1 **Uncovering Direct Targets of MiR-19a Involved in Lung Cancer Progression**

2

3 **Short title:** Direct targets of miR-19a related to lung cancer

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18 # Abstract

19 Micro RNAs (miRNAs) regulate the expression of target genes
20 posttranscriptionally by pairing incompletely with mRNA in a sequence-specific
21 manner. About 30% of human genes are regulated by miRNAs, and a single miRNA is
22 capable of reducing the production of hundreds of proteins by means of incomplete
23 pairing upon miRNA–mRNA binding. Lately, evidence implicating miRNAs in the
24 development of lung cancers has been emerging. In particular, miR-19a, which is highly
25 expressed in malignant lung cancer cells, is considered the key miRNA for
26 tumorigenesis. However, its direct targets remain underreported. In the present study,
27 we focused on six potential miR-19a target genes selected by miRNA target prediction
28 software. To evaluate these genes as direct miR-19a target genes, we performed
29 luciferase, pull-down, and western blot assays. The luciferase activity of plasmids with
30 each miR-19a–binding site was observed to decrease, while increased luciferase activity
31 was observed in the presence of anti-miR-19a locked nucleic acid (LNA). The
32 pull-down assay showed biotinylated miR-19a to bind to AGO2 protein and to four of
33 six potential target mRNAs. Western blot analysis showed that the expression levels of
34 the four genes changed depending on treatment with miR-19a mimic or
35 anti-miR-19a-LNA. Finally, *FOXP1*, *TP53INP1*, *TNFAIP3*, and *TUSC2* were identified
36 as miR-19a targets. To examine the function of these four target genes in lung cancer
37 cells, LK79 (which has high miR-19a expression) and A549 (which has low miR-19a
38 expression) were used. The expression of the four target proteins was higher in A549

39 than in LK79 cells. The four miR-19a target cDNA expression vectors suppressed cell
40 viability, colony formation, migration, and invasion of A549 and LK79 cells, but LK79
41 cells transfected with *FOXP1* and *TP53INP1* cDNAs showed no difference compared to
42 the control cells in the invasion assay.
43

Introduction

Micro RNAs (miRNAs) are ~22-bp non-coding small RNAs that posttranscriptionally regulate gene expression in a sequence-specific manner [1]. miRNAs are encoded by either their own genes or embedded into introns of the "host" genes and are transcribed by RNA Polymerase II as a part of a long capped and polyadenylated transcript (pri-miRNA) [2]. Pri-miRNAs undergo further processing that involves excision of a hairpin structure along with flanking sequences by a member of RNAse III family Drosha to create pre-miRNA [3-4]. Pre-miRNAs are exported into the cytoplasm by Exportin-5 where they are further cleaved by Dicer that removes terminal loop creating an imperfect RNA duplex [3-5]. One of the strands is preferentially bound by the RNA-induced silencing complex (RISC), which contains Argonaute (AGO) family proteins. Although both strands can become stably associated with AGO family proteins (loading step) only one strand (guide strand; miRNA) is retained by the AGO protein, while the other strand (passenger strand; miRNA*) is degraded. The human AGO proteins (AGO1 to 4) are characterized by a conserved PIWI domain that is structurally similar to the RNAse H. The PIWI domain interacts with the 5'end of mature miRNA and is involved in cleavage of target mRNAs. All four human AGO proteins show remarkably similar structural preferences for small-RNA duplexes: central mismatches (guide position 8–11) promote RISC loading, and mismatches in the seed (guide position 2–7) or 3′-mid regions (guide position 12–15) are required for unwinding [6]. It is difficult for small RNA duplexes bearing mismatches in the seed region to load into

65 AGO proteins [6-12]. On the other hand, the recognition of one miRNA with target
66 mRNAs requires complete or nearly complete matches with the seed region. More than
67 2,500 miRNAs are reported in humans (GRCh38,
68 http://www.mirbase.org/cgi-bin/browse.pl?org=has), and 30% of human genes are
69 considered to be regulated by miRNAs [13].

70 Lung cancer is responsible for 19.4% of all cancer-related deaths, which
71 constituted approximately 1.59 million deaths worldwide in 2012
72 (http://www.who.int/mediacentre/factsheets/fs297/en/). Lung cancer progression is
73 associated with multiple genetic and epigenetic changes that affect gene expression of a
74 wide variety of genes. In particular, alterations in expression of more than two dozen
75 miRNA has been reported in lung cancer patients [14], including recently reported
76 overexpression of the miR-17-92 cluster (oncomiR-1) that encodes, among others,
77 miR-19a and 19b [14]. OncomiR-1 is involved in the regulation of cell survival,
78 proliferation, differentiation, and angiogenesis [15, 16]. Some genes, such as *STAT3* and
79 *MAPK14*, which are involved in tumorigenesis, have been reported as target genes of
80 miR-17-92 in lung cancer cells [17]. MiR-19a, which is highly expressed in malignant
81 lung cancer cells, is considered the key miRNA in tumorigenesis [18]. Cell growth rate
82 and viability differ between lymphomas transfected with wild or mutated miR-19a;
83 therefore, miR-19a might also be associated with cell growth [18]. Moreover, miR-19a
84 activates the Akt-mTOR pathway by repressing the tumor suppressor *PTEN* [18, 19].
85 Furthermore, *SOCS-1* [20], *THBS1* [21], *IMPDH1*, *NPEPL1* [22], and *TNF-α* are also
86 known as targets of miR-19a [23].

However, as miRNA–mRNA binding depends on seed sequences and imperfect pairing of their strands, miR-19a must have yet-unidentified target genes that influence the onset and progression of lung cancer. In the present study, we identified novel target genes of miR-19a and showed the suppressive ability of the target genes on the growth, migration, and invasion of lung cancer cells.

Materials and Methods

Selection of miR-19a target candidate genes

Potential target genes of miR-19a were predicted by using the following miRNA target prediction software: PicTar (http://pictar.bio.nyu.edu), TargetScan (http://targetscan.org), MiRanda (http://cbio.mskcc.org), and miGTS (Kyowa Hakko Kirin Co. Ltd. Tokyo, Japan). The prediction yielded 3,398 genes. To narrow the range of possible miR-19a targets, genes involved in cancer were extracted by search refinement by including more than two words related to cancer (tumor, suppressor, and apoptosis) in the preliminary literature search. Although more than 10 genes remained as miR-19a target candidates, six genes (excluding the genes already reported as miR-19a targets), namely, forkhead box P1 (*FOXP1*), p53-dependent damage-inducible nuclear protein 1 (*TP53INP1*), TNF-α-induced protein 3 (*TNFAIP3*), tumor suppressor candidate 2 (*TUSC2*), SIVA apoptosis-inducing factor 1 (*SIVA1*), and tumor necrosis factor receptor superfamily 12A (*TNFRSF12A*), were selected for this study.

108 ## Cell culture

109 Human normal lung fibroblast cell line WI-38 and human normal embryonic kidney cell
110 line HEK293 were purchased from American Type Culture Collection (ATCC). Human
111 lung small-cell carcinoma cell lines SBC-5 and LK-79 and human non-small-cell lung
112 cancer cell lines PC3, PC14, LK2, NCI-H23, NCI-H460, NCI-H520, SQ5, Lu65A,
113 Lu-99c, RERF-LCMS, and A549 have been used in our laboratory [24]. HEK293,
114 SBC-5, Lu-65A, PC3, PC14, LK2, NCI-H23, NCI-H460, NCI-H520, SQ5, Lu-99c, and
115 RERF-LCMS were grown in DMEM (Life Technologies, Foster City, CA, USA) or
116 RPMI-1640 (Wako, Osaka, Japan). LK-79 and A549 were grown in mixed DMEM and
117 RPMI-1640 (1:1) supplemented with 10% FBS (Life Technologies), 100 units/mL of
118 penicillin G, and 100 mg/mL of streptomycin (Meiji Seika, Tokyo, Japan). All cells
119 were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

120 Exogenous miRNA transfection experiments were performed with miR-19a
121 mimic (1, 5, and 10 nM) (CosmoBio, Tokyo, Japan) using HiPerFect Transfection
122 Reagent (Qiagen, Venlo, Netherlands). The sequences were as follows: miR-19a mimic,
123 sense 5′-p-AUC CGC GCG AUA GUA CGU AUU-3′ and antisense 5′-p-UAC GUA
124 CUA UCG CGC GGA UUU-3′; random miRNA (control miRNA), sense 5′-p-UGU
125 GCA AAU CUA UGC AAA ACU GA-3′ and antisense 5′-p- UUA GUU UUG CAU
126 AGU UGC AC-3′. The expression of miR-19a was knocked down by transfection with
127 anti-miR-19a locked nucleic acid (LNA) (30 nM; 5′-TCA GTT TTG CAT AGA TTT
128 GCA CA-3′) or a control-LNA oligonucleotide targeting GFP (5′-ATC ACT CTC GGC
129 ATG GAC GAG C-3′) (Gene Design Inc., Osaka, Japan) using HiPerFect Transfection

130 Reagent (Qiagen). The miR-19a target cDNA expression plasmids were transfected into
131 the cells using Lipofectamine 2000 (Life Technologies). The transfected cells were
132 subjected to cell viability assays and RNA extraction 24 h after transfection, and protein
133 extraction 72 h after transfection. Cell viability was determined using a water-soluble
134 tetrazolium salt assay, namely, the
135 2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium
136 monosodium salt assay (WST-1; Roche Applied Science, Mannheim, Germany).
137

138 Colony formation assay

139 *FOXP1*, *TP53INP1*, *TNFAIP3*, and *TUSC2* cDNA expression plasmids were transfected
140 into A549 and LK79 cells using Lipofectamine 2000 and selected by G418 (100–400
141 μg/mL) (Sigma-Aldrich, St. Louis, MO, USA) in 6-well plates. After 3 weeks, the
142 colonies consisting of more than 200 cells were stained with crystal violet. The number
143 of colonies was then counted, and average values were calculated in triplicate wells.
144

145 Cell growth

146 A549 or LK79 cells transfected with *FOXP1*, *TP53INP1*, *TNFAIP3*, or *TUSC2* cDNA
147 expression plasmid vector were selected by G418 to obtain cells with stable expression.
148 After 3 weeks, the bulk population of G418-resistant cells was collected and used for
149 the cell growth assay, migration assay, and invasion assay. For the cell growth assay,
150 cells with stable expression were cultured at 500 cells/well in a 96-well plate. After 24,
151 48, and 72 h, the cells were counted using Hoechst 33342 staining and microscopy.

152 Average values of the cells with clearly stained nuclei were calculated in triplicate
153 wells.
154

155 Migration assay

156 Cells were grown to 95% confluence in a 60-mm plate and scratched with a pipette tip.
157 The floating cells were removed by washing with phosphate-buffered saline (PBS), and
158 microscopic images of the wounds were obtained at 24 h.
159

160 Invasion assay

161 A Corning Matrigel Invasion Chamber (24-well plate 8.0 micron; Corning, NY,
162 USA) was used for the invasion assay. A549 (1×10^5) or LK79 (2.5×10^4) cells were
163 cultured with serum-free DMEM in the upper chamber separated from the lower
164 chambers, which were supplied with DMEM with 10% FBS by permeable translucent
165 PET membranes. After 24 h, the cells were fixed and stained with Cyto Quick A and B
166 Solution (Muto Pure Chemicals, Tokyo, Japan). Non-invasive cells on the upper side of
167 the membrane were gently removed by wiping, and cells on the lower side were stained
168 and observed by a microscope. The number of invasive cells was counted, and average
169 values were calculated in seven fields per chamber.
170

171 Pull-down assay of target mRNAs of miR-19a

172 Semi-confluent HEK293 cells on 90-mm culture dishes were washed with cold PBS,
173 harvested by a scraper, and treated with 0.5 mL of 25 mM Tris-HCl (pH 7.5), 70 mM

174 KCl, 2.5 mM EDTA, 0.05% NP-40, 80 U/mL RNase Inhibitor (Life Technologies), and
175 1× protease inhibitor cocktail (Sigma-Aldrich) on ice for 20 min and centrifuged at
176 12,000 ×*g* for 15 min at 4°C. The supernatant was transferred to a new tube.
177 Biotinylated double-stranded RNA (8 nmoles) of miR-19a (sense 5′-p-UGU GCA AAU
178 CUA UGC AAA ACU GA-biotin-3′ and antisense 5′-p-AGU UUU GCA UAG AUU
179 UGC AUA AG-3′) or control random RNA (sense 5′-p-AUC CGC GCG AUA GUA
180 CGU AUU-biotin-3′ and antisense 5′-p-UAC GUA CUA UCG CGC GGA UUU-3′)
181 was added to the supernatant (500 μL) and incubated at 4°C for 30 min with 8-rpm
182 shaking and then at 30°C for 1 h with 30-rpm shaking. The extract was incubated at 4°C
183 for 1 h with 10 μL Streptavidin Mutein Matrix (Roche Applied Science), which was
184 pretreated with extraction buffer [250 μg RNase-free BSA and 100 μg yeast tRNA in
185 500 μL of 25 mM Tris-HCl (pH 7.5), 70 mM KCl, 2.5 mM EDTA, and 0.05% NP-40]
186 for 3 h and washed with the same buffer twice. The Streptavidin/biotin–miRNA/mRNA
187 complex was collected after a spin at 5,000 ×*g* for 30 s and washed 5 times at 4°C for 5
188 min with 8-rpm shaking using 20 mM Tris-HCl (pH 7.4), 400 mM KCl, and 0.5%
189 NP-40. The biotin–miRNA/mRNA complex was eluted with 250 μL of 20 mM
190 Tris-HCl (pH 7.4), 400 mM KCl, 0.5% NP-40, 5 mM biotin, and 80 U/mL RNase
191 inhibitor at 42°C for 5 min. The *PTEN* gene, reported to be an miR-19a target, was used
192 as the positive control, and *DAPK1*, whose sequence did not match with miR-19a seed
193 sequences at the 3′ untranslated region (UTR) of its mRNA, was used as the negative
194 control.
195

196
Plasmids

197 The cDNAs of *FOXP1*, *TP53INP1*, *TNFAIP3*, and *TUSC2* were amplified using PCR
198 with gene-specific primers and human normal cDNA reverse-transcribed from HEK293
199 mRNA using ReverTra Ace-α kit (Toyobo, Osaka, Japan). The cDNAs were cloned into
200 pBluescript and confirmed by DNA sequencing using the ABI 3100 sequencer
201 (Applied Biosystems, Foster City, CA, USA). FLAG tag sequences were fused at the
202 5′-end of the cDNA in frame, and they were inserted into the pIRES2-EGFP vector
203 (Clontech, Mountain View, CA, USA) with *Nhe*I and *Sal*I restriction sites. These
204 sequences were reconfirmed by DNA sequencing.

205

206
Luciferase reporter assay

207 The 3′-UTR fragments containing a possible miR-19a–binding region in the candidate
208 genes were synthesized as oligonucleotides for both strands, which can produce *Xba*I
209 cohesive ends after annealing. The annealed double strands were cloned into the 3′-UTR
210 *Xba*I site of *Renilla* luciferase in the pTK-hRG vector, which was a phRG-B vector
211 (Promega, Madison, WI, USA) bearing luciferase cDNA downstream of the herpes
212 thymidine kinase promoter that we had inserted. The inserted fragments were sequenced,
213 and their orientation and fragment number were determined. For the luciferase assay,
214 the pTK-hRG constructs (180 ng) were co-transfected with the firefly luciferase reporter
215 plasmid pOA-SRα-luciferase (20 ng) as an internal control into HEK293 cells. The
216 pTK-hRG construct with no insert was used as a control. The luciferase activity was
217 measured 48 h after transfection using a dual luciferase reporter assay system

218 (Promega) on a Labosystems Luminoskan RT instrument (Thermo Fisher Scientific,
219 Waltham, MA USA). The relative luciferase activity was calculated by normalizing
220 *Renilla* luminescence to firefly luminescence. For each experimental trial, each sample
221 was assayed in duplicate. The *p*-value was calculated using the two-tailed *t*-test. For the
222 other luciferase assay, the pTK-hRG constructs were co-transfected with anti-miR-19a
223 LNA or control LNA (100 nM) under the same conditions. The pTK-hRG construct
224 bearing a mismatch with the central region of miR-19a seed sequences was used as a
225 negative control.
226

227 Quantitative reverse-transcription PCR

228 The pulled-down biotin–miRNA/mRNA complex was treated with the
229 guanidium-phenol chloroform extraction procedure with ISOGEN-LS (Nippon Gene,
230 Tokyo, Japan), followed by DNase I (Sigma-Aldrich) and ISOGEN-LS treatment. The
231 mRNA was reverse-transcribed in a total volume of 20 μL using the ReverTra Ace-α kit.
232 The cDNA of the biotin–miRNA/mRNA complex was amplified by PCR in a 20-μL
233 volume containing 0.2 μL of each reverse transcription reaction and 10 μL of the
234 TaqMan 2× Universal PCR Master Mix (Life Technologies) in a 7300 Fast Real-Time
235 PCR System (40 cycles of 95°C for 15 s and 58°C for 5 min). Primers to detect
236 candidate miR-19a target cDNAs were designed flanking a possible miR-19a–binding
237 region in the target mRNA (S1 Table). The TaqMan probe sequences with 5′-FAM and
238 3′-TAMRA labeling for real-time PCR are shown in S2 Table. The expression level, i.e.,
239 cycle threshold (CT) value, of each target mRNA in the pull-down sample with

240 miR-19a–biotin was compared to the CT value of that in the pull-down sample with
241 miR-random–biotin and shown as the relative ratio. Each PCR was performed four
242 times, and the *p*-value was calculated using the two-tailed *t*-test.

243 For miR-19a expression analysis, total cellular RNA was extracted using
244 ISOGEN (Nippon Gene). The quantitative real-time PCR analysis of miR-19a was
245 performed with a TaqMan MicroRNA Reverse Transcription Kit, TaqMan 2× Universal
246 PCR Master Mix and TaqMan MicroRNA Assay (Life Technologies). Total RNA (10
247 ng) was reverse-transcribed in a total volume of 15 μL using a TaqMan MicroRNA
248 Reverse Transcription kit. Aliquots of each reverse transcription reaction were
249 amplified by PCR in a 20-μL total volume containing 10 μL of the TaqMan 2×
250 Universal PCR Master Mix. The PCR was performed on a 7300 Fast Real-time PCR
251 System with 50 cycles of 95°C for 15 s and 60°C for 60 s. The expression level (CT
252 value) of miR-19a was normalized to the CT value of a small nuclear RNA, U6B, which
253 was co-amplified as an endogenous control. The ΔCT was calculated as the difference
254 in the CT values between miR-19a and the internal control U6B in one sample. The
255 comparisons of miRNA expression levels were conducted using the ΔΔCT method,
256 where ΔΔCT was the difference in the ΔCT values of a test sample compared to that of
257 the control sample, and $2^{-\Delta\Delta CT}$ represents the fold change in miRNA expression.

258 For quantitative real-time PCR analysis of the miR-19a target genes, *FOXP1*,
259 *TP53INP1*, *TNFAIP3*, and *TUSC2* cellular mRNA was reverse transcribed with the
260 ReverTra Ace First Strand cDNA Synthesis Kit (Toyobo), and the cDNA was amplified
261 by PCR in a 20-μL total volume containing 0.2 μL of each reverse transcription reaction

262 and 10 μL of the TaqMan 2× Universal PCR Master Mix (Life Technologies) in a 7300
263 Fast Real-Time PCR System with 40 cycles of 95°C for 15 s and 58°C for 5 min. The
264 primers and probes used to detect these cDNAs were the same as those for detecting
265 cDNAs of the biotin–miRNA/mRNA complex described above. The CT was calculated
266 as the difference in the CT values between each miR-19a target gene and the β-actin
267 gene in one sample. The expression levels of the target mRNA were also measured
268 using the ΔΔCT method.
269

270 ## Western blotting analysis

271 For confirmation of AGO2 protein binding with the biotin–miRNA/mRNA upon *in*
272 *vitro* pull-down, the complex was combined with gel loading buffer, heated to 95°C for
273 10 min, and then separated on 12% SDS-polyacrylamide gels and electrotransferred to
274 polyvinylidene difluoride (PVDF) membranes (Life Technologies). Membranes were
275 blocked overnight at 4°C in 3% BSA/PBS and then incubated for 4 h at room
276 temperature with anti-AGO2 monoclonal antibody (No.016-20861, Wako, Osaka,
277 Japan). The filters were washed with PBS/0.05% Tween-20 and then incubated with
278 alkaline phosphatase-conjugated antibodies. The protein signal was visualized using
279 FLA-3000 (Fujifilm, Tokyo, Japan).

280 For the effect of miR-19a on protein expression, at 72 h after transfection of
281 HEK293 and A549 with the miR-19a mimic or anti-miR-19a-LNA, the protein samples
282 (25 μg) were separated on 8% or 12% SDS-polyacrylamide gels, electrotransferred onto
283 PVDF membranes, and incubated overnight at 4°C with the following antibodies:

284 anti-TP53INP1 (T-17; Santa Cruz Biotechnology, Dallas, TX, USA), anti-FOXP1
285 (ab16645; Abcam, Cambridge, UK), anti-TNFAIP3 (ab74037; Abcam), anti-TUSC2
286 (ab70182; Abcam), anti-SIVA1 (ab67620; Abcam), and anti-TNFRSF12A (ITEM-4;
287 Santa Cruz Biotechnology). Anti-β-actin (AC-15; Sigma-Aldrich) was used as a loading
288 control.
289

290 Statistical analysis

291 The relative ratios in luciferase, RNA expression, cell viability, cell growth, colony
292 formation, and invasion experiments are expressed as the mean values ± SD and were
293 analyzed by the two-tailed *t*-test. A value of $p < 0.05$ was considered statistically
294 significant.
295
296

297 Results

298 Evaluation of miR-19a target candidate genes by the 299 luciferase reporter assay

300 We focused on six miR-19a target gene candidates, *FOXP1*, *TP53INP1*, *TNFAIP3*,
301 *TUSC2*, *SIVA1*, and *TNFRSF12A*, which had been selected using miRNA target
302 prediction software. The nucleotide sequences of the possible miRNA-binding site on
303 the 3′-UTR of their mRNAs were obtained from miRNA target prediction software (Fig.
304 1A). To verify whether the miRNA-binding sites are regulated by miR-19a *in vivo*, we

305 constructed luciferase reporter plasmids with each miR-19a binding site in the 3′-UTR
306 of the *Renilla* luciferase gene (Fig. 1B). Luciferase reporter plasmids with the sequences
307 mismatched with the miR-19a seed region comprised the negative control. We
308 transfected *Renilla* luciferase reporter plasmids bearing possible miR-19a–binding sites
309 of miR-19a target candidates and internal control firefly luciferase plasmids into
310 HEK293 cells in which miR-19a expression was confirmed. The plasmids were then
311 examined for luciferase activity by the dual-luciferase system. The luciferase activity of
312 all plasmids with the miRNA-binding sites was significantly lower than that of the
313 empty vector control (Fig. 1C), suggesting that miR-19a–binding sequences in each
314 miR-19a target candidate mRNA are recognized correctly by endogenous miR-19a in
315 HEK293 cells. Furthermore, we examined the luciferase activity of the plasmids bearing
316 the miRNA-binding sites under miR-19a knockdown by co-transfection with
317 anti-miR-19a LNA. The luciferase activity of all plasmids with the miRNA-binding
318 sites increased significantly in the cells treated with anti-miR-19a LNA as compared
319 with that of the cells treated with control LNA (Fig. 1D). These results suggest the
320 possibility that these genes are miR-19a target genes. We then performed other
321 experiments to evaluate the possibility of these being miR-19a target genes.
322

323 **Figure 1. Verification of miR-19a candidate target genes by luciferase assays**
324 (A) Overview of miR-19a target candidate mRNAs. The miR-19a–binding sites
325 identified using PicTar, TargetScan, or MiRanda software are shown in the
326 3′-untranslated (UTR) region of miR-19a target candidate mRNAs. (B) Construction of

327 luciferase vectors. The miR-19a–binding sites of the candidate genes and negative
328 control sequences (Negative Ctrl) were cloned downstream of the luciferase ORF at the
329 *Xba*I restriction site of the pTK-hRG vector. Sense (*upper*) and antisense (*lower*)
330 strands of complementary sequences indicate the miRNA target site of the mRNA
331 3′-UTR and miR-19a sequences, respectively. Underlines indicate miR-19a seed
332 sequences. The negative control plasmid has mismatches in the center of miR-19a seed
333 sequences. (C) The luciferase activity of constructs with miR-19a–binding sites was
334 compared with that of the empty vector, which had no insert at the *Xba*I site (No-Insert
335 Ctrl), and was statistically analyzed. (D) Luciferase plasmids with the miR-19a–binding
336 site and negative control plasmid were transfected with anti-miR-19a-LNA or
337 control-LNA (Ctrl). The luciferase activity of cells treated with anti-miR-19a-LNA was
338 compared with that of cells treated with the control-LNA. *, $p < 0.05$ and **, $p < 0.005$
339 using two-tailed *t*-tests.

340

341 Evaluation of candidate genes by an *in vitro* pull-down assay 342 using biotinylated miR-19a

343 MiRNA target genes were recently recovered by a two-step procedure wherein the
344 mRNA/miRNA/FLAG-AGO2 complex was first pulled down with an anti-FLAG
345 antibody, and then, the mRNA/biotinylated miRNA complex was purified by
346 Streptavidin beads in the extract of the cells transfected with biotinylated miRNA and
347 FLAG-AGO2 cDNA expression vector [25]. Therefore, to evaluate our candidate genes
348 as miR-19a targets, we adopted the improved *in vitro* pull-down assay (Fig. 2A). At

349 first, the cell extract was mildly prepared from HEK293 cells in which sufficient
350 expression of each candidate mRNA was detected (Fig. 2B). The biotinylated
351 double-stranded miR-19a or control random RNA was incubated in cell extract to
352 produce a complex of the biotinylated miRNA single strand with RISC and mRNA. The
353 biotinylated miRNA/mRNA/RISC complex was incubated with Streptavidin beads in
354 the extract, collected by short centrifugation, and eluted with elution buffer containing 5
355 mM biotin from Streptavidin beads. The biotinylated miRNA/mRNA complex was
356 treated by phenol–chloroform extraction, DNase I, and reverse transcription. Before
357 RT-PCR analysis of the target candidate genes, we confirmed whether the biotinylated
358 miR-19a/mRNA complex contains the RISC component AGO2 by western blotting.
359 The pull-down complex with biotinylated miR-19a exhibited the signal of the AGO2
360 protein (Fig. 2C, lane 1), while no signal was noted for the pull-down complex with
361 biotinylated random RNA (Fig. 2C, lane 2), suggesting that biotinylated miR-19a, target
362 mRNA, and AGO2 might form a complex in this pull-down assay. Then, we compared
363 the quantity of candidate target mRNAs in the complexes pulled down using
364 biotinylated miR-19a and control random RNA by quantitative real-time PCR. PCR
365 primers for the candidate target genes were designed to amplify the region (100~300
366 bp) containing miR-19a–binding sites in their 3′-UTRs (S1 Table). Among the six
367 candidates, *FOXP1*, *TP53INP1*, *TNFAIP3*, and *TUSC2* mRNA levels were significantly
368 higher in the complex pulled down with biotinylated miR-19a than those of the control
369 (Fig. 2D). For the confirmation of this pull-down system, we examined a positive
370 control miR-19a target gene, *PTEN*, as well as a negative control gene, whose sequence

371 did not match the miR-19a seed sequences. We detected an increased level of *PTEN*
372 mRNA and decreased level of the negative control gene mRNA in the pull-down
373 complex by using biotinylated miR-19a. Therefore, we focused on evaluating *FOXP1*,
374 *TP53INP1*, *TNFAIP3*, and *TUSC2* genes as miR-19a targets.
375

376 **Figure 2. Pull-down assay using biotinylated miRNA**
377 (A) Overview of the *in vitro* pull-down assay. The biotinylated double-stranded
378 miR-19a or control random RNA was incubated in cell extract (step 1) to yield a
379 complex of the biotinylated miRNA single strand with target mRNA and RISC (step 2).
380 The biotinylated miRNA/target mRNA complex was incubated with streptavidin beads
381 and pulled down (step 3). The complex was treated by DNase I and reverse-transcribed
382 (step 4). (B) The expression of miR-19a target candidate mRNAs in HEK293 cells.
383 *PTEN*, known as one of miR-19a target genes, was used as a positive control. The gene,
384 whose sequence did not match with miR-19a seed sequences, was used as the negative
385 control. (C) Confirmation of AGO2 protein in biotinylated miRNA/target mRNA
386 complex by western blotting with the AGO2 antibody. Biotinylated miR-19a (lane 1),
387 biotinylated control random RNA (lane 2), and biotin (lane 3) were used for the
388 pull-down assay and subjected to SDS-polyacrylamide gel electrophoresis and western
389 blotting. Total cell extract was used as the positive control (lane 4). (D) Detection of
390 target mRNAs in biotinylated miRNA/target mRNA complex by real-time RT-PCR.
391 The relative level of each target mRNA in the complex pulled down by using
392 biotinylated miR-19a was compared to that of the complex pulled down by using the

393 biotinylated control random RNA. **, $p < 0.005$ using two-tailed *t*-tests.

394

395

396 Evaluation of four candidate genes by western blotting

397 Genes *FOXP1*, *TP53INP1*, *TNFAIP3*, and *TUSC2* were evaluated as miR-19a targets
398 using another analysis for examining posttranscriptional regulation by miR-19a in the
399 cells. At first, miR-19a mimic or control random miRNA was transfected into HEK293
400 cells to examine whether miR-19a reduced the expression of the four miR-19a target
401 proteins. By western blotting at 72 h after transfection, decreased expression of the four
402 target proteins was observed in miR-19a mimic–treated cells as compared to that of
403 control miRNA–treated cells (Fig. 3A). Secondly, anti-miR-19a LNA or control LNA
404 was transfected into HEK293 cells to knock down endogenous miR-19a, and the
405 expression of the four candidate proteins was analyzed by western blotting. Increased
406 expression of the four target proteins was observed in anti-miR-19a LNA–treated cells
407 as compared to that of control LNA–treated cells (Fig. 3B). Taken together, *FOXP1*,
408 *TP53INP1*, *TNFAIP3*, and *TUSC2* were confirmed to be miR-19a target genes. No
409 change in SIVA1 or TNFRSF12A expression was observed in anti-miR-19a
410 LNA–treated cells as compared to that in control LNA–treated cells (S1 Fig).

411

412 **Figure 3. Verification of miR-19a target candidate genes by western blot analysis**

413 (A) Expression analysis of candidate proteins by western blot analysis using proteins of
414 HEK293 cells transfected with miR-19a mimic or control oligo RNA. –, control oligo

415 RNA; +, miR-19a mimic. (B) Expression analysis of candidate proteins by western blot
416 analysis using proteins of HEK293 cells transfected with anti-miR-19a LNA or control
417 LNA. –, control LNA; +, anti-miR-19a LNA.
418

419 Effect of miR-19a in lung cancer cell lines

420 To examine the function of the newly identified miR-19a target genes in lung cancer
421 cells, we analyzed the expression level of miR-19a among 13 human lung cancer cell
422 lines using TaqMan RT-PCR analysis. LK79 and A549 showed the highest and lowest
423 level of miR-19a expression, respectively (Fig. 4A). LK79 and A549 cells were then
424 selected for the following analyses, to compare the function of these four genes between
425 two lung cancer cell lines showing noticeably different expression levels of miR-19a.
426 Western blotting analysis with antibodies for four miR-19a targets showed that the
427 protein expression level of the four targets was higher in A549 than in LK79 cells (Fig.
428 4B).
429

430 **Figure 4. Expression of miR-19a and miR-19a target genes in human lung cancer**
431 **cell lines**
432 (A) Relative expression of miR-19a in various lung cancer cell lines is shown as
433 compared with that in human normal lung cell line WI-38. (B) Expression of miR-19a
434 target proteins in WI-38, A549, and LK79, as determined by western blot analysis.
435

436 At 24 h after transfection with anti-miR-19a LNA into LK79 cells, the cells
437 treated with anti-miR-19a LNA showed a decrease in the level of miR-19a to
438 one-thirtieth that in the cells treated with control LNA (Fig. 5A) and exhibited
439 decreased cell viability (Fig. 5B), although the transfection efficiency of LK79 was very
440 low. The mRNA level of *TUSC2* increased significantly, while the mRNA level of
441 *FOXP1*, *TP53INP1*, and *TNFAIP3* showed no significant difference between control
442 and anti-miR-19a LNA–treated LK79 cells (Fig. 5C). We could not observe clear
443 differences among the protein levels of the four miR-19a targets by western blotting
444 between control and anti-miR-19a LNA–treated LK79 cells (data not shown).
445

446 **Figure 5. Effect of quantitative change of miR-19a on LK79 cells**
447 (A) Relative expression of miR-19a in LK79 cells 24 h after transfection of
448 anti-miR-19a LNA or control LNA. (B) Cell viability of LK79 cells 24 h after
449 transfection of anti-miR-19a LNA or control LNA. (C) Relative expression of miR-19a
450 target mRNAs in LK79 cells 24 h after transfection of anti-miR-19a LNA and control
451 LNA. *, $p < 0.05$ and **, $p < 0.005$ using two-tailed *t*-tests.
452

453 At 24 h after transfection of A549 cells, the cells transfected with miR-19a
454 mimic (10 nM) showed an increase of about 7,000-fold of miR-19a as compared with
455 that of the cells treated with control RNA (Fig. 6A) and exhibited increased cell
456 viability (Fig. 6B). Moreover, the mRNA levels of all four targets decreased
457 significantly in A549 cells treated with the miR-19a mimic compared to control cells

458 (Fig. 6C). Western blotting analysis showed a decrease in all four miR-19a target
459 proteins in A549 cells treated with the miR-19a mimic compared to those of the cells
460 treated with control miRNA (Fig. 6D). Furthermore, the cells transfected with the
461 miR-19a mimic (1 and 5 nM) showed an increase of about 300- and 1,700-fold,
462 respectively, in miR-19a (S2 Fig). Then, the mRNA levels of all four targets decreased
463 in a dose-dependent manner in A549 cells treated with the miR-19a mimic. The levels
464 of four miR-19a target proteins in A549 cells treated with the miR-19a mimic tended to
465 be lower than those in cells treated with control miRNA.
466

467 **Figure 6. Effect of quantitative change in miR-19a on A549 cells**
468 (A) Relative expression of miR-19a in A549 cells 24 h after transfection of miR-19a
469 mimic or control miRNA (10 nM). (B) Cell viability of A549 cells 24 h after the
470 transfection of miR-19a mimic or control miRNA. (C) Relative expression of miR-19a
471 target mRNAs in A549 cells 24 h after transfection of miR-19a mimic and control
472 miRNA. (D) Relative expression of miR-19a target proteins in A549 cells 72 h after
473 transfection of miR-19a mimic and control miRNA. **, $p < 0.005$ using a two-tailed
474 *t*-test.
475

476 miR-19a target genes suppress cell viability, migration, 477 and invasion of lung cancer cells

478 Expression plasmids for the miR-19a target proteins FOXP1, TP53INP1, TNFAIP3,
479 and TUSC2 fused with the FLAG tag were constructed, and the protein expression

480 derived from the plasmids was confirmed by western blotting using the anti-FLAG
481 antibody (S3 Fig). The expression plasmids were transfected into A549 or LK79 cells,
482 and cell viability was measured 24 h after the transfection. A549 showed a significantly
483 decreased viability in all dishes with the miR-19a target cDNA plasmids compared to
484 the control (Fig. 7A), while there was no significant difference in the cell viability of
485 LK79 (Fig. 7B). Therefore, to exclude the effect of the low transfection efficiency of
486 LK79, colony-formation assay was performed to examine the effect of the target genes
487 on tumor cell growth. The number of colonies after selection using G418 was smaller in
488 A549 and LK79 cells transfected with the cDNA plasmids as compared to those of the
489 control (Fig. 7C, D), although the transfection efficiency of LK79 was poorer than that
490 of A549.
491
492 **Figure 7. Effect of miR-19a target genes on lung cancer cells**
493 (A) Relative cell viability of A549 cells 24 h after transfection of expression plasmids
494 with miR-19a target gene cDNAs. (B) Relative cell viability of LK79 cells 24 h after
495 transfection of expression plasmids with miR-19a target gene cDNAs. (C) Colony
496 formation of A549 cells (*upper*) and LK79 cells *(lower)* 3 weeks after transfection with
497 miR-19a target cDNA plasmids and G418 selection. Each assay was performed in 3
498 wells of 6-well plates, and the representative images of the results are shown. (D)
499 Average colony number of A549 cells (*left*) and LK79 cells *(right)* in the colony
500 formation assay. **, $p < 0.005$ using a two-tailed *t*-test.
501

502 After A549 or LK79 cells were transfected with the expression plasmids and
503 selected with G418, G418-resistant cells were collected and used for examining the
504 effect of the target genes on cell growth, migration, and invasion abilities. Cell count
505 assays at 24, 48, and 72 h showed significantly decreased cell growth in all the
506 cDNA-transfected A549 and LK79 cells as compared to the control cells with the empty
507 plasmids (Fig. 8A). All A549 and LK79 cells transfected with the cDNA showed
508 decreased migration as compared with the control cells by an *in vitro* migration assay.
509 The representative images of the results are shown in Fig. 8B. In an *in vitro* invasion
510 assay, the cDNA-transfected A549 cells showed significantly decreased invasion in all
511 four targets as compared with the control cells (Fig. 8C, D). The LK79 cells transfected
512 with *TNFAIP3* and *TUSC2* genes showed significantly decreased invasion, while the
513 LK79 cells transfected with *FOXP1* and *TP53INP1* showed no difference compared to
514 the control cells (Fig. 8C, D).
515
516 **Figure 8. Stable cell lines with miR-19a target genes exhibit suppression of cell**
517 **growth, migration, and invasion**
518 (A) Cell growth of G418-resistant stable cells of A549 *(upper)* and LK79 *(lower)*
519 transfected with miR-19a target cDNA expression vectors or empty vector (Ctrl). Cells
520 were counted using Hoechst 33342 staining at 24, 48, and 72 h. The number of each
521 miR-19a target expression cell was significantly less than control cells at 24, 48, and 72
522 h. (B) Migration assays used A549 *(upper)* and LK79 *(lower)* stable cells with miR-19a
523 target genes. Scale bar, 100 μm. (C) *In vitro* invasion assay used A549 *(upper)* and

LK79 *(lower)* stable cells with miR-19a target genes. (D) Average number of invading A549 *(left)* and LK79 *(right)* stable cells with miR-19a target genes for the invasion assay shown in (C). **, $p < 0.005$ using a two-tailed t-test.

Discussion

Recently, in addition to some reports that miR-19a represses the expression of cell signaling pathway genes such as *PTEN* or *TNF-α* [18, 19, 23], the overexpression of miR-19a in gastric cancer has been demonstrated to promote the epithelial–mesenchymal transition by activating the PI3K/AKT pathway [26]. Furthermore, patients with multiple myeloma and low miR-19a levels have been found to have an improved response to bortezomib compared to those with high miR-19a expression, and they experience a significantly extended survival upon bortezomib-based therapy [27]. Although there are many reports on the role of miR-19a and malignant tumors, miR-19a has been considered a clinical biochemical marker for acute myocardial infarction (AMI) diagnosis because circulating miR-19a expression is high in AMI patients [28]. Thus, miR-19a also has potential applications in basic cancer studies, therapeutic strategies and decision-making, and as a biochemical marker for prognosis prediction of many kinds of malignant tumors.

In the present study, we investigated the effect of miR-19a on lung cancer cells by transfecting an miR-19a mimic or anti-miR-19a LNA into cells with high (LK79 cells) or low (A549 cells) miR-19a expression. The changes in the viability of

545 the transfected cells demonstrated that miR-19a participates in the regulation of the cell
546 cycle as dictated by miR-19a levels, and these cells are responsible for the difference in
547 miR-19a quantities (Fig. 5B, 6B). However, the transfection efficiency of LK79 was
548 very low compared to that of A549. The miR-19a relative ratio showed a 1/30-fold
549 decrease in LK79 transfected with anti-miR-19a LNA, while the miR-19a relative ratio
550 showed a drastic 7,000-fold increase in A549 transfected with the miR-19a mimic. No
551 significant difference in cell viability was observed in LK79 transfected with the target
552 cDNA expression vectors (Fig. 7B), suggesting that low transfection efficiency of LK79
553 might be one of the causes. Fig. 4 shows differences in the amounts of miR-19a and the
554 four target proteins between LK79 and A549 cells. Knockdown of miR-19a to 1/30 of
555 the control level by LNA in LK79 cells (Fig. 5A) may be expected to lead to increase in
556 mRNA and protein expression of the target genes. However, the expression ratio of
557 miR-19a and the target mRNAs might be extremely different in the two cell lines. In the
558 different cell lines, which have differing expression ratios of miRNA and the target
559 mRNAs, the effective knockdown ratio of miRNA to target mRNAs may be different
560 and may vary according to the cell line. In LK79 cells, miR-19a might be expressed at
561 much more higher levels than the target mRNAs. Therefore, the knockdown of miR-19a
562 to 1/30 of the control level might not have much effect on the increase in the target
563 mRNAs in LK79 cells, as seen in most cases in Fig. 5C. However, these data do not
564 preclude these four genes as miR-19a targets because significant differences were
565 observed in target mRNA and protein analyses of A549 transfected with the miR-19a

566 mimic (Fig. 6C, D) and in cell viability of A549 transfected with the target cDNA
567 expression vectors (Fig. 7A, S4 Fig).

568 To evaluate the effect of *FOXP1*, *TP53INP1*, *TNFAIP3*, and *TUSC2* on LK79
569 cells independently from transfection efficiency, G418-resistant LK79 and A549
570 colonies were collected and used for colony formation, cell growth, cell migration, and
571 cell invasion assays. All four miR-19a target genes showed a suppression effect for
572 colony formation, cell growth, and migration in both LK79 and A549 lines **(**Fig. 7C, D;
573 Fig. 8A, B). In the invasion assay, *TNFAIP3* and *TUSC2* showed a suppression effect in
574 LK79, while the 4 target genes showed suppression of invasion in A549 **(**Fig. 8C, D**)**.
575 The underlying reason might be the difference in malignancy between A549 (non-small
576 cell lung carcinoma, NSCLC) and LK79 (SCLC, small cell lung carcinoma). It is also
577 reported that LK79 cells overexpress chemokine CCL2, which promotes migration and
578 metastasis [29, 30]. Another reason might be that strong overexpression of miR-19a in
579 LK79 caused decreased protein level of not the targets but also other unidentified
580 targets of miR-19a. Therefore, decreased expression of some other target genes might
581 affect the suppressive effect of invasion in LK79.

582 We used an *in vitro* pull-down assay for screening the target genes with
583 biotinylated miR-19a and HEK293 cells. HEK293 transfected with the miR-19a mimic
584 primarily showed increased cell viability, and HEK293 transfected with anti-miR-19a
585 LNA showed decreased cell viability (data not shown), suggesting that HEK293 was
586 available for evaluation assays of miR-19a target genes. In the assay, when *DAPK1*,
587 whose sequence did not match with that of the seed region of miR-19a, was used as a

588 negative control, the relative ratio of *DAPK1* mRNA was significantly lower than that
589 of the positive control *PTEN* in mRNA samples pulled down by using biotinylated
590 miR-19a (Fig. 2D). These results suggest that this assay is suitable for searching
591 miRNA target genes.

592 The functions of FOXP1, TP53INP1, TNFAIP3, and TUSC2 and the scope or
593 availability of gene-based therapies for certain diseases are described in the following
594 paragraphs.

595 *TUSC2* maps to the human chromosome 3p21.3 region, which is frequently
596 lost or reduced in lung cancer [31]. *TUSC2* is reported to decrease cell growth,
597 migration, invasion, and colony formation and to be able to lead tumors to apoptosis
598 [32]. A loss or reduction in TUSC2 protein expression is associated with poor overall
599 survival. It has been shown to function as a potent proapoptotic factor. The first human
600 systemic gene therapy clinical trial of *TUSC2* has also been reported recently [33-36].
601 Although multiple miRNAs downregulate TUSC2 protein through the mRNA 3′-UTR,
602 *pseudogene TUSC2P* mRNA has the same 3′-UTR as TUSC2 mRNA. Consequently,
603 *pseudogene TUSC2P* promotes *TUSC2* function by binding with multiple miRNAs [32].
604 MiR-19a would be one of the multiple miRNAs regulating TUSC2 through the
605 pseudogene, because we found possible miR-19a–binding sequences on the 3′-UTR of
606 *pseudogene TUSC2P*.

607 FOXP1 is a forkhead transcriptional repressor that regulates the development
608 of lung, lymphocytes, monocyte differentiation, and macrophage activities. In cancers,
609 FOXP1 can act as a tumor suppressor [37, 38]. Genetic aberrations of Foxp1 loci at

610 chromosome 3p14.1 have been reported in many cancers including lung cancer and
611 neuroblastoma. Indeed, deletions at 3p have been observed in the majority of small
612 (SCLCs) and of non-small (NSCLCs) cell lung cancers and the extent of deletions
613 correlated with tumor progression [39]. Similarly, the loss of heterozygosity at 3p has
614 been reported in neurobalstomas where it correlated with poor prognosis. Taking
615 together these observations led to the hypothesis that *FOXP1* is a tumor suppressor gene.
616 Indeed re-introduction of *FOXP1* into neuroblastoma cells inhibited cell proliferation
617 and colony formation in soft agar, and resulted in cell cycle arrest and apoptosis [38,
618 40].

619 *TP53INP1* is a p53-inducible tumor suppressor gene. The protein complex
620 interacting with TP53INP1 is shown to phosphorylate Ser46 of p53, leading cells to
621 apoptosis by double-stranded breaks in DNA [41-42]. *TP53INP1* is also targeted by
622 miR-155, a likely oncomiR, in esophageal squamous cell carcinoma [43] and liver
623 cancer stem cells [44]. Our results suggest that miR-19a participates in the progression
624 of lung cancer through *TP53INP1* downregulation.

625 *TNFAIP3* was identified as a gene whose expression is rapidly induced by
626 TNF-α. This gene encodes a zinc finger protein that has both ubiquitin ligase and
627 deubiquitinase activities. TNFAIP3 inhibits the activity of NF-κB, which strongly
628 facilitates the tumor progression through MMP9 expression, and represses cell division
629 and inflammation [45-49]. As *TNF-α* is a target of miR-19a [23], and our results
630 suggest that *TNFAIP3* is a target of miR-19a, miR-19a might affect TNF-α signaling.

631 Our results revealed that miR-19a downregulates the target genes *FOXP1*,

632 *TP53INP1*, *TNFAIP3*, and *TUSC2* and that these genes might play important roles in
633 the tumorigenesis of lung cancer; however, the invasion inhibition potency was found to
634 differ among the four genes. Our study had a limitation in that it involved *in vitro*
635 analyses for identifying miRNA target genes based on cultured cells. Therefore, further
636 studies will be required for determining the association of the target genes with tumor
637 malignancy and/or miR-19a expression in clinical studies using pathological specimens
638 and tissues from patients with lung cancer. Information regarding the mechanisms
639 underlying tumorigenesis mediated by the target genes may help provide promising
640 potential targets for effective diagnostic applications and targeted therapies for lung
641 cancer.

642

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787

788 # Supporting Information

789 **S1 Fig. Analysis of SIVA1 and TNFRSF12A proteins by western blot analysis.**
790 Expression of SIVA1 and TNFRSF12A was analyzed by western blotting using
791 proteins from HEK293 cells transfected with anti-miR-19a LNA or control LNA. –,
792 control LNA; +, anti-miR-19a LNA.
793
794 **S2 Fig. Effect of quantitative change in miR-19a on A549 cells.** (A) Relative
795 expression of miR-19a in A549 cells at 24 h after transfection of miR-19a mimic or
796 control miRNA (1 and 5 nM). (B) Relative expression of miR-19a target mRNAs in
797 A549 cells at 24 h after transfection of miR-19a mimic and control miRNA. (C)
798 Relative expression of miR-19a target proteins in A549 cells at 48 h after transfection of
799 miR-19a mimic and control miRNA. *, $p < 0.05$; **, $p < 0.005$ using a two-tailed *t*-test.
800
801 **S3 Fig. Protein expression of cDNA expression plasmids coding miR-19a target**
802 **proteins fused with FLAG tag peptides.** At 72 h after transfection of HEK293 with
803 cDNA expression plasmids (*right*) or empty plasmid (*left*), the protein samples (25 μg)
804 were analyzed by western blotting using the anti-FLAG antibody (*upper*) and
805 anti-β-actin antibody (*lower*).
806
807 **S4 Fig. Cell growth of a single colony of G418-resistant A549 cells with each**
808 **miR-19a target gene.** *FOXP1*, *TP53INP1*, *TNFAIP3*, and *TUSC2* cDNA expression

809 plasmids and empty plasmids were transfected into A549 cells and selected with G418.
810 Single colonies were isolated 3 weeks after transfection and used for the cell growth
811 assay. After 24, 48, and 72 h, the cells were counted using Hoechst 33342 staining and
812 microscopy. Average values of the cells with clearly stained nuclei were calculated in
813 triplicate wells.
814
815 **S1 Table. Primers Used to Detect miR-19a Target cDNA.**
816
817 **S2 Table. TaqMan Probe Sequences for Real-Time PCR.**