Biochemical and Biophysical Research Communications 464 (2015) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

miR-29a promotes scavenger receptor A expression by targeting QKI (quaking) during monocyte-macrophage differentiation

Shuai Wang [1], Jie Zan [1], Mingjie Wu, Wenting Zhao, Zhenwei Li, Yanyun Pan, Zewei Sun, Jianhua Zhu*

Department of Cardiology, First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, PR China

ARTICLE INFO

Article history:
Received 22 April 2015
Accepted 7 May 2015
Available online 6 June 2015

Keywords:
Differentiation
QKI
SRA
miR-29a and lipid uptake

ABSTRACT

Monocyte differentiation into macrophages results in upregulation of miR-29a and scavenger receptor A (SRA) expression, while the expression of RNA binding protein, QKI is suppressed. Since SRA is a functionally important protein in atherosclerosis, it is imperative to understand the various mechanisms involved in its regulation specially the mechanism involving miR-29a. There are individual studies linking miR-29a to SRA or QKI to monocyte differentiation but there is no evidence of any linkage among them. Therefore, we intend to investigate the association among these three, if any, in terms of regulation of SRA expression. Hence, in this study, the differentiated macrophages were initially transfected with miR-29a or its inhibitor and it was shown that QKI is a direct target of mir-29a. In addition, it was also observed by bioinformatics analysis that 3′UTR in SRA mRNA has QKI binding site. So, we attempted to further understand the role of QKI in SRA regulation. The macrophages were manipulated either with overexpression of QKI or by its ablation and it was observed that QKI suppressed SRA at the transcriptional level. Moreover, with the help of luciferase reporter vector, it was shown that QKI inhibited SRA transcription by binding to QRE region in its 3′UTR mRNA. Furthermore, to link the QKI mediated regulation of SRA expression with its functional activity; we analyzed lipid uptake capacity of macrophages transfected with either ectopic OKI plasmid or ablated for QKI. It was observed that, indeed, QKI upregulation inhibits lipid uptake by repressing SRA expression. Overall, our study demonstrates that miR-29a inhibits QKI, which in turn results in upregulation of SRA and lipid uptake.

1. Introduction

Macrophages play an important role in atherosclerosis. They impact atherosclerotic lesions by incorporating modified lipoproteins and transform themselves into lipid rich foam cells [1], which are the hallmark of atherosclerosis. The important pathway that contributes to this transformation of macrophages is scavenger receptor pathway. Scavenger receptor A (SRA) is a membrane protein, mainly expressed in macrophages, endothelial cells and smooth muscle cells [2]. Atherosclerotic lesions are significantly decreased in mice that are fed on western diet when they lack SRA [3]. In previous study, it has been suggested that during monocyte-macrophage differentiation, SRA expression is regulated by signal transduction pathways involving, *ras*, ets and AP-1 domain proteins [4]. But, recently it has been suggested that miR-29a can also result in SRA upregulation but the exact mechanism is missing [5].

The RNA binding protein Quaking (QKI), a member of highly conserved signal transduction and activator of RNA (STAR) family of RNA-binding proteins, participate in various physiological activities including gastric cancer [6], myocardial ischemia [7], oligodendrocyte [8] and gastrointestinal epithelium [9] differentiation, and myelination of Schwann cells [10]. During monocyte differentiation into macrophage, its expression is down regulated [11]. Bioinformatics data suggest that mRNA of SRA gene contains QRE (quaking response element) at its 3′UTR region. Now, if QKI has any regulatory function with respect to SRA expression during monocyte-macrophage differentiation, requires further investigation.

The microRNAs, which are about 22 nucleotide RNA molecules, are mainly involved in suppressing gene expression through

* Corresponding author.
E-mail address: zjh_john@medmail.com.cn (J. Zhu).
[1] Contributed equally to this study.

http://dx.doi.org/10.1016/j.bbrc.2015.05.019
0006-291X/

binding to 3′UTR of target mRNA and inhibit protein synthesis [12,13]. Among different microRNAs, miR-29a is involved in the functioning of multiple biological processes. By targeting HBP1 (HMG box-containing protein-1) [14] or PTEN [15], miR-29a promotes angiogenesis. It has also been shown to influence myogenesis in chronic kidney disease (CKD) [16]. Moreover, mir-29a also regulates insulin secretion [17], manifestation of amyotrophic lateral sclerosis (ALS) disease [18], myocardial ischaemia-reperfusion injury [19] and immune responses to intracellular bacterial infection [20]. During GM-CSF, IL-6 and Flt-3L mediated monocyte-macrophage differentiation, miR-29a levels are increased [21].

Therefore, based on information in the literature, one can conclude that during monocyte-macrophage differentiation, miR-29a and SRA expression were increased whereas QKI levels were decreased. But the exact regulatory mechanism and the cause and consequence type of correlation between these three candidates, if any, is missing. Therefore, we wanted to test that if, a) QKI down regulation has any correlation with both miR-29a and SRA up-regulation, and b) QKI contribution to monocyte-macrophage differentiation process involves SRA regulation. Hence, in this study, we have used macrophages that were differentiated from human primary monocytes by GM-CSF treatment, to investigate the relationship among miR-29a, SRA and QKI genes.

2. Materials and methods

All the experiments involving human blood samples had prior approval from the ethics committee of experimental research at Zhejiang University and were in conformation with the principles emphasized in the declaration of Helsinki. In addition, we also had the written consent forms signed by the healthy donors.

3. Cell culture and transfection

Human primary monocytes were isolated from the peripheral blood of healthy donors using lymphocyte separation kit (Sigma, CA, USA). Cells were cultured in complete RPMI-1640 (Gibco, USA) medium supplemented with 10% fetal bovine serum (Sigma, CA, USA) and 100 ng/ml of GM-CSF (RD, USA) for 7 days to differentiate into macrophages. Cell culture medium was replaced with fresh complete medium every other day. 293T cells were also cultured in RPMI-1640 complete medium including 10% fetal bovine serum and were passaged every other day.

The siRNA transfection was performed according to manufacturer's protocol. 1×10^6 cells in each well were cultured before transfection into six-well plates in 1 ml serum-free medium. The 5 μl RNAiMax (Life Technologies, USA) and 10 μl siRNA (50 nmol, Baiao Shanghai, China) were mixed in 200 μl RPMI-1640 medium. After 15 min incubation, mixture was added to the target cells. Finally, cells were harvested after 24 h and analyzed further. For transfection of plasmid cDNAs, 3 μl of P3000TM enhancer reagent (Life Technologies, USA) was mixed with 2.5 μg of plasmid cDNA in a 100ul RPMI-1640 medium. In a separate tube, 3 μl of lipofectamine3000 (Life Technologies, USA) was also added in 100ul RPMI-1640 medium. After 5 min incubation, the mixture from both the tubes were combined and added to the target cells in six-well plates with complete medium. After, 24 h, cells were harvested and analyzed further.

4. Western blotting

Harvested cells were lysed to extract protein using RIPA lysis buffer (Beyotime China). After estimating the protein concentration from the lysates, 30 μg of total protein was loaded into 10% SDS-PAGE gel. Following electrophoresis, proteins were transferred onto PVDF membranes (Millipore, USA), which were incubated with primary antibodies at 4 °C overnight. After washing three times with PBS for a total of 15 min, membranes were incubated with specific secondary antibodies (1:10000, Kangwei, China) for 1 h at room temperature. Later the signals were detected by chemiluminescence using ECL kit (Pierce, USA). Primary antibodies used were as follows: rabbit anti-QKI (1:1000, Abcam, USA), mouse anti-SRA (1:1000, Millipore, USA) and rabbit anti-GAPDH (1:1000, CST, USA).

5. RT-PCR analysis

Total RNA was extracted by using the RNA elution kit (Tiangen, China) for mRNA isolation and miRcute miRNA isolation kit (Tiangen, China) for microRNA isolation. Subsequently, cDNA was generated by using Primescript 1st strand cDNA synthesis kit (Takara, Japan) or the one step primescript miRNA cDNA synthesis kit (Takara). SYBR premix Ex taq II (Takara, Japan) master mix was used for performing RT-PCR reactions and the amplicons were detected through ABI PRISM 7500 Detection System (ABI USA). 18S or U6 RNA was measured as endogenous control for mRNA detection or microRNA detection respectively. The following primers were used, 18S F: GTAACCCGTTGAACCCCATT, R: CCATCCAATCGGTAGTAGCG; QKI F: ACGGCTTCGAGGATCAGATTC R: TACAGGATCATTGGCTACACACC; U6 F: ACTTGCTCATCAAGGTGTCAG R: TGACCAGCGTTTGTTCAATGT. F: miR-29a ACUGAUUUCUUUUGGUGUUCAG R: uni-miR qPCR primer (TTTTTTTTTTTTTTTTTTTT) (Takara Japan).

6. Luciferase reporter assay

The 3′UTR region of QKI or SRA were first amplified by PCR. Next, the PCR products of QKI and SRA 3′UTR regions containing wild-type and mutant binding sites [11]of miR-29a or QRE respectively were gel purified. After purification, they were ligated into pGL3 vector (Promega) between HindIII and SacI restriction sites. Thereafter, macrophages were co-transfected with either 750 ng of PGL3-QKI-3′UTR alone or in combination with scramble miRNA or miR-29a (50 nM each) using Hiperfect transfection reagent (Life Technologies, USA). Cells transfected with 750 ng of PGL3-basic alone or in combination with scramble miRNA or miR-29a (50 nM each) were used as controls. In addition, 20 ng of renilla luciferase plasmid was also co-transfected as the endogenous control. After 48 h of transfection, cells were washed and lysed with reporter gene lysis Buffer (Promega USA), and firefly luciferase activities were detected using a luminometer. The luciferase reporter activity was normalized to the control renilla luciferase activity.

7. RNA-binding protein immunoprecipitation (R–IP)

R–IP was performed using Magna RIP kit (Millipore, USA). Briefly, the cells were harvested after washing two times with 1X PBS and then lysed with RIP lysis buffer. The proteins were immunoprecipitated with antibodies against Ago2 (Abcam, USA), IgG, or GFP (CST, USA) for 24 h. The 50 μl A/G magnetic beads were added to the supernatant. After immobilizing magnetic beads bound complexes with a magnetic separator (Millipore USA), supernatant were used to extract RNAs with PCA (phenol:-chloroform:isoamyl alcohol) reagent at a ratio of 125:24:1 (all chemicals were purchased from Aladdin USA). CDNA synthesis kit (ABI USA) was used to synthesize the 1st cDNA strand. Finally, quantitative RT-PCR was performed for analysis using SYBR green master mix (ABI USA).

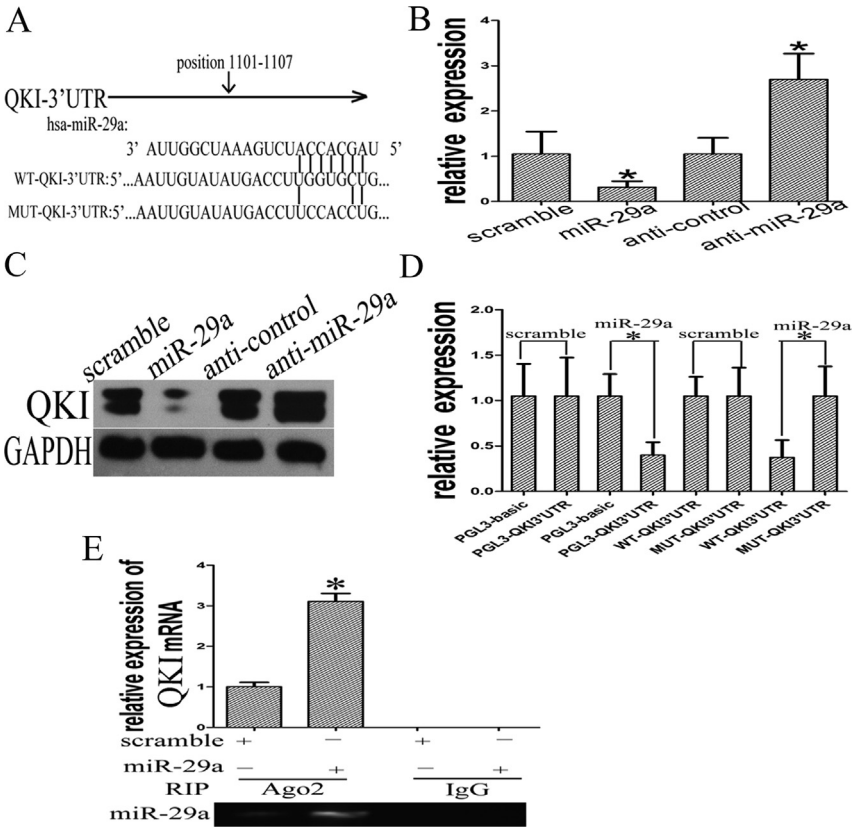


Fig. 1. Identification and validation of QKI as miR-29a target. A, Bioinformatics analysis confirm the presence of miR-29a binding site in the 3′UTR of QKI mRNA; B, Analysis of QKI mRNA expression in the presence of miR-29a or its inhibitor with relevant controls by RT-PCR ($p < 0.05$); C, Expression of QKI at protein level under similar conditions. GAPDH expression is a loading control; D, RT-PCR analysis of QKI expression in the cells transfected with either wild type or miR-29a binding site mutant 3′UTR plasmid construct with relevant controls ($P < 0.05$); E, RNA-binding protein immunoprecipitation (RIP) analysis with Ago2 or IgG antibodies in lysates of macrophages transfected with scramble sequence or miR-29a ($P < 0.05$).

8. Oil red staining

After transfection, cells were treated with oxLDL (40 μg/ml) for 24 h. Following the treatment, cells were washed twice by PBS and then stained with 1 ml working solution of oil red stain (composed of 70% oil red solution and 30% alcohol). Later, the cells were again washed twice with PBS at 37 °C and were incubated with 1 ml of hematoxylin solution per well. Finally, after PBS washing, cells were observed under light microscope (Olympus Japan). All chemicals were purchased from Jiancheng company (China).

9. High performance liquid chromatography (HPLC)

Macrophages were transfected with QKI plasmid or siRNA for 24 h. After transfection, cells were incubated with oxLDL (40 μg/ml) for another 24 h and were then harvested for sterol analyses using high performance liquid chromatography (HPLC) system (Milford, USA). A photodiode array detector was used for detecting sterols. After elution with acetonitrile-isopropanol (30v/70v), total and esterified cholesterol were detected by absorbance at 210 nm.

10. Statistical analysis

Data are presented as mean ± standard deviation (SD). The differences between two groups were compared by t-test. The P value of <0.05 was considered statistically significant and all experiments were repeated at least three times.

11. Results

11.1. Analysis of miR-29a and QKI expression in differentiated macrophages

First, the macrophages differentiated from monocytes isolated from peripheral blood were analyzed for MiR-29a expression by RT-PCR. As seen in S-Fig. 1A, miR-29a expression increased over a period of 7 days, when monocytes were treated with 100 ng/ml of GM-CSF. Furthermore, in the same time frame, QKI protein expression as analyzed by western blotting was suppressed as seen in S-Fig. 1B.

11.2. Identification of QKI as a target of miR-29a

Bioinformatics prediction programs, TargetScan and PicTar, identified that 3′UTR region of QKI has mir-29a target sequences as shown in Fig 1A. To confirm if miR-29 can regulate QKI expression directly, miR-29a mimic (cggactgatttcttttggtgttcag) and siRNA (cggtgactaaagaaaaccacaagtc), which imitate ectopic expression or knockdown of miR-29a respectively, were used to treat differentiated macrophages. RT-PCR results showed that miR-29a mimic decreased QKI mRNA levels whereas miR-29a siRNA enhanced as observed in Fig 1B. In addition, we also analyzed the protein expression of QKI with different miR-29a manipulations and the results as seen in Fig 1C, are consistent with expectations. Next, we analyzed if miR-29a based regulation of QKI expression involved targeting of 3′UTR. Luciferase reporter plasmid carrying WT-QKI-

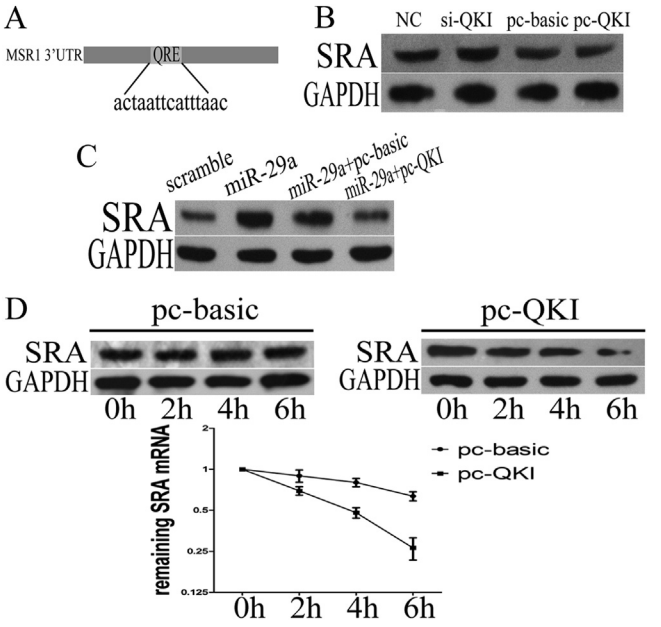


Fig. 2. Negative regulation of SRA expression by QKI. A, Bioinformatics analysis confirm QKI binding site in 3′UTR of SRA mRNA; B, Analysis of SRA protein expression by QKI manipulation (overexpression or ablation). GAPDH signal is a loading control; C, Analysis of SRA protein expression in the presence of either miR-29a alone or in combination with QKI overexpression with relevant controls. GAPDH signal is a loading control; D, Analysis of SRA expression at protein and mRNA levels at different time points in cells transfected with either pc-GFP vector or pc-QKI plasmid and treated with actinomycin D to inhibit new mRNA synthesis ($p < 0.05$).

3′UTR or mut-QKI-3′UTR (mutation at miR-29a target) sequence as shown in Fig 1A, were transfected to macrophages. These cells were then further transfected with scramble miRNA or mir-29a. As observed in Fig 1D, the relative luciferase activity was reduced by 60% when miR-29a was transfected with WT-QKI-3′UTR plasmid as compared to control. In addition, miR-29a transfection to mut-QKI-3′UTR plasmid containing cells, did not show any difference in the luciferase activity in comparison to the control transfection. Furthermore, we analyzed the step in the RNA-induced silencing complex (RISC), where miR-29a can regulates QKI. Herein, we used Ago2 antibody to co-precipitate the complex including QKI mRNA, as Ago2 is the essential component of RISC, which contains target mRNA and Ago2 [22]. As seen in Fig 1E, Ago2 immunoprecipitation resulted in pulling more QK1 mRNA in the presence of mir-29a than the scramble sequence. This suggests that miR-29a promotes the formation of QKI mRNA with Ago2 and regulates QKI post-transcriptionally.

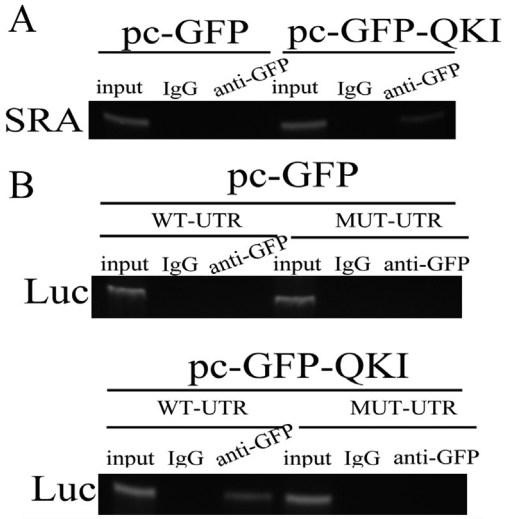


Fig. 3. Confirmation of QKI binding to 3′UTR of SRA mRNA. A, R–IP analysis with IgG and anti-GFP antibody in the cells ectopically expressing either GFP vector or QKI-GFP fusion protein; B, Similar R–IP analysis with IgG and anti-GFP antibody in 293T cells expressing either GFP vector or QKI-GFP fusion protein along with SRA 3′UTR reporter vector either with wild-type or mutant QRE. The wild-type QRE luciferase mRNA was only immunoprecipitated with GFP-QKI, where as there is no signal with mutant QRE 3′UTR SRA mRNA.

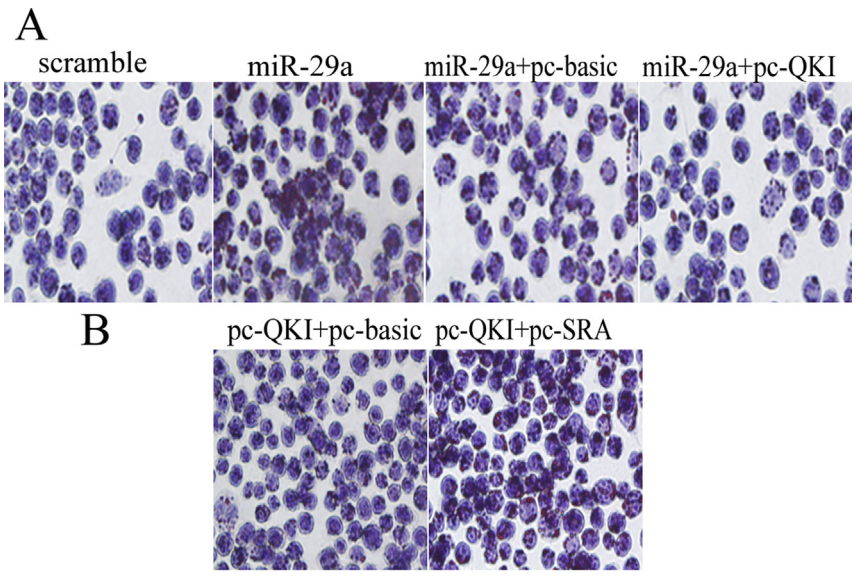
11.3. QKI negatively regulates SRA expression

It has been observed earlier that SRA expression is increased by mir-29a in dendritic cells [5]. But the exact mechanism of miR-29a regulation of SRA expression is not clear. Thus, we explored the possibility of QKI mediated regulation of SRA, because QKI is a direct target of miR-29a. Herein, we first analyzed 3′UTR of SRA gene by bioinformatics analysis and identified that it contains a QRE site, a potential binding target of QKI gene (Fig 2A). Next, the ectopic expression or ablation of QKI altered the SRA protein expression as expected and shown in Fig 2B. Since QKI is a target of miR-29a, we intend to find if mir-29a mediated regulation of SRA expression involves QKI. To address this issue we transfected miR-29a alone or in combination with QKI plasmid. The miR-29a enhanced SRA expression as seen in Fig 2C, but over expression of QKI with miR-29a reversed this enhanced expression, thus suggesting that SRA regulation by miR-29a, does involve QKI. Moreover, as QKI is an RNA binding protein, we wanted to test the mechanism of its regulation of SRA expression. To test this, we transfected macrophages with vector alone or QKI plasmid. Post transfection, cells were treated with Actinomycin D, which is used to inhibit mRNA transcription. Thereafter, we detected the half-life of SRA mRNA and protein by doing RT-PCR and western blotting at different time points post Actinomycin D treatment. RT-PCR results showed that expression of QKI in macrophages promotes degradation of SRA mRNA as seen in Fig 2D, with a half life of about 4 h. In addition, the western blot data in Fig 2D also suggested that even the protein expression was inhibited with half life of little more than 4 h. This eventually confirmed that QKI destabilize SRA mRNA and thus regulating its protein expression.

To further elucidate if QKI mediated regulation of SRA expression involve direct targeting of QRE at 3′UTR sequence of SRA mRNA, we constructed the luciferase reporter vectors containing 3′UTR sequence of SRA mRNA with wild-type or mutant QRE region. Also, QKI was expressed in GFP vector. RNA immunoprecipitation with anti-GFP antibody pulled SRA mRNA only when GFP was in fusion with QKI gene as shown in Fig 3A. In addition, GFP-only or GFP-QKI ectopic expression plasmid were cotransfected with reporter vector having either wild-type or mutant QRE 3′UTR region of SRA mRNA in 293T cells. The R–IP analysis showed that only SRA mRNA with wild type QRE sequence was pulled with anti GFP antibody and not QRE mutant 3′UTR mRNA (Fig 3B). This suggested that QKI bind to the QRE site in the SRA mRNA 3′UTR.

11.4. QKI inhibited lipid uptake through targeting SRA in macrophages

Since we showed that miR-29a promotes SRA expression and involves QKI in this regulation, we wanted to correlate the changes in SRA expression with its lipid uptake function. As shown in Fig 4A, miR-29a promotes lipid uptake in macrophages, but ectopic expression of QKI reversed the miR-29a induced lipid uptake. Furthermore, when QKI plasmid was cotransfected with SRA



C

	Total Cholesterol	Esterified Cholesterol	EC/TC%
NC	571±13.6	336±15.6	59±1.83%
si-QKI	725±9.4*	514±11.9*	71±2.15%
pc-basic	559±18.7	313±16.0	56±1.79%
pc-QKI	386±10.5*	166±7.9*	43±1.54%

Fig. 4. QKI suppressed lipid uptake by macrophages. Panel A, suggest that miR-29a expression promotes lipid uptake while ectopic expression of QKI reverse miR-29a mediated lipid uptake; Panel B, depicts that SRA overexpression reversed the inhibitory effect of QKI on lipid uptake by macrophages; Panel C, depicts the levels of total and esterified cholesterol in oxLDL treated macrophages either overexpressing QKI or ablated for its expression ($P < 0.05$).

plasmid in macrophages, we observed that SRA overexpression was able to reverse the inhibitory effect of QKI on lipid uptake (Fig 4B). Moreover, this inhibitory effect of QKI on lipid uptake was further verified by HPLC analysis of the levels of total cholesterol and esterified cholesterol in macrophages either overexpressing or inhibiting QKI. As observed in Fig 4C, knockdown of QKI expression induced lipid uptake significantly, while its ectopic expression suppressed it in macrophages. These experiments thus, suggested that QKI inhibit lipid uptake by targeting SRA.

12. Discussion

Since SRA protein play an important role in lipid uptake by macrophages and subsequently in the progression of atherosclerosis, it is important to understand the different pathways contributing to its gene regulation. In this study, we have tried to elucidate a novel mechanism that regulates SRA expression. Based on the previously published studies by us and other labs, it has been shown that during monocyte differentiation into macrophages, a) QKI expression is inhibited [11,5], and, b) miR-29a enhanced SRA expression [5,18]. So, this led us to ask a question, how miR-29a is regulating SRA expression and is it possible that these three genes are inter-related in terms of their regulation of each other. The other reason to understand miR-29a mediated regulation of SRA is due to the importance of miRNAs as novel therapeutic targets.

Thus, in this study we first confirmed that indeed during monocyte differentiation to macrophages by GM-CSF treatment, there is an upregulation of miR-29a and inhibition of QKI expression. This observation was consistent with previous published studies involving miR-29a and QKI [11,21]. On further analysis, it was identified that QKI has mir-29a binding sites in 3′UTR of its mRNA. Based on miR-29a mimic and inhibitor studies and also the studies involving mutation of its target sequence in 3′UTR of QKI, we confirmed that miR-29a directly inhibits QKI expression at post-transcriptional level. Similarly, bioinformatics study also pointed towards SRA gene having QRE sequence in its 3′UTR mRNA. Here again, by using miR-29a and QKI ectopic expression and ablation, we established that QKI inhibits SRA expression by destabilizing its mRNA. It was further proved with the help of QRE mutant, that QKI effect on SRA are specifically through its binding to QRE region. Finally, to understand if this manipulation of SRA expression by miR-29a or QKI has any functional relevance, we measured the lipid uptake by oil red staining and sterol analysis by HPLC. It was observed that miR-29a enhanced lipid uptake and this was reversed by QKI overexpression. Similarly, the transfection of SRA along with QKI can also enhance lipid uptake. Also, ectopic expression and ablation of QKI modulates levels of sterols as expected. Therefore, it was suggested that QKI can modulate SRA functional activity in terms of lipid uptake.

However, there are still some important questions that remain to be answered. For example, in one of our previous study we have shown that Wnt1 protein regulate SRA expression by activating the canonical Wnt pathway in macrophages. So the question remains, if Wnt1 mediated regulation of SRA does involve QKI or miR-29a. Moreover, QKI has been shown to mediate the nuclear export of certain mRNAs [23]. So it needs further investigation if QKI restricts the SRA mRNA in the nucleus in addition to destabilization and blocks its movement to the cytoplasm for further protein synthesis. Also the detailed investigation into the QKI role in modulating lipid uptake by macrophages needs attention and we intend to follow through these studies in future.

In conclusion, our study demonstrated for the first time, the detailed mechanism of miR-29a mediated upregulation of SRA

expression during monocyte differentiation to macrophages. The miR-29a effect is mediated via regulation of QKI protein, which in turn regulates not only SRA expression but also its functional activity like lipid/sterols uptake in macrophages.

Conflict of interest

None.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.019.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.019.

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