Detection and Validation of Novel Human Targets for EBV-miR-BART10-3p

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Detection and validation of novel human targets for EBV-miR-BART10-3p in colon cancer

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Abstract

Epstein-Barr virus (EBV) miRNAs are expression in various human tissues, including the human colon. We have previously shown that the EBV miRNA miR-BART10-3p is higher expressed in colon cancer tissue compared to adjacent normal tissue. Here, we aim to identify and validate novel human targets of miR-BART10-3p. MiR-BART10-3p mimics were transfected into the two colon cancer cell lines, SW620 and LS411N, and changes in RNA expression were evaluated by RNA-seq. Several genes showed significant changes in gene expression upon miR-BART10-3p transfection, and genes that were significantly differentially expressed in both cell lines were more often down-regulated in miR-BART10-3p transfected cells, indicating regulation by miR-BART10-3p. Candidate human targets of

miR-BART10-3p were predicted using TargetScan, and two genes with predicted target sites, MAT2B and CCND1, were functionally validated as miR-BART10-3p targets. Together, we here present several novel candidate human targets of miR-BART10-3p, thereby increasing the understanding of miR-BART10-3p in colon cancer.

Introduction

Epstein-Barr virus (EBV) was the first oncovirus to be discovered, and was originally identified in Burkitt lymphoma cells (69, 79). EBV is shown to be linked to several human cancers such as Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma, gastric cancers and also colon cancer (1-4). EBV miRNAs are expressed in all phases of the viral life cycle and are transcribed and generated in the same way as human miRNAs (5). 25 EBV miRNA precursors and 44 mature EBV miRNAs have been identified, four of which are encoded from the BamHI fragment H rightward open-reading frame (BHRF) region and the remainders are from the BamHI-A region rightward transcript (BART) region (6).

The EBV miRNA EBV-miR-BART10-3p is previously shown to promote cell proliferation and migration in a gastric carcinoma cell line by targeting DKK1 (7). Moreover, another study showed that EBV-miR-BART10-3p promote metastasis in gastric carcinoma by activating the canonical Wnt signaling pathway through targeting of adenomatous polyposis coli (APC) and DKK1 (8). Yan et al. showed that EBV-miR-BART10-3p facilitates epithelial-mesenchymal transition and promotes metastasis of nasopharyngeal carcinoma by targeting BTRC (9). They also showed that EBV-miR-BART10-3p was highly expressed in NPC tissues, as compared to adjacent non-tumor nasopharyngeal epithelial (NPE) tissues.

Our group has previously shown that EBV-miR-BART10-3p is elevated in colon cancer tissue compared to adjacent normal tissue (4). Despite several studies showing that miR-BART-10-3p can target specific human genes, the extent of such host gene targeting is still unclear, and only a few of the predicted targets are likely to be functional.

Here, we aimed to identify and validate human target genes of EBV-miR-BART10-3p in colon cancer. We over-expressed EBV-miR-BART10-3p in two colon cancer cell lines, SW620 and LS411N, by transfecting EBV-miR-BART10-3p mimics. Global changes in gene expression were evaluated by RNA-seq following EBV-miR-BART10-3p transfection. Two candidate genes, CCND1 and MAT2B were further validated using reporter vector systems and were shown to be direct targets of EBV-miR-BART10-3p. Together, the study identifies several putative EBV-miR-BART10-3p targets in colon cancer.

Results

Over-expression of miR-BART-10-3p shows major changes in gene expression in colon cancer cell lines

Our group has previously shown that miR-BART-10-3p is over-expressed in colon cancer tissue compared to adjacent normal tissue from early stage colon cancer patients (4). Here, we wanted to identify and validate novel human targets of the miRNA miR-BART-10-3p by over-expressing the miRNAs in human colon cancer cell lines and investigate changes in RNA expression. The miRNA miR-BART-10-3p was over-expressed in the two colon cancer cell lines SW620 and LS411N using artificial miRNA mimics, and mRNA-sequencing

(mRNA-seq) was performed and gene expression changes were compared between samples transfected with miR-BART-10-3p and non-targeting control miRNA mimic.

A principal component analysis of the mRNA-seq data showed clear grouping of miR-BART-10-3p transfected cells and negative control transfected cells, for both cell lines (Figure 1A). Principal component 1 (PC1) separated the two groups indicating that the major changes in the data were due to the transfection of miRNA mimics.

In LS411N, 539 genes were significantly differentially expressed between miR-BART-10-3p and non-targeting control transfected cells, of which 336 were up-regulated and 203 were down-regulated (Figure 1B). In SW620, 87 genes were significantly differentially expressed, of which 50 were up-regulated and 37 were down-regulated (Figure 1B). 21 genes were differentially expressed in both cell-lines, of which 15 genes were significantly downregulated in both cell lines, five were significantly up-regulated in both cell lines, and one gene (ALS2CL) was significantly up-regulated in LS411N and significantly down-regulated in SW620 (Figure 1B-D). Of the genes that were differentially expressed in both LS411N and SW620, six genes were up-regulated in both cell lines and 15 genes were down-regulated in both cell lines (Figure 1E).

Next, we investigated if genes that were consistently up-or down-regulated in the two cell lines were related to specific biological processes. Since only 21 genes were commonly differentially expressed between the two cell lines, we also considered genes that were significant before multiple testing. We detected no enriched gene ontology term among the 76 genes that were commonly down-regulated between the two cell lines, however, among the 80 genes that were commonly up-regulated we detected enrichment of biological

processes related to cell-adhesion, cell-junction, wound healing and several other similar terms (Figure 1F).

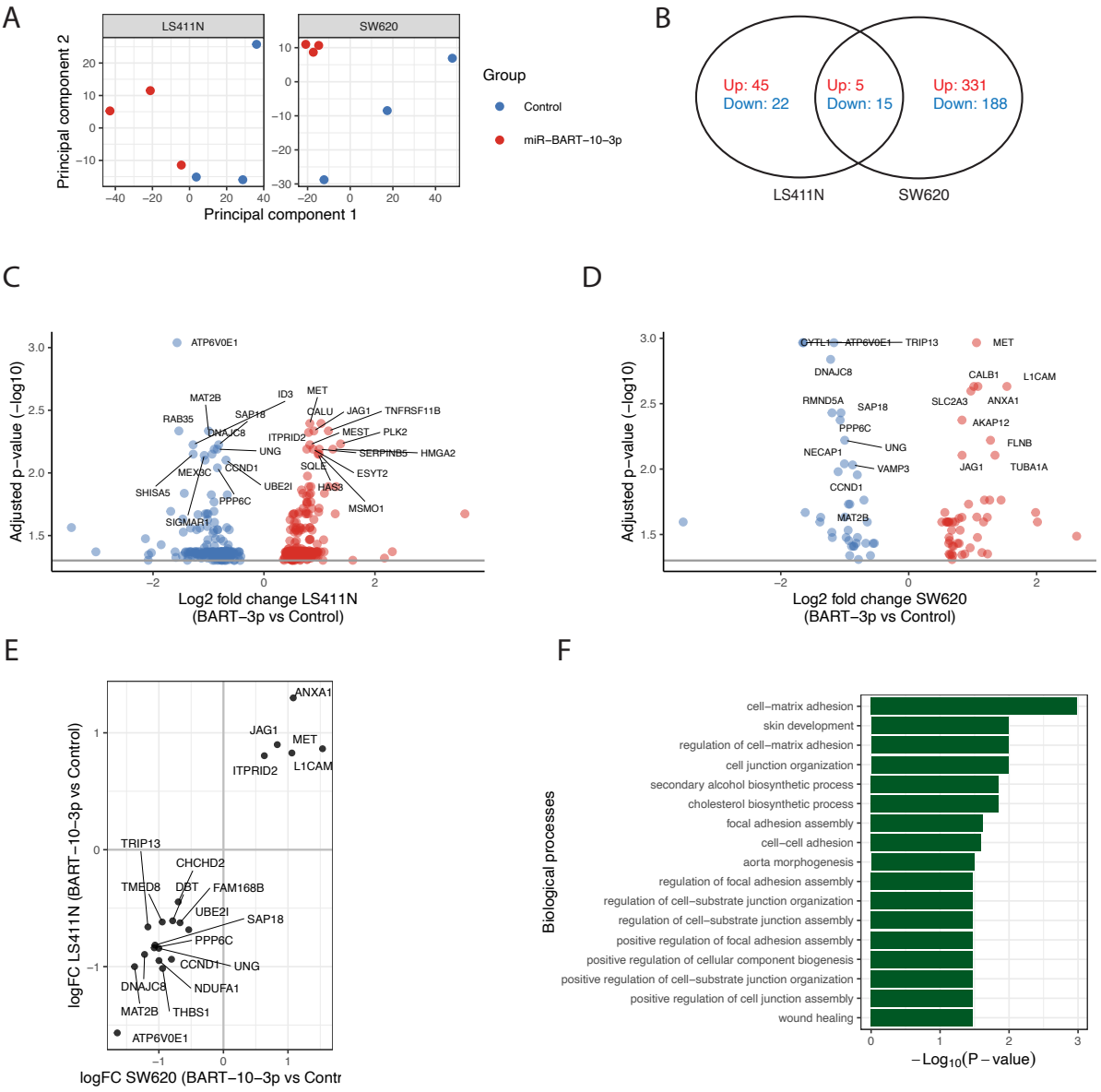

Figure 1: Overexpression of miR-BART-10-3p in SW620 and LS411N colon cancer cells. **A)** PCA plot of the gene expression results showing miR-BART-10-3p transfected cells and negative control transfected cells in LS411N and SW620. **B)** Venn diagram showing the number of up-regulated (red) and down-regulated (blue) genes in LS411N and SW620 upon transfection of miR-BART-10-3p. **C)** Volcano plot showing of the significantly differentially

expressed genes from the RNA-seq in LS411N cells. The y-axis shows the inverted benjamini hochberg adjusted p-value (-log10) and the x-axis shows the log2 fold change between miR-BART-10-3p transfection and negative control transfection. Genes with adjusted p-value less than 0.01 are indicated with gene names **D)** Similar as in C) for the cell line SW620. **E)** Scatterplot showing log2 fold change values of genes differentially expressed in both LS411N and SW620 and changing in the same direction in both cell lines. **F)** Gene ontology analysis (biological processes) of genes commonly up-regulated in both LS411N and SW620 before multiple testing. The plot is ordered by q-value from ClusterProfiler.

Human genes MAT2B and CCND1 are direct targets of miR-BART-10-3p

Having shown that several genes are differentially expressed upon miR-BART-10-3p over-expression, we wanted to focus on potential mRNA-targets of miR-BART-10-3p. To identify potential miR-BART-10-3p targets we used the TargetScan algorithm to find genes with predicted sites for the 6-nts seed region of miR-BART-10-3p. First, using conserved predicted target sites from TargetScan (see Methods) we identified 395 genes with conserved target sites of miR-BART-10-3p of which 238 genes were identified in our RNA-seq experiment. Four of these genes were significantly down-regulated in both cell lines and one gene (JAG1) was significantly up-regulated in both cell lines (Figure 2A). Next, we looked at non-conserved targets predicted by TargetScan and detected 4818 genes in the RNA-seq data that were predicted targets of miR-BART-10-3p. 15 of these genes were significantly down-regulated in both cell lines and three genes were significantly up-regulated in both cell lines (Figure 2A). Of note, all genes that contained conserved target sites also contained additional

non-conserved target sites. No enriched gene ontology terms were detected for genes that were consistently up- or down-regulated and predicted targets.

Next, we selected the genes CCND1 and MAT2B for further functional validation. The genes were selected based on their consistent changes in both cell lines and because both genes had strong predicted target sites in TargetScan. Moreover, both CCND1 and MAT2B are implicated in cancer (see Discussion). CCND1 encodes the cyclin D1 protein which is a key protein in cell cycle regulation (10). MiR-BART-10-3p has two TargetScan predicted target sites within the 3'UTR of CCND1, one 8mer and one 6-mer site (Supplementary table 1). MAT2B has two predicted non-conserved target sites within its 3'UTR (one 8mers and one 7mer) and one conserved 8mer (Supplementary table 2). First, we validated the RNA-seq data by over-expressing miR-BART-3p in the SW620 cell line and measuring the mRNA level of CCND1 and MAT2B by real-time qPCR (rt-qPCR). We observed a significant down-regulation of both CCND1 and MAT2B upon miR-BART-10-3p transfection compared to the negative control miRNA (Figure 2B). Next, we wanted to validate direct targeting between miR-BART-10-3p and CCND1 and MAT2B by constructing luciferase plasmids containing either the whole 3'UTR of the genes or the specific target sites only. First, the whole 3'UTR of CCND1 and MAT2B were cloned in the LightSwitch reporter vector which were co-transfected into SW620 cells together with miR-BART-10-3p mimics and negative control miRNA mimics. Both CCND1 and MAT2B were significantly down-regulated when co-transfected with miR-BART-3p compared to co-transfections with negative control miRNA mimics (Figure 2C). Next, we wanted to validate the exact target sites of miR-BART-3p within the 3'UTRs or CCND1 and MAT2B. We utilized the Dual-Luciferase Reporter Assay System and created one vector containing the MAT2B 8-mer target sites and one vector containing the 8-mer target site of CCND1 (Figure

2D). We observed significant down-regulation of both the MAT2B and the CCND1 genes indicating that miR-BART-10-3p is a direct target of these two genes by targeting the 8mer sites (Figure 2E). In summary, we show that the expression of both CCND1 and MAT2B are reduced upon miR-BART-10-3p transfection.

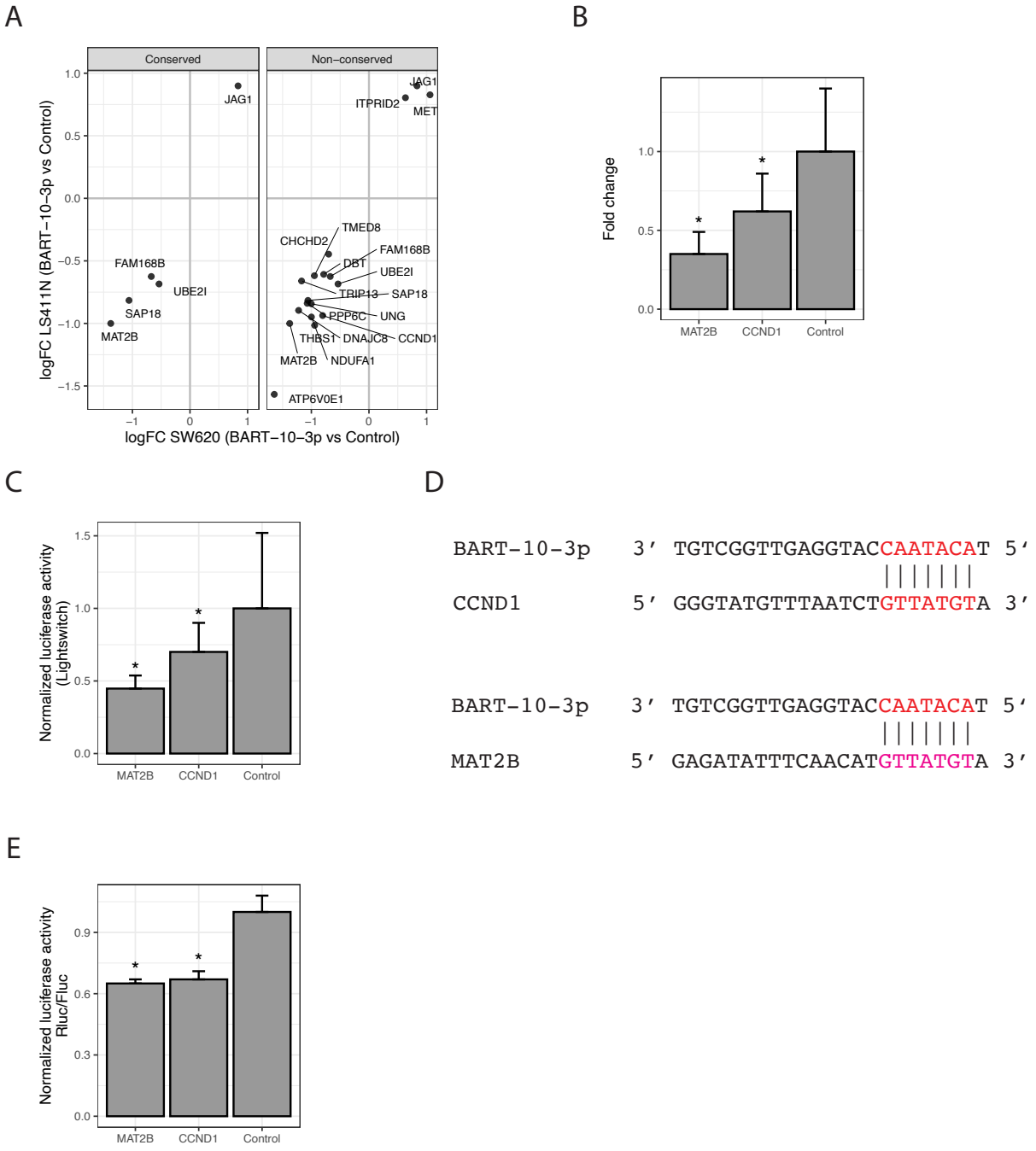

Figure 2: Validation of MAT2B and CCND1 targets. **A)** Log2 fold change values for genes with consistent changes in gene expression in SW620 and LS411N that are predicted by TargetScan to contain either conserved or non-conserved target sites, or both types of target sites. **B)** Expression of CCND1 and MAT2B as measured by rt-qPCR upon transfection of miR-BART-10-3p mimics and non-targeting control mimics in SW620 cells. The aesthetics indicate significance ($p<0.05$) from a one-tailed student's t-test compared to non-targeting control mimics. The error bars indicate standard deviation from three biological replicates. **C)** Normalized luciferase activity of lightswitch vectors containing either the 3'UTR of CCND1 or MAT2B upon co-transfection of the lightswitch vectors and miR-BART-10-3p mimics or non-targeting control mimics in SW620 cells. The aesthetics indicate significant down-regulation ($p<0.05$) of vectors co-transfected with miR-BART-10-3p mimics compared to vectors co-transfected with non-targeting control mimics. The error bars indicate standard deviation from three biological replicates. **D)** Illustration of the predicted TargetScan 8mer sites within the 3'UTR of CCND1 and MAT2B that were validated as functional target sites. **E)** Normalized Renilla/Firefly activity of PsiCheck-2 vectors containing either the 8mer site of CCND1 or MAT2B upon co-transfection of the PsiCheck-2 vector and miR-BART-10-3p mimics or non-targeting control mimics in SW620 cells. Aesthetic and error bars as explained in C).

Discussion

In the current study we aimed to validate predicted human targets of the EBV miRNA miR-BART-10-3p in colon cancer. The performed transfection experiments with miR-BART-10-3p mimics in colon cancer cell lines followed by RNA-seq to investigate changes in predicted target genes. Interestingly, we found that genes that showed significant changes in expression

in both cell lines upon miR-BART-10-3p transfection, were more often down-regulated, indicating that miR-BART-10-3p has negative effect on gene expression, as expected in miR-BART-10-3p function as a miRNA in human host cells. When we looked at genes predicted to be targeted by miR-BART-10-3p, only three genes were up-regulated and 15 down-regulated. Genes that show significant down-regulation that were not predicted to be targets could still be targeted by miR-BART-10-3p as the prediction algorithms do not predict all potential targets. However, we do observe some up-regulated genes, both genes predicted to be targeted by miR-BART-10-3p and genes not predicted to be targeted. These changes could be explained by indirect effects of miR-BART-10-3p, for instance that miR-BART-10-3p down-regulates negative regulators of these genes.

For the validation of CCND1 and MAT2B we chose to include reporter vectors containing both the full-length 3'UTR and a short region spanning only the predicted target site. Since both CCND1 and MAT2B were predicted to bind multiple sites, we chose to validate the strongest target site type as predicted by TargetScan, in this case an 8mer site for both CCND1 and MAT2B. By using reporter vectors with the whole 3'UTR we can validate the interaction between miR-BART-10-3p and the genes as it takes place in the cells, which could include more target sites than those predicted by TargetScan. Further, by creating reporter vectors with the exact target site only, we limit potential effects from other miRNAs binding to the same 3'UTR and are able to validate the exact target site. However, the disadvantage of using reporter vectors with only the target site is that potential secondary structures important for targeting will not be present.

The EBV miRNA miR-BART-10-3p has been suggested to promote cell proliferation in gastric carcinoma and to facilitates epithelial-mesenchymal transition and promote metastasis

of nasopharyngeal carcinoma by targeting the genes DKK1 and BTRC, respectively ((7, 9)). In our study, we identify the cell cycle gene CCND1 as target of miR-BART-10-3p. Since CCND1 is regarded as a proto-oncogene, down-regulation of CCND1 by miR-BART-10-3p could imply that miR-BART-10-3p acts as a tumor-suppressor, by reducing expression of CCND1. Furthermore, MAT2B is also shown to promote proliferation in other cancers ((11, 12) further suggesting that miR-BART-10-3p acts as a tumor-suppressor by down-regulating MAT2B. However, it should be noted that the net effect of miR-BART-10-3p on gene expression in colon cancer is not fully investigated in this study and other interactions could potentially be more important in promoting tumor growth. However, our results at least suggest that miR-BART-10-3p could have tumor-suppressor effects in colon cancer.

Overall, this study is the first to investigate global changes in gene expression upon miR-BART-10-3p over-expression in colon cancer. We identify several potential human targets of miR-BART-10-3p and successfully validate two novel target genes.

Methods

Cell culture of LS411N and SW620 cells

The cell line LS411N (ATCC® CRL-2159™) was grown in RPMI-1640 medium modified to contain L-glutamine and sodium bicarbonate. The medium was supplemented with 10% fecal bovine serum (FBS), 1% Penicillin-Streptomycin and L-glutamine. The cell line SW620 (ATCC® CCL-227™) was grown in Dulbecco's Modified Eagle's Medium (DMEM) modified to contain 4500 mg/L glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate. The medium was supplemented with 10% FBS, 1% Penicillin-Streptomycin and L-glutamine.

RNA-sequencing experiments

The colon cancer cell lines SW620 and LS411N were transfected in three biological replicates with miR-BART10-3p mimic (ThermoFisher #4464066/MC12577) and negative miRNA mimic (mirVana™ miRNA Mimic, Negative Control #1, #4464058) in 6 well plates for 24h followed by RNA isolation using the Total RNA Purification Kit from Norgen Biotek (#17200). The RNA was used for library preparation using SENSE mRNA-Seq Lib Prep Kit V2 Illumina from Lexogen (#001.96). The libraries were sequenced on a NEXTSeq 500 flow cell using 75bp single read.

Real-time quantitative PCR

The real-time qPCR (rt-qPCR) reactions were carried out on a Step One Plus Real-Time PCR system (Applied biosystems) with TaqMan Universal Master Mix II (Applied Biosystems) and TaqMan Gene Expression assays for CCND1 (#Hs00765553_m1), MAT2B (Hs00203231_m1) and actin as housekeeping control (#Hs99999903_m1). All rt-qPCR experiments were performed on SW620 cells. The cells were then transfected with miR-BART10-3p (ThermoFisher #4464066/MC12577) or a negative miRNA control (mirVana™

miRNA Mimic, Negative Control #1, #4464058) by using Lipofectamine RNAiMAX (InvitrogenTM) according to the manufacturer's instructions. After 24 hours of incubation, the cell suspensions were trypsinized, resuspended in a 1.5 mL tube with PBS, and then centrifuged at 4000 x g for 5 minutes. Cell pellets were frozen and kept at -20 ℃ until use for RNA isolation. All rt-qPCR-reactions samples were loaded in triplicate. qPCR analysis was performed on the StepOne™ Real-Time PCR System (Applied Biosystems) under the following reaction conditions: 95 °C for 10 minutes for polymerase activation, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. The fold change of SW620 treated with miR-BART10-3p versus negative miRNA was determined using the $2^{-\Delta\Delta Ct}$ (Livak) method.

Luciferase assays

Pre-cloned 3'UTR GoClone vector were purchased from Switchgear Genomics that includes one optimized luciferase gene, Renilla (RenSP), and a coding region for the β-lactamase resistant gene (Ampr), and whole 3'UTR inserts (MAT2B: #S807438; CCND1: #S813994). The inserts of the entire 3'UTR of CCND1 and MAT2B are 3400 bp and 1065 bp respectively and are cloned downstream of the luciferase gene. The 3'UTR GoClone vector (100ng) and mimics (100 nM) were co-transfected in SW620 cells using DharmaFECT® Duo Transfection Reagent (#T-2010-02) in a 96 well plate with 100uL media, and incubated for 24h followed by addition of LightSwitch Luciferase Assay Reagent and luminescence measurements on a plate luminometer. The degree of knockdown was measured as the ratio of miR-BART10-3p mimic (ThermoFisher #4464066/MC12577) transfected cells and negative miRNA mimic (mirVana™ miRNA Mimic, Negative Control #1, #4464058) transfected cells.

Construction of the psiCHECK™-2 vector (Promega) with target-site inserts (see supplementary for sequence information) of the CCND1 8mer and MAT2B 8mer site is performed using the following steps: restriction cutting of psiCHECK™-2 vector, PCR cleanup or gel extraction of restriction cut psiCHECK™-2 vector, ligation of psiCHECK™-2 vector and CCND1 target site insert, transformation by heat shock using DH5-alpha cells, and verification of inserts by sanger sequencing. The inserts were ordered as DNA oligos from idtDNA. The co-transfection of the psiCHECK™-2 vector containing the target sites were performed as described for the 3'UTR GoClone vector above, in SW620 cells, followed by luminescence measurements using the Dual-Luciferase® Reporter Assay System protocol (Promega, #E1910).

Data analysis and statistics

The sequencing data was aligned to the human genome (GRCh38.p7 from NCBI) using STAR aligner (13) with the parameter: *--readFilesCommand zcat --outFileNamePrefix $* --chimSegmentMin 30 --runThreadN 12 --alignSJoverhangMin 8 --alignSJDBoverhangMin 1 --outFilterMismatchNmax 10 --outFilterMultimapNmax 20 --outFilterMismatchNoverLmax 0.04 --alignIntronMin 20 --alignIntronMax 1000000*. Reads were counted using htseq-count (14) with parameters: *-s no -i Parent -t exon*, using the corresponding GFF file of the GRCh38.p7 genome.

The sequencing data was analyzed in R using limma-voom. P-values were adjusted using the Benjamini-hochberg method. We required genes to be expressed with at least 1 count per million (cpm) in 50% of the samples. TargetScan (15) files were downloaded from TragetScanHuman release 7.2 (http://www.targetscan.org/vert_72/) and analyzed in R. The seed sequence of miR-BART10-3p was used to predict targets. Gene ontology-analyses were

performed in R using ClusterProfiler (16). Expressed genes were used as background in the Gene ontology-analyses. For the rt-qPCR and luciferase experiments, the p-values were calculated from three biological replicates using one-tailed student's t-test.

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Author contributions

JTJ: Lab work related to validation of target genes. VS: Lab work related to validation of target genes: EH: Manuscript preparation, supervision. RM: Manuscript preparation, lab work, data analysis.

Competing interests

The authors declare no competing interests.

Data availability

The RNA-seq data is submitted to SRA with accession PRJNA770482

Supplementary Files

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- Supplementarydatacombined.pdf