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ORIGINAL ARTICLE

miR-574-5p negatively regulates *Qki6/7* to impact β -catenin/Wnt signalling and the development of colorectal cancer

Shunlong Ji,¹ Gengtai Ye,² Jun Zhang,¹ Linpei Wang,² Tao Wang,¹ Zhen Wang,¹ Tiantian Zhang,¹ Guanghui Wang,¹ Zongsheng Guo,¹ Yu Luo,³ Jianchun Cai,² James Y Yang^{1,4}

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¹State Key Laboratory of Cellular Stress Biology and Department of Biomedical Sciences, School of Life Sciences, Xiamen University, Xiamen, People's Republic of China

²Department of Surgical Oncology the First Affiliated Hospital of Xiamen University and Xiamen Cancer Center, Xiamen, People's Republic of China

³School of Nursing, the Third Military Medical University, Chongqing, People's Republic of China

⁴Fujian Provincial Transgenic Core, Xiamen University Laboratory Animal Center, Xiamen, People's Republic of China

Correspondence to

Dr James Y Yang, Department of Biomedical Sciences, School of Life Sciences, Xiamen University, Xiamen, China 361005; jyqy2008@gmail.com or

Dr Jianchun Cai, Department of Surgical Oncology, the First Affiliated Hospital of Xiamen University and Xiamen Cancer Center, Xiamen, China 361003; jianchunfh2@sina.com

SJ, GY and JZ contributed equally to the work.

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ABSTRACT

Objective Deficiency or reduced expression of signal transduction and activation of RNA family protein *Quaking (Qki)* is associated with developmental defects in neural and vascular tissues and the development of debilitating human diseases including colorectal cancer (CRC). However, the mechanisms underlying the aberrant downregulation or deficiency of *Qki* were uncertain.

Design Expression of miR-574-5p, *Qki5/6/7/7b* splicing variants, β -catenin and *p27^{Kip1}* was determined in mouse and human CRC cells and tissues to investigate the post-transcriptional regulation of *Qki* isoforms by miR-574-5p and its impact on β -catenin/*p27^{Kip1}* signalling, cell cycle progression, proliferation, migration, invasion and tumour growth.

Results In the CRC tissues of C57BL/6-*Apc^{min/+}* mice, miR-574-5p was found to be significantly upregulated and negatively correlated with the expression of *Qki* but positively correlated with the expression of β -catenin. In mouse and human CRC cells, miR-574-5p was shown to regulate *Qki* isoforms (*Qki6/7* in particular) post-transcriptionally and caused altered expression in β -catenin and *p27^{Kip1}*, increased proliferation, migration and invasion and decreased differentiation and cell cycle exit. Furthermore, in clinical CRC tissues, miR-574-5p was shown to be greatly upregulated and inversely correlated with the expression of *Qkis*. Finally, inhibition of miR-574-5p was shown to suppress the growth of tumours in the nude mice.

Conclusions Together, these novel findings suggest that miR-574-5p is a potent ribo-regulator for *Qkis* and that aberrant miR-574-5p upregulation can be oncogenic.

INTRODUCTION

Quaking (QKI) is a RNA binding protein belonging to the signal transduction and activation of RNA (STAR) protein family that has been implicated in the regulation of cellular processes such as cell cycle and differentiation,¹ apoptosis,² cell fate decision and development^{3–5} and angiogenesis.^{6,7} Aberrant expression of the STAR proteins has been associated with a number of developmental defects and human diseases.^{8,9} Studies have shown that Src associated in mitosis, of 68 kDa (Sam68), a prototype of the STAR proteins, is a multifunctional player in human cancers.^{10–14} Genetic deletion in

Significance of this study**What is already known on this subject?**

- Downregulation of QKI5 and QKI6 was shown to be associated with the development of colorectal cancer (CRC).
- QKI5/6 might contribute to the development of CRC by controlling β -catenin and *p27^{Kip1}* signalling.
- DNA methylation in the promoter region of *Qki* was suggested to contribute in part to *Qki*'s aberrant down regulation in CRC.

What are the new findings?

- miR-574-5p regulates *Qki6/7* negatively and post-transcriptionally.
- QKI7 is the most predominant isoform in human CRC cells. QKI5/6/7 proteins all regulate β -catenin post-transcriptionally.
- miR-574-5p expression correlates inversely with that of *Qkis* in CRC cells and tissues.
- Aberrant upregulation of miR-574-5p can be oncogenic and its inhibition is beneficial.

How might it impact on clinical practice in the foreseeable future?

- The discovery of this miR-574-5p-QKI- β -catenin/*p27^{Kip1}* axis of signal transduction will enable better understanding of the pathogenic mechanisms in human diseases associated with the abnormal expression of miR-574-5p, QKI and QKI-regulated genes.
- miR-574-5p and *Qkis* might serve as diagnostic or therapeutic targets for CRC.

the *Qki* locus, on the other hand, was found to be associated with human glioblastoma or astrocytic glioma^{15–17} mental retardation and autism.¹⁸ Haploinsufficiency of *Qki* has also been linked with the 6q terminal deletion syndrome.¹⁹ Furthermore, reduced *Qki* expression has been implicated in the pathogenesis of human disorders such as schizophrenia^{20,21} and major depressive disorders.²² Yang *et al* found that QKI5 and QKI6 were greatly reduced or even absent in colorectal cancer (CRC) cells and tissues and that downregulation of QKI5/6 was associated with abnormal regulation of

β -catenin and $p27^{Kip1}$ signalling.²³ More recently, QKI expression was found to be significantly reduced in several types of tumours, including testis, lung, breast, bladder, cervix, ovary and colon cancer, which was shown to be associated with an abnormal decrease in the histone variant macroH2A1.1.²⁴

Despite the potentially dire biological consequences of dysregulation in the expression of *Qki*, the exact genetic and epigenetic mechanisms governing *Qki* expression are poorly understood.^{9, 25} Klempan *et al* studied the effects of genetic and epigenetic variation on the promoter function and expression of *Qki* in the human brains of the suicide victims with major depressive disorders.²² While significant reductions in *Qki* expression were found in the cortical regions, the hippocampus and the amygdala of suicide victims, analysis of variations in and methylation state of the promoter sequence did not identify differences at genetic and epigenetic levels between depressed suicide victims and controls. In a later study with 288 individuals diagnosed with schizophrenia and 288 controls, no association was found between seven genetic markers (including single-nucleotide polymorphism and haplotype) around the promoter region of the *Qki* gene and schizophrenia.²⁶ In the study conducted by Yang *et al*,²³ while characterisations of the *Qki* promoter region in a few CRC cell lines suggested enhanced promoter DNA methylation, increased methylation was found in only 4 out of 10 CRC tissue samples tested. Moreover, methylation was often detected in both CRC tissues and the neighbouring normal tissues from the same patient. Furthermore, DNA methylation is unlikely to be the major mechanism responsible for the downregulation of *Qki* as it cannot adequately explain the temporal, spatial and isoform-specific regulation of *Qki* in many cases of cell development and diseases. Collectively these observations suggest that genetic variations and epigenetic modifications in the *Qki* promoter region might not be the major determinants responsible for reduced *Qki* expression that is associated with human diseases such as schizophrenia, depression and cancers.

MicroRNAs (miRNAs) are non-coding small RNAs that serve as important regulators of gene expression by targeting the 3' untranslated region (UTR) of messenger RNA (mRNA) and inducing mRNA degradation and suppression of translation.^{27–28} The 3'UTRs of *Qki* mRNAs are evolutionally conserved and thus may be important to *Qki*'s post-transcriptional regulation.^{29–31} In this current study, we found that miR-574-5p negatively regulates *Qki6/7/7b* through interactions with miRNA recognition elements (MREs) on the corresponding 3'UTRs. In mouse and human CRC tissues, very significant upregulation of miR-574-5p is correlated with substantial reductions in *Qki6/7/7b*, which might contribute significantly to the aberrant regulation of β -catenin and $p27^{Kip1}$ and to the pathogenesis or progression of CRC. These results unravel a novel and important mechanism for the regulation of *Qkis* and the development of CRC.

MATERIALS AND METHODS

For details of the following, please see the supplemental materials and methods section in online supplementary information: plasmid construction, cell culture, transient transfections, western blot, immunohistochemistry, immunofluorescence staining, in situ hybridisation analyses; cell cycle, proliferation, migration, colony formation and invasion assays; luciferase reporter assays, intestinal alkaline phosphatase (IAP) enzyme assays and real-time quantitative RT-PCR (qPCR) analyses of mRNAs and miRNAs.

Animal experiments

All experimental procedures involving animals were performed in accordance with animal protocols approved by the Institutional Animal Use and Care Committee of Xiamen University. C57BL/6-*Apc*^{min/+} mice and controls were generous gifts from Dr Boan Li, Xiamen University. Five-week-old male athymic BALB/c nude mice were obtained from the SLAC Laboratory Animals Co Ltd (Shanghai, China).

For xenograft tumour growth, 5×10^6 SW480 cells pre-infected with control lentiviruses or lentiviruses carrying shRNA for miR-574-5p were injected subcutaneously into the dorsal flanks of mice. Beginning from the 10th day after the injection, the size of the tumour was measured every 3 days by a Vernier calliper along two perpendicular axes. The volume of the tumour was calculated with the formula: volume (mm³) = length (mm) \times width (mm)² \times 0.52. Twenty-eight days after the injection, the mice were sacrificed and the tumours were dissected for further analyses.

For bioluminescence imaging of tumour growth in live animals, see supplemental materials and methods section in online supplementary information.

Clinical samples

All clinical samples were collected with the informed consent of the patients and study protocols that were in accordance with the ethical guidelines of the Declaration of Helsinki (1975) and were approved by the Institutional Medical Ethics Committee of Xiamen University. CRC pathological diagnosis was verified by at least two pathologists. Sixty human CRC specimens and paired adjacent epithelial tissues and serum samples from seven patients with CRC and seven normal controls were obtained from the First Affiliated Hospital of Xiamen University from 25 January 2010 to December 2011. Tumour and non-cancerous tissues were confirmed histologically by haematoxylin and eosin (H&E) staining.

Bioinformatics, data acquisition, image processing and statistical analyses

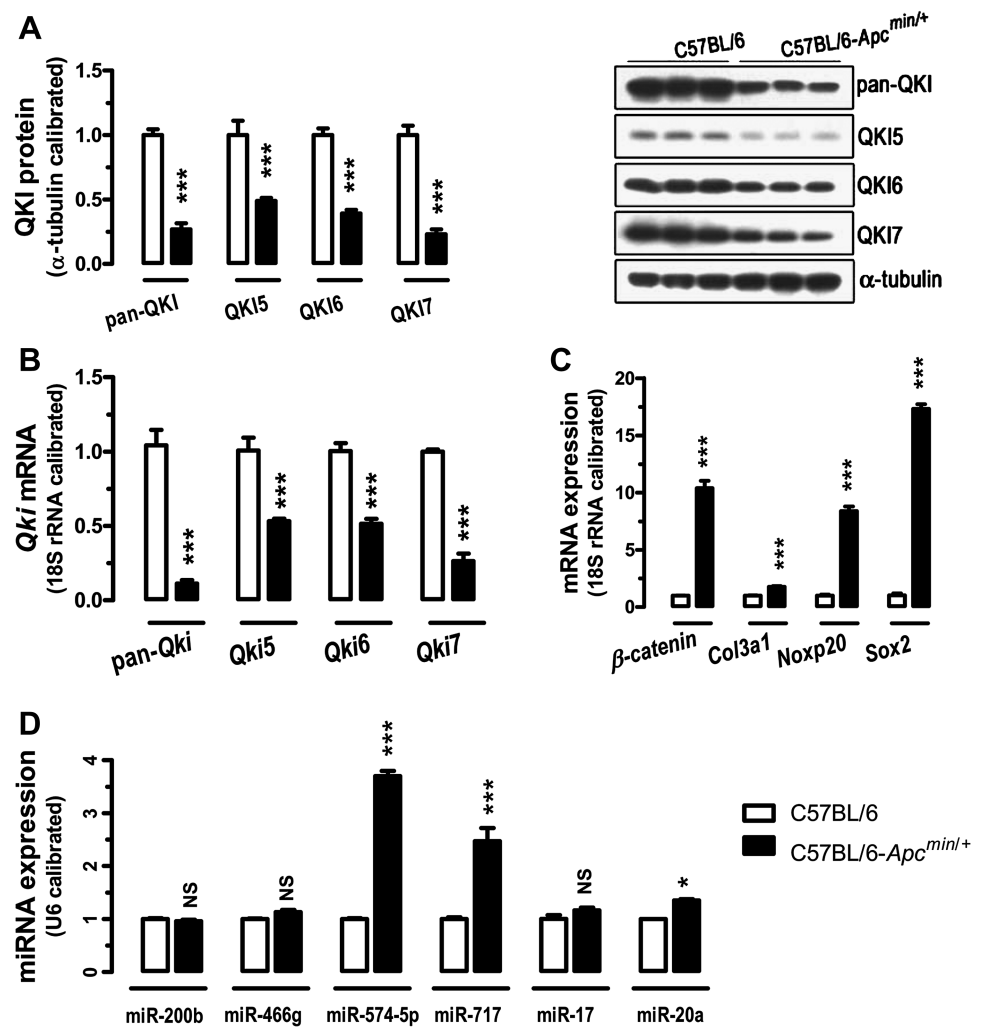
See supplemental materials and methods section in online supplementary information.

RESULTS

Negative correlation between miR-574-5p and *Qki* expression and positive correlation between miR-574-5p and β -catenin in the CRC tissues of C57BL/6-*Apc*^{min/+} mice

Our bioinformatics analyses suggested the presence of multiple MREs on the 3' UTR of mouse or human *Qki5/6/7/7b* mRNAs, including those for miR-574-5p, miR-17 and miR-20a (figure S1, online supplementary information). We therefore performed qPCR and western blotting to analyse the expression levels of *Qki* mRNAs and proteins and miRNAs in the CRC tissues from C57BL/6-*Apc*^{min/+} (*Apc*-mutant) mice and in colorectal tissues from normal control mice. Compared with the expression levels in the normal tissues, total *Qki* mRNA/protein (pan-*Qki*/pan-QKI) and *Qki5/6/7* mRNA/protein isoforms were all greatly downregulated while β -catenin was greatly upregulated in the CRC tissues of the *Apc*-mutant mice (figure 1A–C). Analyses of miRNA expression indicated that miR-574-5p, miR-717 and miR-20a were significantly upregulated in mouse CRC tissues but miR-200b, miR-466g and miR-17 were not (figure 1D). Among the three significantly upregulated miRNAs, miR-574-5p had the highest fold change (~ 3.7 -fold, $p < 0.001$). Interestingly, MREs for miR-574-5p were found on the 3'UTRs of mouse

Figure 1 miR-574-5p expression correlates negatively with the expression of *Qki* but correlates positively with β -catenin in C57BL/6-*Apc*^{min/+} mice (16-week old males, n=3). Data are represented as mean \pm SEM. **p*<0.05; ****p*<0.001. (A) Total QKI and QKI5/6/7 protein expression levels were greatly downregulated in mouse colorectal cancer (CRC) tissues. (B) Total *Qki* and *Qki5/6/7* messenger RNA (mRNA) expression levels were significantly downregulated in mouse CRC tissues. (C) The expression levels of β -catenin, *Noxp20*, *Col3a1* and *Sox2* mRNAs were greatly increased in mouse CRC tissues. *Col3a1*, procollagen type III, α -1; *Noxp20*, nervous system overexpressed protein-20; *Sox2*, SRY-like HMG box-2. (D) The expression levels of miR-574-5p, miR-717 and miR-20a were significantly upregulated in mouse CRC tissues, whereas that of miR-200b, miR-466g and miR-17 were not. See also online figure S1. miRNA, microRNA; NS, not significant.



Qki6/7 but not on that of *Qki5* mRNA (online figure S1), suggesting that miR-574-5p might be involved in the negative regulation of *Qki6/7* in mouse colorectal tissues. Remarkably, the expression levels of two putative intronic miR-574-5p-hosting genes, nervous system overexpressed protein-20 (*Noxp20*,³² and miRBase) and procollagen type III, α -1 (*Col3a1*)³³ and the level of a transcription factor SRY-like HMG box-2 (*Sox2*) were also significantly upregulated in mouse CRC tissues (figure 1C). This result suggests that miR-574-5p might be co-regulated with *Noxp20* or *Col3a1* in the mouse tissues.

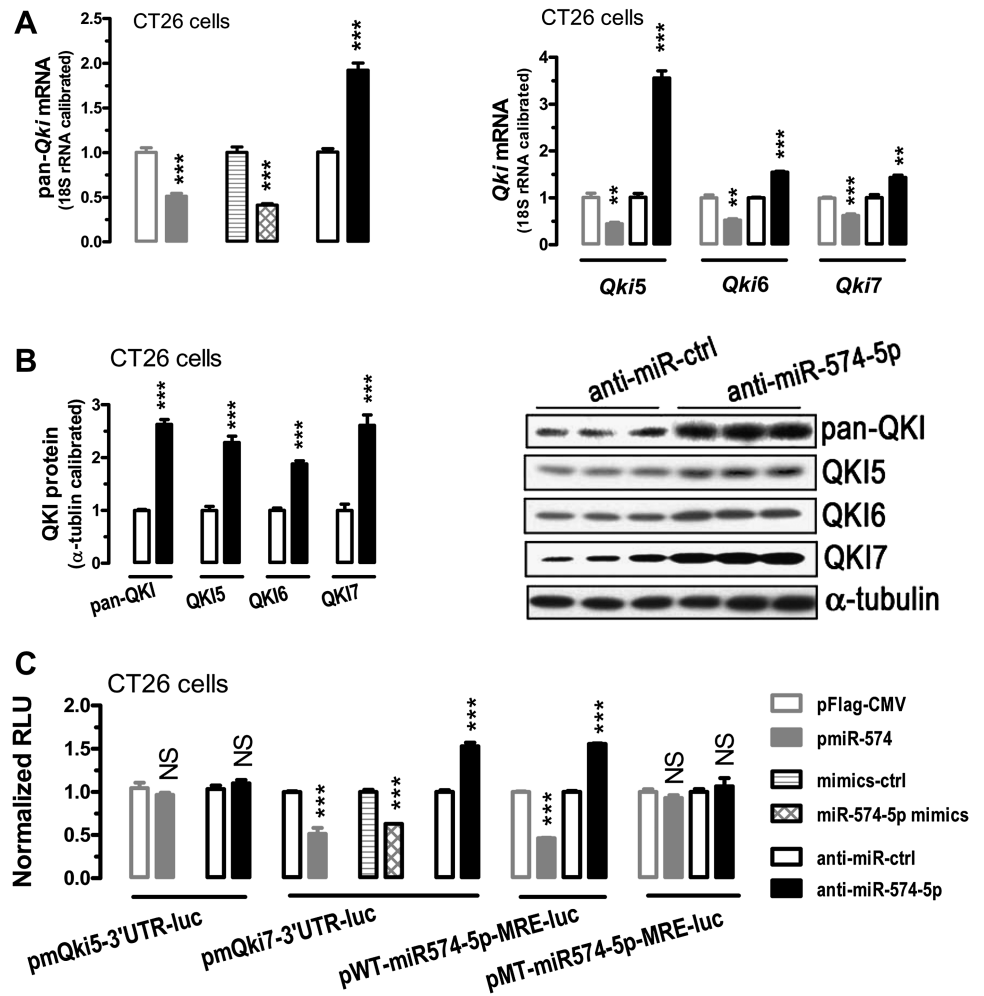
miR-574-5p represses the expression of *Qki6/7/7b* through a post-transcriptional mechanism

To demonstrate the silencing effects of miR-574-5p on *Qkis*, we performed transient transfection studies in cultured CRC cells (figure 2). Our qPCR analyses indicated that, compared with the control group, transfection of CT26 cells with a plasmid over-expressing miR-574 precursor (pmiR-574) or with chemically synthesised mimics for miR-574-5p (see supplemental materials and methods section in online supplementary information) led to a 49–59% reduction in total *Qki* mRNA expression (*p*<0.001, figure 2A). Conversely, inhibition of miR-574-5p with an antisense inhibitor (see supplemental materials and methods section in online supplementary information) resulted in a 92% increase in *Qki* mRNA expression (*p*<0.001). Similar effects were found for *Qki5/6/7* mRNA isoforms. Paralleling the increase in *Qki*

mRNAs following the inhibition of miR-574-5p, total QKI and QKI5/6/7 proteins were all significantly increased as indicated by western blots (*p*<0.001, figure 2B). Moreover, similar effects were observed for human HCT116, SW480 and SW620 cells (figure S2, online supplementary information).

To determine whether miR-574-5p regulates *Qkis* by targeting *Qki*-3'UTR, we constructed mouse *Qki*-3'UTR-luciferase reporter plasmids (see supplemental materials and methods section in online supplementary information) and used them for CRC cell transfections. Plasmid pmQki7-3'UTR-luc carries the 3'UTR for *Qki7* mRNA that contains a putative miR-574-5p MRE, which is also shared by *Qki6*-3'UTR and *Qki7b*-3'UTR (figure S1, online supplementary information). In contrast, plasmid pmQki5-3'UTR-luc carries the *Qki5*-3'UTR that does not contain any MRE for miR-574-5p. Consistent with the bioinformatics prediction, however, increased miR-574-5p or inhibition of miR-574-5p did not lead to significant changes in *Qki5*-3'UTR activity, suggesting that miR-574-5p does not regulate *Qki5* mRNA through 3'UTR-mediated mechanisms (figure 2C). As anticipated, however, increased miR-574-5p led to significant suppression of *Qki7*-3'UTR activity in CT26 cells. Conversely, inhibition of miR-574-5p led to significant activation of *Qki7*-3'UTR (figure 2C), suggesting that *Qki6/7/7b* mRNAs are all under the post-transcriptional control of miR-574-5p. Mutational analyses of the miR-574-5p MRE on *Qki7*-3'UTR (pMT-miR-574-5p-MRE-luc (mutated MRE)

Figure 2 miR-574-5p represses *Qki6/7/7b*. CT26 cells were transfected with a microRNA (miRNA)-overexpressing plasmid or a miRNA mimics or a miRNA inhibitor, in the absence or presence of a luciferase reporter together with pSV40- β -galactosidase (4:3:1). Cells were harvested for quantitative PCR (qPCR), western blotting and luciferase activity assays 24 h after the transfections. For qPCR and the luciferase reporter assays, $n=3-4$. Statistical comparisons were made between a control (pFlag-CMV, mimics control, or inhibitor control)-transfected cells and a particular treatment. Data are represented as mean \pm SEM. ** $p < 0.01$; *** $p < 0.001$. (A) Overexpression of miR-574-5p or transfection with its mimics caused significant downregulation of total *Qki* messenger RNA (mRNA) and *Qki5/6/7* mRNA isoforms, whereas inhibition of miR-574-5p significantly increased the level of total *Qki* and *Qki5/6/7* mRNA isoforms as determined by qPCR. (B) Inhibition of miR-574-5p significantly increased the levels of total QKI and QKI5/6/7 protein as determined by western blots. (C) miR-574-5p regulates *Qki6/7/7b* (but not *Qki5*) by interacting with the 3' untranslated region of *Qki6/7/7b*. β -galactosidase activity was determined and used for the calibration of luciferase reporter activity. NS, not significant. See also online figure S2. RLU, relative light units.



versus pWT-miR-574-5p-MRE-luc (wildtype MRE)) further confirmed the specific interaction between miR-574-5p and *Qki6/7/7b*-3' UTR (figure 2C).

QKI5/6/7 represses β -catenin expression and activity in CRC cells

β -catenin is one of the predicted targets of QKI proteins,³⁴ with two putative QREs on its mRNA (figure S3, online supplementary information). However, a previous study suggested that QKI5/6 suppressed the expression of β -catenin protein but not that of mRNA.²³ We therefore wanted to further determine how QKI5/6/7 would affect the expression of β -catenin mRNA and protein and the β -catenin/Wnt signalling activity in CRC cells. To this end, we first transiently transfected CT26 cells with three plasmids overexpressing mouse *Qki5/6/7* respectively (see supplemental materials and methods section in online supplementary information). Overexpression of each QKI caused significant downregulation of β -catenin mRNA and protein expression (figure 3A,C). To determine whether QKIs regulate β -catenin through post-transcriptional silencing, we constructed a mouse β -catenin-3' UTR-luciferase reporter (pmCtnnb1-3' UTR-luc) that contains two putative QREs (figure S3 and supplemental materials and methods section in online supplementary information). Co-transfection studies indicated that overexpression of QKI5/6/7 all dose dependently reduced the reporter activity (figure 3B), suggesting that these QKIs are all capable of silencing β -catenin expression post-transcriptionally. Consistent with QKI-mediated downregulation of

β -catenin, the TOP/FOP-Flash analyses indicated that these three QKIs could all suppress the activity of TOP-Flash reporter but not the FOP-Flash reporter (figure 3D), suggesting that β -catenin/Wnt signalling is regulated in part by the STAR family proteins QKI5/6/7.

miR-574-5p positively regulates β -catenin but negatively regulates $p27^{Kip1}$

To determine how miR-574-5p might affect β -catenin and $p27^{Kip1}$, two putative targets for QKIs, we performed qPCR and western blot analyses with CRC cells. In CT26 and SW480 cells, overexpression of miR-574-5p caused significant upregulation of mRNA and protein levels in β -catenin, whereas inhibition of miR-574-5p caused significant downregulation (figure 4A–C). In contrast, overexpression of miR-574-5p caused downregulation of $p27^{Kip1}$ mRNA and protein (figure 4D–F). These results suggest that miR-574-5p positively regulates β -catenin but negatively regulates $p27^{Kip1}$, both largely through downregulating the QKIs.

Effects of miR-574-5p on CRC cell proliferation, differentiation, invasion, migration and colony formation

To evaluate the impact of miR-574-mediated regulation of QKIs, β -catenin and $p27^{Kip1}$, we inhibited or overexpressed miR-574-5p in CT26 cells or SW480 cells. In human and mouse cells, inhibition of miR-574-5p greatly reduced cell proliferation as determined by the MTT assays (figure 5A). In SW480 cells, the G1

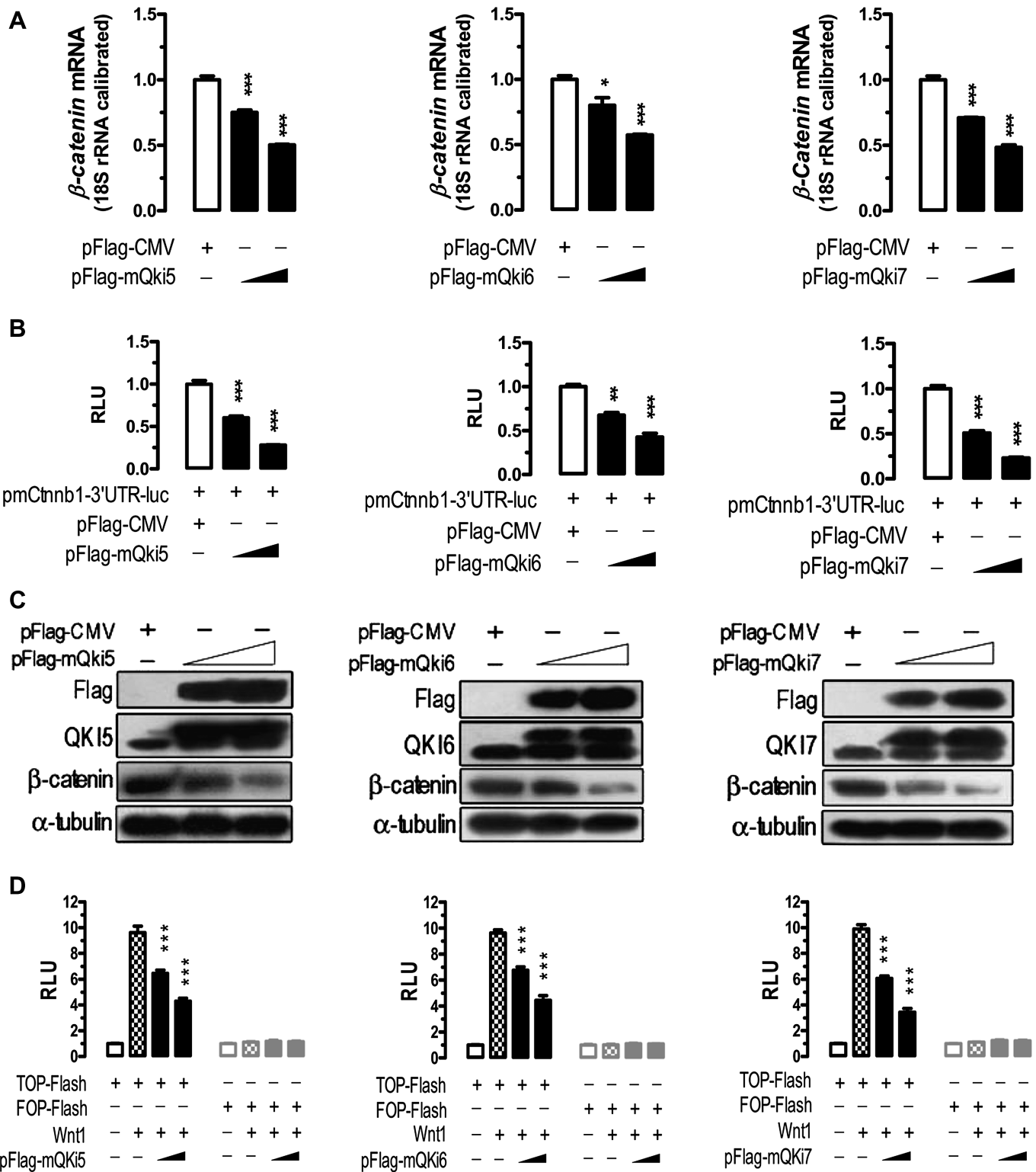
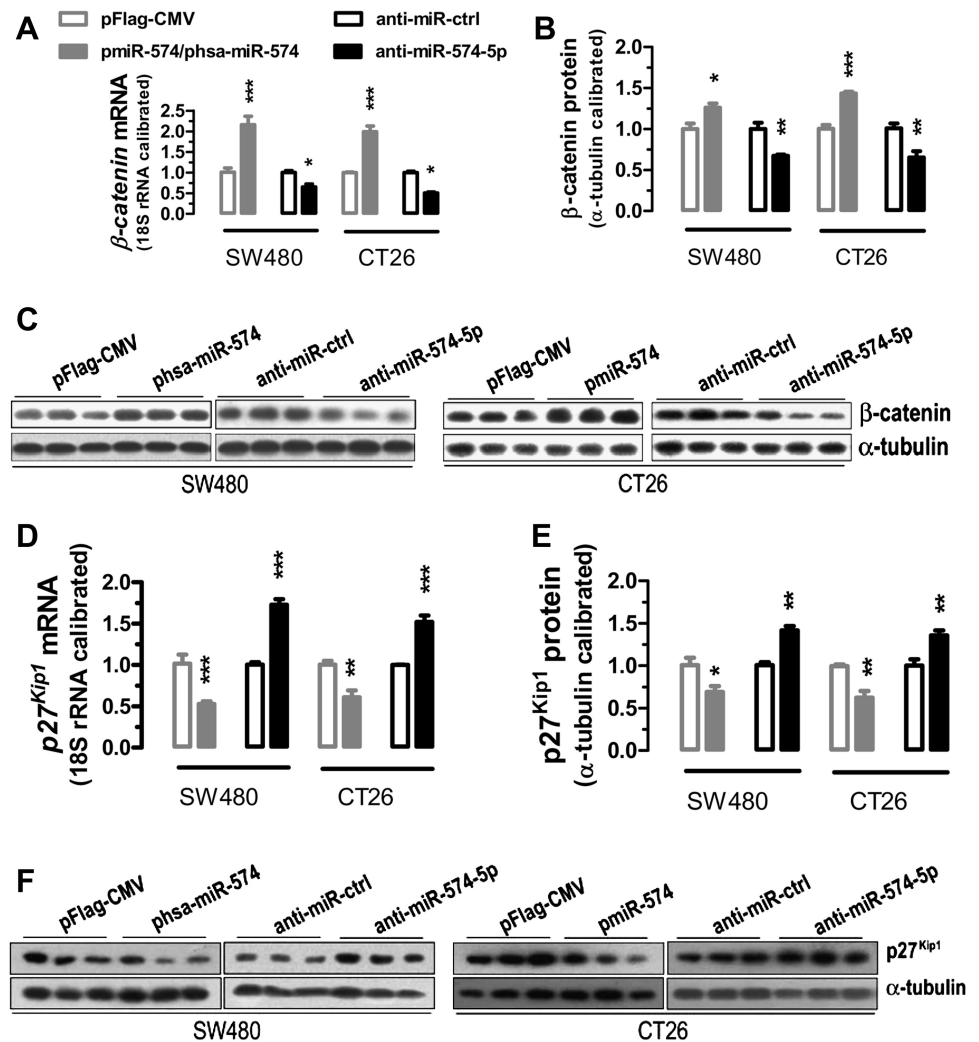


Figure 3 QKI5, QKI6 and QKI7 negatively regulate β -catenin expression and activity. For western blots, CT26 cells were transfected with pFlag-CMV or QKI overexpressing plasmids (pFlag-mQki5 or pFlag-mQki6 or pFlag-mQki7). For luciferase reporter assays, cells were co-transfected with a *Qki*-overexpressing plasmid (pFlag-mQki5 or pFlag-mQki6 or pFlag-mQki7), a luciferase reporter plasmid (pmCtnnb1-3' UTR-luc or TOP-Flash or FOP-Flash), Wnt1, together with pSV40- β -galactosidase (3:2:2:1). Cells were harvested for analyses 24 h after the transfections. α -Tubulin expression and β -galactosidase activity were determined and used for the calibration of western blots and luciferase reporter assay respectively. For the luciferase reporter assays, $n=3$. Data are represented as mean \pm SEM. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. (A) QKI5/6/7 repress the expression of β -catenin messenger (mRNA) in CT26 cells dose dependently. (B) QKI5/6/7 dose-dependently repress the expression of β -catenin by interacting with β -catenin-3' untranslated region. (C) QKI5/6/7 repress β -catenin protein expression dose dependently. Plasmid-derived proteins and endogenous proteins appeared to be resolved as two separate bands. (D) QKI5/6/7 suppress β -catenin/Wnt signalling dose dependently. See also online figure S3. RLU, relative light units.

Figure 4 miR-574-5p positively regulates β -catenin but negatively regulates $p27^{Kip1}$. SW480 and CT26 cells were transfected with a miRNA-overexpressing plasmid or a microRNA (miRNA) inhibitor. Cells were harvested for quantitative PCR and western blot assays 24 h after the transfections ($n=3$). Statistical comparisons were made between a control ((pFlag-CMV or inhibitor control)-transfected cells) and a particular treatment. Data are represented as mean \pm SEM. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. (A, B) Overexpression of miR-574-5p significantly increased messenger RNA (mRNA) or protein expression of β -catenin, whereas inhibition of miR-574-5p significantly reduced the levels of β -catenin mRNA or protein in SW480 and CT26 cells. (C) Western blots for β -catenin in SW480 and CT26 cells. (D, E) Overexpression of miR-574-5p significantly decreased mRNA or protein expression of $p27^{Kip1}$ in SW480 and CT26 cells, whereas inhibition of miR-574-5p significantly increased the levels of $p27^{Kip1}$ mRNA or protein in SW480 and CT26 cells. (F) Western blots for $p27^{Kip1}$ in SW480 and CT26 cells.



cells were significantly increased while the S cells were significantly decreased 72 h after miR-574-5p inhibition, suggesting G1/S arrest (figure 5B). We analysed the expression of *Lactase* mRNA and the enzyme activity of intestinal alkaline phosphatase (IAP), two important markers of intestinal differentiation. In CT26 cells, overexpression of miR-574-5p significantly reduced the expression of *Lactase* mRNA and the activity of IAP, whereas inhibition of miR-574-5p increased both (figure 5C,D). Meanwhile, overexpression of miR-574-5p significantly increased invasion likelihood in CT26 cells, whereas inhibition of miR-574-5p decreased invasion likelihood (figure 5E). Furthermore, overexpression of miR-574-5p in SW480 cells greatly enhanced cell migration, whereas inhibition of miR-574-5p suppressed the migration (figure 5F). Additionally, specific inhibition of miR-574-5p also greatly decreased the ability of colony formation in SW480 cells (figure 6B). Together these data suggest that when overexpressed, miR-574-5p might be oncogenic.

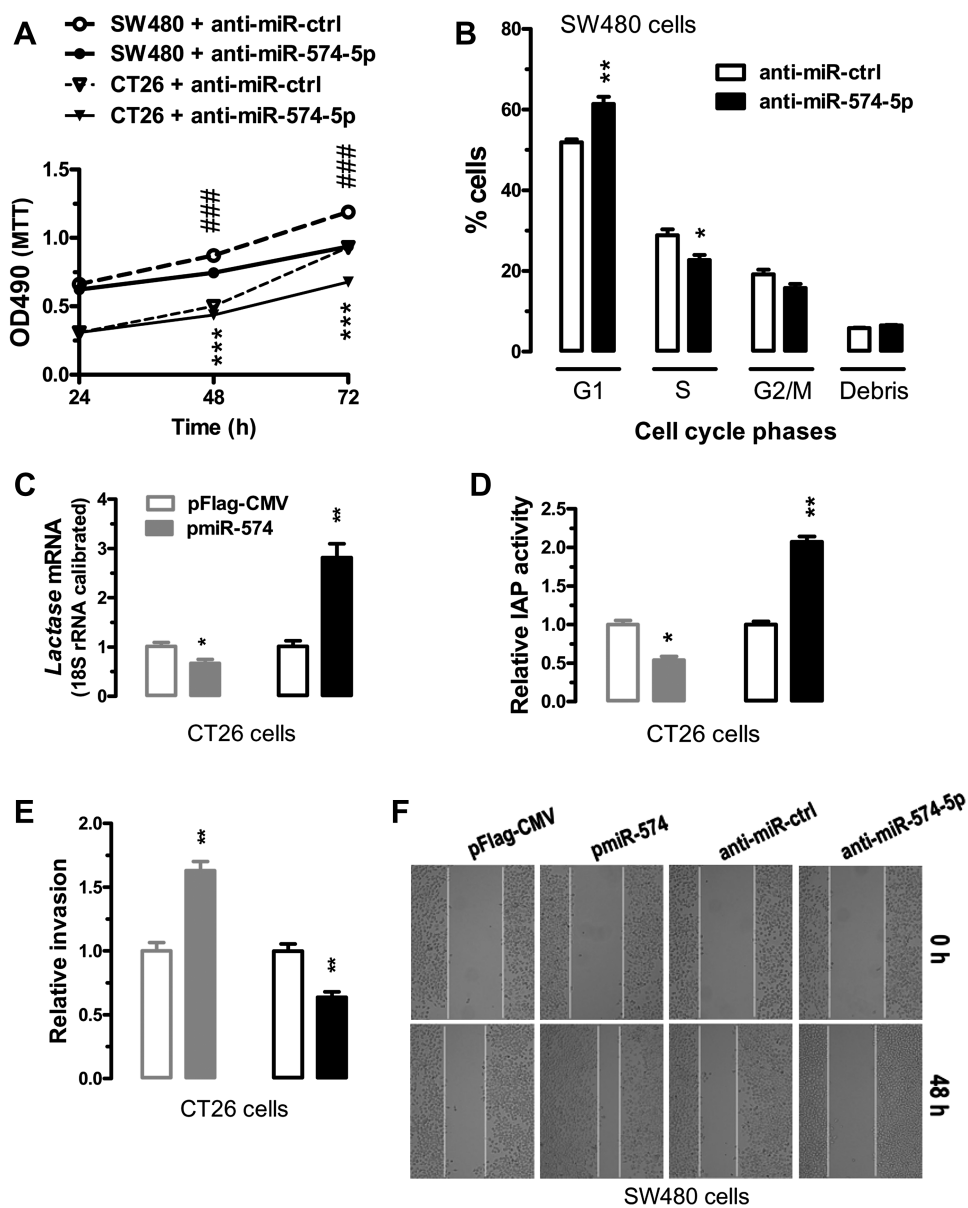
To verify that miR-574-5p-mediated repression of QKIs is important for the oncogenic function of miR-574-5p, we co-expressed one of the three mouse QKIs (QKI5/6/7) with miR-574-5p in CT26 cells (online figure S4). In comparison with cells overexpressing miR-574-5p only, cells co-overexpressing miR-574-5p and QKI5/6/7 in general all appeared to lead to increased mRNA expression of $p27^{Kip1}$, reduced mRNA expression of β -catenin, reduced ability in cell invasion and slightly reduced cell

proliferation, although the re-introduction of QKI5 only caused relatively minor and in some cases not significant effects. These results confirm that the miR-574-5p-mediated repression of *Qkis* contributes significantly to its oncogenic function.

Inverse correlation between the expression of miR-574-5p and the expression of total *Qki* mRNA/protein in CRC clinical samples

To explore the relationship between the expression of miR-574-5p and the expression of total *Qki* mRNA/protein, we performed qPCR and western blots with 60 pairs of clinical samples. In about 76.7% of human CRC tissues (46 out of 60), miR-574-5p was found to be significantly upregulated in comparison with the controls, while total *Qki* mRNA and protein expression levels were greatly reduced (figure 7A–E). These results were further confirmed by immunohistochemistry analyses of QKI protein and in situ hybridisation analyses of miR-574-5p (figure 7F). Largely consistent with the down-regulation in total *Qki*, *Qki5/6/7/7b* mRNA and protein isoforms were all significantly downregulated in most CRC tissues (online figure S5). Pearson's correlation analyses demonstrated significant inverse correlations between the expression of miR-574-5p and the expression of total *Qki* mRNA (Pearson $r=-0.4082$, $p<0.05$) or QKI protein (Pearson $r=-0.4406$, $p<0.05$) in the 60 pairs of CRC samples tested (figure 7D,E). No apparent association, however, was found between miR-574-5p

Figure 5 Effects of miR-574-5p on cell cycle, proliferation, differentiation, invasion and migration. SW480 or CT26 cells were transfected with a microRNA (miRNA)-overexpressing plasmid or a miRNA inhibitor (see supplemental materials and methods section in online supplementary information). Cells were harvested for analyses 24 h after the transfections ($n=3$). Statistical comparisons were made between a control (pFlag-CMV or inhibitor control)-transfected cells and a particular treatment. Data are represented as mean \pm SEM. * $p<0.05$; ** $p<0.01$; *** or ###, $p<0.001$. (A) Inhibition of miR-574-5p significantly reduced the rate of proliferation in CT26 and SW480 cells. ### represents comparisons among SW480 cells whereas *** represents comparisons among CT26 cells. (B) Inhibition of miR-574-5p caused G1/S arrest in SW480 cells. (C) Overexpression of miR-574-5p significantly decreased messenger RNA (mRNA) of *Lactase*, whereas inhibition of miR-574-5p significantly increased the mRNA level of *Lactase* in CT26 cells. (D) Overexpression of miR-574-5p significantly decreased the activity of intestinal alkaline phosphatase (IAP), whereas inhibition of miR-574-5p significantly increased IAP activity in CT26 cells. (E) Overexpression of miR-574-5p significantly enhanced cell invasion, whereas inhibition of miR-574-5p significantly suppressed invasion in CT26 cells as determined by the Matrigel invasion assays. (F) Overexpression of miR-574-5p significantly enhanced migration in SW480 cells, whereas inhibition of miR-574-5p suppressed cell migration as determined by wound-healing assays. Results are typical for three independent experiments. Similar results were obtained for CT26 cells but not included. Original magnification, $\times 100$.



expression and patient gender, patient age, tumour location, TNM staging or lymph node status (online table S1). As a matter of fact, the highest levels (5.737 ± 2.546 -fold of the normal level) of miR-574-5p expression were detected for TNM stage I cancers that decreased subsequently but maintained at moderate levels (2.4–2.6-fold) for TNM stages II, III and IV cancers. A similar trend was observed for lymph node status.

Remarkably, serum miR-574-5p was also found to be significantly elevated in patients with CRC with respect to the controls (2.11 ± 0.39 for patients with CRC vs 1.00 ± 0.22 for controls, $p<0.05$, figure 7G).

***Qki* isoform subcellular localisation and relative expression levels in CRC cells and tissues**

We performed western blot and immunofluorescence analyses to determine the subcellular localisation of QKI isoforms in the CRC cells. Largely consistent with previous studies in neural cells³⁵ and HeLa cells,^{2, 36} QKI5 was found to be present in the

nucleus and cytoplasm of CT26 and SW620 cells, whereas QKI6/7 proteins were exclusively localised in the cytoplasm (online figure S6A,B). To determine the relative expression levels of *Qki* mRNA isoforms, we performed qPCR analyses for mouse CT26, human HCT116, SW480 and SW620 cells (online figure S6C), CRC tissues and normal adjacent epithelial tissues from 10 patients with CRC (online figure S6D). In cultured CT26 cells, *Qki6* mRNA was 210-fold higher than that of *Qki5* mRNA while *Qki7* was 308 times higher. In three lines of cultured human CRC cells and most of the human tissues (CRC and normal colorectal tissues), *Qki7* mRNA was consistently more than 100-fold higher than that of any other isoforms (including *Qki5/6/7b*), with only a few exceptions. These results highlight that *Qki6* and *Qki7* are the predominant isoforms in mouse CRC cells but in human CRC cells and tissues, *Qki7* is the most abundant splicing variant.

To evaluate the effects of increased miR-574-5p on the expression and subcellular distribution of β -catenin, we

Figure 6 Inhibition of miR-574-5p suppresses tumour growth in the nude mice. For a xenograft tumour model, SW480 cells were pre-infected with LV-miR-shRNA-ctrl or LV-miR-574-5p-shRNA (see supplemental materials and methods section in online supplementary information).

Subsequently, the LV-infected cells were harvested and suspended in phosphate-buffered saline and then inoculated subcutaneously ($\sim 5 \times 10^6$ cells/mouse) into the right side of the posterior flank of 5-week-old nude mice. For bioluminescence imaging of tumour growth in live animals, CT26 cells stably overexpressing luciferase or its controls were injected into the nude mice intraperitoneally (5×10^5 cells/mouse). Starting from the third day after CT26 cell inoculation, each mouse was injected with 2×10^7 transducing units of control lentiviruses or lentiviruses carrying short hairpin RNA (shRNA) for miR-574-5p once a week for 2 weeks. Peritoneal tumour growth was monitored by bioluminescence imaging (described in supplemental materials and methods section in online supplementary information). Data are represented as mean \pm SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (A)

Downregulation of miR-574-5p and upregulation of total *Qki* messenger RNA (mRNA) 96 h after lentiviral infection ($n=3$). (B) Lentivirus-miR-shRNA-mediated inhibition of miR-574-5p significantly decreased colony formation in SW480 cells. SW480 cells were transfected with LV-miR-shRNA-ctrl or LV-miR-574-5p-shRNA.

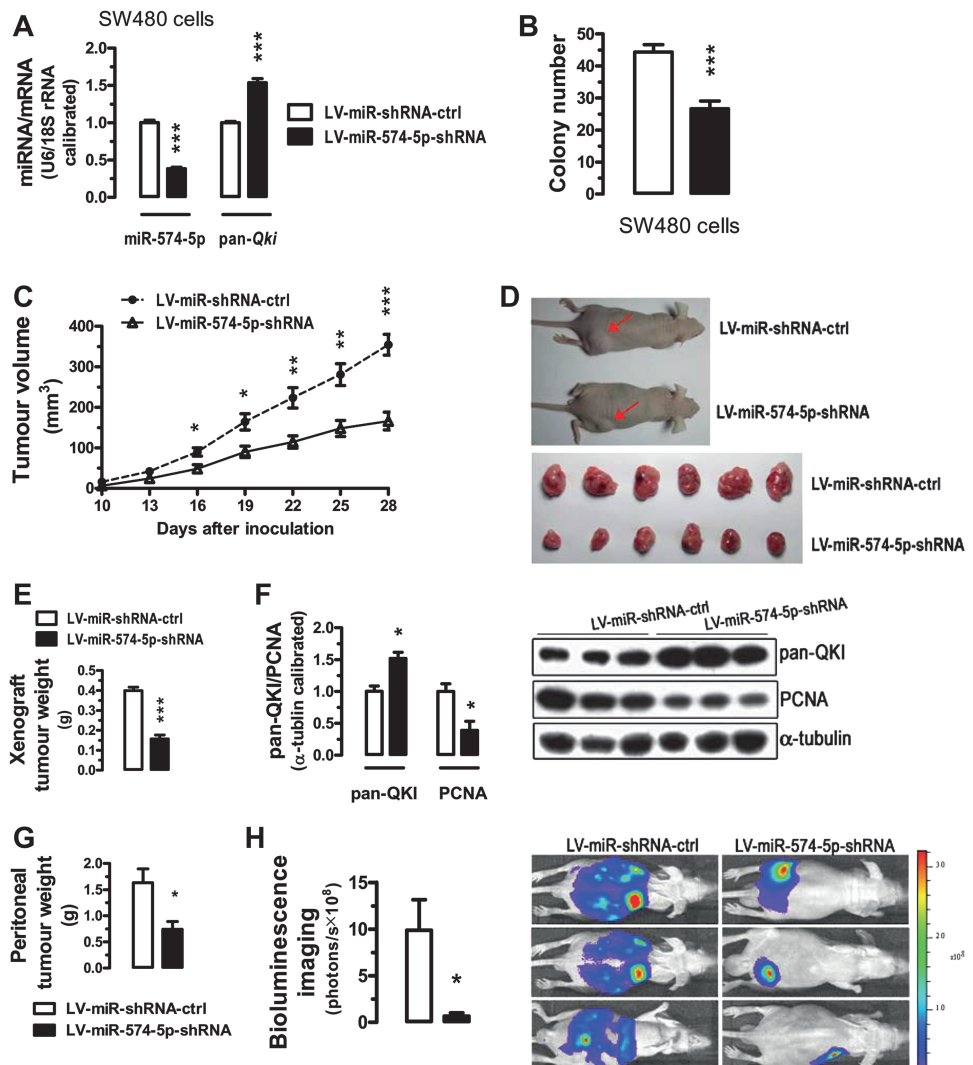
Ninety-six hours after the transfection, 50 transfected cells were seeded on six-well plates and maintained in DMEM containing 10% FBS for 2 weeks before colony counting ($n=3$). (C) Time-dependent growth of xenograft tumour tissues in the nude mice ($n=6$). (D, E) Tumour tissues dissected 28 days after the inoculation and the comparison of the tumour weights ($n=6$). (F) Altered expression of total QKI and proliferating cell nuclear antigen in the dissected tumour tissues ($n=3$). (G) Weights of visible and dissected peritoneal tumours following gene therapy with lentiviruses carrying shRNA for miR-574-5p or its controls for 24 days ($n=3$). (H) Intraperitoneal tumour growth as demonstrated by bioluminescence imaging in the live nude mice inoculated with CT26 cells stably overexpressing a luciferase reporter gene 24 days after treatment with lentiviruses carrying shRNA for miR-574-5p or its control ($n=3$). miRNA, microRNA. DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; PCNA, proliferating cell nuclear antigen.

performed both the western blots and immunofluorescent staining in CT26 cells overexpressing miR-574-5p (online figure S7). Our western blots indicated that following miR-574-5p overexpression, β -catenin protein was significantly increased in the nuclear and membrane fractions while not much increase was found in the cytoplasm. Immunofluorescent staining also confirmed significant increases in β -catenin in the nucleus.

Inhibition of miR-574-5p suppresses tumour growth in the nude mice

To further investigate the role of miR-574-5p in tumour growth, we assessed the effects of inhibition of miR-574-5p on the growth of xenograft tumours in nude mice. Mice were injected with SW480 cells that were pre-infected with control lentiviruses or lentiviruses carrying shRNA for miR-574-5p. Lentivirus-mediated knockdown of miR-574-5p caused significant upregulation in total *Qki* (figure 6A). Probably as a consequence, the ability to form colonies was significantly reduced in miR-574-5p

knockdown cells (figure 6B). In nude mice, tumour growth with miR-574-5p-knockdown cells was much slower than that with the control SW480 cells (figure 6C). When dissected at the end of the study (day 28), the average tumour weight of the miR-574-5p knockdown group was only 39% of that of the control group ($p < 0.001$) (figure 6D,E). In the miR-574-5p knockdown tumour tissues, total QKI protein was greatly increased whereas the proliferating cell nuclear antigen expression was significantly decreased (figure 6F). In a more systemic in vivo miR-574-5p inhibition experiment, CT26 cells stably overexpressing luciferase were injected into the nude mice intraperitoneally. The mice were then treated with control lentiviruses or lentiviruses carrying shRNA for miR-574-5p. As shown in figure 6G,H, miR-574-5p inhibition greatly reduced peritoneal tumour growth in the nude mice as monitored by bioluminescent imaging on the 24th day. Together these results suggest that in vivo inhibition of miR-574-5p suppresses CRC tumour growth and this effect is largely due to the increased expression in QKI proteins.



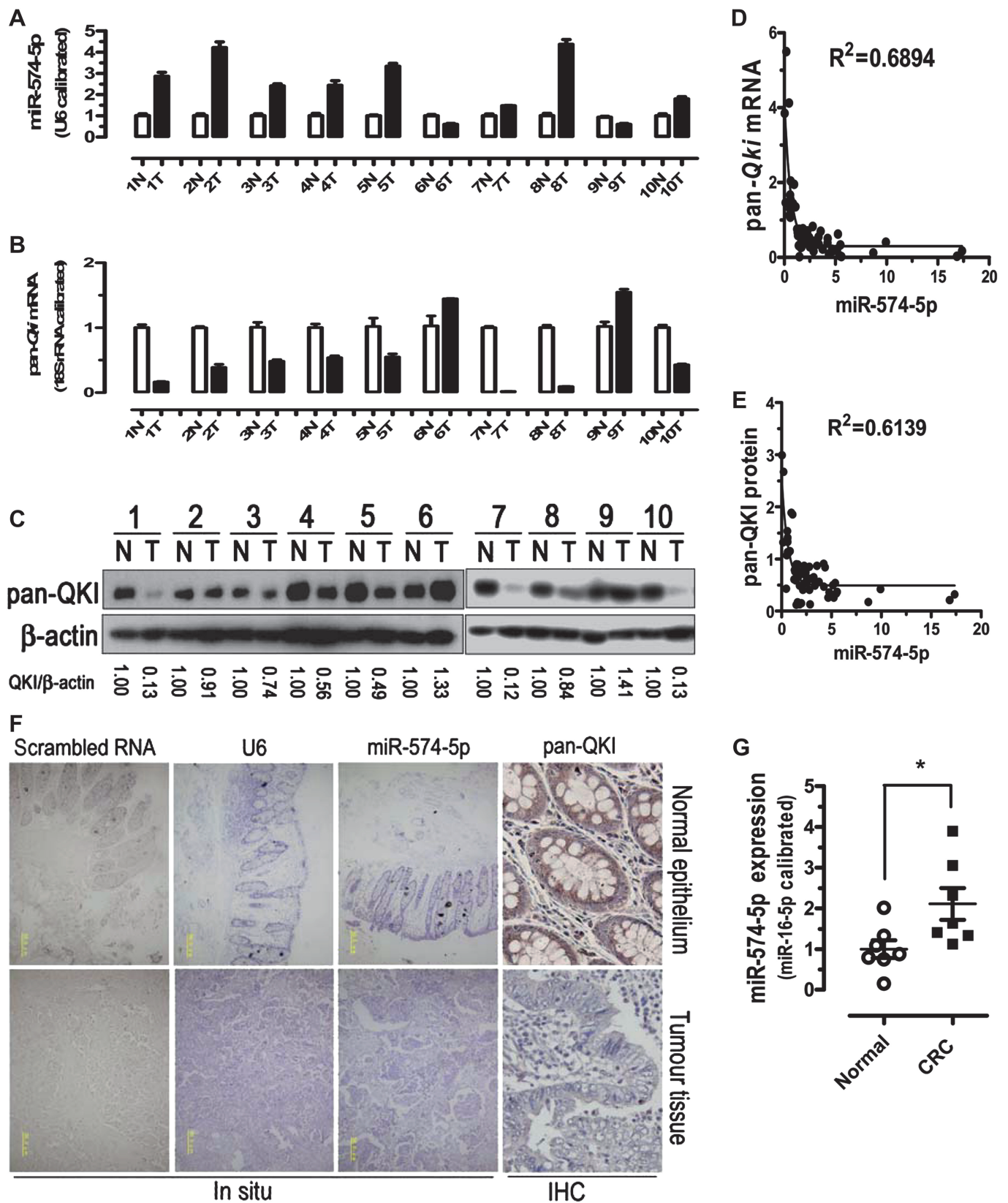


Figure 7 Expression of miR-574-5p and total *Qki* messenger RNA (mRNA)/protein levels in control and colorectal cancer (CRC) samples. Arabic numbers indicate individual sample pairs. (A) miR-574-5p expression was significantly upregulated in human CRC tissues. (B, C) Total *Qki* mRNA and protein were significantly downregulated in human CRC tissues. N, normal adjacent epithelial tissue; T, tumour tissue. (D, E) Inverse correlation between the expression of miR-574-5p and the expression of *Qki* mRNA/protein in 60 pairs of clinical samples. Nonlinear regression (exponential) was indicated. (F) Typical expression patterns of pan-QKI protein in human CRC tissues and normal adjacent epithelial tissues as determined by immunohistochemistry (IHC, original magnification, $\times 1000$) and miR-574-5p expression patterns in human CRC tissues and normal adjacent epithelial tissues as determined by in situ hybridisation (original magnification, $\times 100$; yellow bars represent 50.0 μm). See also online figure S5. (G) miR-574-5p expression was significantly upregulated in serum samples from patients with CRC. Normal, normal controls, $n=7$; CRC, patients with CRC, $n=7$.

DISCUSSION

QKI is emerging as a major regulator for RNA metabolism and cell signalling.^{37–38} Dysregulation of QKIs has been associated with a number of important human diseases such as schizophrenia, ataxia and cancers.^{8–24} However, the regulatory mechanisms underlying the dysregulation of QKIs were elusive. We showed in this current study that miR-574-5p represses the expression of *Qki6/7/7b* mRNAs by interacting with the MREs on their 3'UTRs. As predicted by bioinformatics analyses, however, miR-574-5p did not regulate the expression of *Qki5* through this miR-574-5p-3'UTR interaction. Nevertheless, *Qki5* expression very often was downregulated when miR-574-5p was overexpressed but upregulated when miR-574-5p was inhibited. This suggests that other indirect and unknown mechanisms might be involved in the regulation of *Qki5* that require further investigation. Importantly, we demonstrated in human CRC cells that *Qki7* mRNA expression is usually more than 100-fold higher than any other isoform, with only rare exceptions (figure S6D). This is in contrast to *Qki6* being the most abundant isoforms in the brain³⁹ and probably has important physiological implications. Surprisingly, we did not find increased apoptosis in *Qki7*-overexpressing CT26 cells or human cells with abundant QKI7 protein, contrary to previous reported QKI7-induced apoptosis in fibroblast cells and oligodendrocytes.^{2–40} QKI5 and QKI6 were previously shown to exert a suppressive effect on β -catenin/Wnt signalling by reducing the level of β -catenin protein and affecting its subcellular redistribution, but they did not appear to affect mRNA expression of β -catenin.²³ By contrast, we showed in our current study that QKI5/6/7 proteins are all capable of repressing mRNA and protein expression of β -catenin and suppressing β -catenin/Wnt signalling in CRC cells. The reason for the discrepancy in β -catenin mRNA expression between the previous study and our current study is not clear but might be related to the semi-quantitative real-time PCR used in the previous study²³ compared with the qPCR used in our current study. In light of the relative abundance of QKI7 and its ability to regulate β -catenin/*p27^{Kip1}* expression and their signalling activity, however, our novel results suggest a prominent role for QKI7 in QKI-mediated signal transduction and activation of RNA and in colorectal cell differentiation and the development of CRC which to date had been missed from the research.

In human and mouse, the miR-574 gene is intronic. Initially, miR-574-5p was thought to be hosted by the first intron of the gene encoding *Noxp20³²* on human chromosome 4 or mouse chromosome 5. A recent study, however, also suggested that miR-574-5p might be encoded by the first intron of *Col3a1*. Interestingly, alterations in the expression of miR-574-5p have been found to be associated with a variety of diseases, including many types of cancers (online table S2). Ranade *et al* found that miR-574-5p is one of a few miRNAs that were associated with chemoresistance and decreased survival in patients with small cell lung cancer.⁴¹ In serum samples of patients with early stage non-small cell lung cancer, they also found that miR-1254 and miR-574-5p were significantly elevated in the patients with cancer compared with controls,⁴² which suggests miR-574-5p might be used as a biomarker for non-small cell lung cancer. Very recently, Meyers-Needham *et al* also showed that miR-574-5p mediated the silencing of tumour suppressor ceramide synthase-1 (*CerS1*) in head and neck squamous cell carcinoma by selectively targeting one of its alternatively spliced mRNA variants, *CerS1-2*.⁴³ Moreover, inhibition of miR-574-5p was shown to reconstitute *CerS1-2* expression and C₁₈-ceramide generation in multiple lines of cancer cells, which subsequently led to

suppressed proliferation and anchorage-independent growth of cancer cells. Together these results strongly suggest that miR-574-5p is oncogenic and its inhibition might be beneficial.

We demonstrated in our current investigation that miR-574-5p was significantly elevated in colorectal tissues and serum samples from patients with CRC, especially for cancers at TNM stage I. Consistent with the lack of association between miR-92a and TNM stage in CRC,⁴⁴ the abnormal upregulation of miR-574-5p in the CRC tissues of our current study was not found to be associated with TNM stage or lymph node status. One possible explanation for the lack of association between miR-574-5p and TNM stage or lymph node status is that miR-574-5p probably promotes CRC tumourigenesis or progression primarily at the early stages. Moreover, it is probably not necessary for miR-574-5p to be continuously increased with CRC progression because a moderate twofold to threefold increase appeared to be sufficient to knockdown *Qki* significantly (figure 7D,E). Indeed, QKI protein expression was maintained at low levels for cancers at TNM stages II, III and IV. We further demonstrated that aberrantly upregulated miR-574-5p probably contributes to the development of CRC in part by selectively targeting tumour suppressors *Qki6/7/7b* post transcriptionally, leading to the dys-regulation of β -catenin/Wnt signalling. Whether miR-574-5p also regulates macroH2A1.1 and *CerS1-2* in the CRC cells to contribute to CRC development or not, however, remains to be determined. Despite this, findings from our current study have important implications for the elucidation of pathogenic mechanisms and for clinical practice. Theoretically, the identification of this novel regulatory mechanism of *Qki* isoforms mediated by miR-574-5p sheds lights on the elucidation of pathogenic mechanisms underlying the development of important human diseases associated with the aberrant expression of miR-574-5p, QKIs and more than 1400 putative QKI-regulated genes (online figure S8). From a clinical perspective, our demonstration that serum miR-574-5p is significantly elevated in most patients with CRC suggests that miR-574-5p might serve as a novel diagnostic biomarker for early detection of CRC. As importantly, our demonstration that inhibition of miR-574-5p ameliorated CRC development or progression suggests that miR-574-5p might be an important therapeutic target for the prevention or treatment of CRC and other miR-574-5p-associated cancers.

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Contributors SJ, GY, JZ, LW, TW, ZW, TZ and GW performed the experiments, collected and analysed the data; YL and ZG designed the experiments, analysed the data and revised the paper; JC designed and supervised the project, analysed the data and revised the paper; JYY conceived, designed and supervised the project, analysed the data, wrote and revised the paper.

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SUPPLEMENTARY INFORMATION

1. SUPPLEMENTAL DATA

Fig. S1 miR-574-5p MREs on human *Qki6/7/7b* mRNAs (related to Fig. 1). **A**, Human *Qki* gene structure, major transcripts, protein isoforms and 3'UTRs. Located at 6q26-q27, the human *Qki* gene contains eight exons. Due to alternative splicing, at least 4 major transcripts, namely *Qki5/6/7/7b*, can be produced. Each of these transcript isoforms has its own unique 3'UTR derived from the alternatively-spliced exons. However, a long stretch of overlapping sequences is found in the 3'UTRs for *Qki6/7/7b* respectively. Furthermore, a putative MRE for miR-574-5p was found in the 3'UTRs of *Qki6/7/7b* but not that of *Qki5* mRNA. Four major QKI proteins (QKI5/6/7/7b) can be produced from the four mRNA isoforms, which differ in their carboxyl termini encoded for by the alternatively spliced exons. A common N-terminal 311 amino acid peptide fragment encoded by the first six exons is shared by all four protein isoforms. This region contains a KH domain flanked by the QUA1 and the QUA2 motifs respectively. QUA1 is involved in protein homodimerization or heterodimerization while the KH and QUA2 domains might be responsible for RNA binding. The Y-motif is a tyrosine-rich region that serves as a site for tyrosine phosphorylation. In addition, there is a P-motif for the proline-rich region. The C-terminal of QKI5 (but not of the other three isoforms) contains a nuclear localization signal (NLS) that directs its entry into the nucleus. The figure was redrawn based on previous reports¹⁻³. The putative MREs for miR-574-5p were indicated on the 3'UTRs for *Qki6/7/7b* respectively. **B**, Alignment of putative miR-574-5p seed-binding sites of mouse *Qki6/7-3'UTR* and human *Qki6/7/7b-3'UTR*. Mature human miR-574-5p (hsa-miR-574-5p) and mouse miR-574-5p (mmu-miR-574-5p) share 100% sequence similarity, with the sequence being 5'-ugagugugugugugagugugu-3'. **C**, MREs for miR-17, miR-20a and miR-200b on mouse *Qki5-3'UTR* as predicted by the miRanda algorithm (www.microna.org). Data sets on www.microna.org so far contain more complete 3'UTRs for specific genes and the miRanda algorithm distinguishes the 3'UTRs. **D** & **E**, MREs for miR-17, miR-20a, miR-200b, miR-466g, miR-574-5p and miR-717 on mouse *Qki6/7-3'UTRs* as predicted by the miRanda algorithm. Surprisingly, no MRE for miR-574-3p was predicted in the 3'UTRs for mouse or human *Qki5* or *Qki6/7/7b* mRNA.

Fig. S2 Effects of miR-574-5p on *Qki* expression in human HCT116, SW480 and SW620 cells (related to Fig. 2). Human CRC cells were transfected with a miR-574 overexpressing plasmid, a miR-574-5p inhibitor. Cells were harvested for qPCR or western blot assays 24 hours after the transfections ($n = 3-4$). Statistical comparisons were made between a control (pFlag-CMV or inhibitor control)-transfected cells and a particular treatment. NS, not significant; **, $p < 0.01$; ***, $p < 0.001$. **A**, Inhibition of miR-574-5p significantly increased the levels of *Qki5/6/7/7b* mRNA isoforms in SW480 and SW620 cells, but only *Qki6/7* were increased in HCT116 cells. **B**, Overexpression of miR-574-5p significantly reduced the level of total QKI protein in SW480 cells. **C**, Inhibition of miR-574-5p significantly increased the levels of total QKI and QKI5/6/7 proteins in SW480 cells.

Fig. S3 Putative QREs on β -catenin mRNAs from a few mammals (related to Fig. 3). hsa, *Homo sapiens*; pab, *Pongo abelii*; mmu, *Mus, musculus*; bta, *Bos taurus*; ssc, *Sus scrofa*. Two putative QREs were indicated by QRE1 and QRE2 respectively. The distal QRE1 for β -catenin mRNAs from orangutans, mice, cows and pigs completely satisfies the bipartite consensus sequence of NACUAAAY-N₁₋₂₀-UAAY (Galarneau and Richard, 2005; Hafner et al., 2010). With human β -catenin mRNAs, however, the number of the intervening nucleotides in the QRE1 between the core site NACUAAAY and the half site UAAY is 22 rather than smaller than or equal to 20. Similarly, the proximal QRE2 might also be functional⁴. However, this putative QRE is also slightly different from the consensus sequence, with the half-site being “CAAY” rather than “TAAY” and 22 intervening nucleotides between the putative core site and the putative half site.

Fig. S4 Co-overexpression of *Qki5/6/7* significantly attenuated the oncogenic effects of miR-574-5p. CT26 cells were grown and transfected with pmiR-574 or pFlag-CMV in the presence or absence of pFlag-*Qki5/6/7* for 24 hours. Cells were subsequently harvested for mRNA or MTT analyses. For invasion analysis, similar transfection was performed with CT26 cells for 12 hours. Subsequently, 5×10^4 transfected cells were seeded per upper chambers in serum-free RPMI 1640 medium whereas the lower chambers were loaded with RPMI 1640 medium containing 5% FBS. After 48 hours of incubation, cells were harvested for analysis. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.001$. **A**, Re-introduction of *Qki5/6/7* significantly decreased β -catenin and increased *p27^{kip1}* mRNA expression in miR-574-5p-overexpressing cells ($n = 3-4$). **B** and **C**, Re-introduction of *Qki5/6/7* significantly reduced cell viability and cell invasion in miR-574-5p-overexpressing cells ($n = 3-4$).

Fig. S5 Expression levels of *Qki5/6/7/7b* mRNA and protein isoforms in the human CRC tissues and the adjacent normal epithelial tissues. T, tumor tissue; N, normal adjacent epithelial tissue. **A**, *Qki5* expression from ten pairs of clinical samples (#11-20) as determined by qPCR. **B**, *Qki6* expression from ten pairs of clinical samples (#11-20) as determined by qPCR. **C**, *Qki7* expression from ten pairs of clinical samples (#11-20) as determined by qPCR. **D**, *Qki7b* expression from ten pairs of clinical samples (#11-20) as determined by qPCR. **E**, QKI5/6/7/7b protein isoform expression in five pairs of clinical samples (#16-20) as determined by western blotting. $n = 5$, ***, $p < 0.001$.

Fig. S6 Subcellular localization of QKI5/6/7 proteins in human SW620 or mouse CT26 cells and the relative expression of *Qki5/6/7/7b* mRNAs in CRC cells, clinical CRC tissue samples and normal adjacent epithelial tissue samples. **A**, Subcellular localization of QKI5/6/7 proteins in SW620 or CT26 cells as determined by western blotting. **B**, Subcellular localization of QKI5/6/7 proteins in CT26 cells as determined by immunofluorescence assays. Original magnification, $\times 1,000$. **C**, Relative expression of *Qki* mRNA isoforms in CT26, HCT116, SW480 and SW620 cells. Total RNA samples were isolated and qPCR was performed ($n = 3-4$). All values were normalized with *Qki5* expression. **D**, Relative expression of *Qki* mRNA isoforms in clinical CRC and normal adjacent epithelial samples from 10 patients. Both the values for tumor tissues (T, black-colored) and corresponding adjacent noncancerous epithelial tissues from a particular patient (N, grey-colored) were normalized with the expression of *Qki5* mRNA in normal adjacent epithelial tissue from the same patient.

Fig. S7 Effects of miR-574-5p overexpression on β -catenin subcellular localization as determined by western blots and immunofluorescent staining. For western blots, CT26 cells were transfected with a miR-574-5p overexpressing plasmid (pmiR-574) or its control (pFlag-CMV) for 24 hours and harvested for western blot analyses. For immunofluorescent staining, CT26 cells were seed on the coverslips and transfected with pmiR-574 or pFlag-CMV for 24 hours. Cells were then fixed for immunofluorescence analyses. **A**, Subcellular distribution of β -catenin protein as determined by western blots; **B**, Subcellular distribution of β -catenin protein as determined by immunofluorescent staining. Original magnification, $\times 1000$.

Fig. S8 The miR-574-5p-QKI- β -catenin/p27^{Kip1} axis of signal transduction in the development of CRC. miR-574-5p derived from the first intron of either *Col3a1* or *Noxp20* regulates the expression of *Qki6/7/7b*

directly and negatively. *Qki5* may also be regulated by miR-574-5p indirectly. Down-regulation of QKIs will cause the overactivation of β -catenin and the suppression of p27^{Kip1}. As a result, activities in colorectal epithelial cell proliferation, migration and invasion will be enhanced, whereas cell cycle arrest and differentiation will be suppressed. Additionally, miR-574-5p might also repress the expression of tumor suppressor ceramide synthase-1 isoform 2 (*CerS1-2*) posttranscriptionally.⁵ QKIs, on the other hand, might also control the expression of macroH1A1.1 to impact tumorigenesis.⁶ miR-574-5p thus appears to be oncogenic and may contribute to the dysregulation of colorectal epithelial cell differentiation and tumorigenesis or cancer progression through the suppression of tumor-suppressive QKIs. miR-574-5p expression might be co-regulated with its hosting-genes (either *Noxp20* or *Col3a1*), although this needs to be verified with further study. Additionally, Sox2 is a transcriptional factor implicated in the maintenance of the pluripotency of stem cells⁷⁻⁹, neurogenesis¹⁰⁻¹³ and development of cancers.¹⁴⁻¹⁷ In glioblastoma multiforme cells, the knockdown of *Sox2* caused significant down-regulation of miR-574-5p.¹⁸ Coincidentally or not, aberrant Sox2 up-regulation and miR-574-5p up-regulation are often co-detected in diseases such as CRC (¹⁵ and current study), glioblastoma,¹⁸ lung cancer,¹⁹⁻²¹ esophageal squamous carcinoma^{16,22} etc. Together these observations suggest that Sox2 might regulate miR-574-5p positively. The possible regulation of *Noxp20*, *Col3a1* and miR-574-5p and *Qkis* by *Apc*, however, is not clear and warrant further research. *Apc*, adenomatous polyposis coli; *Col3a1*, procollagen Type III, alpha-1; *Noxp20*, nervous system overexpressed protein-20; *Sox2*, SRY-like HMG box-2.

Fig. S9 Schematic graphs for twelve plasmids used in the current study. See the Materials and Methods section for more details. hGH-pA, poly-A for human growth hormone gene; Luc, luciferase; SV40-pA, SV40 poly-A.

Table S1 The expression of miR-574-5p and pan-*Qki* mRNA and protein and the clinicopathological features of 60 CRC patients.

Table S2 Alterations of miR-574-5p expression in diseases.

Fig. S1

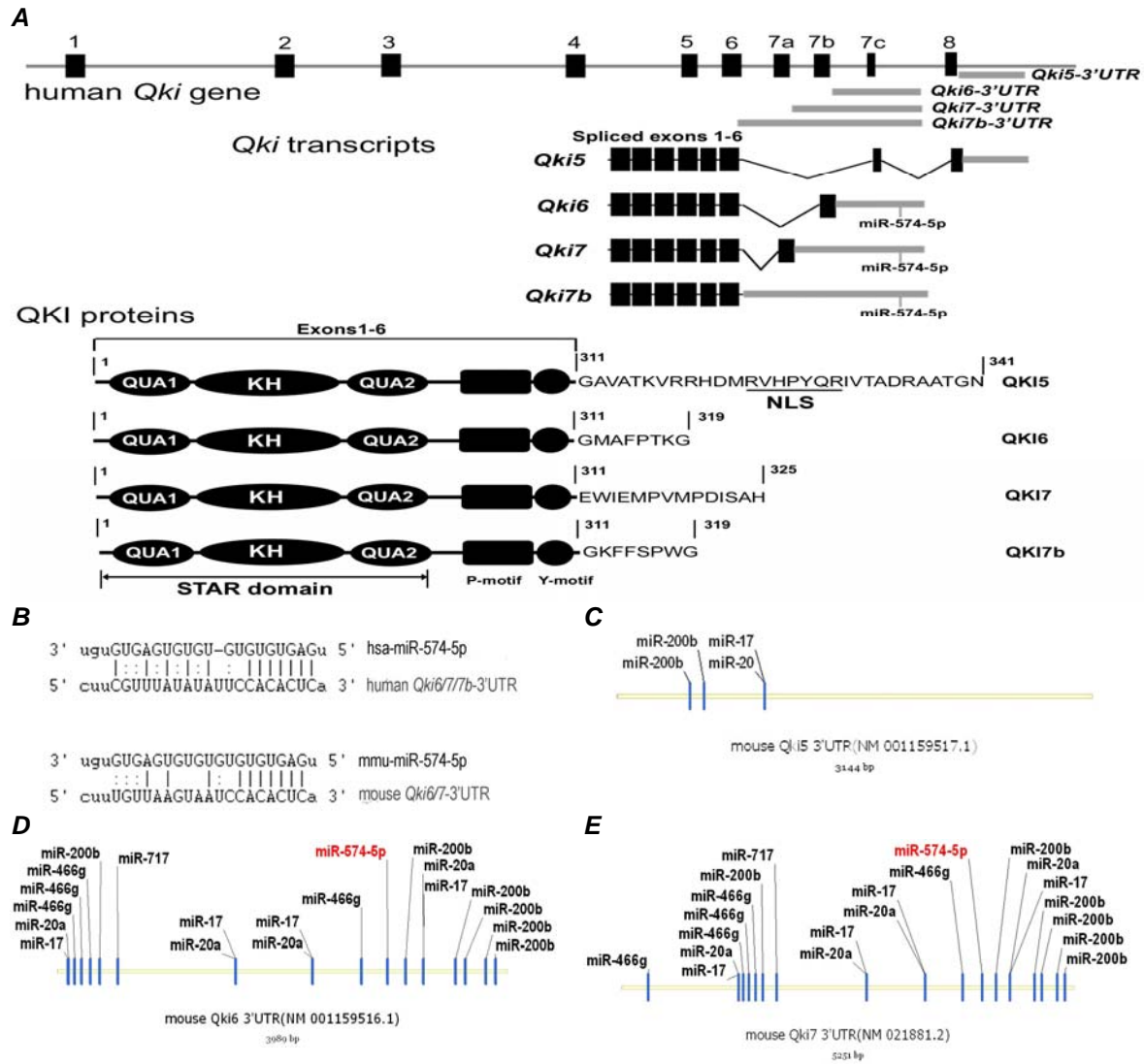
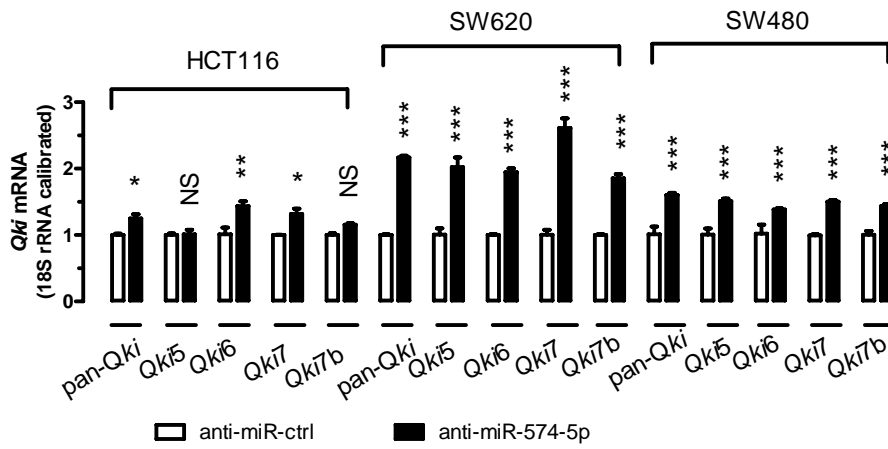
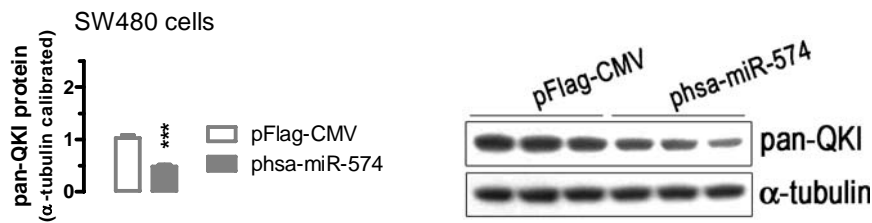


Fig. S2

A



B



C

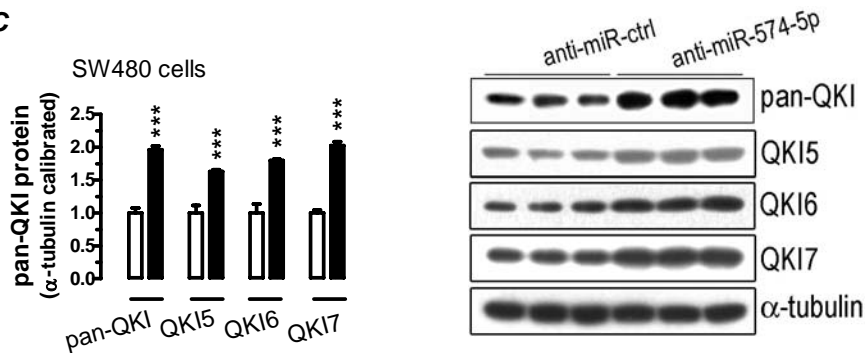


Fig. S3

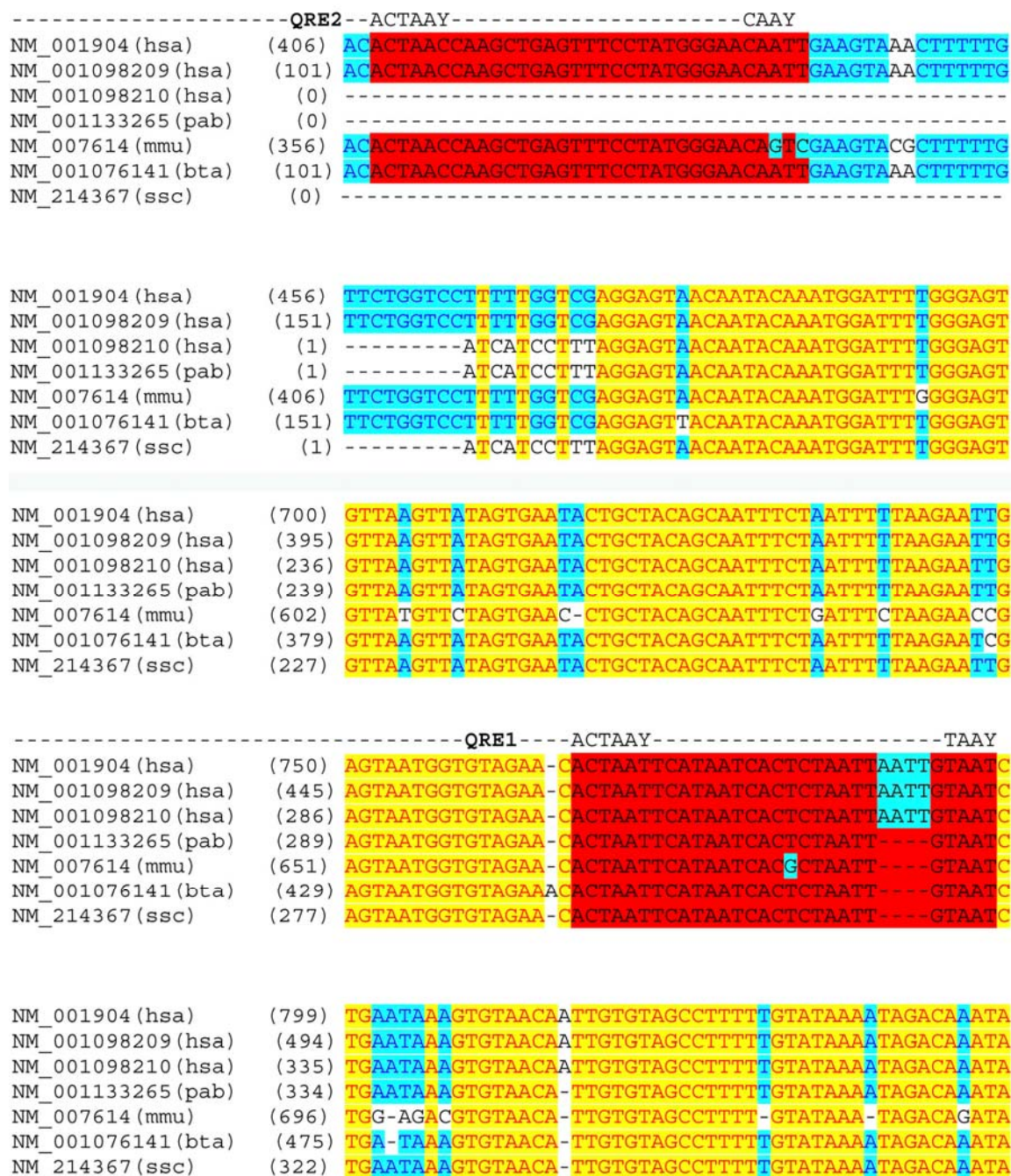
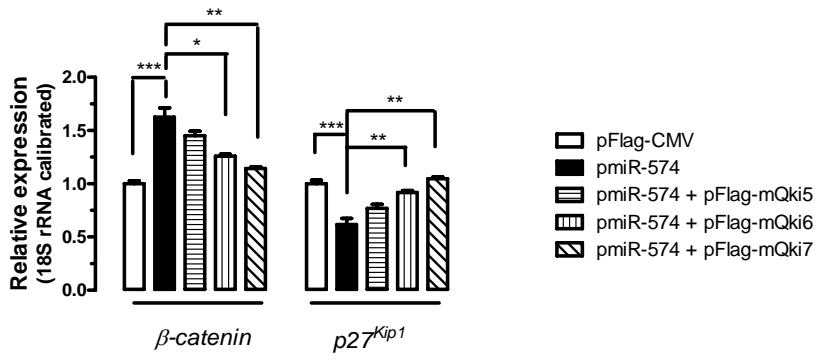
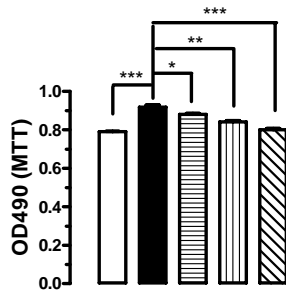


Fig. S4

A



B



C

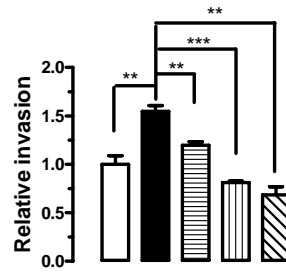
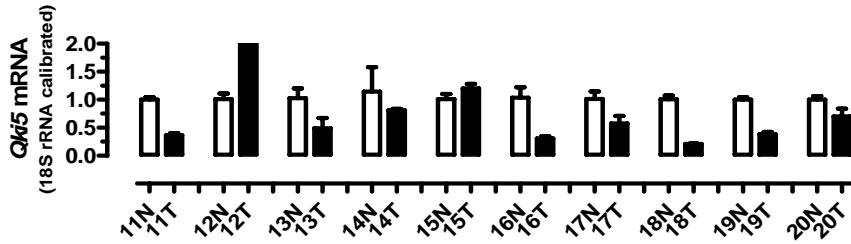
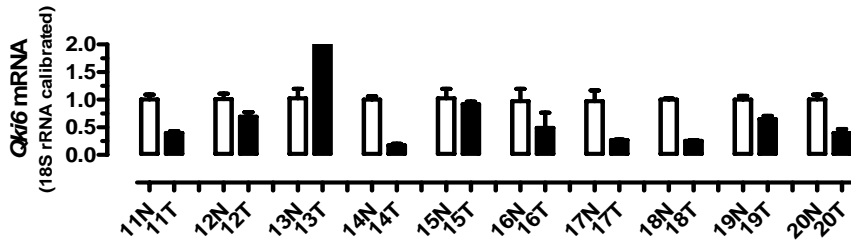


Fig. S5

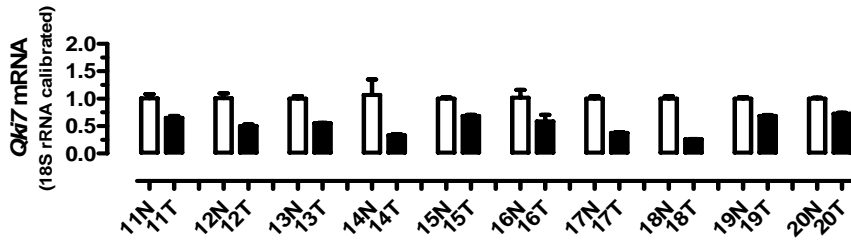
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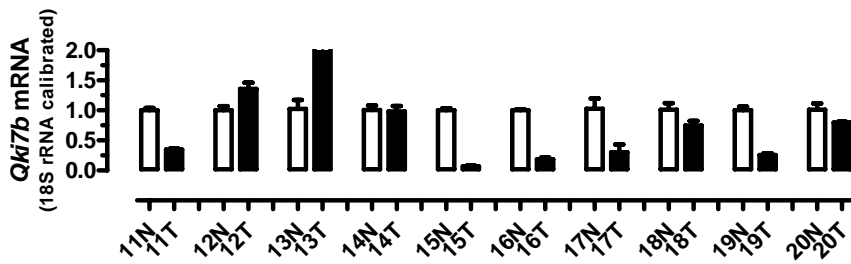
B



C



D



E

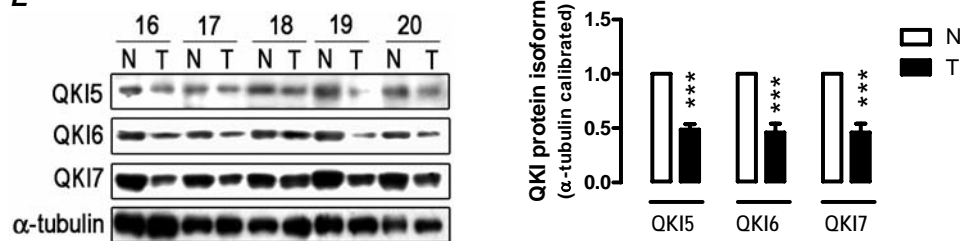


Fig. S6

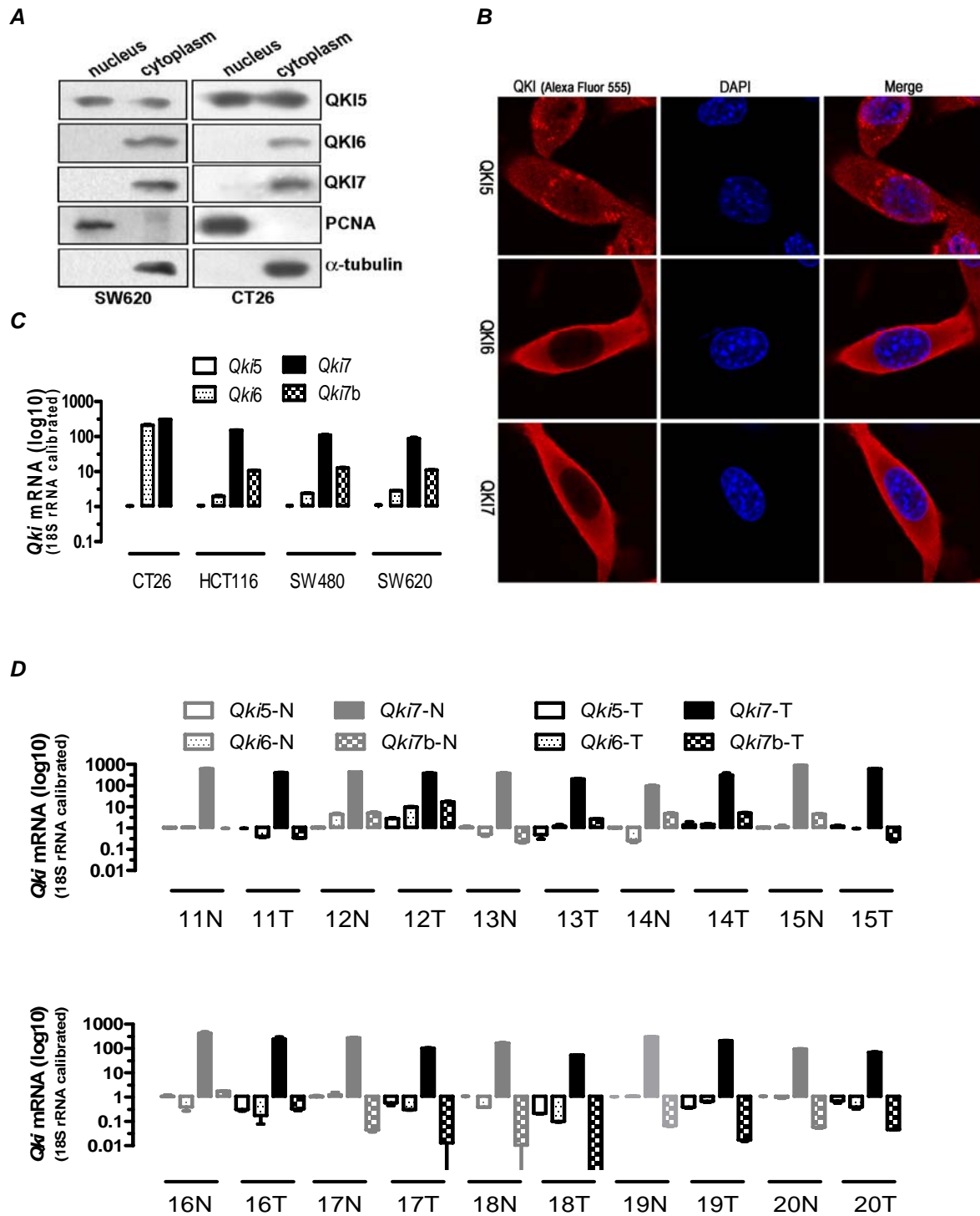


Fig. S7

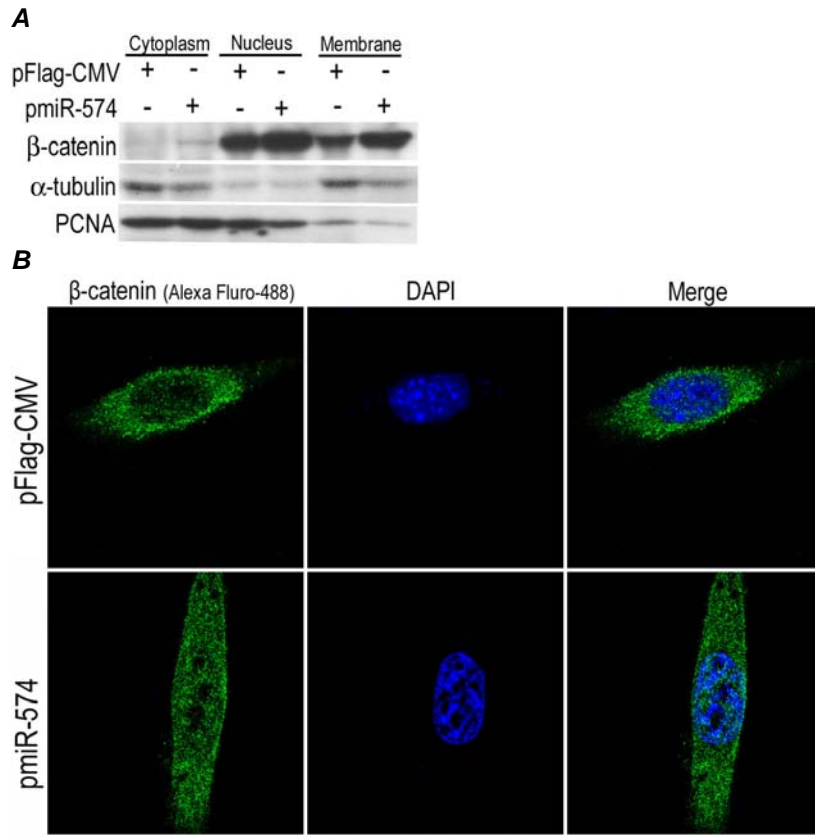


Fig. S8

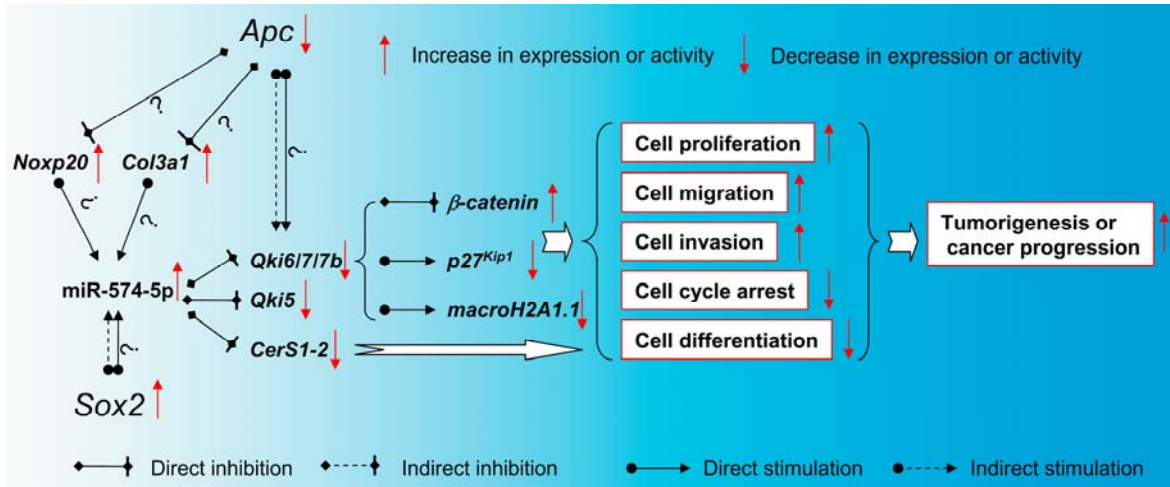


Fig. S9

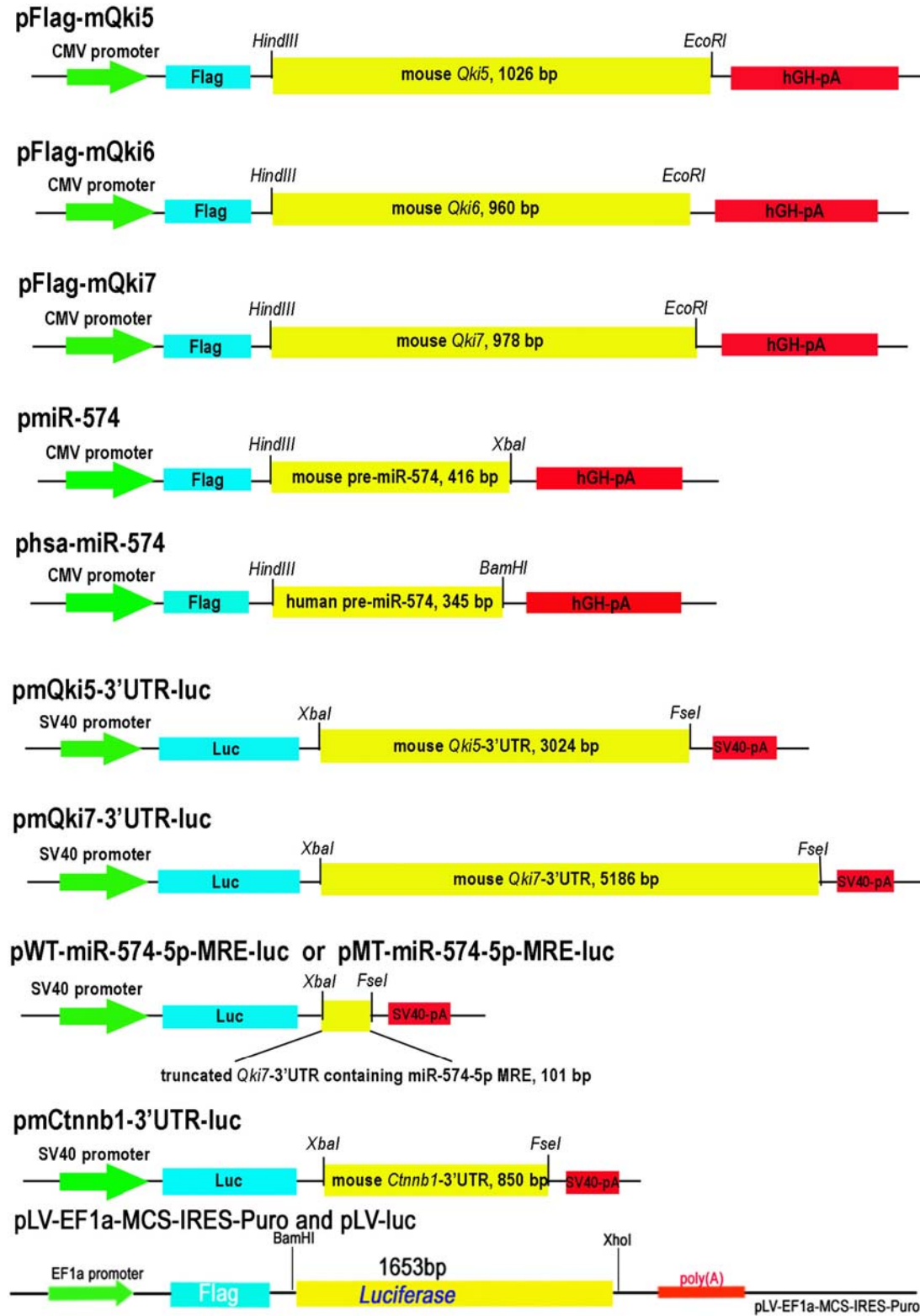


Table S1

		<i>n</i>	%	miR-574-5p (Normalized)	Pan- <i>Qki</i> mRNA (Normalized)	Pan-QKI protein (Normalized)
Gender	M	40	67.7	3.136 ± 0.5718	0.8483 ± 0.1774	0.7554 ± 0.0977
	F	20	33.3	2.793 ± 0.532	0.7278 ± 0.1335	0.7882 ± 0.0929
Age	≤60	28	46.7	3.238 ± 0.8269	0.9206 ± 0.2304	0.7509 ± 0.094
	>60	32	53.3	2.843 ± 0.3409	0.7134 ± 0.1315	0.7789 ± 0.1097
TNM stage	I	8	13.3	5.737 ± 2.546	0.9503 ± 0.4494	0.8613 ± 0.3316
	II	15	25	2.666 ± 0.5652	1.115 ± 0.412	0.8046 ± 0.1773
	III	36	60	2.593 ± 0.3291	0.6608 ± 0.082	0.7292 ± 0.0674
	IV	1	1.7	2.415	0.4825	0.74
Lymph node status	N0	23	38.4	3.734 ± 0.9734	1.058 ± 0.3051	0.8243 ± 0.1589
	N1	17	28.3	2.549 ± 0.5684	0.7131 ± 0.1462	0.7564 ± 0.0935
	N2	20	33.3	2.621 ± 0.3574	0.6075 ± 0.0828	0.7066 ± 0.0935
	N3	0				

Table S2

Organism	Condition	miR-574-5p expression	Affected cells or tissues	Fold change	References
Human	CRC	Up	Colorectal cells	Very significant	Current study
Human	Pituitary adenomas	Up	Pituitary tissue	2.89	²³
Human	Systemic lupus erythematosus	Up	T lymphocyte	> 2	²⁴
Human	Pancreatic cancer	Up	Pancreatic tissue	51	²⁵
Human	Lymphoma	Altered	Lymphocyte	?	²⁶
Human	Myocardial infarction	Up	Heart	Very significant	²⁷
Human	Non-small cell lung cancer	Up	Lung	2.17	²⁰
Human	Small cell lung cancer	Altered	Lung	?	²¹
Human	Eosophageal squamous cell carcinoma	Up	Eosophageal squamous cells	1.72	²²
Human	Alzheimer's	Up	Gray matter	?	²⁸
Human	Schizophrenia	Altered	Brain tissue	?	²⁹
Human	Mitral stenosis	Up	Right atrial appendage	1.96	³⁰
Human	Ovarian cancer	Up	Ovary tissue	1.24	³¹
Mouse	CRC	Up	Colorectal cells	3.7	Current study
Mouse	Vesicular stomatitis virus infection	Up	Macrophage	?	³²
Mouse	Asthma	Up	Lung cells	13	³³
Mouse	SARS infection	Altered	Bronchoalveolar stem cells	?	³⁴
Mouse	Liver injury	Up	Liver/plasma	3.4/1.49	³⁵

2. SUPPLEMENTAL MATERIALS AND METHODS

Plasmids. TOP/FOP-Flash plasmids and the Wnt1-overexpressing (Wnt1) plasmid were kind gifts from Prof. Qiao Wu, Xiamen University.

For *Qki* overexpression plasmids, primers were designed based on the genomic sequences from the NCBI databases, with the forward primer carrying a *HindIII* site and the reverse primer carrying an *EcoRI* respectively. Mouse *Qki5*, *Qki6*, *Qki7* were individually PCR-amplified using mouse brain tissue cDNA and appropriate primers (see List of primers used for miRNA or *Qki* overexpression plasmids), with the LA Taq DNA Polymerase (TaKaRa, Dalian). The resultant DNA fragments were subcloned into pFlag-CMV (Sigma Aldrich, St. Louis, MO, USA) using the *HindIII* and *EcoRI* sites.

For mouse miRNA overexpressing plasmids, primers were designed based on the genomic sequences from the miRBase (microna.sanger.ac.uk), with the forward primers carrying a *HindIII* site and the reverse primers carrying a *XbaI* site respectively. Pre-miRNA gene fragments were individually PCR-amplified using genomic DNA prepared from mouse inner medullary collecting duct epithelial mIMCD3 cells (mmu-miR-574) or human CRC SW480 cells (hsa-miR-574) and appropriate primers (see List of primers used for miRNA or *Qki* overexpression plasmids), with Pyrobest DNA Polymerase (TaKaRa). The resultant DNA fragments were subcloned into pFlag-CMV, using the *HindIII* and *XbaI* (mmu-miR-574) or *HindIII* and *BamHI* (hsa-miR-574) sites. The insertion sequences of the resultant plasmids were confirmed by sequencing.

For the construction of mouse *Qki5*-3'UTR, wildtype *Qki7*-3'UTR miR-574-5p MRE, mutant *Qki7*-3'UTR miR-574-5p MRE and *Ctnnb1* luciferase reporters, mouse 3'UTR regions were amplified from mouse brain tissue cDNA through PCR amplification with the LA Taq DNA Polymerase (TaKaRa) and appropriate primers (see List of primers used for the construction of five luciferase reporters). The resultant PCR fragments carrying a *NheI* site and a *FseI* site was subcloned into the pGL3-control vector (Promega), using the *XbaI* and *FseI* sites immediately downstream of the stop codon of the luciferase cDNA, generating pmQki5-3'UTR-luc, pmQki7-3'UTR-luc, pWT-miR574-5p-MRE-luc, pMT-miR574-5p-MRE-luc and pmCtnnb1-3'UTR-luc respectively.

For the construction of lentiviral plasmid carrying a luciferase gene, the luciferase reporter gene on plasmid pGL3 (Promega, USA) was PCR amplified and a *BamHI-XhoI* insert gene cassette was inserted

into a lentiviral vector pLV-EF1a-MCS-IRES-Puro (A gift from Prof. Jiahuai Han) to obtain a plasmid pLV-luc.

The resulting twelve plasmids are shown in Fig. S9 in the previous section.

Cell culture and transient transfections. Mouse CT26, human SW480 and SW620 CRC cells were obtained from ATCC (Manassas, VA, USA), human HCT116 CRC cells was obtained from the Chinese Academy of Sciences (Shanghai, China). CT26 cells and HCT116 cells were normally cultured in RPMI 1640 with 10% fetal bovine serum (FBS). SW480 and SW620 cells were cultured in DMEM supplemented with 10% FBS. Plasmids, miRNA mimics and inhibitor transfections were performed with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols.

Western blot, immunohistochemistry, immunofluorescence and in situ hybridization analyses.

Western blots were performed according to standard protocols. The detection was achieved using the Immobilon Western Chemiluminescent HRP Substrate kit (Millipore, Billerica, MA, USA). Antibodies and the dilutions are anti- α -tubulin (sc-5286, Santa Cruz, 1:10000); anti- β -actin (sc-47778, Santa Cruz, 1:20000); anti-pan-QKI (sc-103851, Santa Cruz, 1:2000); anti-QKI5 (AB9904, Millipore, 1:2000); anti-QKI6 (AB9906, , Millipore, 1:2000); anti-QKI7 (AB9908, Millipore, 1:2000); anti-p27^{Kip1} (sc-528, Santa Cruz, 1:2000); anti- β -catenin (sc-7199, Santa Cruz, 1:2000); anti-PCNA (sc-7907, Santa Cruz, 1:2000); Alexa Fluor 555-labeled goat Anti-Rabbit IgG (H+L) (A0452, Beyotime Institute of Biotechnology, 1:500) respectively.

Immunohistochemistry was performed with an instant-type SABC immunohistochemistry kit purchased from the Boster Bioengineering Company (Wuhan, China) as instructed. Briefly, 5 μ m fixed tissue sections were microwave-heated for 10 min for antigen retrieval. Slides were washed and incubated with primary antibody for 1 hour followed by incubation with instant-type secondary antibody for 30 minutes at room temperature. After being thoroughly washed with PBS (pH 7.5), the tissues were incubated with SABC (Strept-Avidin-Biotin Complex) for 20 minutes at room temperature. After washing thoroughly, 100-200 μ l freshly-made diaminobenzidine (DAB) solution was added to each slide. After incubation for ~10 minutes, sections were lightly counter-stained with hematoxylin.

For immunofluorescence analyses, cells seeded on cover glass overnight were fixed in 4% paraformaldehyde. Fixed cells were incubated with anti-QKI5, QKI6 or QKI7 primary antibody

respectively, followed by incubation with Alexa Fluor® 555-conjugated secondary antibody or Alexa Fluor® 488-conjugated secondary antibody (Beyotime Institute of Biotechnology, Jiangsu, China). Cell nuclei were stained by DAPI (Beyotime Institute of Biotechnology). Stained cells were visualized and photographed with a Leica-TCS-SP2-SE confocal microscope.

In situ hybridization of miRNAs was performed as described³⁶. miRCURY™ LNA miRNA probes (hsa-miR-574-5p, 5'-ACACACTCA-CACACACACTCA-3'; hsa-miR-200b, 5'-TCATCATTACCAGGCAGTATTA-3'; U6, 5'-CACGAATTTGCGTGTCATCCTT-3'; Scramble-miR, 5'-GTGTAACACGTCTATACGCCCA-3') were purchased from Exiqon (Copenhagen, Denmark).

qPCR analyses of mRNAs and miRNAs. For qPCR mRNA quantification, reverse transcription was performed with TRIzol (Invitrogen)-extracted total RNAs using a ReverTra Ace-α-® Kit as instructed (TOYOBO, Shanghai). qPCR was performed by using the SYBR Green qPCR Master Mix (TOYOBO) and the StepOne Plus qPCR system (Applied Biosystems Inc., Foster City, CA) according to the manufacturer's protocols and with the primer pairs listed below.

For miRNAs, qPCR was performed with the stem-loop primers as described previously³⁷ using the miRNA-specific reverse transcription primers, the universal primer and the miRNA-specific reverse locked nucleic acid (LNA)-primers as described below.

Cell cycle, proliferation, migration, invasion and colony formation assays. For cell cycle analysis, 5×10^5 cells were synchronized by serum starvation for 24 hours and induced to re-enter the cell cycle by replacing old media with a fresh media containing 10% fetal bovine serum (FBS) for 24 hours. Cells were harvested and fixed in 75% ethanol at 4 °C overnight. Cells were incubated with RNase A at 37 °C for 30 min, and then stained with propidium iodide. The cell cycle phases were measured by flow cytometry.

Cell proliferation was analyzed by the MTT assay. A total of 4×10^3 cells was seeded in 96-well plates and MTT was added to each well every 24 hours. The plates were incubated for 4 hours before the addition of 10% SDS (in 0.01M HCl) (Sigma Aldrich, St. Louis, MO, USA). The absorbance was measured at 490 nm using a microplate reader.

Cell migration was analyzed by the wound-healing assays. SW480 cells were seeded onto 6-well plates. After transfection, a wound was incised in the center of the confluent culture, followed by careful washing to remove detached cells and the addition of fresh medium. Phase contrast images of the wounded

area were recorded using an inverted microscope at indicated time points.

Matrigel invasion assays were performed using Millicell inserts coated with matrigel (BD Biosciences, Sparks, MD, USA). 5×10^4 CT26 cells were seeded per upper chambers in serum-free RPMI1640 whereas the lower chambers were loaded with RPMI1640 containing 5% FBS. After 48 hours, the non-migrating cells on the upper chambers were removed by a cotton swab, and cells invaded through the matrigel layer to the underside of the membrane were stained with a 0.1% crystal violet solution and counted manually in eight random microscopic fields.

For colony formation assays, control and miRNA inhibitor or LV-miR-shRNA (Supplemental Materials and Methods section, Supplementary Information) transfected SW480 cells were seeded on six-well plates and maintained in DMEM containing 10% FBS for 2 weeks. Cells were fixed with methanol and stained with 0.5% crystal violet in 50% methanol for 1 hour and colonies larger than 100 μm in diameter were counted.

Luciferase reporter assays and IAP enzyme activity assays. Luciferase reporter activities were determined using a Luciferase Reporter Gene Assay System (Promega, Madison, WI, USA) as instructed. For all luciferase assays, β -galactosidase activities were determined to calibrate the transfection efficiency. The calibrated value for a proper control was used to normalize all other values to obtain the normalized relative luciferase units (RLU) representing the activities of 3'UTRs.

The enzyme activity of IAP was measured by an IAP kit (Jiancheng, Nanjing, China) according to the manufacturer's instructions. Protein concentration was measured by the Pierce BCA kit (Pierce Biotechnology, Rockford, USA).

Bioluminescence imaging of tumor growth in live animals. Briefly, CT26 cells were transfected with a luciferase-overexpressing plasmid or its control vector (pLV-luc or pLV-EF1a-MCS-IRES-Puro). Stable clones of cells overexpressing luciferase were selected with the addition of 8-10 $\mu\text{g}/\text{ml}$ puromycin in the media for 3 weeks (Invitrogen, Carlsbad, CA, USA). Luciferase-overexpressing cells or its controls were subsequently injected into the nude mice intraperitoneally (5×10^5 cells/mouse). Starting from the third day after CT26 cell inoculation, each mouse was injected with 2×10^7 transducing units of control lentiviruses or lentiviruses carrying shRNA for miR-574-5p once a week for 2 weeks. Three days after lentiviral injection, the mice were subjected to bioluminescence imaging under an IVIS Lunina II *in vivo* imaging system

(Xenogen, Hopkinton, MA, USA) as described by Lan et al.³⁸ every other three days. Ten minutes prior to imaging, each mouse was injected with 100 μ l D-luciferin solution (15 mg/ml in PBS, Promega #E1602) by i.p. injection. Immediately before imaging taking, the experimental mice were anesthetized using an XGI-8 Gas Anesthesia System (Xenogen). Images and amount of bioluminescent signals were analyzed using Living Image software (Xenogen).

Serum miRNA preparations and analyses. Total RNA from serum was extracted using a miRVana miRNA isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. RNA was eluted in nuclease free water at 95°C. Five microliters of total RNA was reverse transcribed using the ReverTra Ace- α -[®] Kit as instructed (TOYOBO, Shanghai) and miRNA-specific stem-loop primers as shown in the Materials and Methods section. has-miR-16-5p served as an internal control. Visible and dissectable peritoneal tumors were dissected and weighted.

Bioinformatics, data acquisition, image processing and statistical analyses. Mature and pre-miRNA sequences were based on miRBase (microrna.sanger.ac.uk). miRNA target predictions were performed with the miRanda (www.microrna.org) algorithm. Western blot images were captured by Biosense SC8108 Gel Documentation System with GeneScope V1.73 software (Shanghai BioTech, Shanghai, China). Gel images were imported into Photoshop for orientation and cropping. The digital density values were acquired by Image-Pro Plus software (Media Cybernetics) and analyzed by Graphpad Prism 5.0. Data are the means \pm SEM. One-way ANOVA with Bonferonni's post-test was used for multiple comparisons and the Student's *t*-test (two-tailed) for pair-wise comparisons. The correlation analyses were performed with Pearson's test.

List of primers used for the construction of miRNA or Qki overexpression plasmids

Plasmid	Primer sequence (from 5'→3')	Gene ID	Amplicon (bp)
pFlag-mQki5 (mouse)	Forward:CCCAAGCTTATGGTCGGGGAAAT GGAAAC Reverse:CCGGAATTCCTAGTTGCCGGTGGC GGCTC	NM_001159517 (Genbank)	1026
pFlag-mQki6 (mouse)	Forward:CCCAAGCTTATGGTCGGGGAAAT GGAAAC Reverse:CCGGAATTCCTAGCCTTTCGTTGG GAAAG	NM_001159516 (Genbank)	960
pFlag-mQki7 (mouse)	Forward:CCCAAGCTTATGGTCGGGGAAAT GGAAAC Reverse:CCGGAATTCCTCAATGGGCTGAAAT ATCAG	NM_021881 (Genbank)	978
pmiR-574 (mouse)	Forward: CCCAAGCTTTGTCCGCTGTAGGGTGTGAG AA Reverse: TGCTCTAGAATCAGGATGGAGGTCAAGGC CT	MI0005518 (miRBase)	416
phsa-miR-574 (human)	Forward: CCCAAGCTTCCTCTGCGTTAGTGAGAAGC AG Reverse: GCGGATCCTCTGTCTTACAGGGACCTGC TC	MI0003581 (miRBase)	345

List of primers used for the construction of six luciferase reporters.

Plasmid	Primer sequence (from 5'→3')	Gene ID	Amplicon length and location
pmQki5-3'UTR-luc	Forward: TGCGCTAGCTATGACCTTCTGACCTCTGAACTCT Reverse: ACTGGCCGGCCTATGGGTTAATAGAAACAGCAAAGA	<i>mQki5</i> NM_001159517	(NTs 1517-4540) 3024 bp
pmQki7-3'UTR-luc	Forward: TGCGCTAGCCTTGCTGGATGAAGGACTAGA Reverse: ACTGGCCGGCCTTGGCCTCATGATACAAAGCAATAC	<i>mQki7</i> NM_021881	(NTs 1467-6652) 5186 bp
pWT-miR574-5p-MRE-luc	Forward: TGCTCTAGACTTTGTTAAGTAATCCCACTC Reverse: ACTGGCCGGCCAACGGTTGTCCCATAGTCTTAA	<i>mQki7</i> NM_021881	(NTs 5650-5750) 101bp
pMT-miR574-5p-MRE-luc	Forward: TGCTCTAGACTTTGTTAAGTAATCCGCACGC Reverse: ACTGGCCGGCCAACGGTTGTCCCATAGTCTTAA	<i>mQki7</i> NM_021881	(NTs 5650-5750) 101 bp
pmCnnb1-3'UTR-luc	Forward: TGATCTAGAAAGACTTGGTAGGGTGGGAATGG Reverse: ACTGGCCGGCCGCAGGTTACAACAACCTTTGGGAT	Mouse β -catenin NM_001904	(NTs 2707-3557) 850 bp
pLV-luc	Forward: AGAGAATTCGGATCCATGGAAGACGCCAAAAACATAA Reverse: CCATGGCTCGAGCCCTTACACGGCGATCTTTCCG	pGL3 (Promega)	1365 bp

List of miR-mimics, anti-miRs, lentiviral miR-shRNAs and LNA-probes for miRNA in situ hybridization.

Name	Sequence or target (5'→3')	Catalog #	Supplier
mimics-ctrl	Scrambled		GenePharma, Shanghai
miR-574-5p mimics	UGAGUGUGUGUGUGUGAGUGUGU ACACUCACACACACACUCAUU		GenePharma, Shanghai
anti-miR-ctrl	Scrambled		GenePharma, Shanghai
anti-miR-574-5p	UGAGUGUGUGUGUGUGAGUGUGU	AM17000	Ambion
LV-miR-shRNA- ctrl	TTCTCCGAACGTGTCACGT	pLVT4	Sunbio, Shanghai
LV-miR-574-5p- shRNA	ACACACTCACACACACACTCA	pLVT278	Sunbio, Shanghai
Scrambled miRNA	GTGTAACACGTCTATACGCCCA/3Dig	99004-05	Exiqon, Copenhagen
U6 probe	CACGAATTTGCGTGTCATCCTT/3Dig	99002-05	Exiqon, Copenhagen
hsa-miR-574-5p probe	ACACACTCACACACACACTCA//3Dig	38674-05	Exiqon, Copenhagen

List of primers used for qPCR analyses of mRNAs.

Organism	Gene	Gene ID	Primer sequence (5'→3')	Amplicon (bp)
Human	pan- <i>Qki</i>	NM_006775		
		NM_206853	Forward:CATCAGCTGCATCTTCTTCAG	121
		NM_206854	Reverse:CACTGTGGAAGATGCTCAGAA	
		NM_206855		
	<i>Qki5</i>	NM_006775	Forward:GCCCTACCATAATGCCTTTGA Reverse:AACTTTAGTAGCCACCGCAACC	
	<i>Qki6</i>	NM_206853	Forward:GCCCCAAGCTGGTTTAATCTATA Reverse:TCGTTGGGAAAGCCATACCTAAT	118
	<i>Qki7</i>	NM_206854	Forward:GCTGGTTTAATCTATACACCCTATG A	113
			Reverse:GACTGGCATTCAATCCACTCTA	
	<i>Qki7b</i>	NM_206855	Forward:AATGCCTTTGATCAGACAAATACA G Reverse:TGGGGAGAAGAAGCTTACCTAATAC A	198
	<i>β-catenin</i>	NM_001904	Forward:AGCCACAAGATTACAAGAAACGG Reverse:ATCCACCAGAGTGAAAAGAACGA	173
<i>p27^{Kip1}</i>	NM_004064	Forward:GGGGCTCCGGCTAACTCTGA Reverse:AGGCTTCTTGGGCGTCTGCT	215	
18S rRNA	NR_003286.2	Forward:CGACGACCCATTCGAACGTCT Reverse:CTCTCCGGAATCGAACCTGA	102	
Mouse	pan- <i>Qki</i>	NM_001159517	Forward:TAGAGGACTTACAGCTAAACAACCT	288
		NM_001159516	Reverse:ATTCAGAATTGCAAGCTCCATCA	
		NM_021881		
	<i>Qki5</i>	NM_001159517	Forward:GCCCTACCATAATGCCTTTGA Reverse:AACTTTAGTAGCCACCGCAACC	211
	<i>Qki6</i>	NM_001159516	Forward:GCCTGAAGCTGGGTTAATCTACA Reverse:TCGTTGGGAAAGCCATACCTAAC	118
	<i>Qki7</i>	NM_021881	Forward:GCTGGGTTAATCTACACACCCTAT GA	113
Reverse:GACTGGCATTCAATCCACTCTA				
<i>β-catenin</i>	NM_007614	Forward:TGGACCCCAAGCCTTAGTAAACA Reverse:GTCTGTGATGAAGCCCCAGTG	159	

<i>Lactase</i>	NM_001081078	Forward:GAGACCCAGAACTCAATGACACC Reverse:GGTCAGAGCGGTTACAAAAGT	165
<i>p27^{Kip1}</i>	NM_009875	Forward:GCGGTGCCTTTAATTGGGTCT Reverse:TCTTGGGCGTCTGCTCCACA	225
<i>Col3a1</i>	NM_009930	Forward:GTTTCTTCTCACCTTCTTCATCCC Reverse:GCAGTCTAGTGGCTCCTCATCACA G	196
<i>Noxp20</i>	NM_026667	Forward:AGGGAGACACCGGATCTGAAATA Reverse:GAATTGGCAGTGTGGATTCGTAG	199
<i>Sox2</i>	NM_011443	Forward: GCGGAGTGGAACTTTTGTCC Reverse: GGGAAGCGTGTACTTATCCTTCT	156

List of conventional or LNA-primers for qPCR analyses of U6 and miRNAs.

RNA or miRNA	Genbank or miRBase seq#	Primer sequence (5'→3')
Mouse and human U6	NR_004394.1	Reverse transcription: CGCTTCACGAATTTGCGTGTCAT Forward: GCTTCGGCAGCACATATACTAAAAT Reverse: CGCTTCACGAATTTGCGTGTCAT (LNA)
hsa-miR-16-5p	MI0000070	Reverse transcription: GTCGTATCCAGTGC GTGTCGTGGAGTCGGCAATTGCA CTGGATACGACTCGCCAA Forward: GGGTAGCAGCACGTAAA Reverse: TCGTGTCGTGGAGTC (LNA)
mmu-miR-574-5p	MI0005518	Reverse transcription: GTCGTATCCAGTGC GTGTCGTGGAGTCGGCAATTGC ACTGGATACGACTACACAC Forward: GGGTGAGTGTGTGTGTG Reverse: TCGTGTCGTGGAGTC (LNA)
hsa-miR-574-5p	MI0003581	Reverse transcription: GTCGTATCCAGTGC GTGTCGTGGAGTCGGCAATTGC ACTGGATACGACTACACAC Forward: GGGTGAGTGTGTGTGTG Reverse: TCGTGTCGTGGAGTC (LNA)
mmu-miR-200b	MI0000243	See Huang et al. ³⁷
mmu-miR-717	MI0004704	See Huang et al. ³⁷
mmu-miR-466g	MI0005510	Reverse transcription: GTCGTATCCAGTGC GTGTCGTGGAGTCGGCAATTGC ACTGGATACGACTGTGTGT Forward: GGGATACAGACACATGC Reverse: TCGTGTCGTGGAGTC (LNA)
mmu-miR-17	MI0000687	Reverse transcription: GTCGTATCCAGTGC GTGTCGTGGAGTCGGCAATTGC ACTGGATACGACTCTACCT Forward: GGGCAAAGTGCTTACAG Reverse: TCGTGTCGTGGAGTC (LNA)
mmu-miR-20a	MI0000568	Reverse transcription: GTCGTATCCAGTGC GTGTCGTGGAGTCGGCAATTGC ACTGGATACGACTCTACCT

Forward: GGGGTAAAGTGCTTATAG

Reverse: TGC GTGTCGTGGAGTC (LNA)

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SUPPLEMENTARY INFORMATION

1. SUPPLEMENTAL DATA

Fig. S1 miR-574-5p MREs on human *Qki6/7/7b* mRNAs (related to Fig. 1). **A**, Human *Qki* gene structure, major transcripts, protein isoforms and 3'UTRs. Located at 6q26-q27, the human *Qki* gene contains eight exons. Due to alternative splicing, at least 4 major transcripts, namely *Qki5/6/7/7b*, can be produced. Each of these transcript isoforms has its own unique 3'UTR derived from the alternatively-spliced exons. However, a long stretch of overlapping sequences is found in the 3'UTRs for *Qki6/7/7b* respectively. Furthermore, a putative MRE for miR-574-5p was found in the 3'UTRs of *Qki6/7/7b* but not that of *Qki5* mRNA. Four major QKI proteins (QKI5/6/7/7b) can be produced from the four mRNA isoforms, which differ in their carboxyl termini encoded for by the alternatively spliced exons. A common N-terminal 311 amino acid peptide fragment encoded by the first six exons is shared by all four protein isoforms. This region contains a KH domain flanked by the QUA1 and the QUA2 motifs respectively. QUA1 is involved in protein homodimerization or heterodimerization while the KH and QUA2 domains might be responsible for RNA binding. The Y-motif is a tyrosine-rich region that serves as a site for tyrosine phosphorylation. In addition, there is a P-motif for the proline-rich region. The C-terminal of QKI5 (but not of the other three isoforms) contains a nuclear localization signal (NLS) that directs its entry into the nucleus. The figure was redrawn based on previous reports¹⁻³. The putative MREs for miR-574-5p were indicated on the 3'UTRs for *Qki6/7/7b* respectively. **B**, Alignment of putative miR-574-5p seed-binding sites of mouse *Qki6/7-3'UTR* and human *Qki6/7/7b-3'UTR*. Mature human miR-574-5p (hsa-miR-574-5p) and mouse miR-574-5p (mmu-miR-574-5p) share 100% sequence similarity, with the sequence being 5'-ugagugugugugugagugugu-3'. **C**, MREs for miR-17, miR-20a and miR-200b on mouse *Qki5-3'UTR* as predicted by the miRanda algorithm (www.microrna.org). Data sets on www.microrna.org so far contain more complete 3'UTRs for specific genes and the miRanda algorithm distinguishes the 3'UTRs. **D** & **E**, MREs for miR-17, miR-20a, miR-200b, miR-466g, miR-574-5p and miR-717 on mouse *Qki6/7-3'UTRs* as predicted by the miRanda algorithm. Surprisingly, no MRE for miR-574-3p was predicted in the 3'UTRs for mouse or human *Qki5* or *Qki6/7/7b* mRNA.

Fig. S2 Effects of miR-574-5p on *Qki* expression in human HCT116, SW480 and SW620 cells (related to Fig. 2). Human CRC cells were transfected with a miR-574 overexpressing plasmid, a miR-574-5p inhibitor. Cells were harvested for qPCR or western blot assays 24 hours after the transfections ($n = 3-4$). Statistical comparisons were made between a control (pFlag-CMV or inhibitor control)-transfected cells and a particular treatment. NS, not significant; **, $p < 0.01$; ***, $p < 0.001$. **A**, Inhibition of miR-574-5p significantly increased the levels of *Qki5/6/7/7b* mRNA isoforms in SW480 and SW620 cells, but only *Qki6/7* were increased in HCT116 cells. **B**, Overexpression of miR-574-5p significantly reduced the level of total QKI protein in SW480 cells. **C**, Inhibition of miR-574-5p significantly increased the levels of total QKI and QKI5/6/7 proteins in SW480 cells.

Fig. S3 Putative QREs on β -catenin mRNAs from a few mammals (related to Fig. 3). hsa, *Homo sapiens*; pab, *Pongo abelii*; mmu, *Mus, musculus*; bta, *Bos taurus*; ssc, *Sus scrofa*. Two putative QREs were indicated by QRE1 and QRE2 respectively. The distal QRE1 for β -catenin mRNAs from orangutans, mice, cows and pigs completely satisfies the bipartite consensus sequence of NACUAAY-N₁₋₂₀-UAAY (Galarneau and Richard, 2005; Hafner et al., 2010). With human β -catenin mRNAs, however, the number of the intervening nucleotides in the QRE1 between the core site NACUAAY and the half site UAAY is 22 rather than smaller than or equal to 20. Similarly, the proximal QRE2 might also be functional⁴. However, this putative QRE is also slightly different from the consensus sequence, with the half-site being “CAAY” rather than “TAAY” and 22 intervening nucleotides between the putative core site and the putative half site.

Fig. S4 Co-overexpression of *Qki5/6/7* significantly attenuated the oncogenic effects of miR-574-5p. CT26 cells were grown and transfected with pmiR-574 or pFlag-CMV in the presence or absence of pFlag-Qki5/6/7 for 24 hours. Cells were subsequently harvested for mRNA or MTT analyses. For invasion analysis, similar transfection was performed with CT26 cells for 12 hours. Subsequently, 5×10^4 transfected cells were seeded per upper chambers in serum-free RPMI 1640 medium whereas the lower chambers were loaded with RPMI 1640 medium containing 5% FBS. After 48 hours of incubation, cells were harvested for analysis. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.001$. **A**, Re-introduction of *Qki5/6/7* significantly decreased β -catenin and increased $p27^{kip1}$ mRNA expression in miR-574-5p-overexpressing cells ($n = 3-4$). **B and C**, Re-introduction of *Qki5/6/7* significantly reduced cell viability and cell invasion in miR-574-5p-overexpressing cells ($n = 3-4$).

Fig. S5 Expression levels of *Qki5/6/7/7b* mRNA and protein isoforms in the human CRC tissues and the adjacent normal epithelial tissues. T, tumor tissue; N, normal adjacent epithelial tissue. **A**, *Qki5* expression from ten pairs of clinical samples (#11-20) as determined by qPCR. **B**, *Qki6* expression from ten pairs of clinical samples (#11-20) as determined by qPCR. **C**, *Qki7* expression from ten pairs of clinical samples (#11-20) as determined by qPCR. **D**, *Qki7b* expression from ten pairs of clinical samples (#11-20) as determined by qPCR. **E**, QKI5/6/7/7b protein isoform expression in five pairs of clinical samples (#16-20) as determined by western blotting. $n = 5$, ***, $p < 0.001$.

Fig. S6 Subcellular localization of QKI5/6/7 proteins in human SW620 or mouse CT26 cells and the relative expression of *Qki5/6/7/7b* mRNAs in CRC cells, clinical CRC tissue samples and normal adjacent epithelial tissue samples. **A**, Subcellular localization of QKI5/6/7 proteins in SW620 or CT26 cells as determined by western blotting. **B**, Subcellular localization of QKI5/6/7 proteins in CT26 cells as determined by immunofluorescence assays. Original magnification, $\times 1,000$. **C**, Relative expression of *Qki* mRNA isoforms in CT26, HCT116, SW480 and SW620 cells. Total RNA samples were isolated and qPCR was performed ($n = 3-4$). All values were normalized with *Qki5* expression. **D**, Relative expression of *Qki* mRNA isoforms in clinical CRC and normal adjacent epithelial samples from 10 patients. Both the values for tumor tissues (T, black-colored) and corresponding adjacent noncancerous epithelial tissues from a particular patient (N, grey-colored) were normalized with the expression of *Qki5* mRNA in normal adjacent epithelial tissue from the same patient.

Fig. S7 Effects of miR-574-5p overexpression on β -catenin subcellular localization as determined by western blots and immunofluorescent staining. For western blots, CT26 cells were transfected with a miR-574-5p overexpressing plasmid (pmiR-574) or its control (pFlag-CMV) for 24 hours and harvested for western blot analyses. For immunofluorescent staining, CT26 cells were seed on the coverslips and transfected with pmiR-574 or pFlag-CMV for 24 hours. Cells were then fixed for immunofluorescence analyses. **A**, Subcellular distribution of β -catenin protein as determined by western blots; **B**, Subcellular distribution of β -catenin protein as determined by immunofluorescent staining. Original magnification, $\times 1000$.

Fig. S8 The miR-574-5p-QKI- β -catenin/p27^{Kip1} axis of signal transduction in the development of CRC. miR-574-5p derived from the first intron of either *Col3a1* or *Noxp20* regulates the expression of *Qki6/7/7b*

directly and negatively. *Qki5* may also be regulated by miR-574-5p indirectly. Down-regulation of QKIs will cause the overactivation of β -catenin and the suppression of p27^{Kip1}. As a result, activities in colorectal epithelial cell proliferation, migration and invasion will be enhanced, whereas cell cycle arrest and differentiation will be suppressed. Additionally, miR-574-5p might also repress the expression of tumor suppressor ceramide synthase-1 isoform 2 (*CerS1-2*) posttranscriptionally.⁵ QKIs, on the other hand, might also control the expression of macroH1A1.1 to impact tumorigenesis.⁶ miR-574-5p thus appears to be oncogenic and may contribute to the dysregulation of colorectal epithelial cell differentiation and tumorigenesis or cancer progression through the suppression of tumor-suppressive QKIs. miR-574-5p expression might be co-regulated with its hosting-genes (either *Noxp20* or *Col3a1*), although this needs to be verified with further study. Additionally, Sox2 is a transcriptional factor implicated in the maintenance of the pluripotency of stem cells⁷⁻⁹, neurogenesis¹⁰⁻¹³ and development of cancers.¹⁴⁻¹⁷ In glioblastoma multiforme cells, the knockdown of *Sox2* caused significant down-regulation of miR-574-5p.¹⁸ Coincidentally or not, aberrant Sox2 up-regulation and miR-574-5p up-regulation are often co-detected in diseases such as CRC (¹⁵ and current study), glioblastoma,¹⁸ lung cancer,¹⁹⁻²¹ esophageal squamous carcinoma ^{16,22} etc. Together these observations suggest that Sox2 might regulate miR-574-5p positively. The possible regulation of *Noxp20*, *Col3a1* and miR-574-5p and *Qkis* by *Apc*, however, is not clear and warrant further research. *Apc*, adenomatous polyposis coli; *Col3a1*, procollagen Type III, alpha-1; *Noxp20*, nervous system overexpressed protein-20; *Sox2*, SRY-like HMG box-2.

Fig. S9 Schematic graphs for twelve plasmids used in the current study. See the Materials and Methods section for more details. hGH-pA, poly-A for human growth hormone gene; Luc, luciferase; SV40-pA, SV40 poly-A.

Table S1 The expression of miR-574-5p and pan-*Qki* mRNA and protein and the clinicopathological features of 60 CRC patients.

Table S2 Alterations of miR-574-5p expression in diseases.

Fig. S1

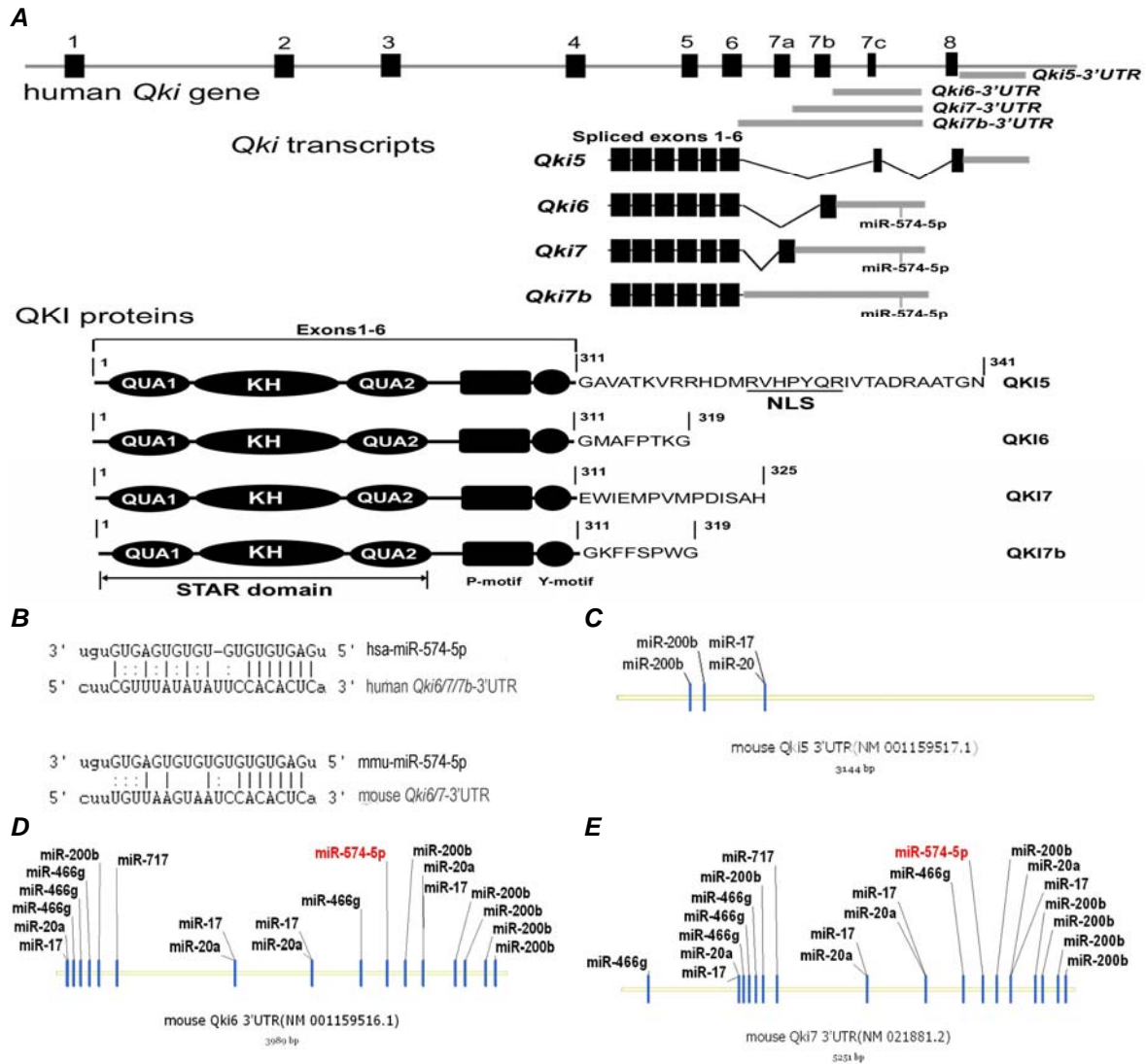
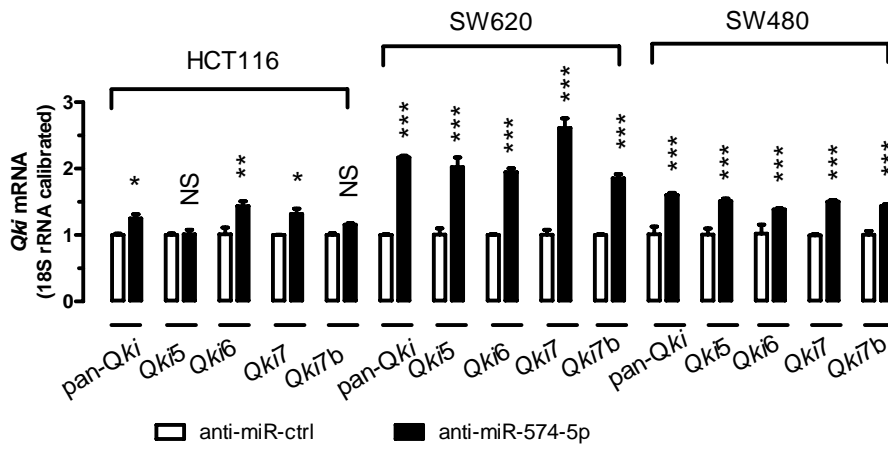
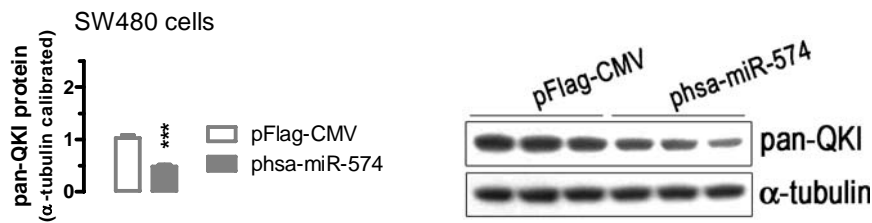


Fig. S2

A



B



C

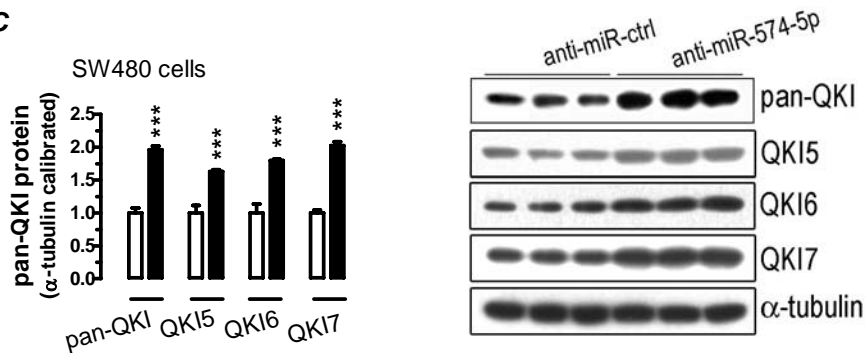


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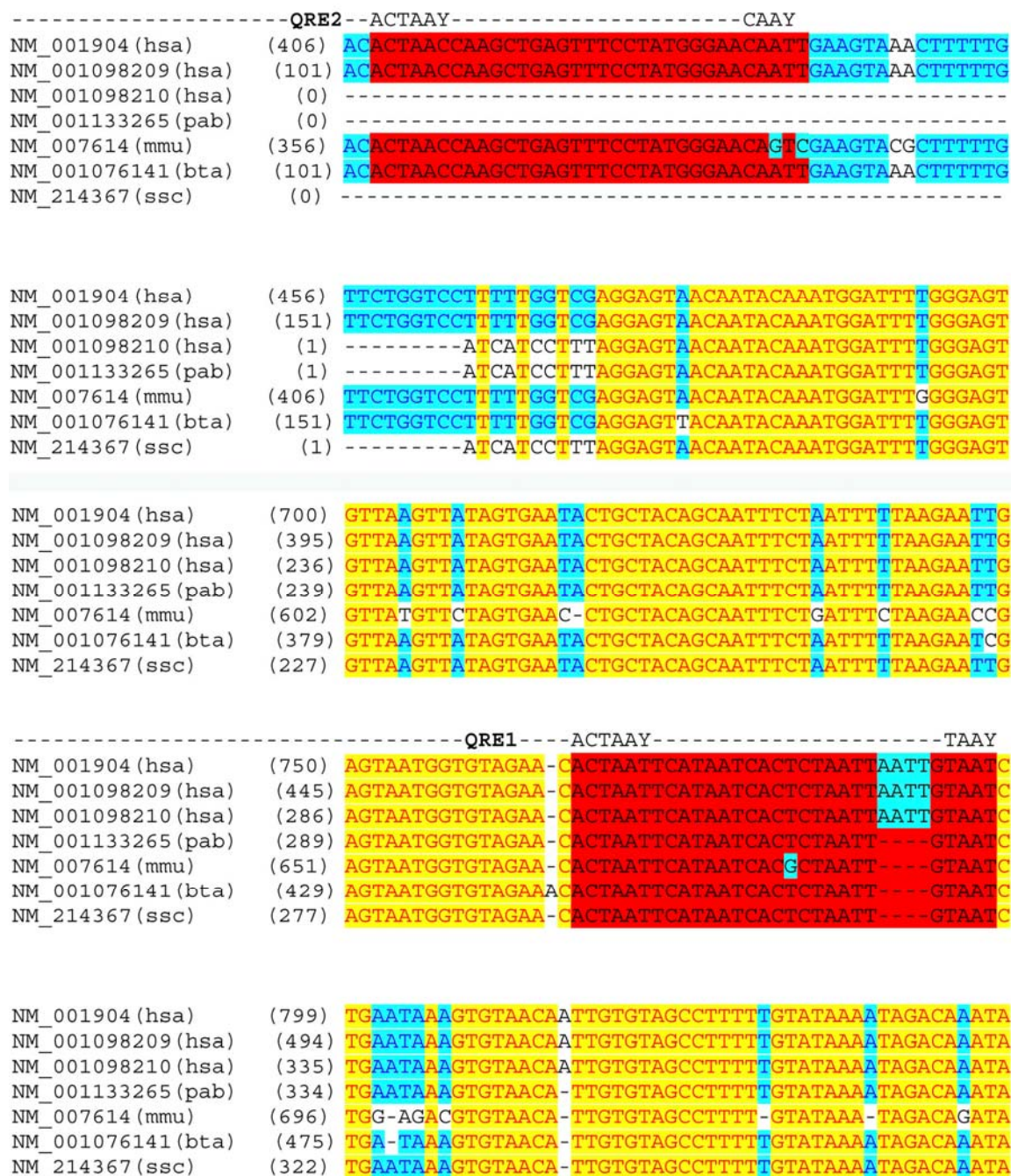
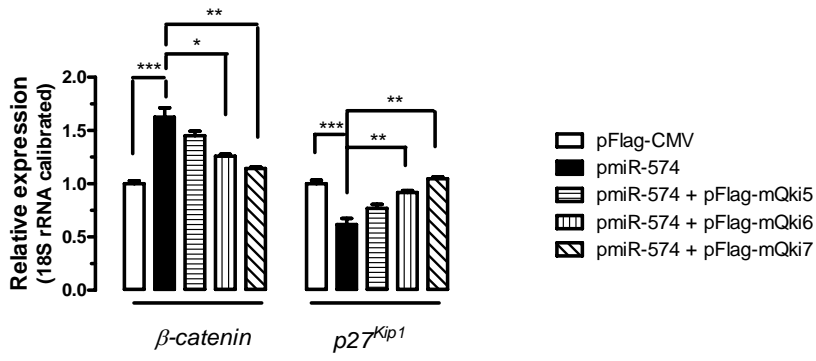
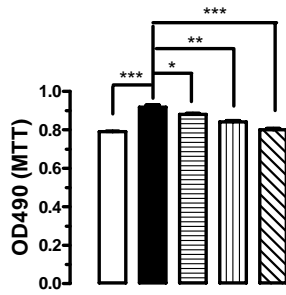


Fig. S4

A



B



C

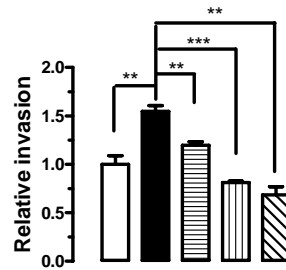
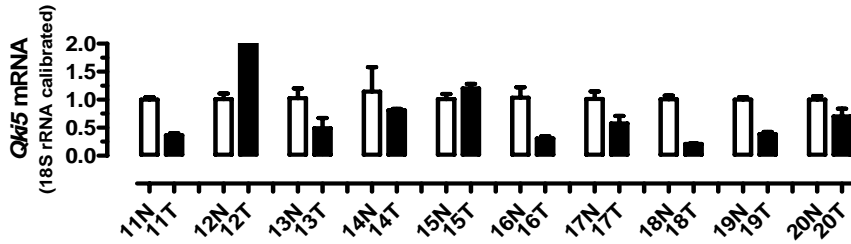
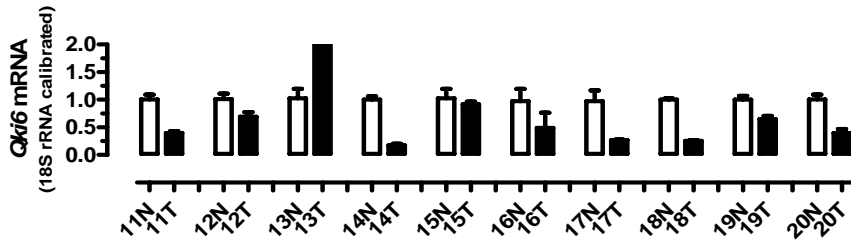


Fig. S5

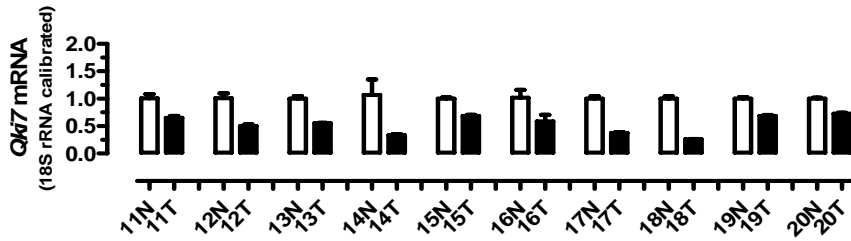
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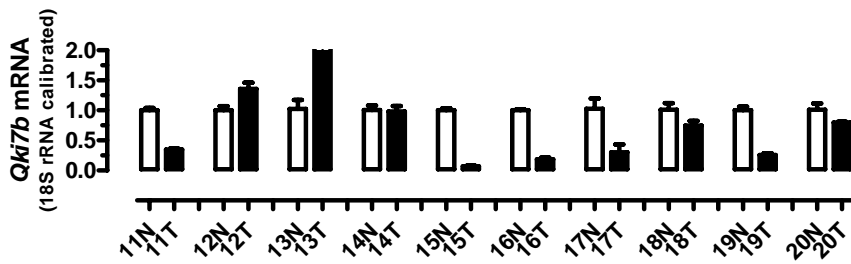
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C



D



E

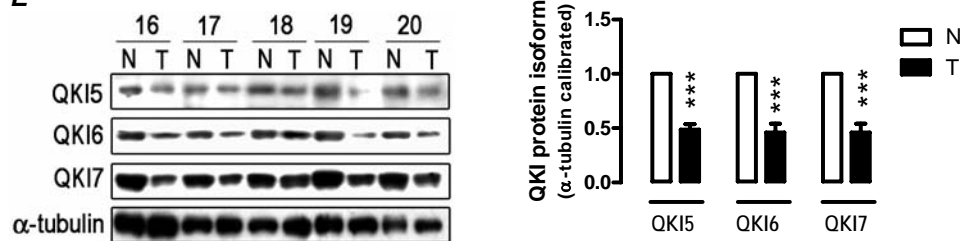


Fig. S6

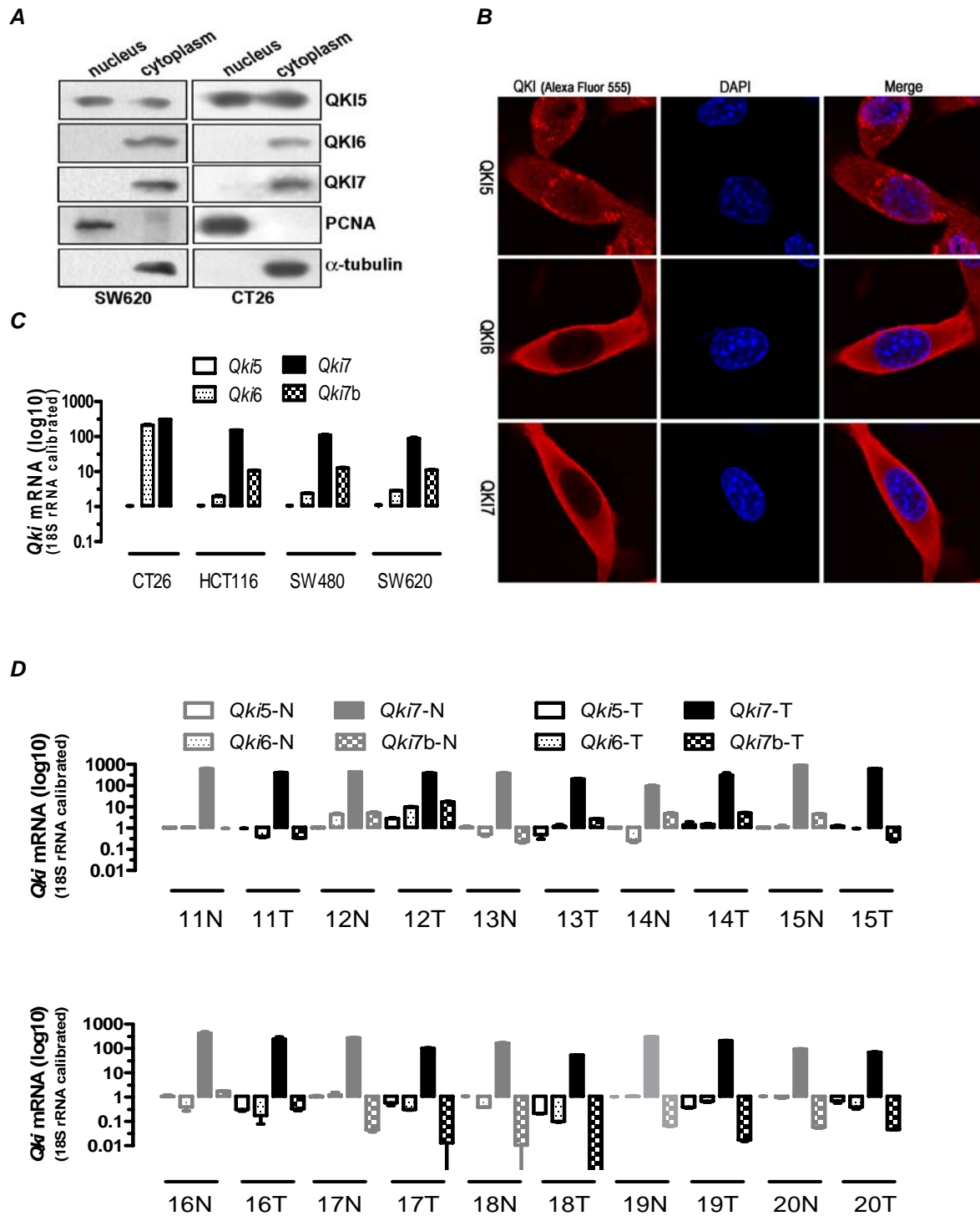


Fig. S7

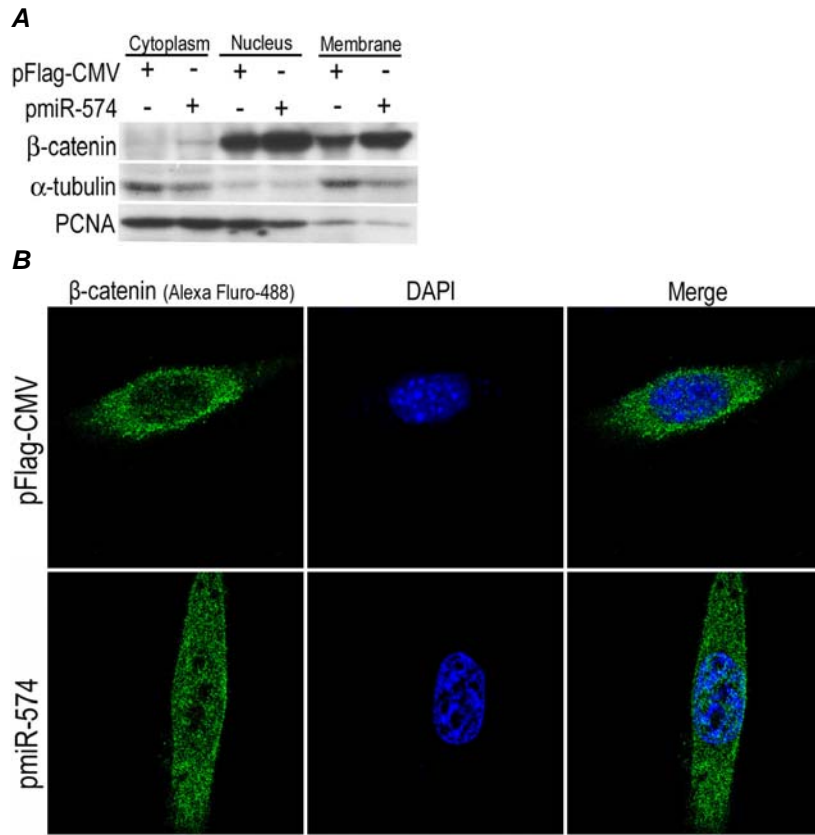


Fig. S8

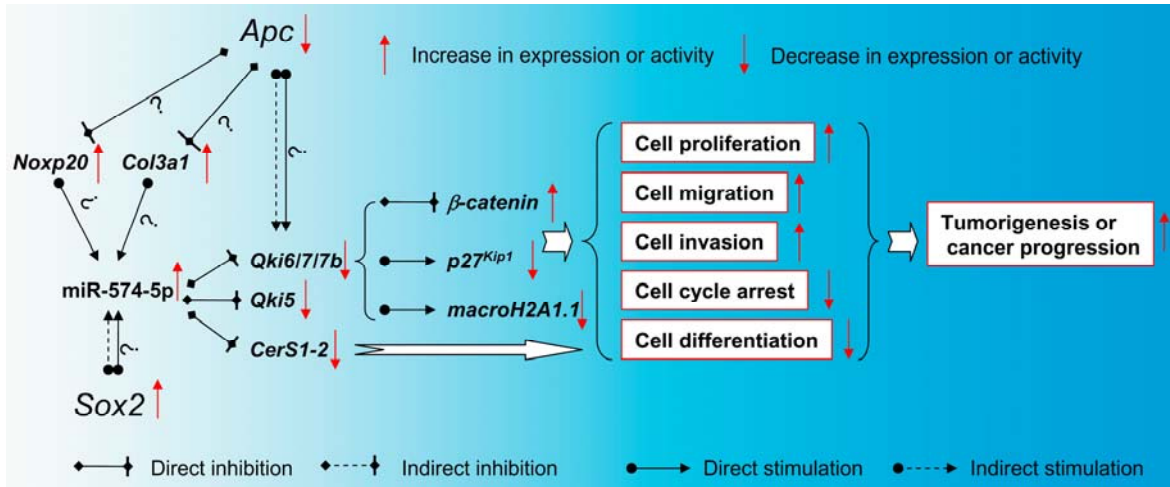


Fig. S9

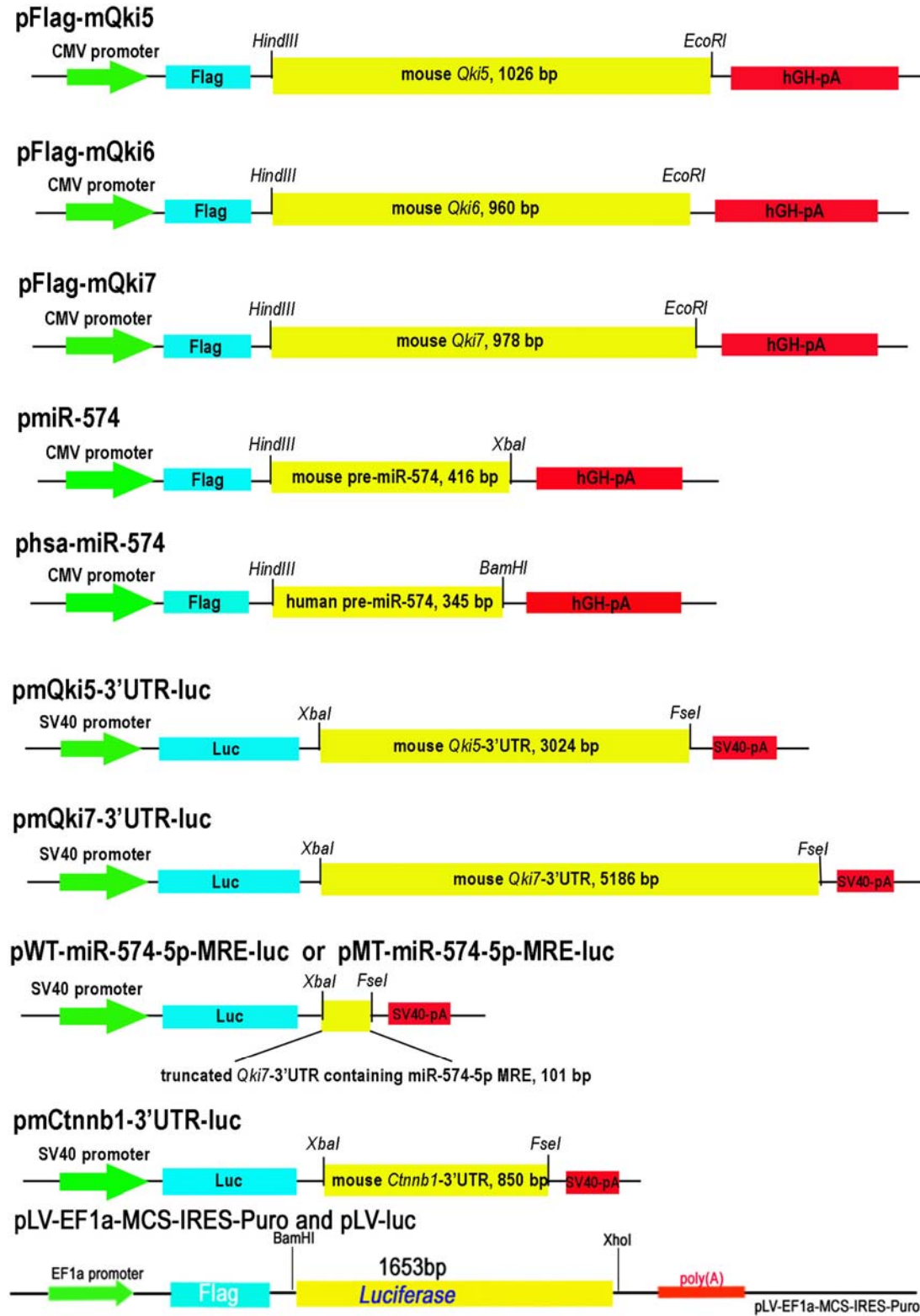


Table S1

		<i>n</i>	%	miR-574-5p (Normalized)	Pan- <i>Qki</i> mRNA (Normalized)	Pan-QKI protein (Normalized)
Gender	M	40	67.7	3.136 ± 0.5718	0.8483 ± 0.1774	0.7554 ± 0.0977
	F	20	33.3	2.793 ± 0.532	0.7278 ± 0.1335	0.7882 ± 0.0929
Age	≤60	28	46.7	3.238 ± 0.8269	0.9206 ± 0.2304	0.7509 ± 0.094
	>60	32	53.3	2.843 ± 0.3409	0.7134 ± 0.1315	0.7789 ± 0.1097
TNM stage	I	8	13.3	5.737 ± 2.546	0.9503 ± 0.4494	0.8613 ± 0.3316
	II	15	25	2.666 ± 0.5652	1.115 ± 0.412	0.8046 ± 0.1773
	III	36	60	2.593 ± 0.3291	0.6608 ± 0.082	0.7292 ± 0.0674
	IV	1	1.7	2.415	0.4825	0.74
Lymph node status	N0	23	38.4	3.734 ± 0.9734	1.058 ± 0.3051	0.8243 ± 0.1589
	N1	17	28.3	2.549 ± 0.5684	0.7131 ± 0.1462	0.7564 ± 0.0935
	N2	20	33.3	2.621 ± 0.3574	0.6075 ± 0.0828	0.7066 ± 0.0935
	N3	0				

Table S2

Organism	Condition	miR-574-5p expression	Affected cells or tissues	Fold change	References
Human	CRC	Up	Colorectal cells	Very significant	Current study
Human	Pituitary adenomas	Up	Pituitary tissue	2.89	²³
Human	Systemic lupus erythematosus	Up	T lymphocyte	> 2	²⁴
Human	Pancreatic cancer	Up	Pancreatic tissue	51	²⁵
Human	Lymphoma	Altered	Lymphocyte	?	²⁶
Human	Myocardial infarction	Up	Heart	Very significant	²⁷
Human	Non-small cell lung cancer	Up	Lung	2.17	²⁰
Human	Small cell lung cancer	Altered	Lung	?	²¹
Human	Eosophageal squamous cell carcinoma	Up	Eosophageal squamous cells	1.72	²²
Human	Alzheimer's	Up	Gray matter	?	²⁸
Human	Schizophrenia	Altered	Brain tissue	?	²⁹
Human	Mitral stenosis	Up	Right atrial appendage	1.96	³⁰
Human	Ovarian cancer	Up	Ovary tissue	1.24	³¹
Mouse	CRC	Up	Colorectal cells	3.7	Current study
Mouse	Vesicular stomatitis virus infection	Up	Macrophage	?	³²
Mouse	Asthma	Up	Lung cells	13	³³
Mouse	SARS infection	Altered	Bronchoalveolar stem cells	?	³⁴
Mouse	Liver injury	Up	Liver/plasma	3.4/1.49	³⁵

2. SUPPLEMENTAL MATERIALS AND METHODS

Plasmids. TOP/FOP-Flash plasmids and the Wnt1-overexpressing (Wnt1) plasmid were kind gifts from Prof. Qiao Wu, Xiamen University.

For *Qki* overexpression plasmids, primers were designed based on the genomic sequences from the NCBI databases, with the forward primer carrying a *HindIII* site and the reverse primer carrying an *EcoRI* respectively. Mouse *Qki5*, *Qki6*, *Qki7* were individually PCR-amplified using mouse brain tissue cDNA and appropriate primers (see List of primers used for miRNA or *Qki* overexpression plasmids), with the LA Taq DNA Polymerase (TaKaRa, Dalian). The resultant DNA fragments were subcloned into pFlag-CMV (Sigma Aldrich, St. Louis, MO, USA) using the *HindIII* and *EcoRI* sites.

For mouse miRNA overexpressing plasmids, primers were designed based on the genomic sequences from the miRBase (microna.sanger.ac.uk), with the forward primers carrying a *HindIII* site and the reverse primers carrying a *XbaI* site respectively. Pre-miRNA gene fragments were individually PCR-amplified using genomic DNA prepared from mouse inner medullary collecting duct epithelial mIMCD3 cells (mmu-miR-574) or human CRC SW480 cells (hsa-miR-574) and appropriate primers (see List of primers used for miRNA or *Qki* overexpression plasmids), with Pyrobest DNA Polymerase (TaKaRa). The resultant DNA fragments were subcloned into pFlag-CMV, using the *HindIII* and *XbaI* (mmu-miR-574) or *HindIII* and *BamHI* (hsa-miR-574) sites. The insertion sequences of the resultant plasmids were confirmed by sequencing.

For the construction of mouse *Qki5*-3'UTR, wildtype *Qki7*-3'UTR miR-574-5p MRE, mutant *Qki7*-3'UTR miR-574-5p MRE and *Ctnnb1* luciferase reporters, mouse 3'UTR regions were amplified from mouse brain tissue cDNA through PCR amplification with the LA Taq DNA Polymerase (TaKaRa) and appropriate primers (see List of primers used for the construction of five luciferase reporters). The resultant PCR fragments carrying a *NheI* site and a *FseI* site was subcloned into the pGL3-control vector (Promega), using the *XbaI* and *FseI* sites immediately downstream of the stop codon of the luciferase cDNA, generating pmQki5-3'UTR-luc, pmQki7-3'UTR-luc, pWT-miR574-5p-MRE-luc, pMT-miR574-5p-MRE-luc and pmCtnnb1-3'UTR-luc respectively.

For the construction of lentiviral plasmid carrying a luciferase gene, the luciferase reporter gene on plasmid pGL3 (Promega, USA) was PCR amplified and a *BamHI-XhoI* insert gene cassette was inserted

into a lentiviral vector pLV-EF1a-MCS-IRES-Puro (A gift from Prof. Jiahui Han) to obtain a plasmid pLV-luc.

The resulting twelve plasmids are shown in Fig. S9 in the previous section.

Cell culture and transient transfections. Mouse CT26, human SW480 and SW620 CRC cells were obtained from ATCC (Manassas, VA, USA), human HCT116 CRC cells was obtained from the Chinese Academy of Sciences (Shanghai, China). CT26 cells and HCT116 cells were normally cultured in RPMI 1640 with 10% fetal bovine serum (FBS). SW480 and SW620 cells were cultured in DMEM supplemented with 10% FBS. Plasmids, miRNA mimics and inhibitor transfections were performed with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols.

Western blot, immunohistochemistry, immunofluorescence and in situ hybridization analyses.

Western blots were performed according to standard protocols. The detection was achieved using the Immobilon Western Chemiluminescent HRP Substrate kit (Millipore, Billerica, MA, USA). Antibodies and the dilutions are anti- α -tubulin (sc-5286, Santa Cruz, 1:10000); anti- β -actin (sc-47778, Santa Cruz, 1:20000); anti-pan-QKI (sc-103851, Santa Cruz, 1:2000); anti-QKI5 (AB9904, Millipore, 1:2000); anti-QKI6 (AB9906, , Millipore, 1:2000); anti-QKI7 (AB9908, Millipore, 1:2000); anti-p27^{Kip1} (sc-528, Santa Cruz, 1:2000); anti- β -catenin (sc-7199, Santa Cruz, 1:2000); anti-PCNA (sc-7907, Santa Cruz, 1:2000); Alexa Fluor 555-labeled goat Anti-Rabbit IgG (H+L) (A0452, Beyotime Institute of Biotechnology, 1:500) respectively.

Immunohistochemistry was performed with an instant-type SABC immunohistochemistry kit purchased from the Boster Bioengineering Company (Wuhan, China) as instructed. Briefly, 5 μ m fixed tissue sections were microwave-heated for 10 min for antigen retrieval. Slides were washed and incubated with primary antibody for 1 hour followed by incubation with instant-type secondary antibody for 30 minutes at room temperature. After being thoroughly washed with PBS (pH 7.5), the tissues were incubated with SABC (Strept-Avidin-Biotin Complex) for 20 minutes at room temperature. After washing thoroughly, 100-200 μ l freshly-made diaminobenzidine (DAB) solution was added to each slide. After incubation for ~10 minutes, sections were lightly counter-stained with hematoxylin.

For immunofluorescence analyses, cells seeded on cover glass overnight were fixed in 4% paraformaldehyde. Fixed cells were incubated with anti-QKI5, QKI6 or QKI7 primary antibody

respectively, followed by incubation with Alexa Fluor® 555-conjugated secondary antibody or Alexa Fluor® 488-conjugated secondary antibody (Beyotime Institute of Biotechnology, Jiangsu, China). Cell nuclei were stained by DAPI (Beyotime Institute of Biotechnology). Stained cells were visualized and photographed with a Leica-TCS-SP2-SE confocal microscope.

In situ hybridization of miRNAs was performed as described³⁶. miRCURY™ LNA miRNA probes (hsa-miR-574-5p, 5'-ACACACTCA-CACACACACTCA-3'; hsa-miR-200b, 5'-TCATCATTACCAGGCAGTATTA-3'; U6, 5'-CACGAATTTGCGTGTCATCCTT-3'; Scramble-miR, 5'-GTGTAACACGTCTATACGCCCA-3') were purchased from Exiqon (Copenhagen, Denmark).

qPCR analyses of mRNAs and miRNAs. For qPCR mRNA quantification, reverse transcription was performed with TRIzol (Invitrogen)-extracted total RNAs using a ReverTra Ace-α-® Kit as instructed (TOYOBO, Shanghai). qPCR was performed by using the SYBR Green qPCR Master Mix (TOYOBO) and the StepOne Plus qPCR system (Applied Biosystems Inc., Foster City, CA) according to the manufacturer's protocols and with the primer pairs listed below.

For miRNAs, qPCR was performed with the stem-loop primers as described previously³⁷ using the miRNA-specific reverse transcription primers, the universal primer and the miRNA-specific reverse locked nucleic acid (LNA)-primers as described below.

Cell cycle, proliferation, migration, invasion and colony formation assays. For cell cycle analysis, 5×10^5 cells were synchronized by serum starvation for 24 hours and induced to re-enter the cell cycle by replacing old media with a fresh media containing 10% fetal bovine serum (FBS) for 24 hours. Cells were harvested and fixed in 75% ethanol at 4 °C overnight. Cells were incubated with RNase A at 37 °C for 30 min, and then stained with propidium iodide. The cell cycle phases were measured by flow cytometry.

Cell proliferation was analyzed by the MTT assay. A total of 4×10^3 cells was seeded in 96-well plates and MTT was added to each well every 24 hours. The plates were incubated for 4 hours before the addition of 10% SDS (in 0.01M HCl) (Sigma Aldrich, St. Louis, MO, USA). The absorbance was measured at 490 nm using a microplate reader.

Cell migration was analyzed by the wound-healing assays. SW480 cells were seeded onto 6-well plates. After transfection, a wound was incised in the center of the confluent culture, followed by careful washing to remove detached cells and the addition of fresh medium. Phase contrast images of the wounded

area were recorded using an inverted microscope at indicated time points.

Matrigel invasion assays were performed using Millicell inserts coated with matrigel (BD Biosciences, Sparks, MD, USA). 5×10^4 CT26 cells were seeded per upper chambers in serum-free RPMI1640 whereas the lower chambers were loaded with RPMI1640 containing 5% FBS. After 48 hours, the non-migrating cells on the upper chambers were removed by a cotton swab, and cells invaded through the matrigel layer to the underside of the membrane were stained with a 0.1% crystal violet solution and counted manually in eight random microscopic fields.

For colony formation assays, control and miRNA inhibitor or LV-miR-shRNA (Supplemental Materials and Methods section, Supplementary Information) transfected SW480 cells were seeded on six-well plates and maintained in DMEM containing 10% FBS for 2 weeks. Cells were fixed with methanol and stained with 0.5% crystal violet in 50% methanol for 1 hour and colonies larger than 100 μm in diameter were counted.

Luciferase reporter assays and IAP enzyme activity assays. Luciferase reporter activities were determined using a Luciferase Reporter Gene Assay System (Promega, Madison, WI, USA) as instructed. For all luciferase assays, β -galactosidase activities were determined to calibrate the transfection efficiency. The calibrated value for a proper control was used to normalize all other values to obtain the normalized relative luciferase units (RLU) representing the activities of 3'UTRs.

The enzyme activity of IAP was measured by an IAP kit (Jiancheng, Nanjing, China) according to the manufacturer's instructions. Protein concentration was measured by the Pierce BCA kit (Pierce Biotechnology, Rockford, USA).

Bioluminescence imaging of tumor growth in live animals. Briefly, CT26 cells were transfected with a luciferase-overexpressing plasmid or its control vector (pLV-luc or pLV-EF1a-MCS-IRES-Puro). Stable clones of cells overexpressing luciferase were selected with the addition of 8-10 $\mu\text{g}/\text{ml}$ puromycin in the media for 3 weeks (Invitrogen, Carlsbad, CA, USA). Luciferase-overexpressing cells or its controls were subsequently injected into the nude mice intraperitoneally (5×10^5 cells/mouse). Starting from the third day after CT26 cell inoculation, each mouse was injected with 2×10^7 transducing units of control lentiviruses or lentiviruses carrying shRNA for miR-574-5p once a week for 2 weeks. Three days after lentiviral injection, the mice were subjected to bioluminescence imaging under an IVIS Lunina II *in vivo* imaging system

(Xenogen, Hopkinton, MA, USA) as described by Lan et al.³⁸ every other three days. Ten minutes prior to imaging, each mouse was injected with 100 ul D-luciferin solution (15 mg/ml in PBS, Promega #E1602) by i.p. injection. Immediately before imaging taking, the experimental mice were anesthetized using an XGI-8 Gas Anesthesia System (Xenogen). Images and amount of bioluminescent signals were analyzed using Living Image software (Xenogen).

Serum miRNA preparations and analyses. Total RNA from serum was extracted using a miRVana miRNA isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. RNA was eluted in nuclease free water at 95°C. Five microliters of total RNA was reverse transcribed using the ReverTra Ace- α -[®] Kit as instructed (TOYOBO, Shanghai) and miRNA-specific stem-loop primers as shown in the Materials and Methods section. has-miR-16-5p served as an internal control. Visible and dissectable peritoneal tumors were dissected and weighted.

Bioinformatics, data acquisition, image processing and statistical analyses. Mature and pre-miRNA sequences were based on miRBase (microrna.sanger.ac.uk). miRNA target predictions were performed with the miRanda (www.microrna.org) algorithm. Western blot images were captured by Biosense SC8108 Gel Documentation System with GeneScope V1.73 software (Shanghai BioTech, Shanghai, China). Gel images were imported into Photoshop for orientation and cropping. The digital density values were acquired by Image-Pro Plus software (Media Cybernetics) and analyzed by Graphpad Prism 5.0. Data are the means \pm SEM. One-way ANOVA with Bonferonni's post-test was used for multiple comparisons and the Student's *t*-test (two-tailed) for pair-wise comparisons. The correlation analyses were performed with Pearson's test.

List of primers used for the construction of miRNA or Qki overexpression plasmids

Plasmid	Primer sequence (from 5'→3')	Gene ID	Amplicon (bp)
pFlag-mQki5 (mouse)	Forward:CCCAAGCTTATGGTCGGGGAAAT GGAAAC Reverse:CCGGAATTCCTAGTTGCCGGTGGC GGCTC	NM_001159517 (Genbank)	1026
pFlag-mQki6 (mouse)	Forward:CCCAAGCTTATGGTCGGGGAAAT GGAAAC Reverse:CCGGAATTCCTAGCCTTTCGTTGG GAAAG	NM_001159516 (Genbank)	960
pFlag-mQki7 (mouse)	Forward:CCCAAGCTTATGGTCGGGGAAAT GGAAAC Reverse:CCGGAATTCCTCAATGGGCTGAAAT ATCAG	NM_021881 (Genbank)	978
pmiR-574 (mouse)	Forward: CCCAAGCTTTGTCCGCTGTAGGGTGTGAG AA Reverse: TGCTCTAGAATCAGGATGGAGGTCAAGGC CT	MI0005518 (miRBase)	416
phsa-miR-574 (human)	Forward: CCCAAGCTTCCTCTGCGTTAGTGAGAAGC AG Reverse: GCGGATCCTCTGTCTTACAGGGACCTGC TC	MI0003581 (miRBase)	345

List of primers used for the construction of six luciferase reporters.

Plasmid	Primer sequence (from 5'→3')	Gene ID	Amplicon length and location
pmQki5-3'UTR-luc	Forward: TGCGCTAGCTATGACCTTCTGACCTCTGAACTCT Reverse: ACTGGCCGGCCTATGGGTTAATAGAAACAGCAAAGA	<i>mQki5</i> NM_001159517	(NTs 1517-4540) 3024 bp
pmQki7-3'UTR-luc	Forward: TGCGCTAGCCTTGCTGGATGAAGGACTAGA Reverse: ACTGGCCGGCCTTGGCCTCATGATACAAAGCAATAC	<i>mQki7</i> NM_021881	(NTs 1467-6652) 5186 bp
pWT-miR574-5p-MRE-luc	Forward: TGCTCTAGACTTTGTTAAGTAATCCACACTC Reverse: ACTGGCCGGCCAACGGTTGTCCCATAGTCTTAA	<i>mQki7</i> NM_021881	(NTs 5650-5750) 101bp
pMT-miR574-5p-MRE-luc	Forward: TGCTCTAGACTTTGTTAAGTAATCCGACGCG Reverse: ACTGGCCGGCCAACGGTTGTCCCATAGTCTTAA	<i>mQki7</i> NM_021881	(NTs 5650-5750) 101 bp
pmCnnb1-3'UTR-luc	Forward: TGATCTAGAAAGACTTGGTAGGGTGGGAATGG Reverse: ACTGGCCGGCCGCAGGTTACAACAACCTTTGGGAT	Mouse β -catenin NM_001904	(NTs 2707-3557) 850 bp
pLV-luc	Forward: AGAGAATTCGGATCCATGGAAGACGCCAAAAACATAA Reverse: CCATGGCTCGAGCCCTTACACGGCGATCTTTCCG	pGL3 (Promega)	1365 bp

List of miR-mimics, anti-miRs, lentiviral miR-shRNAs and LNA-probes for miRNA in situ hybridization.

Name	Sequence or target (5'→3')	Catalog #	Supplier
mimics-ctrl	Scrambled		GenePharma, Shanghai
miR-574-5p mimics	UGAGUGUGUGUGUGUGAGUGUGU ACACUCACACACACACUCAUU		GenePharma, Shanghai
anti-miR-ctrl	Scrambled		GenePharma, Shanghai
anti-miR-574-5p	UGAGUGUGUGUGUGUGAGUGUGU	AM17000	Ambion
LV-miR-shRNA- ctrl	TTCTCCGAACGTGTCACGT	pLVT4	Sunbio, Shanghai
LV-miR-574-5p- shRNA	ACACACTCACACACACACTCA	pLVT278	Sunbio, Shanghai
Scrambled miRNA	GTGTAACACGTCTATACGCCCA/3Dig	99004-05	Exiqon, Copenhagen
U6 probe	CACGAATTTGCGTGTCATCCTT/3Dig	99002-05	Exiqon, Copenhagen
hsa-miR-574-5p probe	ACACACTCACACACACACTCA//3Dig	38674-05	Exiqon, Copenhagen

List of primers used for qPCR analyses of mRNAs.

Organism	Gene	Gene ID	Primer sequence (5'→3')	Amplicon (bp)
Human	pan- <i>Qki</i>	NM_006775		
		NM_206853	Forward:CATCAGCTGCATCTTCTTCAG	121
		NM_206854	Reverse:CACTGTGGAAGATGCTCAGAA	
		NM_206855		
	<i>Qki5</i>	NM_006775	Forward:GCCCTACCATAATGCCTTTGA Reverse:AACTTTAGTAGCCACCGCAACC	
	<i>Qki6</i>	NM_206853	Forward:GCCCCAAGCTGGTTTAATCTATA Reverse:TCGTTGGGAAAGCCATACCTAAT	118
	<i>Qki7</i>	NM_206854	Forward:GCTGGTTTAATCTATACACCCTATG A	113
			Reverse:GACTGGCATTCAATCCACTCTA	
	<i>Qki7b</i>	NM_206855	Forward:AATGCCTTTGATCAGACAAATACA G Reverse:TGGGGAGAAGAAGCTTACCTAATAC A	198
	<i>β-catenin</i>	NM_001904	Forward:AGCCACAAGATTACAAGAAACGG Reverse:ATCCACCAGAGTGAAAAGAACGA	173
<i>p27^{Kip1}</i>	NM_004064	Forward:GGGGCTCCGGCTAACTCTGA Reverse:AGGCTTCTTGGGCGTCTGCT	215	
18S rRNA	NR_003286.2	Forward:CGACGACCCATTCGAACGTCT Reverse:CTCTCCGGAATCGAACCTGA	102	
Mouse	pan- <i>Qki</i>	NM_001159517	Forward:TAGAGGACTTACAGCTAAACAACCT	288
		NM_001159516	Reverse:ATTCAGAATTGCAAGCTCCATCA	
		NM_021881		
	<i>Qki5</i>	NM_001159517	Forward:GCCCTACCATAATGCCTTTGA Reverse:AACTTTAGTAGCCACCGCAACC	211
	<i>Qki6</i>	NM_001159516	Forward:GCCTGAAGCTGGGTTAATCTACA Reverse:TCGTTGGGAAAGCCATACCTAAC	118
	<i>Qki7</i>	NM_021881	Forward:GCTGGGTTAATCTACACACCCTAT GA	113
Reverse:GACTGGCATTCAATCCACTCTA				
<i>β-catenin</i>	NM_007614	Forward:TGGACCCCAAGCCTTAGTAAACA Reverse:GTCTGTGATGAAGCCCCAGTG	159	

<i>Lactase</i>	NM_001081078	Forward:GAGACCCAGAACTCAATGACACC Reverse:GGTCAGAGCGGTTCACAAAGT	165
<i>p27^{Kip1}</i>	NM_009875	Forward:GCGGTGCCTTTAATTGGGTCT Reverse:TCTTGGGCGTCTGCTCCACA	225
<i>Col3a1</i>	NM_009930	Forward:GTTTCTTCTCACCTTCTTCATCCC Reverse:GCAGTCTAGTGGCTCCTCATCACA G	196
<i>Noxp20</i>	NM_026667	Forward:AGGGAGACACCGGATCTGAAATA Reverse:GAATTGGCAGTGTGGATTCGTAG	199
<i>Sox2</i>	NM_011443	Forward: GCGGAGTGGAACTTTTGTCC Reverse: GGGAAGCGTGTACTTATCCTTCT	156

List of conventional or LNA-primers for qPCR analyses of U6 and miRNAs.

RNA or miRNA	Genbank or miRBase seq#	Primer sequence (5'→3')
Mouse and human U6	NR_004394.1	Reverse transcription: CGCTTCACGAATTTGCGTGTTCAT Forward: GCTTCGGCAGCACATATACTAAAAT Reverse: CGCTTCACGAATTTGCGTGTTCAT (LNA)
<u>hsa-miR-16-5p</u>	<u>MI0000070</u>	Reverse transcription: <u>GTCGTATCCAGTGCCTGTGCTGGAGTCGGCAATTGCA</u> <u>CTGGATACGACTCGCCAA</u> Forward: GGGGTAGCAGCACGTAAA Reverse: TGCCTGTGCTGGAGTC (LNA)
mmu-miR-574-5p	MI0005518	Reverse transcription: GTCGTATCCAGTGCCTGTGCTGGAGTCGGCAATTGC ACTGGATACGACTACACAC Forward: GGGGTGAGTGTGTGTGTG Reverse: TGCCTGTGCTGGAGTC (LNA)
hsa-miR-574-5p	MI0003581	Reverse transcription: GTCGTATCCAGTGCCTGTGCTGGAGTCGGCAATTGC ACTGGATACGACTACACAC Forward: GGGGTGAGTGTGTGTGTG Reverse: TGCCTGTGCTGGAGTC (LNA)
mmu-miR-200b	MI0000243	See Huang et al. ³⁷
mmu-miR-717	MI0004704	See Huang et al. ³⁷
mmu-miR-466g	MI0005510	Reverse transcription: GTCGTATCCAGTGCCTGTGCTGGAGTCGGCAATTGC ACTGGATACGACTGTGTGT Forward: GGGGATACAGACACATGC Reverse: TGCCTGTGCTGGAGTC (LNA)
mmu-miR-17	MI0000687	Reverse transcription: GTCGTATCCAGTGCCTGTGCTGGAGTCGGCAATTGC ACTGGATACGACTCTACCT Forward: GGGGCAAAGTGCTTACAG Reverse: TGCCTGTGCTGGAGTC (LNA)
mmu-miR-20a	MI0000568	Reverse transcription: GTCGTATCCAGTGCCTGTGCTGGAGTCGGCAATTGC ACTGGATACGACTCTACCT

Forward: GGGGTAAAGTGCTTATAG

Reverse: TGC GTGTCGTGGAGTC (LNA)

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