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Behzad Mansoori ORCID iD: 0000-0001-9444-7134

Behzad Baradaran ORCID iD: 0000-0002-8642-6795

MiR-330 suppresses EMT and induces apoptosis by down-regulating HMGA2 in human colorectal cancer

Behzad Mansoori [1,2,3], Ali Mohammadi [4], Sanaz Naghizadeh [1], Morten Gjerstorff [4], Dariush Shanehbandi [1], Solmaz Shirjang [1], Souzan Najafi[1], Uffe Holmskov [4], Vahid Khaze [1], Pascal H.G. Duijf [5*], Behzad Baradaran [1,6*]

1. Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

2. Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran.

3. Aging Research Institute, Physical Medicine and Rehabilitation Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

4. Department of Cancer and Inflammation Research, Institute for Molecular Medicine, University of Southern Denmark, Odense, Denmark.

5. University of Queensland Diamantina Institute, The University of Queensland, Translational Research Institute, Brisbane, Australia

6. Department of Immunology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.

Corresponding authors: Behzad Baradaran, Ph.D., Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. Phone: +98-41-33371440 Fax: +98-41-33371311, Email: baradaranb@tbzmed.ac.ir

This is the author manuscript accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jcp.29007.

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Pascal H.G. Duijf, Ph.D., University of Queensland Diamantina Institute, The University of Queensland, Translational Research Institute, Brisbane, Australia. Phone: +61 7 344 36937, Email: p.duijf@uq.edu.au

Author contribution statement

Behzad Mansoori developed the hypotheses for this research.

Behzad Baradaran and Pascal H.G. Duijf planned the methodology.

Behzad Baradaran and Behzad Mansoori organized, supervised and were responsible for the course of the project and the article.

Behzad Baradaran provided personnel, environmental and financial support, tools, and instruments that were vital for the project.

Behzad Mansoori and Ali Mohammadi provided biological materials, reagents and referred patients.

Behzad Mansoori, Sanaz Naghizadeh, Solmaz Shirjang, Dariush Shanehbandi, Vahid Khaze and Souzan Najafi were responsible for the execution of the experiments, patient follow-up, data management and reporting.

Behzad Mansoori, Pascal H.G. Duijf, Morten F. Gjerstorff were responsible for the interpretation and presentation of the results.

Behzad Baradaran was responsible for overall supervision of this work.

Behzad Mansoori was responsible for the construction of the whole or body of the manuscript.

Pascal H.G. Duijf, Morten F. Gjerstorff, and Uffe Holmskov reviewed and edited the article before submission.

Funding statement

Iran national science foundation [grant number 93041394].

Abstract

MicroRNAs (miRNAs) are important molecular regulators of cellular signaling and behavior. They alter gene expression by targeting mRNAs, including those encoding transcriptional regulators, such as HMGA2. While HMGA2 is oncogenic in various tumors, miRNAs may be oncogenic or tumor suppressive. Here, we investigate

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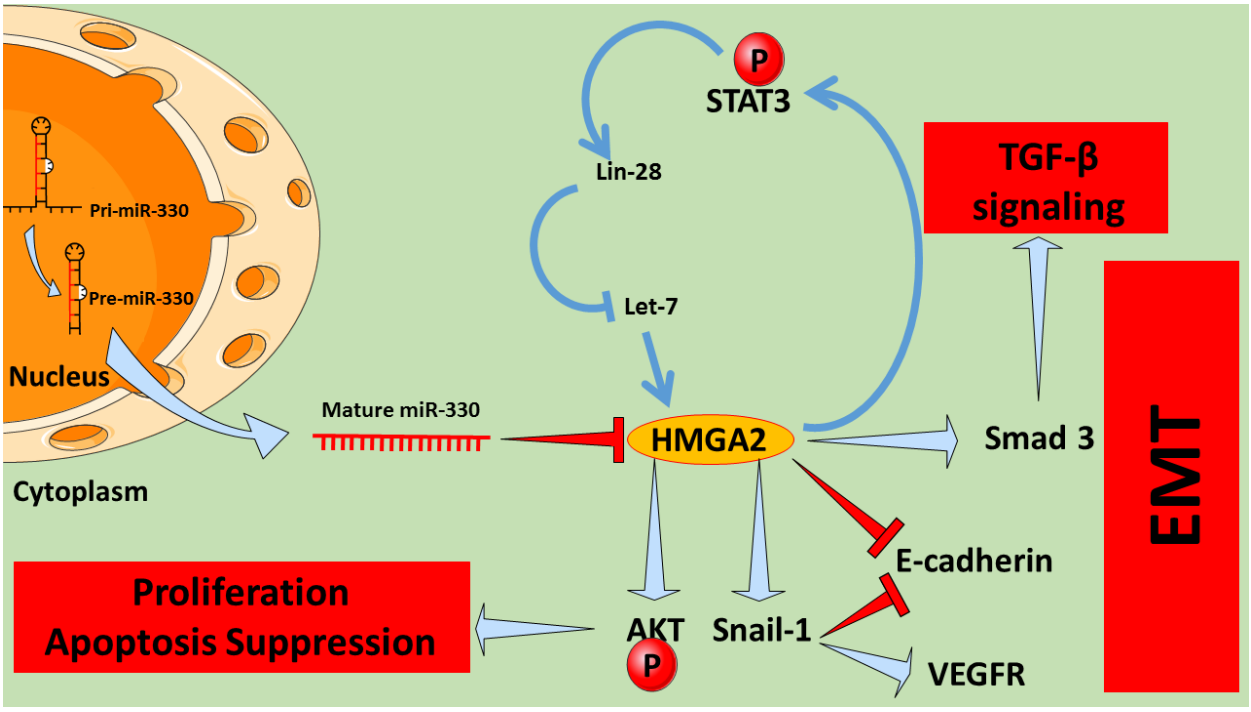
expression of HMGA2 and the miRNA miR-330 in colorectal cancer patient samples and their effects on oncogenic cellular phenotypes. We found that HMGA2 expression is increased and miR-330 expression is decreased in colorectal cancers and each predicts poor long-term patient survival. Stably increased miR-330 expression in HCT116 and SW480 colorectal cancer cell lines downregulates oncogenic expression of HMGA2, a predicted miR-330 target. Additionally, this promotes apoptosis and decreases cell migration and viability. Consistently, it also decreases protein-level expression of markers for epithelial-to-mesenchymal transition (Snail-1, E-cadherin, VEGFR) and TGF-β signaling (SMAD3), as well as phospho-AKT and phospho-STAT3 levels. We conclude that miR-330 acts as a tumor suppressor miRNA in colorectal cancer by suppressing HMGA2 expression and reducing cell survival, proliferation and migration. Thus, we identify miR-330 as a promising candidate for miRNA replacement therapy for colorectal cancer patients.

Graphical Abstract

In this study, we show that increasing miR-330 expression in colorectal cancer cells induces apoptosis and suppresses cell viability and migration. We also show that miR-330 suppresses HMGA2 expression and leads to constitutively suppressed Smad3, VEGFR, Snail-1, p-AKT and p-STAT3 and increase E-cadherin proteins level in CRC cells. Altogether, these results specify that miR-330 could be as a TS-miRNA in colorectal cancer



Keywords: miR-330, HMGA2, Snail-1, Smad3, Apoptosis, Colorectal cancer

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Introduction

With more than one million newly diagnosed cases annually, colorectal cancer (CRC) is ranked third most common in worldwide (Davudian et al., 2016). Although the 5-year survival rate of early-stage of this disease is around 90%, this decreases to less than 5% for advanced stage metastatic CRC (Yoo et al., 2016). CRC treatments include chemotherapy, colectomy, radiotherapy, as well as neoadjuvant, adjuvant and targeted therapies (Chen et al., 2018).

MicroRNAs (miRNA) are a 18-24 nucleotides non-coding small RNAs involved in many biological processes, such as cell proliferation, differentiation, apoptosis and invasion. Depending on the target gene(s) that is/are suppressed, miRNAs could act as tumor suppressors or oncogenes (Karimi et al., 2017; Mansoori et al., 2015).

As a family member of miRNAs, miR-330 first explain by Weber in 2005 (Weber, 2005). The gene of this miRNA is located on chromosome 19q12.32 in the first intron of the *EML2* gene. This gene has two subservient strands, 3p, and 5p (Liu et al., 2017; Qu et al., 2012). This miRNA was found to have a paradoxical role in carcinogenesis. It has been demonstrated that miR-330 is an oncomiR in esophageal cancer (Meng et al., 2015) and glioblastoma (Helmke et al., 2012), whereas it acts as a tumor suppressor in prostate cancer (Lee et al., 2009), cutaneous malignant melanoma (Yoo et al., 2016) and colorectal cancer (Li et al., 2013). These conflicting results suggest that miR-330 promotes or suppresses tumor development in a tissue-dependent manner (Meng et al., 2015). MiR-330 overexpression alleviated the capacity of cells to proliferate, migrate and invade to other tissues in murine colon cancer cells (Ai et al., 2015). Another study showed that miR-330 targets TYMS in CRC cells and enhances chemosensitivity to 5-FU by simultaneously inducing apoptosis and suppressing cell proliferation. These reports propose that miR-330 could be a tumor suppressor miRNA (TS miRNA) in CRC (Xu et al., 2017).

HMGA2, also called HMGI-C, encodes a non-histone chromosomal protein belonging to the high mobility group A family genes and it is located on chromosome 12q24 (Wang et al., 2011). Via its three AT-hook DNA binding domains, HMGA2 binds to the minor groove of the DNA at AT-rich sites and alters the chromatin structure to recruit components that either promote or suppress downstream gene expression. HMGA2 is involved in a wide variety of cell biological processes, such as cell growth, differentiation and proliferation. HMGA2 overexpression has been found in a range of malignant epithelial tumors, including breast, gastric, lung, ovarian, pancreas, melanoma and colorectal cancers (Hawsawi et al., 2018; Mansoori et al., 2016a; Mohammadi et al., 2016; Sun et al., 2017; Thanasupawat et al., 2017; Zhao et al., 2016).

Our previous study, showed that HMGA2 promotes epithelial to mesenchymal-transition (EMT), cell invasion and cell growth (Esmailzadeh et al., 2017). Wang et al. showed that HMGA2 overexpression is associated with reduced CRC patient survival and promotes distant metastasis of colon cancer cells (Wang et al., 2011). These findings are in concordance with results of another research study, which found that high expression of HMGA2 induces EMT and invasion of colon cancer cells *in vitro* and *in vivo* (Li et al.,

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2014). In addition, HMGA2 is overexpressed in epithelial and mesenchymal transformed cells. Previous studies showed that HMGA2 regulates expression of EMT transcription factors, such as SNAIL1, ZEB1, ZEB2, TWIST and SLUG, which directly links altered HMGA2 expression to tumor invasiveness and metastasis. Finally, another study reported that high expression of HMGA2 leads to induction of the ERK/EMT signaling pathway and this promotes migration and invasion of ovarian cancer cells (Huang et al., 2017).

In this study, we show that increasing miR-330 expression in colorectal cancer cells induces apoptosis and suppresses cell viability and migration. We also show that miR-330 suppresses HMGA2 expression and leads to constitutively suppressed Smad3, VEGFR, Snail-1, p-AKT and p-STAT3 and increase E-cadherin proteins level in CRC cells. All together, these results specify that miR-330 could be as a TS-miRNA in colorectal cancer.

Materials and methods

2.1. Gene expression and survival analyses

Clinical details, gene expression RNAseq (HiSeqV2) and miRNA mature strand expression RNAseq (HiSeq) from The Cancer Genome Atlas (TCGA) were from the colon adenocarcinoma (COAD) and rectal adenocarcinoma (READ) cohorts (Nature, 2012) and samples were combined into the COADREAD cohort. Both mRNA and miRNA expression levels were processed as described (Thangavelu et al., 2016; Vaidyanathan et al., 2016a). They were shown as log2(norm_RPM+1), i.e., the log2 of the normalized reads-per-million (RPM), and expression levels were compared using Mann-Whitney *U* tests. Overexpression and underexpression were called if the expression level of the gene or miRNA was, respectively, above or below the range of expression observed in normal tissue, with 95% confidence interval. Overall and recurrence-free survival curves were generated for high and low expression as described (Vaidyanathan et al., 2016b), using the median expression levels as cut-offs and log-rank tests for statistical analyses.

2.2. Bioinformatics analysis

miR-330-3p and -5p sequences were extracted from the miRbase database (http://www.mirbase.org/). Binding of miR-330-3p and -5 to the 3'UTR sequences of HMGA2 was predicted by miRWalk (http://mirwalk.umm.uni-heidelberg.de/), miRTarbase (http://mirtarbase.mbc.nctu.edu.tw/php/index.php), Targetscan (http://www.targetscan.org/vert_72/) and miRmap (https://mirmap.ezlab.org/) online software and the sequences analyzed by snap gene software (GSL Biotech; available at snapgene.com).

2.3. Cell lines and construct transfection

Human colorectal cancer cells (HCT116 and SW480) were obtained from the cell bank collection of Pasteur Institute (Tehran, Iran). The cells growth in RPMI-1640 supplemented with10% FBS medium (GIBCO, Carlsbad, CA, USA) in cell culture incubator with condition includes 37ºC, 95% humidity and 5% Co2. All the experiment

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preformed when the cells reached the logarithmic phase. Before transfection the cells seeded at total number of 2×10^5 cells per 6-well cell culture plate. The cells transfected by Empty PCMV vector and miR-330 PCMV vector were obtained from OriGene (OriGene, Rockville, MD) at the 60% cell confluence. Briefly, 6 µl transfection reagent (jetPEI®, Polyplus Co., Illkirch, France) was diluted in 94 µl 150 mM NaCl solution. In the other tube 6000 µg/ml of pCMV vector was diluted in 150mM NaCl solution. The two solution mixed together and incubated in RT for 30 min. Afterward, the mixture contained DNA complex were added to the corresponded well and shake the plate gently. The procedure did in same way for negative control (NC CTRL) or mock group. Then the plate incubated in cell culture incubator for 6 hours. The vectors contain antibiotic resistance gene for Geneticin. After 6 hours of transfection the vector positive cells selected with 8µl of Geneticin (50 mg/ml) antibiotic (Gibco, USA) for 14 days. The selection efficacy was evaluated by a flow cytometer, a fluorescent microscope with a live-cell imaging system (Cytation 5, Biotek, Winooski, VT) and its software Gen 5. Finally, the expression level of miR-330 was measured by qRT-PCR (see below).

2.4. mRNA and miRNA expression analysis

Total cellular RNA were isolated using RiboEX reagent (GeneAll Biotechnology, Korea). One microgram of total RNA used for cDNA synthesis using BioFACT RT-Kit (BioFACT, Korea) for mRNA and miScript II RT Kit (Qiagen) for miRNA. The qRT-PCR preform using SYBR green method using 2X SYBR green master mix (BioFACT, Korea) and specific primers (SinaClon, Iran)(Table 1), via LightCycler 96 instrument (Roche Diagnostics, Mannheim, Germany).

2.5. Cell proliferation assay

The proliferation of CRC cells were measured by MTT assay. 10×10^3 number of stable miR-330 and empty vector cells were seeded in 96-well culture plates. After 48 hours, 50 µl of MTT solution (Sigma-Aldrich, St. Louis, MO) (2 mg/ml in PBS) was added to each well contain 100 µl media and incubated the plate for 4 h in cell culture incubator and dark condition. After that the media contain MTT solution discard and the formazan crystals were dissolved by 100 µl of DMSO and shaking in 1000 rpm. The optical density of each well measured using an ELISA reader (Sunrise; Tecan Co., Salzburg, Austria) at a wavelength of 570 nm.

2.6. Apoptosis assay

2.6.1. Annexin/PI assay

The percentage of apoptosis and necrosis of HCT116 and SW480 cells in miR-330 and mock group were determined by annexinV/PI assay. Briefly, 2×10^5 cells seeded per well of 6-well cell culture plate. After 48 h the cells detached via trypsin EDTA 0/05% and stained with ApoFlowEx ® FITC Kit; was bought from EXBIO (EXBIO, Vestec, Czech Republic) according to the EXBIO instructions. The percentage of apoptotic cells were assessed through MACS Quant 10 flow cytometry instrument (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). For analysis, the cells were gated from FCS and SSC

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chart and the gated cells were selected to investigate the percentage of FITC-/PI-, FITC+/PI-, FITC+/PI+, and FITC-/PI+ cells determined in the FITC relative to the PI channel. The obtained data were analyzed using the FlowJo software package (Treestar, Inc., San Carlos, CA). The sum of percentages of FITC+/PI- cells (early apoptosis) and FITC+/PI+ cells (late apoptosis) was reported as total apoptosis.

2.6.2. DAPI staining

To assess the effect of miR-330 induction on nucleus fragmentation, the cells stained with DAPI dye. In this regard, the cells were fixed with 4% of paraformaldehyde for 4 h. After that, the permeabilization of cells performed by incubation with Triton X-100 (0.1%) for 5 min. Subsequent, the cells were stained with DAPI solution (0.1% in PBS) for 5 min. Finally, nucleus fragmented cells were monitored using fluorescence imaging microscope (Cytation 5, Biotek, Winooski, VT).

2.7. Migration assay

To evaluate the effect of miR-330 on the cellular mobility of HCT116 and SW480 cells, $5x10^5$ cells were seeded. After cells filled the entire bottom of wells, a scratch was generated to each well via a yellow tip. The number of migrated cells into the scratched area evaluated after 48 hours by an inverted light microscope (Optika, XDS-3, Bergamo, Italy).

2.8. Western blot analysis

Total cellular protein was extracted after generating the stable miR-330 expressing HCT116 and SW-480 using RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) according to Santa Cruz's instructions. The vertical electrophoresis performed by SDS-PAGE method. Fifty microgram of total protein from each sample loaded into the gel (4% stacking and 10% running). After that the proteins blotted into PVDF membrane (Roche, Germany) by semi-dry western blot transfer system (Bio-Rad). The membrane blocked with tween-20 (0.5% in PBS) for 2h. Subsequently, the membrane incubated with goat monoclonal antibody against HMGA2, Snail-1, and b-actin, and mouse monoclonal anti-AKT, -P-AKT, -STAT3, -P-STAT3,-E-cadherin and -VEGFR antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and mouse anti-Smad3 monoclonal antibody was bought from Abnova Corporation (Taipei, Taiwan). Then the membrane incubated with rabbit and mouse anti-goat secondary antibody conjugated with HRP. The protein bands visualized by ECL kit (Roche, Germany) via Western blot imaging instrument (Sabz.co, Iran). The proteins bond density measured using ImageJ software (NIH, Bethesda, Maryland) and normalized with beta-actin bands.

2.9. Statistical analyses

All experiments were repeated three times. The data were expressed as mean ± standard deviation (SD). The data analyzed via GraphPad Prism software (Graph Pad Prism version7; San Diego, CA, USA) or the *R* statistical environment (*R* Core Team, Vienna,

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Austria). The Student's t-test and ANOVA were used to understanding the significance between the groups. *P* values less than 0.05 were considered significant differences.

3. Results

3.1. Overexpression of HMGA2 and underexpression of miR-330 associate with poor colorectal cancer patient survival

Statistical analysis of mRNA expression data from The Cancer Genome Atlas (TCGA) showed that HMGA2 is overexpressed in human colorectal cancer compared to adjacent normal tissues ($p=1.1\times10^{-9}$) (Figure 1A). Also, both miR-330-3p and miR-330-5p are downregulated in tumor tissues compared to their adjacent normal tissues (p=0.0011 and $p=6.7\times10^{-4}$, respectively) (Figure 1B, C). Colorectal cancers that show underexpression of either miR-330-3p or miR-330-5p significantly show overexpression of HMGA2 ($p=7.2x10^{-15}$ and $p=2.2x10^{-7}$, respectively; Fisher's exact test) (Figure 1D, E).

Over 300 patients were included for overall survival (OS) and recurrence-free survival (RFS) analyses using clinical data and HMGA2, miR-330-3p and miR-330-5p expression data from TCGA. This showed that there is a significant difference in RFS between patients whose tumors express low and high HMGA2, with the latter showing a poorer outcome (p= 0.0258, Figure 1F-G). We did not observe significant differences in OS or RFS for patients showing low versus high miR-330-3p or miR-330-5p expression (p>0.05) (Figure 1H-K). However, we note that from the 4-year survival mark onward, the survival curves consistently diverge, consistent with the prediction that patients with low miR-330-3p or miR-330-5p expression levels do more poorly than those with high miR-330-3p or miR-330-5p expression levels, respectively (Figure 1H-K). In addition, around 40% of patients with high miR-330-3p or miR-330-5p expression levels show recurrence-free events, whereas this percentage is nearly 60% for patients with low miR-330-3p or miR-330-5p expression levels (Figure 1I, K). Finally, for both miR-330-$3p^{low}$ and miR-330-$5p^{low}$ patients, the median survival time is 9.0 years (108.9 months), whereas by 12.3 years (148 months) still less than 50% of miR-330-$3p^{high}$ and miR-330-$5p^{high}$ patients show recurrence. Thus, these data indicate that HMGA2 is overexpressed in colorectal cancer and this predicts poor patient survival, while miR-330-3p or miR-330-5p are underexpressed and this associates with poor long-term clinical outcome for colorectal cancer patients.

3.2. Stable miR-330 overexpression increases miR-330 expression in colorectal cancer cells

We next aimed to increase miR-330 expression in a stable manner in the colorectal cancer cell lines HCT116 and SW480. Following transfection of the pCMV-GFP-miR-330 vector and empty pCMV-GFP vector as a control into these CRC cell lines, we treated the cells with Genticin for 14 days to select for stably transfected cells. We then used flow cytometry, fluorescence microscopy and qRT-PCR to evaluate the number of vector-positive cells. This showed that 58% and 69% of HCT116 and SW480 cells were positive for the pCMV-miR-330 construct, respectively (Figure 2A-D). Then, qRT-PCR analysis showed a significant induction in miR-330 expression in each of the stable cell

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lines (Figure 2E, F). Thus, collectively, these data indicate that we generated two CRC cell lines, HCT116 and SW480, that stably expressed miR-330.

3.3. miR-330 downregulates HMGA2 expression

Bioinformatics analysis showed that miR-330-3p and miR-330-5p may target HMGA2 mRNA with prediction scores of 31.87 and 84.35, respectively (Figure 3A). Consistently, qRT-PCR analysis of our stable miR-330-expressing HCT116 and SW480 cells showed that HMGA2 mRNA levels were decreased to fractions of 0.34±0.03 and 0.45±0.03 in miR-330-replaced cells, respectively (Figure 3B). In addition, Western blot analysis showed that increased miR-330 expression reduced HMGA2 protein expression to fractions of 0.53±0.04 and 0.45±0.03 in HCT116 and SW480 cells, respectively (Figure 3C, D). These data indicate that miR-330 downregulates HMGA2 at both mRNA and protein level.

3.4. miR-330 replacement promotes apoptosis in colorectal cancer cells

To demonstrate whether the induction of miR-330 induce apoptosis or not, the annexin-V/PI assays was performed on the HCT116 and SW480 cells. This showed that in miR-330-expressing cells, apoptosis was increased to 15.57±2.34 and 21.56±2.6 in HCT116 and SW-480, respectively, compared to scrambled miRNA treated cells, which respectively showed 1±0.45% and 1.6±0.25% apoptosis (Figure 4A, B). Additionally, DAPI staining of the cells also confirmed chromatin fragmentation, a hallmark feature of apoptotic cells, in miR-330-expressing cells compared to scramble miRNA control cells (Figure 4C). Jointly, these results indicate miR330 induces apoptosis in these two colorectal cancer cell lines.

3.5. miR-330 decreases colorectal cancer cell migration and viability

To investigate whether miR-330 transfection affects colorectal cancer cell migration, we performed wound healing assays. This showed that increased miR-330 expression leads to reduced cell migration of colorectal cancer cells into the scratch area (Figure 5A, B). At 48 hours after applying the scratch, the numbers of HCT116 and SW480 cells in the scratch area were 39.3±8.2 and 62.6±6.33, compared to 109.6±6 and 156±6 for the respective negative control cells (Figure 5A, B). We also performed MTT assays to assess the effect of increased miR-330 expression on cell viability. This showed that increased miR-330 expression inhibited cell viability by 35±4.46% and 42±5.74% in HCT116 and SW480 cells, respectively (Figure 5C). Importantly, these levels of reduction in cell viability are considerably lower than the reduced levels of migrated cells into the scratch area measured above, which involved reductions of $100 \times (1 - \frac{39.3}{109.6}) = 64.1\%$ and $100 \times (1 - \frac{62.6}{156}) = 59.9\%$, respectively. This indicates that the reduced migratory capacity following increased miR-330 expression cannot be solely explained by a reduction in cell viability. Therefore, we conclude that a cancerous reduction of miR-330 expression in colorectal cancer cells promotes both cell viability and cell migration.

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3.6. miR-330 transfection in colorectal cancer cells decreases protein-level expression of markers for cell survival, proliferation, migration, invasion, metastasis and angiogenesis

Above, we found that HMGA2 expression is increased and miR-330 expression is decreased in colorectal cancer, and that miR-330 reduces HMGA2 expression (Figures 1A-C, 3B-D). Since HMGA2 is involved in metastasis, we next studied if the induction of miR-330 expression could impact the expression of oncogenic and epithelial-to-mesenchymal transition (EMT)-related proteins in our HCT116 and SW480 cell lines. Hence, we performed Western blot analyses to probe for Smad3, Snail-1, E-cadherin, VEGFR, AKT, p-AKT, STAT3, and p-STAT3 protein levels. We found that the relative protein expression levels of AKT and STAT3 were not significantly different after increased miR330 expression (Figure 6A-C). However, their phosphorylation levels were significantly reduced to 0.63±0.04 and 0.47±0.02 for p-AKT and to 0.68±0.02 and 0.43±0.02 for p-STAT3, respectively (Figure 6A,D,E). Consistent with our data above, these reduced phosphorylation levels mark decreased cell survival, proliferation and migration.

Further, we found that increased miR-330 expression reduced Smad3 expression to 0.82±0.03 and 0.51±0.01, VEGFR expression to 0.72±0.03 and 0.34±0.03, Snail-1 expression to 0.76±0.01 and 0.31±0.02, in contrast, E-cadherin increased to 1.65±0.03 and 1.51±0.01, respectively in our colorectal cancer cell lines (Figure 6F-H). The reduced expression of these markers indicates a reduced potential of cell migration, invasion, metastasis and angiogenesis following increased miR-330 expression. Taken together, our results indicate that increasing miR-330 expression markedly reduces the expression of a broad range of oncogenic markers.

Discussion

MiR-330 was identified as a tumor suppressor that promotes apoptosis and inhibits metastasis in most solid tumors. Studies have shown that this miRNA could target several genes involved in cell growth and proliferation, including E2F1 (Sebastiani et al., 2017), NOB1 (Kong et al., 2017), SP1 (Mao et al., 2013), MUC1 (Tréhoux et al., 2015), SRPR (Kim et al., 2016), PDIA3 (Kim et al., 2015), CTNNB1 (Zhan et al., 2017b) and MMP13(Ai et al., 2015).

Using clinical data from TCGA, our results showed that HMGA2 is overexpressed in colorectal tumor samples compared to normal colorectal tissues. Although most prior studies have reported that HMGA2 is increased in various cancers, outcomes of studies comparing expression levels of HMGA2 in tumor and non-tumor colon cells are conflicting. For instance, it has been shown that HMGA2 is elevated in all colon cancer cell lines (HT-29, COLO 205, LS 174T, SW480, SW620) in comparison to the normal human colon epithelial cell line CCD-18Co (Li et al., 2014). On the other hand, Burkhard Maria Helmke and colleagues showed that in CRC samples, HMGA2 expression varied over a 132-fold range in comparison to matched normal tissue samples. Also, another study determined that HMGA2 expression in the DUKES stage A and B groups in tumor and non-tumor colorectal cancer presented no significant differences, whereas in the

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DUKE C and D stages groups, HMGA2 expression was significantly higher in tumorous cells (Huang et al., 2009). Here, aside from showing that HMGA2 is overexpressed in colorectal cancers, we also find that overexpression predicts poor recurrence-free patient survival.

Our analyses of TCGA samples also showed that miR-330-3p and miR-330-5p were downregulated in colorectal tumor tissues compared to normal tissues. Reduced expression of this miR-330 in human colon cancer cell lines has been reported previously. This observation is concordant with results of another study, which reported that mir330-5p expression was remarkably decrease in CRC tissues compare to adjusted normals. miR-330 downregulation in CRC cell lines in comparison to normal colon cell line (FHC) is also consistent with this (Ai et al., 2015; Xu et al., 2017; Yoo et al., 2016). Additionally, we here found that both low miR-330-3p and low miR-330-5p expression associate with poor long-term clinical outcome for colorectal cancer patients. Our bioinformatics analysis further suggested that miR-330-3p, and particularly miR-330-5p, could target HMGA2 mRNA. Consistently, we demonstrated that both HMGA2 mRNA and protein expression levels decreased after miR-330 induction in colorectal cancer cell lines.

Proliferation assays showed that transfection of miR-330 decreases proliferation of two colorectal cancer cell lines. We also performed flow cytometry analyses to show that miR-330 induction increases apoptosis in the colorectal cancer cells.

Our migration assays showed that induction of miR-330 expression inhibits colorectal cancer cell migration. In a study conducted by Zhan et al., transfection of miR-330-5p mimic inhibited migration of A549 lung carcinoma cells. The authors suggested that the anti-metastatic effect of curcumin is due to modulation of miR-330 levels in these cells (Zhan et al., 2017a). In another study, miR-330-5p was found to directly bind to the 3'UTR of the ITAG5 mRNA in CRC tumors, leading to downregulation of this gene and decreased migration and invasion capacity (Yoo et al., 2016). miR-330 was also shown to regulate MMP13 expression and this attenuated proliferation, migration and invasion of murine colon cancer cells (Ai et al., 2015).

Our current study also demonstrates that increased miR-330 expression reduces the expression levels of Snail-1, VEGFR, Smad3 and the phosphorylated forms of AKT (p-AKT) and STAT3 (p-STAT3), and increases E-cadherin expression level. This indicates that the reduced miR-330 expression that is seen in colorectal tumors contributes to the acquisition of a wide variety of oncogenic features, including cell survival, proliferation, migration, invasion, metastasis and angiogenesis.

There are multiple reports portending AKT pathway activation by HMGA2. In one of those, Yu et al. demonstrated that HMGA2 induced AKT/mTOR/P70S6K phosphorylation, which in turn repressed p16 and p21 expression in human umbilical cord blood-derived stromal cells and thereby promoted cell proliferation (Yu et al., 2013). Similarly, another study unveiled a significant role of HMGA2 in the induction of the PI3K/AKT/mTOR pathway, thereby stimulating cell proliferation in the context of acute myeloid leukemia (Tan et al., 2016). Also, miR-26a was found to negatively

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regulate HMGA2 expression in NSCLC cells at the post-transcriptional level and this caused a reduction in both the expression of E2F1 and the phosphorylation of AKT, thereby repressing cell proliferation (Yang et al., 2016). Restoration of miR-211 expression was also shown to cause negative regulation of HMGA2 and this subsequently inactivated the AKT/β-catenin pathway, thus inhibiting glioblastoma cell growth and invasion (Mansoori et al., 2017).

Various other studies show that Snail genes are upregulated by HMGA2. HMGA2 overexpression in tongue cancer upregulated Snail expression and induced EMT (Zhao et al., 2016). Conversely, HMGA2 knockdown reduced the amount of HMGA2 that directly binds to the Snail promoter and this reduced Snail expression, as well as cell proliferation and EMT in human pancreatic cancer cells (Watanabe et al., 2009). HMGA2 overexpression and consequent Snail overexpression also associated with EMT and depth of invasion in esophageal squamous cell carcinoma tissues (Liu et al., 2014). Thuault and colleagues confirmed that HMGA2 is a transcriptional activator that directly binds to the Snail-1 promoter and they showed that HMGA2 does so in cooperation with the TGFβ/SMAD pathway (Thuault et al., 2008a). We note, however, that the latter finding is inconsistent with prior studies showing that HMGA2 is a gene downstream of TGFβ/Smad signaling. For example, Wang et al. determined that HMGA2 is a transcription factor induced by the TGFβ1/Smad3 axis during the EMT process (Pandit et al., 2010; Wang et al., 2016) and Li et al. found that the miRNA let7-a modulates TGFβ/Smad pathway activity by targeting HMGA2, which subsequently suppresses proliferation and invasion of glioma cells (Mansoori et al., 2016b). In fact, TGFβ binds to its type 1 and type 2 serine/threonine kinase receptors, activates the receptors, which then phosphorylate the 'R-Smads', e.g., Smad2/Smad3, and finally Smad/R-Smad complexes regulate gene transcription in the nucleus (Tan et al., 2014). HMGA2 is one of these downstream genes. In turn, HMGA2 regulates other EMT-induced transcription factors, including Snail-1, TWIST1 ZEB1, ZEB2 and etc. (Hawsawi et al., 2018; Tan et al., 2014; Thuault et al., 2006a; Xia et al., 2015), although there are also studies indicating that Snail is activated downstream of VEGFRs (Li et al., 2015). In addition, the Snail family has been linked to tumor angiogenesis, as Snail promotes VEGFR3 expression and deep vascular network formation (Lomelí et al., 2009; Park et al., 2015). Besides, Qian et al showed Snail regulation via HMGA2 resulting in repression of E-cadherin expression and causing EMT by TGF-β signaling pathway (Qian et al., 2009). Also, consistent with our study, Shi et al. showed that suppression of HMGA2 via siRNA increased E-cadherin expression (Shi et al., 2016). HMGA2 directly binds to the Snail gene promoter for transcriptional activation, probably leading to transcriptional repression of the E-cadherin gene. In addition, the authors showed an inverse correlation between HMGA2 and E-cadherin in cancerous tissue, and also demonstrated that Smads promote the binding of HMGA2 to the Snail promotor (Foubert et al., 2010; Thuault et al., 2008b).

HMGA2 mediates EMT by TGF-β, whereas ectopic HMGA2 causes irreversible EMT by severe E-cadherin suppression (Thuault et al., 2006b). Ectopic HMGA2 increased the TGF-β response. TGF-β increased Snail1 expression in breast and lung cancer cells (Park et al., 2017). Additionally, TGFβ inhibits E-cadherin expression and promotes the expression of Snail1 in prostate cancer (Cantelli et al., 2017). Altogether, HMGA2 has positive feedback with TGFβ and both HMGA2 and TGFβ promote Snail1 expression.

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Various observations link HMGA2 and STAT3. For instance, Yuan et al. demonstrated that STAT3 protein co-localized with HMGA2 in the nucleus of differentiating adipocytes. Hence, they suggested that HMGA2 and STAT3 cooperatively promote adipogenesis (Yuan et al., 2017). In two other studies, STAT3 was found to promote the LIN28-LET7-HMGA2 cascade, in which STAT3 directly binds to the lin28 promoter and induces its expression. This, in turn quenches let7 transcription and leads to upregulation of let7, which targets HMGA2 (Guo et al., 2013; Thanasupawat et al., 2017). On the other hand, other work demonstrated that STAT3 is located downstream of HMGA2, as Zhang et al. showed that HMGA2 overexpression reverts inhibition of STAT3 phosphorylation following treatment with a curcumin analog (Zhang et al., 2015). Finally, another study reported that HMGA2 directly binds to the IL-11 promoter, induces its expression and this is accompanied by STAT3 phosphorylation (Wu et al., 2016).

Taken together, our observations here, along with a large body of previously published work, suggest that miR-330 acts as a key suppressor of colorectal cancer development by negatively regulating HMGA2 expression (Figure 7). In turn, lower HMGA2 expression reduces AKT and STAT3 phosphorylation and downregulates Smad3, SNAIL1 and VEGFR. As a result, miR-330 inhibits cell survival, proliferation, EMT, migration, invasion, metastasis and angiogenesis (Figure 7). Thus, these observations also suggest that miR-330 is a promising candidate for microRNA replacement therapy to treat colorectal cancer patients (Hosseinahli et al., 2018).

Acknowledgments

This work was supported financially by the Iran national science foundation [grant number 93041394].

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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Figures

Figure 1. **HMGA2 overexpression and miR-330 down-regulation in human colorectal cancer predict poor recurrence-free patient survival.** (A-C) The expression levels of HMGA2 (A), miR-330-3p (B) and miR-330-5p (C) were analyzed in colorectal cancer and their matched adjacent normal tissues. Data are shown as log2 of the normalized reads-per-million (norm_RPM) from either RNAseq (A) or miRNAseq (B,C). These data were extracted from The Cancer Genome Atlas (TCGA). (D-E) Colorectal tumors that show underexpression of miR-330-3p or -5p significantly show HMGA2 overexpression (p values: Fisher's exact test). (F-L) Kaplan–Meier survival curves for RFS and OS comparing colorectal cancer patients whose tumors express high- and low-level HMGA2 (F-G), miR-330-3p (H-I), and miR-330-5p (J-K). The median expression levels were used as the cut-offs between low and high expression levels. The log-rank test is used for statistical analysis. RFS: recurrence-free survival; OS: overall survival.

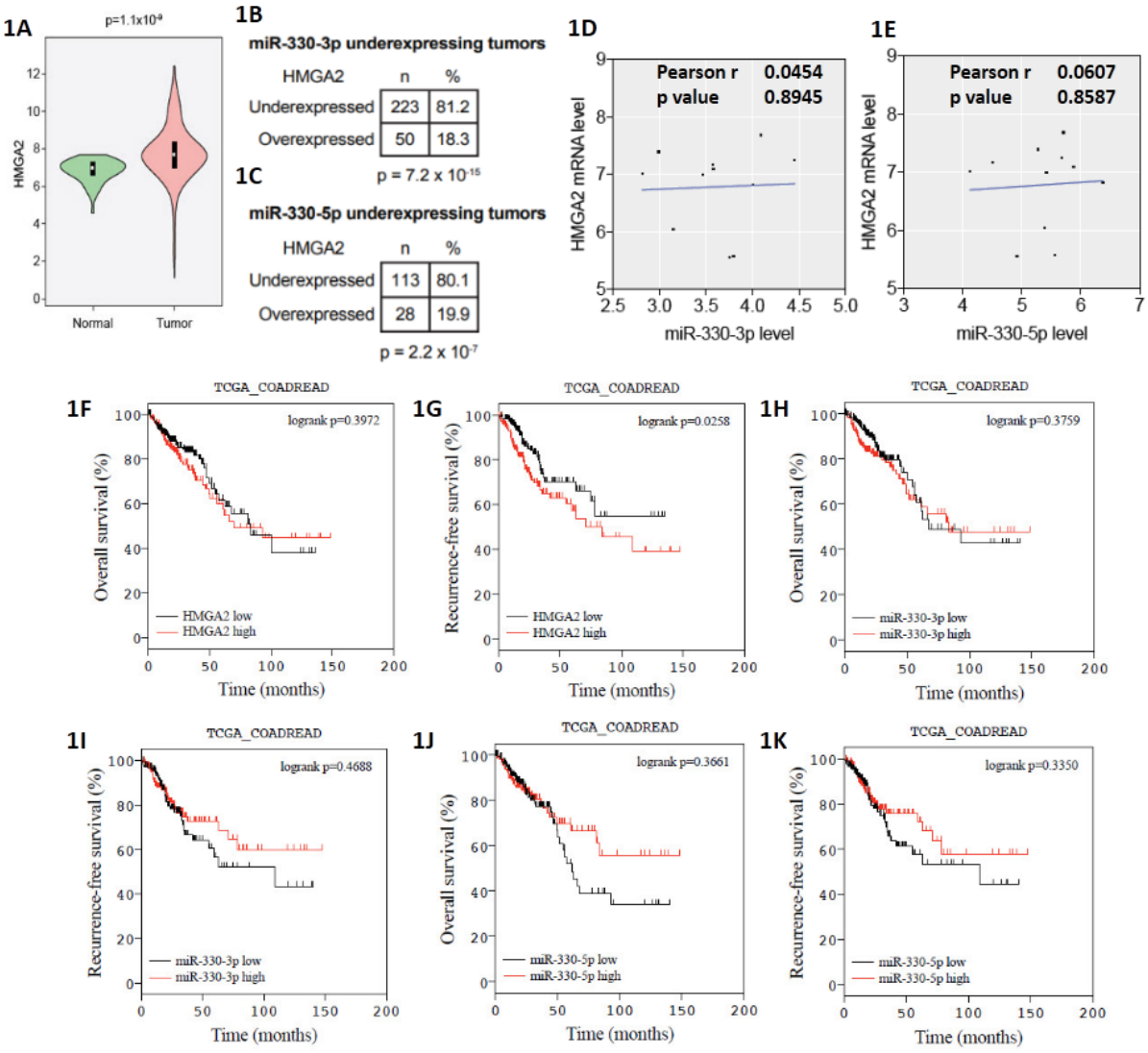

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Figure 2. **Generation of colorectal cancer cell lines with stable increased miR-330 expression.** (A-D) The vector pCMV-GFP-miR330 – or empty pCMV-GFP vector as a control (ctrl) – was transfected into the HCT116 and SW480 cell lines. After 14 days of selection with Genticin, stable miR-330 expression in these cell lines was confirmed by flow cytometry (A, B) and fluorescent microscopy (C, D). (E, F) MiR-330 expression levels were also measured in these colorectal cancer cell lines by qRT-PCR. ****P < 0.0001 compared to negative control cells (NC).

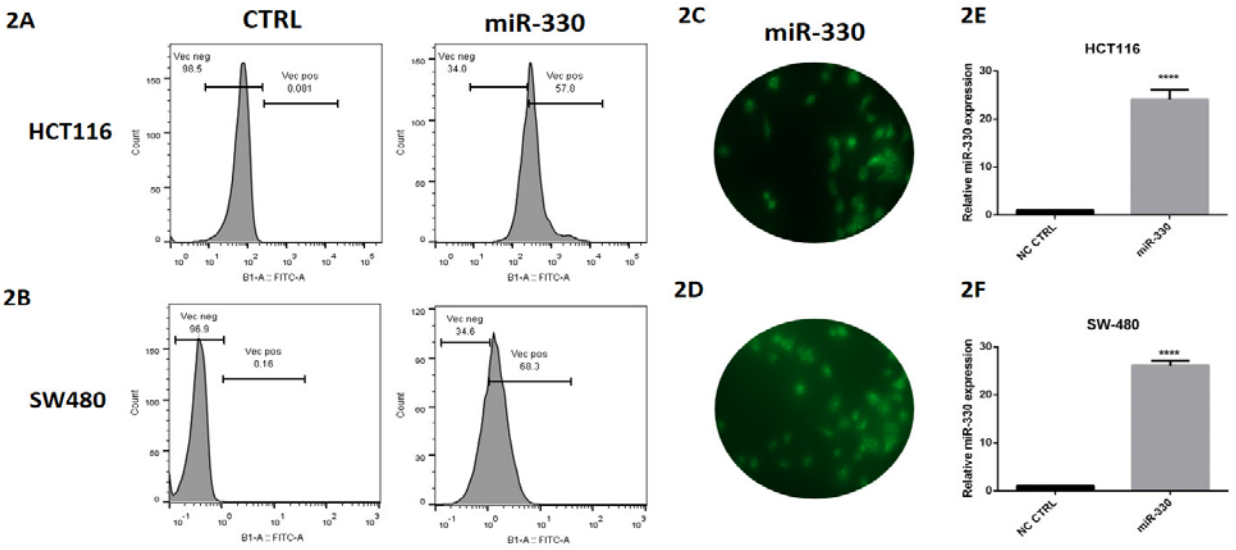

Figure 3. **miR-330 downregulates HMGA2 mRNA expression in colorectal cancer cells.** (A) Bioinformatics analyses showing the HMGA2 mRNA sequences that miR-330-3p and miR330-5p are predicted to target. (B) qRT-PCR analysis showing HMGA2 mRNA expression in both HCT116 and SW480 cell lines in response to increased miR330 expression. (C) Western blots showing the protein levels of HMGA2 following increased miR-330 expression. (D) Quantification of Western blots shown in (C). ****P < 0.0001 versus negative control cells (NC).

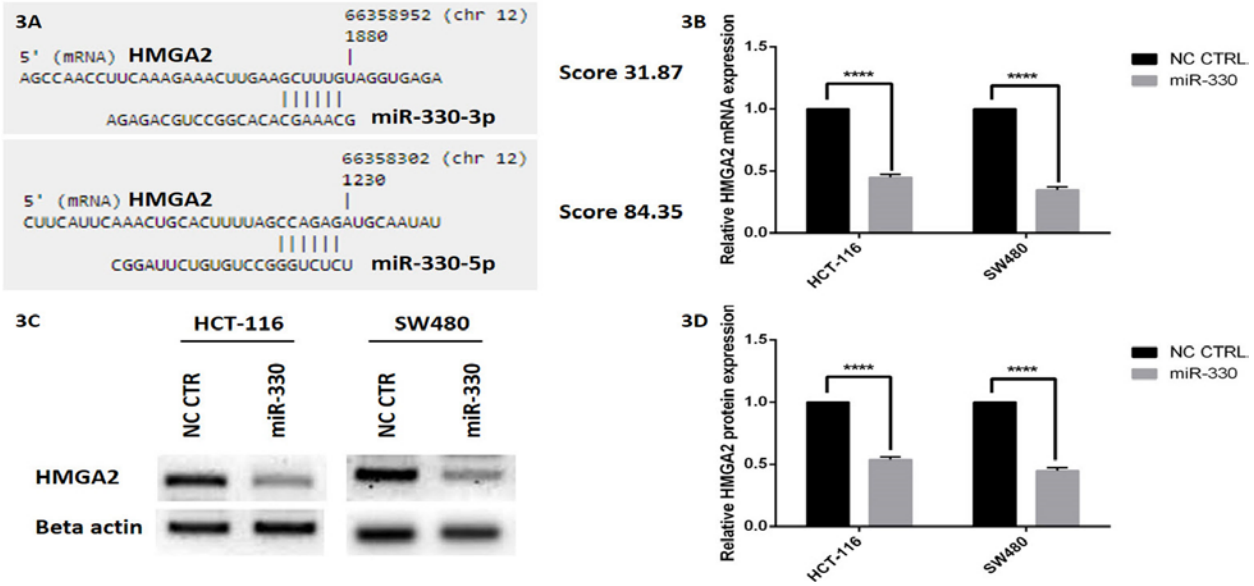

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Figure 4. **miR-330 replacement induces apoptosis in colorectal cancer cells.** (A) FACS analysis of annexin V/PI assay showing the fractions of apoptotic HCT116 and SW480 following increased miR-330 expression. (B) Quantification of the fractions of apoptotic cells determined in (A). (C) Nucleus fragmentation was assessed by DAPI staining. **P < 0.01, and ***P < 0.001 versus the negative control (NC).

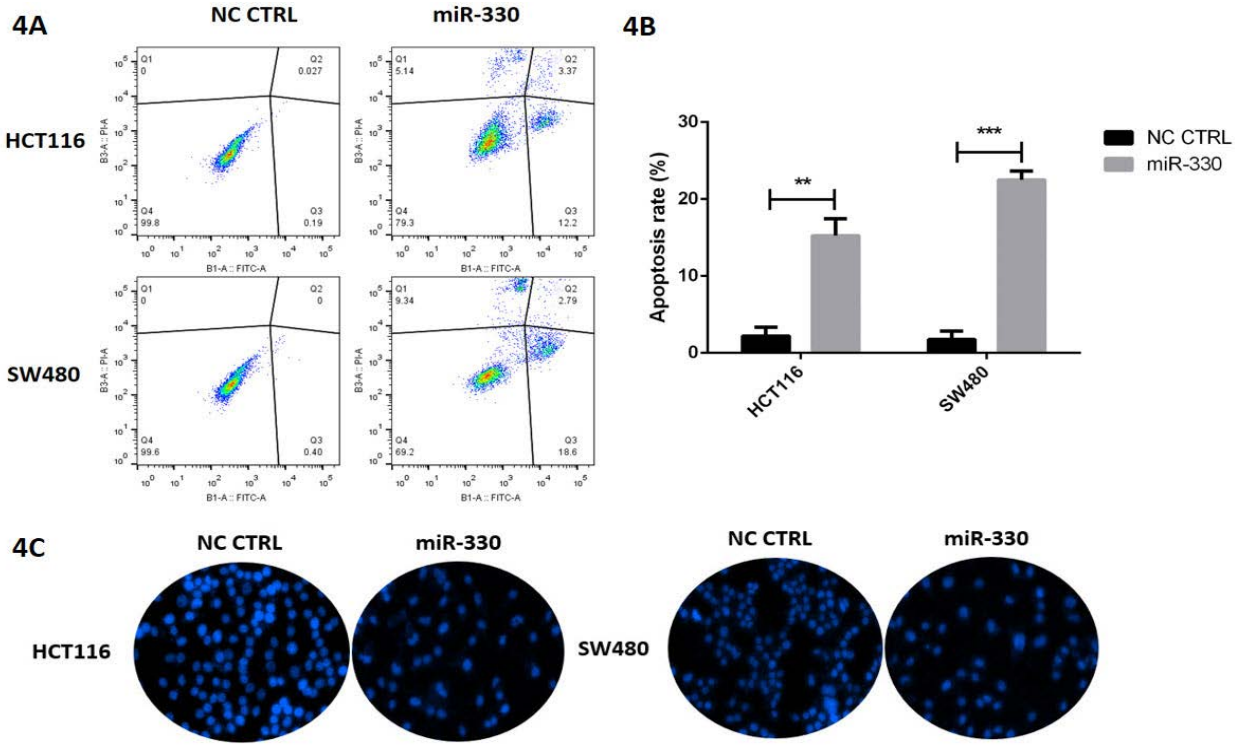

Figure 5. **miR-330 reduces colorectal cancer cell migration and viability.** (A) Wound healing assays. Images of the scratch areas were taken 48 hours after scratching the monolayer in miR-330-expressing and negative control (NC) cells. (B) The numbers of cells that migrated into the scratched areas shown in (A) were quantified. (C) The normalized rates of cell proliferation over a 48-hour period. ***P < 0.001, ****P < 0.0001 versus negative control cells.

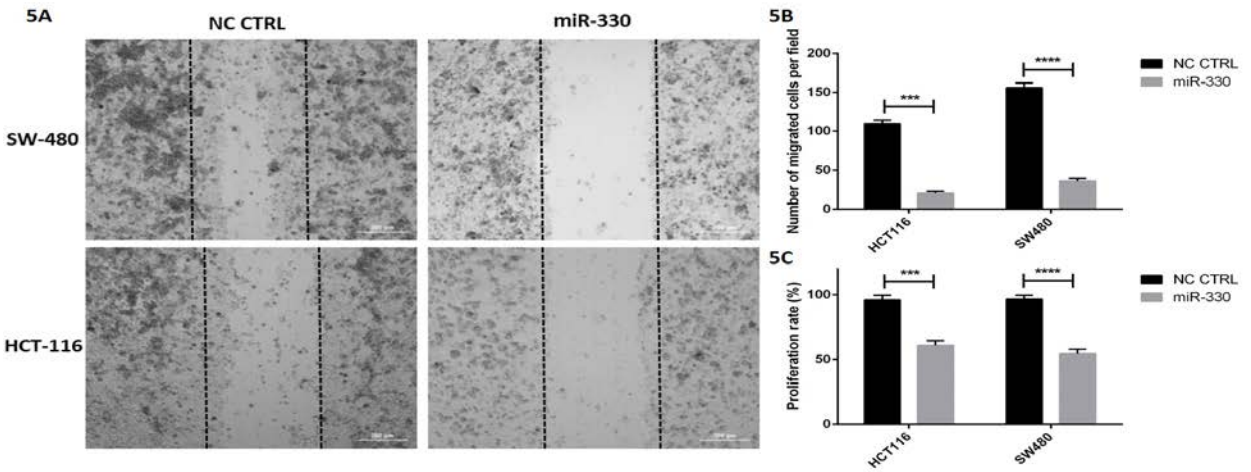

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Figure 6. **Increasing miR-330 expression in colorectal cancer cell lines reduces the expression of a wide range of oncogenic markers.** (A) Western blot analysis comparing the protein and phospho-protein expression levels of indicated markers, and beta-actin as a loading control, in HCT116 and SW480 control and miR-330-overexpressing cells. (B-H) Protein and phospho-protein expression levels of the markers shown in (A) relative to beta-actin expression. ** p<0.01, *** p<0.001 and **** p<0.0001 versus negative control (NC).

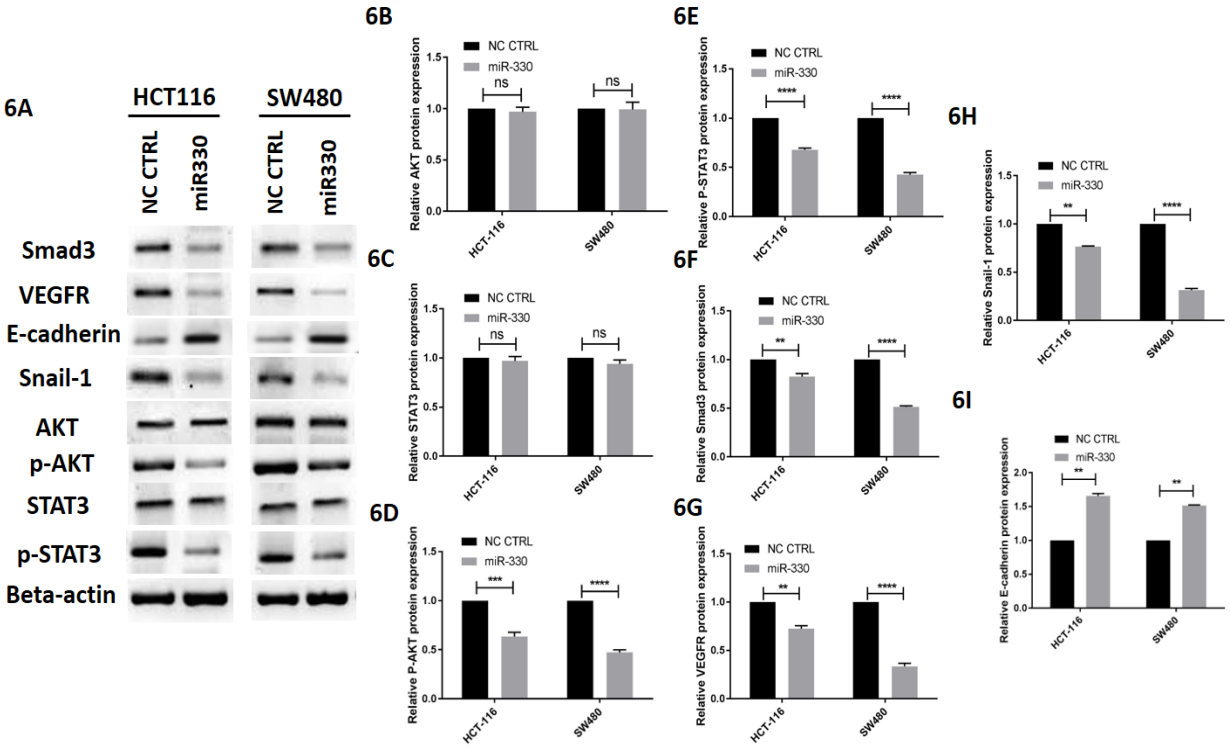

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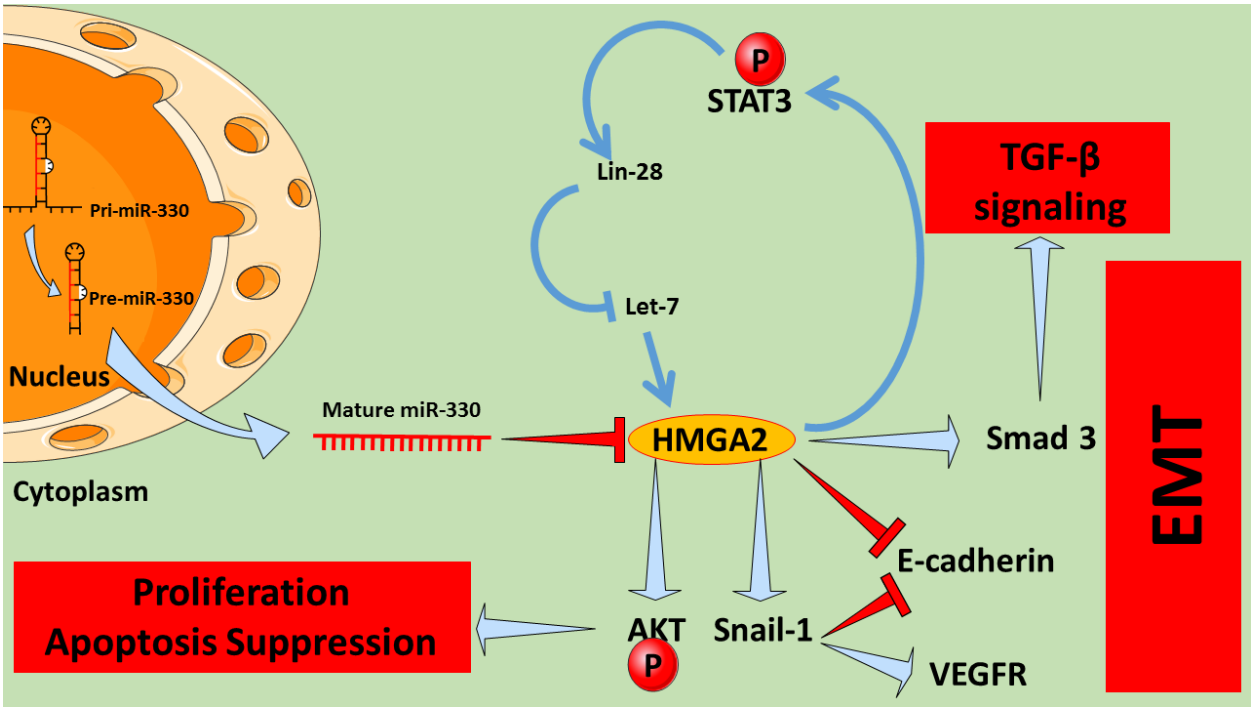
Figure 7. **Schematic diagram of miR-330 regulations after targeting HMGA2.** Induction of miR-330 inhibits HMGA2 expression and subsequently reduces expression of EMT-related proteins including Snail-1 and VEGFR, increase E-cadherin and reduce TGF-β pathway-related proteins (Smad3). Phosphorylation of STAT3 and AKT also decrease following miR-330-mediated silencing of HMGA2. Overall, miR-330 acts as a tumor suppressor by inducing apoptosis and inhibiting proliferation and EMT.



Table

Table 1. Primer sequences

Name		Sequences
Beta-Actin	Forward	5’- TCCCTGGAGAAGAGCTACG -3’
	Reverse	5’- GTAGTTTCGTGGATGCCACA-3’
HMGA2	Forward	5’-TGGGAGGAGCGAAATCTAAA-3’
	Reverse	5’-TCCCTGGAGAAGAGCTACG-3’
U6 snRNA	Forward	5’- CTTCGGCAGCACATATACTAAAATTGG -3’
	Reverse	5’- TCATCCTTGCGCAGGGG -3’
Has-mir-330	Forward	5’- TCTCTGGGCCTGTGTC -3’
	Reverse	5’- CCAGTTTTTTTTTTTTTTGCCTAAG -3’

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