miR-27a-3p is a master regulator of metabolic reprogramming in colorectal cancer

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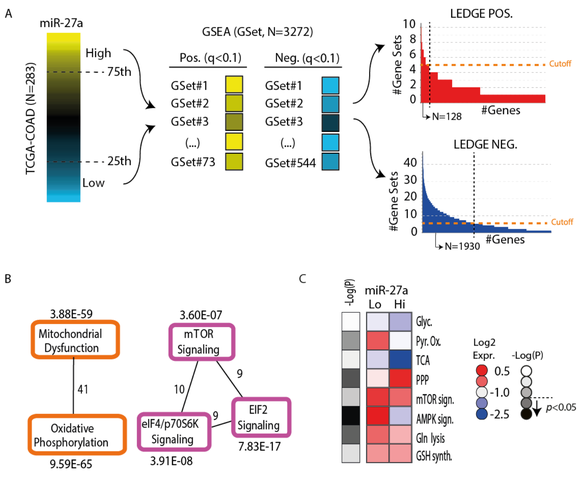
Background

Reprogramming energy metabolism is a hallmark of cancer, the molecular bases of which are still undefined. Cancer cells redirect their energy metabolism by restricting it largely to glycolysis, even in the presence of oxygen, leading to a process called "aerobic glycolysis". Such a metabolic reprogramming can be orchestrated by activated oncogenes such as K-RAS, TP53, AMPK, PI3K and their downstream signaling which enhance the glycolytic and glutamine pathways to support biosynthesis, redox homeostasis and, ultimately, cell growth and survival. An emerging new class of modulators of cellular processes is represented by microRNAs (miRNA), short non-coding RNAs that regulate gene expression at the post-transcriptional level. miRNA dysregulation has been correlated and functionally linked to various types of human cancers. Recently, we have reported that miR-27a-3p influences colorectal cancer (CRC) initiation/progression.

Aim of this study was to investigate whether miR-27a has any role in cancer metabolic reprogramming, to delineate the pathways affected and to define whether this metabolic rewiring is associated with chemoresistance.

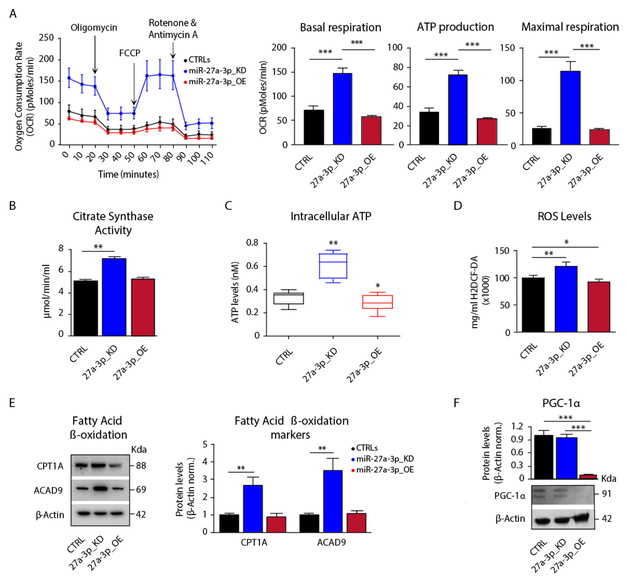
Results

BIOINFORMATIC ANALYSIS OF GENES AND PATHWAYS REGULATED BY miR-27A EXPRESSION



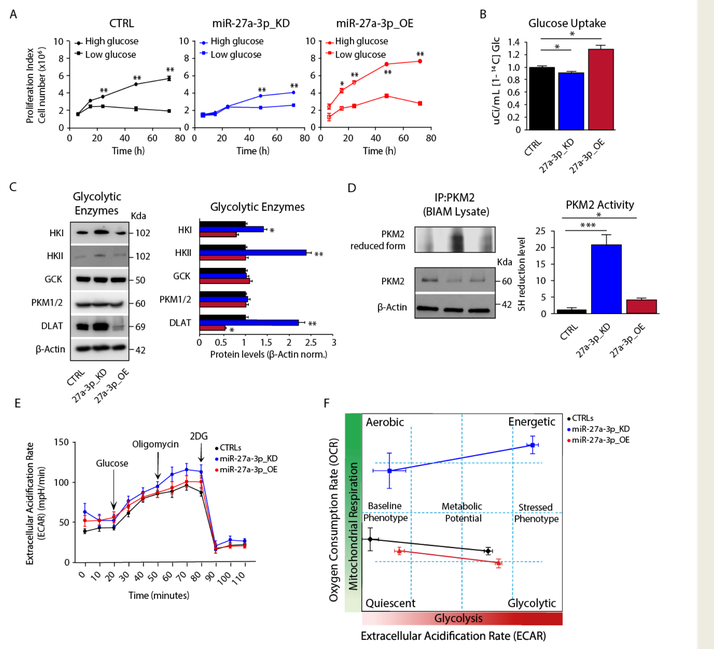
To explore the impact of miR-27a expression in CRC, we retrieved RNA-seq data (miRNA and mRNA) of 283 patients from the TCGA-COAD repository and ordered the samples according to miR-27a expression considering only the highest and lowest percentiles (>75th and <25th). We, then, performed Gene Set Enrichment Analysis (GSEA) using ~3300 curated gene-sets and identified 73 gene-sets significantly enriched in high- miR-27a and 544 in low-miR-27a tumors (**A**). We refined this result by performing Leading-Edge analysis (LEDGE) and derived a list of 128 overlapping genes in high-miR-27a and 1930 in low-miR-27a CRCs (**A**). Next, we used Ingenuity Pathway Analysis (IPA) to recapitulate the pathways influenced by miR-27a expression in CRC: overexpression is associated with gene expression signatures of mitochondrial dysfunction, deregulated oxidative phosphorylation and mTOR signaling activation (**B**). Of note, we found significant differential expression of several enzymes and pathways including: i) AMPK, ii) glutaminolytic enzymes, and iii) pentose phosphate pathway (PPP) (**C**).

miR-27A-3p MODULATES BASAL RESPIRATION AND AFFECTS OVERALL MITOCHONDRIAL FUNCTIONS



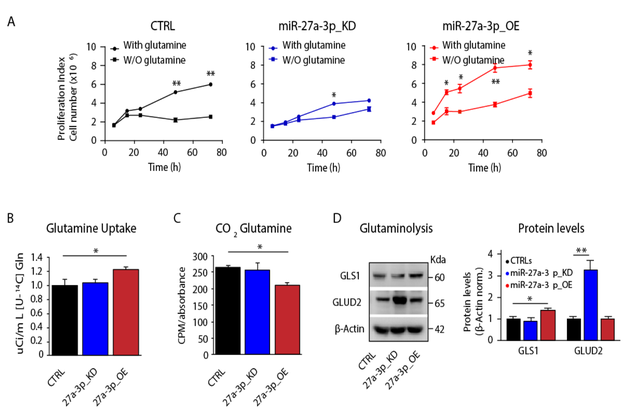
To confirm the impact of miR-27a on metabolism predicted *in silico*, we employed an *in vitro* model system represented by HCT116 cells, endogenously expressing miR-27a-3p, as control (CTRL), and two stable derived clones in which we either silenced or overexpressed miR-27a-3p, the predominant mature form in CRC, henceforward named miR-27a-3p_KD and miR-27a-3p_OE, respectively. In an Oxygen Consuming Rate (OCR) assay, miR-27a-3p_KD cells showed high values of both basal and maximal respiration with a remarkable production of ATP and a moderate respiratory capacity (**A**). Contrariwise, miR-27a-3p_OE cells exhibited a very low basal respiration level, similar to CTRL, with no further maximal respiration increase and minimal ATP production (**A**). These data suggest that miR-27a-3p loss of expression significantly unleashes mitochondrial respiration. In line we found that: i) citrate synthase activity, that is the tricarboxylic acids (TCA) cycle gatekeeper enzyme, increased by about 30% with respect to OE or CTRL cells (**B**); ii) intracellular ATP levels increased with a similar trend (**C**); iii) reactive-oxygen species (ROS) production increased paralleling the mitochondrial respiration vs CTRL and OE cells (**D**). Next, Carnitine Palmitoyl-Transferase 1A (CPT1A), the transporter of acyl-CoA into mitochondria, and Acyl-CoA Dehydrogenase Family Member 9 (ACAD9), the first enzyme of fatty acid β-oxidation, were higher in miR-27a-3p_KD vs OE or CTRL cells (**E**), corroborating the results of the OCR assay. PPAR gamma co-activator-1α (PGC-1α), a nodal regulator of mitochondrial activity and biogenesis was strongly reduced in OE with respect to CTRL and KD cells (**F**). We carried out a bioinformatic analysis for putative miR-27a targets and identified several components of Complex 1 and Complex 5 of the electron transport chain and PGC-1α as predicted targets of miR-27a-3p that may explain these results.

miR-27A-3p CONTROLS THE DESTINY OF GLUCOSE IN CRC CELLS



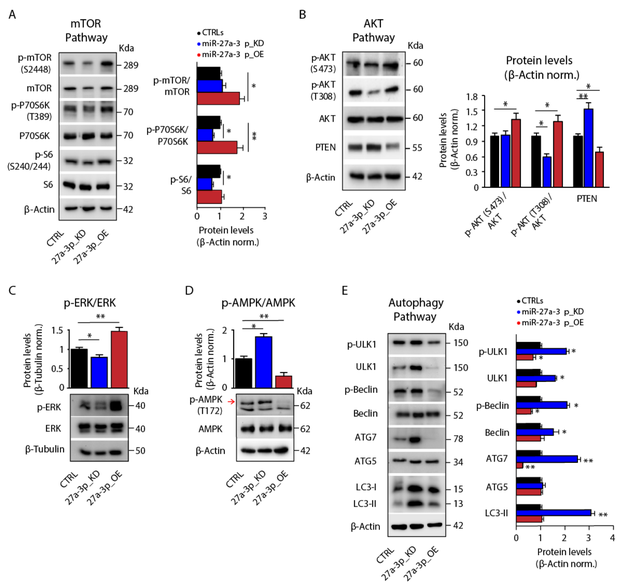
We then tested the glucose-dependent cell growth and found that miR-27a_OE cells were strongly dependent on glucose supply, while KD cells only slightly influenced (**A**). Consistently, glucose uptake was about 30% higher in miR-27a-3p_OE cells than KD or CTRL cells (**B**). Analysis of the glycolytic enzymes showed that DihydroLipoamide S-Acetyl-Transferase (DLAT), i.e. the E2 component of the pyruvate dehydrogenase complex (PDH) which converts pyruvate into acetyl-CoA, was strongly downregulated in miR-27a-3p_OE cells (**C**). Conversely, DLAT, Hexokinase 1 and 2 (HK1-HK2) were upregulated in miR-27a-3p_KD cells along with the tetrameric and reduced form of Pyruvate Kinase Muscle Isoform 2 (PKM2) (**C-D**). Consistent with these results, HK1, HK2 and DLAT were identified as predicted targets of miR-27a-3p in our bioinformatic analysis; thus, their downmodulation may account for the effects reported here. The extracellular acidification rate (ECAR) assay displayed a similar profile in all three cells, suggesting that they already operate at submaximal glycolytic conditions (**E**). By correlating OCR to ECAR values, we observed that miR-27a-3p_KD cells were more aerobic and energetic than CTRL and OE cells (**F**). We, therefore, reasoned that miR-27a-3p expression may favor the availability of glycolytic intermediates, which can be diverted into different biosynthetic processes to support unrestricted cancer cell proliferation.

miR-27A-3p TIGHTLY REGULATES GLUTAMINE METABOLISM IN CRC CELLS



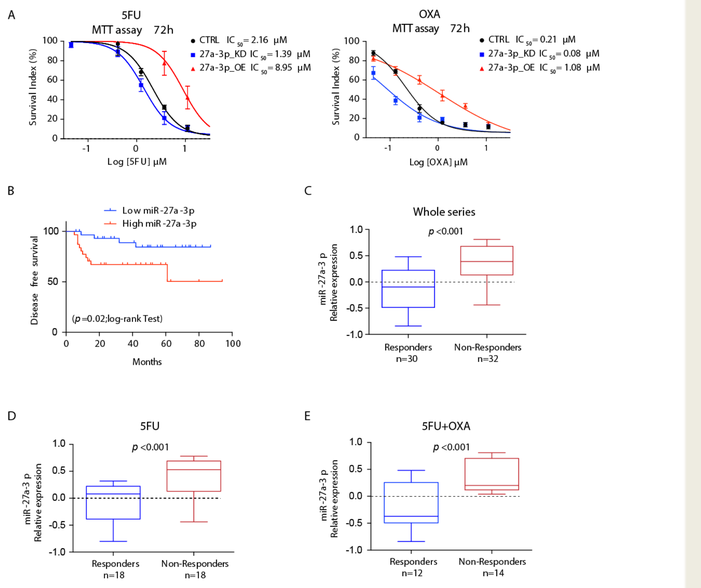
miR-27a-3p_OE cells are more glutamine-dependent than CTRL or KD cells, as they were remarkably impaired when propagated for 72h in a glutamine-free medium (**A**). Moreover, glutamine uptake in miR-27a-3p_OE cells was 20% higher (p<0.01) than CTRL and KD cells (**B**), whereas the amount of ^{14}C labelled CO_2, produced in glutamine-supplied medium as the sole carbon source, was reduced in miR-27a-3p_OE with respect to CTRL and KD cells (**C**). Glutaminase 1 (GLS1), the first enzyme of glutaminolysis, was increased in miR-27a-3p_OE, while glutamate dehydrogenase (GLUD2), the subsequent enzyme of the pathway, was upregulated in miR-27a-3p_KD cells (**D**), in line with the notion that it is a hallmark of quiescent cells and associated with decreased synthesis of non-essential amino acids (NEAA). GLUD2 down-modulation by miR-27a-3p, reported here, is consistent with its identification as a predicted target in our bioinformatic analysis and with the reduced expression of key glutaminolytic enzymes found in miR-27a overexpressing CRCs in the TGCA-COAD dataset.

miR-27A-3p REGULATES THE mTOR PATHWAY



In keeping with our *in silico* prediction, we asked whether miR-27a-3p could modulate mTOR to increase tumor biomass in the contest of aerobic glycolysis. Notably, mTOR activation is an essential step for tumor growth and progression; in line mTOR and downstream protein levels were higher in miR-27a-3p_OE than CTRL and KD cells (**A**), indicating that it activates mTOR pathway and the associated protein synthesis. mTOR is regulated by mitogen-responsive signaling, such as the PI3K/AKT-dependent and RAS/MEK/ERK (AKT-independent) pathways and we found that AKT phosphorylation at positions Thr308 and Ser473 was higher in miR-27a-3p_OE than CTRL or KD cells (**B**). Instead, PTEN, the PI3K/AKT inhibitor, that is a validated target of miR-27a-3p, was downmodulated in OE cells accounting for the inverse correlation detected here (**B**). ERK phosphorylation was also elevated in miR-27a-3p_OE cells powering mTOR signaling (**C**).
The mTOR pathway is also regulated by the intracellular energy status and nutrients content . Accordingly, we looked at the AMP-activated protein kinase (AMPK) which regulates the eukaryotic cells metabolism acting as the main sensor of the energetic status. Strikingly, phospho-AMPK (p-AMPK Thr172) was strongly downregulated in miR-27a-3p_OE cells (**D**). By inhibiting the mTOR complex 1, AMPK regulates also autophagy, an alternative and possible source of nutrients and energy for biosynthetic processes. miR-27a-3p is a critical regulator of the entire autophagy pathway through its several putative targets all increased in miR-27a-3p_KD cells in comparison to CTRL and downregulated in OE cells (**E**).

miR-27A-3p AFFECTS THE RESPONSE TO CHEMOTHERAPY

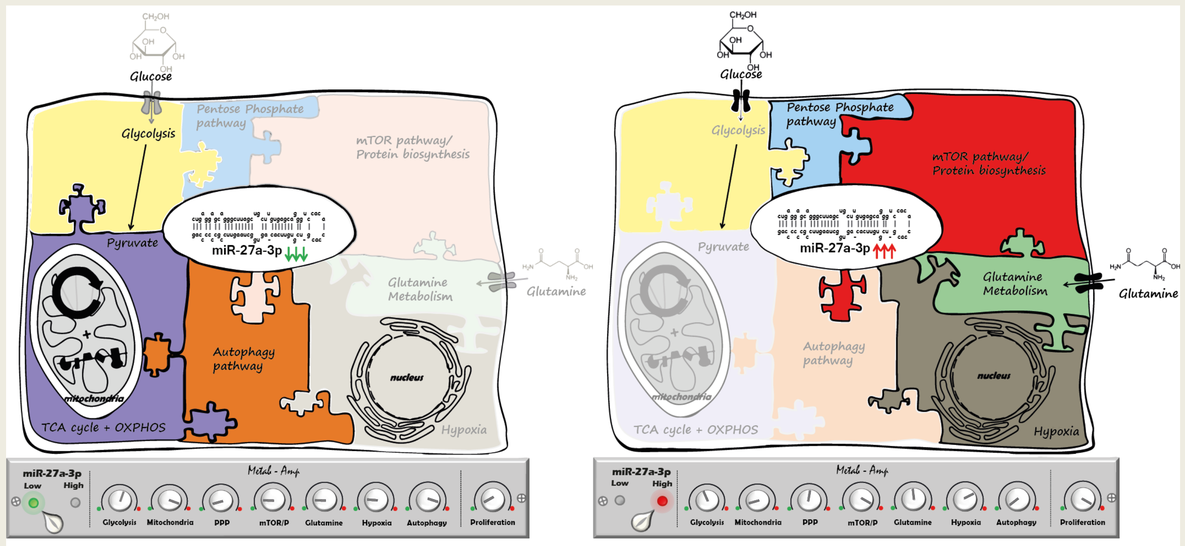


We investigated the impact that miR-27a-3p has on drug sensitivity in CRC. miR-27a overexpressing CRCs do significantly correlate with chemotherapy resistance *in silico*. Similarly, we verified this result in our *in vitro* model system by evaluating the relative IC_{50} in HCT116 CTRL, miR-27a-3p_KD and OE cells treated with 5-Fluorouracil (5FU) and Oxaliplatin (OXA), two of the major drugs routinely used for CRC chemotherapy. Both drugs inhibited the survival, but the IC_{50} was greater in miR-27a-3p_OE than in CTRL and KD cells (**A**). We further confirmed such a relation in our independent cohort of CRC patients (N=62) subjected to surgical resection and treated by adjuvant chemotherapy (5FU and OXA). Overall, patients with miR-27a-3p overexpressing CRCs correlated with an increased risk of recurrence (**B**) and with reduced drug response (5FU alone or in combination with OXA) (**C-E**). Remarkably, dihydropyrimidine dehydrogenase (DPYD), the rate-limiting enzyme of the uracil catabolism crucial in the pharmacokinetics of 5FU, is a direct target of miR-27a-3p influencing the treatment success. Likewise, we predicted ERCC1 and ERCC4, involved in repairing OXA-induced DNA damages, as targets of miR-27a-3p, explaining the accumulation of DNA damages that contribute to chemoresistance Collectively, these results confirmed that miR-27a-3p overexpression induces resistance to chemotherapy and favors tumor progression.

Conclusions

miR-27a-3p is a determinant key with a profound impact on CRC metabolic reprogramming modulating multiple metabolic checkpoints:

- *it down-regulates HK1 and HK2, the first two unidirectional glycolytic enzymes and the DLAT subunit of the PDH complex leading to lower amounts of acetyl CoA, the main source of the TCA cycle;*
- *inhibits PKM2 leading to a strong impairment of mitochondrial functions and forcing aerobic glycolysis;*
- *downmodulates PGC-1α a pivotal protein in metabolism and a PPARG co-regulator, explaining the impairment of overall mitochondrial biogenesis and activities, including fatty acid β-oxidation through direct and indirect effects;*
- *ensures an efficient supply of nutrients via upregulation of glucose and glutamine uptake and downregulation of downstream glutaminolytic enzymes (GLUD2) so to divert intermediates towards biosynthetic pathways;*
- *downmodulates AMPK, the main sensor of the energetic status of the cell, thus relieving the negative control on mTOR pathway that becomes upregulated;*
- *reduces ROS production by hampering oxidative phosphorylation and activates scavenger enzymes to maintain the redox state of the cells and reduce cell death;*
- *high miR-27a-3p expression associates with higher resistance to chemotherapy and hence with a worse prognosis.*
- *thus, miR-27a-3p acts as a master regulator of CRC metabolism and it can be recognized as a possible target of interventions paving the way for personalized therapeutic strategies.*



The drawing illustrates the metabolic pathways influenced by miR-27a-3p in CRC, depicted as individual puzzle pieces; the color intensity is proportional to the pathways activation as further illustrated by switches positions in conditions of high or low miR-27a-3p.