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MiR-29b Downregulates Canonical Wnt Signaling by Suppressing Coactivators of β-Catenin in Human Colorectal Cancer Cells

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ABSTRACT

The β-catenin/Wnt signaling pathway is activated in many cancers and its constitutive activation has a central role in colorectal cancer pathogenesis. Recent studies have highlighted the role of microRNAs as novel regulators of gene expression including that of signaling intermediates from the Wnt signaling pathway. The purpose of our study was to determine the role of miR-29b in the regulation of Wnt signaling in human colorectal cancer cells. TOPFlash/FOPFlash reporter assays, gene expression studies by quantitative RT-PCR and western blot analysis were used to study the effect of ectopic expression of miR-29b on canonical Wnt signaling. miR-29b antagonized transactivation of β-catenin target genes by downregulating coactivators of β-catenin (TCF7L2, Snail, and BCL9L) in SW480 cells. miR-29b targeted the 3′UTR of BCL9L and decreased its expression with a consequent decrease in nuclear translocation of β-catenin. Ectopic expression of miR-29b inhibited anchorage independent cell growth, promoted reversal of epithelial to mesenchymal transition and reduced the ability of conditioned medium from SW480 cells to induce in vitro tube formation in endothelial cells. These results have unraveled a novel role of miR-29b in Wnt signaling in human colorectal cancer cells with implications in the treatment of colorectal cancer. J. Cell. Biochem. 115: 1974–1984, 2014.

KEY WORDS: miR-29b; Wnt SIGNALING; COLORECTAL CANCER; TCF7L2; SNAIL; BCL9L; ANGIOGENESIS

The canonical Wnt signaling pathway is highly conserved across the animal kingdom and plays an important role in embryonic development, stem cell maintenance, and tissue homeostasis [Clevers, 2006; Petersen and Reddien, 2009]. Canonical Wnt signaling is dependent on the protein β-catenin (CTNNB1), which is present in three distinct pools in the cell—plasma membrane-bound, cytoplasmic, and nuclear. There is a homeostasis maintained between these fractions by the degradation complex consisting of AXIN1, GSK3B, and APC, which together with the casein kinases in the cytoplasm, phosphorylate the free β-catenin and mark it for ubiquitination by the E3 ubiquitin protein ligase, β-TrCP, and subsequent proteolytic degradation. When the Wnt pathway is activated by the binding of Wnt ligands to the Frizzled receptors and the low-density lipoprotein coreceptors, the degradation complex is inactivated resulting in the stabilization of β-catenin. This leads to the translocation of β-catenin into the

Abbreviations: CRC, colorectal carcinoma; miRNA, microRNA; snRNA, small non-coding RNA; UTR, untranslated region; RT-PCR, reverse transcriptase-polymerase chain reaction; EMT, epithelial to mesenchymal transition; TCM, tumor cell conditioned medium.

The concept of this study was from MS and DK. All authors contributed to the study design. MS was primarily responsible for experimentation and data analysis. SRR contributed to cloning and luciferase reporter assays and PT contributed to the immunoblotting assays. DK and SC contributed to the research implementation of the study. MS was primarily responsible for paper preparation. DK and SRR helped draft and critically revise the paper. All authors reviewed and approved the final version of the paper.

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nucleus where it associates with lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors, histone acetylase CREB-binding protein (CBP)/p300 and coactivators like pygopus (PYG) and B-cell lymphoma proteins (BCL9 and BCL9L/BCL9-2) to activate many downstream target genes [MacDonald et al., 2009]. The β-catenin-dependent downstream gene expression is enhanced by the binding of Snail (SNAI1) to the N-terminus of β-catenin [Stemmer et al., 2008] and by downregulation of E-cadherin (CDH1), which is known to selectively bind and inhibit the transcriptionally active pool of β-catenin [Gottardi et al., 2001]. Constitutive activation of this pathway is central to colorectal cancer (CRC) pathogenesis with inactivating mutations of APC or Axin or activating mutation of β-catenin reported in almost every case [Morin et al., 1997; Liu et al., 2000]. Constitutive Wnt signaling in itself may not be sufficient to drive tumorigenesis [Wong et al., 1998] but it contributes greatly to tumor growth and progression by modulating cell proliferation, migration, invasion, tumor angiogenesis, and maintaining the stemness of cancer stem cells and therefore is a good candidate for therapeutic intervention [Le et al., 2008; Takada et al., 2012].

In recent years, there has been a great deal of interest in studying the role of microRNAs (miRNAs) as regulators of gene expression that influence various cellular processes. They are emerging as novel therapeutic targets and intervention tools [Pereira et al., 2013]. MiRNAs are 22–25 nucleotides long evolutionarily conserved RNA molecules, which act by binding to complementary sequences on the target mRNAs repressing the gene expression post transcription [Shukla et al., 2011]. Every miRNA has multiple targets and every mRNA has binding sites for multiple miRNAs. The extent to which an mRNA is modulated by a specific miRNA in a system depends on the binding affinity between the two and on the relative levels of other available targets. This makes the effect of any miRNA on a signaling pathway very context dependent [Salmena et al., 2011]. Many miRNAs have been reported to modulate Wnt Signaling by targeting the 3′UTR of the mRNAs of various pathway intermediaries and downregulating their expression [Huang et al., 2010; Schepeler, 2013]. miR-29a upregulates Wnt Signaling, by directly targeting and repressing its antagonists such as Dkk1, Kremen2, and sFRP2 [Kapinas et al., 2010]. miR-29b has been shown to target CTNNBIP1, and GSK3B, antagonists of the Wnt pathway, in murine osteoblasts and 293T cells, respectively [Li et al., 2009, 2011]. Many studies have reported the tumor suppressor role of miR-29b and a recent study suggested a strong correlation between downregulation of miR-29a and miR-29c and early recurrence of colorectal cancer (CRC) [Pekarsky et al., 2006; Mott et al., 2007; Park et al., 2009; Kuo et al., 2012] but the role of miR-29 (if any) in Wnt signaling in CRC has not been studied so far. Here we report for the first time that miR-29b negatively regulates Wnt signaling in human colorectal cancer cells and targets B-cell CLL/lymphoma 9-like (BCL9L), Transcription factor 7-like 2 (TCF7L2), and SNAI1. We also followed up the functional consequences of Wnt signaling downregulation by miR-29b and observed that it decreases anchorage-independent cell growth in human colorectal cells and tumor cell condition medium (TCM)-induced in vitro tube formation in endothelial cells.

MATERIALS AND METHODS

CELL CULTURE

HEK293, HCT116-WT, HCT116 p53 −/−, SW480, Ea.hy926, and ECV304 cells were cultured in complete Dulbecco's Modified Eagle Medium (cDMEM) composed of DMEM supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml), and 10% fetal bovine serum (FBS). The cultures were maintained in a humidified incubator at 37 °C and 5% CO_2. HCT116-WT and HCT116 p53−/− cells were kind gifts from Dr. Bert Vogelstein, Johns Hopkins University.

PLASMIDS, miRNA MIMIC AND miRNA INHIBITOR

pEGFP-N3-miR-29b expression construct was kindly provided by Dr. Beena Pillai, IGIB, New Delhi. The expression vectors for miRNA-29a/b/c were constructed by cloning the pre-mir sequences with additional flanking sequences amplified from human genomic DNA into pcDNA3.1(+) Neomycin construct using the BamH1 and Xba1 sites. For the *BCL9L* 3′UTR sensor construct, partial *BCL9L* 3′UTR sequence including the putative binding site for miR-29 family was amplified from human genomic DNA and cloned into the pMIR-Report Luciferase construct from Ambion using MluI and HindIII sites. The primer sequences used are given in Supplementary Table 1. The M50 Super8x TOPFlash (β-catenin/TCF reporter) and its mutant control FOPFlash [Veeman et al., 2003] were provided by Dr. Randal Moon, Howard Hughes Medical Institute, Seattle (Addgene plasmid no. 12456). pMIR-Report-β-galactosidase vector (Ambion) and pRL-TK vector (Promega) were used as the internal controls for luciferase experiments in HEK293 and CRC cells, respectively. The miRIDIAN miR-29b mimic, miRIDIAN miR-29b hairpin inhibitors and their negative controls were obtained from Thermo Scientific.

TRANSFECTIONS

HEK293 cells were transfected by the calcium phosphate method as described before [Lin et al., 2004]. The cancer cells were transfected using Lipofectamine 2000 (Invitrogen). Plasmid transfection with Lipofectamine 2000 was carried out according to manufacturer's protocol with minor modifications. For transfection of miRNA mimic, miRNA inhibitor, and their respective controls, a ratio of 1:4 of transfection mix to plating medium was used. The concentration of RNA oligos used for transfection was calculated for the total volume of plating medium and transfection mixture taken together. This total volume was fixed at 1.0 ml for a 35 mm dish and scaled accordingly to the well size used for each experiment. The cells were transfected with 80 nM of miRNA mimic unless otherwise indicated.

LUCIFERASE ASSAYS

HEK293 cells grown in 24 well plates were cotransfected with expression vector or empty vector, TOPFlash or FOPFlash plasmid and pMIR-report β galactosidase plasmid. Twenty four hours after transfection, the cell culture medium was changed to 25 mM LiCl in cDMEM and cells were incubated for 18 h followed by cell lysis in phosphate lysis buffer (100 mM KPO_4 (pH 7.5), 1 mM DTT, 0.1% Triton X-100). Firefly luciferase and β-galactosidase activities were assayed using methods previously described with D-luciferin (Promega) and ortho-nitrophenyl-β-D-galactopyranoside/ONPG (Sigma) as the respective substrates [Dyer et al., 2000; Sambrook

and Russell, 2006] For TOPFlash/FOPFlash assays, HCT116-WT, HCT116 p53−/−, and SW480 cells were seeded in 96 well-plates and cotransfected with miR-29a/b/c expression vector or empty vector, TOPFlash or FOPFlash plasmid and pRL-TK plasmid using Lipofectamine 2000 (Invitrogen). Medium was changed to cDMEM after 6 h of transfection and cells were lysed 24 h post transfection and firefly and renilla luciferase activities were assayed using the Dual-Glo Luciferase kit (Promega). For the *BCL9L* 3′UTR sensor luciferase assay, SW480 cells seeded in 12 well plates were cotransfected with *BCL9L* 3′UTR luciferase construct or empty pMIR-report luciferase construct and pRL-TK plasmid using Lipofectamine 2000. After 24 h, the cells were transfected again with different concentrations of miR-29b mimic (20nM, 40nM, or 80 nM) or control. Cells were lysed 24 h after the second transfection and firefly and renilla luciferase activities were assayed. Each transfection was repeated thrice in triplicates and firefly luciferase values were normalized to β-galactosidase or renilla luciferase activities. Values are expressed as percentage of control.

SEMI-QUANTITATIVE AND QUANTITATIVE REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was extracted from cells 24 h post transfection using TRIzol reagent (Invitrogen) and cDNA synthesis was carried out using MMLV-RT enzyme (Invitrogen). Oligo-dT was used for cDNA synthesis from mRNA and stem-loop RT primers were used for cDNA synthesis from miRNA or U6 snRNA (*RNU6*). One microgram of total RNA was taken for cDNA synthesis in a 20 μl reaction of which 1 μl was used as template in a 10 μl PCR reaction. For semi-quantitative RT-PCR, number of cycles of PCR was set at 25 for cyclin D1 (*CCND1*), cMyc (*MYC*), *TCF7L2 and BCL9L*, 22 for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and 30 for miR-29b and *RNU6*. *GAPDH* and *RNU6* were used as the internal controls. Quantitative RT-PCR was carried out using SensiMix SYBR green/low-ROX Kit (Bioline). Real-Time Miner [Zhao and Fernald, 2005] was used to calculate individual threshold cycle (Ct) values and PCR efficiency and the ΔΔCt method was used for relative quantification of gene expression in samples using β-2-microglobulin (*B2M*) and *RNU6* as internal controls for mRNA and miRNA, respectively. Results are expressed as fold change with respect to control (gene expression in control oligo transfected cells) whose value is set at 1.0 and indicated by the dotted line. The quantitative PCR reactions were run in duplicates and the results represent average of three independent experiments. The primer sequences are given in Supplementary Tables 2 and 3.

WESTERN BLOT ANALYSIS

Cells were washed with phosphate-buffered saline (PBS), lysed in ice-cold RIPA lysis buffer (20mM Tris (pH 7.4), 150mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate) containing protease inhibitors and centrifuged at 14000 rpm for 15 min at 4 °C to remove debris. Cell lysate containing 30 μg of protein was subjected to SDS-PAGE separation followed by transfer onto PVDF membrane (GE Healthcare-Amersham). The membrane was incubated with primary antibodies against CTNNB1, TCF7L2, BCL9-2, VIM, CDH1, GSK3B, phosphoGSK3B (Ser9) (Santa Cruz; 1:1000 dilution), FN1, SNAI1, CCND1 (Cell Signaling; 1:1000 dilution) or β-actin/ACTB (Sigma, 1:10,000 dilution), followed by incubation with an HRP-conjugated secondary antibody (Jackson ImmunoResearch Inc, 1:15,000 dilution). Protein bands were detected using an enhanced chemiluminescence detection kit (GE Healthcare-Amersham).

IMMUNOFLOURESCENCE

Cells were fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized with 1% Triton X-100 in PBS for 10 min. They were then washed with PBS and incubated with 1% BSA in PBS with for 30 min. followed by incubation in antibody against CTNNB1 (1:200 dilution) overnight at 4 °C. After multiple washes with PBS, they were incubated with FITC or rhodamine conjugated secondary antibody in PBS (Jackson ImmunoResearch Inc., 1:200 dilution) for 1 h. The cells were washed with PBS after removal of the secondary antibody and stained with 1 μg/ml of diamidino-2-phenylindole (DAPI) solution for 3 min. The cells were washed again with PBS and layered with 50% glycerol solution in

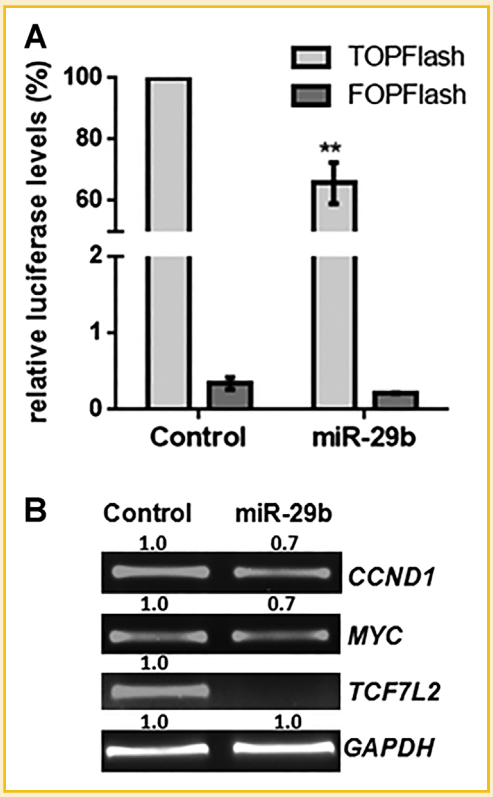

Fig. 1. Effect of ectopic expression of miR-29b on LiCl-induced Wnt signaling in HEK293 cells. (A) HEK293 cells were transiently transfected with pEGFP-N3 (control) or pEGFP-N3-miR-29b plasmids along with TOPFlash or FOPFlash construct and pMIR-β-galactosidase plasmid. After 24 h, both the control and test sample were treated with 25 mM LiCl for 18 h and luciferase activity was normalized to β-galactosidase activity in the cell lysates ($n = 3$, mean ± S.E; $^{**}P < 0.01$ vs. control). (B) HEK293 cells were transiently transfected with pEGFP-N3 (control) or pEGFP-N3-miR-29b plasmid. After 24 h, both the control and test sample were treated with 25 mM LiCl for 18 h and analysed for expression of CCND1, MYC, TCF7L2, and GAPDH (control) by SQ-RTPCR as described in Materials and Methods. These results were confirmed in another independent experiment.

PBS. Images were taken at 20x magnification using Nikon Eclipse TS-100 microscope.

CELL VIABILITY ASSAY

Transfected SW480 cells were reseeded into a 96 well plate (5000 cells/well). At each time point, Resazurin sodium dissolved in PBS was added to the wells to a final concentration of 0.1 mg/ml. Cells were incubated till a purple color developed. The supernatant was then diluted 20 times and absorbance readings were taken at 570 nm (resorufin) and 595 nm (resazurin). Readings were taken at 24 h, 48h, and 72 h in triplicates. The cell viability was calculated as previously described [Larson et al., 1997] and expressed as percentage of cell viability at 24 h. Results represent average of three independent experiments. EA.hy926 cells suspended in TCM from miR-29b transfected or control transfected SW80 cells, were seeded in a 96 well plate with or without growth factor reduced (GFR) matrigel (15 000 cells/well) and incubated for 16 h before addition of resazurin. Readings were taken in duplicates and cell viability is expressed as percentage of control. Results represent average of three independent experiments.

SOFT AGAR COLONY FORMATION ASSAY

Transfected SW480 cells were resuspended in 0.3% agarose in cDMEM and layered onto solidified 0.5% agarose in cDMEM in 12 well plates (2000 cells/well). After solidification, a feeder layer of cDMEM was added and cells were grown for 15 days with changing of feeder layer every 3 days. The images of colonies formed, were taken at 10x magnification and colony size was measured using Image J. The percentage of colonies with radius greater than the average radius of control oligo transfected cells was calculated in each sample. At least 75 colonies were measured per sample in each experiment and results represent the average of two independent experiments.

ADHESION ASSAY

Transfected SW480 cells were reseeded onto uncoated bacteriological plastic plates (~5 lakh cells/35 mm dish) and incubated for 48 h at the end of which the live and dead cells in the supernatant and adherent fractions were counted using a hemocytometer after trypan blue staining. Total number of dead cells, adherent cells, and non-adherent cells were expressed as percentage of total number of cells

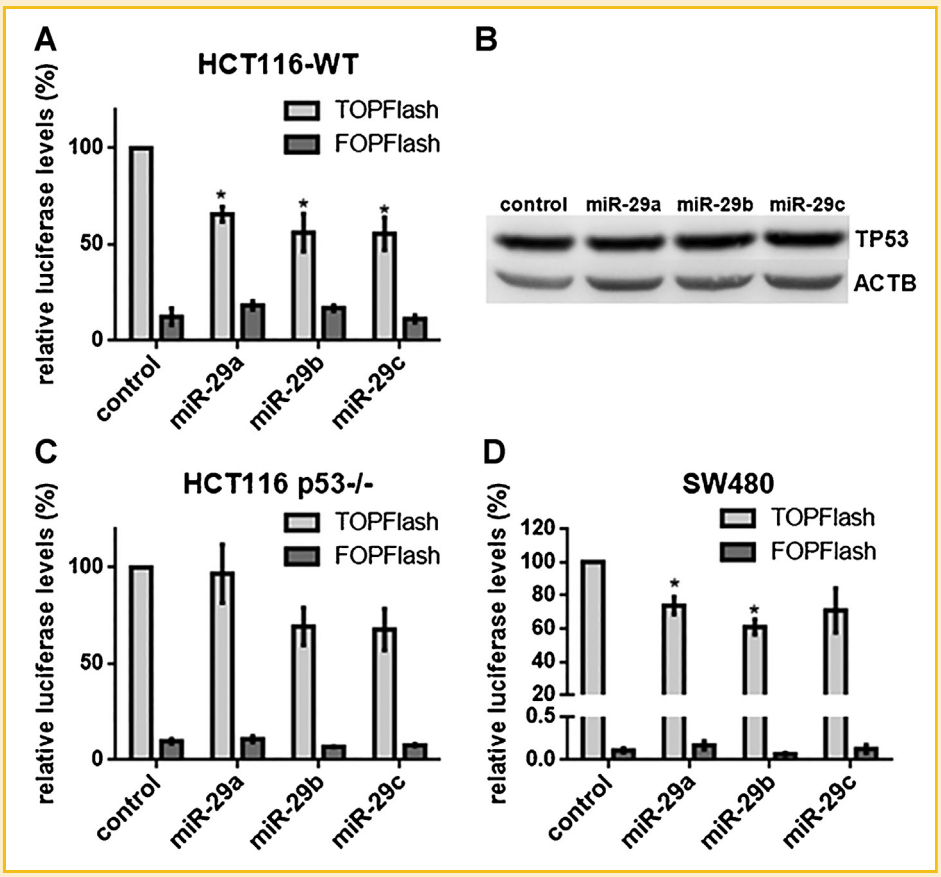

Fig. 2. Effect of ectopic expression of miR-29b on constitutive Wnt signaling in human colorectal cancer cells. (A) HCT116-WT (C) HCT116 p53-/- and (D) SW480 human colorectal cancer cells were co-transfected with pcDNA3.1 empty vector (control) or pcDNA 3.1-pre-mir-29a/b/c, TOPFlash or FOPFlash construct and pRL-TK plasmid. After 24 h, the cell lysates were assayed for firefly and renilla luciferase (internal control) activities ($n = 3$, mean $\pm$ S.E; $^*P < 0.05$ vs. control). (B) Whole cell lysates of HCT116-WT cells transfected with pcDNA3.1 empty vector (control) or pcDNA 3.1-pre-mir-29a/b/c were subjected to SDS/PAGE and immunoblotting for the detection of TP53 and ACTB.

in each sample. Results represent average of four independent experiments.

TUBE FORMATION ASSAY

SW480 cells were transfected with control oligo or miR-29b mimic. 24 h after transfection, the cells were washed with DMEM and incubated in DMEM with protease inhibitors for another 24 h. The conditioned medium (TCM) was then collected and centrifuged at 12 000 rpm to remove cell debris and used for the experiment. A suspension of EA.hy926 cells or ECV304 cells was diluted 10 times with TCM and seeded on solidified GFR matrigel (15 000 cells/well) in a 96 well plate. The cells were incubated for 16 h to allow tube formation. Images were taken at 4x magnification and the tubes formed were highlighted with the help of Image J using the Sobel edge detector function before quantification of total number of tubes, total number of branching points and total tube length by WimTube (Wimasis GmbH) online analysis tool. Results represent average of three independent experiments.

WOUND HEALING ASSAY

EA.hy926 cells were grown as a monolayer and scratch was made in the monolayer. The cells were gently washed and treated with 250 μl TCM from SW480 cells transfected with control oligo or miR-29b mimic. Images of the scratch were taken at 4X magnification immediately after addition of TCM and after a 16 h incubation period. The extent of migration in each sample was measured as the area covered by the cells in 16 h using WimScratch (Wimasis GmbH) and expressed as percentage of control. Results represent average of three independent experiments.

STATISTICAL ANALYSIS

Two tailed student's *t*-test was used evaluate the significance of difference between the control and test samples. A '*' indicates P value <0.05 and a '**' indicates P value <0.01.

RESULTS

miR-29b INHIBITS LiCl-INDUCED β-CATENIN/Wnt SIGNALING PATHWAY IN HEK293 CELLS

Some of the experimentally validated targets of miR-29 family of miRNAs and predictions of those from TargetScan 6.2 [Lewis et al., 2005] include both activators and repressors of Wnt signaling (Supplementary Table 4) suggesting that members of the miR-29 family may act as regulators of Wnt signaling. LiCl is known to activate Wnt signaling by inhibiting GSK3B [Stambolic et al., 1996] and stabilizing β-catenin. Hence, we first tested the effect of miR-29b on LiCl-induced activation of β-catenin/Wnt signaling in non-tumorigenic HEK293 cells. The regulation of β-catenin/Wnt signaling by miR-29b was evaluated using the TOPFlash/FOPFlash reporter system as a read-out assay [DasGupta et al., 2005] and by the expression analysis of its downstream targets, *CCND1* and *MYC* [He et al., 1998; Tetsu and McCormick, 1999]. Ectopic expression of miR-29b by transient transfection of pEGFP-N3-miR-29b plasmid decreased LiCl-induced TOPFlash activity in HEK293 cells (Fig. 1A)

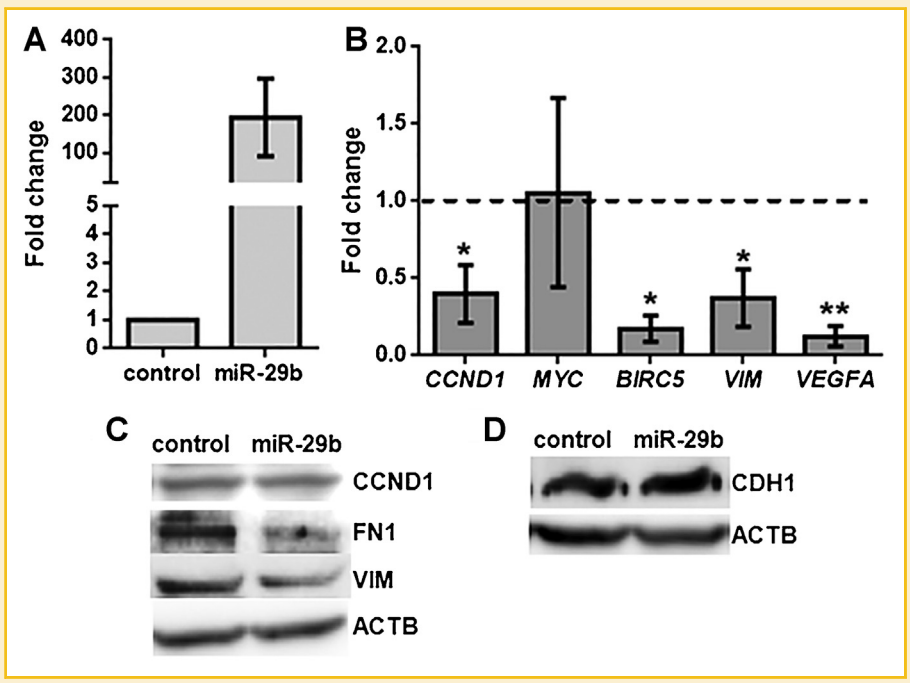

Fig. 3. Effect of ectopic expression of miR-29b on down-stream targets of Wnt signaling and epithelial and mesenchymal markers in SW480 cells. Total RNA was isolated from SW480 cells, 24 h after transfection with control oligo or miR-29b mimic, and was subjected to Quantitative RT-PCR, for relative quantification of expression of (A) miR-29b and (B) mRNA of *CCND1*, *MYC*, *BIRC5*, *VIM* and *VEGFA*. miRNA expression was normalized to *RNU6* expression and mRNA expression was normalized to β-2-microglobulin mRNA expression ($n = 3$, mean ± S.E; $^{**}P < 0.01$/$^{*}P < 0.05$ vs. control). Whole cell lysates of SW480 cells transfected with control oligo or miR-29b mimic were subjected to SDS/PAGE and immunoblotting for the detection of (C) CCND1, FN1, VIM, and ACTB (D) CDH1 and ACTB, 48 h post transfection. Similar results were obtained in another independent experiment.

and downregulated the expression of *CCND1*, *MYC* (Fig. 1B) and *BCL9L* (Supplementary Fig. 1) mRNA. TCF7L2 is the main interacting partner of β-catenin required for transactivation of downstream target genes [Graham et al., 2001]. A very strong downregulation of *TCF7L2* mRNA upon ectopic expression of miR-29b was also observed (Fig. 1B). These results demonstrate that miR-29b inhibits the transactivation of β-catenin/Wnt signaling pathway thereby downregulating the target genes, *CCND1* and *MYC* in HEK293 cells, potentially through the inhibition of TCF7L2 expression.

miR-29b INHIBITS CONSTITUTIVE β-CATENIN/Wnt SIGNALING PATHWAY IN HUMAN COLORECTAL CANCER CELLS EVEN IN THE ABSENCE OF p53

To study the effect of the miR-29 family on constitutive Wnt signaling in CRC cells, the ectopic expression of miR-29a/b/c was achieved by transient transfection with the pcDNA 3.1-pre-mir-29a/b/c constructs. Overexpression of miR-29a/b/c individually in HCT116-wild type p53 (HCT116-WT) cells, significantly downregulated CTNNB1-TCF/LEF dependent transactivation as demonstrated by the TOPFlash/FOPFlash assay (Fig. 2A). miR-29 is known to induce p53 (TP53) [Park et al., 2009], a known repressor of TCF7L2 expression [Rother et al., 2004]. Since miR-29b also lowered TCF7L2 expression (Fig. 1B), it was of interest to determine if the inhibition of Wnt signaling in colorectal cancer cells is p53-dependent. At first we ectopically expressed miR-29a/b/c and they did not increase TP53 levels in HCT116-WT, 24 h after transfection (Fig. 2B). Next, miR-29a/b/c were ectopically expressed in HCT116 p53−/− cells and SW480 colorectal cancer cells expressing a mutant form of p53 [Rodrigues et al., 1990] and tested for their effect on Wnt signalling and the results show that miR-29b consistently downregulated TOPFlash activity in both the cell lines (Fig. 2C and D). These results suggest that miR-29b downregulates Wnt signaling in human colorectal cancer cells (HCT116-WT, HCT116 p53−/−, and SW480) and that miR-29b is not exclusively dependent on wild type p53 for this effect.

miR-29b DECREASES THE EXPRESSION OF DOWNSTREAM TARGETS OF β-CATENIN/Wnt SIGNALING IN SW480 CELLS

To confirm the downregulation of β-catenin/Wnt signaling by miR-29b in SW480 cells, the effect of miR-29b on the expression of Wnt signaling-activated genes, *CCND1*, *MYC*, survivin (*BIRC5*) [Kim et al., 2003], vimentin (*VIM*) [Gilles et al., 2003] and Vegf-α (*VEGFA*) [Zhang et al., 2001] and fibronectin (*FN1*), was studied by quantitative RT-PCR. Transient transfection of miR-29b mimic resulted in ~100–300-fold overexpression of this miRNA as estimated by quantitative reverse transcriptase PCR using SYBR green chemistry (Fig. 3A). Transient transfection of SW480 cells with miR-29b mimic downregulated the mRNAs of *CCND1*, *BIRC5*, *VIM*, and *VEGFA* but *MYC* mRNA levels were highly variable (Fig. 3B).

miR-29b DECREASES THE EXPRESSION OF MESENCHYMAL MARKERS AND INCREASES EXPRESSION OF E-CADHERIN (EPITHELIAL MARKER) IN SW480 CELLS

We next looked at the effect of miR-29b on the expression of mesenchymal markers (VIM and Fibronectin) and an epithelial marker, E-cadherin (CDH1), at the protein level in SW480 cells. Both *VIM* and Fibronectin (*FN1*) are direct targets of Wnt signaling [Gradl et al., 1999] but a change in protein level and function which determine the phenotype, depend on post-translational modifications and protein stability and do not always correspond to changes in mRNA levels. Here we observed that miR-29b decreased the expression of VIM and Fibronectin (FN1) at the protein level but CCND1 protein was not significantly altered (Fig. 3C). E-cadherin (CDH1) protein, a negative regulator of canonical Wnt signaling is known to be repressed by SNAI1 [Gottardi et al., 2001]. An increase in CDH1 protein on ectopic expression of miR-29b was apparent when compared to the corresponding ACTB levels (Fig. 3D). These results confirm that miR-29b downregulates mesenchymal markers and upregulates epithelial marker indicating a reversal of epithelial to mesenchymal transition.

miR-29b DECREASES THE EXPRESSION OF COACTIVATORS OF β-CATENIN IN SW480 CELLS

To investigate the mechanism of downregulation of Wnt signaling by miR-29b, we looked at its effect on β-catenin (CTNNB1) and its coactivators, TCF7L2 and SNAI1. Overexpression of mir-29b in SW480 cells significantly repressed the expression of SNAI1 and TCF7L2 both at the mRNA (Fig. 4A) and protein levels without affecting the levels of total β-catenin protein (Fig. 4B). GSK3B and its inactive phosphorylated form (Ser9 phosphorylation) were also downregulated on ectopic expression of miR-29b (Supplementary

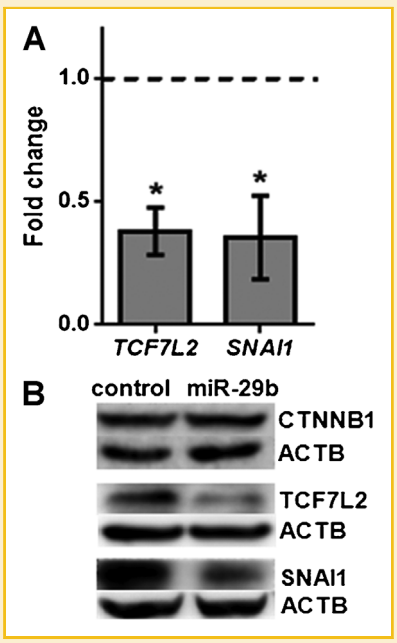

Fig. 4. Effect of ectopic expression of miR-29b on CTNNB1 and its coactivators in SW480 cells. (A) Total RNA was isolated from SW480 cells, 24 h after transfection with control oligo or miR-29b mimic, and was subjected to Quantitative RT-PCR, for relative quantification of expression of mRNA of *TCF7L2* and *SNAI1*. mRNA expression was normalized to β-2-microglobulin mRNA expression ($n = 3$, mean ± S.E; $^{**}P < 0.01$ vs. control). (B) Whole cell lysates of SW480 cells transfected with control oligo or miR-29b mimic were subjected to SDS/PAGE and immunoblotting for the detection of CTNNB1, TCF7L2, SNAI1 and ACTB, 24 h post transfection. Similar results were obtained in another independent experiment.

Fig. 2A) keeping with the trend seen in 293T and ovarian cancer cells [Liu et al., 2011; Yu et al., 2013]. We also observed that ectopic expression of miR-29b did not induce the expression of mutant p53 protein in SW480 cells (Supplementary Fig. 2B).

BCL9L, A POSITIVE REGULATOR OF β-CATENIN/Wnt SIGNALING, IS A NOVEL TARGET FOR miR-29b

As stated earlier, there is a strong possibility that miR-29b could have downregulated Wnt signaling by directly targeting some of its intermediates and we noticed that *BCL9L* 3′UTR has a single putative high affinity binding region for the miR-29 family (Fig. 5A) as predicted by miRanda [John et al., 2004] and Targetscan 6.2 [Lewis et al., 2005; Friedman et al., 2009]. A luciferase sensor to assess the regulatory effect of miR-29b on *BCL9L* 3′UTR was constructed by cloning a part of the 3′UTR into a reporter plasmid, downstream of a luciferase gene under a constitutive promoter. The *BCL9L* 3′UTR luciferase sensor activity was downregulated by miR-29b mimic in a concentration-dependent manner in SW480 cells whereas miR-29b inhibitor did not have any significant effect on the sensor activity (Fig. 5B). The BCL9L protein was downregulated by miR-29b in these cells (Fig. 5C and Supplementary Fig. 3A). A decreased nuclear translocation and increased membrane localization of β-catenin on knock-down of BCL9L in SW480 cells as demonstrated previously [Brembeck et al., 2004] was also observed on ectopic expression of miR-29b in SW480 cells (Fig. 5D and Supplementary Fig. 3B). These results suggest that *BCL9L* is a novel functional target for miR-29b and that the inhibitory effect of miR-29b on Wnt signaling is at least partially mediated through the down regulation of BCL9L expression.

miR-29b DECREASES ANCHORAGE INDEPENDENT GROWTH OF SW480 CELLS AND THE ABILITY OF TCM TO INDUCE IN VITRO TUBE FORMATION OF ENDOTHELIAL CELLS

Resistance to anoikis and ability to survive and colonize without anchorage are essential for cancer metastasis and tumor cells secrete factors that promote angiogenesis to ensure the blood supply required for their growth. We initially determined the effect of miR-29b on cell viability using the resazurin reduction assay and the results show that there was no significant change in cell viability up to 72 h (Fig. 6A). But SW480 cells transfected with miR-29b mimic formed smaller colonies on soft agar over 15 days indicating a downregulation of anchorage independent cell growth (Fig. 6B). miR-29b overexpression also resulted in a significant decrease in the

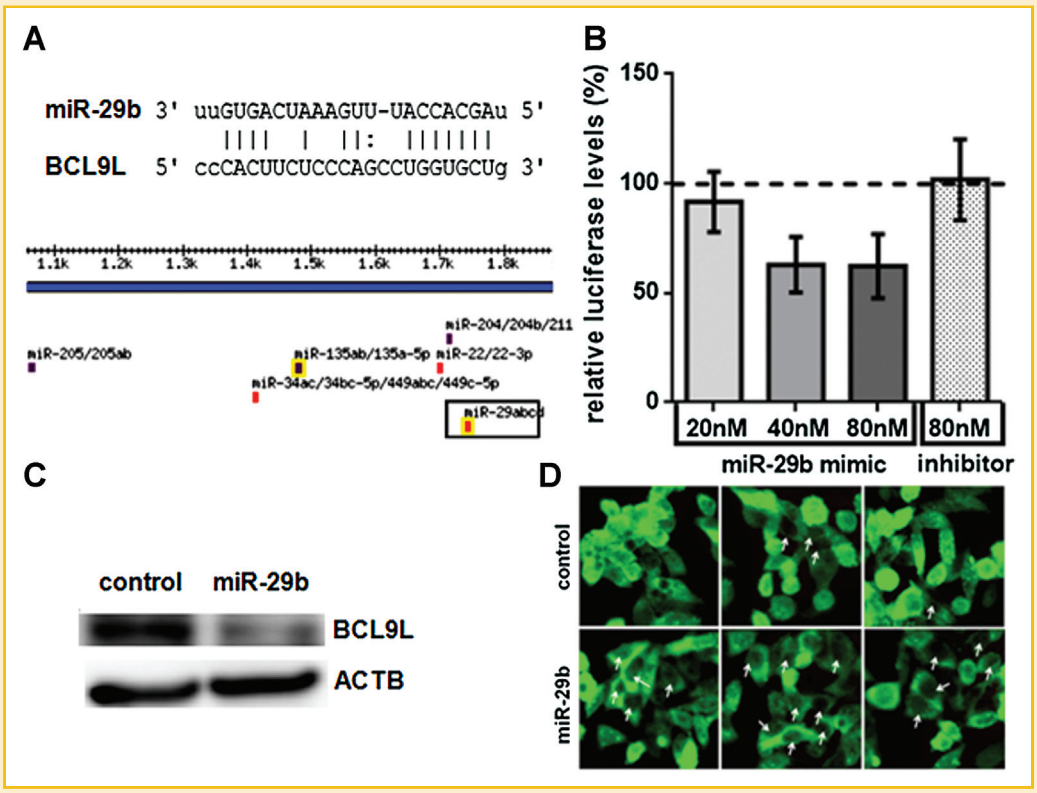

Fig. 5. Effect of ectopic expression of miR-29b on BCL9L expression and CTNNB1 localization in SW480 cells. (A) miRanda algoritm prediction of base pairing between miR-29b and BCL9L > 3′UTR and TargetScan6.2 prediction of miRNA binding sites on the BCL9L > 3′UTR. (B) SW480 cells were cotransfected with the BCL9L > 3′UTR luciferase construct or pMIR-report Luciferase vector and pRL-TK plasmid. This was followed by a second transfection after 24 h, with increasing concentrations of miR-29b mimic (20 nM, 40 nM, or 80 nM) or 80 nM of miR-29b inhibitor. After another 24 h, the cell lysates were assayed for the firefly and renilla luciferase activities. The firefly luciferase activity was normalized against the renilla luciferase activity and the corresponding pMIR-report Luciferase control for each concentration. Results are represented as percentage of luciferase activity of control oligo transfected cells (n = 2, mean ± S.D). (C) Whole cell lysates of SW480 cells transfected with control oligo or miR-29b mimic were prepared 24 h post transfection and subjected to SDS/PAGE and immunoblotting for detection of BCL9L and ACTB. The same results were observed in another independent experiment. (D) SW480 cells transfected with control oligo or miR-29b mimic were fixed, permeabilized and stained with anti-CTNNB1 primary antibody followed by FITC-conjugated secondary antibody. Images of FITC-fluorescing cells (three fields) were taken at 20x magnification using Nikon Eclipse TS-100 microscope with B-2A fluorescence filter set.

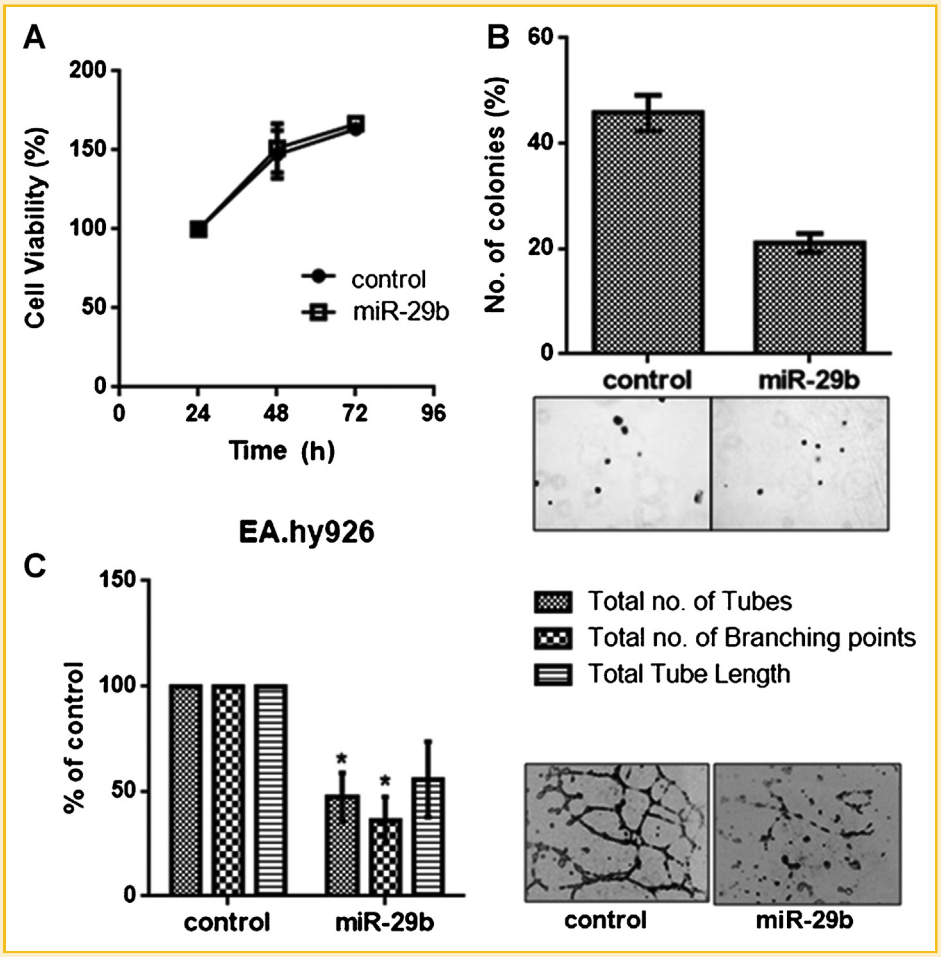

Fig. 6. Effect of ectopic expression of miR-29b in SW480 cells on anchorage dependent and independent cell growth and TCM-induced in vitro endothelial tube formation. (A) Anchorage dependent cell viability of SW480 cells transfected with control oligo or miR-29b was assayed using resazurin reduction method. Cell viability at 24, 48, and 72 h was expressed as percentage of cell viability at 24 h ($n = 3$, mean ± S.E). (B) SW480 cells transfected with control oligo or miR-29b mimic were grown in 0.3% agarose in cDMEM for 15 days. Images of colonies were taken under microscope at 10x magnification and ImageJ was used to measure colony size. Colonies with > average radius of control oligo transfected colonies were counted for each sample ($n = 2$, mean ± S.D). (C) EA.hy926 cells were suspended in the TCM collected from SW480 cells transfected with control oligo or miR-29b mimic and seeded on matrigel in 96 well plates and images were taken under microscope at 4x magnification after 18 h incubation. Total number of tubes and branching points and total tube length in each sample were estimated using WimTube (Wimasis GmbH) online analysis tool ($n = 3$, mean ± S.E; *$P < 0.05$ vs. control).

adhesion of SW480 cells to uncoated plastic plates and increased cell death under this low adherent condition at 48 h post transfection (Supplementary Fig. 4). The serum free TCM of miR-29b-transfected SW480 cells had a greatly reduced potential to induce in vitro tube formation on matrigel by EA.hy926, an endothelial cell line derived from human umbilical vein (Fig. 6C), and ECV304, a bladder carcinoma cell line which retains tube forming properties of endothelial cells (Supplementary Fig. 5). There was no significant difference in the proliferation or migration of EA.hy926 cells treated with TCM from miR-29b-transfected SW480 cells as compared to control (Supplementary Fig. 6). Together these results suggest that miR-29b plays a role in inhibiting anchorage independent cell growth of SW480 cells and reduces the ability of TCM (derived from SW480 cells) to induce in vitro tube formation in endothelial cells.

Thus we propose that miR-29b inhibits the expression of many coactivators and downstream targets of CTNNB1/Wnt signaling in colorectal cancer cells resulting in the inhibition of anchorage independent cell growth, tumor angiogenesis and EMT (Fig. 7).

DISCUSSION

Our results in HEK293 and colorectal carcinoma cells clearly indicate for the first time that miR-29b acts as a negative regulator of β-catenin/Wnt signaling. However, in previous studies it was reported that the miR-29 family activates canonical Wnt signaling by directly targeting inhibitors of the pathway in osteoblasts and HEK293T cells [Kapinas et al., 2010; Liu et al., 2011]. This apparent contradiction is not very surprising as many miRNAs are known to act in a context-dependent manner depending on the relative availability of their targets in any cell type with miR-29 family itself being a prime example of this phenomenon [Pekarsky and Croce, 2010]. In

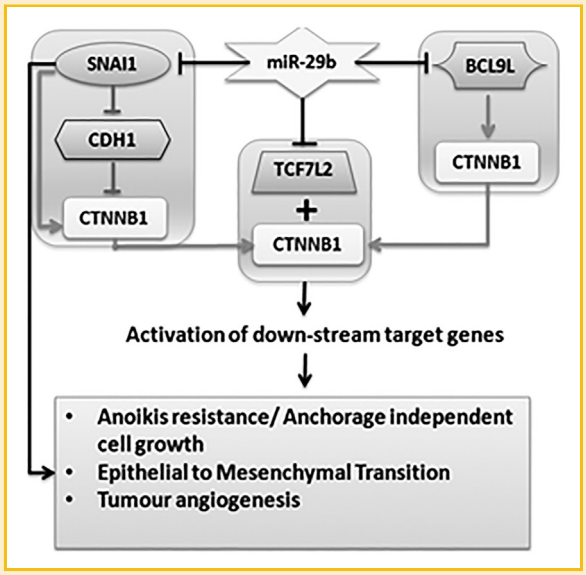

Fig. 7. Proposed mechanism of CTNNB1/Wnt signaling regulation by miR-29b in human colorectal cancer cells. The present work provides evidence that miR-29b inhibits the expression of many coactivators of CTNNB1/Wnt signaling and its downstream targets including BCL9L, which is a novel target of miR-29b. Consequently, anchorage independent cell growth, tumor angiogenesis and EMT are inhibited by miR-29b.

addition, miR-29b can directly target and repress GSK3B expression or repress GSK3B inhibition by phosphorylation but in SW480 cells, the presence of a mutant APC prevents CTNNB1 degradation irrespective of its phosphorylation status [Yang et al., 2006]. Our study therefore emphasizes the need to determine the roles of microRNAs in specific contexts. Our data showing decrease in the expression of TCF7L2, key interacting partner, and SNAI1 and BCL9L, coactivators of β-catenin by miR-29b, are novel and support the downregulation of β-catenin/Wnt signaling by miR-29b. The decrease in nuclear β-catenin with a concurrent increase in its membrane localization in SW480 cells is likely to be a consequence of decreased expression of BCL9L by miR-29b since it fits with the proposed role for BCL9L in the switch between the roles of β-catenin in adhesion and transcription [Brembeck et al., 2004]. It is also relevant to note that TCF7L2 and BCL9L are also Wnt-inducible genes [de la Roche et al., 2008; Yang et al., 2010] and SNAI1 protein is stabilized by GSK3B inhibition [Zhou et al., 2004]. Therefore, they may be involved in a feedback loop where their downregulation by miR-29b may both be the cause and consequence of β-catenin/Wnt signaling inhibition. Our observation that miR-29b decreases the anchorage independent cell growth and angiogenic potential of conditioned medium of SW480 can be partly attributed to the downregulation of β-catenin transactivation potential and the concomitant repression of its downstream targets or a repression of the β-catenin independent function of SNAI1. SNAI1 promotes tumorigenicity, epithelial to mesenchymal transition (EMT) and tumor angiogenesis independent of β-catenin [de Herreros et al., 2010; Hwang et al., 2011]. Increase in the expression of epithelial marker CDH1, and decrease in expression of the mesenchymal markers, VIM and FN1, observed in this study indicate a reversal of EMT. The Wnt signaling inhibition by miR-29b in p53 null cells and in SW480 cells with a mutant oncogenic p53 as observed in this study suggests that wild type p53 is not entirely required for this regulation. miR-29b-mediated decrease in the mRNA of *CCND1, BIRC5, VIM, FN1* and *VEGFA* observed in this study correlates with the downregulation of Wnt signaling by it although these genes are not exclusively regulated by β-catenin/Wnt signaling. That miR-29b did not affect cell growth in adherent conditions but decreased the anchorage independent cell growth is consistent with the inability of miR-29b in inducing cell death under adherent conditions demonstrated earlier (Park et al., 2009).

The loss of adhesion to the substratum by SW480 cells upon ectopic expression of miR-29b could be due to the repressive effect of miR-29b on the expression of FN1 (Fig. 3E) and also presumably because of its similar effects on the expression of laminin and collagen [Luna et al., 2009; Steele et al., 2010]. An inverse correlation between miR-29b and *BIRC5, VIM, VEGFA* and *FN1* has been demonstrated [Villarreal et al., 2011; Amodio et al., 2012; Chou et al., 2013] and *VEGFA* is a known direct target of the miR-29 family [Chou et al., 2013]. The reduced potential of the tumor cell conditioned medium to promote in vitro tube formation upon over expression of miR-29b observed in this study is in agreement with similar effects of miR-29b in hepatocellular carcinoma and breast cancer cells in which it was mediated through downregulation of MMP2, MMP9, PDGF family of proteins and other pro-angiogenic factors which are direct targets of miR-29b [Fang et al., 2011; Chou et al., 2013]. Our data suggest that mir-29b has the potential to target cancer epithelium and endothelial cells (angiogenesis) by interfering with canonical Wnt signaling. Our results have thus unravelled a novel role of miR-29b in Wnt signaling in human colorectal cancer cells with implications in the treatment of colorectal cancer.

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