ATAD2 silencing decreases VEGFA secretion through targeting has-miR-520a to inhibit angiogenesis in colorectal cancer

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Complete List of Authors:	Hong, Sen; The First Hospital of Jilin University Chen, Si; The First Hospital of Jilin University Wang, Xu; The First Hospital of Jilin University Sun, Di; The First Hospital of Jilin University Yan, Zhenkun; China-Japan Union Hospital of Jilin University Tai, Jiandong; The First Hospital of Jilin University Bi, Miaomiao; China-Japan Union Hospital of Jilin University, Department of Ophthalmology
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3 Sen Hong¹, Si Chen¹, Xu Wang¹, Di Sun¹, Zhenkun Yan², Jiandong Tai^{1,*}, Miaomiao
4 Bi^{3,*}

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Abstract

ATPase family AAA domain-containing protein 2 (ATAD2) is involved in various types of cancers, including colorectal cancer. This study aimed to determine the role of ATAD2 in angiogenesis in colorectal cancer. Here, we downregulated ATAD2 expression in HCT116 and SW480 cells, and collected the conditioned medium (CM) from control and ATAD2-sienced cells. The effect of CM on human umbilical vein endothelial cells (HUVEC) was evaluated by using CCK-8, wound healing, tube formation, western blot, and dual-luciferase reporter assays. Our results showed that the proliferation, migration and tube formation of HUVEC were reduced in presence of ATAD2-sienced CM, and the levels of phosphorylated vascular endothelial growth factor receptor 2 (P-VEGFR2), CD31, and CD34 were down-regulated. Mechanism studies showed that ATAD2 silencing regulated the expression of vascular endothelial growth factor A (VEGFA) and miR-520a. Moreover, we found that miR-520a could bind to ATAD2, and its inhibitor partly reversed the alterations in HUVEC induced by CM from ATAD2-sienced cells. In addition, we demonstrated that miR-520a directly bound to 3'-UTR of VEGFA and inhibited its expression. Collectively, our results indicate that ATAD2 inhibition suppresses VEGFA secretion by increasing miR-520a levels. Our study suggests ATAD2 as a potential therapeutic target for angiogenesis in colorectal cancer.

Key words: ATAD2; colorectal cancer; angiogenesis; miR-520a; VEGFA

40 **Introduction**

41 Colorectal cancer is the third most common malignant tumor and the fourth leading
42 cause of cancer death worldwide (Ferlay et al. 2015). The etiology of this cancer is
43 multifactorial and is thought to be associated with genetic and environmental factors
44 (Kim et al. 2013). Currently, the main treatment of colorectal cancer consists of
45 surgery, chemotherapy and radiation therapy, but the survival of colorectal cancer
46 patients with advanced disease remains poor (Chen et al. 2014; Kou et al. 2015).
47 Hence, it is necessary to develop new therapeutic strategies for the treatment of
48 colorectal cancer.

49 ATAD2 (ATPase family, AAA domain containing 2), a member of ATPase family,
50 contains an AAA domain that is important in regulating ATPase activity and protein
51 multimerization, and a bromodomain that is capable of binding to acetylated histones
52 (Krakstad et al. 2015). The structure of ATAD2 indicates that it has functions related
53 to the regulation of cell proliferation, differentiation and apoptosis (Zheng et al. 2015).
54 Previous studies demonstrated that ATAD2, as a cofactor for MYC, could promote
55 tumor progression (Ciro et al. 2009). Overexpression of ATAD2 has been reported to
56 be associated with poor prognosis in patients with cancer, such as breast cancer
57 (Caron et al. 2010), lung cancer (Caron et al. 2010), gastric carcinoma (Zhang et al.
58 2016), ovarian cancer (Wan et al. 2014), and colorectal cancer (Hou et al. 2016).
59 Moreover, our previous study demonstrated that knockdown of ATAD2 significantly
60 inhibited migration, invasion, and epithelial-mesenchymal transition of colorectal
61 cancer cells (Hong et al. 2016). However, the effect of ATAD2 on angiogenesis in
62 colorectal cancer has not been yet characterized.

63 In the present study, we silenced the expression of ATAD2 in HCT116 and SW480
64 cells and evaluated the effect of the conditioned medium (CM) from ATAD2-silenced
65 colorectal cancer cells on cell proliferation, migration, and tubule formation in human
66 umbilical vein endothelial cells (HUVEC). Furthermore, we demonstrated that
67 silencing of ATAD2 suppressed vascular endothelial growth factor A (VEGFA)
68 secretion by upregulating miR-520a in colorectal cancer.

69 **Materials and methods**

70 **Cells and cell culture**

71 HCT116, SW480, and HUVEC were purchased from Shanghai Zhong Qiao Xin Zhou
72 Biotechnology Co., Ltd. (Shanghai, China). All cells were cultured in Dulbecco's
73 modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented
74 with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and placed in an
75 incubator at 37°C and 5% CO₂.

76 **Vector construction and stable transfection**

77 The sequences of ATAD2 shRNA and non-target (negative control, NC) shRNA were
78 as follows: ATAD2 shRNA, 5'-GATCCCCGGCGATGGGTCATCAGTTATTCAAG
79 AGATAACTGATGACCCATCGCCTTTTT-3'; NC shRNA: 5'-GATCCCCTTC
80 TCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCCGAGAATTTTT-3'.
81 They were cloned into the BamHI/HindIII site of pRNA H1.1 vector. Thereafter, the
82 recombinant plasmid was transfected into HCT116 and SW480 cells by
83 Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The drug resistant pooled cell
84 clones were obtained by G418 (Invitrogen) selection. The conditioned medium (CM)

85 was collected from untreated cells, or cells stably transfected NC shRNA, or cells
86 stably transfected ATAD2 shRNA.

87 **Transient transfection and VEGFA treatment**

88 MiR-520a mimics and inhibitor were synthesized by GenePharma (Shanghai, China)
89 and transfected into HCT116 and SW480 cells by Lipofectamine 2000 (Invitrogen).
90 VEGFA was obtained from Sino Biological Inc. (Beijing, China) and dissolved in
91 sterile water. 20 ng/ml VEGFA was mixed with CM from HCT116 with ATAD2
92 knockdown to culture HUVEC.

93 **Real-time PCR**

94 Total RNA was isolated from cells using TriPure reagent (Biotek, Beijing, China)
95 and converted to cDNA by reverse transcription-PCR. Mir-520a RT primer:
96 GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACACAGTC.
97 Then the cDNA was amplified by real-time PCR. The primer sequences were as
98 follows: ATAD2 forward, GTGGGAAATAGTTGGACCGACA, reverse,
99 CGGAATGGGGTTTGTCTAGAAG; mir-520a forward:
100 GTACGAAAGTGCTTCCCTTTGG, reverse, GTGCAGGGTCCGAGGTATTC;
101 CD31 forward: AAGATAGCCTCAAAGTCGGA, reverse,
102 TCAAAGTGGGCATCATAAGA; CD34 forward: GTCTTCCACTCGGTGCGTCTC,
103 reverse, GTTCCCTGGGTAGGTAAGTCT; β -actin forward,
104 CTTAGTTGCGTTACACCCTTTCTTG, reverse,
105 CTGTCACCTTCACCGTTCCAGTTT. The reaction was carried out using an
106 Exicycler 96 Bioneer (Bioneer Corporation, Daejeon, Korea). The relative expression
107 of gene was calculated by the $2^{-\Delta\Delta C_t}$ method.

108 Western blot assay

109 Total protein was exacted from cells using RIPA buffer (Beyotime Institute of
110 Biotechnology, Beijing, China). A BCA protein assay kit (Beyotime Institute of
111 Biotechnology) was employed to determine protein concentration. Afterward, samples
112 were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gel
113 electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes
114 (Millipore, Billerica, MA, USA). Then membranes were blocked with 5% defatted
115 milk and incubated with primary antibodies against ATAD2 (1:1000, Abcam,
116 Cambridge, MA, UK), vascular endothelial growth factor receptor 2 (VEGFR2; 1:500,
117 Abcam), P-VEGFR2 (1:1000, Abcam), CD31 (1:1000, Proteintech, Wuhan, China),
118 CD34 (1:10000, Abcam), VEGFA (1:1000, Proteintech), and β -actin (1:500, Keygen,
119 Nanjing, China) overnight at 4°C, followed by incubation with horseradish
120 peroxidase-labeled secondary antibodies. The blots were visualized with enhanced
121 chemiluminescence (Beyotime Institute of Biotechnology) by a gel imaging system
122 (Beijing Liuyi Biotechnology Co., Ltd., Beijing, China). The intensity of the bands
123 was evaluated by densitometric analysis and each band was normalized to β -actin
124 signal.

125 Enzyme-linked immunosorbent assay (ELISA)

126 The content of VEGFA was detected using a commercial ELISA kit (MultiSciences
127 Biotech Co., Ltd., Hangzhou, China) according to the manufacturer's instructions.

128 Cell counting kit (CCK)-8 assay

129 Cell proliferation was determined by a CCK-8 kit (Beyotime Institute of
130 Biotechnology). In brief, cells (3×10^3 per well) were seeded into 96-well plates in

131 quintuplicate. After treatment for 24, 48, 72, and 96 h. The supernatant was discarded
132 at the indicated time point. Then 100 μ l of complete medium and 10 μ l of CCK-8
133 solution added to each well for 1 h at 37°C. The optical density was detected at 450
134 nm with a microplate reader (Biotek, Winooski, VT, USA).

135 **Scratch wound healing assay**

136 Cell migration was evaluated by scratch wound healing assay. Briefly, cells were
137 stimulated with 1 μ g/ml mitomycin C (Sigma Aldrich, St Louis, USA) for 1 h when
138 they reached full confluence. After treatment for 24 h. Scratch wounds were made
139 with sterile 200 μ l pipette tips. Cells were then cultured in serum-free medium and
140 photographed with an inverted phase-contrast microscope (Olympus, Tokyo, Japan) at
141 0 and 24 h. The cell migration rate was calculated based on the distance between the
142 two fronts of the wound.

143 **Tube formation assay**

144 Angiogenesis was assessed by tube formation assay. Briefly, cells (3×10^4 per well)
145 were dispensed into 96-well plates coated with Matrigel (BD Bioscience, Franklin
146 Lakes, NJ, USA). After treatment for 18 h. The image was captured using a
147 microscope (Olympus) and the number of tube formation was counted.

148 **Dual-luciferase reporter assay**

149 293T cells were co-transfected with pmirGLO vector including the 3' UTR fragment
150 or 3' untranslated region (UTR) mutant fragment of ATAD2, and miR-520 mimics or
151 miR-520 NC mimics for 48 h. Renilla and firefly luciferase activities were assayed

152 using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA).
153 The ratios of firefly and renilla luciferase activity were calculated.

154 **Statistical analysis**

155 Each experiment was repeated three times and the results were presented as mean $\pm$
156 standard deviation (SD). Statistical differences were analyzed using one or two-way
157 ANOVA by GraphPad prism 7 software (GraphPad Software, La Jolla, CA, USA).
158 Values of P less than 0.05 were considered statistically significant.

159

160 **Results**

161 **Silencing of ATAD2 in colorectal cancer cells**

162 We stably knocked down the expression of ATAD2 in HCT116 and SW480 cells. As
163 shown in Figure 1A and B, results of western blot and real-time PCR assays showed
164 that ATAD2 expression was significantly decreased in ATAD2-silenced colorectal
165 cancer cells.

166 **Proliferation and migration of HUVEC were attenuated after being cultured in** 167 **CM from ATAD2-silenced colorectal cancer cells**

168 We collected CM from HCT116 or SW480 cells with or without ATAD2 knockdown.
169 As shown in Figure 2A, results from CCK-8 assay revealed that the proliferation
170 ability of HUVEC was inhibited when cultured in CM from ATAD2-silenced
171 colorectal cancer cells. Moreover, the migration ability of HUVEC was suppressed
172 when cultured in CM from ATAD2-silenced HCT116 or SW480 cells (Figure 2B).

173 Tube formation of HUVEC was inhibited after being cultured in CM from
174 ATAD2-silenced colorectal cancer cells

175 Furthermore, we evaluated the influence of CM on the tube formation of HUVEC. As
176 shown in Figure 3A, the number of tube formation in HUVEC cultured in ATAD2-
177 silenced CM was reduced. In addition, western bolt analysis revealed a decreased
178 expression of P-VEGFR2, CD31, and CD34 in HUVEC exposed to ATAD2-silenced
179 CM (Figure 3B). The results from real-time PCR indicated that the alterations in the
180 mRNA levels of CD31 and CD34 were in accordance with their protein levels (Figure
181 3C).

182 Knockdown of ATAD2 suppressed the level of VEGFA through targeting miR-
183 520a

184 We subsequently studied the underlying molecular mechanisms of ATAD2 in
185 colorectal cancer cells. As shown in Figure 4A, we found that the level of VEGFA in
186 the supernatants of ATAD2-silenced CT116 and SW480 cells was decreased. Real-
187 time PCR analysis showed an increase in miR-520a in colorectal cancer cells after
188 knockdown of ATAD2 (Figure 4B). Similarly, VEGFA expression in ATAD2-
189 silenced colorectal cancer cells was inhibited following ATAD2 silencing (Figure 4C).
190 Moreover, the results of western blot showed that miR-520a mimics could inhibit the
191 expression of VEGFA, and miR-520a inhibitor could promote VEGFA expression in
192 HCT116 and SW480 cells (Figure 4D). Furthermore, we demonstrated that miR-520a
193 directly targeted the 3'-UTR of ATAD2 in 293T cells by dual-Luciferase reporter
194 assay (Figure 4E). Moreover, we found that miR-520a inhibitor could reverse the
195 inhibition of VEGFA level and cell migration and tube formation caused by CM from
196 ATAD2-silenced HCT116 cells (Figure 5A-C).

197 Administration of VEDFA relieved the inhibiting effect of CM on HUVEC

198 Lastly, we demonstrated that miR-520a directly targeted the 3'-UTR of VEGFA in
199 293T cells (Figure 6A). Moreover, as shown in Figure 6B-D, administration of
200 VEDFA reversed the inhibition of proliferation, migration, and tube formation caused
201 by CM from ATAD2-silenced HCT116 cells in HUVEC.

202

203 Discussion

204 Metastasis is a main cause of mortality in patients with colorectal cancer (Cierniewski
205 et al. 2010), and angiogenesis plays a critical role in this process (Harris et al. 2012).
206 Numerous studies have demonstrated that ATAD2 was involved in cell growth in
207 several cancers, such as cervical cancer (Zheng et al. 2015), hepatocellular carcinoma
208 (Lu et al. 2015), breast cancer (Kalashnikova et al. 2010), and colorectal cancer
209 (Hong et al. 2016). However, the role of ATAD2 in angiogenesis during tumor
210 development has not been reported yet. In the present study, we demonstrated that the
211 proliferation, migration, and tube formation in HUVEC cultured in CM from
212 ATAD2-silenced colorectal cancer cells were significantly inhibited, which was
213 accompanied with downregulation of vascular endothelial cell markers, including P-
214 VEGFR2, CD31, and CD34, indicating that ATAD2 plays a role in the angiogenesis
215 in colorectal cancer.

216 Multiple signaling pathways are implicated in the regulation of angiogenesis, such as
217 VEGF (Hicklin and Ellis 2005), Delta-like 4 (Dll4)/Notch (Liu et al. 2014), Wnt
218 (Choi et al. 2012), and dimethylarginine dimethylaminohydrolase/asymmetric
219 dimethylarginine (DDAH/ADMA) (Fiedler and Wojciak-Stothard 2009) pathways.

220 Among angiogenic factors, VEGF family is the most important one involved in the
221 angiogenesis, which includes VEGFA, VEGFB, VEGFC, VEGFD and a structurally
222 related molecule, placental growth factor (PlGF) (Murukesh et al. 2010; Varinska et al.
223 2015). The most intensively studied member of VEGF family is VEGFA, which can
224 bind to both VEGF receptor 1 (VEGFR-1) and -2 (VEGFR-2) to promote
225 angiogenesis (Shibuya 2013). VEGFA is up-regulated in a variety of cancers,
226 including colorectal cancer, and it is used as an indicator for poor prognosis (Martins
227 et al. 2013). Moreover, numerous VEGFA inhibitors have been reported in the
228 treatment of cancers through inhibition of tumor angiogenesis, such as bevacizumab
229 and ranibizumab (Ferrara et al. 2007). In the present study, we found that knockdown
230 of ATAD2 decreased the level of VEGFA in colorectal cancer cells. Moreover, the
231 effect of CM from ATAD2-silenced colorectal cancer cells on HUVEC could be
232 reversed by adding exogenous VEGFA.

233 MicroRNAs are a class of non-coding RNAs of about 22 nucleotides in length, and
234 they function as posttranscriptional gene regulators (Diederichs and Haber 2007).
235 Previous studies have shown that miRNAs, such as miR-15a, miR-16, and
236 miR-101, contribute to angiogenesis by targeting VEGF (Sun et al. 2013; Zhang et al.
237 2013). Recent studies show that miR-520a can suppress cell proliferation in non-small
238 cell lung cancer (Yu et al. 2015) and hepatocellular carcinoma (Dong et al. 2015),
239 suggesting it as a tumor suppressor. In this study, we found miR-520a as a miRNA
240 regulated by ATAD2. Our data showed that ATAD2 inhibition lead to miR-520a
241 upregulation. Similar phenomenon was observed in hepatocellular carcinoma by
242 Huang and co-workers (Huang et al. 2016). Our study together with a study from Ye
243 et al. (Ye et al. 2015) demonstrated VEGFA as a direct target of miR-520a. Although
244 how ATAD2 regulates miR-520a is unclear, by analyzing the sequence of ATAD2

(TargetScan, Version 7.2), we find that ATAD2 possesses sequence for miR-520a recognition. Our additional dual-Luciferase reporter assay shows that ATAD2 can bind to miR-520a. It is possible that the mRNA of *ATAD2* gene may act as a competitive RNA to sponge miR-520a from targeting VEGFA. Further experiments will be performed in the near future to validate this hypothesis.

In summary, our data demonstrate that the proliferation, migration, and tube formation of HUVEC was suppressed after being cultured in CM from ATAD2-silenced colorectal cancer cells. Moreover, knockdown of ATAD2 downregulates the level of VEGFA by upregulating miR-520a. Our results suggest ATAD2 as a potential therapeutic target to inhibit angiogenesis in colorectal cancer.

Conflict of interest

All authors declare that they have no conflicts of interest.

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377

378

379 **Figure legends**

380 **Figure 1. Knockdown of ATAD2 in colorectal cancer cells.** The expression of
381 ATAD2 was evaluated in HCT116 and SW480 cells following silencing of ATAD2
382 by western blotting (A) and real-time PCR (B). Data were present as mean $\pm$ SD.
383 ***P<0.001.

384 **Figure 2. HUVEC proliferation and migration were inhibited after culture in**
385 **CM from ATAD2-silenced colorectal cancer cells.** Proliferation and migration
386 ability of HUVEC cultured in CM was determined by CCK-8 assay (A) and wound
387 healing assay (B), respectively. H-CM, HCT116 conditioned medium; H-NC,
388 HCT116 negative control conditioned medium; H-ATAD2-shRNA, HCT116
389 ATAD2-shRNA conditioned medium. S-CM, SW480 conditioned medium; S-NC,
390 SW480 negative control conditioned medium; S-ATAD2-shRNA, SW480 ATAD2-
391 shRNA conditioned medium. Data were present as mean $\pm$ SD. *P<0.05; **P<0.01;
392 ***P<0.001.

393 **Figure 3. The angiogenesis of HUVEC was suppressed after culture in CM from**
394 **ATAD2-silenced colorectal cancer cells.** The capillary tube formation of HUVEC
395 cultured in CM was evaluated by tube formation assay (A). Expression levels of P-
396 VEGFR2, VEGFR2, CD31, and CD34 were detected by western blotting (B). The
397 mRNA expression levels of CD31 and CD34 were determined through real-time PCR
398 (C). H-SFM, HCT116 serum free medium; H-CM, HCT116 conditioned medium; H-
399 NC, HCT116 negative control conditioned medium; H-ATAD2-shRNA, HCT116
400 ATAD2-shRNA conditioned medium. S-SFM, SW480 serum free medium; S-CM,
401 SW480 conditioned medium; S-NC, SW480 negative control conditioned medium; S-

402 ATAD2-shRNA, SW480 ATAD2-shRNA conditioned medium. Data were present as
 403 mean $\pm$ SD. *P<0.05; **P<0.01; ***P<0.001.

404 **Figure 4. ATAD2 regulated the level of VEGFA via miR-520a in colorectal**
 405 **cancer cells.** Levels of VEGFA in the supernatant of ATAD2-silenced HCT116 or
 406 SW480 cells were determined by ELISA kit (A). Real-time PCR was employed to
 407 detect miR-520a expression in colorectal cancer cells with or without ATAD2
 408 knockdown (B). The expression of VEGFA in HCT116 or SW480 cells following
 409 silencing of ATAD2 was assessed through western blotting (C). The effect of miR-
 410 520a mimics or inhibitor on the expression of VEGFA in HCT116 or SW480 cells
 411 was evaluated by western blotting (D). The effect of miR-520a mimics on luciferase
 412 activity of ATAD2 3'-UTR was determined (E). Data were present as mean $\pm$ SD.
 413 *P<0.05; **P<0.01; ***P<0.001.

414 **Figure 5. MiR-520a inhibitor reversed the inhibiting effect of CM from ATAD2-**
 415 **silenced HCT116 cells on HUVEC.** Levels of VEGFA in the supernatant of
 416 ATAD2-silenced HCT116 were determined by ELISA kit (A). Migration and
 417 capillary tube formation of HUVEC were assessed by wound healing (B) and tube
 418 formation assays (C), respectively. H-NC, HCT116 negative control conditioned
 419 medium; H-ATAD2-shRNA, HCT116 ATAD2-shRNA conditioned medium. H-
 420 ATAD2-shRNA + NC inhibitor, HCT116 ATAD2-shRNA conditioned medium + .
 421 negative control inhibitor. H-ATAD2-shRNA + mir-520a inhibitor, HCT116
 422 ATAD2-shRNA conditioned medium + mir-520a inhibitor. Data were present as
 423 mean $\pm$ SD. *P<0.05; **P<0.01; ***P<0.001.

424 **Figure 6. Treatment with VEGFA reversed the inhibiting effect of CM from**
 425 **ATAD2-silenced HCT116 cells on HUVEC.** The effect of miR-520a mimics on

426 luciferase activity of VEGFA 3'-UTR was assessed (A). Proliferation, migration, and
 427 capillary tube formation of HUVEC was determined by CCK-8 (B), wound healing
 428 (C), and tube formation assays (D), respectively. H-SFM, HCT116 serum free
 429 medium; H-NC, HCT116 negative control conditioned medium; H-ATAD2-shRNA,
 430 HCT116 ATAD2-shRNA conditioned medium. H-ATAD2-shRNA + VEGFA,
 431 HCT116 ATAD2-shRNA conditioned medium + 20 ng/ml VEGFA. Data were
 432 present as mean $\pm$ SD. **P<0.01; ***P<0.001.

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Figure 1. Knockdown of ATAD2 in colorectal cancer cells. The expression of ATAD2 was evaluated in HCT116 and SW480 cells following silencing of ATAD2 by western blotting (A) and real-time PCR (B). Data were present as mean $\pm$ SD. ***P<0.001.

205x166mm (300 x 300 DPI)

Figure 2. HUVEC proliferation and migration were inhibited after culture in CM from ATAD2-silenced colorectal cancer cells. Proliferation and migration ability of HUVEC cultured in CM was determined by CCK-8 assay (A) and wound healing assay (B), respectively. H-CM, HCT116 conditioned medium; H-NC, HCT116 negative control conditioned medium; H-ATAD2-shRNA, HCT116 ATAD2-shRNA conditioned medium. S-CM, SW480 conditioned medium; S-NC, SW480 negative control conditioned medium; S-ATAD2-shRNA, SW480 ATAD2-shRNA conditioned medium. Data were present as mean $\pm$ SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

217x186mm (300 x 300 DPI)

Figure 3. The angiogenesis of HUVEC was suppressed after culture in CM from ATAD2-silenced colorectal cancer cells. The capillary tube formation of HUVEC cultured in CM was evaluated by tube formation assay (A). Expression levels of P-VEGFR2, VEGFR2, CD31, and CD34 were detected by western blotting (B). The mRNA expression levels of CD31 and CD34 were determined through real-time PCR (C). H-SFM, HCT116 serum free medium; H-CM, HCT116 conditioned medium; H-NC, HCT116 negative control conditioned medium; H-ATAD2-shRNA, HCT116 ATAD2-shRNA conditioned medium. S-SFM, SW480 serum free medium; S-CM, SW480 conditioned medium; S-NC, SW480 negative control conditioned medium; S-ATAD2-shRNA, SW480 ATAD2-shRNA conditioned medium. Data were present as mean ± SD. *P<0.05; **P<0.01; ***P<0.001.

250x242mm (300 x 300 DPI)

Figure 4. ATAD2 regulated the level of VEGFA via miR-520a colorectal cancer cells. Levels of VEGFA in the supernatant of ATAD2-silenced HCT116 or SW480 cells were determined by ELISA kit (A). Real-time PCR was employed to detect miR-520a expression in colorectal cancer cells with or without ATAD2 knockdown (B). The expression of VEGFA in HCT116 or SW480 cells following silencing of ATAD2 was assessed through western blotting (C). The effect of miR-520a mimics or inhibitor on the expression of VEGFA in HCT116 or SW480 cells was evaluated by western blotting (D). The effect of miR-520a mimics on luciferase activity of ATAD2 3'-UTR was determined (E). Data were present as mean $\pm$ SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

243x181mm (300 x 300 DPI)

Figure 5. MiR-520a inhibitor reversed the inhibiting effect of CM from ATAD2-silenced HCT116 cells on HUVEC. Levels of VEGFA in the supernatant of ATAD2-silenced HCT116 were determined by ELISA kit (A). Migration and capillary tube formation of HUVEC were assessed by wound healing (B) and tube formation assays (C), respectively. H-NC, HCT116 negative control conditioned medium; H-ATAD2-shRNA, HCT116 ATAD2-shRNA conditioned medium. H-ATAD2-shRNA + NC inhibitor, HCT116 ATAD2-shRNA conditioned medium + . negative control inhibitor. H-ATAD2-shRNA + mir-520a inhibitor, HCT116 ATAD2-shRNA conditioned medium + mir-520a inhibitor. Data were present as mean $\pm$ SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

250x186mm (300 x 300 DPI)

Figure 6. Treatment with VEGFA reversed the inhibiting effect of CM from ATAD2-silenced HCT116 cells on HUVEC. The effect of miR-520a mimics on luciferase activity of VEGFA 3'-UTR was assessed (A). Proliferation, migration, and capillary tube formation of HUVEC was determined by CCK-8 (B), wound healing (C), and tube formation assays (D), respectively. H-SFM, HCT116 serum free medium; H-NC, HCT116 negative control conditioned medium; H-ATAD2-shRNA, HCT116 ATAD2-shRNA conditioned medium. H-ATAD2-shRNA + VEGFA, HCT116 ATAD2-shRNA conditioned medium + 20 ng/ml VEGFA. Data were present as mean $\pm$ SD. ** $P < 0.01$; *** $P < 0.001$.

233x190mm (300 x 300 DPI)