

Roles of miR-29a in the Antifibrotic Effect of FXR in Hepatic Stellate Cells

Jiang Li, Yifei Zhang, Ramalinga Kuruba, Xiang Gao,
Chandrashekhhar R. Gandhi, Wen Xie, and Song Li

Center for Pharmacogenetics, Department of Pharmaceutical Sciences, School of Pharmacy,
University of Pittsburgh, Pittsburgh, PA (J.L., YF.Z., R.K., X.G., W.X., S.L.); Department of
Surgery, Thomas E. Starzl Transplantation Institute, University of Pittsburgh, Pittsburgh, PA
(C.R.G.)

Running title: regulation of miRNA-29a by FXR in hepatic stellate cells

Corresponding author:

Dr. Song Li, Center for Pharmacogenetics, School of Pharmacy, University of Pittsburgh,
Pittsburgh, PA 15261. Tel: 412-383-7976. Fax: 412-648-1664. E-mail: sol4@pitt.edu

Number of text pages: 33

Number of tables: 0

Number of figures: 6

Number of words in Abstract: 248

Number of words in Introduction: 731

Number of words in Discussion: 959

Abbreviations: ECM, extracellular matrix; HSCs, hepatic stellate cells; FXR, farnesoid X receptor; miR-29a, microRNA-29a; PPAR- γ , peroxisome proliferators-activated receptor gamma; PXR, pregnane-X-receptor; BAs, bile acids; SHP, short heterodimer partner; miRNAs, microRNAs; RISC, RNA-induced silencing complex; FXRE, FXR response element; RXR, retinoid X receptor; COL1A1, collagen 1A1; ELN 1, elastin 1; FBN 1, fibrillin 1; PBC, primary biliary cirrhosis; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation.

Abstract

Liver fibrosis is a chronic disorder that is characterized by an alteration of the balance between fibrogenesis and fibrolysis, resulting in accumulation of excessive amounts of extracellular matrix (ECM) and distortion of the normal liver architecture. The activation and transformation of quiescent hepatic stellate cells (HSCs) into myofibroblast-like cells constitute a major mechanism for the increased production of ECM in the liver. The nuclear receptor farnesoid X receptor (FXR) shows potent antifibrotic activity in HSCs and protects animals in rodent models of liver fibrosis. However, the detailed mechanism remains incompletely understood. We report in this study that treatment with GW4064, a synthetic FXR ligand, led to upregulation of microRNA-29a (miR-29a) in HSCs isolated from wild-type mice, rats, and humans but not from *FXR*^{-/-} mice. MiR-29a appears to play an inhibitory role in the regulation of ECM production as: 1) transfection of HSCs with miR-29a mimic resulted in drastic downregulation of the mRNA expression of a number of genes encoding ECM proteins; and 2) miR-29a significantly inhibited the expression of a reporter expression plasmid that contains the 3'-UTR of the corresponding ECM genes. Our results suggest that miR-29a is a FXR target gene because miR-29a promoter activity was significantly increased by pharmacological or genetic activation of FXR. Functional analysis of human miR-29a promoter identified an imperfect inverted repeat DNA motif, IR1 (-AGGTCAcAGACCT), as a likely FXR-responsive element that is involved in miR-29a regulation. Our study uncovers a new mechanism by which FXR negatively regulates the expression of ECM in HSCs.

Introduction

Liver fibrosis is a chronic disorder that is characterized by an alteration of the balance between fibrogenesis and fibrolysis, resulting in accumulation of excessive amounts of extracellular matrix (ECM) and distortion of the normal liver architecture (Jiao et al., 2009). The activation and transformation of quiescent hepatic stellate cells (HSCs) into myofibroblast-like cells constitute a major mechanism for the increased production of ECM in the liver (Parsons et al., 2007; Friedman, 2008). The mechanism of HSC activation is not completely understood and involves the alterations of a number of intracellular pathways such as TGF- β and PDGF signaling (Parsons et al., 2007; Friedman, 2008). Several studies have shown that nuclear receptors such as peroxisome proliferators-activated receptor gamma (PPAR- γ) and pregnane-X-receptor (PXR) also play an important role in the modulation of HSC activation and their biological functions (Miyahara et al., 2000; Galli et al., 2002; Marek et al., 2005).

FXR (farnesoid X receptor, NR1H4) is a member of the nuclear receptor superfamily that is highly expressed in liver, kidney, adrenals, and intestine (Forman et al., 1995). FXR plays a key role in the homeostasis of cholesterol and bile acids (BAs) by regulating the expression of genes involved in the synthesis and transport of BAs (Chiang, 2002; Bertolotti et al., 2008). In addition to the potential of its ligands in the treatment of cholestasis (Clausel et al., 2002), FXR has been shown to be expressed in HSCs and negatively regulate the activation of HSCs and the associated overproduction of ECM in rodent models of liver fibrosis (Fiorucci et al., 2004; Fiorucci et al., 2005a; Fiorucci et al., 2005b). A study by Fiorucci et al. suggested that short heterodimer partner (SHP) played a role in the FXR-mediated antifibrotic effect. SHP is one of the FXR target genes and it effectively blocks the AP-1 signaling that is critically involved in

ECM production (Fiorucci et al., 2004). However, the detailed mechanism that is involved in the antifibrotic effects of FXR remains incompletely understood.

MicroRNAs (miRNAs) are short non-coding RNA molecules that control gene expression via modulating the stability and/or the translational efficiency of target messenger RNAs (Lee et al., 1993; Ghildiyal & Zamore, 2009). MiRNAs play a role in the control of a wide range of biological functions and processes such as development, differentiation, metabolism, carcinogenesis, immune response etc (Ghildiyal & Zamore, 2009). MiRNAs are initially transcribed as long primary transcripts that undergo several processing steps to form the mature 22-nt miRNA:miRNA* duplex. The complementary strand miRNA* is typically degraded, while the mature single-stranded form is incorporated into the RNA-induced silencing complex (RISC). Most of miRNA binding sequences are found in the 3'-UTR of the mRNAs (Ghildiyal & Zamore, 2009). However, increasing evidence suggests that miRNAs can also be targeted to both coding-regions and 5'-UTR of mRNAs (Forman et al., 2008). Several studies showed that miRNAs were involved in the activation of HSCs: a number of miRNAs species are upregulated while many others are downregulated during the process (Ji et al., 2009; Guo et al., 2009; Venugopal et al., 2010). A recent study by Roderburg and colleagues showed that all three members (a, b, and c) of the miR-29 family were significantly downregulated in mouse liver in both CCl₄ and common bile ligation models (Roderburg et al., 2011). Downregulation of miR-29a/b/c was also shown in activated HSCs *in vitro*. A likely role of miR-29 members in liver fibrosis is suggested by: a) overexpression of miR-29 in mouse HSCs led to downregulation of the expression of several ECM genes; and b) treatment with TGF- β or LPS-mediated activation of NF- κ B signaling led to downregulation of the expression of miR-29a/b/c (Roderburg et al.,

2011). The clinical significance is further supported by the observation that serum levels of miR-29a in patients with cirrhosis are inversely correlated with the stages of the disease (Roderburg et al., 2011). However, it is unknown if miRNAs are also involved in the antifibrotic effect of FXR in HSCs.

In this study, we showed that activation of FXR by a synthetic ligand, GW4064, led to significant upregulation of the expression of miR-29a in mouse, rat, and human HSCs. Functional and reporter assays suggested that miR-29a is a potent mediator that negatively regulates the expression of several ECM genes. Furthermore, a functional FXR response element (FXRE) was found in the miR-29a promoter. Our study unveils a new mechanism by which FXR negatively regulates the expression of ECM in HSCs.

Materials and Methods

Reagents & Chemicals. GW4064 (3-[2-[2-Chloro-4-[[3-(2,6-dichlorophenyl)-5-(1-methylethyl)-4-isoxazolyl]methoxy]phenyl]ethenyl]benzoic acid) was synthesized following a published protocol (Maloney et al., 2000). MiR-29a mimic and non-specific control miRNA mimic were purchased from ABI (Applied Biosystems, Foster City, CA). All products for cell culture were purchased from Invitrogen (Carlsbad, CA). pCMX, pCMX-FXR, and pCMV- β gal were described previously (Xie et al., 2001). pCMX-vpFXR (a gift from Drs Enrique Saez and Ronald Evans at the Salk Institute) was generated by fusing the VP16 activation domain from the herpes simplex virus to the N terminus of the FXR (Xie et al., 2001).

Animals and HSC Isolation. Retired male Sprague-Dawley rats and C57BL/6 mice were from Charles River Laboratories. *FXR*^{-/-} mice (Sinal et al., 2000) were purchased from Jackson Laboratories and bred in the Central Animal Facility of University of Pittsburgh. HSCs were isolated via *in situ* proteinase/collagenase perfusion followed by density gradient centrifugation as described (Thirunavukkarasu et al., 2005). Primary cells were more than 95% pure. Cells were grown on standard tissue culture plastic dishes in DMEM with 10% fetal bovine serum and antibiotics. HSCs were used following activation via culturing for 7 days. All studies conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

LX-2, an immortalized human hepatic stellate cell line, was kindly provided by Dr. Scott L. Friedman (Mount Sinai School of Medicine, New York, NY, USA) (Xu et al., 2005). The cells were maintained in DMEM with 10% fetal bovine serum and antibiotics.

Real Time RT-PCR. Total RNA was extracted from cells with TRIzol reagent (Invitrogen) and the first-strand cDNA was synthesized by use of SuperScript III reverse transcriptase (Invitrogen). Real-time PCR analysis of rat, mouse, and human fibrosis-related genes and miR-29 precursor was performed by use of SYBR Green-based assays with the ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) (Li et al., 2008). Transcript abundance, normalized to β -glucuronidase expression, was expressed as fold increase over a calibrated sample.

For detection of mature miRNA, total RNA was reversely transcribed into cDNA using miScript Reverse Transcriptase Kit (Qiagen) according to the manufacturer's protocol. cDNA samples (2 μ l) were used for real-time PCR in a total volume of 25 μ l using miScript SYBR Green PCR Kit (Qiagen) and miRNA specific primers (Qiagen) on a qPCR machine (Applied Biosystems, Foster City, CA). The sequences of primers for all of the RT-PCR analysis were shown in Supplemental Tables 1 and 2.

Western Blot Analysis. Protein extraction and Western blot analysis were performed as described (Li et al., 2008). FXR antibody (sc-13063) and collagen 1A1 (COL1A1) antibody (sc-25974) were purchased from Santa Cruz Biotechnology. Horseradish peroxidase-labeled goat anti-rabbit IgG and the ECL chemiluminescence kit were purchased from Amersham Biosciences (Piscataway, NJ).

Plasmid Construction. A fragment spanning 1.98 kb of 5'-flanking sequence of the human miR-29a gene was PCR-amplified from human genomic DNA using primers 5'-cgacgcgtgtgggtaaggagagggaag-3' and 5'-cgacgcgttgctgactgatgagaggaaa-3' and cloned into *MluI* of pGL3-basic vector (Promega). A mutated construct that lacks a putative IR-1 (miR-29a/IR-1) binding site was similarly generated via cloning a fragment spanning 1.87 kb of 5'-flanking sequence of the miR-29a gene that was amplified using primers 5'-cgacgcgtctggtgttcgcagctttcac-3' and 5'-cgacgcgttgctgactgatgagaggaaa-3'. A TK-Luc construct that contains three copies of miR-29a/IR-1 sequence was generated by annealing the oligonucleotides 5'-agcttcttgagggtcacag-acctcgagggtcacagacctcgagggtcacagacctcgttg-3' and 5'-caacgagggtctgagacctcgagggtctgagacctcgag-gtctgagacctccaaga-3' followed by ligation into *HindIII/BamHI*-digested TK-Luc. For functional analysis of miR-29a, 3'-UTR segments containing the miR-29a-binding sequences or without binding sequences for COL1A1, collagen 3A1(COL3A1), and elastin-1(ELN1) genes were PCR-amplified from human genomic DNA utilizing the corresponding forward and reverse primers (Supplemental Table 1). The PCR product was then subcloned into the *SacI-MluI* site downstream of the stop codon in the pMIR-REPORT Firefly Luciferase reporter vector (Ambion).

Transfection Assays. Normal monkey kidney fibroblast cells (CV-1 line) were grown to 60–70% confluence in 48-well plates. Cells were transiently transfected using Lipofectamine2000 (Invitrogen) with pGL3-miR-29a in the presence or absence of pCMX-vpFXR or pCMX-FXR. pCMX was added to ensure identical amounts of DNA in each well. Transfection efficiency was monitored by co-transfection of pCMV- β gal plasmid. Twenty-four h later, cells were treated with GW4064 or DMSO vehicle. Cell extracts were prepared 24 h after GW4064 treatment and the

luciferase and β -galactosidase assays were performed as described (He et al., 2006) and luciferase activity was normalized against β -galactosidase activity. Transfection experiments were performed on at least three occasions and in each case were done in triplicate. Data were represented as fold induction over reporter gene alone.

In a separate study, CV-1 cells were transfected with pMIR-COL1A1-3'-UTR, pMIR-COL3A1-3'-UTR, or pMIR-ELN1-3'-UTR in the presence of miR-29a mimic or non-specific control miRNA mimic. The reporter expression was then similarly examined as described above 24 h following transfection.

Electrophoretic Mobility Shift Assay (EMSA). FXR and retinoid X receptor (RXR) proteins were generated *in vitro* by coupled *in vitro* transcription/translation (TNT system, Promega) with pCMX-FXR and pCMX-RXR plasmids. The DNA probe miR-29a/IR1 (5'-gaag**aggtcacagac**ctctgg -3') was derived from a region in the human miR-29a promoter that contains a putative FXR response element (bold). It was labeled with [γ - 32 P]-ATP by using the Klenow fragment of DNA polymerase. DNA-binding reactions were carried out as follows: aliquots of *in vitro* translation mixture were incubated in 20 μ L binding buffer (10 mM Hepes, pH 7.9, 10 mM EGTA, 10 mM EDTA, 0.25 mM DTT, and 10% glycerol) containing 2 μ g polydI: dC (Sigma) and 6–20 $\times 10^3$ cpm of DNA probe at room temperature for 20 min. For supershift assays, 0.2 μ g rabbit anti-FXR IgG was added and the samples were incubated for another 10 min. The binding mixture was then applied onto a 5% polyacrylamide gel (0.5 x TrisBorateEDTA buffer) for electrophoresis. The gels were dried and exposed at -80°C for autoradiography.

Chromatin Immunoprecipitation (ChIP) Assay: ChIP assay was performed using a ChIP assay kit (Millipore, Massachusetts, USA) according to the instructions of the manufacture. Soluble chromatin was prepared from LX-2 cells treated with 1 μ M GW4064 for 24 hours. Chromatin was immunoprecipitated with antibodies (2 μ g) directed against FXR (sc-13063). Final DNA extractions were PCR amplified using primer pairs that cover an IR-1 consensus sequence in the miRNA-29a promoter as follows: forward, 5'-GTGGGTAAGGGAGAGGGAAG-3'; antisense, 5'-ACATTGCCTTCTCCCCAAAG-3'.

Statistical Analysis: All data are expressed as means \pm S.E.M. unless otherwise stated. Comparisons between two groups were made with unpaired Student's *t*-test. Comparisons between three or more groups were made with analysis of variance followed by Tukey-Kramer post hoc analysis. In all cases, $P < 0.05$ was considered statistically significant.

Results

Treatment of rat HSCs with GW4064 led to significant inhibition of the mRNA expression of several ECM genes. Using 6-ECDC as a specific ligand, Fiorucci and colleagues (2004) have previously shown that activation of FXR leads to a significant inhibition of collagen 1A1 (COL1A1) expression in both primary rat HSCs and an immortalized human hepatic stellate cell line HSC-T6. In this experiment, we examined if GW4064 could similarly inhibit the expression of COL1A1 in rat HSCs. GW4064 is also a synthetic ligand that is highly specific for FXR and has been widely used in studying FXR-mediated gene regulation *in vitro* and *in vivo* (Maloney et al., 2000; Li et al., 2009). Fig. 1 shows that GW4064 treatment resulted in a significant downregulation of the expression of COL1A1 mRNA in rat HSCs. GW4064 also significantly inhibited the expression of several other fibrosis-related genes including COL1A2, COL3A1, COL4A1, COL5A1, elastin 1 (ELN 1), and fibrillin 1 (FBN 1) (Fig. 1). Inhibition of ECM protein expression by GW4064 was also confirmed as shown in Western analysis of COL1A1 expression (Supplemental Figure 1).

GW4064 treatment led to upregulation of miR-29a in rat and mouse HSCs. Following the demonstration of the inhibition of the mRNA expression of several ECM genes by GW4064, we went on to explore the potential mechanism involved. We hypothesized that a miRNA might be involved based on the fact that a cluster of ECM-related genes were affected by GW4064 treatment. Multiple algorithms were used to screen for miRNAs that may be involved in the regulation of ECM including Microcosm Targets, TargetScan, and PITA (Lewis et al., 2003; Xin et al., 2009; Dong et al., 2010). Members of miR-29 family including miR-29a, miR-29b, and miR-29c were identified by all three programs to be the best candidates as ECM-targeting

miRNAs (Supplemental Table 4). As an initial step to study a potential role of miR-29a in GW4064-mediated effects, we examined if the expression of miR-29a is regulated by GW4064 in HSCs. Fig. 2A shows that GW4064 treatment resulted in a significant increase in the expression of miR-29a gene as assessed by real-time RT-PCR analysis of miR-29a precursor. A similar induction of miR-29a expression by GW4064 was also observed in LX-2 cells (an immortalized human hepatic stellate cell line) (Fig. 2B) and HSCs isolated from wild-type mice (Fig. 2C). However, no induction of miR-29a was observed in HSCs prepared from *FXR*^{-/-} mice (Fig. 2D), suggesting that induction of miR-29a by GW4064 was mediated by FXR. Interestingly, GW4064 treatment had no effect on the expression of miR-29b or miR-29c in both rat and mouse HSCs (Supplemental Figure 2). A similar result was obtained when miR-29a expression was examined by RT-PCR analysis of mature miR-29a (data not shown). Induction of miR-29a in mouse liver was also observed following oral delivery of GW4064 (Supplemental Figure 3).

MiR-29a negatively regulated the expression of ECM in HSCs. Following demonstration of induction of miR-29a by GW4064 we then examined the effect of miR-29a overexpression on the expression of several ECM genes in HSCs to further establish a role of miR-29a in the GW4064/FXR-mediated antifibrotic effect. HSCs were transfected with a miR-29a mimic, a control sequence or a miR-29a inhibitor and the mRNA expression of several ECM genes was examined 24 h later. As shown in Fig. 3, overexpression of miR-29a mimic in HSCs resulted in a significant inhibition of the mRNA expression of all six ECM genes examined. Higher levels of ECM mRNAs were observed in HSCs treated with miR-29a inhibitor compared to cells treated with control sequence. This might be due to the inhibition of endogenous miR-29a that is

involved in the control of the basal levels of ECM expression. The effect of miR-29a appears to be target sequence-specific as it showed no effect on the expression of FXR and SHP expression at both mRNA and protein level over a wide range of concentration (Supplemental Figure 4 & 5). MiR-29a treatment had no effect either on GW4064-mediated induction of SHP expression, suggesting that the function of FXR was well-retained in miR-29a-treated HSCs (Supplemental Figure 6). In contrast, miR-29a inhibited the expression of COL1A1 at both mRNA and protein levels in a dose-dependent manner (Supplemental Figure 4 & 5).

To further establish a role of miR-29a in the regulation of ECM expression we examined the effect of miR-29a on the expression of several reporter constructs that contain the respective 3'-UTRs from COL1A1, COL3A1 and ELN1 genes. All of the three 3'-UTRs contain a putative miR-29a target sequence as analyzed by TargetScan algorithm. As shown in Fig. 4A, transfection of cells with miR-29a mimic significantly inhibited the expression of the reporter construct with an intact COL1A1 3'-UTR. Such inhibitory effect was completely lost for a mutant reporter construct lacking the miR-29a target sequence. These results suggest that the presence of the miRNA target site in the COL1A1 3'-UTR of the reporter construct is necessary for the inhibition by miR-29a. Similar results were observed with the construct with a 3'-UTR from either COL3A1 or ELN1 gene (Fig. 4B & C).

Human miR-29a is a likely target gene of FXR. Upregulation of miR-29a expression by GW4064 suggests that activation of FXR modulates miR-29a expression at the transcriptional level. We then hypothesized that activation of FXR enhances miR-29a expression via exerting its stimulatory activity on miR-29a promoter. To test this hypothesis, we constructed a luciferase

reporter expression plasmid (pGL3-miR-29a) that is driven by a 1.98 kb sequence of human miR-29a promoter. To examine promoter activation, CV-1 cells were co-transfected with pGL3-miR-29a and pCMX-FXR expression vector, followed by GW4064 treatment. CV-1 cells instead of HSCs were used for transfection due to low transfection efficiency in the HSCs. As shown in Fig. 5, treatment with GW4064 resulted in a significant increase in the transcriptional activity of the miR-29a promoter. To further elucidate a role of FXR in regulating miR-29a promoter activity, we then co-transfected pGL3-miR-29a with an expression plasmid encoding a constitutively activated FXR, vpFXR. vpFXR was generated by fusing the VP16 activation domain to the N terminus of FXR cDNA (Xie et al., 2001). Fig. 5 shows that co-expression of vpFXR in CV-1 cells significantly enhanced the miR-29a promoter activity, clearly demonstrating that a genetic activation of FXR enhances miR-29a promoter activity.

To search for FXR responsive elements (FXREs) that may mediate miR-29a induction by GW4064, the 2 kb miR-29a promoter sequence was subjected to *in silico* analysis with a Web-based algorithm (NUBIScan). One imperfect inverted repeat spaced by one nucleotide (IR1) site was identified, and its sequences and location are shown in Fig. 6A. To determine whether miR-29a/IR1 is necessary and sufficient in mediating FXR transactivation a heterologous tk-luciferase reporter gene that contain three copies of miR-29a/IR1 element was generated and tested for FXR transactivation in CV-1 cells. As shown in Fig. 6B, the synthetic tk reporter gene was activated by FXR in the presence of its agonist GW4064. To further determine whether this IR1 sequence is responsible for FXR-mediated transactivation of miR-29a promoter, we generated a mutant pGL3-miR-29a in which this putative FXR binding site was eliminated. The wild-type and mutated plasmids were then similarly transfected into the CV-1 cells, and their transfection

efficiency was compared. Fig. 6C shows that the FXR-mediated activation of miR-29a promoter was substantially diminished when the miR-29a/IR1 site was eliminated. The above studies strongly suggested a likely role of miR-29a/IR1 element in FXR-mediated transactivation of human miR-29a promoter.

Fig. 6D shows the result of an EMSA with a 20 bp oligonucleotide that contains the putative FXRE (lanes 1 ~ 5). A typical IR1/FXRE oligonucleotide was also included as a positive control (lanes 6 ~ 10) because FXR is known to bind to IR1 with high specificity and affinity. Interaction of the oligonucleotide with *in vitro* translated FXR/RXR yielded a DNA/protein band of expected mobility (lane 1). This binding was specific, as it was inhibited by addition of excess unlabelled (cold) miR-29a/IR1 (lane 2) or IR1/FXRE (lane 4) but not a mutated miR-29a/IR1 (lane 3). Addition of antibody against FXR to the reaction mixture resulted in the disappearance of the radiolabeled band (lane 5) (Fig. 6D). This supershifting confirms the identity of the protein that interacts with the DNA as being FXR.

To further demonstrate the binding of FXR to miR-29a/IR1, chromatin immunoprecipitation (ChIP) assay was then performed. In this experiment, LX-2 cells were treated with vehicle (DMSO) or GW4064 for 24 h before ChIP analysis using an anti-FXR antibody or the control mouse IgG. As shown in Fig. 6E, treatment of LX-2 cells with GW4064 led to a significant increase in the recruitment of FXR to miR-29a/IR1. Together these results suggested that miR-29a is a direct transcriptional target of FXR.

Discussion

We have demonstrated in this study that miR-29a was significantly upregulated in HSCs following treatment with GW4064, a synthetic FXR-specific agonist. Overexpression of miR-29a in HSCs resulted in a significant inhibition of the mRNA expression of a number of ECM genes including collagens, elastin and fibrillin. MiR-29a also significantly inhibited the expression of a reporter expression plasmid that contains a full-length 3'-UTR from COL1A1, COL3A1 or ELN1 gene. These results are consistent with the notion that miR-29a may be critically involved in the FXR-mediated inhibition of ECM expression in HSCs.

Induction of miR-29a in HSCs is likely to be mediated by FXR as: a) GW4064 is highly specific for FXR and is often used as a "chemical tool" to show that BA target genes are regulated in a FXR-specific manner (Maloney et al., 2000); and b) the induction of miR-29a was abolished in *FXR*^{-/-} mouse HSCs. We have also identified an imperfect IR1 as a FXRE that appears to be involved in GW4064-mediated upregulation of miR-29a in HSCs. IR1 is a typical FXRE that has been implicated in the regulation of a number of FXR target genes. Thus, miR-29a is a likely target gene of FXR.

Various biological functions have been reported for members of miR-29 family including miR-29a, b & c. These include modulations of self-renewal in hematopoietic progenitor cells (Han et al., 2010), regulations of WNT signaling in human osteoblasts (Kapinas et al., 2010), control of host-HIV-1 interactions (Nathans et al., 2009), and regulation of the expression of ECM in fibroblasts and stellate cells (Jiang et al., 2010) etc. Clearly, the biological functions of miRNAs

are complex and may be tissue- or cell type-specific. A study from van Rooij et al. (2008) has shown that dysregulation of miR-29 plays an important role in cardiac fibrosis following myocardial infarction. All three members of miR-29 family were downregulated in the region of heart adjacent to the infarct. Systemic delivery of a cholesterol-conjugated miR-29b inhibitor led to increased expression of collagen in liver, kidney and heart, suggesting a role of miR-29 in regulating the expression of ECM *in vivo* (van Rooij et al., 2008). Recently, downregulation of miR-29 has also been shown in rodent models of liver fibrosis and in liver biopsies from patients with HCV infection (Pogribny et al., 2010; Kwiecinski et al., 2009). The role of miR-29 members in liver fibrosis was further demonstrated in a recent study by Roderburg et al. in which all members of miR-29 were significantly downregulated in mouse liver in both CCl₄ and common bile ligation models (Roderburg et al., 2011). They have further shown that TGF- β or NF- κ B signaling negatively regulated the expression of miR-29, suggesting TGF- β \uparrow \rightarrow miR-29 \downarrow or NF- κ B \uparrow \rightarrow miR-29 \downarrow as an important mechanism in the development of liver fibrosis (Roderburg et al., 2011). A similar role of TGF- β \uparrow \rightarrow miR-29 \downarrow was also demonstrated in a mouse model of lung fibrosis (Cushing et al., 2010). These data, together with our observation that activation of FXR led to upregulation of miR-29a, strongly support an important role of miR-29 in the control of ECM expression under both physiological and pathophysiological conditions. They also suggest the potential of miR-29 as a novel therapeutics for the treatment of various fibrotic diseases including liver fibrosis.

It is likely that other mechanisms are involved in the antifibrotic effect of FXR/FXR ligands in addition to a direct activation of the miR-29a gene. A number of studies including our work have suggested that many nuclear receptors such as FXR and SHP inhibit various inflammatory

signalings including NF- κ B, AP-1, and TGF- β via competition for essential cofactors or recruitment of corepressors (He et al., 2006; Zhang et al., 2010). Therefore, it is possible that treatment of HSCs with FXR ligands at quiescent stage may prevent the NF- κ B- and/or TGF- β -mediated downregulation of miR-29 expression during their transactivation process. Studies are currently ongoing in our laboratory to test this hypothesis. Alternatively, FXR and/or SHP may directly inhibit the expression of fibrosis-related genes through the interference of AP-1 and/or TGF- β signaling as suggested by Fiorucci et al. in their study (Fiorucci et al., 2004). Clearly, more studies are required in the future to better understand the contribution of each potential mechanism to the overall antifibrotic effect of FXR.

It should be noted that, despite a similar function in regulation of ECM expression, miR-29a, b and c may be differentially up- or down-regulated under different physiological or pathophysiological conditions. MiRNA-29a and miR-29b1 are clustered together and are located in chromosome 7 in humans, whereas the miR-29b2/miR-29c cluster is found on chromosome 1. A study by Pogribny et al. (2010) shows that miR-29c was downregulated in a mouse model of dietary nonalcoholic steatohepatitis. In contrast, in a rat model of common bile duct ligation and in liver biopsies from HCV patients, prominent downregulation of miR-29a and moderate downregulation of miR-29b were observed (Kwiecinski et al., 2009). We only observed a significant upregulation of miR-29a in both mouse and rat HSCs following GW4064 treatment, which is likely due to lack of functional FXR response element in the miR-29b2/miR-29c transcriptional unit. This is in contrast to downregulation of all members of miR-29 family in mouse models of liver fibrosis in the study by Roderburg et al., which may be due to the fact that the expression of both miRNA-29a/miR-29b1 and miR-29b2/miR-29c is negatively regulated by

NF- κ B and/or TGF- β signaling. This may reflect a complex mechanism in the regulation of miRNA expression.

In summary, we have shown for the first time that activation of FXR led to expression of miR-29a in HSCs, which may play an important role in FXR-mediated antifibrotic effect. Our study provides new insight into the mechanism by which FXR/FXR ligands control the expression of ECM in HSCs. It also suggests a miR-29a-based new therapy for the treatment of liver fibrosis.

Acknowledgements

We would like to thank Dr. Scott L. Friedman at Mount Sinai School of Medicine, New York, NY for generously providing the LX-2 cell line.

Authorship Contributions

Participated in research design: J. Li, and S.Li.

Conducted experiments: J. Li, Zhang, and Gao.

Contributed new reagents or analytic tools: Kuruba, Gandhi, Xie, and Gao.

Performed data analysis: J. Li, Zhang, S.Li.

Wrote or contributed to the writing of the manuscript: J. Li, Zhang, and S.Li.

Other: S. Li acquired funding for the research.

REFERENCES

- Bertolotti M, Gabbi C, Anzivino C, Carulli L, Loria P and Carulli N (2008) Nuclear receptors as potential molecular targets in cholesterol accumulation conditions: Insights from evidence on hepatic cholesterol degradation and gallstone disease in humans. *Curr Med Chem* **15**(22):2271-2284.
- Chiang JYL (2002) Bile acid regulation of gene expression: Roles of nuclear hormone receptors. *Endocr Rev* **23**(4):443-463.
- Claudel T, Sturm E, Duez H, Torra IP, Sirvent A, Kosykh V, Fruchart JC, Dallongeville J, Hum DW, Kuipers F and Staels B (2002) Bile acid-activated nuclear receptor FXR suppresses apolipoprotein A-I transcription via a negative FXR response element. *J Clin Invest* **109**(7):961-971.
- Cushing L, Kuang PP, Qian J, Shao F, Wu J, Little F, Thannickal VJ, Cardoso WV, Lu J (2010) Mir-29 is a major regulator of genes associated with pulmonary fibrosis. *Am J Respir Cell Mol Biol* doi:10.1165/rcmb.2010-0323OC
- Dong H, Paquette M, Williams A, Zoeller RT, Wade M, Yauk C (2010) Thyroid hormone may regulate mRNA abundance in liver by acting on microRNAs. *PLoS One* **5**(8):e12136.
- Fiorucci S, Antonelli E, Rizzo G, Renga B, Mencarelli A, Riccardi L, Orlandi S, Pellicciari R and Morelli A (2004) The nuclear receptor SHP mediates inhibition of hepatic stellate cells by FXR and protects against liver fibrosis. *Gastroenterology* **127**(5):1497-1512.
- Fiorucci S, Rizzo G, Antonelli E, Renga B, Mencarelli A, Riccardi L, Morelli A, Pruzanski M and Pellicciari R (2005a) Cross-talk between farnesoid-X-receptor (FXR) and peroxisome proliferator-activated receptor gamma contributes to the antifibrotic activity of FXR ligands in rodent models of liver cirrhosis. *J Pharmacol Exp Ther* **315**(1):58-68.

- Fiorucci S, Rizzo G, Antonelli E, Renga B, Mencarelli A, Riccardi L, Orlandi S, Pruzanski M, Morelli A and Pellicciari R (2005b) A farnesoid X receptor-small heterodimer partner regulatory cascade modulates tissue metalloproteinase inhibitor-1 and matrix metalloproteinase expression in hepatic stellate cells and promotes resolution of liver fibrosis. *J Pharmacol Exp Ther* **314**(2):584-595.
- Forman BM, Goode E, Chen J, Oro AE, Bradley DJ, Perlmann T, Noonan DJ, Burka LT, McMorris T, Lamph WW, Evans RM and Weinberger C (1995) Identification of a nuclear receptor that is activated by farnesol metabolites. *Cell* **81**(5):687-693.
- Forman JJ, Legesse-Miller A and Collier HA (2008) A search for conserved sequences in coding regions reveals that the let-7 microRNA targets Dicer within its coding sequence. *Proc Natl Acad Sci U S A* **105**(39):14879-14884.
- Friedman SL (2008) Hepatic stellate cells: Protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev* **88**(1):125-172.
- Galli A, Crabb DW, Ceni E, Salzano R, Mello T, Svegliati-Baroni G, Ridolfi F, Trozzi L, Surrenti C and Casini A (2002) Antidiabetic thiazolidinediones inhibit collagen synthesis and hepatic stellate cell activation *in vivo* and *in vitro*. *Gastroenterology* **122**(7):1924-1940.
- Ghildiyal M and Zamore PD (2009) Small silencing RNAs: an expanding universe. *Nat Rev Genet* **10**(2):94-108.
- Guo CJ, Pan Q, Li DG, Sun H and Liu BW (2009) miR-15b and miR-16 are implicated in activation of the rat hepatic stellate cell: An essential role for apoptosis. *J Hepatol* **50**(4):766-778.

- Han YC, Park CY, Bhagat G, Zhang JP, Wang YL, Fan JB, Liu MF, Zou YR, Weissman IL and Gu H (2010) microRNA-29a induces aberrant self-renewal capacity in hematopoietic progenitors, biased myeloid development, and acute myeloid leukemia. *J Exp Med* **207**(3):475-489.
- He FT, Li J, Mu Y, Kuruba R, Ma Z, Wilson A, Alber S, Jiang Y, Stevens T, Watkins S, Pitt B, Xie W and Li S (2006) Downregulation of endothelin-1 by farnesoid X receptor in vascular endothelial cells. *Circ Res* **98**(2):192-199.
- Ji JL, Zhang JS, Huang GC, Qian J, Wang XQ and Mei S (2009) Over-expressed microRNA-27a and 27b influence fat accumulation and cell proliferation during rat hepatic stellate cell activation. *Febs Lett* **583**(4):759-766.
- Jiang X, Tsitsiou E, Herrick SE and Lindsay MA (2010) MicroRNAs and the regulation of fibrosis. *Febs J* **277**(9):2015-2021.
- Jiao JJ, Friedman SL and Aloman C (2009) Hepatic fibrosis. *Current Opinion in Gastroenterology* **25**(3):223-229.
- Kapinas K, Kessler C, Ricks T, Gronowicz G and Delany AM (2010) MiR-29 modulates Wnt signaling in human osteoblasts through a positive feedback loop. *J Biol Chem* **285**(33):25221-25231.
- Kwiecinski M, Strack I, Noetel A, Schievenbusch S, Scheffler M, Elfimova N, Dienes HP and Odenthal M (2009) MicroRNA-29: a novel antifibrogenic mediator in liver fibrogenesis. *J Hepatol* **50**:S110.
- Lee RC, Feinbaum RL and Ambros V (1993) The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* **75**(5):843-854.

- Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB (2003) Prediction of mammalian microRNA targets. *Cell* **115**(7):787-98.
- Li J, Wilson A, Kuruba R, Zhang Q, Gao X, He F, Zhang LM, Pitt BR, Xie W and Li S (2008) FXR-mediated regulation of eNOS expression in vascular endothelial cells. *Cardiovasc Res* **77**(1):169-177.
- Li J, Wilson A, Gao X, Kuruba R, Liu YH, Poloyac S, Pitt B, Xie W and Li S (2009) Coordinated regulation of dimethylarginine dimethylaminohydrolase-1 and cationic amino acid transporter-1 by farnesoid X receptor in mouse liver and kidney and its implication in the control of blood levels of asymmetric dimethylarginine. *J Pharmacol Exp Ther* **331**(1):234-243.
- Maloney PR, Parks DJ, Haffner CD, Fivush AM, Chandra G, Plunket KD, Creech KL, Moore LB, Wilson JG, Lewis MC, Jones SA and Willson TM (2000) Identification of a chemical tool for the orphan nuclear receptor FXR. *J Med Chem* **43**(16):2971-2974.
- Marek CJ, Tucker SJ, Konstantinou DK, Elrick LJ, Haefner D, Sigalas C, Murray GI, Goodwin B and Wright MC (2005) Pregnenolone-16 alpha-carbonitrile inhibits rodent liver fibrogenesis via PXR (pregnane X receptor)-dependent and PXR-independent mechanisms. *Biochem J* **387**:601-608.
- Miyahara T, Schrum L, Rippe R, Xiong SG, Yee HF, Motomura K, Anania FA, Willson TM and Tsukamoto H (2000) Peroxisome proliferator-activated receptors and hepatic stellate cell activation. *J Bio Chem* **275**(46):35715-35722.
- Nathans R, Chu CY, Serquina AK, Lu CC, Cao H and Rana TM (2009) Cellular microRNA and P bodies modulate host-HIV-1 interactions. *Mol Cell* **34**(6):696-709.

- Parsons CJ, Takashima M and Rippe RA (2007) Molecular mechanisms of hepatic fibrogenesis. *J Gastroenterol Hepatol* **22**:S79-S84.
- Pogribny IP, Starlard-Davenport A, Tryndyak VP, Han T, Ross SA, Rusyn I and Beland FA (2010) Difference in expression of hepatic microRNAs miR-29c, miR-34a, miR-155, and miR-200b is associated with strain-specific susceptibility to dietary nonalcoholic steatohepatitis in mice. *Lab Invest* doi: 10.1038/labinvest.2010.1113.
- Roderburg C, Urban GW, Bettermann K, Vucur M, Zimmermann H, Schmidt S, Janssen J, Koppe C, Knolle P, Castoldi M, Tacke F, Trautwein C, Luedde T (2011) Micro-RNA profiling reveals a role for miR-29 in human and murine liver fibrosis. *Hepatology* **53**(1):209-18.
- Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G, Gonzalez FJ (2000) Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. *Cell* **102**(6):731-744.
- Thirunavukkarasu C, Uemura T, Wang LF, Watkins SC and Gandhi CR (2005) Normal rat hepatic stellate cells respond to endotoxin in LBP-independent manner to produce inhibitor(s) of DNA synthesis in hepatocytes. *J Cell Physiol* **204**(2):654-665.
- van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, Hill JA and Olson EN (2008) Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. *Proc Natl Acad Sci U S A* **105**(35):13027-13032.
- Venugopal SK, Jiang J, Kim T-H, Li Y, Wang S-S, Torok NJ, Wu J and Zern MA (2010) Liver fibrosis causes downregulation of miRNA-150 and miRNA-194 in hepatic stellate cells, and their overexpression causes decreased stellate cell activation. *Am J Physiol Gastrointest Liver Physiol* **298**(1):G101-106.

- Xie W, Radominska-Pandya A, Shi YH, Simon CM, Nelson MC, Ong ES, Waxman DJ and Evans RM (2001) An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. *Proc Natl Acad Sci U S A* **98**(6):3375-3380.
- Xin F, Li M, Balch C, Thomson M, Fan M, Liu Y, Hammond SM, Kim S, Nephew KP (2009) Computational analysis of microRNA profiles and their target genes suggests significant involvement in breast cancer antiestrogen resistance. *Bioinformatics* **25**(4):430-4.
- Xu L, Hui AY, Albanis E, Arthur MJ, O'Byrne SM, Blaner WS, Mukherjee P, Friedman SL, Eng FJ (2005) Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. *Gut* **54**(1):142-151.
- Zhang Y, Hagedorn CH, Wang L (2010) Role of nuclear receptor SHP in metabolism and cancer. *Biochim Biophys Acta* doi:10.1016/j.bbadis.2010.10.006

Footnotes

This work was supported by the National Institutes of Health [Grants HL68688, HL091828-01], and a grant from University of Pittsburgh Central Research Development Fund (CRDF).

Address for reprint requests:

Dr. Song Li, Center for Pharmacogenetics, Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, 639 Salk Hall, 3501 Terrace Street, Pittsburgh, PA 15261, U.S.A. Email: sol4@pitt.edu

Legends for figures.

Fig. 1. GW4064 treatment led to downregulation of the mRNA expression of ECM genes in rat HSCs. Rat HSCs were isolated as described in Materials & Methods and cultured for 7 days to allow transactivation. HSCs were then treated with GW4064 (1 μ M) or DMSO vehicle. The mRNA expression levels of several ECM genes were determined by real-time RT-PCR 24 h following the treatment. N = 3. $P < 0.05$ (vs. DMSO).

Fig. 2. GW4064 treatment led to upregulation of miR-29a expression in HSCs. Rat HSCs (A) or LX2 cells (B) were treated with GW4064 or DMSO vehicle. The expression level of miR-29a was determined by real-time RT-PCR 24 h following the treatment. The effect of GW4064 on miR-29a expression was similarly examined in HSCs isolated from wild-type mice (C) or *FXR*^{-/-} mice (D). N = 3. $P < 0.05$ (vs. DMSO).

Fig. 3. Overexpression of miR-29a resulted in downregulation of the mRNA expression of ECM genes in rat HSCs. HSCs were transfected with miR-29a mimic, non-specific control miRNA mimic, or miR-29a inhibitor (25 pmol per well in 6-well plates by 10 μ l of Lipofectamine) for 24 h. The mRNA expression levels of several ECM genes were then determined by real-time RT-PCR. N = 3. $P < 0.05$ (vs. control miRNA).

Fig. 4. MiR-29a regulates the expression of ECM genes via targeting at the 3'-UTR of their mRNAs. CV-1 cells were transfected with a luciferase construct with Col1A1-3'-UTR (A), Col3A1-3'-UTR (B) or ELN-3'-UTR (C) in the presence of miR-29a mimic or non-specific

control miRNA mimic. Luciferase assay was performed 24 h following the transfection. Data shown in the panels represent mean (SD) from triplicate assays. $N = 3$. $P < 0.05$ (vs. control miRNA).

Fig. 5. FXR enhances the transcriptional activity of the miR-29a gene promoter. A

luciferase reporter driven by a human miR-29a promoter of 1984 bp was used to study the miR-29a promoter activity. CV-1 cells were transiently transfected with pGL3-miR-29a in the presence or absence of pCMX-FXR. Five hours later, the transfection medium was replaced with complete medium and cells were incubated for 20 h. Cells were then cultured in the presence of GW4064 or vehicle DMSO for 24 h. Luciferase assay was then performed. Data shown in the panels represent mean (SD) from triplicate assays. To examine the effect of genetic activation of FXR on miR-29a promoter activity, CV-1 cells were transfected with pGL3-miR-29a in the presence or absence of pCMX-vpFXR. Luciferase assays were then examined as described above. $N = 3$. $P < 0.05$ (vs. DMSO).

Fig. 6. Analysis of putative FXR-responsive elements (FXREs) in human miR-29a promoter. (A)

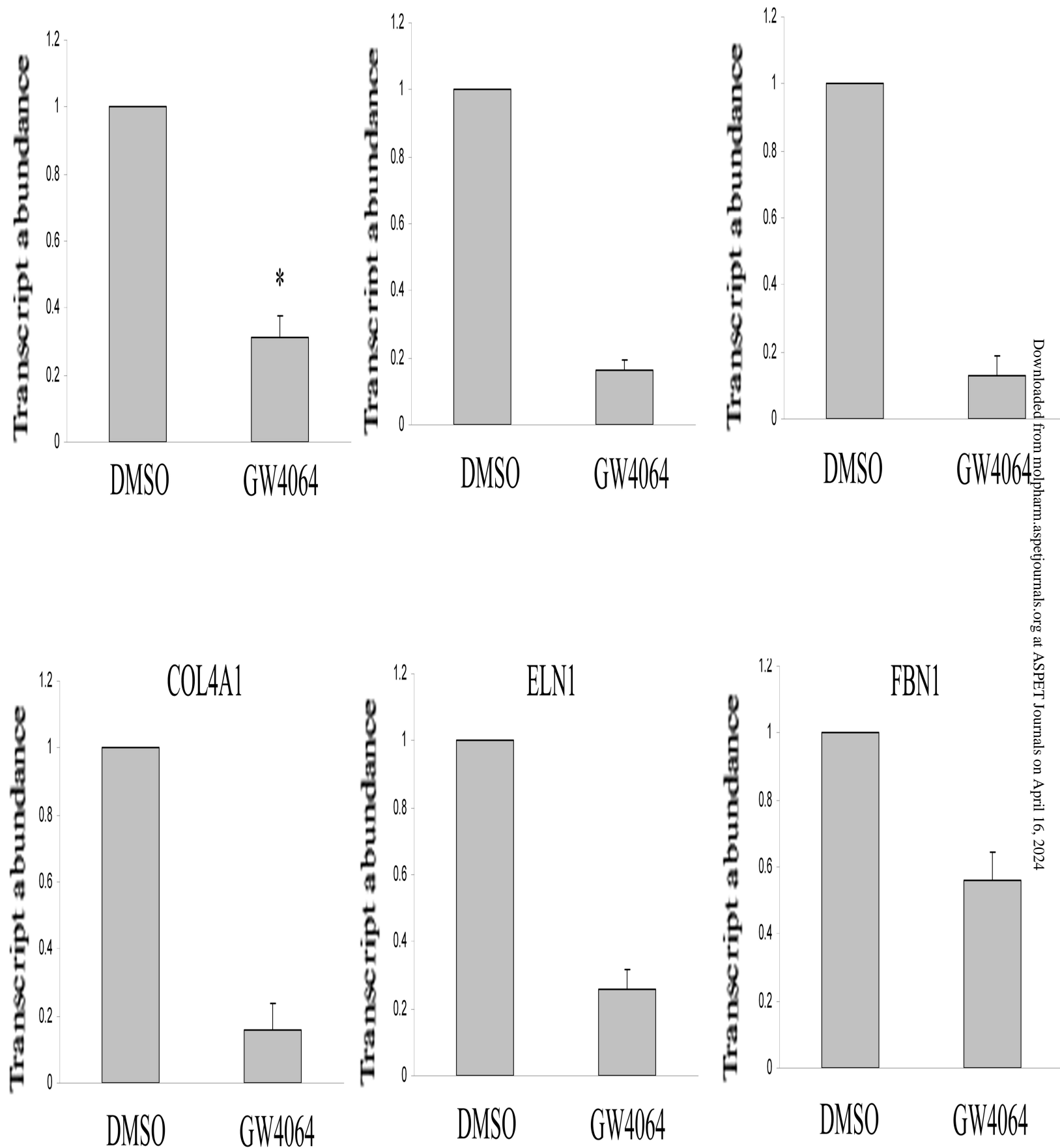
Identification of a putative FXRE in human miR-29a promoter via an *in silico* analysis with a Web-based algorithm (NUBIScan). **(B)** miR-29a/IR1 mediates FXR transactivation of a heterologous tk-luciferase reporter gene. $N = 3$. $P < 0.05$ (vs. DMSO). **(C)** Mutation of miR-29a/IR1 on the miR-29a promoter eliminates activation by FXR. $N = 3$. $P < 0.05$ (vs. DMSO). **(D)** EMSA analysis of the binding of FXR/RXR to miR-29a/IR1 in human miR-29a promoter. Double-stranded oligonucleotides (–1924/–1936) were end-labeled with [γ - 32 P]-ATP using T4 polynucleotide kinase. The labeled probe was incubated with *in vitro*-translated RXR/FXR for

20 min. The reactions were analyzed by electrophoresis in a non-denaturing 5% polyacrylamide gel followed by autoradiography. In some studies, the samples were pre-incubated with anti-FXR antibody prior to gel electrophoresis. (E) ChIP analysis of the binding of FXR to miR-29a/IR1 in human miR-29a promoter in LX-2 cells. Soluble chromatin was prepared from LX2 cells treated with 1 μ M GW4064 or DMSO for 24 hours. Chromatin was immunoprecipitated with antibodies directed against FXR. The extracted DNA was PCR amplified using primer pairs that cover an IR-1 consensus sequence in the miRNA-29a promoter.

COL1A1

COL1A2

COL3A1



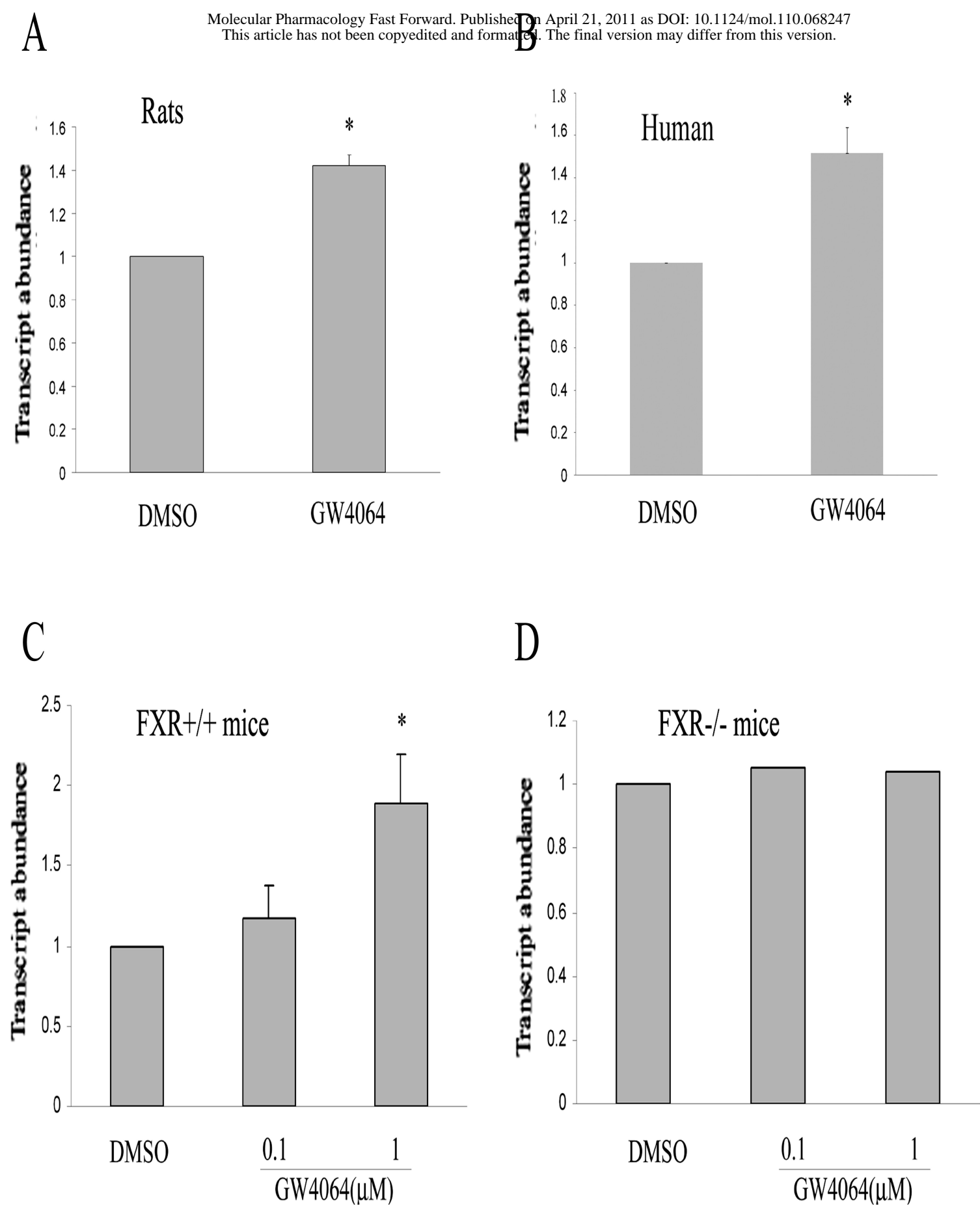
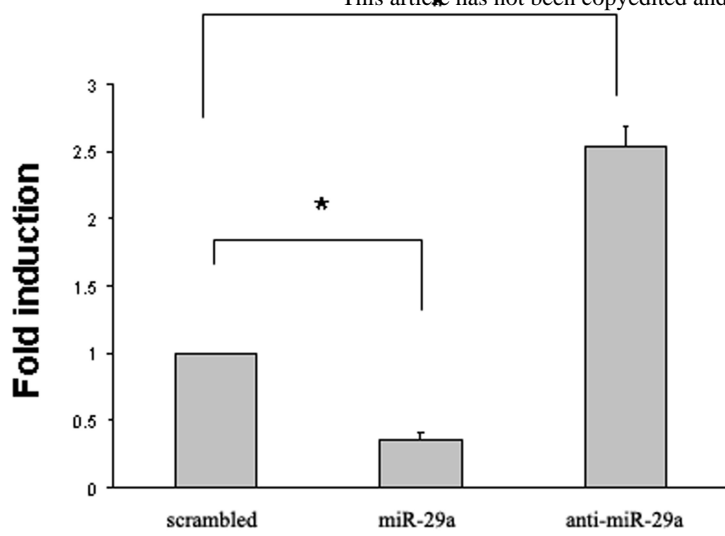
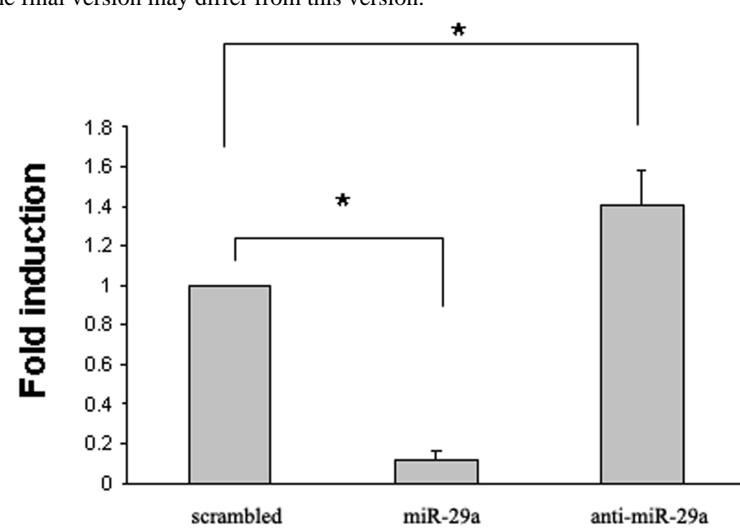


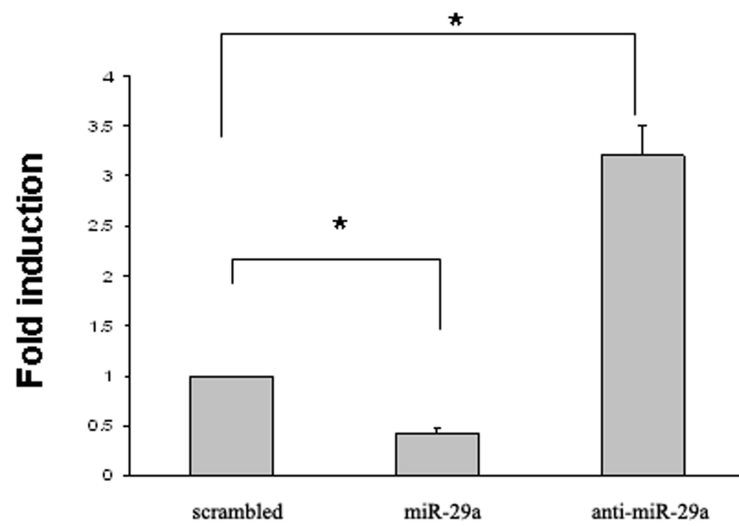
Fig. 2



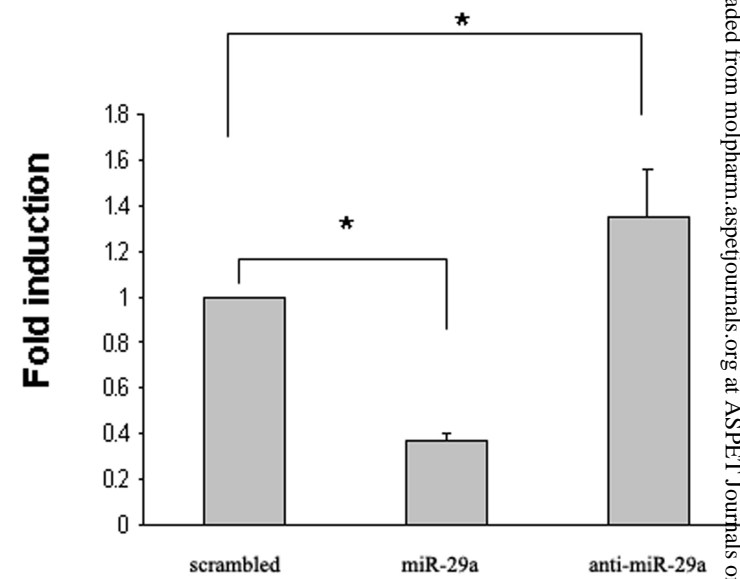
COL1A1



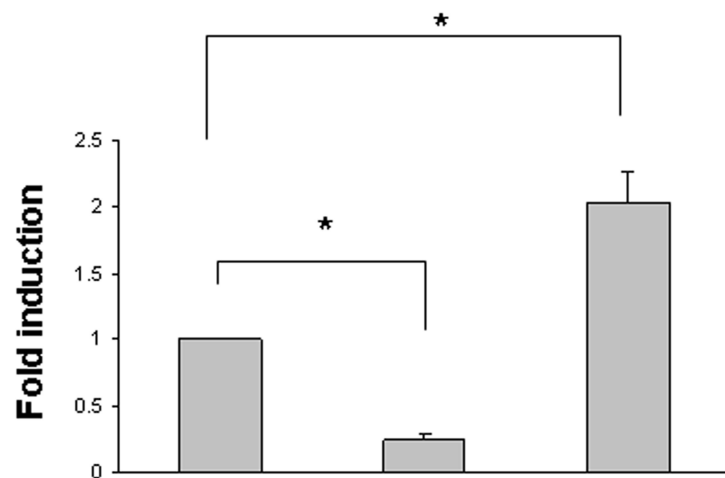
COL1A2



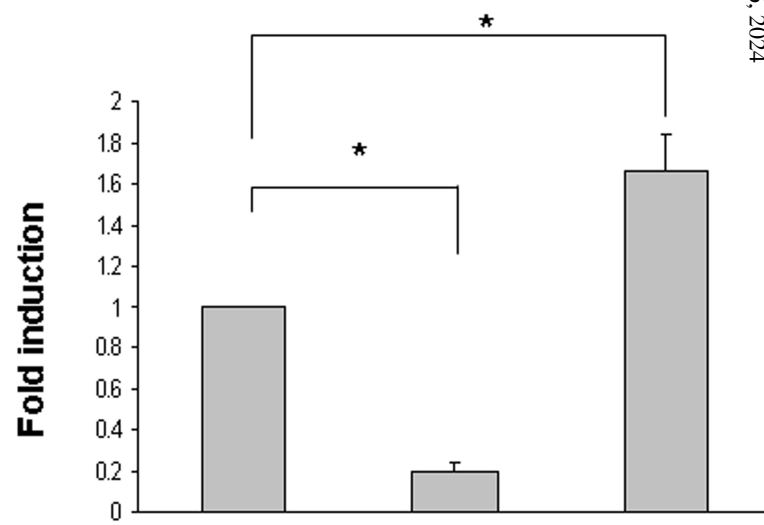
COL3A1



COL4A1



ELN1



FBN1

Fig. 3

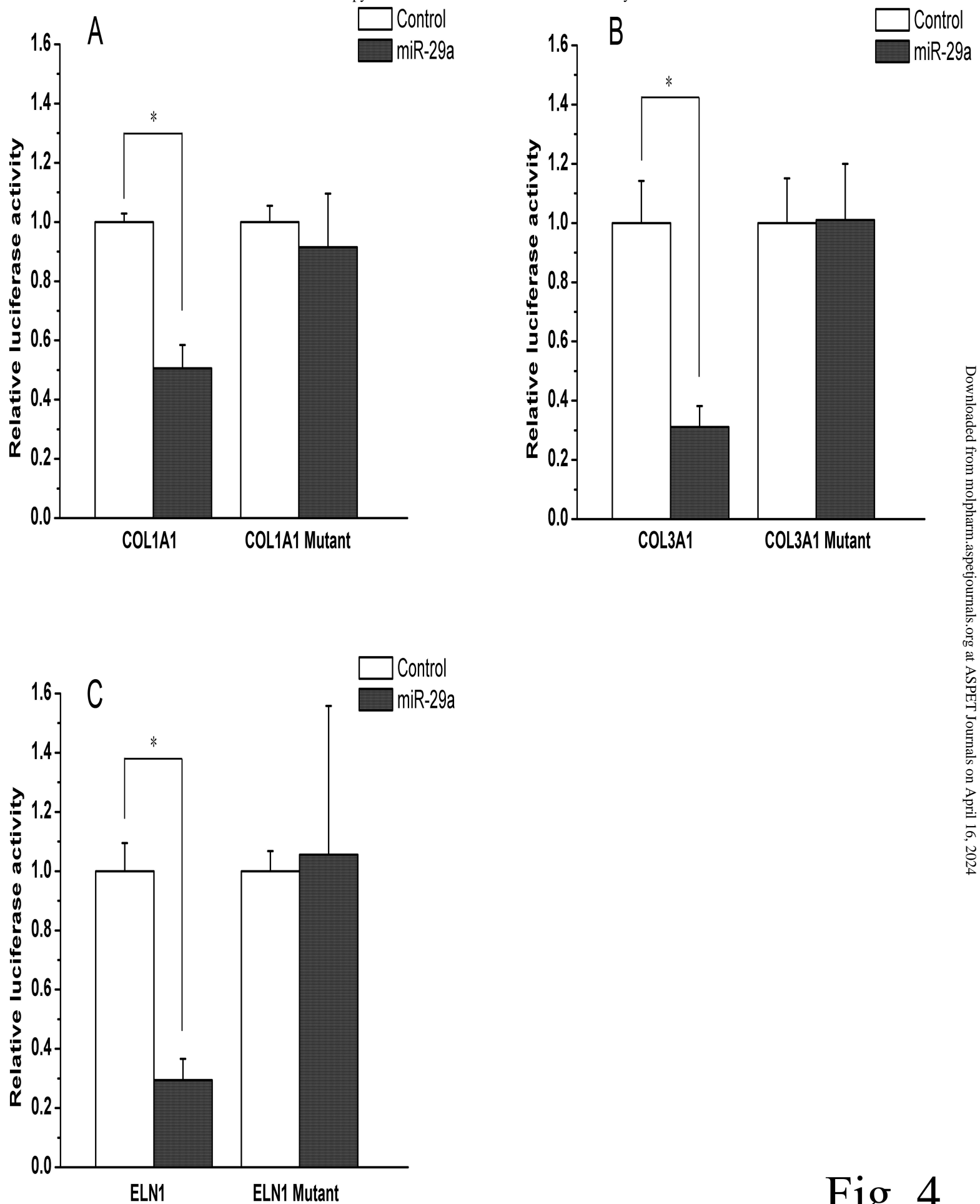


Fig. 4

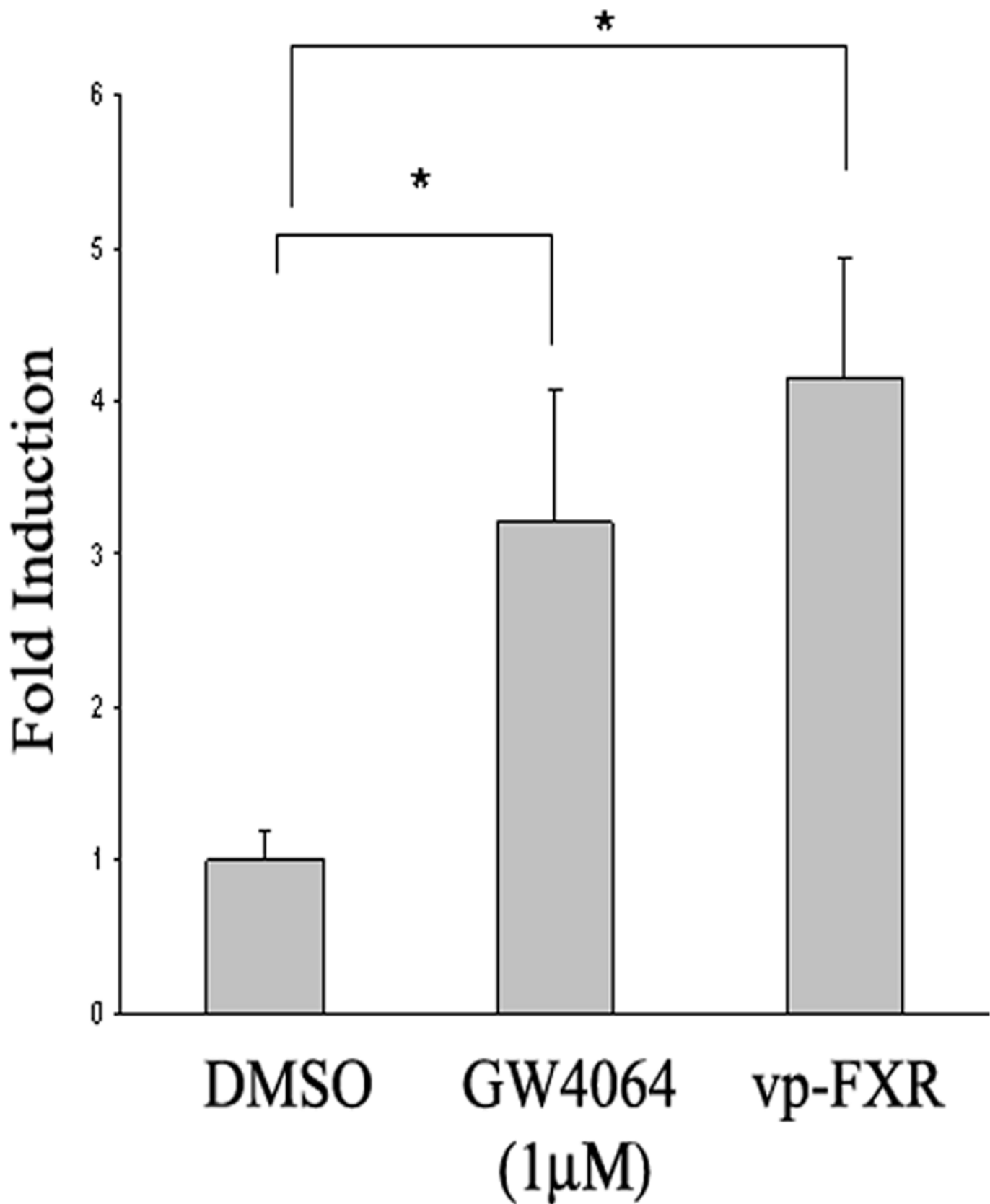
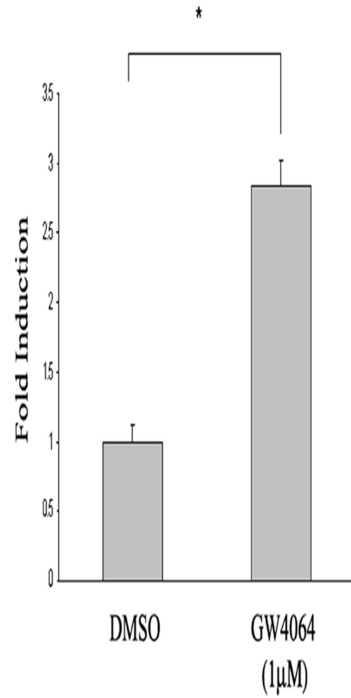


Fig. 5

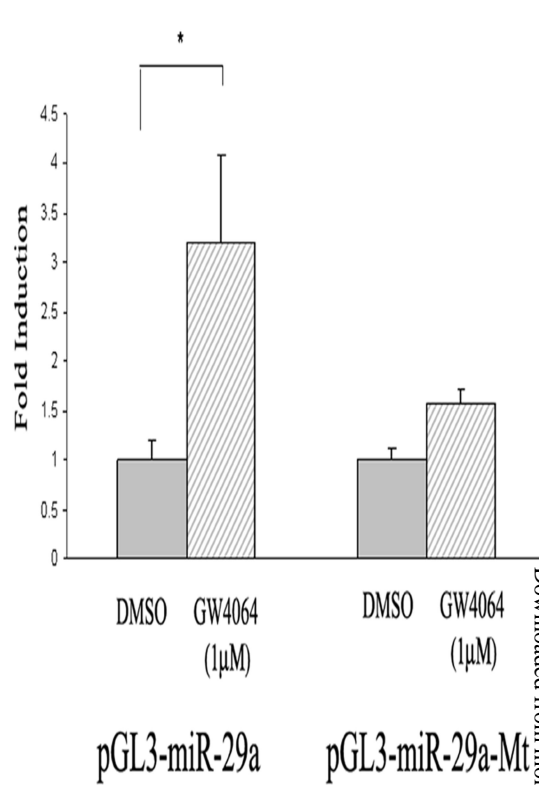
A

Binding site	Core sequence
IR1 (consensus)	AGGTCA n TGACCT
miR-29a IR1	AGGTCA c AGACCT
Mutated IR1	AGTGCA c AGATCT

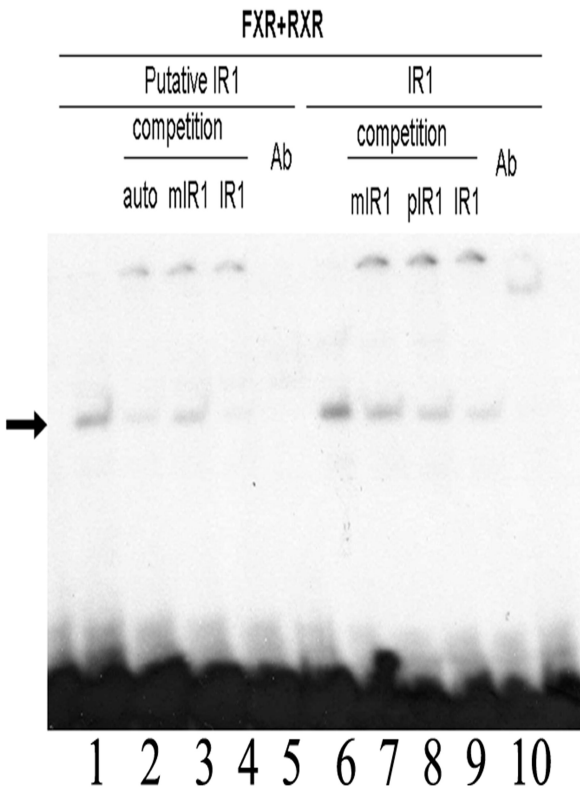
B



C



D



E

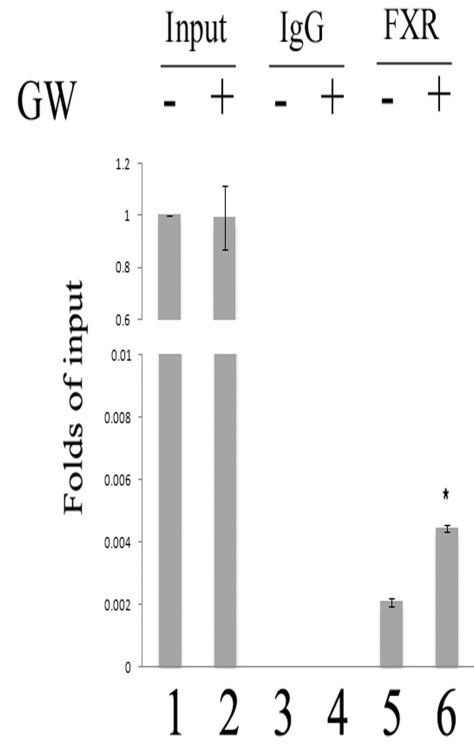


Fig. 6