3].

For the transfected HKE3 and HCT116 cell lines, two cell lines were created of each; cells that were treated with 4 µg Puromcyin/ ml in culture, and another with 2 µg Puromcyin/ ml in culture.

The HKE3 and HCT116 shRNA positive cells and the negative controls were revived by being cultured in complete media for 48 hours. Then puromycin (Puromycin dihydrochloride, sc-108071B 250mg) (Santa Cruz Biotechnology, Inc.) was added to all experimental cells and negative controls, to select for successfully transfected cells by killing off non-transfected cells. For the transfected HKE3 and HCT116 experimental and control cell lines, two cell lines were created of each; cells that were treated with 4 µg Puromcyin/ ml in culture, and another with 2 µg Puromcyin/ ml in culture.

Western Blotting was used to confirm knockdown of the cell lines using Anti-Brg-1 Antibody (H-10) HRP sc-374197 HRP 200 µg/ml (Santa Cruz Biotechnology, Inc.), which is an anti-human mouse monoclonal antibody (Ab). Anti β-Actin antibody was used as a housekeeping antibody. In order to perform a Western Blot, protein was extracted from the cells, and the concentration of the protein was measured so that the correct amount of protein could be

loaded into the gel. qPCR analysis was used to assess expression of KRAS, by extracting RNA from the cells, then measuring the concentration and A260/280 with Nanodrop ND-1000 Spectrophotometer. The A260/280 ratio had to be not lower than 1.6 and not higher than 2.2, or else the extracted RNA could not be used. Then cDNA was created from the RNA, and qPCR was performed with the cDNA. Primers for KRAS3 and GAPDH were used, GAPDH was used as a housekeeping gene.

3.2 Detailed Methods

3.2.1 Cell Culture

A. For cell culture, we will use RPMI with 1% pen strep, 0.4% gentamycin, and 10% FBS.

B. Cell cultures in flasks and petri dishes were kept in an incubator, with an internal environment of 37^0C and 5% CO_2. To prevent infection, replace the water and clean water tray with alcohol weekly.

C. To passage cells, all media was aspirated from the dish or flask, the dish was washed with PBS that was then aspirated, trypsin was added, and the dish was incubated for 5 minutes or until the cells were dislodged. The contents of the dish were then pipetted into a 15 ml tube and centrifuged for 3 minutes at 3,000 rpm. The media and trypsin were then aspirated, and the pellet of cells at the bottom of the tube was resuspended in complete medium.

3.2.2 Cell Counting

To count cells, the cells were resuspended in complete medium as per the procedure for passaging, and 15 µL of the media with cells and 15 µL of trypan blue were transferred to a 1.5 ml Eppendorf tube. The remaining cells were plated in a flask or petri dish. Then the Eppendorf tube was vortexed, 10 µL of media and trypan blue mixture were added to each end of a cell counting plate, and the samples in each end of the counting plate were counted separately using an Invitrogen Countess® II, as per the manufacturer's instructions. The average of the two samples in the counting plate was used as the average number of cells per 10 µL of media with resuspended cells.

3.2.3 Electroporation

To transfect cells with BRG-1 shRNA by electroporation, a known quantity of cells (6 million) was resuspended in fresh RPMI with no gentamycin, pen strep or FBS, and was added to an electroporation cuvette. The shRNA was kept in ice until it was used. 1 µg of the shRNA was added to the media with cells for the experimental group, but no shRNA was added to the control group. The cells were then shocked using a Mirus® Ingenio® EZporator with a 1200 V 25 µF shock lasting 25 milliseconds, and the cuvettes were left in ice for 10 minutes. The cells were then reconstituted directly in complete medium, without centrifugation, and cultured in a flask for 48 hours to recover from electroporation.

3.2.4 Puromycin Selection:

After 48 hours, transfer cells to medium with 1µg Puromycin/ ml. Many dead cells will be observed, but if the transfection was successful some will survive. Later, Puromycin dosage can be raised to 2 µg/ ml, and then 4 µg/ ml.

3.2.5 Protein Standard Curve and Protein Estimation Protocol

1. Turn spectrophotometer on and open software before to allow software to load.
2. Perform 1 to 2 dilutions of BSA: add 20 mg of BSA to 10 ml distilled water in a 15 ml tube, vortex tube. Add 2 ml of distilled water to four other 15 ml tubes. Transfer 2 ml of the solution from the first tube to the second and vortex the second tube, repeat this for the rest of the tubes, for a total of 5 tubes.
3. To 5 Eppendorf tubes, add 500 µL Bradford dye, 5 µL of each BSA sample, and 495 µL of water. Then transfer 500 µL of this mixture to spectrophotometer cuvettes.
4. Turn on the Beckman Coulter DU 800 spectrophotometer, open the software “DU 800 spectrophotometer” on the computer.
5. Click “visible” in bottom left corner of screen, then in top left select “Protein Assay Analysis,” which will set the absorbance to 595 nm. Put cuvette of purified water in machine with flat side facing left, click “BLK” in top left of screen to blank. To measure absorbance of a sample first load sample into spectrophotometer, then press “go” in top left of the screen. Once the absorbance has been measured twice, the machine will display the average absorbance.

6. In Microsoft® Excel®, graph the concentration of the sample added to each cuvette against its average absorbance, then add a line of best fit with an equation. The line of best fit is the standard curve.

3.2.6 Protein Estimation Protocol

To find the concentrations of future samples of protein, prepare the samples as above, except use 5 µL of the protein sample instead of BSA. Measure the absorbances as above, and use the equation of the standard curve to calculate the concentration of the sample.

3.2.7 Protein Isolation

1. Keep cell samples on ice in Eppendorf tubes.
2. Resuspend the cell pellets in 250 µL of Freeze-Thaw Lysis buffer mixed with 10 µL of Halt Protease and Phosphatase Inhibitor Cocktail for every 1 ml of Freeze-Thaw Lysis buffer.
3. Keep Freeze-Thaw Lysis buffer and Protease and Phosphatase Inhibitor Cocktail on ice when not in use. Store them in -20^{o} C.
4. Place microcentrifuge tubes with the cell samples in a floating Eppendorf holder.
5. Fill the insulated black box with liquid nitrogen to one inch deep
6. Place the floating Eppendorf holder in the liquid nitrogen for 10 seconds
7. Place Eppendorfs on the work bench until they have thawed, to induce heat shock.
8. Perform the above 2 steps 2 more times.
9. Note: for the HCT cell line, the Eppendorf tubes were placed in a floating Eppendorf holder, the holder was placed in a metal tube with a mesh bottome, and the end of the tube was submerged directly into a tank of liquid nitrogen.
10. Incubate samples on ice for 30 minutes.
11. Pass the lysate 5-10 times through an 18 to 21-gauge syringe needle. Use a new syringe and needle for each sample.
12. Centrifuge the Eppendorf tubes in the cold centrifuge at max speed for 10 minutes at 4^{o} C
13. Aspirate supernatants and place them in new microcentrifuge tubes. The pellet may be disposed.
14. Store the purified proteins in -80^{o} C or proceed immediately to protein estimation. Avoid freeze-thaw cycles of protein. After estimation, store proteins in -80^{o} C

3.2.8 Freezing Cells

1. Make cells into a pellet as in "To Passage Cells."
2. Add 200 µL of dimethyl sulfoxide (DMSO) to each freezing vial that will be used.
3. Aspirate supernatant and reconstitute pellet in cell growth medium, add 1 ml of medium with cells per freezing vial and mix with pipette.
4. Wrap freezing vials with clothe, wrap clothe with tape, write your initials, cell line, and date on tape. Place freezing vials in -80^{o} C freezer for at least a day to a week, then transfer to liquid nitrogen.

3.2.9 Reviving Frozen Cells

1. Remove freezing vial of cells from liquid nitrogen. If multiple vials are being removed, leave the vials that are not being revived at first in -80^{o} C.
2. Wait until medium in freezing vial is half melted, add 1 ml of warm medium to the freezing vial, and pipette contents of freezing vial into a 15 ml tube.
3. Centrifuge the 15 ml tube, resuspend in new medium, and plate as written in specified in the protocol for passaging cells.

Western Blot [11]

3.2.10 Sample preparation

1. Place a beaker of water on a hot plate to boil.
2. Add 60 µg of each protein sample obtained by protein isolation to locking Eppendorf tubes. If 60 µg is not available then equal amounts of each protein should be used. Then add a volume of 2X Laemmli buffer to each tube that is equal to the volume of the protein sample used.
3. Before placing samples in boiling water, use a thermometer to check that temperature of water is 100^{o} C, or wait until the water begins boiling.
4. Place Eppendorf tubes in a floating tube holder, then place the holder in the boiling water for 10 minutes.

5. Turn off hot plate.
6. Cool samples on ice for a minute to cool them, then centrifuge samples in microcentrifuge for a few seconds.

3.2.11 Running pre-cast gels to separate proteins by size:

7. This protocol uses Pierce® Fast Western Blot Kit.
8. To make running buffer dilute 10X Tris/Glycine/SDS buffer to 1X. Make 1 L of running buffer.
9. Remove the green tape from the bottoms of the pre-cast gels
10. Assemble two gels opposite each other in the Bio-Rad electrophoresis apparatus. Load gel so that both gels' numbers can be read, which means that the 2 gels will face in opposite directions.
11. Fill the inner and outer chambers half way with running buffer.
12. Remove the comb carefully so as to not damage the wells
13. Spin the Eppendorf tubes with the protein samples in microcentrifuge for 1 minute.
14. Load 30 µL of the sample and the protein ladder in the wells of the gel carefully. Use gel-loading pipette tips. Take care not to puncture the wells
15. Fill the inner and outer chambers all the way with running buffer.
16. Run gel at 125 V until the bromophenol blue dye front has run off the gel, and the lowest band of the ladder has almost reached the end of the gel, which will take about 1.5 hours.
17. Stop running the gel

3.2.12 Transferring the proteins from gel to Nitrocellulose membrane

1. To make transfer buffer: 100 ml of Tris/Glycine buffer, 200 ml methanol, 700 ml water, for a total of 1 L.
2. Fill a glass casserole tray with transfer buffer.
3. Soak all parts of the pre-assembled transfer sandwich in transfer buffer.
4. After the gel has finished running, carefully disassemble the two plates: use a spatula to pry open the plates at the corners marked with arrows

5. Assemble the transfer sandwich in the transfer chamber in the following order from top to bottom: black side of holder, black fiber pad, blotting paper, gel, nitrocellulose membrane, blotting paper, and black fiber pad.
6. After laying down the last Whatman paper sheet on top of the membrane, roll out any bubbles with a 5mL or 10mL plastic pipette
7. Close the transfer sandwich and soak it in the transfer buffer
8. Place the transfer sandwich in the Bio-Rad transfer apparatus, black side of the sandwich holder against the black side of the transfer apparatus.
9. Fill gel tank with transfer buffer.
10. Place transfer apparatus in a bucket of ice.
11. Place lid on machine, making sure the black socket in the lid is aligned with the black part of the transfer apparatus, and the red socket in the lid with the red part of the transfer apparatus.
12. Run transfer for 1 hour at 80 V. The transfer device will be hot after running.

3.2.13 Blocking Membrane and adding antibodies

13. To make blocking solution: 10 mg BSA/ml in 1X TBS. Only use 10 ml per blot
14. Put nitrocellulose membrane in a dish with blocking solution, place on rocker for 20 minutes.
15. To make primary antibody solution: 7 ml of Fast Western Antibody Diluent and 10 µL of primary antibody.
16. Drain off blocking solution.
17. Add all of the primary antibody solution to the dish, then leave dish in the fridge overnight. From now on, the dish with the membrane must remain covered with aluminum foil.
18. The next day, place membrane on rocker for 1 hour.
19. Make 5 ml of HRP reagent working dilution with 500 µL of HRP reagent and 5 ml antibody diluent.
20. Drain primary antibody off dish, add wash buffer, place, on rocker for 5 minutes. Always use 20 ml of wash buffer per wash.
21. Drain off wash buffer and add HRP reagent working dilution.

22. Place dish on rocker for 15 minutes.
23. Perform 3 washes with wash buffer, and transfer nitrocellulose membrane to a new dish before 3rd wash.
24. Make 5 ml detection reagent: 1:1 mixture of detection reagent 1 and detection reagent 2.
25. Drain off wash buffer, place membrane in new dish, add detection reagent, and place on rocker for 5 minutes.
26. Image the blot with the Bio-Rad Chemi-Doc imaging system.

3.2.14 Imaging the Blot with Bio-Rad Chemi-Doc imaging system.

27. Turn on the Bio-Rad Chemi-Doc imaging system at least an hour before use, or imaging will take longer than otherwise.
28. Open Image Lab 5.0 on the computer connected to the Chemi-Doc machine.
29. Place the white light conversion screen on the tray in the machine, place the nitrocellulose membrane on the screen, and remove air bubbles with a roller or a pipette.
30. On computer, select "New Protocol," "single channel," "Select," "Blots," "Chemi," then click "Run protocol."
31. To save raw luminescence image to a flash drive, plug the flash drive into the computer, click "Save," select "external drive," name the image and click "save."
32. To save the image as it appears on the computer screen, click "screenshot" in top left, save as file, save to flash drive.

3.2.15 To image with a second housekeeping antibody:

33. For the housekeeping protein: add 10 µL of the housekeeping antibody to 5 ml of Fast Western antibody diluent, this is the "Primary Antibody Working Dilution"
34. Add the Primary Antibody Working Dilution to the dish the nitrocellulose membrane is in.
35. Place dish on rocker for 1 hour.
36. Drain off Primary Antibody Working Dilution and wash membrane with wash buffer for 5 minutes.
37. Drain off wash buffer, add HRP Reagent Working Dilution, and place on rocker for 15 minutes.

38. Then perform three 5 minute washes, each with 20 ml of wash buffer and with blot on rocker. Before third wash, transfer membrane to a new dish.
39. Drain wash buffer off of dish, add Detection Reagent Working dilution, and place dish on rocker for 5 minutes.
40. Image nitrocellulose membrane.

3.2.16 Performing Densitometric Calculation for Western Blot

1. Open the ImageJ software, open the image of the Western Blot, select the heart-shaped icon, then use the mouse to trace the outline of the band in the Western Blot image.
2. Select analyze -> measure -> the software will display the area and mean of the protein band. Calculate the relative intensity of band by multiplying the area by the mean.

1. **3.2.17 DNA and RNA isolation [12]**

1. **Cite** Quick-DNA/RNA™ Miniprep Plus Kit Manual https://files.zymoresearch.com/protocols/_r1057t_r1057_r1058_quick-rna_miniprep_plus_kit.pdf
2. This protocol uses Zymo Research Quick-DNA/RNA™ Miniprep
3. Obtain a cell pellet.
4. Resuspend cell pellet in 400 µl of DNA/RNA Lysis Buffer.
5. Transfer sample to a yellow Spin-Away™ Filter Column.
6. Centrifuge the Spin-Away™ Filter Column in microcentrifuge at 10,000 RPM for 1 minute. DNA is now on the top above the filter, and RNA is on the bottom in the flow-through. The DNA may be discarded.
7. Transfer the Spin-Away™ Filter into a new collection tube, which is the same kind of tube it was just in.
8. For the flow through: Add 1 volume of ethanol to the flow through. Then pipette the ethanol with RNA into a green Zymo-Spin™ Green Column that is in a collection tube.
9. Microcentrifuge the sample for 1 minute at same speed as above. Discard the flow through.
10. Add 400 µl DNA/RNA Prep Buffer to column and microcentrifuge for 1 minute. Discard the flow through.
11. Add 700 µl DNA/RNA Wash Buffer to the column and centrifuge for 1 minute. Discard the flow-through.
12. Add 400 µl DNA/RNA Wash Buffer and centrifuge the column for 2 minutes to ensure complete removal of the wash buffer.
13. Label Eppendorfs to receive the samples and cut off caps.
14. Put the top section of the filter column into the Eppendorfs that do not have caps, place Eppendorf caps on the filter columns, dispose of the collection tubes.
15. Elute sample with 100 µL of nuclease free water, make sure to pipette directly onto the filter, not onto the walls. Let stand for 5 minutes.
16. Microcentrifuge for 1 minute, the RNA is in the flow through.
17. Store the RNA in -80° C.
18. Measure concentration and A260/280 of RNA with the Nanodrop Spectrophotometer (see below).

3.2.18 Measuring Concentration and A260/280 with Nanodrop ND-1000 Spectrophotometer

1. Select “Nanodrop” software on computer.
2. Cover the tiny hole under the arm with 2 µL of nuclease free water.
3. Press “nucleic acids” then press “initialize”.
4. Wipe hole with Kim Wipe
5. Again, put 2 µL of nuclease free water on hole.
6. Click “blank”
7. 2 µL of RNA solution on hole.
8. Select “DNA”. Note the A260/280 ratio, which represents the purity of the RNA. A260/280 needs to be not lower than 1.6 and not higher than 2.2, or the RNA cannot be used.
9. 2 µL water on hole again, click “blank.”
10. When done, put a Kim Wipe under the arm of the machine

3.2.19 cDNA Preparation

1. Use up to 2 µg of total RNA per 20-µL reaction
2. Allow the RNA solution and the following kit components to thaw on ice: N9 (10µM) : 1µL (Random Primer), and dTVN (10µM): 1µL.
3. Mix and run in thermal cycler under the protocol (10 min at 80^{o}C followed by 4^{o} C hold) set for 8 µL.
4. 2X first strand reaction mix: 10 µL
5. Superscript enzyme mix: 2 µL
6. Total reaction volume: 20 µL
7. Mix and run thermal cycler under the protocol (60 min at 42^{o} C followed by 10 min at 80^{o} C and 4^{o} C hold).
8. Transfer resultant cDNA solution into an Eppendorf tube and add 100 µL of nuclease free water, mix thoroughly.

3.2.20 Performing qPCR

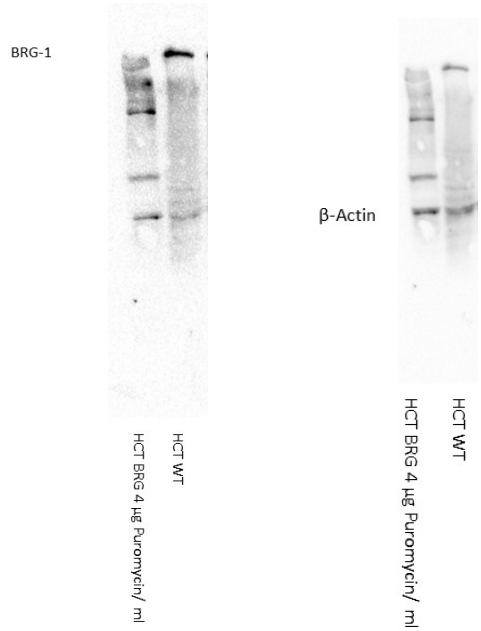
1. Use two 8-well qPCR strips for each cell line, 3 wells for each primer per cell line.

2. To make master mixes: for each primer use 14 µL of that primer and 42 µL of nuclease free water. Make one master mix per primer. Keep the amount of primer and nuclease free water at this proportion, more or less of the master mix can be prepared as needed.
3. To make cDNA master mixes: for each cell line combine 13 µL of cDNA and 65 µL of SYBR green. More or less of the master mix can be prepared as needed.
4. Then: 10 µL of wax into each well.
5. 4 µL of primer master mix and 6 µL of cDNA master mix per well. Each well should only have the cDNA from one cell line and one primer in it.
6. Then add 10 µL of liquid wax to each well.
7. Put qPCR strips in the Applied Biosystems 7300 Real Time PCR System.
8. Turn qPCR machine on.
9. On the computer connected to the qPCR machine, 7300 System SDS software.
10. Select file -> new-> absolute quantification of standard curve -> 96 well -> next -> SYBR green -> add -> next
11. Highlight all the wells, check the box towards the top of the window to select all the wells
12. Select finish.
13. Select the instrument tab-> set last step to 58^{o}C for 30 minutes.
14. Then add step-> set step to 72^{o}C for 30 minutes.
15. Add dissociation stage -> leave preset values for dissociation stage.
16. Set sample volume to 20 µL
17. Select file -> save as -> name and save the qPCR
18. Run the qPCR.
19. When qPCR is done, the data can be exported onto a flash drive.

4. Results

The Western blot in Figure 1a confirmed knockdown of SMARCA4. This Western blot image, which was prepared with anti-BRG-1 antibody, has a signal for BRG-1 in the HCT WT cell line but not for the HCT BRG 4 µg Puromycin/ ml cell line. Figure 1b is an image of the same Western blot with anti-β-actin antibody, β-actin being used as the housekeeping gene. Both cell lines show a β-actin signal.

Figure 1. Western Blot Images of HCT WT and HCT BRG 4 µg Puromycin/ ml

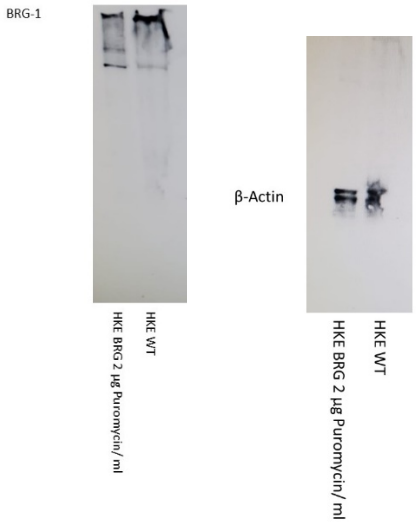


A. B.

A. Western blot image with anti-BRG-1 antibody B. With anti-β-actin antibody

Knockdown of HKE BRG 2 µg Puromycin/ ml was confirmed by Western Blotting. Figure 2a shows the Western Blot image of HKE WT and HKE 2 µg Puromycin/ ml with anti-BRG-1 antibody. Figure 2b. shows the Blot with anti-β-actin antibody, β-actin being used as the housekeeping gene.

Figure 2. Western Blot Images of HKE WT and HKE BRG 2 µg Puromycin/ ml



A. B.

A. Western blot image with anti-BRG-1 antibody B. With anti-β-actin antibody

However, the qPCR was only performed on the HKE 4 µg Puromycin/ ml cell line, further research is needed to perform qPCR on an HKE cell line that was confirmed to have BRG1 knocked down.

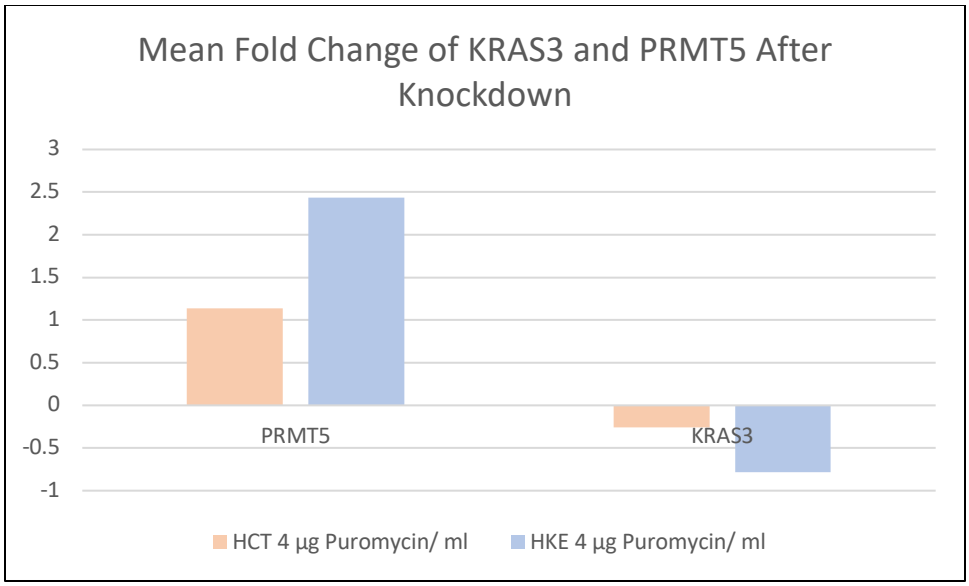
Densitometric calculation of the Western blots was performed. As shown in Table 1, HCT WT had a signal from the anti-BRG1 antibody, while HCT BRG1 4 µg Puromcyin/ ml has a visibly weaker signal in the western blot image, yet has a higher intensity in the calculations. Perhaps the band in the HCT BRG1 4 µg Puromcyin/ ml is not really a BRG1 signal, and if not then the method used for densiometric calculations must be flawed. Both cell lines had signals for the anti-β-actin antibody of approximately the same intensity. The HKE WT cell line had an anti-BRG1 signal approximately twice as intense as that of the HKE BRG 2 µg Puromycin/ml cell line. The two HKE cell lines had anti-β-actin antibody signals of approximately the same intensity.

Table 1. Densitometric Calculations for the Western Blots

	Western Blot Densitometric Calculations		
	With Anti-BRG-1 Antibody		
	Area	Mean	Intensity=Area*Mean
HCT BRG 4 µg Puromcyin/ ml aka lane 2	0.076	189.716	14.418416
HCT WT aka lane 1	0.042	78.956	3.316
HKE BRG 2 µg Puromycin/ml aka leftmost	229	89.341	20459.089
HKE WT aka second from left	708	56.444	39962.352
	With Anti-β Actin Antibody		
	Area	Mean	Intensity=Area*Mean
HCT BRG 4 µg Puromcyin/ ml aka lane 2	576	182.533	105139.008
HCT WT aka lane 1	553	180.51	99822.030
HKE BRG 2 µg Puromycin/ml aka leftmost	432	73.076	31568.832
HKE WT aka second from left	451	80.694	36392.994

The CT, ΔCt, ΔΔCT, and fold change from the qPCR are shown in Appendix A, and the fold change values are shown in Figure 3. The results show that knockdown of SMARCA4 results in a decrease in the expression of KRAS3, though, as written above, knockdown of the HKE cell line was not confirmed. The results also show that knockdown of SMARCA4 results in an upregulation of PRMT5.

Figure 3. Fold Change of KRAS3 and PRMT5 As Per qPCR



To calculate the fold change, the qPCR was performed at least 3 times per cell line per primer, the mean of the CT values was taken, then

ΔCT= CT (gene of interest)- CT(housekeeping gene).

Then

ΔΔCT = ΔCT treated – ΔCT control,

and fold change was $2^{-\Delta\Delta CT}$. For example: for HCT in qPCR 1, mean CT of HCT WT for KRAS3 was 24.5, mean CT of GAPDH, the housekeeping protein, was 16.3. Thus

ΔCT of HCT WT was 24.5-16.3=8.2.

Mean CT of KRAS3 for HCT 4 µg Puromycin/ ml was 28.69 and for GAPDH was 16.39, so

ΔCT of HCT 4 µg Puromycin/ ml was 28.69-16.39 = 12.3.

Then

ΔΔCT = 12.3 - 8.2 = 4.1,

and the fold change was

$2^{-\Delta\Delta CT}=2^{-4.1}$ = 0.058314562.

5. Discussion

It would appear from the results that the oncogene KRAS does bind to and regulate SMARCA4 function. Knockdown of SMARCA4 resulted in a decrease in the expression of KRAS3, so it would appear that SMARCA4 promotes the expression of KRAS3, or vis versa. Also, Knockdown of SMARCA4 resulted in an upregulation of PRMT5, so it would appear that SMARCA4 suppresses expression of PRMT5.

The fold changes from the qPCR for both KRAS3 and PRMT5 were greater in magnitude in HKE3, the KRAS wt, than in HCT116, the KRAS mutant cell line. Perhaps the mechanisms by which these 3 genes, KRAS, SMARCA4, and PRMT5, interact are damaged in the KRAS mutant, and thus the affect of downregulation of SMARCA4 resulted in a smaller fold change than in the WT cell line, which had these gene-regulation mechanisms intact.

6. Conclusion

Knockdown of SMARCA4 resulted in an upregulation of PRMT5. Also, given that KRAS appears to regulate SMARCA4, there is the potential to create a SMARCA4 targeted therapy for colorectal cancers expressing KRAS. This could help improve the prognoses of many patients world wide suffering from many types of cancers.

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Appendix A. CT, ΔCT, ΔΔCT, and Fold Change Values from qPCR

Mean CT for KRAS3

	qPCR 1	qPCR 2	qPCR 3
HCT WT	24.5	20.96	24.77
HCT 4 µg Puromycin/ ml	28.69	21.85	23.02
HKE WT	23.27	22.03	23.25333
HKE 4 µg Puromycin/ ml	24.87	24.55	24.77

Mean CT for GAPDH

	qPCR 1	qPCR 2	qPCR 3
HCT WT	16.3	14.28	16.53667
HCT 4 µg Puromycin/ ml	16.39	14.8	15.25333
HKE WT	15.04	14.09	14.54
HKE 4 µg Puromycin/ ml	14.17	14.14	14.27667

ΔCT for KRAS3

	qPCR 1	qPCR 2	qPCR 3
HCT WT	8.2	6.68	8.23333
HCT 4 µg Puromycin/ ml	12.3	7.05	7.76667
HKE WT	8.23	7.94	8.71333
HKE 4 µg Puromycin/ ml	10.7	10.41	10.49333

ΔΔCT for KRAS3

	qPCR 1	qPCR 2	qPCR 3
HCT 4 µg Puromycin/ ml	4.1	0.37	-0.46666
HKE 4 µg Puromycin/ ml	2.47	2.47	1.78

KRAS 3 Fold Change $2^{-\Delta\Delta CT}$ For:

	qPCR 1	qPCR 2	qPCR 3	Mean Fold Change
HCT 4 µg Puromycin/ ml	0.058314562	0.773782497	1.381906494	0.738001184
HKE 4 µg Puromycin/ ml	0.180491149	0.180491149	0.291183397	0.217388565

Mean CT for PRMT5

	qPCR 1	qPCR 2	qPCR 3
HCT WT	20.87	17.33	26.7
HCT 4 µg Puromycin/ ml	21.2	18.09	24.645
HKE WT	19.16	16.65	21.95667
HKE 4 µg Puromycin/ ml	16.76	16.04	20.18667

ΔCT for PRMT5

	qPCR 1	qPCR 2	qPCR 3
HCT WT	4.57	3.05	10.16333
HCT 4 µg Puromycin/ ml	4.81	3.29	9.39167
HKE WT	4.12	2.56	7.41667

HKE 4 µg Puromycin/ ml	2.59	1.9	5.91

ΔΔCT for PRMT5

	qPCR 1	qPCR 2	qPCR 3
HCT 4 µg Puromycin/ ml	0.24	0.24	-0.77166
HKE 4 µg Puromycin/ ml	-1.53	-0.66	-1.50667

PRMT5 Fold Change $2^{-\Delta\Delta CT}$ For:

	qPCR 1	qPCR 2	qPCR 3	Mean Fold Change
HCT 4 µg Puromycin/ ml	0.846745312	0.846745312	1.707233038	1.133574554
HKE 4 µg Puromycin/ ml	2.887858391	1.580082624	2.841534044	2.436491686