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miR-221 targets QKI to enhance the tumorigenic capacity of human colorectal cancer stem cells.

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Abstract

MicroRNAs (miRNAs) are key players in the integrated regulation of cellular processes, and shape many of the functional properties that define the *"cancer stem cell"* (CSC) phenotype. Little is known, however, about miRNAs that regulate such properties in human *colorectal carcinoma* (CRC). In this study, we compared the expression levels of 754 miRNAs between paired samples

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Conflicts of interest statement:

Y.S. and P.D. are co-inventors on patents and patent applications filed by *Stanford University* and licensed to Quanticel Pharmaceuticals Inc. (US-9329170, US-9850483, US-20110021607). P.D. is a co-inventor on patents and patent applications filed by the *University of Michigan* and licensed to Oncomed Pharmaceuticals Inc. (US-7723112, US-20140030786). J.M., T.I., Q.H., T.H., T.W., M.M., H.Y., X.Q., K.Y., H.M., K.M., D.S., Y.K., A.S. declare no competing interests.

of EpCAM^{+}/CD44^{+} cancer cells (enriched in CSCs) and EpCAM^{+}/CD44^{neg} cancer cells (with CSC depletion) sorted in parallel from human primary CRCs, and identified *miR-221* as the miRNA that displayed the highest level of preferential expression in EpCAM^{+}/CD44^{+} cancer cells. High levels of *miR-221* expression were associated with Lgr5^{+} cells in mouse colon crypts and reduced survival in CRC patients. Constitutive over-expression of *miR-221* enhanced organoid-forming capacity of both conventional CRC cell lines and *patient-derived xenografts* (PDXs) *in vitro*. Importantly, constitutive downregulation of *miR-221* suppressed organoid-forming capacity *in vitro* and substantially reduced the tumorigenic capacity of CSC populations from PDX lines *in vivo*. Finally, the most abundant splicing isoform of the human *Quaking* (*QKI)* gene, QKI-5, was identified as a functional target of *miR-221*; overexpression of *miR-221* reduced QKI-5 protein levels in human CRC cells. As expected, overexpression of QKI-5 suppressed organoid-forming capacity *in vitro* and tumorigenic capacity of CRC PDX cells *in vivo*. Our study reveals a mechanistic link between *miR-221* and QKI, and highlights their key role in regulating CSC properties in human colorectal cancer.

Keywords

colorectal cancer; cancer stem cells; miR-221; QKI; tumorigenic capacity

INTRODUCTION

Advanced stage, metastatic *colorectal carcinomas* (CRCs) are difficult to cure, as they often display limited sensitivity to conventional anti-tumor therapies. Among the key sources of tumor resistance to cytotoxic agents is the heterogeneous cell composition of malignant tissues, which originates not only from the divergent somatic mutations within the transformed population, but also from the capacity to recapitulate the multi-lineage differentiation processes that enable adult stem cell populations to sustain the formation of different cell types (1, 2). The cell sub-populations that, within a specific tumor, retain tumorigenic capacity upon serial transplantation and are able to sustain the formation of tumors that recreate the cellular diversity of the parent lesions, are operationally defined as *"cancer stem cells"* (CSCs) (3). Despite important progress in the understanding of the molecular identity of CSC populations in human CRCs, the molecular regulation of their tumor-initiation capacity remains only partially understood.

A large body of experimental evidence indicates that, among the key molecular regulators of CSC properties are microRNAs (miRNAs), non-coding RNAs that contribute to the post-transcriptional regulation of messenger RNAs (mRNAs) (3, 4). For example, in epithelial malignancies such as breast and pancreatic cancer the self-renewal ability of malignant cells is negatively regulated by *miR-200c*, which suppresses the expression of *BMI1* (5, 6). Similar inhibitory effects are exerted, in human CRCs, by *miR-34a*, which suppresses *NOTCH1* (7). On the other hand, selected miRNA species can act as positive regulators of tumorigenic capacity, as observed in the case of *miR-22*, which suppresses *TET2* in mammary epithelia (8), and of *miR-142*, which suppresses *APC* in breast cancer (9).

In this study, we aimed to identify miRNAs involved in the regulation of CSC properties in human CRCs. Our results identified *miR-221* as a positive regulator of tumorigenic capacity in human CRCs and an RNA binding protein QKI, as one of its key functional targets.

MATERIALS AND METHODS

Supplementary Appendix.

A comprehensive and detailed description of all methods used in this study is also provided in the Supplementary Information.

Ethics statements.

Human primary CRCs were obtained from patients admitted to the *Division of Gastrointestinal Surgery* of *Kobe University Hospital*. The research was pre-approved by Kobe University's *Institutional Review Board* (permission number: 1299) and was conducted in accordance with recognized ethical guidelines (Declaration of Helsinki, CIOMS). All patients included in the study provided written informed consent. Animal experiments were performed with the approval of Kobe University's *Animal Care and Use Committee* (permission number: 150802).

Flow cytometry.

Primary tumor specimens, *patient-derived xenografts* (PDXs) and normal colon epithelia were dissociated and analyzed as previously described (1). Dissociated cells were stained with monoclonal antibodies (mAbs) conjugated to fluorescent dyes. A complete list of all antibodies used in this study is provided in the *Supplementary Information*.

Analysis of miRNA expression by multiplex semi-quantitative real-time PCR.

RNA was extracted from 100 cells purified from primary CRCs, and directly collected into TRIzol (Invitrogen). The expression level of 754 miRNAs was measured by multiplex semi-quantitative real-time PCR (TaqMan™ Array Human MicroRNA A+B Cards Set v3.0 with Megaplex™ RT Primers, Human Pool Set v3.0; Thermo Fisher Scientific) as previously described (5). Results were normalized to RNU48 small nuclear RNA (snRNA) and analyzed for statistical significance using the Mann-Whitney U-test.

miRNA-sequence experiments.

Total RNA was isolated from sorted normal murine colon epithelial cells using the NucleoSpin miRNA kit (MACHEREY-NAGEL, Germany). The microRNA-seq profiling was performed on three pairs of biological replicates by LC Sciences (Houston, TX). Results were analyzed using two different mapping and normalization pipelines (LC Sciences, ENCODE) and evaluated for statistical significance using a one-tailed t-test for paired samples.

Bioinformatics analysis of RNA-sequencing (RNA-seq) datasets.

Associations were tested on a dataset downloaded from the *Broad Institute Firehose* (www.gdac.broadinstitute.org), containing mature miRNA expression data of 293 colon

cancer patients from the *colon adenocarcinoma* (COAD) collection of *The Cancer Genome Atlas* (TCGA) database. Patients were stratified into two groups based on miR-221 expression levels (*miR-221*^{low} vs. *miR-221*^{high}) using the minimum *P*-value approach. *Overall survival* and *disease-free survival* rates were estimated using Kaplan-Meier survival curves and tested for statistical differences using the log-rank test and the Cox proportional hazards model. The presence of linear correlations between the expression levels of miR-221 and *QKI* was tested in an expanded release of the TCGA-COAD database (n=439, the TCGA public repository, https://cancergenome.nih.gov; March 8, 2016) (10). Correlations were evaluated using Pearson's correlation coefficients, and tested for statistical significance using a two-tailed t-test (null hypothesis: r=0).

Cell lines.

All cell lines used in this study were obtained from the *American Type culture Collection* (ATCC; http://www.atcc.org) and include: HCT116 human colon cancer cells (ATCC catalog: CCL-247) and HEK293 human embryonic kidney cells (ATCC catalog: CRL-1573). All cell lines were cultured in RPMI-1640 (Sigma-Aldrich) containing 10% *fetal bovine serum* (FBS), penicillin (100 U/mL) and streptomycin (100 mg/mL; Nacalai, Japan). Early passage cells were used in all experiments. All cell lines were tested to be Mycoplasma free by PCR and authenticated using short tandem repeat profiling (BEX, Japan).

Lentivirus plasmids.

The full-length sequence of *miR-221* and the full-length coding region of the *QKI-5* mRNA (NM_001301085) were amplified by PCR (Table S1) and cloned into the pEIZ-HIV-ZsGreen lentivirus vector and the pLentiLox3.7-EF1α-mCherry vector, a derivative of pLentiLox3.7 (Addgene: #11795), respectively (5). The lentivirus vectors encoding for the *anti-miR-221* construct (miRZip-221) and a non-targeting pre-miRNA (negative control) were purchased from *System Biosciences* (USA).

Organoid assays.

Cells were infected with either test or control lentivirus constructs, and seeded on Matrigel™ in 96-well plates (3×10^3 cells/well), and cultured at 37°C with 5% CO_2, as previously described (11). The number of organoids larger than 100 μm in diameter was counted 10 days after seeding. Results were tested for statistical significance using Student's t-test (two-tailed) and/or, a two-way ANOVA test.

Xenotransplantation assays.

PDX-KUC1 cells were infected with lentivirus constructs at a multiplicity of infection (MOI) of 20, mixed with Matrigel and injected subcutaneously into NOD/SCID/IL2R$\gamma^{-/-}$ (NSG) mice (Charles River) as previously described (12). Results were analyzed for statistical significance using Fisher's exact test.

Cloning and mutagenesis of the QKI-5 3'UTR.

A 415-bp fragment of the *QKI-5* 3'UTR (nucleotides 2395–2809 of NM_001301085 (GenBank)) was amplified by PCR (Table S1) and cloned into the pGL3-MC vector, at the 3'-end of the firefly *Luciferase* gene (5). Mutations in the putative miR-221 target sequence within the *QKI-5* 3'UTR were introduced using a *PrimeSTAR Mutagenesis Basal Kit* (Takara Bio) (Table S1).

Luciferase reporter assays.

Cells were co-transfected with: 1) a pGL3-MC luciferase expression construct; 2) the pRL-TK *Renilla* luciferase vector (Promega); and 3) a pEIZ expression plasmid, containing either miR-221 or an empty backbone, using Lipofectamine 3000 (Invitrogen). *Luciferase* activity was quantified and normalized to *Renilla* luciferase activity, using the *Dual-Luciferase Reporter Assay System* (Promega). Results were analyzed for statistical significance using a two-tailed t-test and a two-way ANOVA test.

RESULTS AND DISCUSSION

miR-221 is over-expressed in EpCAM^{+}/CD44^{+} as compared to EpCAM^{+}/CD44^{neg} CRC cells.

To identify miRNAs involved in the regulation of CSC properties in human CRCs, two autologous pairs of EpCAM^{+}/CD44^{+} (enriched in cells with CSC properties) and EpCAM^{+}/CD44^{neg} (depleted in cells with CSC properties) cancer cells were isolated by *fluorescence activated cell sorting* (FACS) and purified in parallel from two independent primary CRC specimens (CRC1, CRC2). A screening of the expression levels of 754 miRNAs identified *miR-221* as the miRNA that showed the highest level of preferential expression in EpCAM^{+}/CD44^{+} as compared to EpCAM^{+}/CD44^{neg} cells (Fig. 1A), a finding subsequently validated on 6 primary CRC tissues (Fig. 1B). In normal mouse colon epithelia, Epcam^{+}/Cd44^{+} cells contain both Kit^{neg} Lgr5^{+} stem cells and Kit^{+} goblet cells (13) (Fig. 1C). A comparison of miR-221 expression levels between Epcam^{+}/Cd66a^{low}/Cd44^{+}/Kit^{neg} cells (enriched in Lgr5^{+} stem cells) and Epcam^{+}/Cd66a^{low}/Cd44^{+}/Kit^{+} cells (enriched in goblet cells) confirmed over-expression of both miR-221–3p and miR-221–5p in Kit^{neg} (Lgr5^{+}) stem cells (Fig. 1C, Fig. S1, Fig. S2). These findings were in agreement with our previous studies in human breast cancer, which identified *miR-221* among the miRNA species over-expressed in mammary CSC populations (5), and with previous studies in several malignancies, which identified *miR-221* as over-expressed in cancerous as compared to normal tissues (14).

High levels of *miR-221* expression are associated with reduced survival in CRC patients.

In many forms of human cancer, tumors characterized by a gene-expression profile similar to that of phenotypic sub-populations enriched in CSCs are associated with reduced survival outcomes (1, 15, 16). Indeed, analysis of a public miRNA-sequencing database from human colon carcinomas revealed that *miR-221^{high}* tumors were associated with worse clinical outcomes than *miR-221^{low}* tumors, with regard to both 5-year overall survival (54.6% vs. 73.6%, n=293; p<0.001) and 5-year disease-free survival rates (57.9% vs. 83.4%, n=275; p=0.012) (Fig. 1D). Importantly, the association between high *miR-221* expression levels and worse clinical outcomes did not appear to be confounded by major clinical or

pathological variables (Fig. 1D), and remained associated with a statistically significant reduction in overall survival rates in a multivariable analysis based on the Cox proportional hazards method (HR=2.44. 95%CI=1.19–5.19, p=0.009) (Fig. 1D), in agreement with results from an independent cohort (17).

Over-expression of *miR-221* enhances the *in vitro* clonogenicity and *three-dimensional* (3D) organoid-forming capacity of human CRC cells.

To understand whether *miR-221* had a direct mechanistic role in supporting the capacity of CSC populations to initiate tumor growth, we tested whether *miR-221* was able to affect the 3D organoid-forming capacity of human colon cancer PDX lines (PDX-KUC1, PDX-KUC2) (Fig. 2A–B) (18). Their clinico-pathological characteristics and gene mutation status are summarized in Fig. S3, S4 and Table S2. Infection of PDX-KUC1 cells with a lentivirus encoding for *miR-221* significantly enhanced their capacity to grow as 3D organoids (Fig. 2A), while infection with a lentivirus encoding for an *anti-miR-221* construct significantly suppressed it (Fig. 2B, Fig. S5A). Similar results were obtained in PDX-KUC2 cells and a human CRC cell line (HCT116) (Fig. 2B–C). In HCT116 cells, forced expression of the *anti-miR-221* construct caused a reduction in the percentage of proliferating cells, an increase in the percentage of apoptotic cells, and a reduction of the expression levels of stem cell-related genes, such as LGR5, SOX2 and OCT4 (Fig. 2C, Fig. S5B). These findings were in agreement with previous observations on the oncogenic effects of constitutive *miR-221* expression (19), which has a capacity to inhibit tumor suppressor genes involved in the regulation of cell-cycle progression, apoptosis and WNT signaling, such as *CDKN1B/p27*, *CDKN1C/p57*, *PTEN, DKK2*, and *AXIN2* (14).

Inhibition of *miR-221* reduces the *in vivo* tumorigenic capacity of human CRC cells from PDX lines.

We tested whether inhibition of miR-221 was able to suppress tumorigenic capacity in immuno-deficient NSG mice (Fig. 2D). The results showed that infection of PDX-KUC1 cells with the lentivirus vector encoding for the *anti-miR-221* construct caused a statistically significant reduction of their *in vivo* tumorigenic capacity (Fig. 2D). Importantly, lack of tumor growth associated with forced *anti-miR-221* expression did not appear to be caused by a delay in growth kinetics, but rather by a lack of tumor engraftment, as revealed by anatomical dissection of the injection sites, which showed lack of even small tumor masses (Fig. 2D). Our data, therefore, suggested that *miR-221* acts not simply as a positive modulator, but as necessary element of the molecular machinery that enables *in vivo* CRC growth.

QKI is a direct molecular target of *miR-221*.

We used the TargetScan 6.2 algorithm (http://www.targetscan.org) to search for putative *miR-221* target genes that play important roles in both oncogenesis and the regulation of stem cell functions. The search identified the *Quaking* (*QKI*) gene as one of the most promising candidates (20). Importantly, QKI is a transcriptional target of p53, often functions as a tumor-suppressor, and is a marker associated with better patient prognosis (20). In addition, QKI plays important roles in both the normal development and epigenetic regulation of stem cells (20, 21), and regulates *epithelial-to-mesenchymal transition* (EMT)

processes (22), WNT signaling (23), and the expression of the transcription factors *SOX2*, *NANOG* and *OCT4* (3, 21).

Among the four known splice variants of the human *QKI* gene (*QKI-5*, *QKI-6*, *QKI-7*, and *QKI-7b*) (20), *QKI-5* is the variant most abundantly expressed in normal human colon tissues, and preferentially down regulated in human CRCs (23, 24). Within its 3'UTR, *QKI-5* carries a predicted 8-mer *miR-221* target site that is highly conserved across mammalian species (Fig. 3A). Indeed, *miR-221* over-expression suppressed the luciferase activity driven by the *Luc-QKI-5 (3'UTR)* construct, and this effect was completely abrogated by the introduction of mutations restricted to the target sequence itself (Fig. 3A–B). Moreover, forced expression of *miR-221* caused a reduction of QKI-5 protein levels in human CRC cells, while inhibition of *miR-221* led to their increase (Fig. 3C). As predicted based on our *in vitro* data, the expression of the two genes was inversely correlated in the TCGA COAD database (n=439) (Fig. 3D) (20).

QKI-5 suppresses the *in vitro* three-dimensional (3D) organoid-forming and tumor formation capacities of human CRC PDX cells.

The constitutive expression of QKI-5 suppressed the 3D organoid-formation capacity of human CRC cells from both classical cell lines (HCT116) and PDX lines (PDX-KUC1) (Fig. 4A, Fig. S6A). When QKI-5 and *miR-221* and were co-expressed together, QKI-5 completely abrogated the capacity of *miR-221* to up-regulate organoid-forming ability (Fig. 4B, Fig. S6B).

Finally, to understand whether *QKI* acted as a negative regulator of *in vivo* tumor engraftment, we infected PDX-KUC1 cells with the lentivirus encoding QKI-5 and evaluated their *in vivo* tumorigenic capacity (Fig. 4C). We found that over-expression of QKI-5 caused a statistically significant reduction of their growth *in vivo* (Fig. 4C).

In summary, we identified *miR-221* as one of the miRNA species that displays the highest degree of preferential expression in the $EpCAM^{+}/CD44^{+}$ population of human CRCs. *miR-221* is not simply a positive regulator, but rather a necessary component of the molecular machinery involved in sustaining *in vivo* tumor growth. Furthermore, our study identified QKI-5 as a functional target of *miR-221* and a suppressor of *in vivo* tumor growth. Taken together, our findings suggest that the functional interaction between *miR-221* and QKI represents one of the key molecular networks involved in the regulations of CSC biology in human CRCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

CSC	cancer stem cell
CRC	colorectal carcinoma
FACS	fluorescence activated cell sorting
mAb	monoclonal antibody
MOI	multiplicity of infection
miRNA	microRNA
mRNA	messenger RNA
NSG	NOD/SCID/IL2R$\gamma^{-}/^{-}$
PDX	patient-derived xenograft
PI	propidium iodide
QKI	quaking
RNA-seq	RNA-sequencing
snRNA	small nuclear RNA

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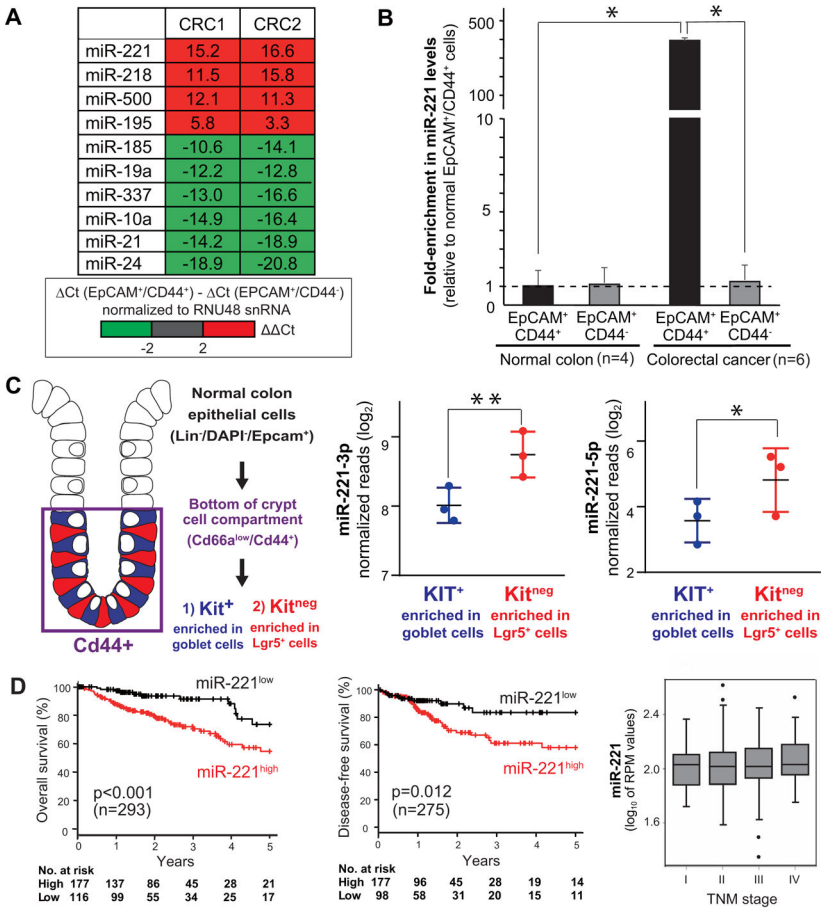
Significance.

Findings uncover molecular mechanisms underlying the maintenance of cancer stem cell properties in colon cancer.



Variable	Univariate analysis HR	95% CI	P value	Multivariate analysis HR	95% CI	P value
Age (≥60 years vs <60)	2.14	1.30-4.36	**0.0174**	2.99	1.56-6.23	**0.002**
Gender (Female vs Male)	0.89	0.52-1.48	0.65	1.03	0.57-1.81	0.92
Histology (MUC vs ADC)	0.97	0.34-2.20	0.95	1.58	0.52-3.85	0.39
T stage (T3/4 vs T1/2)	1.24	0.62-2.83	0.57	0.65	0.29-1.68	0.36
N stage (N1/2/3 vs N0)	2.89	1.69-5.12	**<0.0001**	3.11	1.61-6.19	**0.0007**
M stage (M1 vs M0)	2.52	1.26-4.66	**0.0103**	1.73	0.83-3.46	0.14
miR-221 (High vs Low)	2.82	1.55-6.57	**0.0005**	2.44	1.19-5.19	**0.009**

Figure 1. In human CRCs, *miR-221* is preferentially expressed in EpCAM$^+$/CD44$^+$ cancer cells and associates with reduced survival outcomes.

(A) List of miRNAs displaying the highest degree of differential expression between paired samples of EpCAM$^+$/CD44$^+$ cancer cells (enriched in CSCs) and EpCAM$^+$/CD44^{neg} cancer cells (depleted in CSCs) isolated in parallel from human primary CRCs. The expression level of 754 miRNAs was measured by real-time qPCR. Numbers represent the difference in threshold-cycle (ΔCt) values. **(B)** Relative *miR-221* expression levels in EpCAM$^+$/CD44$^+$ and EpCAM$^+$/CD44^{neg} cells from mouse normal primary colon epithelia (n=4) and primary human CRCs (n=6; *p<0.05). **(C)** Comparison of *miR-221* expression levels in Epcam$^+$/Cd66a^{low}/Cd44$^+$/Kit$^+$ cells (enriched in Lgr5$^+$ stem cells) and Epcam$^+$/ Cd66a^{low}/Cd44$^+$/Kit^{neg} cells (enriched in goblet cells) sorted in parallel from the normal colon epithelium (n=3; *p<0.05, **p<0.01). **(D)** Relationship between *miR-221* expression levels, 5-year overall survival and disease-free survival rates, and tumor stage (TNM) in CRC patients from the TCGA-COAD dataset. Box-plots display 10th, 25th, 50th, 75th, and 90th percentiles

of *miR-221* expression levels. The association between *miR-221* expression levels and 5-year survival outcomes remained statistically significant in multivariable analysis. HR: hazard ratio; CI: confidence interval; MUC: mucinous adenocarcinoma; ADC: adenocarcinoma.

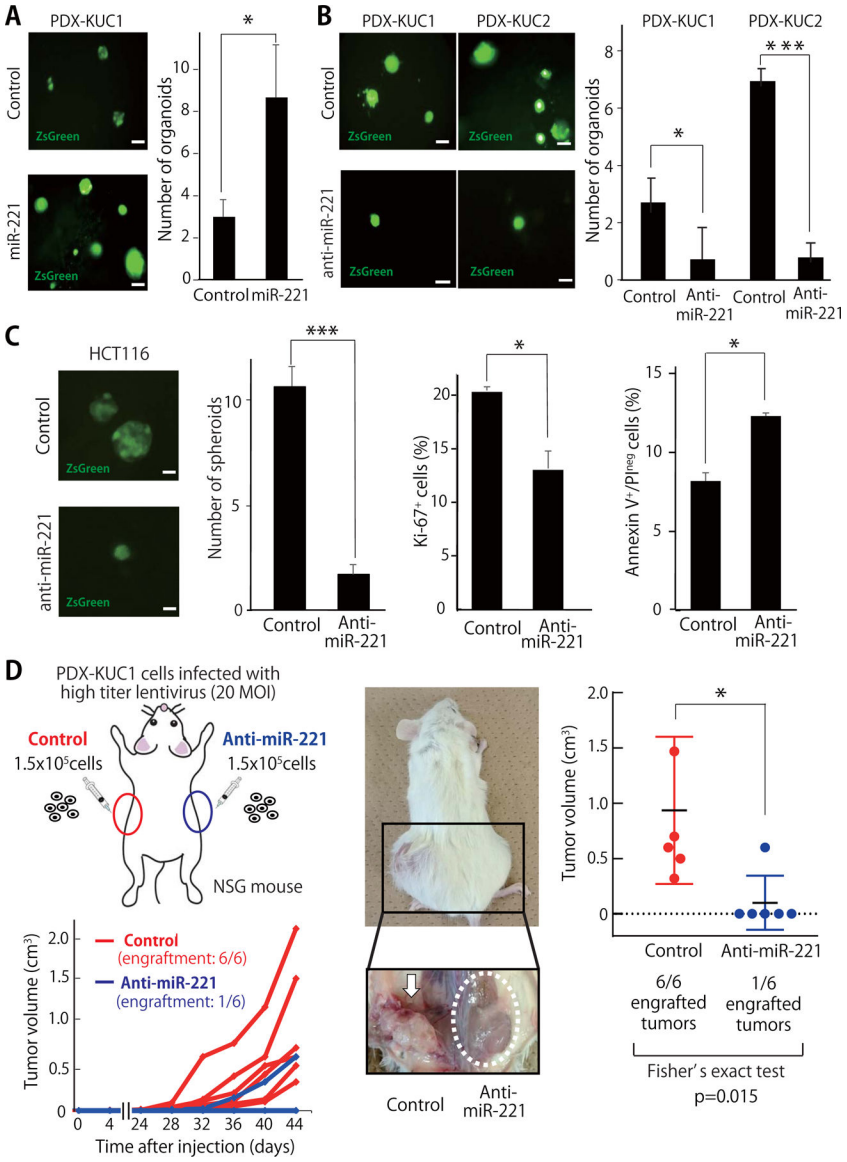


Figure 2. Inhibition of *miR-221* reduces both the *in vitro* three-dimensional (3D) organoid-forming capacity and *in vivo* tumorigenicity of human CRC cells.

(A-B) Representative appearance and number of organoids formed by colorectal cancer PDX cells following infection with lentivirus vectors encoding either *miR-221* (A) or an *anti-miR-221* construct (B) (n=5; *p<0.05, ***p<0.001). scale bar: 100 μm. **(C)** Infection of HCT116 cells with a lentivirus vector driving constitutive miR-221 expression was associated with a reduction in 3D spheroid forming capacity (n=3; ***p<0.001), a reduction in the percentage of Ki67^{+} cells (n=3; *p<0.05), and an increase of the percentage of Annexin-V^{+}/Propidium Iodide^{neg} (PI^{neg}) cells (n=3; *p<0.05). **(D)** Schematic illustration of *in vivo* xenotransplantation experiments and growth curves of tumors originated from PDX-KUC1 cells infected with either a lentivirus vector encoding for the *anti-miR-221* construct or an empty vector used as negative control (n=6; 1.5×10^{5} cells/injection). Two months after xenotransplantation, PDX-KUC1 cells infected with the control vector formed tumors in 6 out 6 cases (100%), while those with the *anti-miR-221* construct formed tumors in only 1

out 6 cases (17%; *p<0.05). All sub-cutaneous injection sites were dissected and visually inspected.

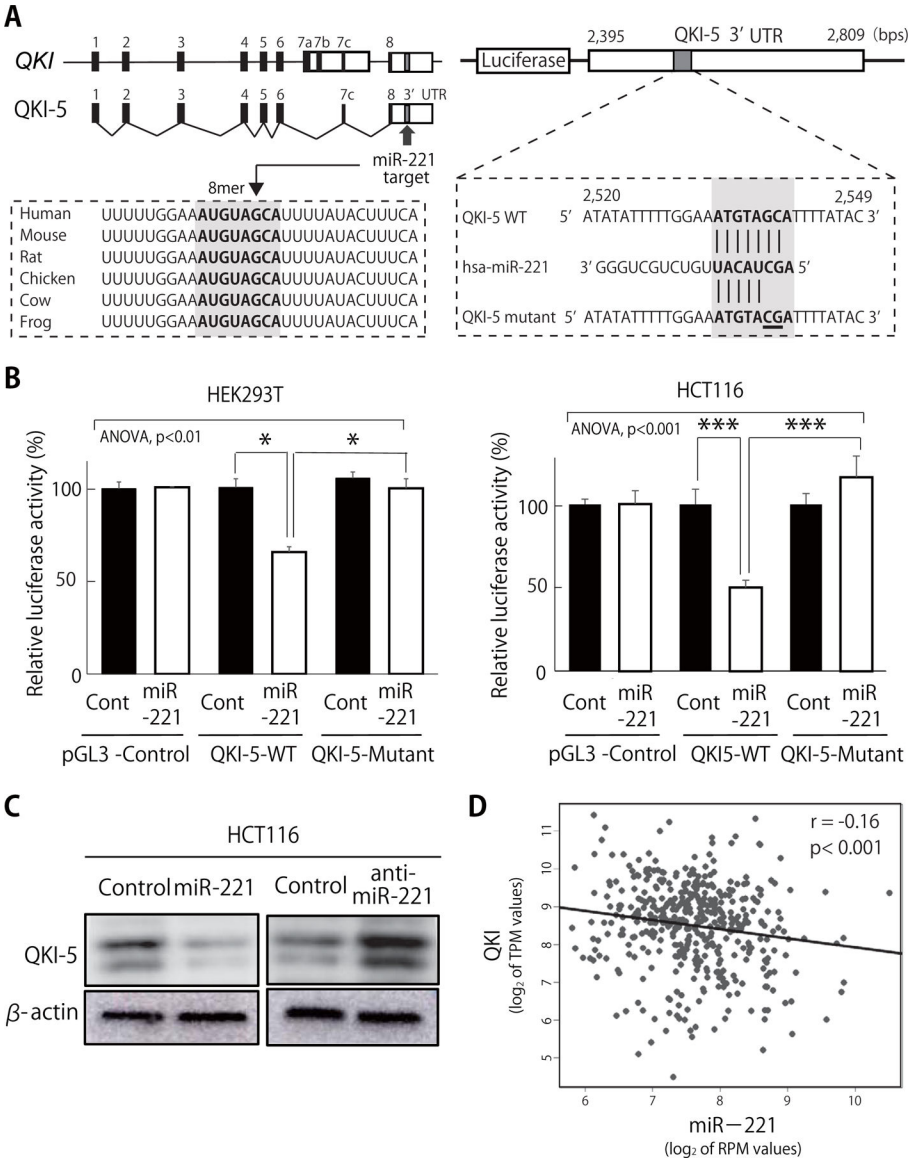


Figure 3. *QKI-5* is a direct molecular target of *miR-221*.

(A) Schematic representation of the predicted *miR-221* target recognition sequence within the 3'UTR of the *QKI-5* mRNA, and of the two mutations introduced to functionally disable it. Numbers correspond to nucleotide positions in *QKI-5* sequence (GenBank: NM_001301085). **(B)** Suppression of the luciferase activity of pGL3 constructs encoding the WT version of the QKI-5 3'UTR, but not that encoding mutant QKI-5 3'UTR by *miR-221* (n=3; *p<0.05, ***p<0.005). **(C)** Forced expression of *miR-221* down-regulated endogenous QKI-5 protein levels in human HCT116 cells, while forced expression of an anti-*miR-221* construct up-regulated them. Expression of β-actin was used as a control. **(D)** *QKI* and *miR-221* expression levels are inversely correlated (r=−0.16, p<0.001) in the TCGA COAD database of human primary CRCs (n=439).

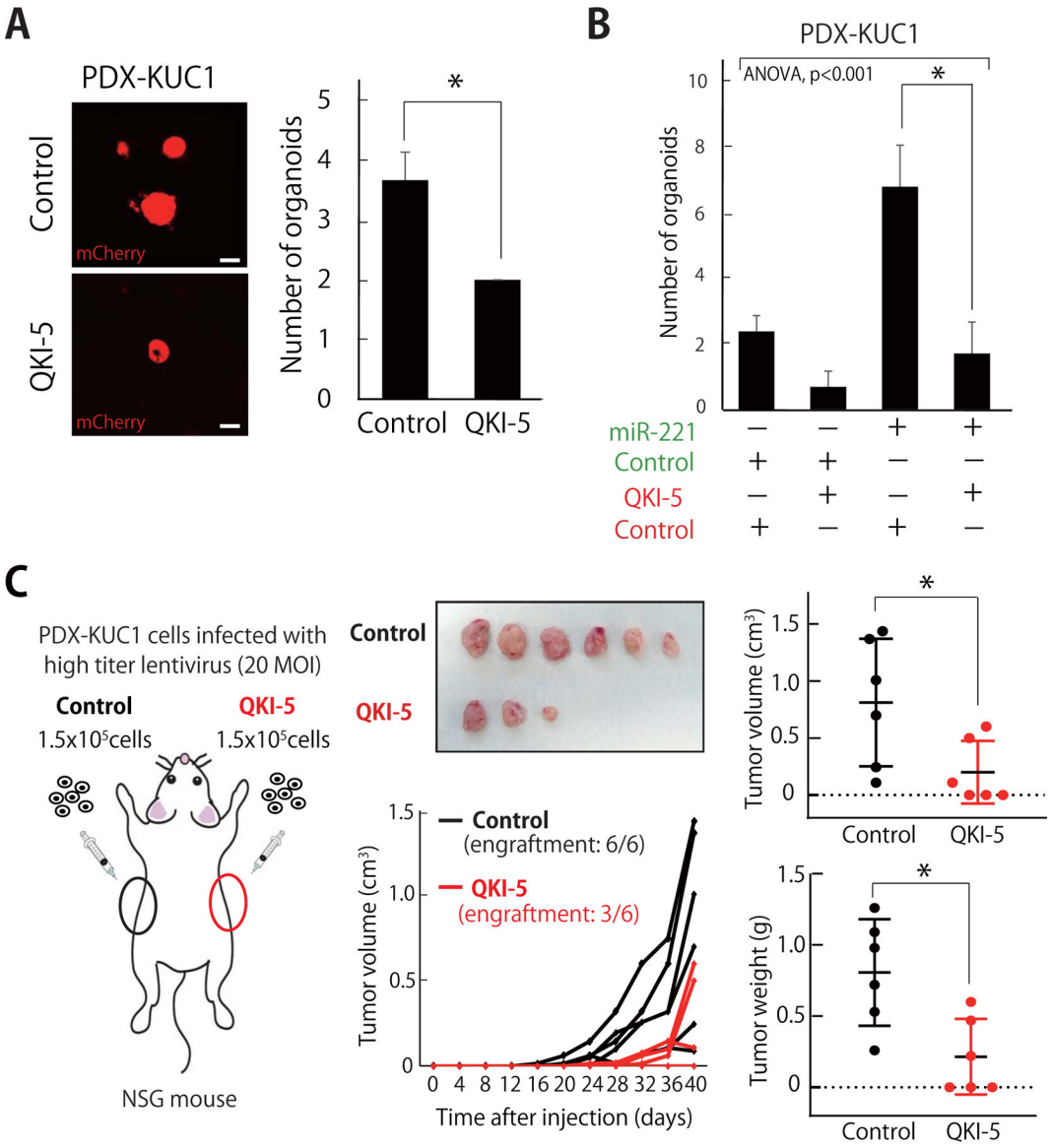


Figure 4. QKI-5 suppresses the *in vitro* three-dimensional (3D) organoid-forming and tumor formation capacities of human CRC PDX cells.

(A) The 3D organoid-forming capacity of PDX-KUC1 cells was reduced following infection with a lentivirus vector driving constitutive expression of the *QKI-5* cDNA (n=3; *p<0.05). Scale bar: 100 μm. **(B)** Forced expression of QKI-5 abrogated *miR-221*'s ability to enhance the 3D organoid forming capacity of PDX-KUC1 cells. Histograms report the number of organoids larger than 100 μm in diameter (n=3; *p<0.05). **(C)** Schematic illustration of *in vivo* xenotransplantation experiments and growth curves of tumors originated from PDX-KUC1 cells infected with either a lentivirus vector encoding for QKI-5 or an empty vector used as negative control (n=6; *p<0.05, 1.5×10^5 cells/injection).