Published in final edited form as:

Biochem Biophys Res Commun. 2011 December 9; 416(1-2): 135–139. doi:10.1016/j.bbrc.2011.11.011.

Regulation of miR-200c by Nuclear Receptors PPAR α , LRH-1 and SHP

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Abstract

We investigated regulation of miR-200c expression by nuclear receptors. Ectopic expression of miR-200c inhibited MHCC97H cell migration, which was abrogated by the synergistic effects of PPAR α and LRH-1 siRNAs. The expression of miR-200c was decreased by PPAR α /LRH-1 siRNAs and increased by SHP siRNAs, and overexpression of the receptors reversed the effects of their respective siRNAs. SHP siRNAs also drastically enhanced the ability of the LRH-1 agonist RJW100 to induce miR-200c and downregulate ZEB1 and ZEB2 proteins. Co-expression of PPAR α and LRH-1 moderately transactivated the miR-200c promoter, which was repressed by SHP co-expression. RJW100 caused strong activation of the miR-200c promoter. This is the first report to demonstrate that miR-200c expression is controlled by nuclear receptors.

Keywords

nuclear receptor; microRNA; transcription; cell migration

1. Introduction

MicroRNA-200c was initially identified in the lung [1]. Its functional association with cancer invasion resulted from a study showing that overexpressing miR-200c downregulated TCF8 but increased E-cadherin expression in non-small-cell lung cancer and breast cancer cells [2]. Further studies demonstrated that miR-200c was downregulated in cells that had undergone epithelial to mesenchymal transition (EMT) and that miR-200c regulated EMT by targeting ZEB1, SIP1 and class III beta-tubulin (TUBB3) [3; 4; 5]. Thus, the loss of miR-200c expression was associated with an invasive cancer phenotype [6]. Subsequent studies observed miR-200c overexpression in human colon cancer [7], ovarian cancer [8], and pancreatic ductal adenocarcinomas [9]. Interestingly, miR-200c expression was downregulated in renal cell carcinoma [10]. miR-200c also sensitized cells to CD95-

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Competing interests

The authors declare that they have no competing interests.

mediated apoptosis by targeting FAP-1 [11]. Downregulation of miR-200c was found in breast cancer stem cells (BCSCs) and ectopic expression of miR-200c inhibited tumor formation by BCSCs [12]. Overall, miR-200c appears to be decreased in some cancers and increased in others.

Despite its important function in cancer invasion, how the transcription of miR-200c is regulated remains largely unknown. ZEB1 was shown to repress miR-200c expression in a negative feedback loop [13]. Silencing of miR-200c by DNA methylation [14] correlated with the invasive capacity of human breast cancer cell lines [15] and bladder cancer [16]. This study for the first time investigated transcriptional control of miR-200c expression and function by nuclear receptors in liver cancer cells.

2. Materials and methods

2.1. Plasmids, siRNAs and antibodies

PPAR α expression plasmid and adenovirus were provided by Drs. Thurl Harris [17] and Clay Semenkovich [18], respectively. Antibodies to LRH-1 (ab18293), PPAR α (ab2779), ZEB1 (ab64098), and ZEB2 (ab25837) were purchased from Abcam. Western blots were performed following standard procedures [19; 20]. Small interfering RNAs (siRNAs) for PPAR α (SASI_Mm02_00319988) and non-specific RNAi (SIC001) were purchased from Sigma. SiRNAs for SHP (ONTARGETplus SMARTpool NR0B2, L-003410) and LRH-1 (NR5A2 ONTARGET plus SMART pool [L-003430-00-0005]) were purchased from Thermo Scientific Dharmacon RNAi Technologies.

2.2. Cell migration assays

MHCC 97H cells were transfected with the indicated siRNAs and miR-200c for 6 hr. The cells were then serum starved for 24 hr and 5×10^4 cells were seeded on Transwell inserts (8 μ m pore size; culterx 96 well cell migration assay, cat#3465-095-K). Cells were allowed to migrate for 16 hr, and the non-migratory cells were removed from insert with a cotton swab. The migrated cells were fixed for 10 min (3.7% v/v formaldehyde in PBS) before staining with 0.1% crystal violet for 15 min, followed by washing with PBS. Pictures were taken by Microfire/Qcam CCD Olympus 1 $\times$ 81 microscope. Crystal violet stained cells were lysed with 1%SDS for 30 min and absorbance was measured at 595 nm.

2.3. Analysis of miRNA expression in agonist treated cells

Huh7 cells were grown to 60–70% confluence and treated with 25 μ M of GW7647 (Sigma) or RJW100 [21; 22] for 24 hr. Cells treated with ethanol served as negative control. Total RNAs including miRNAs were isolated from cells using a miRNeasy mini kit and reverse transcribed using a miRNA reverse transcription kit. miR-200c levels were quantified by qPCR using miRNA primer assays Kit (Qiagen, Valencia, CA). All the kits were used according to the manufacturer's instructions. U6 transcript was used as an internal control to normalize RNA input.

2.4. Transient transfection and luciferase assays

The luciferase reporters containing upstream fragments (~1 kb, 2 kb, and 3 kb) of pri-miR-200c (hmiR-200c Luc) were engineered in our laboratory. Dr. Muneesh Tewari provided us a 0.9 kb hmiR-200c promoter reporter [23], which was used as a control. In brief, human hepatoma Huh7 cells were transfected with the plasmids as indicated in the figure legends. Transfection was carried out using Lipofectamine 2000 (Invitrogen). Luciferase activities were measured and normalized against Renilla activities (Promega). Consistent results were observed in three independent triplicate transfection assays.

2.5. Statistical analysis

All the experiments were repeated at least three times, and the error bars represent the standard error of the mean (SEM). Statistical analyses were carried out using Student's unpaired t test; $p < 0.01$ was considered statistically significant.

3. Results

3.1. Knockdown of PPAR α and LRH-1 antagonizes miR-200c inhibition of HCC cell migration

Because putative DNA binding elements for peroxisome proliferator activated receptor alpha (PPAR α) and liver receptor homolog-1 (LRH-1) were predicted in the has-miR-200c promoter, we first examined the functional association between miR-200c and PPAR α /LRH-1. Cell migration assays were performed using liver cancer MHCC97H cells that have a high invasive potential. Overexpression of miR-200c markedly inhibited MHCC97H cell migration, and the effect of miR-200c was abrogated by its inhibitor (Fig. 1A–1B). Knockdown of endogenous PPAR α or LRH-1 (Fig. 1C) using their respective siRNAs did not alter the basal rate of cell migration but did reverse the inhibitory effect of miR-200c. Co-knockdown of PPAR α and LRH-1 dramatically stimulated basal cell migration as well as antagonized the ability of miR-200c to inhibit MHCC97H cell migration. The results suggest that PPAR α and LRH-1 may act synergistically to repress cell migration that is mediated by miR-200c.

3.2. The expression of miR-200c is activated by PPAR α and LRH-1 and inhibited by SHP

Small heterodimer partner (SHP) is a transcriptional repressor that interacts with PPAR α and LRH-1 [24]. Single or combinational knockdown or overexpression of PPAR α , LRH-1, and SHP were utilized to assess regulation of miR-200c expression by these receptors. Huh7 cells express all three receptors and were thus used for knockdown experiments.

Knockdown of PPAR α alone did not decrease miR-200c (Fig. 2A, #2 vs. 1), whereas overexpression of PPAR α induced miR-200c expression (#3 vs. 1). LRH-1 siRNAs reduced miR-200c expression (#4 vs. 1), and overexpression of LRH-1 counteracted this effect (#5 vs. 4). Co-transfection of siRNAs against both PPAR α and LRH-1 exhibited a more dramatic effect in downregulating miR-200c (#6 vs. 2&4), and the effect was reversed by co-expression of both receptors (#7 vs. 6). Knockdown of SHP upregulated miR-200c (#8 vs. 1), which was reversed by SHP overexpression (#9 vs. 8). SHP siRNAs also antagonized single or combinational effects of siPPAR α and siLRH-1 in inhibiting miR-200c expression (#10, 11&12 vs. 2, 4&6). Interestingly, miR-200c expression was moderately induced by the LRH-1 agonist RJW100 (#15 vs. 13), but not by the PPAR α agonist GW7647 (#14 vs. 13), and siSHP further enhanced the effect of RJW100 (#18 vs. 15). The overexpression and knockdown of PPAR α and LRH-1 proteins is presented in Fig. 2B. Overall, the results suggest that SHP is able to repress miR-200c expression by counteracting the effects of PPAR α and LRH-1.

We also analyzed protein levels of the miR-200c target genes ZEB1 and ZEB2. ZEB1 and ZEB2 proteins were markedly downregulated by RJW/siSHP, but not by GW/siSHP (Fig. 2C). The data suggest that the RJW/siSHP combination could be useful in targeting ZEB1/ZEB2 in cancer intervention, which will be explored in future studies.

3.3. The promoter activity of miR-200c is inhibited by SHP via PPAR α and LRH-1

Several putative PPAR α binding sites (PPRE) and LRH-1 binding sites (LRE) were predicated within a 3 kb genomic region upstream of pre-hsa-miR-200c. We cloned luciferase reporters containing upstream fragments (~1 kb, 2 kb, and 3 kb) of pri-miR-200c.

Strong activation of hmiR-200c promoters by individual expression of PPAR α /RXR α or LRH-1 was not observed. A half PPRE site overlapped with a LRE (–835 nt ~ –816 nt) in the 1 kb miR-200c promoter, suggesting that both receptors may be required to bind to the same response elements. Indeed, co-expression of PPAR α /RXR α and LRH-1 was able to activate the promoter (Fig. 3A, lane 4 vs. 1), although the activation appeared to be moderate. Nonetheless, EID1 (a known repressor of nuclear receptor) [25] or SHP decreased the reporter activity to basal levels (lane 5 & 6 vs. 4). Surprisingly, the LRH-1 agonist RJW100, but not PPAR α agonist GW7647, strongly induced hmiR-200 promoter activity in Huh7 cells (Fig. 3B). A more dramatic effect of RJW100 was observed in Hela cells (not shown). The results suggest that a weak response element for PPAR α and/or LRH-1 may exist in this promoter region.

4. Discussion

Our recent studies identified SHP as a transcriptional repressor of miR-433 and miR-127 expression through inhibition of the activity of ERR γ [26; 27]. PPAR α also exhibited a moderate activation of the miR-433 promoter [26]. The present study suggests that SHP represses miR-200c expression, at least in part, by inhibiting the activity of PPAR α and LRH-1. Thus, SHP may inhibit the expression of miRNAs through common interacting partners and regulatory pathways.

Although functional and expression studies demonstrate that PPAR α and LRH-1 are both activators of miR-200c, results from promoter studies are rather disappointing. One possibility is weak binding of PPAR α and LRH-1 to the several non-consensus PPRE and LRE predicted within the 3 kb miR-200c promoter. Therefore the presence of both receptors is necessary for the activation of miR-200c promoter. The other possibility is that the upstream region of pre-miR-200c that we studied may not serve as the endogenous miR-200c promoter. At present, the transcriptional start site (TSS) of the full length primary miR-200c transcript remains unclear, and its identification may be necessary to resolve the role of nuclear receptors in activation of the miR-200c promoter.

Several studies have reported indirect regulation of miRNA expression by nuclear receptor antagonists or agonists [28; 29; 30]. It is worth noting that the expression of miR-200c can be induced by the pharmacologically active compound for LRH-1. This is of particular importance concerning the critical function of LRH-1 in metabolic diseases. It is plausible that miR-200c may play a role in metabolic regulation. On the other hand, despite the important function of miR-200c in tumor invasion, no report has identified synthetic agonists to activate miR-200c. Our study may provide a means to design such compounds to target miR-200c for cancer therapeutic applications.

Highlights

- Knockdown of PPAR α and LRH-1 abolishes miR-200c inhibition of HCC cell migration
- SHP represses miR-200c expression via inhibition of the activity of PPAR α and LRH-1
- RJW100 exhibits strong ability to downregulate ZEB1 and ZEB2 proteins

Acknowledgments

We are very grateful to Drs. Thurl Harris (University of Virginia), Clay Semenkovich (Washington University), and Muneesh Tewari (Fred Hutchinson Cancer Research Center) for generously providing the plasmids and

adenoviruses. Y.Z. is supported by NIH T32CA092347 Multidisciplinary Cancer Research Training Program (MCRT). This work is in part supported by National Institutes of Health DK080440 to L.W.

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Fig. 1. Effects of siRNA knockdown of PPARα and LRH-1 on MHCC97H cell migration mediated by miR-200c

(A–B) MHCC97H cells were transfected with miR-200c plasmid (1 μg, 12 well plate) without or with its inhibitor (inh, 40 pmol), in the absence or presence of siRNAs against PPARα (siPa, 40 pmol) and LRH-1 (siL, 40 pmol). Cells were seeded onto Transwell inserts and allowed to migrate for a period of 16 hr. Non-migratory cells were removed, and cells that had migrated through the membrane were fixed and stained with crystal violet. Cell migration was visualized by microscopy (A) and quantified by measuring the absorbance of cell lysates at 595 nm (B). Data are expressed as means ± SEM of duplicate assays (n=3/assay). *p<0.01 vs. negative control siRNA (neg); †p<0.01 vs. miR-200c alone; ‡p<0.01 vs.

negative control and miR-200c alone. (C) Western blots (WB) of PPAR α and LRH-1 proteins to confirm the knockdown efficiency of either receptor by their respective siRNAs.

Fig. 2. Induction of miR-200c expression by PPARα and LRH-1 and inhibition by SHP

(A) Relative miR-200c expression in Huh7 cells that were transfected with siRNAs against PPARα (25 pmol), LRH-1 (25 pmol), or SHP (25 pmol), or with expression vectors for PPARα (1 μg), LRH-1 (1 μg), and SHP (1 μg), alone or in combination, in the absence or presence of GW7647 (25 μM) and RJW100 (25 μM). Real-time PCR was used to assess the miR-200c levels which were normalized by U6 expression. Statistical results represent mean ± SEM of triplicate assays (n=3). *p<0.01 vs. control (#1); †p<0.01 vs. siRNAs against PPARα, LRH-1, or SHP (#2, 5, 7, 9). ‡p<0.01 vs. siRNAs against PPARα and LRH-1 (#2, 4, 6); ¶p<0.01 vs. ethanol control (EtOH, #13); §p<0.01 vs. RJW100 (RJW) (#15). (B) Western blots of PPARα and LRH-1 proteins in Huh7 cells transfected with plasmids or siRNAs for either receptor. (C) Western blots of ZEB1 and ZEB2 proteins in Huh7 cells transfected with control (siNeg) or SHP siRNAs (siSHP) in the absence or presence of GW7647 (25 μM) or RJW100 (25 μM).

Fig. 3. Promoter activation of miR-200c by PPARα and LRH-1 and inhibition by SHP

(A) Transient transfection assays to determine human miR-200c promoter activity by PPARα/RXRα (200 ng), LRH-1 (200 ng), EID1 (200 ng), and SHP (200 ng) in Huh7 cells.

(B) Transient transfection assays to determine human miR-200c promoter activation by PPARα agonist GW7647 (25 μM) and LRH-1 agonist RJW100 (25 μM) in Huh7 cells. A–B: Luciferase (luc) activities (act) were determined and normalized by Renilla activities.

Data are expressed as means ± SEM of triplicate assays (n=3). The experiments were repeated two to three times, and one representative result is shown. *p<0.01 vs. control.