Differential expression of *miR-1*, a putative tumor suppressing microRNA, in cancer resistant and cancer susceptible mice.

Mus spretus mice are highly resistant to several types of cancer compared to *Mus musculus* mice. To determine whether differences in microRNA (miRNA) expression account for some of the differences in observed skin cancer susceptibility between the strains, we performed miRNA expression profiling of skin RNA for over 300 miRNAs. Five miRNAs, *miR-1, miR-124a-3, miR-133a, miR-134, miR-206*, were differentially expressed by both array and qPCR. *miR-1* was previously shown to have tumor suppressing abilities in multiple tumor types. We found *miR-1* expression to be lower in mouse cutaneous squamous cell carcinomas (cSCCs) compared to normal skin. Based on the literature and our expression data, we performed detailed studies on predicted *miR-1* targets and evaluated the effect of *miR-1* expression on two murine cSCC cell lines, A5 and B9. Following transfection of *miR-1,* we found decreased mRNA expression of three validated *miR-1* targets, *Met*, *Twf1* and *Ets1* and one novel target *Bag4.* Decreased expression of Ets1 was confirmed by Western analysis and by 3' reporter luciferase assays containing wildtype and mutated *Ets1* 3'UTR. We evaluated the effect of *miR-1* on multiple tumor phenotypes including apoptosis, proliferation, cell cycle and migration. In A5 cells, expression of *miR-1* led to decreased proliferation compared to a control *miR*. *miR-1* expression also led to increased apoptosis at later time points (72 and 96 hours) and to a decrease in cells in S-phase. In summary, we identified five miRNAs with differential expression between cancer resistant and cancer susceptible mice and found that *miR-1,* a candidate tumor suppressor, has targets with defined roles in tumorigenesis.

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Running title*: miR-1* in cSCC

Introduction

Mus Spretus mice are resistant to several cancer types including skin, colon, lung and thymic lymphoma (Nagase *et al.* 1995; Manenti *et al.* 1996; Villa-Morales, Santos & Fernandez-Piqueras 2006; Huang *et al.* 2007). Due to its cancer resistant nature *M. Spretus* has been used for quantitative trait locus (QTL) mapping for cancer susceptibility loci (Dejager, Libert & Montaguetlli 2009). To date, most of the QTL studies for cancer susceptibility have focused on identification of candidate genes with amino acid substitutions or differences in mRNA expression. Recent genome-wide association studies for cancer risk in humans have identified cancer-associated polymorphisms with roles in regulation of gene expression (Pomerantz *et al.* 2009; Spain *et al.* 2012).

MicroRNAs (miRNAs) are short RNAs of 20-22 nucleotides with well-documented roles in gene regulation (Siomi & Siomi 2010). They bind to the 3'untranslated region (3'UTR) of genes and may be involved in binding to other parts of the mRNA as well (Wery, Kwapisz & Morillon 2011). Binding results in mRNA degradation or impaired translation and subsequent decreased protein expression (Shyu, Wilkinson & van Hoof 2008; Djuranovic, Nahvi & Green 2011). Increasing evidence indicates that miRNAs play key roles in carcinogenesis. Expression profiling studies have demonstrated that many miRNAs are down-regulated during tumor development, resulting in subsequent up-regulation of their target genes and respective proteins. These miRNAs act as tumor suppressors and target cell cycle, apoptosis, proliferation, invasion and metastasis genes (Croce 2009). Likewise, another subset of miRNAs is up-regulated during

tumorigenesis resulting in down-regulation of their targets which are frequently tumor suppressor genes (Medina & Slack 2008). Previous studies indicate that several miRNAs map in close proximity to mouse QTLs for cancer susceptibility suggesting that variations in miRNA sequence or expression may be important for cancer susceptibility (Sevignani *et al.* 2007).

Because of the strong correlation between miRNA expression and tumorigenesis, we hypothesized that miRNAs showing differential expression between skin cancer resistant *M. spretus* (SPRET/EiJ) mice and skin cancer susceptible *M. musculus* (FVB/NJ) mice could be considered as candidates for cancer susceptibility. To identify miRNAs which may play a role in differences in cancer susceptibility between SPRET/EiJ and FVB/NJ, we performed expression profiling on normal skin samples from these strains of mice. Five differentially expressed miRNAs with described roles in tumorigenesis were identified and validated. Based on our observations and reports in the literature supporting *miR-1* as having tumor suppressor function in a variety of cancer types (Nasser *et al.* 2008; Datta *et al.* 2008; Yan *et al.* 2009; Li *et al.* 2012; Hudson *et al.* 2012; Nohata et al. 2011; Nohata *et al.* 2012a; Nohata et al. 2012b; Tominaga *et al.* 2013), we hypothesized that *miR-1* acts as a tumor suppressor in cutaneous squamous cell carcinoma (cSCC). Here, we describe the effects of expressing *miR-1* in cSCC cells and the identification of Ets1, as a *miR-1* target in the mouse.

Materials and Methods

RNA isolation

Animal studies were approved by the Ohio State University (OSU) and University of California, San Francisco Institutional Animal Care and Use Committees. The OSU IACUC determined that the research performed at OSU was exempt from IACUC review as pre-existing or commercially available animal specimens were used for this study. Fresh frozen skin samples from three FVB/NJ and three SPRET/EiJ female mice aged 4-5 weeks were obtained through the Jackson Laboratory surgical service. RNA was isolated from the skin using standard Trizol extractions (Invitrogen, Grand Island, NY). We isolated RNA a second time from two of the skin samples to generate a replicate control for the microarrays. Samples were DNAase treated. RNA was quantitated using Nanodrop (ThermoScientific, Wilmington, DE). RNA was isolated from eleven cSCCs from chemically treated C57Bl6/FVB F1 mice using standard Trizol methods. RNA samples were DNAse treated.

MicroRNA expression arrays and data analysis

Five μg of total RNA from each sample was sent to the OSU Comprehensive Cancer Center MicroArray Shared Resource for miRNA expression analysis using the miRNA microarray chip OSUCCC version 4.0 (Liu *et al.* 2004; Liu *et al.* 2008). For each strain of mice, a fourth RNA sample of a second independent RNA isolation was also evaluated. The arrays contain over 300 human and mouse miRNAs spotted in triplicate with probes for both precursor and mature miRNAs. Experiments were performed and analyzed as previously described (Liu *et al.* 2004; Liu *et al.* 2008). In brief, a linear model was employed to detect differentially expressed miRNAs. In order to improve the

91 estimates of variability and statistical tests for differential expression, a variance

92 smoothing method with fully moderated t-statistic was employed for this study (Yu *et al.*

93 2011). The significance level was adjusted by controlling the mean number of false

94 positives (Gordon *et al.* 2007).

95

96 **Quantitative PCR**

97 *Quantitative PCR (qPCR) validation of candidate miRNAs*. qPCR was performed using

98 Applied Biosystems Taqman probes to validate expression of the six candidate miRNAs

99 (Foster City, CA). Probes were specific to either mature (*miR-1*, *miR-133a*, *miR-124a-*

100 *3, miR-206*) or precursor (*miR-134, miR-206, miR-9-1*) miRNAs concordant with the

101 array results. Reverse transcription for mature miRNAs was performed using Applied

102 Biosystem's High Capacity cDNA Reverse Transcription reagents according to

103 manufacturer's protocol in 7.5 μl reactions (Carlsbad, CA). Three independent skin RNA

104 samples were used for each strain from samples not used on the original arrays. qPCR

105 of *miR-1* levels from eleven primary mouse cSCCs was conducted as for normal skin.

106 For miRNA quantification, real-time PCR was performed using Applied Biosystem

107 Taqman Assays per manufacturer's recommended conditions. All samples were done

108 in triplicate. qPCR runs included no template and no-reverse transcriptase controls.

109 Expression was normalized to expression levels for *sno202* RNA for mature miRNAs or

110 to *L19* for pri-miRNAs. qPCR was conducted using Applied Biosystems 7900HT

111 instrument. Cycle threshold (CT) values were averaged across triplicates and delta CT

112 values were calculated between each test miRNA and control. The fold expression of

113 SPRET/EiJ miRNA relative to FVB/NJ was calculated. Standard deviations of fold

114 differences of the percentage of reference gene expression were made by comparisons

115 between independent RNA samples of each of the test samples. Student's t-test was

116 used to calculate p-values for the qPCR. A nonparametric Mann-Whitney U test was

117 performed to test for differences between in *miR-1* expression in tumors versus

118 SPRET/EiJ and FVB normal skin .

119

120 *qPCR of predicted mRNA targets*. To measure expression of predicted targets, mRNA

121 expression was measured at 48 and 72 hours post *miR-1* or scrambled precursor miR

122 negative control transfection in A5 cells and at 48, 72 and 96 hours post-transfection in

123 B9 cells using SYBR green quantitative real-time PCR. Reverse transcription of mRNA

124 was performed using a Bio-Rad iScript cDNA synthesis kit according to manufacturer's

125 recommended conditions. Primers were designed using Integrated DNA Technologies.

126 Target gene expression was measured using Bio-Rad SYBR Green Supermix in 10 μl

127 reactions according to manufacturer's protocol. qPCR of each sample was performed in

128 triplicate and was used to measure *Ets1, Met, Bag4, Twf1, Sp1, Taok1, Zfp148* and

129 *Trp53* target gene expression. The following primer sets were used. *Ets1*: Forward: 5'

130 TGTATGAGTGGAGCAGCACTGTGT 3', Reverse: 5' AGGTAGGGTCTCCATTAACCT

131 3', *Met*: Forward: 5' AACGGGTATTGGGAAGACCCTGAA 3', Reverse: 5'

132 ATCCCGTCTAACAGGAAGAAGGCT 3', *Bag4*: Forward: 5'

133 ACTCCACGGAAGTTCCAA ACACCT 3', Reverse: 5'

134 TTCCAGGGTTCTGTGAAGCAGGAT 3', *Twf1*/*Ptk9*: Forward 5'

135 TTCCAGGCTTTGGAGAAGGTGAGT 3', Reverse: 5'

136 AGTCTCCTTCGTGGGAATGCTTGT 3', *Sp1*: Forward 5'

137 TGCGGCAAAGTATATGGCAAGACC 3', Reverse 5'

138 ACTCCTCATGAAACGCTTAGGGCA 3'

139 *Taok1*: Forward 5' GAACCAGGCCAAGTGAAACTTGCT 3', Reverse 5'

140 AAAGGAGGCTTCCTCTCGGCTAAT 3'

141 *Zfp148*: Forward 5' GCACTGCAATGCTGCCTTTAGAAC 3', Reverse 5'

142 TTTCGCCGGTATGGATCTTCTCGT 3'

143 *Trp53*: Forward 5' ACAAGAAGTCACAGCACATGACGG 3', Reverse 5'

144 TTCCTTCCACCCGGATAAGATGCT 3'

145   and *L19*: Forward: 5' ATTCCCGGGCTCGTTGCCGGAAAA 3', Reverse: 5'

146 ATTGGCAGTACCCTTCCTCTTCCCTA 3'. Candidate targets were normalized to *L19*

147 expression and fold difference relative to the scrambled control at 48 hours post-

148 transfection were calculated. Standard deviations of fold differences of the percentage

149 of reference gene expression were made by comparisons between independent RNA

150 samples. Experiments included no template and no reverse transcriptase controls for

151 each gene. Student's t-test was used to calculate p-values.

152

153 *qPCR confirmation of transfections*. RNA was isolated from a duplicate well for each

154 mock, scrambled control or *miR-1* transfected experiment to confirm increased *miR-1*

155 expression in the *miR-1* transfected cells. qPCR for *miR-1* expression was conducted

156 as described above.

157

158 ***Identification of candidate target genes for differentially expressed microRNAs***

159 Target prediction programs, www.microRNA.org (Beta *et al.* 2008) and

160 www.targetscan.org were searched for predicted mRNA targets for *miR-1* in both

161 mouse and human. Targets for *miR-1* were prioritized for further study by the number

162 of predicted binding sites per 3'UTR (> 1 site), strength of binding score, and predicted

163 binding of the miRNA in both human and mouse. A literature scan was also performed

164 to identify targets which had previously been validated experimentally.

165

166 ***Cell culture and transient transfections***

167 A5 and B9, mouse cutaneous spindle and cSCC cell lines respectively, and C5N, a non-

168 tumorigenic mouse keratinocyte cell line, were used for these studies (Zoumpourlis *et*

169 *al.* 2003). Cell lines were grown in Dulbecco's medium supplemented with 10% fetal

170 bovine serum and 1% penicillin/streptomycin in an atmosphere of 5% CO_2. Transient

171 transfections were performed using Invitrogen Lipofectamine 2000 according to

172 manufacturer's protocol (Life Technologies, Grand Island, NY). Mock and negative

173 control (scrambled pre-miR) transfections were carried out for all experiments when

174 cells were at 40-50% confluency. Scrambled pre-miR (AM17110) negative control and

175 *miR-1* (AM17150) precursor were purchased from Ambion (Life Technologies, Grand

176 Island, NY).

177

178 ***Western blot analysis***

179 Protein from whole cell extracts was extracted 24, 48, 72 and 96 hours post-transfection

180 via solubilization of A5 and B9 cells using RIPA buffer (50 mM Tris base pH 8, 150 mM

181 NaCl, 1% NP40, 0.10% SDS) containing a protease inhibitor (Roche). For each

182 sample, 30 μg of protein were run on a 10% polyacrylamide gel in a Bio-Rad mini-gel

183 system at 150V (12V/cm) for ~1.5 hours. Proteins were transferred to nitrocellulose

184 membrane for 70 minutes at 90V (7V/cm). Following transfer, membranes were blocked

185 in 5% milk in TBST (10X TBS: 1.2% Tris, 8.8% NaCl) and were incubated with primary

186 antibody overnight. Antibodies and dilutions were as follows: Ets1, 1:1000 dilution

187 (antibody a gift from Michael Ostrowski) and α-tubulin, 1:100 dilution (T5168, Sigma-

188 Aldrich, St. Louis, MO). Incubation with secondary antibodies was for 2 hours.

189 Secondary antibodies and dilutions were as follows: Anti-mouse HRP-Linked, 1:10000

190 dilution (W4021, Promega, Madison, WI), Anti-Rabbit HRP-Linked Antibody, 1:1000

191 dilution, (7074S, Cell Signaling Technology, Danvers, MA). Enhanced

192 chemiluminescent reagent (Thermo Scientific, Rockford, IL) was applied in a 1:1 ratio.

193

194 ***Luciferase Assays***

195 *Cloning.* Cloning of *Ets1* 3'UTR was performed using In-Fusion Advantage PCR Cloning

196 Kit according to manufacturer's protocol (Clontech Mountain View, CA). Full length

197 *Ets1* plasmid with 3'UTR provided by Dr. Michael Ostrowski was used as a template for

198 PCR. A 1,278 base pair product was generated containing the predicted three *miR-1*

199 binding sites and was cloned into a pGL3 Control Luciferase vector (Promega, Madison,

200 WI). Primers used for cloning were as follows: *Ets1* 3' UTR In-Fusion Forward: 5'-

201 tgtaatactagtccgAGAAGAGAGGCAATTGGCTGAGGT-3', *Ets1* 3' UTR In- Fusion

202 Reverse: 5'-gtctgctcgaagcggACTGAGGCAGTATTCCTGATAGAG-3'. Clones were

203 sequence verified. Site-directed mutagenesis was carried out to mutate two base pairs

204 in each of the three *miR-1* binding sites in the *Ets1* 3'UTR using QuikChange Lightning

205 Multi Site-Directed Mutagenesis kit according to the manufacturer's protocol (Agilent

206 Technologies, Santa Clara, CA). Primers used for site-direct mutagenesis are as

207 follows:

208 *Ets1* 3' UTR SDM 1 Forward: 5'-gttgatggctgactcccactctcccttgaagactctgaat-3',

209 *Ets1* 3' UTR SDM-1 Reverse: 5'-attcagagtcttcaagggagagtgggagtcagccatcaac-3',

210 *Ets1* 3' UTR SDM-2 Forward: 5'-ccacaaggaagcaaaggccaaactctccagctatatattttgatctta-3' ,

211 *Ets1* 3' UTR SDM-2 Reverse: 5'-taagatcaaaatatatagctggagagtttggcctttgcttccttgtgg-3',

212 *Ets1* 3' UTR SDM-3 Forward: 5'-acctgttgaactcttacgtactctccaaagacgtttcaaggaac-3',

213 *Ets1* 3' UTR SDM-3 Reverse: 5'-gttccttgaaacgtctttggagagtacgtaagagttcaacaggt-3'.

214 Clones were sequenced verified.

215

216 *Transfections and luciferase assays.* C5N and A5 cells were plated in triplicate into a

217 12-well dish at 24 hours prior to transfection. At 70% confluency, 0.10 μg Luc-*Ets1*

218 3'UTR and Luc-*Ets1* 3'UTR-mutated were each co-transfected with 10 pmol of a

219 scrambled control miR or *miR-1* into each well. 0.10 μg of a PRL-TK vector (TK-driven

220 *Renilla* luciferase vector, Promega) was also transfected into each well to normalize the

221 firefly luciferase values. All experiments included mock and pGL3-Control empty vector

222 controls. A5 and C5N cell lysates were prepared using M-PER (Pierce Biotechnology,

223 Rockford, IL) and 30 μg of each sample were used for analysis. Firefly and renilla

224 luciferase measurements were acquired 24 hours post-transfection using a Veristas

225 Microplate Luminometer.

226

227 **Cell proliferation assay**

228 At 24 hours post-transfection with *miR-1*, scrambled miR control or mock transfected,

229 A5 and B9 cells were trypsinized and 2000 cells (A5) or 3000 cells (B9) were plated in

230 quadruplicate in a 96-well plate. Proliferation was measured at 24, 48, 72 and 96 hour

231 post-transfection using a MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

232 bromide) cell proliferation kit (Roche, Indianapolis, IN). Experiments were carried out

233 according to manufacturer's protocol. RNA and protein were isolated at each

234 solubilization step. Optimal absorbance of the formazan product was measured at

235 wavelength of 550 nm and a reference wavelength of 690 nm was used. For each

236 sample, absorbance was normalized to the reference wavelength as well as a blank

237 control, which contained media only.

238

239 **Cell cycle assay**

240 A5 and B9 cells were transfected with *miR-1*, scrambled miR control or mock

241 transfected. Cells were trypsinized and counted 48, 72 and 96 hours post-transfection.

242 Two million cells were fixed in 70% ethanol. Cell cycle analysis was evaluated by DNA

243 content. Cells were incubated with a propidium iodide solution (0.1% Triton X-100, 0.2

244 mg/mL DNAse-free RNAse A and 20 μg/mL propidium iodide for 15 minutes at 37ºC

245 (B9 cells) or 30 minutes at 20ºC (A5 cells). Data was acquired on a BD FACS Calibur

246 instrument. ModFit software was used for analysis. Experiments were done in

247 triplicate.

248

249 **Apoptosis assay**

250 A5 and B9 cells transfected with *miR-1* or scrambled miR control were trypsinized and

251 counted at 48, 72 and 96 hours post-transfection. The FITC Annexin V Apoptosis

252 Detection Kit I was used to evaluate cell death according to manufacturer's protocol (BD

253 Biosciences, San Jose, CA). Unstained, FITC Annexin only, and propidium iodide only

254 conditions were used as controls. Data was acquired on a BD FACS Calibur instrument.

255 Cell Quest Pro for was used for data analysis. Experiments were done in triplicate.

256

257 ***Cell motility assay***

258 A5 and B9 cells were plated at a density of 7×10^5 cells/mL in ibidi cell culture inserts

259 according to the manufacturer's protocol (Martinsried, Germany) at approximately 48,

260 72 and 96 hours post-transfection. Transfections of A5 and B9 cells included mock

261 transfection, scrambled miR control (AM17110) or *miR-1* precursor (AM17150) as

262 described above. Eight hours after plating, cell inserts were removed. Images were

263 taken at 5X magnification using AxioVision 4.8 software on an Axiovert 25 microscope

264 at 0 hours and every 5-8 hours until gaps closed. Images were assessed visually by two

265 independent researchers for differences in rate of gap closure.

266

267 **Results**

268 ***miRNA profiling of normal skin of cancer resistant and cancer susceptible mice***

269 We hypothesized that miRNAs differentially expressed between cancer resistant and

270 cancer susceptible mice might have oncogenic- or tumor suppressor-like properties in

271 the skin. To identify miRNAs that were differentially expressed between cancer

272 resistant SPRET/EiJ and cancer susceptible FVB/NJ mice, we performed miRNA

expression profiling of total RNA isolated from four normal skin samples per strain. The miRNA arrays contained probes for precursor and mature miRNAs of approximately 300 miRNAs (Supplementary Table S1; Liu *et al.* 2004; Liu *et al.* 2008). Following analysis, six miRNAs (*miR-1*, *miR-133a*, *miR-124a-3*, *miR-134*, *miR-206,* and *miR-9-1*) showed a significant difference in average expression and had a 2.0 fold or greater difference across one or more probe sets. All of these showed higher expression in SPRET/EiJ (Table 1). Due to the evolutionary divergence between *M. musculus*, for whom the majority of arrays have been designed, and *M. spretus,* lower miRNA expression observed in SPRET/EiJ mice could indicate a sequence polymorphism. However, we observed the opposite effect in which all miRNAs showing differential expression were higher in SPRET/EiJ; these results decrease the likelihood that the observed profiles were due to polymorphic variants between the strains.

To validate the expression differences of the six miRNAs showing differential expression between SPRET/EiJ and FVB/NJ by microarray, we performed qPCR using Applied Biosystems Taqman probes on samples from mice not used in the initial array studies. Probes were specific to either mature (*miR-1*, *miR-133a*, *miR-124a-3, miR-206*) or precursor (*miR-9*, *miR-134*, *miR-206*) miRNAs and were chosen for evaluation based on the specific probe sets (mature versus precursor) that showed differences by microarray (Table 1). Five of the six miRNAs evaluated showed similar fold-differences in expression between microarray and qPCR for FVB/NJ and SPRET/EiJ skin (Figure 1 and data not shown); however, only *miR-1*, *miR-133a* and *miR-206* showed statistically significant differences in expression by qPCR (p-value < 0.05).

296

miRNA-1 expression decreased in mouse cSCCs

We hypothesized that miRNAs expressed at higher levels in SPRET/EiJ mice relative to

FVB/NJ mice would behave similarly to tumor suppressor genes and exhibit reduced

expression in tumors. We chose *miR-1* for expression studies in mouse cSCCs to test

our hypothesis because it showed significant difference in expression between FVB/NJ

and SPRET/EiJ for six probes on the array and showed a significant difference in

expression by qPCR (Table 1). In addition, there was evidence from the literature

showing down-regulation of *miR-1* in a variety of human cancers and a link between

miR-1 down-regulation and cancer phenotypes (Nasser *et al.* 2008; Datta *et al.* 2008;

Yan *et al.* 2009; Nohata *et al.* 2012a; Nohata *et al.* 2012b). From these data, we

expected that *miR-1* expression would be decreased in mouse cSCCs. To evaluate

miR-1 expression in cSCC, we performed qPCR of *miR-1* in eleven cSCCs isolated

from DMBA/TPA chemically-treated C57Bl6/FVB mice. The median *miR-1* expression

was lower in the tumors (3% of control *sno-202*) compared to median *miR-1* expression

in FVB/NJ (22% of control *sno-202*) and SPRET/EiJ (110 of control *sno-202*). The

difference in expression was significant between the tumors and SPRET/EiJ (Figure 2,

p-value=0.012). Based on the expression data in the primary tumors and the strain

specific differences in expression, we chose *miR-1* as a strong candidate miRNA to

evaluate further for a role in cSCC.

316

qPCR of miR-1 candidate target genes

318 miRNAs are thought to impact gene regulation by targeting mRNAs for degradation via

319 complete sequence matches or inhibition of translation when there is imperfect binding

320 between a miRNA and its target sequence (Djuranovic, Nahvi & Green 2011). We

321 hypothesized that if the difference in miRNA expression between the strains of mice

322 was important in cancer susceptibility there would be differences in target mRNA

323 expression of genes associated with carcinogenesis. To identify putative targets of *miR-*

324 *1*, *miR-133a*, *miR-124a-3*, *miR-134*, and *miR-206*, we searched the literature to identify

325 targets of these miRNAs that had been detected by expression arrays in tumors and

326 had been further validated by other methods. We also identified genes predicted to be

327 targets in microRNA databases www.microRNA.org (Beta *et al.* 2008) and

328 www.targetscan.org. We prioritized genes as candidates which had two or more

329 predicted binding sites, had stronger binding scores and those genes previously

330 implicated in cancer. Using these strategies, we identified multiple candidate targets for

331 each miRNA (Supplemental Table S2).

332

333 We chose seven putative *miR-1* targets, *Bag4*, *Ets1*, *Met*, *Sp1*, *Taok1*, *Trp53*, and

334 *Zfp148*, and one positive control, *Twf1*, which previously had been shown to be a *miR-1*

335 target by Applied Biosystems, for testing. These candidate target genes were prioritized

336 based on our database searches and those which had been validated in the literature

337 as being targets of *miR-1* in studies of primary human tumors or cancer cell lines. By

338 qPCR, two of the candidate targets, *Ets1* and *Met,* and our positive control *Twf1*

339 showed significant down-regulation in *miR-1* transfected A5 cells compared to

340 scrambled miR precursor transfected cells at both 48 and 72 hours post-transfection

341 (Figure 3 and Supplemental Figure S1, *Met* p-values 0.0001 and 0.002; *Ets1* p-

342 value=0.0001 and 0.0002; *Twf1* p-values=0.0001 and 0.006 respectively). Our positive

343 control, *Twf1*, showed decreased expression in *miR-1* transfected B9 cells at 48, 72 and

344 96 hours (p-values=0.0004, 0.0002 and 0.03 respectively). In B9 *miR-1* transfected cells

345 *Met* showed significantly decreased expression only at 48 hours (p-value=0.001), and

346 *Ets1* showed significantly decreased expression at 48 and 72 hours post-transfection

347 compared to scramble miR control transfected cells (p-values 0.008 and 0.005

348 respectively). *Bag4* mRNA expression was significantly reduced in B9 cells transfected

349 with *miR-1* at all three time points (Supplemental Figure S1; 48 hour p-value=0.0007, 72

350 hour p-value=0.02 and 96 hour p-value=0.004), but it only showed significant down-

351 regulation of mRNA at 72 hours in A5 cells transfected with *miR-1* compared to

352 scrambled control cells (Figure 3D and Supplemental Figure S1, p-value=0.01). The

353 remaining targets did not show statistically significant differences in expression between

354 control and *miR-1* A5 transfected cells (data not shown).

355

356 ***Evaluation of Ets1 as a miR-1 target***

357 mRNA expression data is only correlative and does not show a direct effect of miRNAs

358 on predicted target gene expression. As *Ets1* had not been confirmed as a target of

359 *miR-1* in the mouse*,* and since several studies support a role of Ets1 in cSCC and

360 cancer phenotypes such as metastasis, apoptosis and proliferation, we decided to

361 further evaluate murine Ets1 as a *miR-1* target (Keehn, Smoller & Morgan 2004; Hahne

362 *et al.* 2009; Nagarajan *et al.* 2009). We first assessed whether Ets1 protein levels were

363 decreased by *miR-1* expression. By Western, Ets1 was down-regulated in the *miR-1*

364 transfected A5 cells at 24, 48 and 72 hours post-transfection and in B9 cells at 48 and

365 96 hours post-transfection compared to scramble control miR transfected cells (Figure

366 4a and 4b; Supplemental Figure S2). As this could be an indirect effect of *miR-1*

367 expression, we assessed the effect of *miR-1* expression on a luciferase construct

368 containing the 3'UTR of *Ets1*. *miR-1* expression resulted in lower luciferase expression

369 with the wildtype *Ets1* 3'UTR compared to a scrambled precursor miRNA. Furthermore

370 a construct containing mutated *miR-1* binding sites did not show reduced luciferase

371 expression providing additional evidence that *miR-1* regulates Ets1 expression via direct

372 binding to the predicted *miR-1* binding sites in the *Ets1* 3'UTR (Figure 4c). Our data is

373 consistent with a publication showing Ets1 to be a target of *miR-1* in a human liver

374 cancer cell line (Wei *et al.* 2012).

375

376 **Functional characterization of miR-1 expression**

377 ***miR-1 expression in cSCC cells decreases cell proliferation***

378   There are several published studies showing *in vitro* and *in vivo* effects of *miR-1* on

379 tumor suppression (Nasser *et al.* 2008; Datta *et al.* 2008; Yan *et al.* 2009; Nohata et al.

380 2011, Nohata *et al.* 2012a, Nohata *et al.* 2012b). Based on these, we anticipated that

381 expression of *miR-1* in cSCC cells would have tumor suppressing abilities (Nohata *et al.*

382 2012b). To determine if *miR-1* decreased cell proliferation, we transfected A5 and B9

383 cSCC cells with *miR-1* or scrambled precursor miRNA. Cell proliferation was measured

384 by an MTT assay at 24, 48, 72 and 96 hours post-transfection. We observed a

385 significant decrease of 1.7-fold in absorbance at 96 hours post-transfection in A5 *miR-1*

386 transfected cells compared to the scrambled control miR transfected cells suggesting

that *miR-1* is playing a role in growth inhibition (Figure 5a, 72 hour p-value=0.01; 96

hour p-value=0.0001). A modest, but significant decrease in proliferation was observed

at the 72 hour time point in the B9 cSCC cell line, but there was no difference in

proliferation at 96 hours (Figure 5b, 72 hour p-value=0.02; 96 hour p-value=0.3).

The effect of miR-1 expression on additional tumor phenotypes

miR-1 has been shown to induce apoptosis in multiple cancer cell lines including

maxillary sinus SCC, head and neck SCC, and renal cell carcinoma (Nohata *et al.*

2011; Kawakami *et al.* 2011; Nohata *et al.* 2012a; Nohata *et al.* 2012b). To determine if

the decreased proliferation seen in the cells transfected with *miR-1* was the result of

increased apoptosis, we performed FACS analysis of AnnexinV and PI stained *miR-1*

transfected and scramble control miR transfected A5 and B9 cells. No significant

differences in apoptosis were observed at 48 or 72 hours post-transfection in the A5

cells; however, there was a significant increase in apoptosis at 96 hours (p-value=0.036;

Figure 6a, Supplemental Figure S3). The B9 cells showed a statistically significant

decrease in apoptosis in the *miR-1* transfected cells at 48 hours (p-value=0.04) and a

statistically significant increase in apoptosis at 72 hours (p-value=0.003). There were no

differences in apoptosis at 96 hours (Figure 6b, Supplemental Figure S3). The

apoptosis data for the A5 and B9 cell lines are consistent with the proliferation results.

Ectopic expression of *miR-1* has also been shown to induce G0/G1 arrest in several

different cancer cell lines (Nohata *et al.* 2012b). We evaluated cell cycle parameters in

miR-1 transfected cells by FACS analysis (Figure 6, Supplemental Figure S4). We

410   observed modest but consistent and statistically significant fewer cells in the S phase

411   for *miR-1* transfected A5 cells compared to scrambled control miR transfected A5 cells

412   at 48, 72, and 96 hours (p-values=0.002, 0.0001 and 0.006 respectively, Figure 6C, 6E,

413   6G) and for *miR-1* transfected B9 cells at 48 and 96 hours (p-values=0.001 and 0.007

414   respectively, Figure 6D and 6H). There was a trend for a modest increase in cells in

415   G0/G1 for *miR-1* transfected A5 cells at all time points, but this was only significant at

416   48 hours post-transfection (p-value=0.04;Figure 6C). This was not observed in the B9

417   cells. There was a trend for more cells in G2-M in the *miR-1* transfected cells for both

418   A5 and B9 for some time points (Figure 6, Supplemental Figure S4), but the absolute

419   differences are quite small and were not significant except for the *miR-1* transfected B9

420   cells at 48 hours (p-value=0.02, Figure 6F). These results suggest that *miR-1* may have

421   an extremely modest effect on cell cycle progression resulting in fewer cells in S phase.

422

423   Previous studies have reported a role of *miR-1* in migration. In addition, Ets1 has a

424   well-documented role in enhancement of migration (Hahne *et al.* 2005; Smith *et al.*

425   2012). To test whether *miR-1* affects migration in cSCC, we performed cell motility

426   assays of *miR-1* transfected and scrambled control transfected A5 and B9 cells at

427   approximately 24, 48, 72 and 96 hours post-transfection. No consistent differences were

428   observed in cell motility between *miR-1* and scrambled control miR transfected cells for

429   any of the time points (Figure 7, Supplemental Figure S5 and data not shown). Closure

430   of gap appeared to be directly proportional to gap width.

431

432 *miR-1* expression following transfection was confirmed for all phenotypic experiments

433 by qPCR and is below detectable limits for the mock or scrambled control transfected

434 A5 and B9 cell lines.

435

436 **Discussion**

437 Exploitation of genetic differences between cancer resistant and cancer susceptible

438 mice has led to the identification of candidate genes important in tumorigenesis, but little

439 has been explored regarding the differences in miRNA expression profiles in these

440 mice. Here, we describe our discovery of five miRNAs showing higher expression in

441 skin cancer resistant mice compared to skin cancer susceptible mice. In more detailed

442 analyses of one miRNA, *miR-1,* we show that *miR-1* expression leads to decreased cell

443 proliferation which may be the result of modestly higher apoptosis in later time points

444 (72 or 96 hours) in combination with a reduced number of cells in S phase. We also

445 observed decreased mRNA expression of previously described human *miR-1* targets,

446 *Met*, *Twf1*, and *Ets1,* in the mouse which establishes that *miR-1* targets these mRNAs

447 in more than one species. Furthermore, we show that *Bag4*, a novel *miR-1* target, is

448 significantly down-regulated in the B9 cell line.

449

450 *miR-1*, *miR-133a*, and *miR-206* are all part of a group of myo-miRs, miRNAs whose

451 expression is enriched in skeletal and cardiac muscle (McCarthy 2008). All three of

452 these miRNAs showed higher expression in cancer resistant SPRET/EiJ compared to

453 FVB/NJ normal skin. Initial studies on *miR-1* focused on its expression in heart and

454 skeletal muscle as it was thought not to be expressed in other tissues. In

455   cardiomyocytes *miR-1* regulates apoptosis via Bcl-2 and IGF-1 (Yu *et al.* 2008; Tang *et*

456   *al.* 2009). In skeletal muscle, *miR-1* has a role in cellular proliferation and differentiation

457   (Chen *et al.* 2006). All of these functions are also important in tumorigenesis and are

458   consistent with a role for *miR-1* in cancer. A connection between expression of myo-

459   miRs and skin cancer susceptibility has previously been made; a gene network for skin

460   cancer susceptibility from the same strains of mice studied here was found to be

461   enriched in muscle related mRNAs (Quigley *et al.* 2009).

462

463   Decreased *miR-1* expression has been observed in a number of cancers including skin,

464   lung, liver, bladder, renal and prostate (Li *et al.* 2012; Hudson *et al.* 2012; Nohata *et al*.

465   2012a; Tominaga *et al.* 2013; Nohata *et al.* 2012b). This is not the first study to look at

466   the role of *miR-1* in the skin or in SCCs. By array analysis, *miR-1* showed about a 2-fold

467   down regulation in human cSCCs compared to matched normal skin (Darido *et al.*

468   2011). *miR-1*, along with *miR-133a*, *miR-205* and *let-7d,* shows decreased expression

469   in SCCs of the head and neck in comparison to normal adjacent tissue (Childs *et al.*

470   2009). Consistent with their down-regulation in tumors, numerous studies have

471   demonstrated tumor suppressor functions of *miR-1*. Increased expression of *miR-1 in*

472   *vitro* has been associated with increased apoptosis, decreased migration and

473   decreased cell growth (Hudson *et al*. 2012; Wei *et al*. 2012; Yamasaki *et al.*

474   2012;Yoshino *et al.* 2012). *miR-1* was also found to target c-Met in rhabdomyosarcomas

475   and colorectal cancer leading to decreased cell motility and proliferation (Yan *et al.*

476   2009; Reid *et al.* 2012). Our results showed decreased *Met* mRNA expression in cells

477   transfected with *miR-1,* but observed no correlation between *miR-1* expression and cell

478   motility. In this study, we identified a correlation with proliferation and *miR-1* expression

479   levels which is consistent with other studies (Yan *et al.* 2009; Reid *et al.* 2012).

480

481   In contrast to previous studies we observed only modest effects of *miR-1* on apoptosis

482   and cell cycle and did not observe any consistent effects on migration. We observed a

483   consistent decrease in the percentage of *miR-1* transfected cells in S phase and a non-

484   significant increase of *miR-1* transfected cells at G2/M compared to scrambled control

485   cells for some time points, but these differences were very modest compared to studies

486   by others (Nohata *et al.* 2011; Hudson *et al.* 2012; Kawakami et al. 2012; Wei *et al.*

487   2012). We also observed increases in apoptosis in both B9 and A5 cells transfected

488   with *miR-1*, but the apoptosis was only observed at later time points and the percentage

489   of cells undergoing apoptosis was less compared to other studies (Hudson *et al.* 2012;

490   Wei *et al.* 2012; Yoshina *et al.* 2012). Finally, we observed no significant differences in

491   cell motility of *miR-1* transfected A5 or B9 cells. There are several potential explanations

492   for the observed results. First, these cell lines may not be as responsive to the effects of

493   *miR-1* because they do not express high levels of other pro-tumorigenic *miR-1* targets

494   not studied here. Some *miR-1* oncogene-like targets have been identified that were not

495   evaluated for expression in our cells. However, many of the targets tested in our study

496   that have been reported to have oncogenic properties, such as *Bag4*, *Ets1* and *Met*,

497   showed decreased expression in A5 and/or B9 cells following *miR-1* transfection.

498   Another possible explanation for the discordant results is that we evaluated the effects

499   of *miR-1* expression in mouse and not human cell lines. Most of the published studies

500   on *miR-1* have been in human cancers and using human cell lines and it is possible that

501 *miR-1* has unique targets in the mouse which confer tumor suppressor activities that

502 ameliorate some of the effects of silencing target oncogenes. Additional studies are

503 warranted to further explore these and other possibilities.

504

505 Ets1 is a transcription factor that has been described as a proto-oncogene due to its

506 pro-tumorigenic effects and its discovery in chickens affected with the avian

507 erythroblastosis virus, E26 (Dittmer 2003). Specifically, Ets1 has been shown to play a

508 role in cSCC tumor development and progression (Pande *et al.* 1999; Nagarajan *et al.*

509 2009). Ets1 acts as a cSCC tumor promoting gene by inducing cSCC cell proliferation in

510 transgenic mice over-expressing Ets1 (Pande *et al.* 1999). Studies show that Ets1

511 regulates genes important in apoptosis, angiogenesis, migration, invasion, and matrix

512 metalloproteinases which enhance cell migration (Pande *et al.* 1999; Keehn, Smoller &

513 Morgan 2004; Hahne *et al.* 2005). In addition, it is over-expressed in human cSCC

514 malignancies (Keehn, Smoller & Morgan 2004; Saeki *et al.* 2000; Saeki *et al.* 2002). A

515 previous study looked at Ets1 expression in 26 primary skin lesions. Ets1 nuclear

516 expression was present in cSCC in situ, but was the highest in poorly differentiated or

517 metatstatic cSCCs (Keehn, Smoller & Morgan 2004). These results suggest the

518 importance of dysregulation of Ets1 expression for progression of cSCC. Consistent

519 with a role for *miR-1* in dysregulation of Ets1 in cancer, a study published after we

520 chose Ets1 as a candidate showed that Ets1 is targeted by *miR-1* in HepG2 cells (Wei

521 *et al.* 2012). Ets1 is involved in regulation of numerous protein tyrosine kinases (PTKs),

522 a class of molecules to which a majority of known oncogenes belong (Hahne *et al.*

523 2009). Some predicted Ets1 targets include EGFR, FGFR4, INSR, MET, PDGFRA, and

524 VEGFR1 (Hahne *et al.* 2009). Ets1 has been shown to be involved in regulation of

525 tumorigenesis by promotion of the EGFR-Ras-MEK1/2 MAPK pathway (Roberts & Der

526 2007; Ciardiello & Tortora 2008; Montagut & Settleman 2009; Knight, Lin & Shokat

527 2010). Our positive control for *miR-1* transfection, *Twf1*, also belongs to the family of

528 PTKs regulated by Ets1. As *miR-1* has multiple experimentally validated targets from

529 this study (*Ets1*, *Met*, *Bag4*) and from published studies (MET, PTK1, HDAC4, ANXA2,

530 BDNF and FOXP1) that have roles in tumorigenesis, it is likely that *miR-1*'s ability to

531 reduce the expression of many tumor promoting genes could have a global influence on

532 the suppression of tumor development (Nasser *et al.* 2008; Datta *et al.* 2008; Yan *et al.*

533 2009; Reid *et al.* 2012).

534

535 Our data suggest that *Bag4* may also be a target of *miR-1* which is a novel finding. Like

536 Ets1 and Met, Bag4 has been shown to have transforming properties (Yang *et al.* 2010).

537 Bag4 is a member of a family of proteins that act as co-chaperones for anti-apoptotic

538 proteins Bcl-2 and Hsp70 (Doong *et al.* 2002). The literature describing the role of

539 BAG4 in apoptosis is mixed as BAG4 is associated with apoptosis in some studies, but

540 appears to be anti-apoptotic in others (Ozawa *et al.* 2000; Annunziata *et al.* 2007).

541 Additional studies are warranted to further evaluate Bag4 as a *miR-1* target and any role

542 it may have in cSCC.

543

544 There are some limitations to this study. Only one of the three significantly differentially

545 expressed miRNAs by qPCR was the subject of further phenotypic studies. *miR-133a*

546 and *miR-206* may play important roles individually or in combination with *miR-1* in the

547 development of cSCC which were not evaluated in this study. Another weakness of this

548 study was that candidate targets of *miRNA-1* were chosen based on the literature and

549 predicted *in silico* screens rather than experimentally. Thus, we may have missed

550 critical tumor suppressing targets of *miR-1* through this approach.

551

552 We identified and validated five miRNAs showing differential expression between

553 cancer resistant and cancer susceptible mice. The mice used in the study are inbred,

554 are age and sex-matched, and should show very similar expression profiles between

555 mice within a strain, but it is possible that the inclusion of additional mice in the study

556 would have led to increased power and the identification of additional miRNAs of

557 interest. In addition, as we used a 2-fold difference in expression as our cutoff more

558 miRNAs may show more modest differences in expression between Spret/EiJ and

559 FVB/NJ. Nonetheless, these data are consistent with a study measuring miRNA

560 expression of 577 miRNAs from livers of 70 different strains of inbred mice which found

561 few to no differences in expression of any of these miRNAs (Gatti *et al.* 2011). A few

562 studies have looked at miRNA expression in human cSCCs, but none of the miRNAs

563 identified in those studies overlap with the ones identified here (Dziunycz *et al.* 2010).

564

565 It is interesting to note that in our original analysis, we identified miRNAs for which the

566 precursor, but not the mature form of the miRNA showed differential expression on the

567 array. The discrepancy may reflect transcription differences between the strains

568 followed by miRNA processing resulting in similar amounts of mature miRNA being

569 produced, or the precursor miRNAs may also be processed into different forms of

570   mature miRNAs, such as 5p and 3p forms, for which there were not probes on the

571   array. These data may be a reflection of our study being statistically underpowered to

572   detect some of the differences in miRNA expression. For example, *miR-206* showed

573   statistical differences in expression of the precursor miRNA but not the mature miRNA

574   by microarray, but by qPCR both the precursor and mature *miR-206* showed very

575   similar differences in expression between FVB/NJ and SPRET/EiJ.

576

577   In summary, we identified five miRNAs that show differences in expression of skin

578   between cancer resistant and cancer susceptible mice. We show evidence that *miR-1*

579   expression is decreased in cSCCs and that its expression *in vitro* leads to decreased

580   proliferation of cSCC cell lines, increased apoptosis and modest effects on cell cycle. In

581   cell lines, *miR-1* expression is correlated with decreased *Met1* and *Bag4* expression

582   and directly targets the oncogene Ets1. This is the first study to show that mouse *Ets1*,

583   like the human *ETS1* gene, is targeted by *miR-1*. Our studies showing similar *miR-1*

584   targets between the mouse and human suggest that mouse models of *miR-1* may be

585   useful in elucidating the role of *miR-1* in cancer. As the endogenous expression of *miR-*

586   *1* differs between skin cancer susceptible and cancer resistant mice, it may play a role

587   in the differences in cancer risk observed in these strains, but the exact mechanism

588   remains to be elucidated.

589

590

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596

597

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607

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791

792

793

794 **FIGURE LEGENDS**

795 **Fig. 1.** Validation of candidate miRNA expression patterns in the skin by qPCR. miRNA

796 expression was measured by qPCR. miRNA expression in SPRET/EiJ skin (gray)

797 relative to FVB skin (black) for five miRNAs are shown. Expression was normalized to

798 *sno-202* for the mature miRNAs and to *L19* for the precursor RNAs. Fold expression

799 was calculated relative to FVB/NJ. (A) *miR-1*, (B) *miR-133a*, (C) *miR-124a*, (D) *miR-206*

800 mature, (E) *miR-206* pri, (F) *miR-134* pri (M, mature, pri-precursor. Error bars

801 represent standard deviation. *, p-values for significance are shown for those

802 microRNAs demonstrating p-values <0.05 for differences in miRNA expression between

803 FVB/NJ and SPRET/EiJ.

804

805 **Fig. 2.** Decreased *miR-1* expression in cSCCs. *miR-1* expression was measured in

806 eleven mouse cSCCs and six normal mouse skin RNA samples (3 FVB/NJ and 3

807 SPRET/EiJ) by qPCR relative to control gene *sno-202*. Median expression for each

808 group is indicated by a line. p-value for significance between the tumors and SPRET/EIJ

809 is indicated and was calculated using a nonparametric Mann-Whitney U test.

810

811 **Fig. 3.** Validation of candidate *miR-1* target genes by qPCR. Fold mRNA expression of

812 predicted targets of *miR-1* (A) *Met*, (B) *Ets1*, (C) *Twf1* and (D) *Bag4* were assessed by

813 qPCR. Expression is plotted as a fold difference in expression compared to the 48 hour

814 SC transfected cells. Expression was normalized to control gene *L19*. A5 cells

815 transfected with a scrambled control miR (SC) are shown in black and A5 cells

816 transfected with *miR-1* are shown in gray. Cells were harvested at 48 and 72 hours

817 post-transfection. Difference in expression was measured by student's T-test. Error bars

818 represent standard deviation. *, p-value < 0.01, **, p-value <0.001.

819

820 **Fig. 4.** Evaluation of Ets1 as a *miR-1* target. Western blot analysis of Ets1 expression

821 (A) 72 hours after transient transfection of A5 cells with scrambled control miR (SC) or

822 *miR-1* and (B) 48 hours after transient transfection of B9 cells with (SC) or *miR-1*. α-

823 tubulin (A5) or Gapdh (B9) was used as a loading control. (C) Wild type (Ets1 3'UTR)

824 and mutated (Ets1 3'UTR SDM) Ets1 3'UTR luciferase constructs were co-transfected

825 with scrambled control miR (SC) or *miR-1* into C5N cells. Data is expressed as relative

826 light units of firefly over renilla luciferase. (C) The mouse *Ets1* 3'UTR has three *miR-1*

827 binding sites. Shown are the two base pairs (bold/underlined) in the seed region of

828 each site that were mutated using site-directed mutagenesis. Error bars represent

829 standard deviation. **, p-value < 0.01.

830

831 **Fig. 5.** *miR-1* reduces cell proliferation *in vitro*. An MTT cell proliferation assay was

832 conducted using precursor miR scramble (SC) and *miR-1* transfected in (A) A5 cells

833 and (B) B9 cells. Hours post-transfection are indicated. Relative absorbance was

834 measured at the indicated time points. We observed significantly decreased cell

835 proliferation in the A5 *miR-1* transfected cells at 96 hours post-transfection (*p*-value, <

836 0.0001) and for the B9 *miR-1* transfected cells at 72 hours post-transfection (p-value

837 <0.01). Error bars represent standard deviation. Black diamond, scrambled control miR

838 transfected; gray square, *miR-1* transfected; *, p-value<0.01; ***, p-value < 0.0001.

839

840 **Fig. 6.** Effect of *miR-1* expression on tumor phenotypes. Apoptosis in *miR-1* or

841 scrambled control miR transfected cells (SC) was measured at 48, 72 and 96 hours by

842 FACS analysis in (A) A5 and (B) B9 cells. The percentage of apoptotic cells staining

843 positive for AnnexinV and propidium iodide is indicated in scrambled control (SC)

844 (Black) and *miR-1* transfected cells (gray) for representative experiments is shown. Cell

845 cycle parameters for SC (black) and *miR-1* transfected A5 cells (gray) were measured

846 in A5 cells at (C) 48 hours, (E), 72 hours and (G) 96 hours and in B9 cells at (D) 48

847 hours, (F) 72 hours, and (H) 96 hours by staining with propidium iodide and sorting via

848 flow cytometry. The percentage of gated cells for G0-G1, S and G2-M phases for

849 representative experiments are indicated. There was a statistically significant difference

850 for S-phase for the A5 cells at all three time points. Error bars represent standard

851 deviation. *, p-value <0.05; **, p-value<0.001; NS, not significant.

852

853 **Fig. 7**: Effect of *miR-1* on cell motility. Cell motility for *miR-1* and scrambled control (SC)

854 transfected A5 cells are shown for indicated time points after removal of insert at

855 approximately (A) 48 (B) 72 hours or (C) 96 hours after transfection. Representative

856 experiments for each time point are shown.

857

858 **Supplementary Table S1**

859 Supplemental Table S1 contains miRNA array data for three FVB/NJ, three SPRET/EiJ

860 samples and one replicate RNA sample (FVB-A or –B and SPRET-A or B) per strain.

861

862 **Supplemental Table S2**

863 Supplemental Table S2 lists genes predicted to be targets of the validated five miRNAs.

864

865 **Supplementary Figure Legends**

866 **S1: Candidate *miR-1* target genes in B9.** mRNA expression of predicted targets of

867 *miR-1* (A) *Met*, (B) *Ets1*, (C) *Twf1* and (D) *Bag4* were assessed by qPCR. B9 cells

868 transfected with a scrambled control miR (SC) are shown in black and B9 cells

869 transfected with *miR-1* are shown in gray. Cells were harvested at 48, 72 and 96 hours

870 post-transfection. Expression is plotted as a fold difference in expression compared to

871 the 48 hour SC transfected cells. Expression was normalized to control gene *L19*.

872 Difference in expression was measured by student's T-test. Error bars represent

873 standard deviation. *, p-value < 0.01, **, p-value <0.001, NS, not significant.

874

875 **S2: Ets1 levels following *miR-1* transfection.** (A) Western blot analysis of Ets1

876 expression 24, 48 and 72 hours after transient transfection of A5 with scrambled control

877 miR (SC) or *miR-1*. (B) Western blot analysis of Ets1 expression 24, 48 or 96 hours

878 after transient transfection of B9 with SC or *miR-1*. Gapdh (B9) was used as a loading

879 control.

880 **S3: AnnexinV analysis of apoptosis**. Apoptosis in *miR-1* or scrambled control miR

881 transfected cells (SC) was measured at 48, 72 and 96 hours by AnnexinV and PI

882 staining measured by FACS analysis. Shown is a representative sample at 72 hours

883 post-transfection for the A5 (A) SC and (B) *miR-1* transfected cells and for the B9 (C)

884 SC and (D) *miR-1* transfected cells. The gating of the cells and the percentage of

885 apoptotic cells staining positive for AnnexinV, propridium iodine or both (upper right

886 quadrant) are shown.

887 **S4: FACS analysis of cell cycle.** Cell cycle parameters were measured at 96 hours

888 post-transfection for A5 (A) scrambled control miRNA (SC) and (B) *miR-1* transfected

889 cells and for B9 (C) SC and (D) *miR-1* transfected cells by staining with propidium

890 iodide and sorting via flow cytometry. The percentage of gated cells for G0-G1, S and

891 G2-M phases are indicated.

892

893 **S5: Effects of *miR-1* on cell motility.** Cell motility for *miR-1* and SC transfected B9

894 cells are shown for 0 and 8, 11, and 13 hours after removal of insert at approximately

895 (A) 48 hours (B) 72 hours, (C) or 96 hours after transfection. Representative

896 experiments for each time point are shown.

miRNAs showing statistically significant differential expression by array

[#] Average fold difference across all significant probe sets. Types of probes indicate whether probes for mature (mature), precursor (prec) or both (both) showed expression differences. *Both mouse and human probe sets were included on the array. Species indicates whether probes for mouse and/or human showed expression differences. ^When multiple probes were significant, an average of the p-value for all the probes was calculated.

Table 1: miRNAs showing statistically significant differential expression by array

miRNA	Average fold difference SPRET/FVB (range)	Types of probe	Species*	# Probes	p-value^
miR-1	2.6 (2.52-2.84)	Both	Both	8	1.8×10^{-5}
miR-124a-3	3.3	Mature	Mouse	1	1.9×10^{-7}
miR-133a	2.8 (2.71-2.92)	Both	Both	6	5.2×10^{-6}
miR-134	2.3	Prec	Human	1	2.4×10^{-6}
miR-206	2.6	Prec	Human	1	3×10^{-7}
miR-9-1	2.1	Prec	Mouse	1	1.7×10^{-5}

[#]Average fold difference across all significant probe sets. Types of probes indicate whether probes for mature (mature), precursor (prec) or both (both) showed expression differences. *Both mouse and human probe sets were included on the array. Species indicates whether probes for mouse and/or human showed expression differences. ^When multiple probes were significant, an average of the p-value for all the probes was calculated.

Figure 1

Validation of candidate miRNA expression patterns in the skin by qPCR

miRNA expression was measured by qPCR. miRNA expression in SPRET/EiJ skin (gray) relative to FVB skin (black) for five miRNAs are shown. Expression was normalized to *sno-202* for the mature miRNAs and to *L19* for the precursor RNAs. Fold expression was calculated relative to FVB/NJ. (A) *miR-1*, (B) *miR-133a*, (C) *miR-124a*, (D) *miR-206* mature, (E) *miR-206* pri, (F) *miR-134* pri (M, mature, pri-precursor. Error bars represent standard deviation. *, p-values for significance are shown for those microRNAs demonstrating p-values <0.05 for differences in miRNA expression between FVB/NJ and SPRET/EiJ.

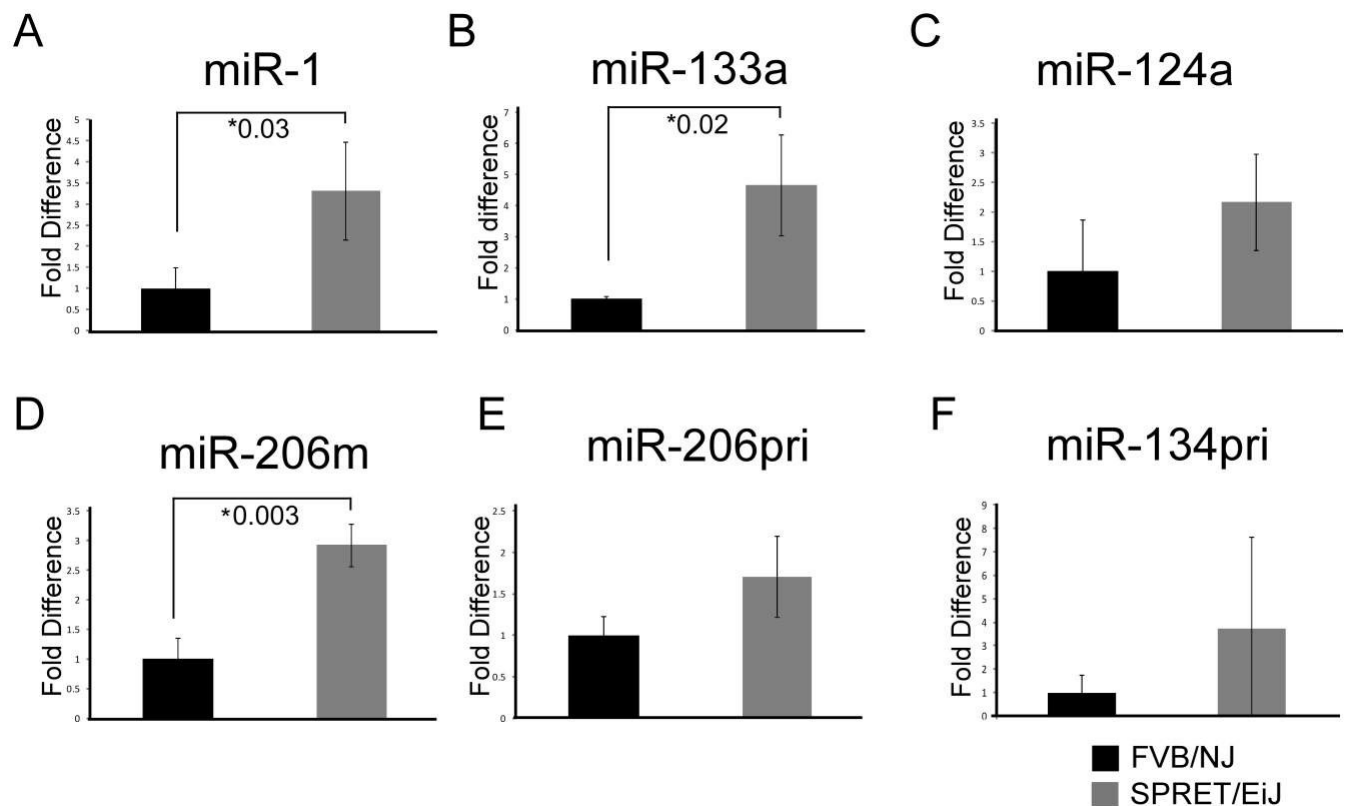



Figure 2

Decreased *miR-1* expression in cSCCs

miR-1 expression was measured in eleven mouse cSCCs and six normal mouse skin RNA samples (3 FVB/NJ and 3 SPRET/EiJ) by qPCR relative to control gene *sno-202*. Median expression for each group is indicated by a line. p-value for significance between the tumors and SPRET/EIJ is indicated and was calculated using a nonparametric Mann-Whitney U test.

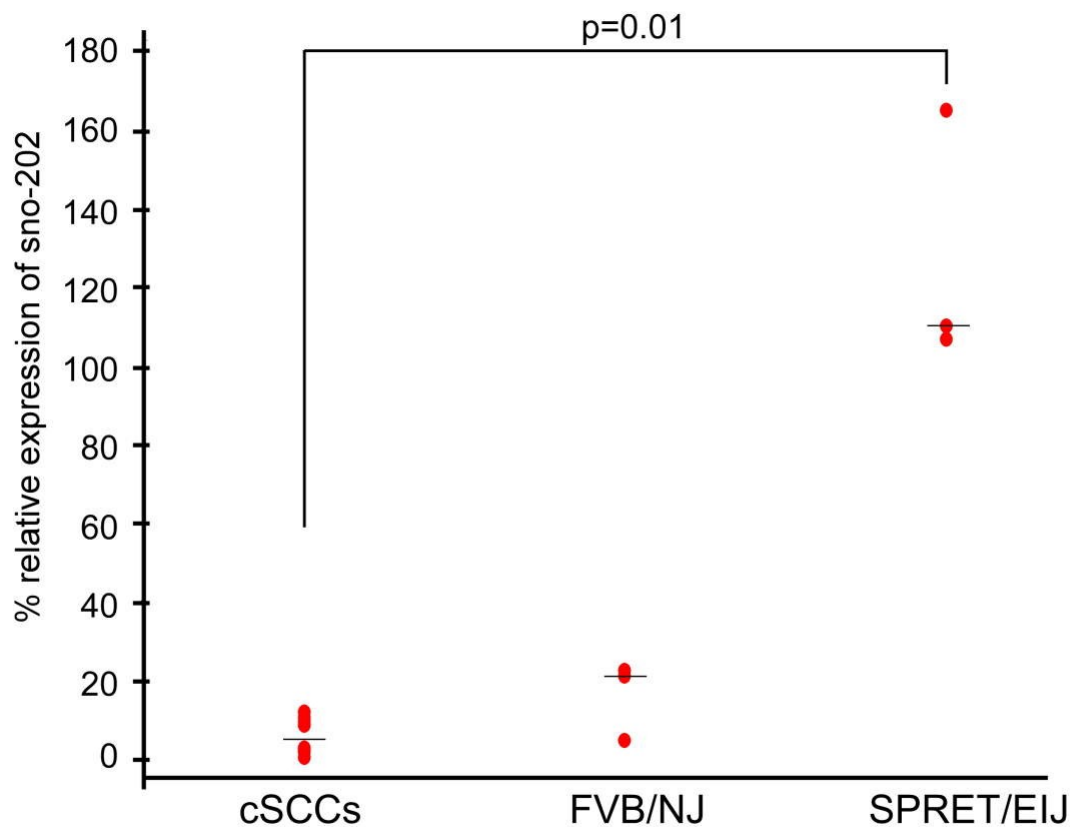



Figure 3

Validation of candidate *miR-1* target genes by qPCR

Fold mRNA expression of predicted targets of *miR-1* (A) *Met*, (B) *Ets1*, (C) *Twf1* and (D) *Bag4* were assessed by qPCR. Expression is plotted as a fold difference in expression compared to the 48 hour SC transfected cells. Expression was normalized to control gene *L19*. A5 cells transfected with a scrambled control miR (SC) are shown in black and A5 cells transfected with *miR-1* are shown in gray. Cells were harvested at 48 and 72 hours post-transfection. Difference in expression was measured by student's T-test. Error bars represent standard deviation. *, p-value < 0.01, **, p-value <0.001.

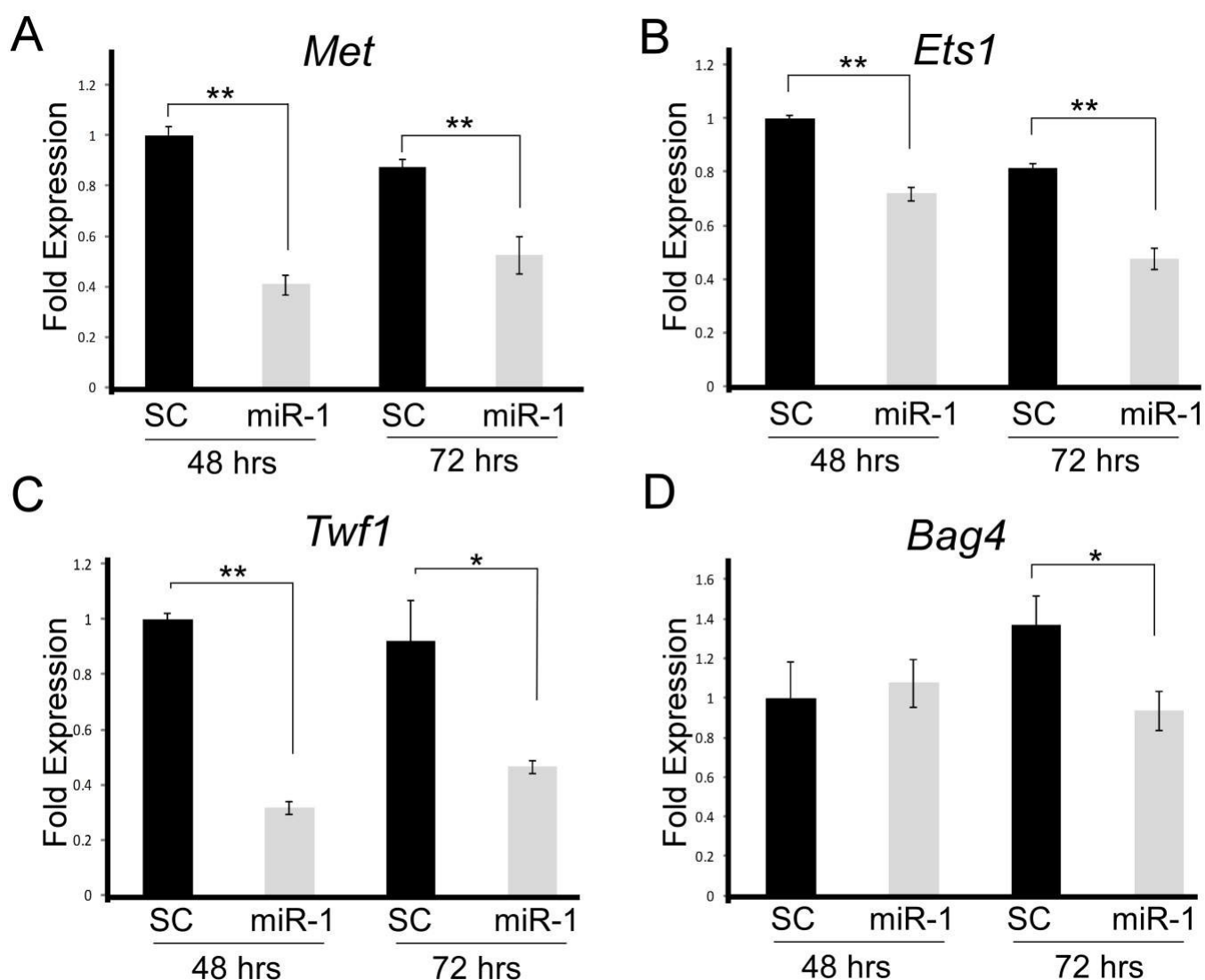



Figure 4

Evaluation of Ets1 as a *miR-1* target

Western blot analysis of Ets1 expression (A) 72 hours after transient transfection of A5 cells with scrambled control miR (SC) or *miR-1* and (B) 48 hours after transient transfection of B9 cells with (SC) or *miR-1*. alpha-tubulin (A5) or Gapdh (B9) was used as a loading control. (C) Wild type (Ets1 3'UTR) and mutated (Ets1 3'UTR SDM) Ets1 3'UTR luciferase constructs were co-transfected with scrambled control miR (SC) or *miR-1* into C5N cells. Data is expressed as relative light units of firefly over renilla luciferase. (C) The mouse *Ets1* 3'UTR has three *miR-1* binding sites. Shown are the two base pairs (bold/underlined) in the seed region of each site that were mutated using site-directed mutagenesis. Error bars represent standard deviation. **, p-value < 0.01.

miR-1 Binding Site	Normal	Mutated
Site 1	uggcugACUCC---CACAUUCCc	uggcugACUCC---CAC**UC**UCCc
Site 2	gaagcaaggccaaACAUUCCa	gaagcaagccaaAC**UC**UCCa
Site 3	uugaacuCUUACGUACAUUCCa	uugaacuCUUACGUAC**UC**UCCa

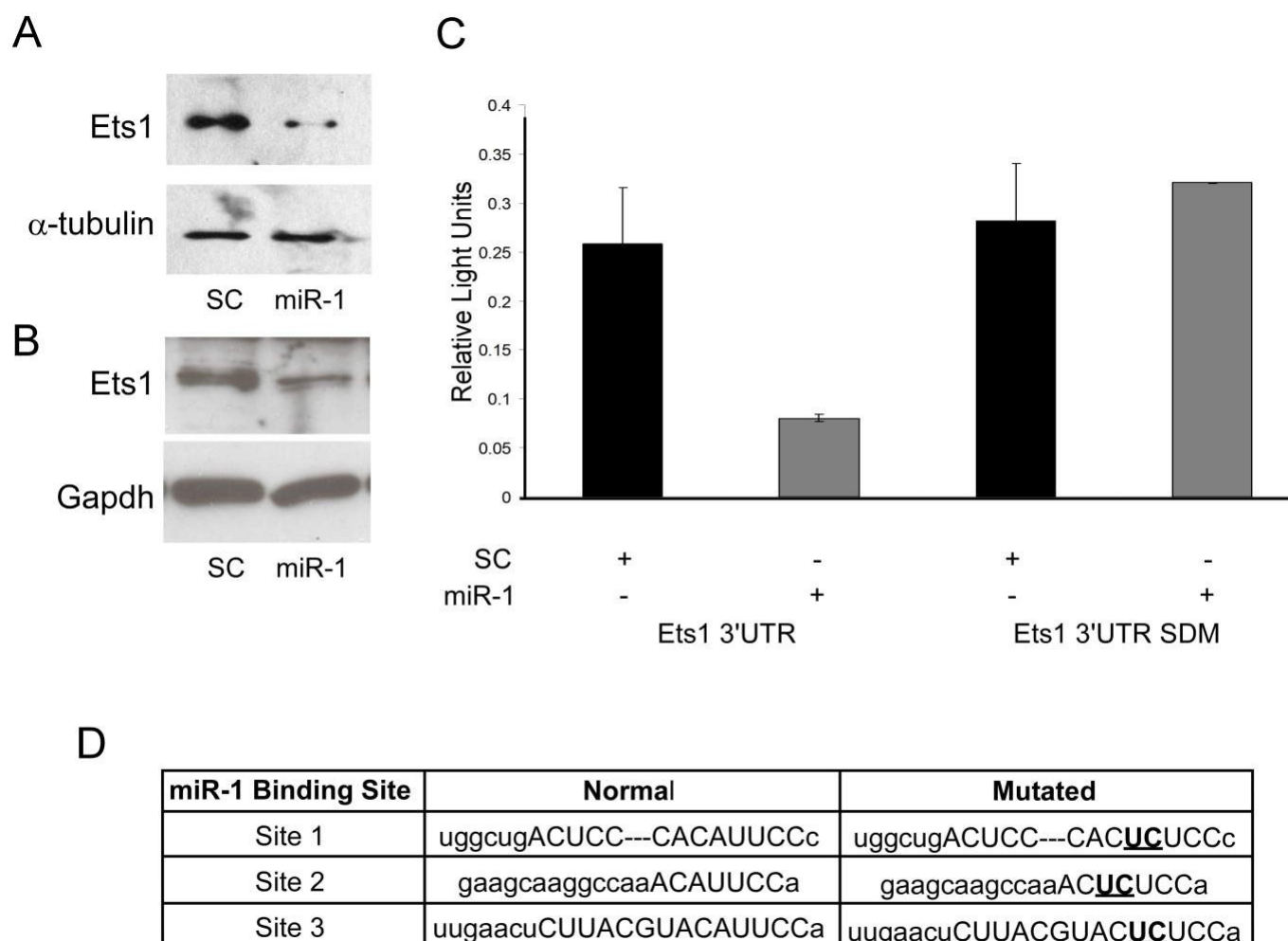



Figure 5

miR-1 reduces cell proliferation *in vitro*

An MTT cell proliferation assay was conducted using precursor miR scramble (SC) and *miR-1* transfected in (A) A5 cells and (B) B9 cells. Hours post-transfection are indicated. Relative absorbance was measured at the indicated time points. We observed significantly decreased cell proliferation in the A5 *miR-1* transfected cells at 96 hours post-transfection (*p*-value, < 0.0001) and for the B9 *miR-1* transfected cells at 72 hours post-transfection (p-value <0.01). Error bars represent standard deviation. Black diamond, scrambled control miR transfected; gray square, *miR-1* transfected; *, p-value<0.01; ***, p-value < 0.0001.

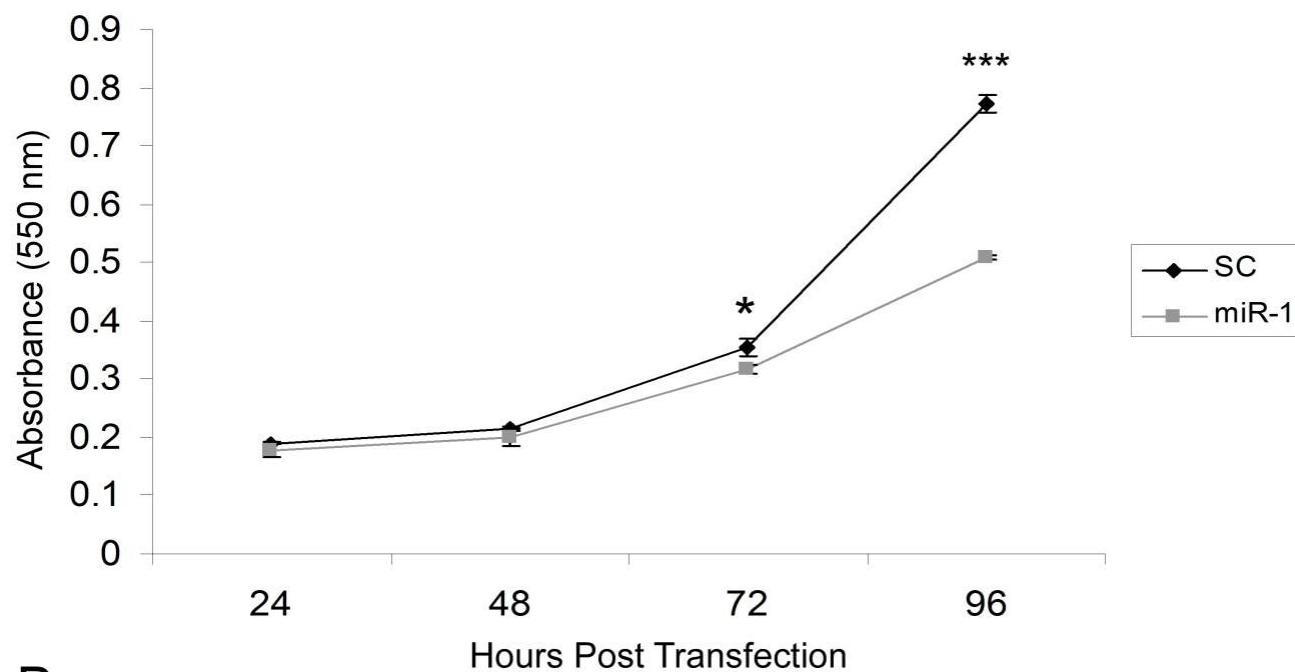
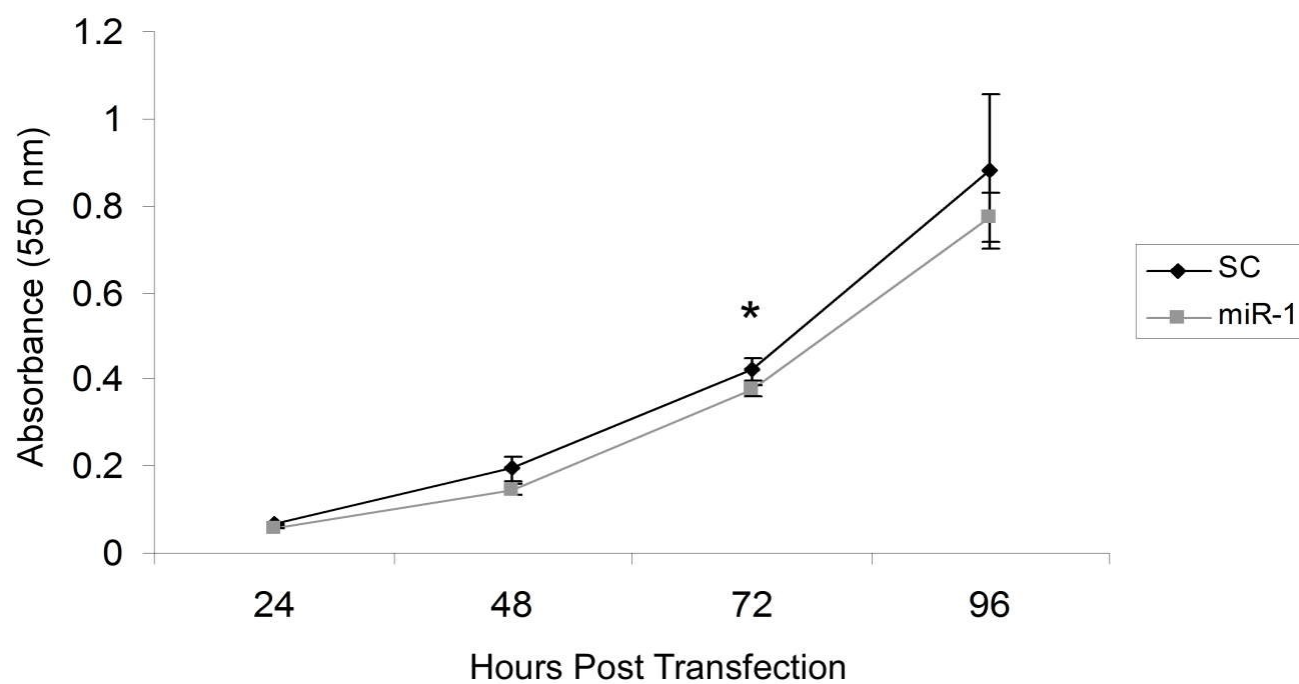



Figure 6

Effect of *miR-1* expression on tumor phenotypes

Apoptosis in *miR-1* or scrambled control miR transfected cells (SC) was measured at 48, 72 and 96 hours by FACS analysis in (A) A5 and (B) B9 cells. The percentage of apoptotic cells staining positive for AnnexinV and propidium iodide is indicated in scrambled control (SC) (Black) and *miR-1* transfected cells (gray) for representative experiments is shown. Cell cycle parameters for SC (black) and *miR-1* transfected A5 cells (gray) were measured in A5 cells at (C) 48 hours, (E), 72 hours and (G) 96 hours and in B9 cells at (D) 48 hours, (F) 72 hours, and (H) 96 hours by staining with propidium iodide and sorting via flow cytometry. The percentage of gated cells for G0-G1, S and G2-M phases for representative experiments are indicated. There was a statistically significant difference for S-phase for the A5 cells at all three time points. Error bars represent standard deviation. *, p-value <0.05; **, p-value<0.001; NS, not significant.

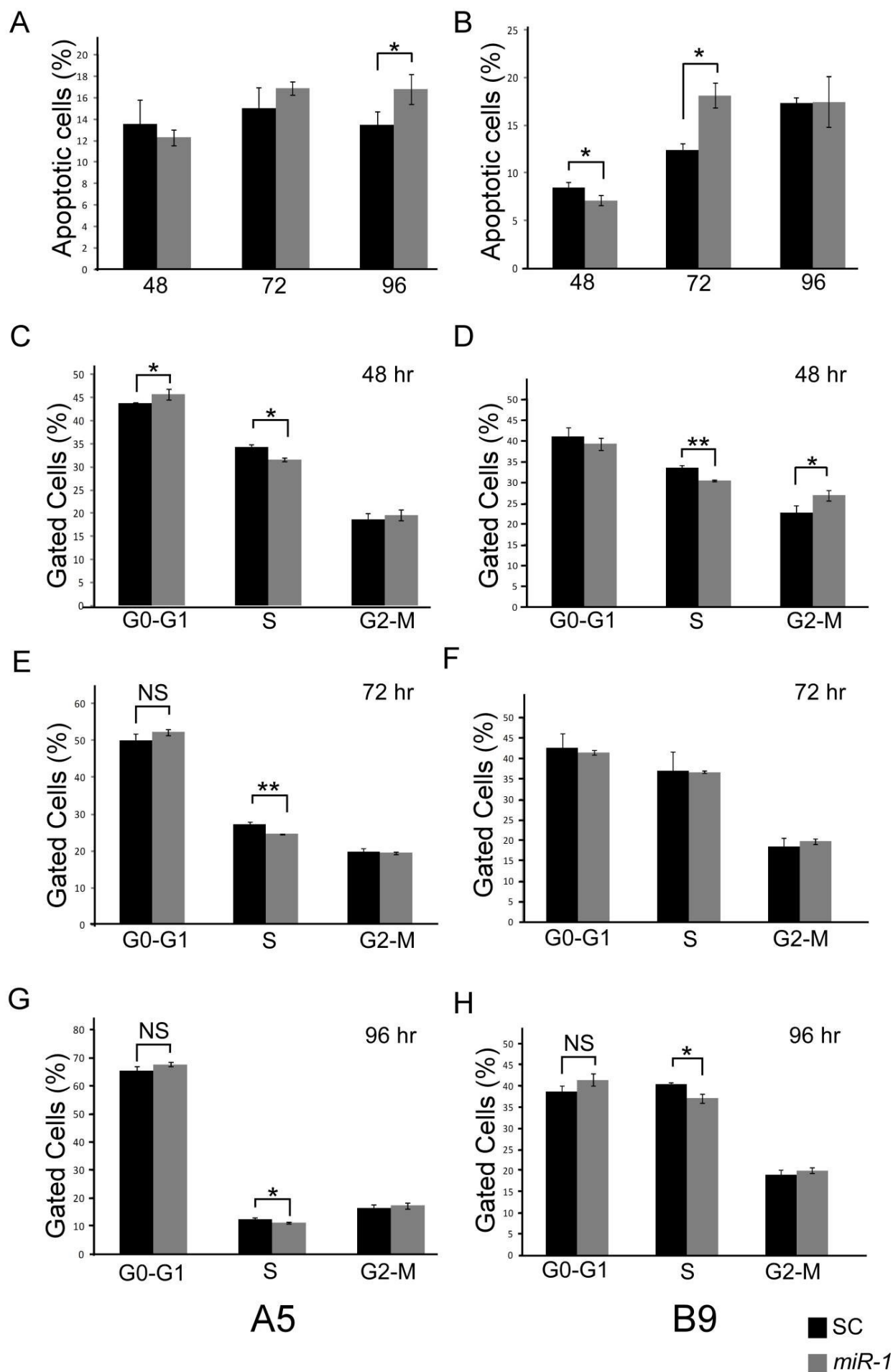
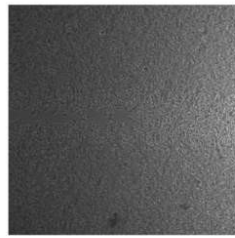
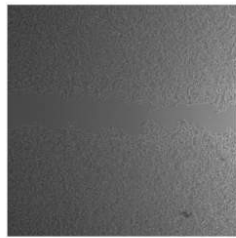
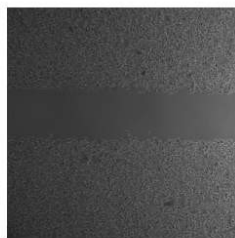
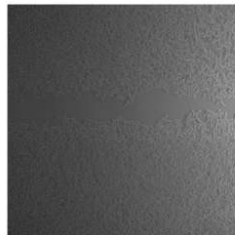
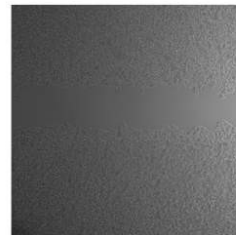
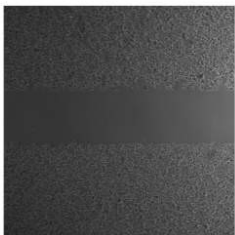



Figure 7

Effect of *miR-1* on cell motility

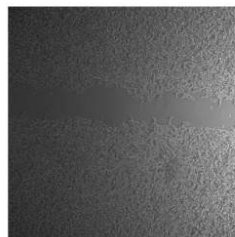
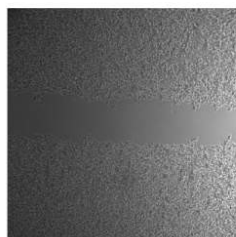
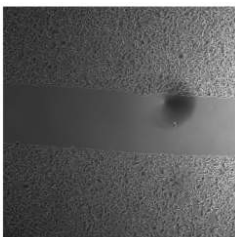
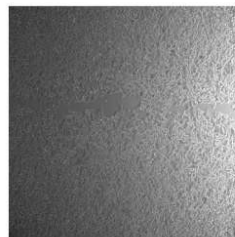
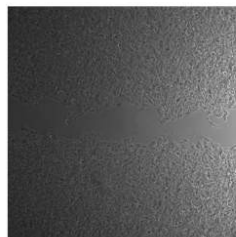
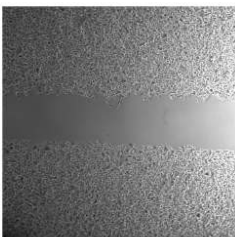
Cell motility for *miR-1* and scrambled control (SC) transfected A5 cells are shown for indicated time points after removal of insert at approximately (A) 48 (B) 72 hours or (C) 96 hours after transfection. Representative experiments for each time point are shown.

A **48 hours post-transfection**

SC

miR-1

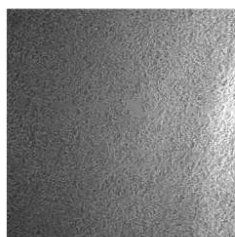
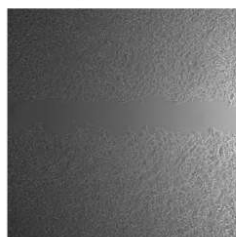
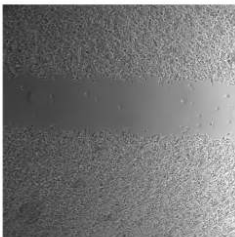
0 hours   13 hours   22 hours

B **72 hours post-transfection**

SC

miR-1

0 hours   13 hours   20 hours

C **96 hours post-transfection**

SC

miR-1

0 hours   11 hours   25 hours