Molecular Pharmacology

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

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Supplemental Table 1: Primers for amplification of 3′-UTR of COL1A1, COL3A1, ELN1 and mature miR-29a

Gene	forward primer	reverse primer
Col1A1 3′-UTR	acgagctcaaactccctccatcccaatc	acacgcgtccaccccagggataaaaact
Mutated Col1A1 3′-UTR	acgagctcaaactccctccatcccaatc	acacgcgtatggccaggctttgaccatttg
Col3A1 3′-UTR	acgagctcttataagccaaactctctgaaac	acacgcgttgatgtttatttattatatcaccatta
Mutated Col3A1 3′-UTR	acgagctcttataagccaaactctctgaaac	acacgcgtgagacagggtgaagttggaattgg
ELN1 3′-UTR	acacgcgtctgactcgcgacctcgtca	acaagcttcacagcgaaacacaaagagg
Mutated ELN1 3′-UTR	cccacgcgtgggggtgctactgcttggtggag	cgaagcttcgcaccaatttctcttggagatgg
miR-29a	tagcaccatctgaaatcggtta	universal primer

Supplemental Table 2: Primers for miR-29 precursor amplification

Gene	forward primer	reverse primer
rno-mir-29a precursor	CCCTTAGAGGATGACTGATTTC	AACCGATTTCAGATGGTGCT
rno-mir-29b precursor	GGAAGCTGGTTTCACATGGT	TCCTAAAACACTGATTTCAAATGG
rno-mir-29c precursor	ACACAGGCTGACCGATTTCT	CCCCTACATCATAACCGATTTC
hsa-mir-29a precursor	TGACTGATTTCTTTTGGTGTTCA	AACCGATTTCAGATGGTGCT
mmu-mir-29a precursor	CCCTTAGAGGATGACTGATTTC	AACCGATTTCAGATGGTGCT

Supplemental Table 3:

Supplemental Table 3A: Prediction of miR-29a target by TargetScan

Target gene	Conserved sites				Poorly conserved sites				Total context score
	total	8mer	7mer-m8	7mer-1A	total	8mer	7mer-m8	7mer-1A	
COL3A1	**2**	2	0	0	**0**	0	0	0	-0.95
FBN1	**2**	1	1	0	**0**	0	0	0	-0.74
ELN	**3**	2	1	0	**0**	0	0	0	-0.7
COL4A1	**2**	1	0	1	**0**	0	0	0	-0.6
COL1A1	**3**	1	0	2	**0**	0	0	0	-0.57

- 8mer: an exact match to positions 2-8 of the mature miRNA (the seed + position 8) followed by an 'A'.
- 7mer-m8: an exact match to positions 2-8 of the mature miRNA (the seed + position 8).
- 7mer-1A: an exact match to positions 2-7 of the mature miRNA (the seed) followed by an 'A'.
- Total context score: the sum of the contribution of four different features including: a) site-type contribution; b) 3' pairing contribution; c) local AU contribution; and d) position contribution.

Supplemental Table 3B: Prediction of miR-29a target by PITA

Gene Name	microRNA	Sites	Score	Position	Seed	dGduplex	Chromosome	Start	End	Strand
COL4A1	hsa-miR-29a	1	-10.31	37	8:0:0	-17.61	13	1.1E+08	1.1E+08	-
COL3A1	hsa-miR-29a	2	-9.61	249	8:0:0	-15.9	2	1.9E+08	1.9E+08	+
ELN	hsa-miR-29a	3	-9.5	45	7:0:0	-16.2	7	73121004	73121010	+
FBN1	hsa-miR-29a	2	-1.46	423	7:0:0	-12.7	15	46490057	46490063	-
COL1A1	hsa-miR-29a	1	0.57	930	7:0:0	-14.4	17	45616933	45616939	+

- columns with the following format: Gene name (column 1), microRNA name (column 2), number of sites (column 3) and total combined energy (column 4), site position (column 5, the base in the 3' UTR where the microRNA 5' end is predicted to bind), seed type (column 6, the seed length, number of mismatches and number of G:U wobbles in that order), microRNA-target hybridization energy (ΔG_{duplex}, column 7), chromosome (column 8), seed start and end positions (columns 9 and 10), and strand (column 11).

Supplemental Table 3C: Prediction of miR-29a target by Microcosm

Gene Name	score	energy	p-value	length	Total sites	No. Cons Species	No. miRNAs
COL1A1	16.7395	-17.4	0.00028	839	17	7	28 [+]
COL3A1	15.9037	-17.14	2.86E-06	972	13	7	15 [+]
COL4A1	15.3034	-18.14	9.28E-09	1399	10	7	11 [+]
ELN	15.1018	-16.68	0.006777	1000	15	4	18 [+]
FBN1	15.8146	-16.21	5.99E-06	1000	19	7	26 [+]

- *Score* derived from the 5' end of the miRNA where multiplied by a scaling factor of 4.0, to reflect the apparent importance of perfect Watson-crick base pairing which has been observed experimentally. The overall score for a hit is the summation of these derived scores across the total miRNA vs UTR alignment.
- *Energy* is computed by the Vienna RNA folding routines and is a measure for the thermodynamic stability of a duplex.
- *P-value* computes an estimated probability of the same microRNA family hitting multiple transcripts for different species in an orthologous group. This is done by taking into account the level of sequence conservation between all the 3' UTRs.
- *Length*: Utr length (bps).
- *Total sites*: Total number of conserved targeted sites found in transcript.
- *No. Cons Species*: Maximum conserved species for which any targeted site is found.
- *No. miRNAs:* miRNA Registry identifiers of miRNAs that are predicted to hit this transcript.

Supplemental Table 4:

Supplemental Table 4A: Putative target miRNA of Human COL1A1 3' UTR

miRNA	conserved sites				poorly conserved sites				Total content
	Total	8mer	7mer-m8	7mer-1A	Total	8mer	7mer-m8	7mer-1A	score
miR-29abc	3	1	0	2	0	0	0	0	-0.57
miR-129/129-5p	2	0	0	2	0	0	0	0	-0.36
miR-218	1	1	0	0	0	0	0	0	-0.34
miR-143	1	0	1	0	0	0	0	0	-0.26
miR-338/338-3p	0	0	0	0	1	0	0	1	-0.23
miR-9	0	0	0	0	2	0	1	1	-0.2
let-7/98	1	0	1	0	0	0	0	0	-0.19
miR-150	0	0	0	0	1	0	0	1	-0.18
miR-133	1	0	0	1	0	0	0	0	-0.15
miR-132/212	0	0	0	0	1	0	1	0	-0.13
miR-103/107	0	0	0	0	1	0	1	0	-0.11
miR-196ab	1	0	1	0	0	0	0	0	-0.11
miR-29abc	3	1	0	2	0	0	0	0	-0.57
miR-129/129-5p	2	0	0	2	0	0	0	0	-0.36

- 8mer: an exact match to positions 2-8 of the mature miRNA (the seed + position 8) followed by an 'A'.
- 7mer-m8: an exact match to positions 2-8 of the mature miRNA (the seed + position 8).
- 7mer-1A: an exact match to positions 2-7 of the mature miRNA (the seed) followed by an 'A'.
- Total context score: the sum of the contribution of following four features: a) site-type contribution; b) 3' pairing contribution; c) local AU contribution; and d) position contribution.

Supplemental Table 4B: Putative target miRNA of Human COL3A1 3' UTR

miRNA	conserved sites				poorly conserved sites				Total content
	Total	8mer	7mer-m8	7mer-1A	Total	8mer	7mer-m8	7mer-1A	score
miR-29abc	2	2	0	0	0	0	0	0	-0.95
let-7/98	1	1	0	0	0	0	0	0	-0.42
miR-128	0	0	0	0	1	0	1	0	-0.31
miR-196ab	1	0	1	0	0	0	0	0	-0.22
miR-129/129-5p	0	0	0	0	1	0	0	1	-0.19
miR-192/215	0	0	0	0	1	0	0	1	-0.17
miR-203	0	0	0	0	1	0	0	1	-0.16
miR-33/33ab	0	0	0	0	1	0	0	1	-0.14
miR-29abc	2	2	0	0	0	0	0	0	-0.95
let-7/98	1	1	0	0	0	0	0	0	-0.42
miR-128	0	0	0	0	1	0	1	0	-0.31
miR-196ab	1	0	1	0	0	0	0	0	-0.22
miR-129/129-5p	0	0	0	0	1	0	0	1	-0.19
miR-192/215	0	0	0	0	1	0	0	1	-0.17

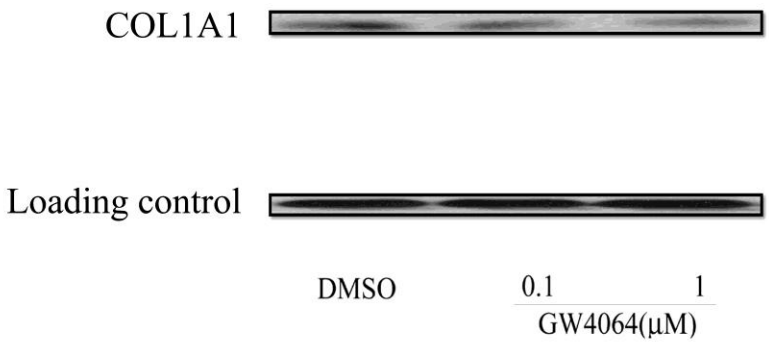
- 8mer: an exact match to positions 2-8 of the mature miRNA (the seed + position 8) followed by an 'A'.
- 7mer-m8: an exact match to positions 2-8 of the mature miRNA (the seed + position 8).
- 7mer-1A: an exact match to positions 2-7 of the mature miRNA (the seed) followed by an 'A'.
- Total context score: the sum of the contribution of following four features: a) site-type contribution; b) 3' pairing contribution; c) local AU contribution; and d) position contribution.

Supplemental Methods and Results of Bioinformatic Analysis of the Putative microRNAs that Regulate ECM Gene Expression:

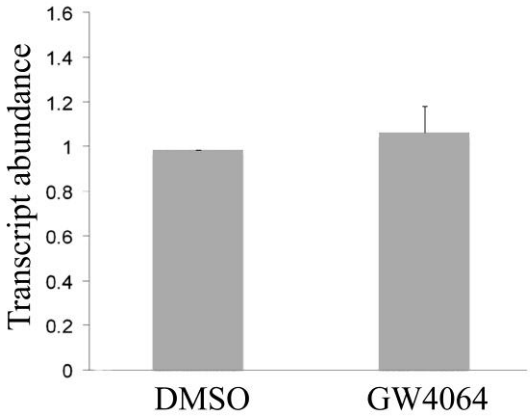
Following the demonstration of the inhibition of the mRNA expression of several ECM genes by GW4064, we went on to explore the potential mechanism involved. We hypothesized that a miRNA might be involved based on the fact that a cluster of ECM-related genes were affected by GW4064 treatment. Multiple algorithms were used to screen for miRNAs that may be involved in the regulation of ECM including Microcosm Targets, TargetScan, and PITA. Supplemental Table 3A shows the results of TargetScan analysis: all of the ECM genes we examined are putative target genes of miR-29a with high predictive scores (-0.57 ~ -0.95). A similar result was obtained when PITA (Supplemental Table 3B) and Microcosm (Supplemental Table 3C) were used for analysis, respectively.

We then went on to analyze the other microRNAs that might be involved in the regulation of the ECM genes examined in our study. Supplemental Table 4A and Supplemental Table 4B show the results of TargetScan analysis for COL1A1 and COL3A1 gene, respectively. As shown in Supplemental Table 4A, more than 10 microRNAs could be potentially involved in the regulation of COL1A1 gene expression. However, miR-29abc shows the highest predictive score. A similar result was obtained for TargetScan analysis of COL3A1 (Supplemental Table 4B) and other four ECM genes (data not shown). Therefore, members of miR-29 family were selected as the top candidate target genes of FXR for subsequent studies.

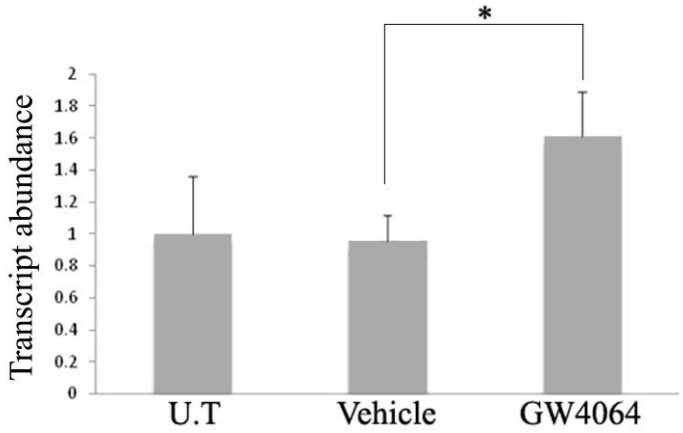
Supplemental Figures



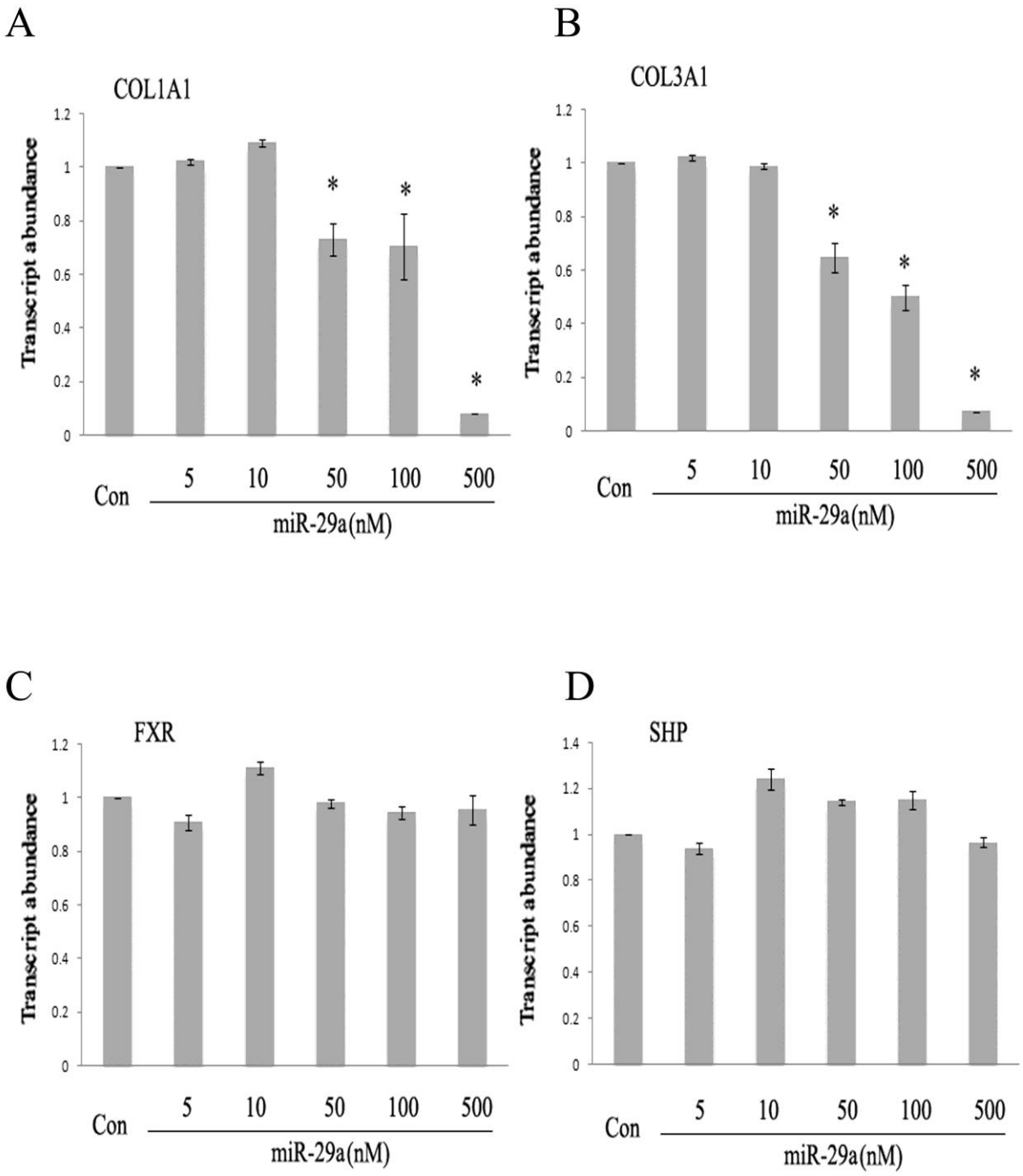
Supplemental Figure 1. GW4064 treatment led to downregulation of COL1A1 protein expression in rat HSCs. Rat HSCs were isolated as described in Materials & Methods and cultured for 7 days to allow transactivation. HSCs were then treated with GW4064 or DMSO vehicle. The expression level of COL1A1 was determined by Western blot 24 h following the treatment. Shown in the figure were representative data from three independent experiments.



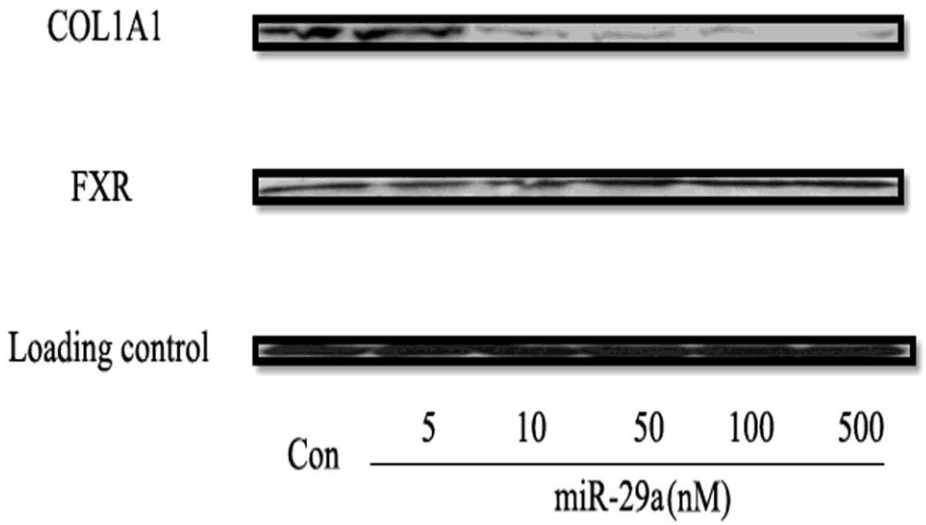
Supplemental Figure 2. MiR-29b expression is not altered in rat HSCs treated with GW4064. Rat HSCs were isolated as described in Materials & Methods and cultured for 7 days to allow transactivation. HSCs were then treated with GW4064 or DMSO vehicle. The expression level of miR-29b was determined by real-time RT-PCR 24 h following the treatment. N = 3. $P > 0.05$ (vs. DMSO).



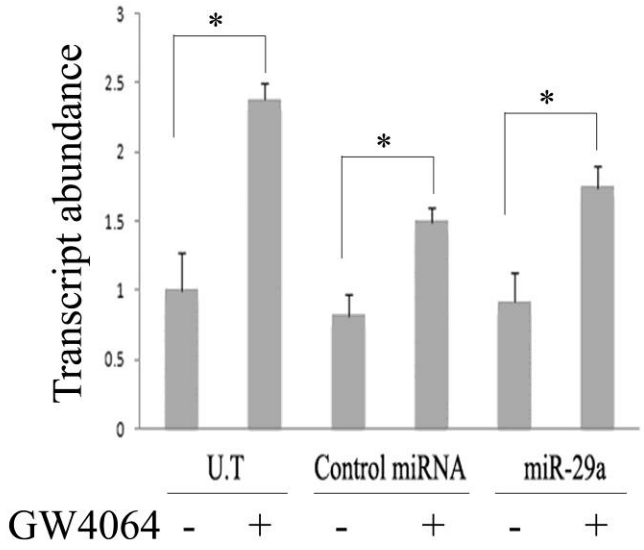
Supplemental Figure 3. miR-29a expression is induced in mouse liver by FXR ligand treatment. Mice were treated for 7 days with vehicle or the synthetic FXR agonist GW4064 (100 mg/kg). The expression level of miR-29a was determined by real-time RT-PCR. N = 3. *P <0.05 (vs. vehicle).



Supplemental Figure 4. Overexpression of miR-29a resulted in downregulation of the mRNA expression of ECM genes in rat HSCs in a dose dependent manner without affecting the mRNA expression of SHP or FXR. HSCs were transfected with different concentrations of miR-29a mimic (5~500 nM) or non-specific control miRNA mimic (500 nM) for 24 h. The mRNA expression levels of COL1A1, COL3A1, FXR and SHP were then determined respectively by real-time RT-PCR. N = 3. $P < 0.05$ (vs. control miRNA).



Supplemental Figure 5. Overexpression of miR-29a resulted in downregulation of the expression of COL1A1 protein in rat HSCs in a dose dependent manner without affecting the protein expression of FXR. Rat HSCs were transfected with different concentrations of miR-29a mimic (5~500 nM) or non-specific control miRNA mimic (500 nM) for 24 h. Protein expression levels of COL1A1 and FXR were determined by Western blot. Shown in the figure were representative data from three independent experiments.



Supplemental Figure 6. Overexpression of miR-29a does not affect FXR-mediated SHP induction following GW4064 treatment. HSCs were transfected with miR-29a mimic or non-specific control miRNA mimic (50 nM) for 24 h followed by treatment with GW4064 or DMSO vehicle for another 24 h. The mRNA expression level of SHP was then determined by real-time RT-PCR. N = 3. $P < 0.05$ (vs. control miRNA).