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microRNA-192 suppresses the expression of the farnesoid X receptor

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Abstract: Farnesoid X receptor (FXR, NR1H4) plays an important role in the regulation of bile acid homeostasis in liver and intestine and may exert protective effects against certain forms of cancer such as colon carcinoma. However, the role of FXR in cell growth regulation, apoptosis, and carcinogenesis is still controversial. Similar to FXR, microRNA-192 (miR-192) is mainly expressed in the liver and colon and plays an important role in the pathogenesis of colon carcinoma. In this study, we investigated the extent to which FXR is regulated by miR-192. Two in silico-predicted binding sites for miR-192-3p within the NR1H4-3' untranslated region (UTR) were examined in vitro by luciferase reporter assays. Wild-type and mutated forms of the NR1H4-3'UTR were subcloned into a pmirGLO vector and cotransfected into Huh-7 cells with miR-192-3p. To study the effects of miR-192 on the expression of FXR, FXR target genes and cell proliferation, Huh-7 and Caco-2 cells were transfected with miR-192-5p and -3p mimics or antagomirs. In addition, the correlation between FXR and miR-192 expression was studied by linear regression analyses in colonic adenocarcinoma tissue from 27 patients. MiR-192-3p bound specifically to the NR1H4-3'UTR and significantly decreased luciferase activity. Transfection with miR-192 led to significant decreases in NR1H4 mRNA and protein levels as well as the mRNA levels of the FXR-inducible bile acid transporters OST-OST and OATP1B3. Significant inverse correlations were detected in colonic adenocarcinoma between NR1H4 mRNA and miR-192-3p expression. In summary, microRNA-192 suppresses the expression of FXR and FXR target genes in vitro and in vivo.

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microRNA-192 suppresses the expression of the farnesoid X receptor

Abbreviated title: miR-192 suppresses the expression of FXR

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Contributorship

Participated in research design: Krattinger, Mwinyi, Kullak-Ublick; Conducted experiments: Krattinger; Data acquisition and analysis: Krattinger, Boström, Thasler; Wrote or contributed to the writing of the manuscript: Krattinger, Mwinyi, Kullak-Ublick, Schiöth.

1 **ABSTRACT**

2 Farnesoid X receptor (FXR, *NR1H4*) plays an important role in the regulation of bile acid
3 homeostasis in liver and intestine, and may exert protective effects against certain forms of
4 cancer such as colon carcinoma. However, the role of FXR in cell growth regulation,
5 apoptosis and carcinogenesis is still controversial. Similar to FXR, microRNA-192 (miR-192)
6 is mainly expressed in the liver and colon, and plays an important role in the pathogenesis of
7 colon carcinoma. In this study, we investigated the extent to which FXR is regulated by miR-
8 192. Two *in silico*-predicted binding sites for miR-192-3p within the *NR1H4*-3' untranslated
9 region (UTR) were examined *in vitro* by luciferase reporter assays. Wild-type and mutated
10 forms of the *NR1H4*-3'UTR were subcloned into a pmirGlo vector and co-transfected into
11 Huh-7 cells with miR-192-3p. To study the effects of miR-192 on the expression of FXR,
12 FXR target genes and cell proliferation, Huh-7 and Caco-2 cells were transfected with miR-
13 192-5p and -3p mimics or antagomirs. In addition, the correlation between FXR and miR-192
14 expression was studied by linear regression analyses in colonic adenocarcinoma tissue from
15 27 patients. MiR-192-3p bound specifically to the *NR1H4*-3'UTR and significantly decreased
16 luciferase activity. Transfection with miR-192 led to significant decreases in *NR1H4* mRNA
17 and protein levels as well as the mRNA levels of the FXR-inducible bile acid transporters
18 OST α -OST β and OATP1B3. Significant inverse correlations were detected in colonic
19 adenocarcinoma between *NR1H4* mRNA and miR-192-3p expression. In summary,
20 microRNA-192 suppresses the expression of FXR and FXR target genes *in vitro* and *in vivo*.

21

22 **KEYWORDS**

23 miR-192 - farnesoid X receptor – bile-acid transporters - drug-induced liver injury – colonic
24 adenocarcinoma

25 INTRODUCTION

26 Nuclear farnesoid X receptor (FXR, *NRIH4*) is a ligand-activated transcription factor that
27 plays a crucial role in the regulation of bile acid, cholesterol, lipid and glucose homeostasis. It
28 is mainly expressed in the liver, intestines, kidney, and adrenal glands [34]. FXR regulates
29 key genes involved in human bile acid synthesis and metabolism, including bile acid
30 transporters [4]. Studies on FXR knockout mice have shown that FXR exerts hepatoprotective
31 effects. Diminished FXR expression has been linked to an increase in inflammatory responses
32 and neoplastic transformation in mice [7,16]. Mice lacking FXR expression show elevations
33 in serum and hepatic bile acid levels and a higher incidence of hepato(cholangio)cellular
34 carcinoma [12,33]. In mouse intestine, loss of FXR and subsequent elevations of intestinal
35 bile acid concentrations lead to earlier mortality caused by increased tumor progression via
36 promotion of Wnt signaling. FXR may play a key role in the intestinal defense against
37 potentially toxic bile acids by regulating their transport, detoxification, and neosynthesis
38 [8,23]. Decreased FXR expression levels in human colon cancer tissue compared with non-
39 neoplastic tissue are associated with adverse clinical outcome [15]. In contrast, strongly
40 enhanced FXR expression, leading to altered expression of FXR-regulated drug uptake
41 transporters, confers chemoresistance in cancer patients [22]. The role of FXR in cell growth
42 regulation, apoptosis and carcinogenesis is controversially discussed in the literature. An
43 immunohistochemical study, for example, showed preserved or enhanced FXR protein
44 expression in tumor cell nuclei of human hepatocellular carcinoma tissue compared with
45 hepatocyte nuclei of normal and diseased liver [14].

46 MicroRNAs (miRs) are short non-coding RNA molecules of 18–25 nucleotides in length,
47 which repress specific target mRNAs by degradation or translational repression [2]. Two
48 important liver-specific miRNAs are miR-122, which is estimated to comprise 70% of the
49 total hepatic miR pool in adults, and miR-192 [28]. The gene encoding hsa-miR-192 is

50 located on chromosome 11. In addition to expression in the liver, miR-192 is found in the
51 kidneys and gastrointestinal tract [20]. Elevated serum levels of miR-192 have been detected
52 in various liver-associated diseases, including drug-induced liver injury, non-alcoholic
53 steatohepatitis, cholangiocarcinoma, and hepatitis-B-related hepatocellular carcinoma, and
54 may serve as a biomarker [24,26,28,31,37]. Thus, several studies have hypothesized that
55 tissue-specific chronic inflammation may trigger increases in miR-192 expression, as in non-
56 alcoholic steatohepatitis [21,24,26]. Because miR-192 shows an inverse correlation with the
57 metastatic potential of colon cancer cells, miR-192 has been suggested to be a predictive
58 biomarker for the risk of developing liver metastasis in colon carcinoma. *In vivo* studies in
59 mice suggest that miR-192 targets B-cell lymphoma 2 (*BCL2*), zinc-finger E-box-binding
60 homeobox 2 (*ZEB2*), and vascular endothelial growth factor A (*VEGFA*), all of which are
61 important anti-apoptotic and angiogenic regulators [9]. The tumor suppressor protein p53 can
62 act as a transcription factor within the miR-192 promoter, whereas miR-192 itself appears to
63 suppress carcinogenesis by promoting p21 accumulation [3,27]. Loss of p53 functions by
64 mutation and consequently decreased expression of miR-192 has been suggested to be a key
65 step in colon carcinogenesis [3,11,25,29]. Although, miR-192 is not considered to be a
66 typically dysregulated microRNA in several studies on hepatocellular carcinoma (HCC), Lian
67 et al. showed a significantly suppressed expression of miR-192 in HCC tissue as compared to
68 non-tumorous tissue [19]. An important role of miR-192 targeting *ZEB2* mRNA has also been
69 found in HCC [13]. In contrast, certain forms of cancer, such as cholangiocarcinoma and
70 esophageal cancer, show increased miR-192 expression during carcinogenesis [21,26].

71 In this study, we investigated to what extent miR-192 modulates the expression of FXR and
72 thereby affects the expression of FXR target genes in liver and colon cancer-derived cell lines.

73

74

75 **MATERIAL AND METHODS**

76 **Bioinformatics.** An *in silico* search for possible miRNA-binding sites in the 3' untranslated
77 region (UTR) of the *NR1H4* gene was performed using miRANDA (Memorial Sloan-
78 Kettering Cancer Center, New York, NY, USA), DIANA-microT-CDS (Biomedical Science
79 Research Center Alexander Fleming, Athens, Greece), and miRBase (Faculty of Life Science,
80 University of Manchester, Manchester, UK). mRNA and miR expression data from 27 colonic
81 adenocarcinoma tissue samples (E-GEOD-29623, Affymetrix GeneChip Humane Genome
82 U133 Plus 2.0 and NIH Taqman microRNA Array v.2 [5]) were retrieved from the openly
83 accessible platform ArrayExpress (EMBL-European Bioinformatics Institute, Wellcome Trust
84 Genome Campus, Hinxton, UK).

85

86 **Cell culture.** The human hepatoma derived Huh-7 and colon carcinoma derived Caco-2 cell
87 lines (American Type Culture Collection, Molsheim, France) were cultured in RPMI-1640
88 and Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, CA, USA),
89 respectively, supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO,
90 USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA).
91 Primary human hepatocytes (PHHs) obtained from three patients suffering from primary or
92 secondary liver carcinoma (ethical approval by the Local Ethical committee of the University
93 of Munich, Germany, and the Ethics Committee of the Canton of Zurich, Switzerland) were
94 isolated from the cancer-adjacent normal tissue and cultured as described [17]. PHHs were
95 kept in maintenance medium including ultraglutamine for 5 h before further procedures. Cell
96 cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂.

97

98 **Transient transfection with miR-192.** To investigate the effect of miR-192 on *NR1H4*
99 mRNA and protein expression, Huh-7 and Caco-2 cells were seeded in 12-well plates (8×10^4

100 and 4×10^5 cells/ml, respectively). After 24 h, cells were transfected with 100 nM hsa-
101 mirVANA[®] miRNA mimics or Anti-miR[™] miRNA inhibitors (hsa-miR-192-3p/-5p and
102 corresponding negative controls, Life Technologies) using Lipofectamine[®] RNAiMax diluted
103 in Opti-MEM I (both purchased from Invitrogen) at a final concentration of 3 mM. Hsa-miR-
104 1 and its known suppressive effect on twinfilin-1 (TWF-1) expression were used as a positive
105 control. After 4 h of incubation, the transfection medium was replaced with fresh complete
106 growth medium. At 24, 48 and 72 h after transfection, total mRNA and protein were isolated
107 using Trizol[®] reagent (Life Technologies) and RIPA buffer [50 mM Tris HCl pH 8, 150 mM
108 NaCl, 1% NP-40, 0.5% sodium deoxycholate (all from Sigma-Aldrich), 1 mM EDTA
109 (Pharmacia Biotech, Uppsala, Sweden), 0.1% SDS, and 10% glycerol (both from Sigma-
110 Aldrich)], respectively. FXR mRNA and protein were quantified in three independently
111 performed experiments.

112 To examine miR-192-dependent expression of FXR target genes, Huh-7 cells were treated
113 with 50 μ M chenodeoxycholic acid (CDCA) (Sigma-Aldrich) for 24 h. Data obtained were
114 merged from four experiments, and the three most representative values were averaged. To
115 prove that miR-192 mimics control the expression of FXR target genes through FXR
116 regulation, 100 nM FXR small interfering RNA (siRNA) or corresponding negative control
117 (Life Technologies) were transfected simultaneously with the miRNA mimics for 48 h.

118

119 **Reverse transcription and quantitative real-time PCR.** Extracted RNA (1.5 μ g) was
120 transcribed into cDNA by a High Capacity cDNA Reverse Transcription Kit (Applied
121 Biosystems, Waltham, MA, USA) according to the manufacturer's protocol. A total of 2 μ l of
122 1:5 diluted cDNA was mixed with 8 μ l RT-PCR Universal Fast Master Mix (Applied
123 Biosystems) including specific primers (TaqMan[®] Gene Expression Assays *NR1H4*,
124 *SLCO1B3*, *SLC51A*, and *SLC51B*; Life Technologies) and subjected to real-time PCR. β -actin

125 was used as an internal control. To measure miR expression, 10 ng of extracted RNA was
126 reverse transcribed into cDNA using a TaqMan[®] miRNA Reverse Transcription Kit (Applied
127 Biosystems) and specific stem-loop reverse transcription primers (TaqMan[®] MicroRNA
128 Assays hsa-miR-192-3p and hsa-miR-192-5p; Life Technologies). RT-PCR was performed
129 using 0.67 μ l cDNA and 9.3 μ l RT-PCR Universal Fast Master Mix (Applied Biosystems)
130 including primers. U6snRNA was used as an internal control. Mean miR expression in three
131 passages of cells were analyzed per cell line. All measurements were performed in triplicate.

132

133 **Western blot analysis.** Protein samples (17–30 μ g) were diluted 1:5 with loading buffer,
134 denatured, and separated by 8% SDS-polyacrylamide gel electrophoresis. The separated
135 proteins were transferred to polyvinylidene fluoride membranes and preincubated for 1 h in
136 blocking buffer and then for 16 h with the primary antibodies (anti-FXR, Santa Cruz
137 Biotechnology, Dallas, TX, USA; anti- β -actin, Abcam, Cambridge, UK) at 4°C. After four
138 washing steps, the membranes were incubated with a horseradish peroxidase-conjugated
139 secondary antibody (goat-anti-rabbit, Thermo Scientific, Waltham, MA, USA) for 1 h at room
140 temperature. Protein bands were visualized using SuperSignal[®] West Femto Maximum
141 Sensitivity Substrate (Thermo Scientific) and Fusion FX7 (Vilber Lourmat, Eberhardzell,
142 Germany). β -actin was used as a housekeeping gene.

143

144 **Transient cotransfection with miR-192-3p (miR-192*) and *NR1H4* 3'UTR plasmid**
145 **constructs, and luciferase reporter assays.** To examine the *NR1H4* 3'UTR as a target of
146 miR-192-3p *in vitro*, luciferase assays were performed using a pmiRGLO Dual-Luciferase
147 miRNA Target Expression Vector (Promega, Fitchburg, WI, USA), containing both the
148 coding sequences of firefly luciferase and Renilla luciferase (internal control). The *NR1H4*
149 3'UTR (accession number: NG_029843.1) was cloned into the pmiRGLO vector system using
150 specific primers (Table 1). Plasmids were verified by Sanger sequencing. Huh-7 cells were

151 seeded in 48-well plates (1.6×10^5 cells/ml). After 24 h, the cells were transfected with 50 nM
152 hsa-mirVANA[®] miRNA mimics or a negative control (hsa-miR-192-3p, Negative Control #1;
153 Life Technologies) and 100 ng/well of plasmid DNA using Lipofectamine[®] 2000
154 (Invitrogen). The activities of firefly and Renilla luciferases were measured at 24 h after
155 cotransfection using the Dual-Luciferase[®] Reporter 1000 Assay System (Promega) according
156 to the manufacturer's protocol. Hsa-miR-21-5p and its effect on the miR-21 target sequence
157 were used as a positive control. Analysis was performed using the GloMax[®] Multi Detection
158 System (Promega). An empty PmiRGLO vector was used as a background control.
159 QuikChange Multi Site-Directed Mutagenesis and QuikChange II XL Site-Directed
160 Mutagenesis Kits (Agilent Technologies, Santa Clara, CA, USA) were used to introduce
161 mutations into the miR-192-3p-binding sites using specific primers (Table 1). Three
162 independent luciferase reporter assays were performed including wild-type and mutated target
163 sequences.

164

165 **Cell proliferation and invasion assays.** Cell proliferation was determined by the
166 alamarBlue[®] (ThermoScientific) cell viability reagent according to the manufacturer's
167 instructions. Briefly, Huh-7 and Caco-2 cells were seeded in 96-well plates (7.5×10^4 and
168 2×10^5 cells/ml, respectively). After 24 h, cells were transfected with 100 nM miR-192
169 mimics, FXR siRNA or the corresponding negative controls. After 48, 72 and 96 h 10 μ l of
170 Alamar Blue was added to each well and cells were incubated for 4 h at 37°C. The absorbance
171 was determined at 560/600nm using GloMax[®] Multi Detection System.

172 Cell invasion was determined with extracellular matrix-coated invasion chambers (QCMTM
173 24-well cell invasion assay, Millipore, Billerica, MA, USA) according to the manufacturer's
174 instructions. Huh-7 and Caco-2 cells were harvested and resuspended in serum-free medium
175 after pretreatment for 48 h with miR-192 mimics or negative control (cell density for

176 transfection: 1×10^5 and 4×10^5 cells/ml, respectively). Then, 1×10^5 cells were plated into the
177 invasion chamber, whereas the bottom well of the chamber contained 500 μ l of the
178 corresponding medium supplemented with 10% FBS. After 48 h of incubation, the invaded
179 cells on the underside of the membrane were detached, lysed and stained with CyQuant[®] GR
180 Dye (Millipore). Fluorescence was measured using a 490/510-570 filter set and GloMax[®]
181 Multi Detection System.

182

183 **Statistical analysis.** Paired one-sample t-tests were performed to compare the effects of miR-
184 192 mimics, antagomirs, FXR siRNA and the corresponding negative controls on FXR
185 mRNA/protein expression levels and on cell proliferation/invasion. Luciferase activities were
186 compared between miR-192-3p- and mock-transfected cells by one-way analysis of variance.
187 All data obtained from transfection experiments were compared with negative control (NC)
188 mimics or inhibitors, in which expression levels in mock-transfected cells were defined as 1
189 (except for cell proliferation assays, where values were normalized to the 48h time point of a
190 particular condition). In general, the negative controls were not expected to bind to the
191 *NR1H4* 3'UTR or to block the activity of endogenously expressed miR-192. Only experiments
192 with verified positive controls were included in statistical analyses. The association between
193 FXR and miR-192 expression levels was investigated in 27 colonic adenocarcinoma tissue
194 samples by linear regression analyses excluding subjects that had undergone chemotherapy
195 (E-GEOD-29623 [5]). Values are shown as averages \pm standard deviation. A p value of less
196 than 0.05 was considered to be statistically significant. Statistical analyses were performed
197 using R-Software (version 2.15.2) and Graph Pad Prism (version 5.04).

198

199

200 **RESULTS**

201 **The mature forms of pre-miR-192 derived from the 5' and 3' strands of the precursor**
202 **are detectable in Huh-7 and Caco-2 cells.** The online database miRBase lists two mature
203 sequences for the human miRNA precursor pre-miR-192, miR-192-5p, and miR-192-3p.
204 Quantitative measurement of the endogenous expression levels showed the presence of both
205 strands at considerable amounts in Huh-7, PHH and Caco-2 cells. MiR-#-3p was expressed
206 10-fold less in Huh-7 cells, 32-fold less in Caco-2 cells, and 39-fold less in PHH cells
207 compared with miR-#-5p. Furthermore, miR-192-3p showed a 9 to 10-fold higher expression
208 in Caco-2 cells than in the hepatoma cell line or the primary human hepatocytes (data not
209 shown).

210

211 **Confirmation of the *NR1H4* 3'UTR as a target of miR-192-3p by luciferase reporter**
212 **assays.** The software tools miRANDA and DIANA-microT-CDS predicted binding of miR-
213 192-3p to *NR1H4* transcript positions 199–227 and 324–352 (position relative to translational
214 stop codon, NG_029843.1, Fig. 1A). No binding site within the *NR1H4* 3'UTR was predicted
215 for miR-192-5p. Cotransfection of miR-192-3p mimics and the *NR1H4* 3'UTR target
216 sequence into Huh-7 cells resulted in a 30% decrease in luciferase activity compared with the
217 empty vector control ($p < 0.01$). In contrast, luciferase activity remained unaffected by
218 cotransfection of the *NR1H4* 3'UTR target sequence carrying the miR-192 binding sites in the
219 mutated form compared with the wild-type construct (Fig. 1B), indicating a negative
220 interaction of miR-192-3p with the predicted binding sites in the *NR1H4* 3'UTR.

221

222 **MiR-192 attenuates endogenous *NR1H4* mRNA levels in Huh-7 and Caco-2 cells.** To
223 investigate the effect of miR-192 on endogenous FXR expression levels, Caco-2 and Huh-7
224 cells were transfected with 100 nM miR-192-3p or -5p mimics for 24 or 48 h. As shown in
225 Fig. 2A, *NR1H4* mRNA levels were repressed to 75% and 65% by miR-192-3p and -5p
226 mimics, respectively, in Huh-7 cells ($p<0.05$). In Caco-2 cells, a decrease by 15% and 28% in
227 *NR1H4* mRNA expression was detected after transfection with miR-192-3p and -5p mimics
228 for 24 h ($p<0.05$). To examine whether transfection of an antagomir could reverse the
229 endogenous miR-192-dependent inhibitory effect on FXR expression, Caco-2 and Huh-7 cells
230 were transfected with 100 nM anti-miR-192 inhibitors for 24 or 48 h, respectively. As shown
231 in Fig. 2B, *NR1H4* mRNA levels were increased significantly by anti-miR-192-3p inhibitors
232 in Caco-2 cells as well as anti-miR-192-5p inhibitors in Huh-7 cells compared with the anti-
233 miR miRNA inhibitor negative control ($p<0.05$). Thus, the miR-192-dependent effect on FXR
234 regulation appeared to be stronger in Huh-7 cells compared with Caco-2 cells.

235

236 **MiR-192 suppresses FXR protein translation in Huh-7 and Caco-2 cells.** Consistent with
237 the miRNA-dependent effects on *NR1H4* mRNA expression, a 63% decrease in FXR protein
238 expression was seen after transfection with miR-192-3p mimic in Huh-7 cells ($p<0.05$).
239 Weaker downregulation by 14% was observed after transfection of miR-192-5p. A decrease
240 by 14% and 43% in FXR protein expression was observed in Caco-2 cells after transfection
241 with miR-192-3p and -5p, respectively (Fig. 3A). As shown in Fig. 3B, the endogenous miR-
242 192-dependent suppressive effect on FXR protein expression was reversed upon transfection
243 with anti-miR-192-3p or -5p inhibitors. In Huh-7 cells, an increase in FXR protein levels was
244 not seen following transfection of the miR-192-3p antagomir, which could be explained by
245 the lower expression of the 3' in relation to the 5' strand in Huh-7 compared to Caco-2 cells.

246 **MiR-192 suppresses the expression of key FXR target genes.** The bile acid transporters
247 organic-anion transporting polypeptide 1B3 (OATP1B3, *SLCO1B3*) and organic solute
248 transporters α/β (OST alpha/beta, *SLC51A/B*) were chosen as model genes to examine the
249 effect of miR-192 on FXR-regulated expression of transport proteins. Huh-7 cells were
250 transfected with 100 nM miR-192-3p or -5p for 48 h. 24 h after transfection, cells were
251 treated with 50 μ M CDCA for 24 h to activate FXR and compared with the miRNA mimic
252 negative control. As shown in Fig. 4, *SLCO1B3*, *SLC51A* and *SLC51B* mRNA levels were
253 decreased by either one or both strands of miR-192, suggesting a reduction of gene
254 transcription secondary to reduced expression of the transcriptional activator FXR. This was
255 confirmed by simultaneous FXR knockdown and miRNA transfection experiment (data not
256 shown).

257

258 **Linear regression analyses reveal an inverse association between the expression levels of**
259 **miR-192 and FXR in colonic adenocarcinoma.** To investigate whether the *in vitro* effects of
260 miR-192 on FXR expression were reproducible *in vivo*, we analyzed 27 tissue samples from
261 chemotherapy-untreated patients with primary colonic adenocarcinoma (Table 2). As shown
262 in Table 3 and Fig. 5, a significant inverse association in expression was observed for the
263 *NR1H4* mRNA transcript coding for FXR α 2(+) and hsa-miR-192-3p. FXR α 2 isomers
264 represent the most abundantly expressed FXR protein forms in colonic tissue, whereas the
265 isomer FXR α 1 is predominantly expressed in liver [30]. No significant associations were
266 found for *NR1H4* mRNA and hsa-miR-192-5p expression, showing that the 5' strand does not
267 confer pronounced *NR1H4* mRNA degradation *in vivo*. These findings support the regulatory
268 effects of miR-192 on FXR expression in hepatoma and colon cancer-derived cell lines
269 observed *in vitro*.

270 **MiR-192 exhibits suppressive effects on proliferation of Huh-7 and Caco-2 cells.** To
271 investigate the functional significance of the observed miR-192/FXR interaction, we
272 performed cell proliferation assays using Alamar Blue. As shown in Fig. 6, transfection with
273 miR-192-3p mimic significantly reduces proliferation of Huh-7 and Caco-2 cells, whereby a
274 stronger effect could be observed in the hepatoma cell line. These findings confirm the
275 previously described suppressive effects of miR-192 on proliferation of the colon cancer cell
276 lines HT-29, RKO and HCT116 [27]. Knockdown of FXR expression causes similar anti-
277 proliferative effects in Huh-7 cells. In contrast, reduction in FXR expression does not seem to
278 have any influence on the proliferative potential of Caco-2 cells. By performing cell invasion
279 assays, we saw a trend towards a suppressive effect for both strands of the miR-192 precursor
280 molecule on cell invasion of Huh-7 cells, however the results were not significant (data not
281 shown). In a previous work, Lian et al. showed that miR-192 could significantly downregulate
282 cell invasion of Huh-7 cells [19].

283

284

285 **DISCUSSION**

286 The aim of this study was to investigate whether FXR is a target of miR-dependent, post-
287 transcriptional gene regulation. Regulation of FXR expression by miR-421 as an oncogenic
288 miR in biliary tract cancer has already been postulated [36]. Our results show an additional, as
289 yet uncharacterized role of miR-192 in regulating FXR expression.

290 Our *in vitro* experiments elucidated the inhibitory effect of miR-192 on FXR expression by
291 transfecting colon and liver cell lines with miR-192 mimics and inhibitors. In the case of the
292 3' strand, we found a significant miR-192-dependent inhibitory effect on *NR1H4*/FXR mRNA
293 and protein expression in Huh-7 cells, whereas in Caco-2 cells only transfection with the anti-
294 miR-192-3p inhibitor showed a relevant effect. A possible explanation for the observed
295 weaker inhibitory effect of the miR-192-3p mimic in Caco-2 cells, compared with that in
296 Huh-7 cells, may be the more abundant expression of miR-192 in the colon cell line. The
297 observed miR-192*-dependent effects on *NR1H4* mRNA levels in both cell lines and our *in*
298 *vivo* findings that showed a strong inverse miR-192* and FXR α 2(+) correlation in colonic
299 adenocarcinoma support our hypothesis of a strong endogenous effect of miR-192 on FXR
300 expression. Degradation of *NR1H4* mRNA transcripts can be explained by perfect
301 complementary interference of the miR-192-3p seed sequence at the two *in silico*-predicted
302 binding positions of the *NR1H4*-3'UTR. The 5' strand of miR-192 also appeared to repress
303 FXR gene expression *in vitro*, albeit through a different mechanism. Transfections with the
304 corresponding antagomirs support our findings with both mimics in our model cell lines.

305 According to the miRBase database, about 80 different human miRNA precursors can yield
306 two abundant mature miRNAs, i.e. the 5' strand (miR-#-5p) and 3' strand (miR-#-3p) with
307 different seed sequences and mRNAs as binding targets. There is increasing evidence for
308 interplay between the 5' and 3' strands of the same precursor molecule targeting the same

309 group of genes, thereby reinforcing a certain phenotype [10], which can be supported by our
310 data.

311 Hsa-miR-192 has been shown to play a crucial role in the pathogenesis of colon carcinoma,
312 the third most common cancer in Western countries [6]. Because of its cancer stage-
313 dependent decline in expression, miR-192 has been suggested to be a potential biomarker to
314 predict metastasis in colon carcinoma patients [9]. Our findings regarding the inverse
315 association of FXR and miR-192* expression in colonic cancer patients suggest that this
316 miRNA-dependent mechanism of FXR regulation could play an important role in
317 carcinogenesis. We additionally showed that miR-192 can suppress expression of the bile acid
318 and anticancer drug transporter OATP1B3 and the bile acid transporter OST α/β in a FXR-
319 dependent manner.

320 It remains unclear whether restoration of miR-192 expression and the consequently
321 diminished expression of FXR target genes would be beneficial for cancer patients, especially
322 considering that miR-192 has been shown to possibly act as an oncogenic miRNA by
323 downregulation of Smad interacting protein 1 in other inflammation-related cancers
324 [21,26,35]. Therefore, it can be speculated that diminished expression of the transport protein
325 OST α /OST β , a heterodimer-forming bile acid transporter important for the excretion of bile
326 acids from hepatocytes and enterocytes, may be a mechanism of intracellular bile acid
327 accumulation promoting inflammation and/or cancer development in certain cases.

328 By performing cell proliferation and invasion assays, we were able to confirm the previously
329 described suppressive role of miR-192 in liver and colon cancer progression. We observed a
330 miR-192-associated anti-proliferative effect in Huh-7 cells that is stronger than in Caco-2
331 cells, where no noteworthy effects of the 3' strand arm could be seen on FXR protein levels.
332 Furthermore, knockdown of FXR did not show any influence on Caco-2 cell proliferation.
333 These observations support the hypothesis that the anti-proliferative effect of miR-192-3p may

334 be to some extent FXR-dependent. Future studies have to elucidate to what extent the miR-
335 192/FXR interplay supports or inhibits tumor pathogenesis. Our results are in line with reports
336 showing that enhanced FXR expression and the associated altered expression of FXR-
337 regulated drug-uptake transporters is related to chemoresistance in cancer patients. Treatment
338 of the colorectal adenocarcinoma cell line LS174T with cisplatin leads to a cisplatin-resistant
339 phenotype that is accompanied by a 350-fold increase in FXR expression [22]. Furthermore,
340 increased expression of OATP1B3 has been suggested to confer anti-apoptotic resistance to
341 paclitaxel by altering p53-dependent pathways [18].

342 It may be of value to confirm the detected association between miR-192 and FXR expression
343 in larger cohorts. We cannot exclude the contribution of other miRs or epigenetic factors to
344 FXR regulation. However, a further step would be to systematically test all *in silico*-predicted
345 miRs for effects on the *NR1H4*-3'UTR.

346 In conclusion, miR-192-5p and -3p negatively regulate the expression of FXR in a synergistic
347 manner, thereby significantly decreasing the expression of FXR target genes OST α/β and
348 OATP1B3. We show a new miR-dependent mechanism of FXR regulation, which could
349 affect the expression of FXR target genes and plays a role in the pathogenesis of liver and
350 colon cancers and their response to anticancer therapies.

351

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356

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361

362 **DISCLOSURES**

363 The authors have no disclosures to report.

364

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502 **FIGURE LEGENDS**

503 Fig. 1. (A) Predicted binding sites for the seed sequence of miR-192-3p in the 3'UTR of
504 *NR1H4* mRNA using the bioinformatic tool miRANDA (www.microrna.org). (B) Relative
505 reporter gene activities at 24 h after cotransfection of Huh-7 cells with the wild-type *NR1H4*-
506 3'UTR target clone or mutated *NR1H4*-3'UTR target clone. The miR target clone control
507 vector was used for normalization. Experiments were performed as triplicates and repeated
508 three times. ** $p < 0.01$, ns = not significant.

509

510 Fig. 2. Effect of miR-192-5p and -3p mimics (A) and inhibitors (B) on *NR1H4* mRNA
511 expression in Huh-7 and Caco-2 cells at 48 h and 24 h, respectively, after transfection.
512 *NR1H4* mRNA expression relative to β -actin was determined by real-time PCR. The miR
513 mimic/inhibitor negative control (NC) was used for normalization. Experiments were repeated
514 three times. * $p < 0.05$, ns = not significant.

515

516 Fig. 3. Effect of miR-192-5p and -3p mimics on FXR protein expression in Huh-7 cells at 72
517 h after transfection and in Caco-2 cells at 48 h after transfection (A). Anti-miRNA-dependent
518 effect on FXR protein levels (B). Caco-2 and Huh-7 cells were treated with anti-miR-192-5p
519 and -3p inhibitors for 48 and 72 h. FXR protein expression relative to β -actin was determined
520 by western blotting and densitometry. Bands of one representative blot are shown for each
521 condition and cell line. Experiments were repeated three times. ** $p < 0.01$, * $p < 0.05$, ns =
522 not significant.

523

524 Fig. 4. Effect of miR-192-5p and -3p mimics on mRNA expression of key FXR target genes
525 important for bile acid homeostasis in Huh-7 cells at 48 h after transfection. *SLCO1B3*,
526 *SLC51A*, and *SLC51B* mRNA expression relative to β -actin was determined by real-time
527 PCR. The miR mimic negative control (NC) was used for normalization. Experiments were
528 repeated four times. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns = not significant.

529

530 Fig. 5. Correlation between FXR and miR-192 expression in colonic adenocarcinoma-derived
531 tissue samples from 27 patients by linear regression analysis. A significant inverse association
532 was detected for miR-192-3p and the *NR1H4* mRNA transcript coding for FXR α 2(+)
533 (coefficient = -1.57/ $p = 0.0259$).

534

535 Fig. 6. Impact of miR-192-5p and -3p (A/C) or FXR siRNA (B/D) on proliferation of Huh-7
536 and Caco-2 cells using Alamar Blue assay. 48h values of each condition were used for
537 normalization. Experiments were repeated four times. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

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546 **TABLE LEGENDS**

547 Table 1. Primer sequences used for subcloning and mutagenesis. Recognition sites for the
548 restriction enzymes are underlined in the primers used for subcloning. *In silico*-predicted
549 binding sites for hsa-miR-192-3p are underlined in the mutagenesis primers, whereas
550 mutations inserted into these sites are indicated in bold. FXRwt_miR192* primers were used
551 to optimize the second binding site for miR-192-3p.

552 |
553 | [Table 2. Clinical characteristics of the 27 patients showing primary colonic adenocarcinoma](#)
554 | [who were included into linear regression analysis.](#)

555 |
556 | Table [32](#). Linear regression analysis of hsa-miR-192-3p with the *NR1H4* mRNA transcripts
557 coding for FXR α 2(+) and FXR α 1(-), and miR-192-5p expression in primary colonic
558 adenocarcinoma tissue obtained from 27 patients. miR-192-3p inversely correlates with
559 FXR α 2(+) expression.

560 |
561 | ~~Table 3. Clinical characteristics of the 27 patients showing primary colonic adenocarcinoma~~
562 | ~~who were included into linear regression analysis.~~

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Table 1: Primer sequences used for subcloning and mutagenesis

Oligonucleotide	Sequence (5' – 3')	Purpose
FXR_3UTR_FW	aggagctctggggattacagggagg	Subcloning of <i>NR1H4</i> 3'prime end into pmirGlo
FXR_3UTR_RV	aggtcgacccaagattgaatacaactct	Subcloning of <i>NR1H4</i> 3'prime end into pmirGlo
FXRmut_miR192*_FW1	ggaatctgcattctgagtcgccgagccctgttgccataataattg	Multi-site directed mutagenesis
FXRmut_miR192*_FW2	gagttgattcaactcgccctcgacctaatacccgcggc	Multi-site directed mutagenesis
FXRwt_miR192*_FW	gagttgattcaactctggcagtcgacctaatacccgcggc	Site-directed mutagenesis
FXRwt_miR192*_RV	gccgcgggattaggtcgactgcccaagattgaatacaactc	Site-directed mutagenesis

Table 2: Linear regression analysis of miR-192-3p expression with FXR α 2(+), FXR α 1(-), and miR-192-5p expression in primary colonic adenocarcinoma

Transcript	Coefficient	Std. Error	P-Value
FXR α 2(+)	-1.57	0.66	2.59E-02
FXR α 1(-)	+1.87	0.70	1.39E-02
hsa-miR-192-5p	0.31	0.14	4.13E-02

Adjusted R-squared: 0.348

Table 23: Clinical characteristics of 27 patients with primary colonic adenocarcinoma

Parameters	Category	N(%)
Gender	male	15(55.6)
	female	12(44.4)
AJCC stage	pT1	6(22.2)
	pT2	13(48.2)
	pT3	4(14.8)
	pT4	4(14.8)
Tumor grade	1	3(11.1)
	2	20(74.1)
	3	4(14.8)
Treatments	Chemotherapy-naive	27(100)

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570

Table 3: Linear regression analysis of miR-192-3p expression with FXR α 2(+), FXR α 1(-), and miR-192-5p expression in primary colonic adenocarcinoma

<u>Transcript</u>	<u>Coefficient</u>	<u>Std. Error</u>	<u>P-Value</u>
<u>FXRα2(+)</u>	<u>-1.57</u>	<u>0.66</u>	<u>2.59E-02</u>
<u>FXRα1(-)</u>	<u>1.87</u>	<u>0.70</u>	<u>1.39E-02</u>
<u>hsa-miR-192-5p</u>	<u>0.31</u>	<u>0.14</u>	<u>4.13E-02</u>

Adjusted R-squared: 0.348

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