Free Fatty acids receptors (FFAR2 and FFAR3) control cell proliferation by regulating cellular glucose uptake

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Abstract

Background Colorectal cancer is a worldwide problem which has been associated with changes in diet and lifestyle pattern. As a result of colonic fermentation of dietary fibres, short chain free fatty acids are generated which activate Free Fatty Acid Receptors 2 and 3 (FFAR2 and FFAR3). FFAR2 and FFAR3 genes are abundantly expressed in colonic epithelium and play an important role in the metabolic homeostasis of colonic epithelial cells. Earlier studies point to the involvement of FFAR2 in colorectal carcinogenesis.

Methods Transcriptome analysis console was used to analyse microarray data from patients and cell lines. We employed shRNA mediated down regulation of FFAR2 and FFAR3 genes which was assessed using qRT-PCR. Assays for glucose uptake and cAMP generation was done along with immunofluorescence studies. For measuring cell proliferation, we employed real time electrical impedance based assay available from xCelligence.

Results Microarray data analysis of colorectal cancer patient samples showed a significant down regulation of FFAR2 gene expression. This prompted us to study the FFAR2 in colorectal cancer. Since, FFAR3 shares significant structural and functional homology with FFAR2, we knocked down both these receptors in colorectal cancer cell line HCT 116. These modified cell lines exhibited higher proliferation rate and were found to have increased glucose uptake as well as increased level of GLUT1. Since, FFAR2 and FFAR3 signal through G protein subunit (Gai), knockdown of these receptors was associated with increased cAMP. Inhibition of PKA did not alter the growth and proliferation of these cells indicating a mechanism independent of cAMP/PKA pathway.

Conclusion: Our results suggest role of FFAR2/FFAR3 genes in increased proliferation of colon cancer cells via enhanced glucose uptake and exclude the role of protein kinase A mediated cAMP signalling. Alternate pathways could be involved that would ultimately result in increased cell proliferation as a result of down regulated FFAR2/FFAR3 genes. This study paves the way to understand the mechanism of action of short chain free fatty acid receptors in colorectal cancer.

Introduction

Colorectal cancer is a disease that is intricately associated with the dietary patterns, metabolism and inflammation[1]. Type of diet intake significantly modifies the risk for development of CRC[2]. There is evidence linking high carbohydrate-low fibre diet to increased risk for CRC[3–6]. The site of this cancer is also the location for processing of food aided by the gut microbiota. The effect of diet on CRC has been studied with different perspectives of associated factors. Metabolism of nutrients, role of gut microbiota and familial factors are being studied to better understand the causal factors in diet related initiation and progression of CRC. Type of food intake, its digestion and metabolism is an upcoming area of research with potential to develop preventive and therapeutic strategies. Characterization of gut microbiota with newer technologies is allowing the possibility of customized probiotic treatment for the prevention of colorectal cancer [7, 8]. Digestion of food results into formation of building blocks which are assimilated in the small intestine. Short Chain Fatty Acids (SCFAs) are produced in the distal gut by bacterial fermentation of macro-fibrous material that escapes digestion in the upper gastrointestinal tract and enters the colon [9, 10]. SCFAs such as butyrate and propionate exert anticancer effect on colon as they

have been shown to induce differentiation, growth arrest and apoptosis, mainly due to their intracellular actions, through inhibition of histone deacetylase (HDAC)[11, 12]. This suggests that SCFAs produced in the gut could have protective properties against development of CRC. SCFAs are cognate ligands for a group of G-protein coupled receptors (GPCRs), FFAR2 and FFAR3 also known as GPR43 and GPR41, respectively[13, 14]. FFAR2 and FFAR3 genes are abundantly expressed in human colon[15]. FFAR2 recognizes all three major SCFAs, but the affinities for FFAR3 are in the order of propionate>butyrate>acetate[16]. Activated FFAR2 initiate signalling through the Gai pathway to suppress cyclic adenosine monophosphate (cAMP) levels, and through the Gαq pathway to enhance calcium mobilization[17]. Negative impact of FFAR2 on cAMP levels results in inhibition of protein kinase A (primary downstream effector of cAMP) and its substrate, cAMP response element binding protein (CREB). Together, this leads to reduced expression of histone deacetylase (HDAC)[18]. Several studies on fatty acid receptors suggested their involvement in the onset and progression of colon cancer[15, 19]. It has been suggested that FFAR2 plays a role in the cancer cell metabolism and growth. Loss of FFAR2 was observed using immunohistochemistry in malignant colon adenocarcinoma tissues by 80% compared to normal human colon mucosa tissue[15]. Transforming activity of loss of FFAR2 and its oncogenic potential was validated in fibroblasts[20]. However, there is no definitive study to suggest the role of FFAR2 as well as FFAR3 in cancer cell growth and metabolism[21].

In order to better understand the connection between gut microbiota and colorectal cancer, we searched for genes encoding for receptors for SCFAs. We therefore analysed the expression level of FFAR2 and FFAR3 genes in our patient cohort. In this study, we provide evidence of reduced expression of FFAR2 gene in colorectal cancer patients using microarray analysis of matched tumour-normal tissues. While some patients had reduced FFAR3 gene expression, collectively we did not observe significant differences in the expression levels in this patient group. To further characterize the mechanistic effect of loss of these receptors, we generated Knockdown (KD) clones of FFAR2 and FFAR3 genes in HCT116 colorectal cancer cell line. While FFAR2 KD exhibited increased cell proliferation, it was further increased with subsequent KD of FFAR3 gene. These engineered cells were found to exhibit a significant increase in their glucose uptake as well as increased expression of GLUT1. Simultaneously, increased levels of cAMP were observed in these cells. These results provide evidence to suggest the role of FFAR2 as well as FFAR3 in progression of colorectal cancer via a previously unknown mechanism of increased glucose uptake.

Methods

Patient samples and microarray analysis

Colorectal cancer patient samples were analysed for gene expression changes using microarray analyses of the dataset reported earlier [22]. This dataset is available on Gene Expression Omnibus, National Center for Biotechnology Information, National Institute of Health, USA (Accession: GSE 50421). The study used to generate the dataset was approved by institutional review board of King Abdullah International Medical Research Center, Saudi Arabia for project RC10/083. All patients were consented

before sample collection. For the analysis of differential gene expression between tumour and normal, we used Transcriptome Analysis Console (TAC) software available from Affymetrix (ThermoFisher Scientific, USA). Sample IDs and respective expression values for FFAR 2 and 3 genes in tumour and normal samples are given in table S1. Gene expression values were generated using TAC and further analysed using GraphPad prism 7 software. Paired t-test was used to compare matched tumor-normal samples and detailed results are provided in table S2.

Ethics, consent and permissions

All ethical consent and permission were obtained as mentioned in our previous publication [23].

Cell line and culture conditions

HCT116 cell line (ATCC® CCL-247™) was obtained from American Type Culture Collection (ATCC), Manassas, VA, USA). Cells were cultured in 5%CO₂ at 37 °C. Cells were grown in advanced Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 100 I. U./mL of penicillin, 100 µg/mL of streptomycin and 2mM L-glutamine (Gibco, Thermo Fisher Scientific, Waltham, MA, USA).

Generation of stable FFAR2 KD HCT116 cell line

FFAR2 Short-hairpin RNA (ShRNA), targeting 4 different sequences of the FFAR2 gene, and a control scrambled shRNA plasmid were obtained from Origene Technologies, Inc. 1x10⁵ HCT116 cells were plated in a 24-well plate and transfected with 0.5 µg of plasmid DNA using Lipofectamine3000 with a ratio of 1:2 (DNA:Lipo3000) according to the manufacturer's protocol. Cells were selected in 0.8 µg/mL of puromycin (As per data available at http://cell-lines.toku-e.com/Cell-Lines_1422.html: Accessed 2July 2018) for 4 weeks then isolated colonies were maintained in 0.8 µg/mL of puromycin antibiotic. Colonies were screened for FFAR2 KD using quantitative real time polymerase chain reaction (qRT-PCR) and one colony with most efficient KD was selected for subsequent experiments. One colony from scrambled shRNA construct was also selected to serve as control in all experiments.

Generation of FFAR2 and FFAR3 double KD cells

We used pre-packed Lentiviral particles harbouring 3 different shRNA sequences against the FFAR3 gene and a control scrambled shRNA which were obtained from GeneTarget, Inc. To generate FFAR2/FFAR3 double KD cells. Earlier selected colony with most efficient KD of FFAR2 were transduced with the FFAR3 Lentiviral particles with a multiplicity of induction (MOI) = 5. Cells were selected in 5 µg/mL of blasticidin antibiotic (Dose was determined for HCT 116 cells using kill curve for blasticidin) for 4 weeks then colonies were maintained in 5 µg/mL of blasticidin and 0.8 µg/mL of puromycin antibiotic. Colonies were

screened for FFAR2 and FFAR3 KD using quantitative real time polymerase chain reaction (qRT-PCR) and one colony with most efficient KD was selected for subsequent experiments.

RNA extraction, cDNA synthesis and real-time PCR

Total RNA from five patients' tumour-normal paired samples was extracted using the QIAGEN RNeasy Mini Kit (Qiagen; Cat# 74104). 2 µg of RNA was used for cDNA synthesis using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific). qRT-PCR was performed by using ABI 7900HT PCR system (Applied Biosystems, Foster City, CA, USA). GAPDH was used as housekeeping control. Sequence of primers used are GAPDH-F: 5' ACAACTTTGGTATCGTGGAAGG 3', GAPDH-R: 5' GCCATCACGCCACAGTTTC 3', FFAR2-F: 5' TGCTACGAGAACTTCACCGAT 3', FFAR2-R: 5' GGAGAGCATGATCCACACAAAAC 3', FFAR3-F: 5' GCGTGGAGGATCTACGTGAC 3', FFAR3-R: 5' TGTGAGTGTTCACTGGTCTTTC 3'. PowerUp SYBR Green Master Mix (Applied Biosystems) was used. All reactions performed in triplicate and the qRT-PCR data was analysed by using the RQ method ($2^{-\Delta\Delta C_t}$) by SDS RQ manager and expression Suite version 1.1.

Complete details with Ct values have been provided in Tables S3 and S4.

Glucose uptake assay

Ten thousand cells from scrambled control, FFAR2 KD and FFAR 2/3 double KD clones of HCT116 cells were plated in each well of a 96 well plate. Next day, cells were washed twice with phosphate buffer saline and incubated in glucose free DMEM. Glucose uptake was determined using Glucose uptake assay kit (Cayman Chemicals, MI, USA) following manufacturers protocol. Briefly, cells were incubated with 100 µg/ml fluorescent 2-N-7-Nitrobenz-2-oxa-1, 3-diazol-4-yl-Amino-2-Deoxyglucose (2-NBDG) in glucose free medium for 1hr. Cells were washed with assay buffer 3 times and analysed immediately. 2-NBDG taken up by cells was detected on a Tecan Infinite 200 pro fluorimeter (Tecan Group Ltd. Männedorf, Switzerland) with fluorescent filters designed to detect fluorescein (excitation/emission = 485/535 nm). Cells without 2-NBDG incubation were used as blank control.

cAMP assay

Thirty thousand scrambled control, FFAR2 KD and FFAR2/FFAR3 KD HCT116 cells were plated on a 48 well plate overnight. Next day, cells were incubated with 500 µM IBMX (3-isobutyl-1-methylxanthine) for 1hr. cAMP in each well was measured using cAMP screen Immunoassay System (Thermo-fisher scientific) by following the manufactures instructions. Briefly, after IBMX incubation, cells were lysed in 100µl lysis buffer (provided with the kit) by incubating at 37°C for 30 minutes. 50µl of cell lysates were added to the pre-coated 96 well plate followed by addition of cAMP-alkaline phosphatase conjugate and cAMP antibodies to each well. A cAMP standard was also prepared ranging from 0.002 to 2000pmol of cAMP. Plate was incubated at room temp for two hours with constant shaking. Each well was washed 6

times with wash buffer followed by addition of CSPD /Sapphire-II™ RTU substrate/enhancer solution and incubation for 30 minutes. Measurements were made using a single-mode luminometer (Molecular Devices). Standard curve was made using the reading from cAMP standards and cAMP was measured in each well with reference to the standard curve. The experiment was repeated twice with 8 technical repeats for each condition.

Cell proliferation assay using XCELLigence

Rate of cell proliferation was measured using xCELLigence real time cell analyser-dual purpose (RTCA-DP) available from ACEA biosciences (San Diego, USA). E-16 plates were used for monitoring cell adhesion and growth. This system works on the principle of electrical impedance. A unitless parameter termed Cell Index (CI) is used to measure the relative change in electrical impedance to represent cell status. CI is a relative and dimensionless value since it represents the impedance change divided by a background value. When there are no cells present in the medium, the sensor's electronic property will not be affected and the impedance will be small. When there are more cells on the electrodes, the impedance will be larger. CI calculation is based on the following formula: $CI = (Z_i - Z_0) / 15 \text{ } \Omega$, where Z_i is the impedance at an individual point of time during the experiment and Z_0 is the impedance at the start of the experiment. CI is a self-calibrated value derived from the ratio of measured impedances. For cell proliferation experiments, we plated 40000 cells from scrambled control, FFAR2 KD and FFAR 2/FFAR3 KD HCT116 cells on E-16 plate in duplicate and cell proliferation was monitored for 60 hrs. The experiment was repeated 3 times and slope values of the growth curve were plotted using in-built software and later analysed on Graph pad prism. H89 (PKA inhibitor) was added at a concentration of 5 μ M after 24 hr of plating and cell growth was monitored up to 60 hrs.

Immunofluorescence for GLUT1 gene expression

HCT116 cells grown on cover slips were washed with PBS and fixed with 4% formaldehyde. Cells were incubated with anti-GLUT1 antibody (Catalogue #PA1-46152, Thermo Fisher Scientific, USA) in PBS containing 5% serum and 0.1% Triton for 30min at 37°C. Cells were washed four times with PBS and incubated with fluorescein isothiocyanate (FITC) conjugated antibodies for 30 minutes. Cells were washed four times and immediately imaged using EVOS FL Auto imaging system (ThermoFisher Scientific). Images were quantified for GLUT1 expression using metamorph image processing software (Molecular devices).

Protein Kinase A Immunoblotting

HCT116 Cells were lysed with Immunoprecipitation buffer (Thermo Fisher Scientific) containing protease inhibitors. The scraped cells were kept on ice for 30 min and centrifuged at 10000g for 5 min and the supernatant was collected. Protein concentrations were determined by Qubit protein assay kit (Thermo

Fisher Scientific). 20 µg of lysate was boiled at 95°C for 5 min and loaded onto ready-made gel 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad), subjected to electrophoresis and transferred to PVDF membranes (Bio-Rad). The membranes were blocked using 5% BSA (MILLIPORE) in Tris buffer saline + Tween 20 (TBST) buffer for 1 hour with shaking at room temperature. The following primary antibodies were used: PKA-PAS (Cell signalling) isotype anti-rabbit at recommended dilution of 1:1000 in 5% BSA with gentle shaking overnight at 4°C. The blots were incubated with the appropriate Horse Radish Peroxidase-conjugated secondary antibody (Bio-Rad), and the signals were detected with Chemiluminiscent HRP Substrate (Bio-Rad). Images were captured and analysed using Chemidoc gel documentation system (Bio-Rad).

Statistical analysis

Data were presented as the mean $\pm$ SD and analysed for statistical significance using two tailed students *t* test available in the GraphPad prism 7 software version 7.03. Each bar represents an average of at least two independent experiments with multiple technical replicates in each experiment. Significance was set for a *P* value of < 0.05.

Results

Reduced expression of free fatty acid receptors in CRC patients

In a selected group of eighteen patients, we used microarray data to compare the expression levels of FFAR2 gene in matched tumour-normal samples and found it to be significantly downregulated (Figure 1A). There was -1.388 ± 0.2065 times difference between means of Normal versus Tumor samples for FFAR2 gene signals (p-value <0.0001). But there was no significant difference in expression levels of FFAR3 gene (Figure 1B). From the available sample of five of these patients, we did qRT-PCR analysis for FFAR2 and FFAR3 genes. In these five patients, there was significant down regulation of both FFAR2 and FFAR3 genes. FFAR2 gene showed 5.388 times down regulation in these samples (p value = 0.0066) and FFAR3 was also down regulated more than two times (Figure 1C&D). A heat map showing FFAR2 and FFAR3 expression in each patient's tumour and normal tissue reflects the inter-patient heterogeneity (Figure 1E). We analyzed other members of FFARs namely FFAR1 and FFAR4 in these patients and found no significant difference in their expression levels (Figure S1 and Table S5)

Loss of FFARs in HCT116 cells results in increased proliferation

We engineered HCT116 cells to down regulate the expression of FFAR2 and FFAR3 genes. First we knocked down FFAR2 gene and obtained 63% reduced stable expression. In this cell line FFAR3 levels were also found to be slightly affected with 32% reduction in expression. Next, we down regulated FFAR3 levels in this cell line. We found stable reduced expression of FFAR2 (77%) and FFAR3 (68%) in this cell clone. These two cell lines were chosen for all further experiments (Figure 2A). We measured the rate of

proliferation of these cells and found increased rate of proliferation as reflected by the cell index values in FFAR2 knock down cells (2.84 times compared to scrambled control). Double KD cells showed even higher increase in cell proliferation with 8.26 times cell index as compared to scrambled control (Figure 2B).

Loss of FFAR2 and FFAR3 leads to increased glucose uptake and GLUT1 expression

We measured the uptake of glucose in the engineered cells. Cell line with FFAR2 KD showed about 1.5 times more glucose uptake whereas double KD exhibited even more with 1.8 times increase over the control cells (Figure 3A). We subsequently measured the levels of GLUT1 expression in these cells and found it to be significantly increased as higher fluorescence intensity was observed in modified cells (Figure 3B). GLUT1 expression pattern correlated with the glucose uptake in the modified cells as reflected in coefficient of determination value of 0.9615 (Figure 3C).

Double KD cells exhibit increased cAMP production

In order to understand the mechanistic role of FFARs in colorectal cancer, we attempted to interrogate the involvement of cAMP pathway. We found highly significant increase in cAMP levels in double KD cells with more than 23 times increased cAMP levels as compared to scrambled control (Figure 4A). There was no significant change in cAMP level in FFAR2 knockdown cells but the rate of cell proliferation was increased suggesting a cAMP independent effect of FFAR2. We further checked if increased cAMP levels are involved in increased cell proliferation. In order to block the effect of cAMP, we tested the rate of cell proliferation in the presence of H89 molecule which functions as protein kinase A (PKA) inhibitor. H89 showed a saturated inhibitory effect on the modified cells above 1 μ M concentration (Figure 4B). We did not observe any difference in rate of cell proliferation with the addition of H89 suggesting PKA independent mechanism of cAMP activity (Figure 4C). H89 also showed sustained inhibition up to 48h which confirms its efficacy during the entire duration of the cell proliferation experiment (Figure S2).

Discussion

As the evidence is supporting the strong connection between diet and colorectal cancer, there is an increased quest to understand the underlying molecular mechanism. Both long and short chain free fatty acids have been shown to be associated with cancer and metastasis [24–28] especially in colorectal cancer where high fat diet has been strongly correlated. Receptors for these fatty acids would be good targets for designing prevention strategies. But the available evidence so far is not clear on establishing their role in different types of cancer. These receptors have shown to mediate increased cancerous activity as well as reduction in growth in different types of cancer cells. Also, these receptors belong to family of GPCRs which are favourable molecules as drug targets [29]. In the present study, we focused on understanding the role of receptors for gut-microbiota derived SCFAs. FFAR2 and FFAR3 are well known receptors for SCFAs. FFAR2 has been implicated in CRC [30] but there is no known evidence for FFAR3 association. There are reports where the heterodimers of these receptors have been suggested to signal the short chain fatty acids. Earlier, we had reported cytogenetic and gene expression profile of colorectal

cancer patients [22, 23, 31]. Using this data we interrogated the expression profile of short chain free fatty acid receptors. While we found significant down regulation of FFAR2 gene in few patients, the variability of expression was high. This further supports the notion of inter-patient heterogeneity observed in cancer especially colorectal cancer [32, 33] and strengthens argument in favour of personalized medicine[8]. We further validated our results using available patient samples and carried out qRT-PCR based expression analysis. Both microarray and qRT-PCR analyses confirmed down regulation of FFAR2 gene in tumour samples. Our data thus confirm previous reports of down regulated FFAR2 gene in colorectal cancer[34]. However, our observations regarding FFAR3 are novel and this study underscores the importance of studying the two receptors together. While we observed no significant difference in expression level of FFAR3 gene in tumour samples, there was significant down regulation observed in qRT-PCR data as reported earlier[30]. This could be due to the variability observed in these selected patient samples and/or due to higher sensitivity of qRT-PCR assay and possible differences in the two techniques[35].

In order to establish the role of short chain free fatty acid receptors, we engineered a colon cancer cell line with reduced expression of FFAR2 and FFAR3 genes. Our hypothesis suggested increased cell proliferation in cells with reduced expression of short chain FFARs. Two cell line clones were generated— One with reduced expression of FFAR2 gene alone and another with reduced expression of both FFAR2 and FFAR3 genes. FFAR2 KD cells showed highly significant increase in cell proliferation whereas double KD showed comparatively enhanced effect in cell proliferation as well as glucose uptake and cAMP production. This is a clear evidence of FFAR2 and FFAR3 function as tumour suppressors via mechanism that needs to be fully understood. A recent report suggested epigenetic dysregulation of inflammation suppressors by FFAR2[36]. Colorectal cancer cells are known to uptake glucose at a higher rate and Warburg effect is a hallmark of cancer cells [37]. Colorectal cancer cells with reduced levels of FFAR2 and FFAR3 also displayed increased uptake of glucose in an additive manner which could be responsible for increased cell proliferation. We could not implicate FFAR2 and FFAR3 in glucose metabolism but this needs to be further studied to better understand the network affected by the reduced expression of these genes. Increased glucose uptake in the engineered cells was accompanied with an increased expression of GLUT1—a well-known glucose transporter. Overexpression of GLUT1 has been suggested as a negative prognostic biomarker in colorectal cancer and indicator of clinically aggressive disease[38]. Our results thus suggest a previously unknown important connection between FFAR2/FFAR3 and glucose metabolism. Increased glucose metabolism has been known to be induced by short chain fatty acids [39].

To further understand the effect of FFAR2/FFAR3 on the signalling pathways, we measured the cAMP levels in engineered cells. Intracellular cAMP level has been shown to regulate cellular motility [40].cAMP has also been shown to suppress apoptosis in colorectal cancer cells [41]. There was a huge increase in cAMP levels in double KD cells which was correlated with increased cell proliferation. However, FFAR2 single KD cells showed an increased cell proliferation and glucose uptake without any changes in cAMP levels. These results suggest that impact of FFAR2/FFAR3 on cell proliferation and glucose uptake are independent of cAMP signalling pathway. To further evaluate the role cAMP pathway, we inhibited PKA, a known downstream target of cAMP signalling by H89 molecule which is a known PKA inhibitor[42]. Inhibition of PKA had no impact on the rate of cell proliferation. Some studies have shown FFAR2/FFAR3

to signal through other pathways like p38 and JNK signalling [43] and Hippo-Yap pathway [44]. These pathways may be involved in mediating FFAR2/FFAR3 effect in our study and are an interesting area of research for future projects.

Our results thus conclusively establish the role of FFAR2 and FFAR3 in increased proliferation of colorectal cancer cells. This study also provide evidence to suggest the involvement of GLUT1 and PKA independent cAMP signalling pathway which needs to be further studied for identifying therapeutic targets and biomarkers for colorectal cancer progression. A schematic of proposed mechanism has been illustrated in Figure 5.

Declarations

Acknowledgement

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Disclosure statement

The authors have no conflict of interest.

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Figures

Figure 1

Gene Expression analysis of FFAR2/FFAR3 genes in patients diagnosed with colorectal cancer. a) Microarray analysis of gene expression of FFAR2 in tumor-normal paired tissues. b) Microarray analysis of gene expression of FFAR3 in tumor-normal paired tissues. c) QPCR gene expression analysis of FFAR2 in tumor-normal paired tissues. d) QPCR gene expression analysis of FFAR3 in tumor-normal paired tissues. e) Heatmap representation of FFAR2 and FFAR3 gene expression using QPCR in tumor-normal

paired tissues. Color intensity ruler is given to represent Ct values with a range of -2 to 2 which is inverse of expression level. More red means less Ct and hence more expression. More blue Each bar represents an average of at least two independent experiments with multiple technical replicates in each experiment. Significance was set for a P value of < 0.05 (paired t test).

Figure 2

Increased proliferation of colorectal cancer cells in single FFAR2 and double FFAR2/3 knockdown colorectal cancer cells. a) Relative mRNA expression in single FFAR2 and double FFAR2/3 knockdown clones compared to scrambled control. b) xCELLigence proliferation assay of FFAR2 and FFAR2/FFAR3 double knockdown clones. Each bar represents an average of at least two independent experiments with 2 technical replicates in each experiment. Significance was set for a P value of < 0.05.

Figure 3

. Single knockdown of FFAR2 and double knockdown of FFAR2/3 increases glucose uptake and GLUT1 expression in colorectal cancer cells. a) Glucose uptake in single FFAR2 and double FFAR2/3 knockdown

clones compared to control. b) Immunofluorescence for GLUT1 in single FFAR2 and double FFAR2/3 knockdown clones compared to scrambled control. Each bar represents an average of at least two independent experiments with multiple technical replicated in each experiment. Significance was set for a P value of < 0.05. c) Correlation plot between GLUT1 immunofluorescence and Glucose uptake. R2 value close to 1 suggest a good correlation.

Figure 4

Increased cAMP levels in double FFAR2/3 knockdown colorectal cancer cells. a) cAMP level in single FFAR2 and double FFAR2/3 knockdown clones compared to scrambled control. b) Western blot analysis of effect of different doses of H89 in inhibiting phosphorylation of protein kinase A. Dose of 5μM and 10 μM showed complete inhibition. c) Cell proliferation of single FFAR2 and double FFAR2/3 knockdown clones compared to scrambled control with and without PKA pharmacologic inhibitor.

Figure 5

An illustration depicting the possible role of FFAR2 and FFAR3 that results into increased cell proliferation. This figure was generated through the use of IPA (QIAGEN Inc., <https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis>)

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