



Research Paper

MEG3 Activated by Vitamin D Inhibits Colorectal Cancer Cells Proliferation and Migration *via* Regulating Clusterin

Yan Zhu ^{a,1}, Peizhan Chen ^{b,1}, Yisha Gao ^a, Na Ta ^a, Yunshuo Zhang ^a, Jialin Cai ^b, Yong Zhao ^d, Shupeng Liu ^{c,*}, Jianming Zheng ^{a,*}

^a Department of Pathology, Changhai Hospital, Secondary Military Medical University, Shanghai 200433, PR China

^b Translational Medicine Research Center, Ruijin Hospital North, Shanghai Jiao Tong University School of Medicine, Shanghai 201821, PR China

^c Clinical Research Center, Changhai Hospital, Secondary Military Medical University, Shanghai 200433, PR China

^d Office of Shanghai Administrative Committee for Laboratory Animal, Shanghai, Laboratory Animals Research Center, Shanghai, 201203, PR China



ARTICLE INFO

Article history:

Received 2 February 2018

Received in revised form 27 March 2018

Accepted 27 March 2018

Available online 31 March 2018

Keywords:

lncRNA

CRC

MEG3

Clusterin

Vitamin D

ABSTRACT

The long non-coding RNA maternally expressed gene 3 (*MEG3*) is frequently dysregulated in human cancers; however, its roles in colorectal cancer (CRC) development are largely unknown. Here, we reported that *MEG3* was down-regulated in CRC tissues and CRC patients with lower *MEG3* showed poorer overall survival and disease-free survival than those with higher *MEG3* level. *MEG3* over-expression represses CRC cells proliferation and migration *in vivo* and *in vitro*, while *MEG3* knockdown leads to the enhanced proliferation and metastasis of CRC cells. In CRC cells, *MEG3* over-expression is related to decreased *Clusterin* mRNA and the corresponding protein levels, and it also directly binds to Clusterin protein through its 732–1174 region. In further, Clusterin over-expression rescues the compromised abilities of proliferation and metastasis induced by *MEG3* over-expression, suggesting that *MEG3* inhibits the CRC progression through regulating the Clusterin activities. Additionally, we found that $1\alpha,25-(OH)_2D$ and vitamin D receptor (VDR) stimulate *MEG3* expression in CRC cells through directly binding to its promoter. These results suggested that *MEG3* functions as a tumor suppressor in CRC *via* regulating the Clusterin activities and may underlie the anticancer activities of vitamin D on CRC cells. The VDR/*MEG3*/Clusterin signaling pathway may serve as potential therapeutic targets and prognosis biomarkers for CRC patients in future.

© 2018 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Colorectal cancer (CRC) is one of the most common malignancies of the digestive system worldwide with the incidence has being increased significantly over the past three decades (Arnold et al., 2017). >700,000 deaths has been estimated to be caused by CRC in 2012, ranking it as the 4th leading cause of cancer-related deaths (Torre et al., 2015). Although much progress has been made for CRC patients for the past decades, such as the advanced chemotherapy methods and targeted therapy including cetuximab or panitumumab, the prognosis of CRC patients is still poor. The 5-year cause specific survival of CRC patients ranges from 90.9% for AJCC 6th TNM stage I to 12.2% for those with stage IV (Lin et al., 2015). Thus, uncovering the underlying mechanisms involved in CRC development and progression may provide potential therapy targets, which may further improve the prognosis of the patients.

The development of colorectal cancer is a multi-stage and multi-step process, involving multiple genomic and epigenetic alterations (Guinney et al., 2015). Clinical studies have reported that mutation in oncogenes or tumor suppressor genes, including APC, KRAS, SMAD4 and TP53 are frequently observed in CRC patients (Yu et al., 2015). Guinney et al. have categorized CRC into four consensus molecular subtypes with distinguishing features and prognoses based on the gene expression pattern and genomic alterations of CRC tissues (Guinney et al., 2015). Recently, increasing evidence has shown that long non-coding RNAs (lncRNAs), the novel regulators in cellular signaling, play vital roles in CRC tumorigenesis and progression (Ohtsuka et al., 2016; Ma et al., 2016). lncRNA is a type of RNA transcripts longer than 200 nucleotides but do not translated into protein in cells. lncRNAs are involved in diverse cellular processes including cell proliferation, migration, invasion and transformation *etc.*, and dysregulation of lncRNAs are associated with various types of diseases (Ponting et al., 2009). lncRNAs regulate the gene functions through multiple levels including the chromatin modification, transcription, post-transcription, interaction with RNA-binding proteins, co-activation of transcription factors and repressors (Marchese et al., 2017). Maternally expressed gene 3 (*MEG3*),

* Corresponding authors at: Changhai Hospital, Secondary Military Medical University, 168 Changhai Road, Shanghai 200433, PR China.

E-mail addresses: lshu_p@aliyun.com, (S. Liu), jmzheng1962@163.com. (J. Zheng).

¹ Authors contributed equally to this article.

located on 14q32.3 of the human genome, encodes a 1.6 kilobase (kb) lncRNA (Zhou et al., 2012). It is expressed in multiple organs, including the brain, stomach, liver, pancreas and ovary. By contrast, *MEG3* expression is frequently repressed in tumor tissues (Zhou et al., 2012). In various types of cancer, genomic deletion and abnormal methylation in the promoter of *MEG3* were noticed, leading to the down-regulation of *MEG3* in tumor tissues (Bando et al., 1999; Yin et al., 2015; Lu et al., 2013). In non-small cell lung cancer (NSCLC) cells, *MEG3* inhibits proliferation and induces cell apoptosis through activating p53 and its downstream signaling pathway (Lu et al., 2013). Interestingly, for tumor cells with p53 deletion, *MEG3* over-expression also inhibits tumorigenesis through targeting the microRNAs such as microRNA-421 and microRNA-184 (Zhang et al., 2017; Li et al., 2018). These studies indicated multiple mechanisms underlying the roles of *MEG3* in tumor development. Previous studies have reported that a lower *MEG3* level was associated with the increased liver metastasis of CRC patients (Kong et al., 2016), and an enhanced CRC cells chemosensitivity to oxaliplatin (Li et al., 2017). However, the underlying mechanisms regarding the tumor suppressor activities of *MEG3* are still largely unknown. In the current study, we evaluated the anticancer activities and the underlying mechanisms of *MEG3* in CRC development and progression, which may provide potential novel intervention methods for CRC in the future.

2. Materials and Methods

2.1. Tissue Microarray Construction

Tumor specimens used in tissue microarrays (TMAs) were obtained from 371 colorectal cancer patients who underwent curative resection at Changhai Hospital of the Second Military Medical University from January 2001 to December 2010. Patients were selected with the following inclusion and exclusion criteria: (i) pathological confirmed as the primary CRC according to the World Health Organization criteria; (ii) with available formalin-fixed, paraffin-embedded (FFPE) CRC tissue samples; (iii) without any pre-operative anti-cancer treatment and no evidence of distant metastases; (iv) with complete clinicopathologic and follow-up data for the patients. Each participant provided the written informed consent and the study was approved by the Changhai Hospital Ethics Committee. The overall survival (OS) time was defined as the length of time between the surgery date and deaths by any causes. For surviving patients, the data were censored at the last following-up. The disease-free survival (DFS) was defined as the length of time between the date of the surgery and the date of tumor recurrence, metastasis or death. The tissue microarrays (TMAs) were constructed with the FFPE tissues by Shanghai Biochip Co, Ltd., Shanghai, China, following the routine protocols (Cai et al., 2017). For each patient, a 0.75-mm diameter core of the FFPE tumor tissue was punched and arranged in the TMA blocks.

2.2. Immunohistological Chemistry Staining and the *in situ* Hybridization

Six-micrometer thick TMA sections were used to perform immunohistochemistry staining and *in situ* hybridization (ISH) following standardized protocols (Pan et al., 2015; Deng et al., 2013). The antibody used for immunohistochemical staining of VDR was purchased from Cell Signaling Technology (Cat# 12550, RRID: AB_2637002). The lncRNA-*MEG3* probes were designed and produced by Exiqon (Vedbaek, Denmark). ISH was performed following the manufacturer's guidelines. The immunohistochemical score for each TMA sample was assessed independently by 2 pathologists.

2.3. Cell Culture

The human colorectal cancer cell lines RKO, SW1116, HT29, HCT116, LoVo, SW620, SW480 and 293 T were purchased from the Shanghai

Institute of Cell Biology, Chinese Academy of Sciences. All cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin), in a humidified atmosphere of 5% CO₂ at 37 °C. Cell lines were authenticated by short tandem repeat polymerase chain reaction (STR-PCR). Mycoplasma infection status was tested by 4', 6-diamidino-2-phenylindole (DAPI) staining in the laboratory. All colorectal cancer cell lines were used to investigate *MEG3* expression, while RKO, SW1116, and LoVo were used to investigate the biological functions of *MEG3*. The SW1116 cell line was used to investigate the effects of *MEG3* on CLU expression.

2.4. Cellular Proliferation Assay

Cellular proliferation was measured using the Cell Counting Kit-8 (CCK-8, Dojindo, Japan) kit. Cells with modified *MEG3* and Clusterin expression or not were seeded at a density of 5×10^3 cells/well in 96-well culture plates and cultured for 24, 48, or 72 h. The cells were then incubated with 10 μ L CCK8 for another 4 h at 37 °C. After incubation, the viability of cells was measured at 450 nm using a microplate reader (BioTek, USA), and all experiments were repeated three times. Down-regulation of *MEG3* or Clusterin (CLU) was performed by small interfering RNA (siRNA) transfection (*MEG3* siRNA, UUAGGUAAGAGGGACA GCUGGCUGG; si-CLU1, CCAGACGGUCUCAGACAAU; si-CLU2, GGUUGA CCAGAAAUAACA; si-CLU3, CCAGGAAGAACCUAUUU). Over-expression of *MEG3* or Clusterin was performed by infection of lentiviruses expressing *MEG3* or Clusterin coding regions, which were obtained from Shanghai Obio technology Company (Shanghai, China).

2.5. Transwell and Invasion Assays

5×10^4 cells in 200 μ L serum-free Dulbecco's Modified Eagle Medium (DMEM) medium were placed in upper surface of the Transwell chambers (8 μ m, Corning Costar Co., MA, USA) with the pre-coated 1:10 diluted Matrigel (BD Biosciences, CA, USA) for invasion assay or without the Matrigel for migration assay. The lower chamber was filled with 500 μ L complete RPMI 1640 medium with 10% FBS. The cells were incubated for 12 to 18 h at 37 °C and then the cells on the top surface of the membrane were removed by wiping with a cotton swab. The cells that had migrated or invaded from the upper surface to the bottom surface of the filter membrane were stained with 0.5% crystal violet solution and photographed in five representative fields per insert. The cell number per field was counted and compared between the groups.

2.6. Xenograft and Metastasis Animal Models

Male BALB/c nude mice aged 4–6 weeks were obtained from Shanghai Laboratory Animal Center of China. For the tumor growth model, 1×10^6 SW1116 cells with or without *MEG3* stable over-expression were injected into the axillary subcutaneous tissues of nude mice. Tumor growth was determined by measuring the tumor volume, $V = \text{tumor length} \times \text{tumor width}^2/2$ every 4 days using calipers.

For the tail vein metastasis models, 1×10^6 cells were suspended in 200 μ L serum-free RPMI1640 were injected into the tail vein of nude mice. After 7 weeks, all of mice were sacrificed and the lung tissues were dissected and fixed with 10% formalin for at least 72 h. The metastasis nodules in lung tissues were analyzed by hematoxylin and eosin (HE) staining methods. All animal experiments were performed according to the guidelines on the care and use of animals for scientific use and approved by the Institutional Animal Care and Use Committee at Second Military Medical University.

2.7. RNA-pull Down Assay

RNA-pull down assay was performed as previously reported (Yuan et al., 2017). *MEG3* was *in vitro*-transcribed from the vector pSPT19-

MEG3 using T7 or Sp6RNA polymerase (Roche) and biotin-labeled with the Biotin RNA Labeling Mix (Roche) and T7 or Sp6 RNA polymerase (Roche). 1 µg whole-cell lysates from SW1116 cells were incubated with 3 µg of purified biotinylated transcripts for 1 h at 25 °C. The complexes were isolated with streptavidin agarose beads (Invitrogen) and send for RT-PCR and western blot analysis.

2.8. Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift analysis (EMSA) was performed as previously reported (Yan et al., 2009). The oligonucleotide 5'-GGGCTTGTCCGTATTTACGTTGAGGCGGGA-3' and 5'-TCCCGCTCAACGTAATA CGGACAAGCCC-3', corresponding to the *MEG3* were synthesized. The annealed oligonucleotide was labeled with [γ -³²P] dATP using T4 polynucleotide kinase and used for the following binding reaction. The Clusterin protein (5 µg, R&D, 2937-HS-050 USA) and Clusterin antibody (Novus Cat# NBP1-68308-0.05 mg, RRID: AB_11027164) were used.

2.9. Luciferase Reporter Assay

The 293 T cells were plated at a subconfluent density in 24-well plate and co-transfected with 0.05 µg of the reporter plasmid with *MEG3* promoter region, 0.5 µg of VDR expression or control vectors, and 0.05 µg of Renilla luciferase pRL-TK as an internal control for transfection efficiency. Cell lysates were prepared 48 h after transfection, and the reporter activity was measured using the Dual-luciferase reporter assay system (Promega).

2.10. Western Blotting

The level of proteins such as secretory Clusterin were investigated using western blotting performed following the general protocols. 20 µg total proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were blocked in 5% non-fat milk in Tris-buffered saline with 0.05% Tween-20 at room temperature for 2 h and then probed with antibodies against Clusterin (sc-6420; RRID: AB_2083323; Santa Cruz, CA, 1:200 dilution) and beta-actin (Abcam Cat# 1854-1, RRID: AB_764434, 1:1000 dilution), and probed with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (Abcam Cat# ab7088, RRID: AB_955595). The bands were observed with the enhanced chemiluminescence western blotting detection kit and visualized with a Tanon luminescent imaging workstation (Tanon, Shanghai, China).

2.11. Statistical Analyses

Quantitative variables were analyzed with the Student's *t*-test or the one-way ANOVA tests. The categorized characteristics between the groups were compared with χ^2 test. The univariate and multivariate Cox regression models were applied to assess the association between the clinical characteristics and the overall survival or DFS of the patients. The Kaplan-Meier plots in together with the log-rank tests were used to compare the survival proportion between the groups. All statistical analyses were performed with SPSS 15.0 for Windows (SPSS, Chicago, IL). Two sided $P < .05$ was considered of statistically significant for all tests.

3. Results

3.1. *MEG3* Expression is Decreased in CRC Tissues and Correlated with Poor Prognosis of CRC Patients

To evaluate *MEG3* expression in CRC tissues, ISH was performed using *MEG3* specific probes (Supplementary Fig. 1). Significantly

decreased *MEG3* expression (22/27) was observed in CRC tissues compared with that in the corresponding adjacent normal tissues (Wilcoxon test, $P = .009$; Fig. 1A). For further validation, *MEG3* expression in another 61 paired CRC tissues was assessed using real-time PCR (RT-PCR). Approximately 77% ($N = 47$) of these CRC samples expressed a lower level of *MEG3* compared with the corresponding normal tissues, while only 23% ($N = 14$) of samples showed a higher level of *MEG3* (Wilcoxon test, $P < .001$; Fig. 1B). These results suggested that *MEG3* was down-regulated in CRC patients and that it may be involved in CRC tumorigenesis and progression.

To investigate the association between *MEG3* expression level and the prognosis of CRC patients, *MEG3* expression was evaluated by ISH in TMAs containing 371 samples of CRC patients. Compared with patients with lower *MEG3* levels (score ≤ 150 ; $N = 225$), patients with higher *MEG3* levels (score > 150 ; $N = 146$) have relative better OS as suggested by the Kaplan-Meier plot (log-rank test; $P = .007$; Fig. 1C). The univariate analyses suggested that the TNM stage, CEA level, CA19-9 level and *MEG3* expression levels were associated with the OS of CRC patients, while multivariate analyses indicated that *MEG3* expression was an independent prognosis factor of OS for CRC patients (Table 1). In addition, Kaplan-Meier analyses also showed that patients with higher *MEG3* expression have better DFS (log-rank test; $P = .003$; Fig. 1D) than those with lower *MEG3* level, and that *MEG3* is also an independent prognostic factor for the DFS of CRC patients (Table 1). These results suggest that *MEG3* expression is decreased in CRC patients, and lower *MEG3* expression level was correlated with poorer prognosis.

3.2. *MEG3* Inhibits Cellular Proliferation and Tumor Growth of CRC Cells *in vitro* and *in vivo*

We next intended to investigate the biological activities of *MEG3* in CRC cells. The mRNA level of *MEG3* in 7 CRC cell lines was determined, among which RKO expressed the highest and SW1116 and LoVo showed the lowest *MEG3* levels (Fig. 2A). In RKO cells transfected with siRNA of *MEG3* significantly reduced its mRNA levels (Supplementary Fig. 2A). *MEG3* knockdown led to a significant increased cellular proliferation compared with the blank or negative control group (Fig. 2B). In contrast, *MEG3* expressing lentiviral transfection for SW1116 and LoVo cells significantly increased the *MEG3* expression levels (Supplementary Fig. 2B), and led to the reduced cellular proliferation compared to control lentivirus as suggested by the CCK-8 assay (Fig. 2C).

To further evaluate the roles of *MEG3* in tumor growth *in vivo*, SW1116 cells with or without stable *MEG3* over-expression (SW1116/*MEG3* or SW1116/con) were subcutaneously injected into nude mice. For every four days, the tumor size was measured and the tumor growth curve suggested that SW1116 cells with *MEG3* over-expression was significantly inhibited compared to control group ($N = 6$ for each group, Fig. 2D). At the end of the experiment, the tumor size was also significantly reduced in the mice injected with SW1116/*MEG3* compared with those injected with control cells in the xenograft animal models (Fig. 2E, Supplementary Fig. 2C). These results indicated that *MEG3* inhibited the cellular proliferation and tumor growth of CRC cells *in vitro* and *in vivo*.

3.3. *MEG3* Suppresses CRC Cell Migration and Metastasis *in vitro* and *in vivo*

We also evaluated the influences of *MEG3* on the migration activities of the CRC cells *in vitro* and *in vivo*. The trans-well migration assays showed that *MEG3* over-expression in SW1116 and LoVo cells significantly inhibited the migration capacities of tumor cells (Fig. 3A, Supplementary Fig. 3A). The invasion assays suggested that *MEG3* over-expression significantly inhibits the invasiveness abilities of the two cell lines (Fig. 3B, Supplementary Fig. 3B). By contrast, when *MEG3* was knocked-down in RKO cells, the migration and invasion abilities

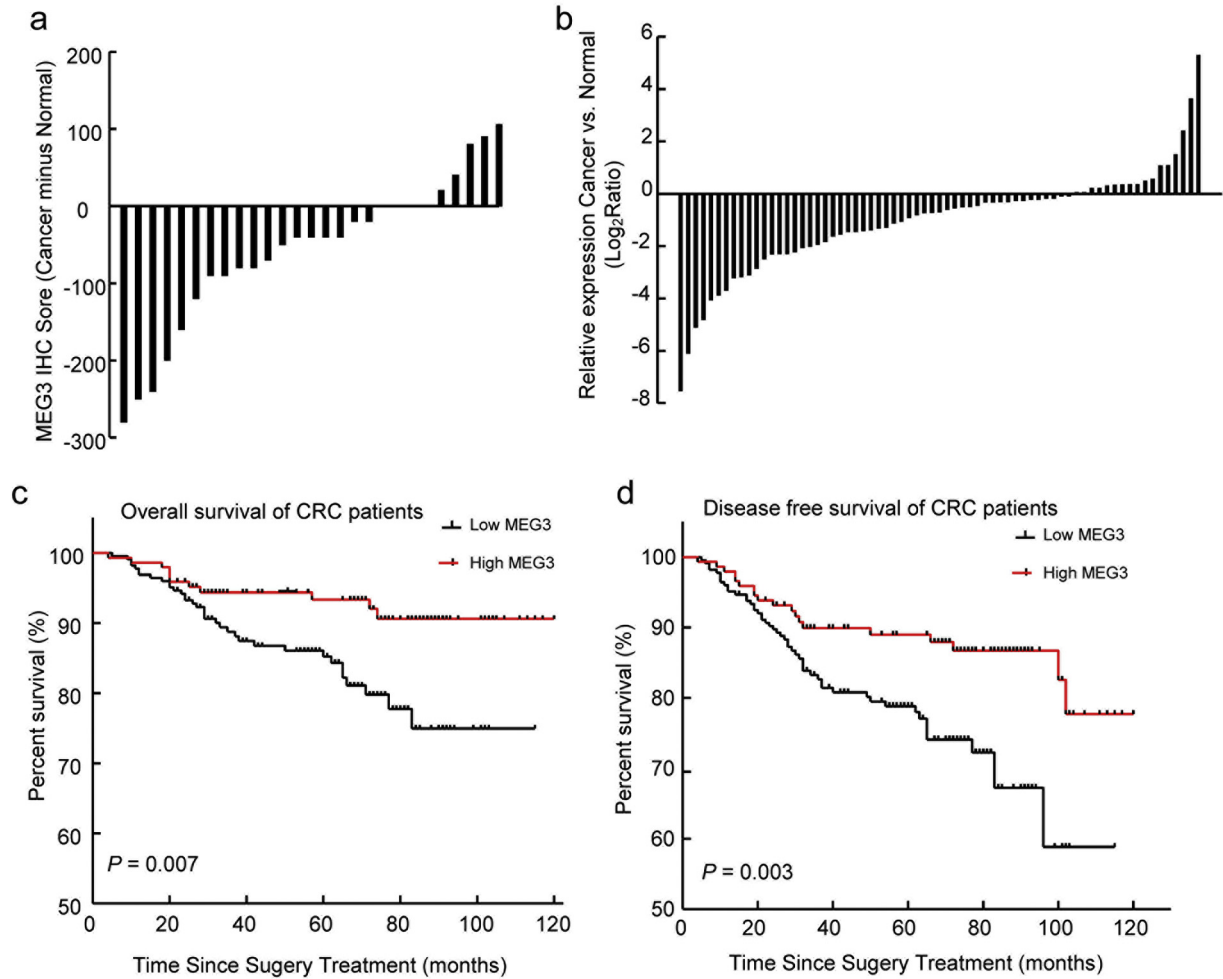


Fig. 1. Association of *MEG3* expression in CRC tissues with the prognosis of CRC patients. (A) Expression level of *MEG3* in CRC (Cancer) and adjacent normal tissues (Normal) was determined by ISH ($N = 27$); (B) Expression level of *MEG3* in CRC (Cancer) relative to adjacent normal tissues (Normal) was determined by RT-PCR ($N = 61$); (C and D) The Kaplan-Meier analysis of the relationship between *MEG3* expression level and overall survival (OS) or disease-free survival (DFS) of CRC patients (Log rank-test, $P = .007$ and $P = .003$, respectively).

of tumor cells were significantly enhanced (Fig. 3C, Supplementary Fig. 3C).

For further verification, the *in vivo* tumor metastasis mice model was established through injecting SW1116/*MEG3* cells into nude mice *via* the tail vein. Seven weeks later, the mice were sacrificed and the

metastasis loci in the lungs were monitored by the HE staining. Fewer metastasis loci were observed in lung tissues from mice injected with SW1116/*MEG3* cells than in those from the control group (Fig. 3D, Supplementary Fig. 3D). These results suggested that *MEG3* inhibits the metastatic features of CRC cells both *in vitro* and *in vivo*.

Table 1

Association between the clinical characteristics and *MEG3* expression level with the overall survival and disease-free survival of CRC patients ($N = 371$).

Variable	Overall survival				Disease-free survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P value	HR (95% CI)	P-value	HR (95% CI)	P value
TNM stage (III vs. I+ II)	2.34 (1.26–4.32)	0.007	2.48 (1.33–4.60)	0.004	2.26 (1.38–3.69)	0.001	2.32 (1.42–3.80)	<0.001
Depth of invasion [(T3 + T4) vs. (T1 + T2)]	1.21 (0.60–2.44)	0.591			1.57 (0.92–2.69)	0.099		
Sex (women vs. men)	1.08 (0.61–1.92)	0.785			0.92 (0.57–1.47)	0.718		
Tumor location (rectum vs.colon)	0.82 (0.42–1.59)	0.554			1.25 (0.76–2.06)	0.379		
Age (>60 vs. ≤60 years)	1.32 (0.74–2.35)	0.352			1.01 (0.63–1.62)	0.967		
Differentiation								
Moderately vs. well	1.74 (0.42–7.25)	0.444			1.25 (0.45–3.48)	0.659		
Poorly vs. well	1.65 (0.27–9.91)	0.585			1.28 (0.34–4.78)	0.713		
Treatment (yes vs. no)	1.85 (0.73–4.68)	0.195			2.13 (0.97–4.67)	0.058		
CEA (>20 ng/mL vs. <20 ng/mL)	2.91 (1.48–5.72)	0.002	2.83 (1.28–6.25)	0.010	2.08 (1.12–3.88)	0.021	2.11 (1.03–4.32)	0.041
CA199 (>22.32 U/mL vs. <22.32 U/mL)	1.79 (1.01–3.18)	0.047	1.41 (0.75–2.67)	0.289	1.30 (0.80–2.12)	0.284		
<i>MEG3</i> (high vs. low)	0.37 (0.19–0.74)	0.005	0.34 (0.16–0.71)	0.004	0.45 (0.27–0.78)	0.004	0.43 (0.25–0.76)	0.004

3.4. *MEG3* Inhibits Proliferation and Metastasis through Regulating Clusterin

lncRNAs could bind to the target proteins to modulate gene expression and biological processes (Zhu et al., 2013). To identify target proteins interacting with *MEG3* in CRC cells, the RNA-pull down assays were performed using biotin labeled *MEG3*, and the precipitated proteins were applied for SDS-PAGE electrophoresis. Those protein bands specifically evident in *MEG3* precipitates, but not in the IgG and *MEG3* antisense controls, were then analyzed by mass spectrometry (Fig. 4A). 68 proteins were identified by mass spectrometry, which may potentially bind to lncRNA *MEG3* (Supplementary Table S1). Among them, secretory Clusterin (CLU) and cell growth-regulating nucleolar protein (LYAR) were selected for further verification due to their tight association with tumors (Wu et al., 2015; Chen et al., 2003). The precipitate from the *MEG3* RNA-pull down was analyzed by western blotting using CLU or LYAR antibody. The secretory CLU was found specifically in the *MEG3* precipitate but not in that from the IgG and *MEG3* antisense control, while LYAR was not found in all three groups (Fig. 4B), suggesting there could be direct physical binding between *MEG3* and CLU. The following RIP using CLU antibody and EMSA also suggested *MEG3* was directly bind with CLU (Fig. 4C, Supplementary Fig. 4A). To identify the binding sites of *MEG3* with CLU, four *MEG3* mRNA fragments (F1:1–389; F2:390–731; F3:732–1174, and F4:1175–1595), truncated based on the predicted RNA structure of *MEG3*, were cloned and used for EMSA analyses (Supplementary Fig. 4B). In the EMSA assays, a significant band shift was noticed for the F3 fragment of *MEG3* mRNA, suggesting that this segment (732–1174 nucleotide acids) contained the binding sites for CLU (Fig. 4D). We next determined whether *MEG3* affects the expression level of secretory CLU in CRC cells. Decreased CLU mRNA level was observed when *MEG3* was over-expressed in SW1116 cells (Fig. 4E) and the corresponding secretory CLU protein level was also significantly decreased in the SW1116 cells as suggested by western blotting (Fig. 4E).

Moreover, whether secretory CLU interferes the anticancer activities of *MEG3* in CRC was determined through knockdown of CLU by siRNAs (Supplementary Fig. 4C). The proliferation and metastasis properties of SW1116 cells with CLU down-regulation (si-CLU2), *MEG3* overexpression (*MEG3*) and *MEG3* and CLU co-overexpression (*MEG3/CLU*) were determined. The CCK-8 assays suggested that SW1116 cells with CLU down-regulation was correlated with reduced proliferation capacities than control cells and that *MEG3* over-expression significantly inhibited SW1116 cells proliferation (Fig. 4F). When *MEG3* and CLU were simultaneously over-expressed, the SW1116 cells show the similar proliferation capacities with the control cells (Fig. 4F). The migration and invasion assays showed that both CLU down-regulation and *MEG3* over-expression could reduce the migration abilities of SW1116 cells and that CLU over-expression weakened the anti-metastasis activities of *MEG3* in SW1116 cells (Fig. 4G; Supplementary Fig. 4D). Collectively, these data suggested that CLU inhibits the anticancer activities of the *MEG3* in CRC cells.

3.5. Vitamin D Signaling Pathway Regulates *MEG3* Expression in CRC Cells

Evidence shows that the vitamin D levels are inversely associated with CRC risk and positively associated with improved overall and CRC-specific survival (Morales-oyarvide et al., 2016). Vitamin D and its nuclear transcriptional receptor VDR have chemo-prevention activities for CRC (Aggarwal and Kallay, 2016). Vitamin D supplementation could decrease the inflammation-associated colorectal tumors incidence in mice, suggesting the pivotal roles of vitamin D in CRC development (Elimrani et al., 2017). To determine whether vitamin D regulates *MEG3* transcription in CRC cells, we analyzed the transcriptional factor binding sites in the promoter region of *MEG3* (–1500 bp to 0 bp) and found two potential VDR binding motifs (site 1: –230~–243; site2: –1373~–1384; Fig. 5A) according to the genome-wide ChIP-seq

study performed by Ramagopalan et al. (2010). To elucidate whether vitamin D could activate *MEG3* transcription, SW1116 cells were treated with $1\alpha,25\text{-(OH)}_2\text{D}$, the VDR ligand in cells. Significantly increased *MEG3* levels were observed 24 and 48 h after the treatment compared with the baseline or ethanol vehicle treatments (Fig. 5B). Similar results were observed in the RKO cells that received $1\alpha, 25\text{-(OH)}_2\text{D}$ treatment (Fig. 5B). In addition, over-expression of VDR increased *MEG3* expression both in SW1116 and RKO cells compared to the vector control with time-dependent manner (Fig. 5C). These results suggested that VDR might stimulate the transcription of *MEG3* in CRC cells. In further, we applied the dual luciferase reporter assay and ChIP assay to verify whether VDR directly binds to the *MEG3* promoter. The promoter of *MEG3* (–1500–1) was cloned into the pGL3 vector (pGL3-*MEG3*-promoter) and then co-transfected with VDR expression vector (pcDNA3.1-VDR) or control vector into 293 T cells. The pGL3-*MEG3*-promoter showed significantly higher relative luciferase activities when co-transfected with pcDNA3.1-VDR (Fig. 5D), suggesting the potential bind of VDR on the *MEG3* promoter. The following ChIP assays using the VDR antibody found that the site 1 segment, but not the site 2 segment, was enriched in the ChIP precipitate (Fig. 5E), suggesting that VDR may binds to the site 1 (–230 ~ –243) region of the *MEG3* promoter. These results suggested that VDR may stimulate *MEG3* transcription through direct binding to its promoter at the –230 ~ –243 region.

Furthermore, we determined the association between the *MEG3* and VDR expression levels in CRC tissues. A significantly positive correlation between the VDR protein level and *MEG3* mRNA levels was noticed ($P < .001$, $N = 371$, Fig. 5F, Supplementary Fig. 5A). Additionally, Kaplan-Meier analysis showed that lower VDR expression was associated with poorer overall survival of CRC patients (log-rank test, $P = .043$; Supplementary Fig. 5B), suggesting that loss of function for VDR may lead to poorer prognosis of CRC patients through down-regulating the *MEG3* levels.

4. Discussion

Although the mortality of CRC has decreased steadily in recent years, it remains the fourth most common cancer deaths globally (Arnold et al., 2017). Uncovering the genetic and epigenetic alterations that are involved in the pathogenesis of CRC may guide the prevention and clinical treatments for patients (Walsh and Terdiman, 2003). Recently, dysregulation of lncRNAs were involved in the tumorigenesis and progression of various types of cancers including CRC, which might serve as potential biomarkers or targets for disease prevention or treatment (Luo et al., 2017). *MEG3* is a novel lncRNA that functions as a tumor suppressor gene in glioma, gastric cancer, lung cancer and liver cancer (Zhang et al., 2003). Bando T et al. firstly reported heterogeneous deletions of the 14q32 region encoding *MEG3* in CRC tissues, suggesting the potential involvement of *MEG3* in CRC development (Bando et al., 1999). Previous studies have reported that *MEG3* served as a prognostic factor for CRC and modulated the chemotherapy sensitivity of CRC cells; however, the underlying mechanisms remain unclear (Kong et al., 2016; Li et al., 2017). In the current study, we confirmed that *MEG3* expression was frequently down-regulated in CRC tissues and *MEG3* knockdown stimulated the migration and proliferation of CRC cell lines. Moreover, we found that *MEG3* was also an independent prognostic factor for the overall survival and disease-free survival of CRC patients as suggested by the multivariate Cox regression models. These results suggested that *MEG3* could serve as a novel prognosis biomarker for CRC and might be a potential therapeutic target for CRC patients.

Clusterin is frequently overexpressed in multiple types of cancer, including prostate, breast, lung and colorectal cancer (Lee et al., 2016). It has been reported that Clusterin inhibits TNF- α induced apoptosis in breast cancer cells through activating the NF- κ B and Bcl-2 signaling pathways (Wang et al., 2012). It also stimulates the migration and metastasis of the cells through activating the EIF31/Akt/MMP13 signaling

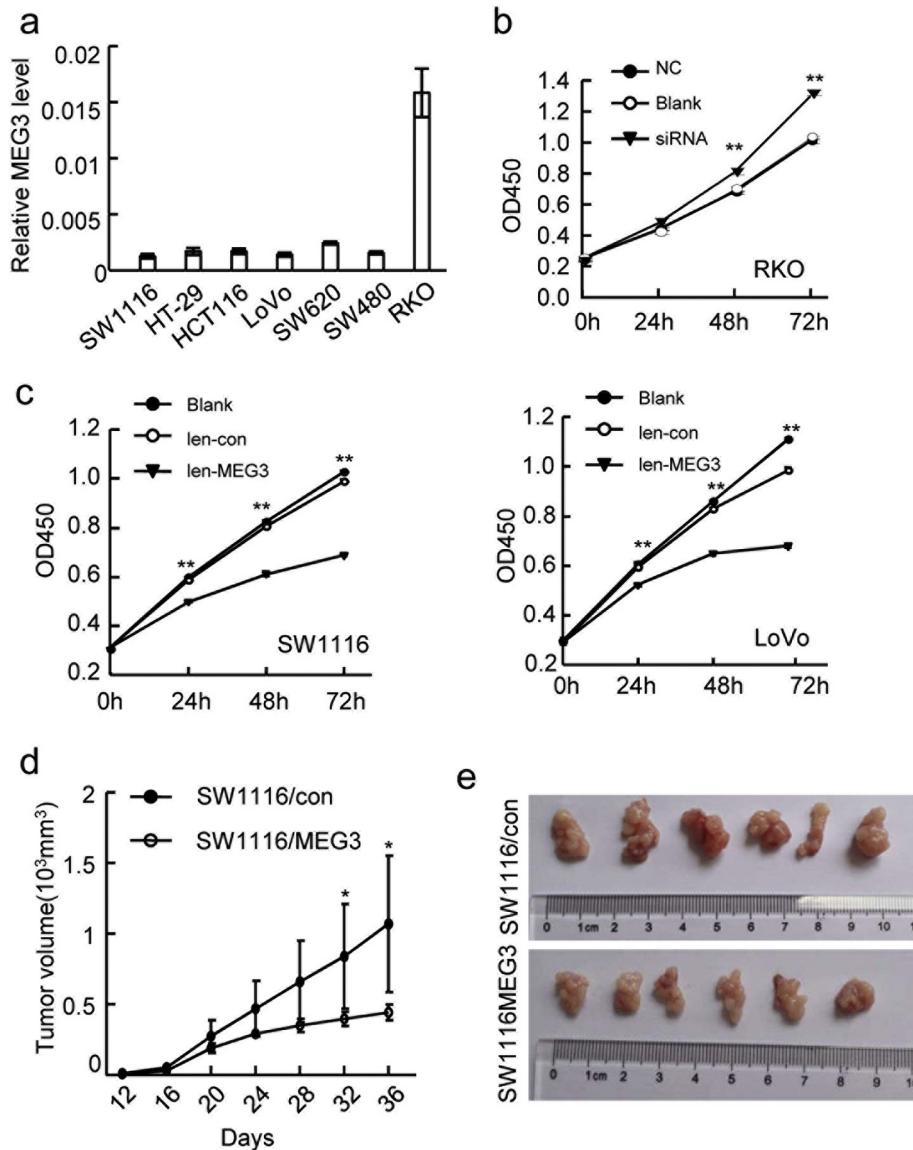


Fig. 2. MEG3 inhibits the cellular proliferation and tumor growth *in vitro* and *in vivo*. (A) Relative expression of MEG3 in 7 CRC cell lines was determined by RT-PCR. (B) The proliferation of RKO cells with siRNA transfection or not was assessed by CCK-8; (C) The proliferation of SW1116 (left) and LoVo (right) cells with MEG3 over-expression or not was assessed by CCK-8 assays. (D) The growth curves of the xenograft tumors among the different groups (SW1116/MEG3 and SW1116/con) (* $P < .05$, ** $P < .01$); (E) The image of tumors derived from SW1116/MEG3 or SW1116/con 36 days after injection. The data are representative of at least three independent experiments.

pathways in hepatocellular carcinoma (Wang et al., 2015). In CRC cells, Clusterin knockdown mimics the anticancer activities of MEG3, and Clusterin over-expression weakens the inhibition activities of MEG3 in cellular proliferation and metastasis, suggesting that Clusterin partially underlies the anti-cancer activities of MEG3 in CRC cells. In the present study, we reported that Clusterin was directly bound to the lncRNA MEG3. EMSA found that MEG3 directly binds to Clusterin at its 732–1174 nucleotide acid region. As the binding between Clusterin and MMP-9 or VEGF is critical in the metastasis in nasopharyngeal carcinoma (Li et al., 2016) and colon cancer (Radziwon-Balicka et al., 2014), MEG3 binding may impede the interactions between Clusterin and its target proteins. However, whether the directly binding with MEG3 could influence the Clusterin function need to be resolved with more investigation. Interestingly, MEG3 over-expression was also found to correlate with Clusterin transcription inhibition, suggesting that MEG3 might inhibit Clusterin activities through multiple mechanisms. As cAMP was reported to stimulate MEG3 expression and down-regulate clusterin expression (Zwain and Amato, 2001; Zhao et al., 2006), MEG3 may possibly rule clusterin regulation through

cAMP signaling pathways. It has also been reported that MEG3 regulates gene expression *via* binding to distal regulatory elements in DNA or recruiting miRNAs or proteins to destabilize target gene mRNA (Mondal et al., 2015; Su et al., 2016; Modali et al., 2015), suggesting a possible regulation of clusterin by MEG3 at transcript level. In addition, other proteins, except CLU and LYAR, were also detected in the MEG3 pull-down assay. Whether these proteins are involved in the anti-cancer activities of MEG3 in CRC remain unclear. Elucidating their roles may provide further insights into the mechanisms of MEG3 in CRC development and progression.

We also reported that VDR may directly regulate the MEG3 level in colorectal cells through binding to its promoter. Vitamin D is an essential vitamin that was found to be associated with CRC development and a poor prognosis (Li et al., 2014). Multiple genes, including lncRNAs encoding genes such as H19 and HOTAIR have been found to be regulated by Vitamin D through VDR in multiple cancers (Jiang and Bikle, 2014a; Jiang and Bikle, 2014b). Here, we found that vitamin D treatment or VDR over-expression up-regulated MEG3 expression in CRC cells. VDR may activate MEG3 expression *via* direct binding to its promoter

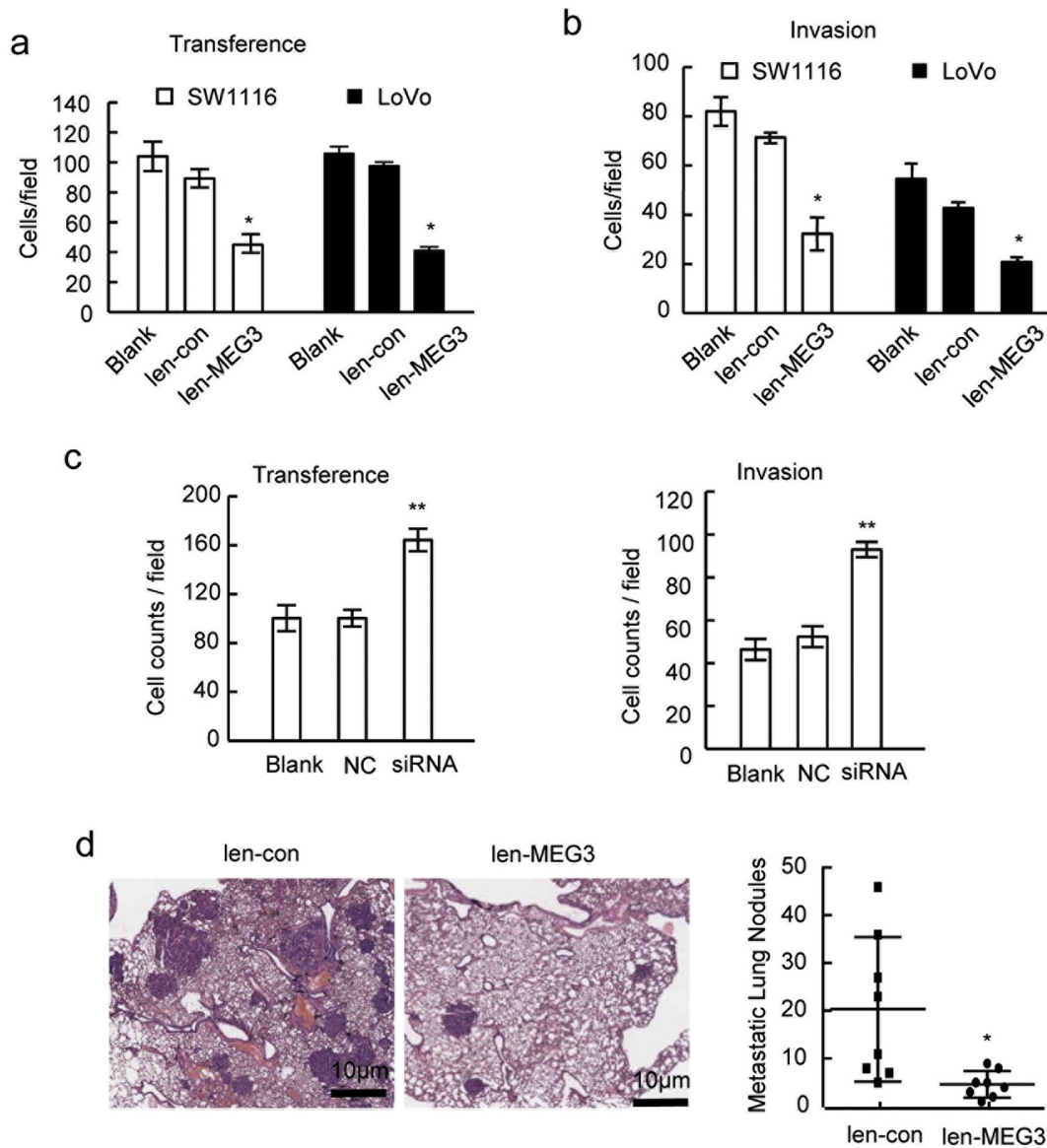


Fig. 3. *MEG3* suppresses the CRC cell migration and metastasis *in vitro* and *in vivo*. The cell migration (A) and invasion (B) ability of SE1116 and LoVo with *MEG3* over-expression was significantly decreased compared with the blank or vector group ($*P < .05$); (C) Cell migration and invasion ability of RKO cells with *MEG3* down-regulation was significantly higher than that of negative control or blank control group ($**P < .01$); (D) the represent image of lung tissues with tumor node (left, HE staining) and the number of lung metastases nodes in groups of SW1116/*MEG3* and SW1116/con (right, $*P < .05$). The data are representative of at least three independent experiments.

at the $-230 \sim -243$ promoter region. Accordingly, Menigatti et al. reported that *MEG3* was epigenetically silenced in precancerous colorectal lesions, possibly leading to the reduced transcriptional level of *MEG3* by VDR (Menigatti et al., 2013). In clinic, VDR level was positively correlated with the *MEG3* level in CRC tissues, and patients with higher VDR were associated with better OS. These results suggested that the regulation of *MEG3* expression by vitamin D/VDR can partially explain why CRC patients with vitamin D deficiency showed poorer prognosis (Duffy et al., 2017). The down-regulation of *MEG3* might also confer the increased risk of CRC development under the vitamin D deficiency status, which needs to be addressed with more investigations.

In conclusion, we found that *MEG3* expression was frequently down-regulated in CRC tissues and CRC patients with lower *MEG3* level showed poorer prognosis. *MEG3* inhibited metastasis and proliferation of CRC tumor cells both *in vivo* and *in vitro*, which may be caused through down-regulating clusterin expression level and its directly binding to CLU protein. In addition, vitamin D activated *MEG3* transcription in CRC tumor cells *via* direct binding to the *MEG3* promoter by VDR. These results suggest the potential roles of *MEG3* in CRC development

and progression and provide further insights into the mechanisms of the anti-cancer activities of vitamin D in CRC development and progression.

Funding Sources

This work was supported by the grants of China National Funds for National Natural Science Fund (No. 81672899), Science and Technology Commission of Shanghai Municipality (16140900703 and 16411966800), Shanghai Municipal Commission of Health and Family Planning (No. 2015ZB0202 and 20164Y0250). The funders had no role in study design, data collection, data analysis, interpretation, and writing of the report.

Conflicts of Interest

None.

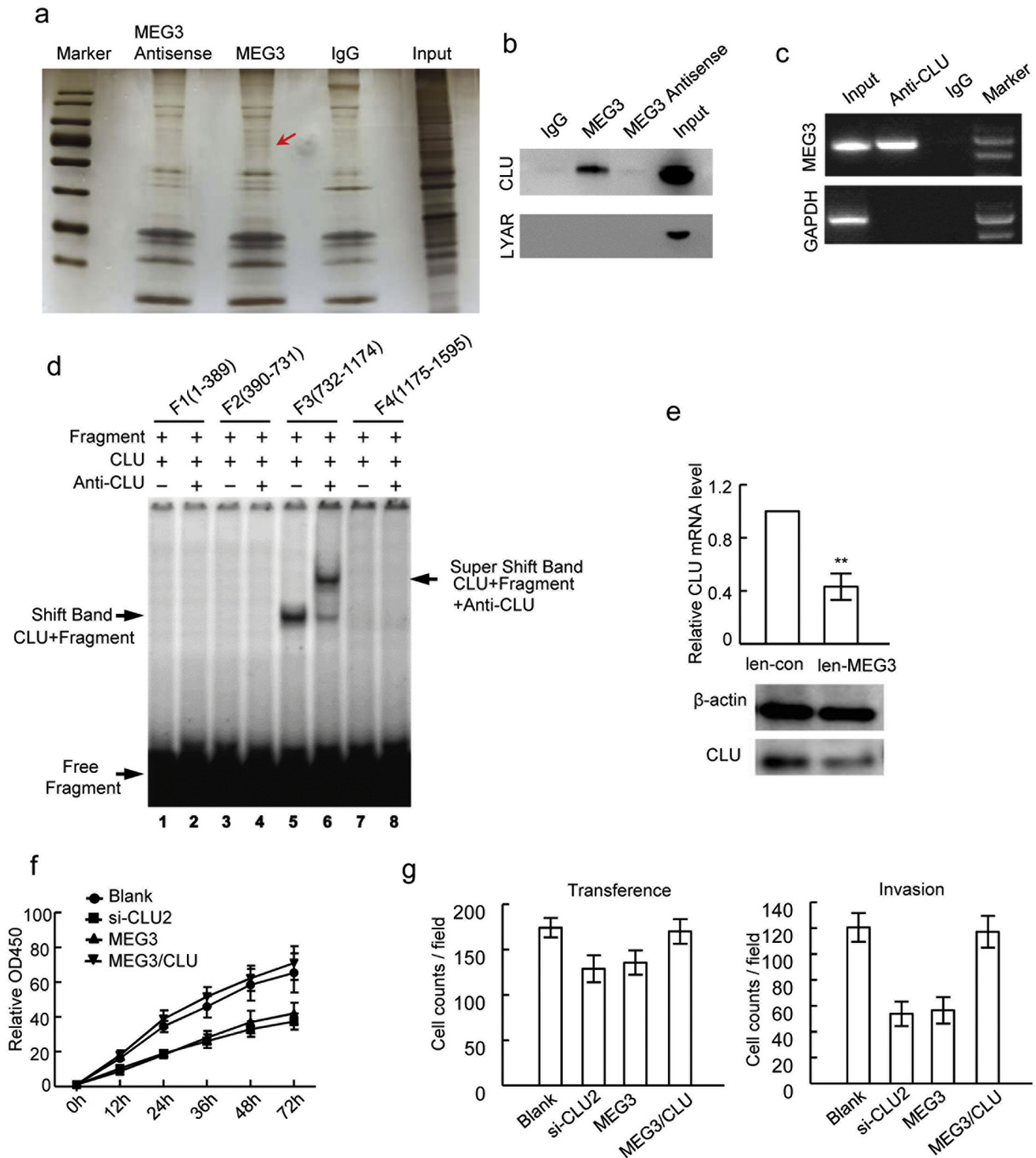


Fig. 4. *MEG3* interacts with Clusterin and inhibits proliferation and metastasis. (A) The represent image of SDS-PAGE with precipitated proteins from *MEG3* RNA pull down. Gel indicated by arrow was obtained for mass spectrometry analysis; (B) The represent image of western blotting for detection of LYAR and CLU in precipitated proteins from *MEG3* RNA pull down; (C) The represent image of agarose gel for *MEG3* detection in the precipitation from RIP by CLU antibody; (D) The represent image of EMSA to investigate the binding site of CLU protein in *MEG3*. *MEG3* was truncated into four fragments: F1–F4; (E) Up-regulation of CLU by *MEG3* over-expression (***P* < .01); (F and G) The cellular proliferation, migration and invasion abilities of SW1116 cells transfected with CLU siRNAs (SW1116/si-CLU), SW1116 cells with *MEG3* over-expression (SW1116/*MEG3*), SW1116 cells with *MEG3* and CLU over-expression (SW1116/*MEG3*/CLU) and blank control cells detected by CCK8 (F) and trans well assays (G). The data are representative of at least three independent experiments.

Author Contributions

The conception and design of the study: Jianming Zheng, Shupeng Liu, Peizhan Chen and Yan Zhu; Acquisition of data: Yan Zhu, Peizhan Chen, Yisha Gao, Na Ta, Yunshuo Zhang, Jialin Cai and Yong Zhao; Analysis and interpretation of data: Yan Zhu, Peizhan Chen and Shupeng Liu; Drafting the article: Yan Zhu, Peizhan Chen and Shupeng Liu; Revising it critically for important intellectual content: Yan Zhu, Peizhan Chen, Shupeng Liu and Jianming Zheng; Final approval of the version to be submitted: Shupeng Liu and Jianming Zheng.

Yan Zhu and Peizhan Chen contributed equally to this article.

Research in context

The mechanisms underlying colorectal cancer (CRC) development and progression are largely unknown. Here, we found that *MEG3* was down-regulated in CRC cells and its expression level was an independent prognostic factor for CRC patients in clinic. *MEG3* over-expression suppressed CRC cell proliferation and metastasis *in vivo* and *in vitro*. *MEG3* reduced the transcription of *Clusterin* and the corresponding

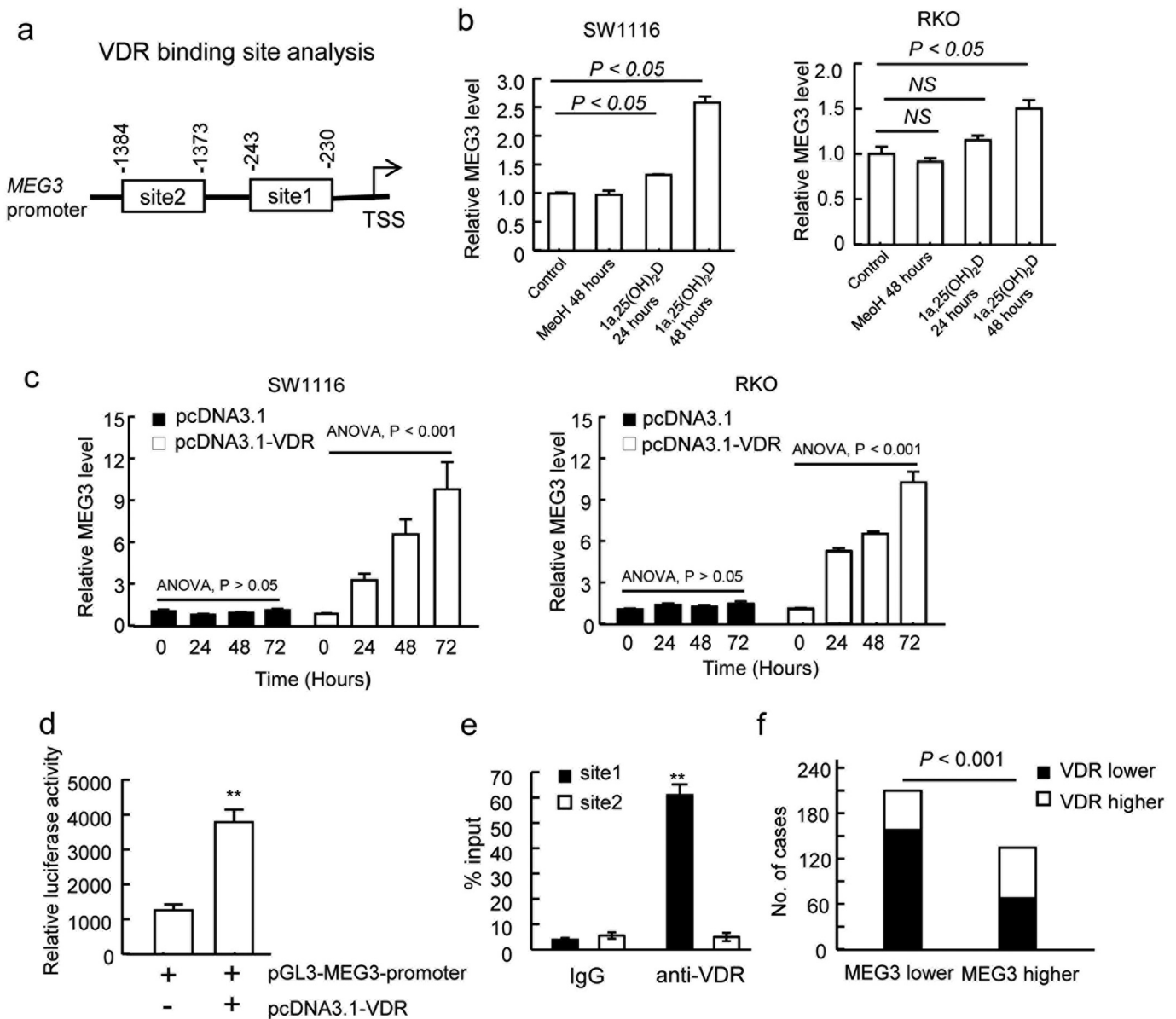


Fig. 5. VDR regulates *MEG3* through directly binding to the promoter region of *MEG3* in CRC cells. (A) Schematic of VDR binding site (site1 and site2) in the promoter of *MEG3* by bioinformatics analysis; (B) up-regulated *MEG3* expression in SW1116 and RKO cells by $1\alpha,25\text{-(OH)}_2\text{D}$ treatment for 24 and 48 h determined by RT-PCR; (C) *MEG3* expression in SW1116 and RKO cells with VDR over-expression (pcDNA3.1-VDR) or not (pcDNA3.1 vector); (D) Enhancement of *MEG3* promoter activity by VDR assessed by the luciferase reporter assay; (E) Binding of VDR with *MEG3* promoter verified by the ChIP assay using VDR antibody (** $P < .01$); (F) Correlation between expression level of VDR and *MEG3* in colorectal cancer tissues assessed by IHC and ISH staining. The data are representative of at least three independent experiments.

protein level in CRC cells. *MEG3* also directly bind to Clusterin protein via its 732–1174 nucleotide acid region. In further, Clusterin overexpression could impede the anti-proliferation and anti-metastasis activities of *MEG3* in CRC cells. Moreover, $1\alpha,25\text{-(OH)}_2\text{D}$ could activate *MEG3* expression in CRC cells via its nuclear receptor VDR (vitamin D receptor), providing further insights into how Vitamin D inhibits CRC development and progression in clinic. These results indicate that VDR/*MEG3*/Clusterin signaling pathway may serve as potential therapeutic targets and prognosis biomarkers for CRC.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2018.03.032>.

References

- Aggarwal, A., Kallay, E., 2016. Cross talk between the calcium-sensing receptor and the vitamin D system in prevention of cancer. *Front. Physiol.* 7, 451.
- Arnold, M., Sierra, M.S., Laversanne, M., Soerjomataram, I., Jemal, A., Bray, F., 2017. Global patterns and trends in colorectal cancer incidence and mortality. *Gut* 66, 683–691.
- Bando, T., Kato, Y., Ihara, Y., Yamagishi, F., Tsukada, K., Isobe, M., 1999. Loss of heterozygosity of 14q32 in colorectal carcinoma. *Cancer Genet. Cytogenet.* 111, 161–165.
- Cai, J., Li, B., Zhu, Y., Fang, X., Zhu, M., Wang, M., Liu, S., Jiang, X., Zheng, J., Zhang, X., Chen, P., 2017. Prognostic biomarker identification through integrating the gene signatures of hepatocellular carcinoma properties. *EBioMedicine* 19, 18–30.
- Chen, X., Halberg, R.B., Ehrhardt, W.M., Torrealba, J., Dove, W.F., 2003. Clusterin as a biomarker in murine and human intestinal neoplasia. *Proc. Natl. Acad. Sci. U. S. A.* 100, 9530–9535.
- Deng, J., He, M., Chen, L., Chen, C., Zheng, J., Cai, Z., 2013. The loss of miR-26a-mediated post-transcriptional regulation of cyclin E2 in pancreatic cancer cell proliferation and decreased patient survival. *PLoS One* e76450, 8.
- Duffy, M.J., Murray, A., Synnott, N.C., O'donovan, N., Crown, J., 2017. Vitamin D analogues: potential use in cancer treatment. *Crit. Rev. Oncol. Hematol.* 112, 190–197.
- Elimrani, I., Koenekoop, J., Dionne, S., Marcil, V., Delvin, E., Levy, E., Seidman, E.G., 2017. Vitamin D reduces colitis- and inflammation-associated colorectal Cancer in mice independent of NOD2. *Nutr. Cancer* 69, 276–288.
- Guinney, J., Dienstmann, R., Wang, X., de Reynies, A., Schlicker, A., Sonesson, C., Marisa, L., Roepman, P., Nyamundanda, G., Angelino, P., Bot, B.M., Morris, J.S., Simon, I.M., Gerster, S., Fessler, E., de Sousa, E.M.F., Missiaglia, E., Ramay, H., Barras, D., Homicsko, K., Maru, D., Manyam, G.C., Broom, B., Boige, V., Perez-Villamil, B., Laderas, T., Salazar, R., Gray, J.W., Hanahan, D., Tabernero, J., Bernards, R., Friend, S.H., Laurent-Puig, P., Medema, J.P., Sadanandam, A., Wessels, L., Delorenzi, M.,

- Kopetz, S., Vermeulen, L., Tejpar, S., 2015. The consensus molecular subtypes of colorectal cancer. *Nat. Med.* 21, 1350–1356.
- Jiang, Y.J., Bikle, D.D., 2014a. LncRNA profiling reveals new mechanism for VDR protection against skin cancer formation. *J. Steroid Biochem. Mol. Biol.* 144 (Pt A), 87–90.
- Jiang, Y.J., Bikle, D.D., 2014b. LncRNA: a new player in 1 α , 25(OH) $_2$ vitamin D(3)/VDR protection against skin cancer formation. *Exp. Dermatol.* 23, 147–150.
- Kong, H., Wu, Y., Zhu, M., Zhai, C., Qian, J., Gao, X., Wang, S., Hou, Y., Lu, S., Zhu, H., 2016. Long non-coding RNAs: novel prognostic biomarkers for liver metastases in patients with early stage colorectal cancer. *Oncotarget* 7, 50428–50436.
- Lee, J.Y., Kim, H.J., Rho, S.B., Lee, S.H., 2016. eIF3f reduces tumor growth by directly interrupting clusterin with anti-apoptotic property in cancer cells. *Oncotarget* 7, 18541–18557.
- Li, M., Chen, P., Li, J., Chu, R., Xie, D., Wang, H., 2014. Review: the impacts of circulating 25-hydroxyvitamin D levels on cancer patient outcomes: a systematic review and meta-analysis. *J. Clin. Endocrinol. Metab.* 99, 2327–2336.
- Li, Y., Lu, J., Zhou, S., Wang, W., Tan, G., Zhang, Z., Dong, Z., Kang, T., Tang, F., 2016. Clusterin induced by N,N'-Dinitrosopiperazine is involved in nasopharyngeal carcinoma metastasis. *Oncotarget* 7, 5548–5563.
- Li, L., Shang, J., Zhang, Y., Liu, S., Peng, Y., Zhou, Z., Pan, H., Wang, X., Chen, L., Zhao, Q., 2017. MEG3 is a prognostic factor for CRC and promotes chemosensitivity by enhancing oxaliplatin-induced cell apoptosis. *Oncol. Rep.* 38, 1383–1392.
- Li, J., Zi, Y., Wang, W., Li, Y., 2018. LncRNA MEG3 inhibits cell proliferation and metastasis in chronic myeloid leukemia via targeting MiR-184. *Oncol. Res.* 26, 297–305.
- Lin, J., Qiu, M., Xu, R., Dobs, A.S., 2015. Comparison of survival and clinicopathologic features in colorectal cancer among African American, Caucasian, and Chinese patients treated in the United States: results from the surveillance epidemiology and end results (SEER) database. *Oncotarget* 6, 33935–33943.
- Lu, K.H., Li, W., Liu, X.H., Sun, M., Zhang, M.L., Wu, W.Q., Xie, W.P., Hou, Y.Y., 2013. Long non-coding RNA MEG3 inhibits NSCLC cells proliferation and induces apoptosis by affecting p53 expression. *BMC Cancer* 13, 461.
- Luo, J., Qu, J., Wu, D.K., Lu, Z.L., Sun, Y.S., Qu, Q., 2017. Long non-coding RNAs: a rising biotarget in colorectal cancer. *Oncotarget* 8, 22187–22202.
- Ma, Y., Yang, Y., Wang, F., Moyer, M.P., Wei, Q., Zhang, P., Yang, Z., Liu, W., Zhang, H., Chen, N., Wang, H., Wang, H., Qin, H., 2016. Long non-coding RNA CCL regulates colorectal cancer progression by activating Wnt/ β -catenin signalling pathway via suppression of activator protein 2 α . *Gut* 65, 1494–1504.
- Marchese, F.P., Raimondi, I., Huarte, M., 2017. The multidimensional mechanisms of long noncoding RNA function. *Genome Biol.* 18, 206.
- Menigatti, M., Staiano, T., Manser, C.N., Bauerfeind, P., Komljenovic, A., Robinson, M., Jiricny, J., Buffoli, F., Marra, G., 2013. Epigenetic silencing of monoallelically methylated miRNA loci in precancerous colorectal lesions. *Oncogene* 2, e56.
- Modali, S.D., Parekh, V.I., Kebebew, E., Agarwal, S.K., 2015. Epigenetic regulation of the lncRNA MEG3 and its target c-MET in pancreatic neuroendocrine tumors. *Mol. Endocrinol.* 29, 224–237.
- Mondal, T., Subhash, S., Vaid, R., Enroth, S., Uday, S., Reinius, B., Mitra, S., Mohammed, A., James, A.R., Hoberg, E., Moustakas, A., Gyllenstein, U., Jones, S.J., Gustafsson, C.M., Sims, A.H., Westerlund, F., Gorab, E., Kanduri, C., 2015. MEG3 long noncoding RNA regulates the TGF- β pathway genes through formation of RNA-DNA triplex structures. *Nat. Commun.* 6, 7743.
- Morales-oyarvide, V., Meyerhardt, J.A., Ng, K., 2016. Vitamin D and physical activity in patients with colorectal Cancer: epidemiological evidence and therapeutic implications. *Cancer J.* 22, 223–231.
- Ohtsuka, M., Ling, H., Ivan, C., Pichler, M., Matsushita, D., Goblirsch, M., Stiegelbauer, V., Shigeyasu, K., Zhang, X., Chen, M., Vidhu, F., Bartholomeusz, G.A., Toyiyama, Y., Kusunoki, M., Doki, Y., Mori, M., Song, S., Gunther, J.R., Krishnan, S., Slaby, O., Goel, A., Ajani, J.A., Radovich, M., Calin, G.A., 2016. H19 noncoding RNA, an independent prognostic factor, regulates essential Rb-E2F and CDK8- β -catenin signaling in colorectal cancer. *EBioMedicine* 13, 113–124.
- Pan, Y., Cao, F., Guo, A., Chang, W., Chen, X., Ma, W., Gao, X., Guo, S., Fu, C., Zhu, J., 2015. Endoplasmic reticulum ribosome-binding protein 1, RRBP1, promotes progression of colorectal cancer and predicts an unfavourable prognosis. *Br. J. Cancer* 113, 763–772.
- Ponting, C.P., Oliver, P.L., Reik, W., 2009. Evolution and functions of long noncoding RNAs. *Cell* 136, 629–641.
- Radziwon-Balicka, A., Santos-Martinez, M.J., Corbalan, J.J., O'Sullivan, S., Treumann, A., Gilmer, J.F., Radomski, M.W., Medina, C., 2014. Mechanisms of platelet-stimulated colon cancer invasion: role of clusterin and thrombospondin 1 in regulation of the P38MAPK-MMP-9 pathway. *Carcinogenesis* 35, 324–332.
- Ramagopalan, S.V., Heger, A., Berlanga, A.J., Maugeri, N.J., Lincoln, M.R., Burrell, A., Handunnetthi, L., Handel, A.E., Disanto, G., Orton, S.M., Watson, C.T., Morahan, J.M., Giovannoni, G., Ponting, C.P., Ebers, G.C., Knight, J.C., 2010. A CHIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. *Genome Res.* 20, 1352–1360.
- Su, L., Han, D., Wu, J., Huo, X., 2016. Skp2 regulates non-small cell lung cancer cell growth by MEG3 and miR-3163. *Tumour Biol.* 37, 3925–3931.
- Torre, L.A., Bray, F., Siegel, R.L., Ferlay, J., Lortet-Tieulent, J., Jemal, A., 2015. Global cancer statistics, 2012. *CA Cancer J. Clin.* 65, 87–108.
- Walsh, J.M., Terdiman, J.P., 2003. Colorectal cancer screening: scientific review. *JAMA* 289, 1288–1296.
- Wang, Y., Wang, X., Zhao, H., Liang, B., Du, Q., 2012. Clusterin confers resistance to TNF- α -induced apoptosis in breast cancer cells through NF- κ B activation and Bcl-2 overexpression. *J. Chemother.* 24, 348–357.
- Wang, C., Jin, G., Jin, H., Wang, N., Luo, Q., Zhang, Y., Gao, D., Jiang, K., Gu, D., Shen, Q., Huo, X., Hu, F., Ge, T., Zhao, F., Chu, W., Shu, H., Yao, M., Cong, W., Qin, W., 2015. Clusterin facilitates metastasis by EIF3/Akt/MMP13 signaling in hepatocellular carcinoma. *Oncotarget* 6, 2903–2916.
- Wu, Y., Liu, M., Li, Z., Wu, X.B., Wang, Y., Wang, Y., Nie, M., Huang, F., Ju, J., Ma, C., Tan, R., Zen, K., Zhang, C.Y., Fu, K., Chen, Y.G., Wang, M.R., Zhao, Q., 2015. LYAR promotes colorectal cancer cell mobility by activating galectin-1 expression. *Oncotarget* 6, 32890–32901.
- Yan, H.L., Xue, G., Mei, Q., Wang, Y.Z., Ding, F.X., Liu, M.F., Lu, M.H., Tang, Y., Yu, H.Y., Sun, S.H., 2009. Repression of the miR-17-92 cluster by p53 has an important function in hypoxia-induced apoptosis. *EMBO J.* 28, 2719–2732.
- Yin, D.D., Liu, Z.J., Zhang, E., Kong, R., Zhang, Z.H., Guo, R.H., 2015. Decreased expression of long noncoding RNA MEG3 affects cell proliferation and predicts a poor prognosis in patients with colorectal cancer. *Tumour Biol.* 36, 4851–4859.
- Yu, J., Wu, W.K., Li, X., He, J., Li, X.X., Ng, S.S., Yu, C., Gao, Z., Yang, J., Li, M., Wang, Q., Liang, Q., Pan, Y., Tong, J.H., To, K.F., Wong, N., Zhang, N., Chen, J., Lu, Y., Lai, P.B., Chan, F.K., Li, Y., Kung, H.F., Yang, H., Wang, J., Sung, J.J., 2015. Novel recurrently mutated genes and a prognostic mutation signature in colorectal cancer. *Gut* 64, 636–645.
- Yuan, J.H., Liu, X.N., Wang, T.T., Pan, W., Tao, Q.F., Zhou, W.P., Wang, F., Sun, S.H., 2017. The MBNL3 splicing factor promotes hepatocellular carcinoma by increasing PXN expression through the alternative splicing of lncRNA-PXN-AS1. *Nat. Cell Biol.* 19, 820–832.
- Zhang, X., Zhou, Y., Mehta, K.R., Danila, D.C., Scolavino, S., Johnson, S.R., Klibanski, A., 2003. A pituitary-derived MEG3 isoform functions as a growth suppressor in tumor cells. *J. Clin. Endocrinol. Metab.* 88, 5119–5126.
- Zhang, W., Shi, S., Jiang, J., Li, X., Lu, H., Ren, F., 2017. LncRNA MEG3 inhibits cell epithelial-mesenchymal transition by sponging miR-421 targeting E-cadherin in breast cancer. *Biomed. Pharmacother.* 91, 312–319.
- Zhao, J., Zhang, X., Zhou, Y., Ansell, P.J., Klibanski, A., 2006. Cyclic AMP stimulates MEG3 gene expression in cells through a cAMP-response element (CRE) in the MEG3 proximal promoter region. *Int. J. Biochem. Cell Biol.* 38, 1808–1820.
- Zhou, Y., Zhang, X., Klibanski, A., 2012. MEG3 noncoding RNA: a tumor suppressor. *J. Mol. Endocrinol.* 48, R45–53.
- Zhu, J., Fu, H., Wu, Y., Zheng, X., 2013. Function of lncRNAs and approaches to lncRNA-protein interactions. *Sci. China Life Sci.* 56, 876–885.
- Zwain, I.H., Amato, P., 2001. cAMP-induced apoptosis in granulosa cells is associated with up-regulation of P53 and bax and down-regulation of clusterin. *Endocr. Res.* 27, 233–249.