

MicroRNA-202 induces myoblast to myocyte differentiation through targeting Rock-1

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Abstract

The expression patterns of microRNAs (small non-coding RNAs) are altered in many biological processes such as myogenesis. In this study, we aimed to investigate the impact of predicted miR-202, its target genes Akt2 and Rock-1 as a potential regulators of myoblast in myocyte differentiation process using C2C12 cell line. After confirmation of the differentiation process induced by 3% horse serum, the expression level of miRNA and its targets were evaluated. In the following, luciferase assay was conducted to approve the effect of miRNA on its target. Our results indicated that miR-202 and Akt2 were significantly up-regulated during differentiation, while Rock-1 was downregulated. Co-transfection of miRNA with psiCHECK2-Rock-1 significantly presented that Rock-1 was directly targeted by miR-202. On the contrary, miR-202 has failed to enforce its inhibitory effect on Akt2 expression. In particular, miR-202 seems to be a regulator of muscle differentiation pathway through targeting Rock-1.

Keywords: miR-202, Rock-1, C2C12, Muscle, Differentiation,

1. Introduction

Myogenesis is a specific multi-step differentiation process that is performed in embryonic and postnatal stages. This process is regulated by a multitude of external and internal markers which activated particular proteins including MyoD and Myf5 and controlled by a diverse group of signaling pathways such as PI3K/AKT and Rho family G proteins pathways [1-3].

The PI3K/AKT signaling pathway is an essential couple of differentiation process which is regulated by IGFs and controls muscle development. Previous data suggest that AKT2 as a downstream component of PI3K cascade plays a vital role in myogenic differentiation [4]. AKT2 motivates MyoD transactivation and consequently increases myogenin transcription [5, 6] or inhibits apoptosis during the differentiation process [5-8].

The Rho family G proteins pathway is the other functional pathway in myogenesis [8, 9]. It has been reported that Rho-associated kinase 1 (ROCK-1), as a downstream effector of Rho GTPases, has a negative regulatory effect on muscle development [10]. Recently, it has been shown that ROCK inhibition allows myoblast fusion, therefore, accelerates myogenesis [8, 10, 11].

Complicated controlling mechanisms of mentioned signaling pathways are applied by various factors during muscle development. Seeking to discover the regulatory effects of noncoding RNAs, investigators are interested in discovering microRNAs and their function in myogenesis [12].

MicroRNAs are small, conserved noncoding RNAs with 18-22 nucleotides [13] which are known as a new class of gene regulatory factors. They bind to the 3' untranslated region (UTR) of targeted mRNA [14, 15]. As a result of complementary binding, translation of their relevant protein is inhibited. Accordingly, multiple molecular and cellular mechanisms have been reported to be under influence of miRNAs and their aberrant expression could be a major reason for cellular dysfunctions [16, 17]. Lately, researchers have determined some of the particular miRNAs as an inhibitory factor of differentiation and some others as an activator. However, several studies are required to find out about the function of miRNAs in myogenesis. This study aims to investigate the impact of microRNA-202 as a regulator of Akt2 and ROCK in myogenesis.

2. Materials and Methods

2.1 Bioinformatics analysis

We used three major algorithms, Target Scan 6.2 [18], miRWalk [19], and RNAhybrid [20] to determine the specific targets of miR-202. Selected targets pick out from those sample targets which had the top score in at least two prediction lists.

2.2 Cell culture

C2C12 cells were grown in growth medium (GM; Dulbecco's Modified Eagle Medium and 10% fetal bovine serum (Gibco, UK)) and when they reach 90% confluency, GM medium was replaced with differentiation medium (DM; Dulbecco's Modified Eagle Medium containing 3% horse serum (Gibco, UK)). The differentiation procedure was continued for 72 hours. Similarly, our control cells were cultured just in GM for 72 hours. It is notable to mention that all types of cell cultures were repeated at least three times.

2.3 Immunocytochemistry (ICC)

After the C2C12 differentiation (72 hours), Immunocytochemical detection of specific markers was performed by using anti-Pax7 or anti-myosin antibody (Sigma, USA) as previously described (data not shown).

2.4 RNA isolation and quantitative real-time PCR

RNA extraction was performed using TRIzol reagent (Invitrogen, USA) and cDNA was synthesized according to reverse transcription kit (Fermentas, USA) using random hexamer for genes and stem-loop primer for miRNAs. Quantitative real-time polymerase chain reaction (PCR) was applied as previously described [21, 22]. *B-actin* and *Snord 47(U47)* were selected as the internal control for the targeted gene and miRNA, respectively. The method to clarify gene expression level was the $2^{-\Delta\Delta Ct}$.

2.5 Comparison of real-time PCR result by a high-throughput method data

GEO, accession #GSE4694 row data was download from NCBI databank [23] and analyzed to compare with our real-time PCR results.

2.6 Luciferase assay

The 3'UTR sequence of Rock-1 was cloned into psiCHECK-2 luciferase reporter plasmid. After seeding 20×10^4 293T cells in 96-well plates, psi-CHECK-Rock-1 and PCDH-miR-202 were co-transfected using lipofectamine 3000 methods, and Luciferase activity was estimated after 48 hours using Dual-Luciferase Reporter Assay System (Promega). Renilla luciferase signal was standardized to the Firefly luciferase signal activity to control transfection effectiveness.

2.7 Statistical analysis

The data are presented as mean \pm standard error. REST analysis and Student's t-test were applied to determine statistical significance and the criterion for significance was set at $P < 0.05$.

3. Results

3.1 Bioinformatics analysis

According to the results of bioinformatics prediction, we selected miR-202 and its targets, Akt2 and Rock-1 for this study, which in our prediction methods had the best miRNA-mRNA interaction (**Table 1**).

Table 1. Characteristics of miR-202 and its predicted targets

Gene Name	Predicted base pair site	Position site/Number of paired bases
Rock-1	5'- CAUAGGAA-3'	1213-1220/8mer
hsa-miR-202	3'- GUAUCCUU-5'	
Akt2	5'- UACCUCA-3'	2617-2623/7mer
hsa-miR-202	3'- AUGGAG-5'	

3.2 Gene expression level

Quantitative real-time PCR (q-RT PCR) was applied to evaluate miR-202, Akt, and Rock-1 expression levels in differentiated (myocyte) vs undifferentiated (myoblast) cell lines. As miR-202 increased in myocytes (**Figure 1a**), Roke-1 was down-regulated, while Akt2 expression level was ascended (**Figure 1b**). Although bioinformatics described Akt2 as the target of miR-202, its increase could be due to other molecular pathways that have helped differentiate muscles [24, 25].

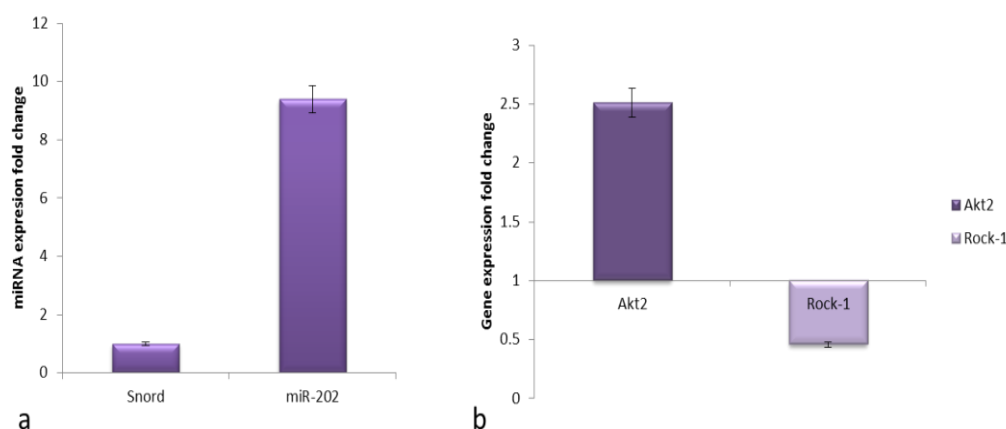


Figure 1: Expression pattern of miR-202 (1a) and its target genes during myoblast differentiation (1b). Based on qRT-PCR results, while Rock-1 was down-regulated (1b), Akt2 was up-regulated during the differentiation process ($P\text{-value} \leq 0.05$). Error bars indicate SEM ($n=3$) ($P < 0.05$).

3.3 Comparison of real-time PCR result by a high-throughput method data

Validation of our q-RT PCR results was achieved by micro-array analysis results. Both analyses were tracing the same way in gene's up/down-regulation (**Figure 2**).

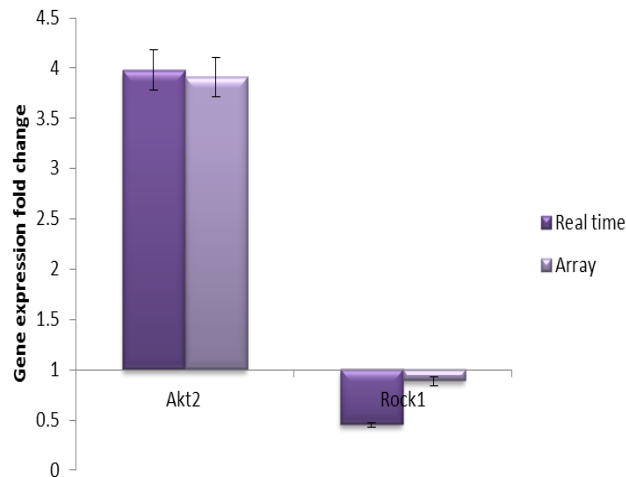


Figure 2. Comparison of the expression levels of predicted targets during myogenesis based on qRT-PCR and microarray analysis. The data were consistent between the two methods.

3.4 Luciferase assay

Based on our bioinformatics results, we hypothesized that miR-202 may have a positive effect on muscle differentiation through ROCK-1 down-regulation. For evaluating the hypothesis, we constructed a Luciferase reporter vector that carries Rock-1 3'UTRs downstream of luciferase stop codon psiCheck2. The results have been shown that co-transfection of miR-202 with psiCheck2-ROCK-1, significantly down-regulated luciferase expression (approximately 40%). The result confirmed that ROCK-1 was directly targeted by miR-202 (**Figure 3**).

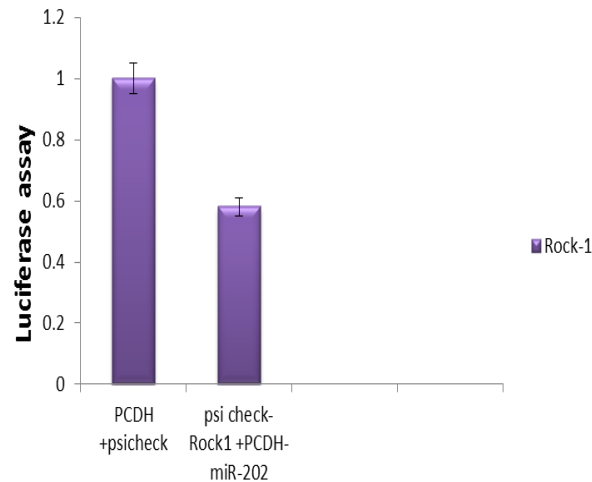


Figure 3. miR-202 has complementary sequences in Rock-1 -3' UTRs and targets it. Co-transfection of miRNA with psiCheck2-target significantly down-regulated luciferase expression. Error bars indicate SEM (n=3). ($P < 0.05$).

4. Discussion

MicroRNAs are small biomolecules regulating different cellular and molecular pathways including muscle cell differentiation [26]. Several studies claim that miRNAs have a vital role in muscle differentiation [1]. Thus we have selected miR-202 as a novel predicted regulator of PI3K/AKT and Rho G proteins signaling components, Akt2 and ROCK-1, and evaluated the expression level of these molecules before and after myoblast to myocyte differentiation.

According to our survey, miR-202 had an increased level during muscle development. Interestingly, ROCK-1 has shown a reduced level, while Akt2 had an opposite expression pattern. To confirm the real-time results, using an Affymetrix cDNA microarray dataset of GEO accession #GSE4694, we compared the fold change of *Akt2* and ROCK-1 in undifferentiated and differentiated C2C12 line. Data analysis revealed that the expression pattern of ROCK-1 and Akt2 were the same in both methods ($P\text{-value} \leq 0.05$), however, the fold change of ROCK-1 was shown to be more decreased in microarray analysis comparison with qRT-PCR analysis (**Figure 2**).

Furthermore, the result of our luciferase assay confirmed that ROCK-1 is directly inhibited through miR-202 induction (**Figure 3**). Recently, it has been shown that preventing ROCK-1 by its inhibitor, increased the phosphorylation level of IRS1/2 tyrosine and consequently activated myogenesis through PI3K activation, a major differentiation pathway in myogenesis [27, 28].

Other studies proved that ROCK-1 is the final inhibitor for differentiating myoblasts to myocyte which report distinguished the association between ROCK-1 and mTOR signaling pathway in suppressing the muscle-cell differentiating process [29, 30].

Moreover, it has been claimed that evaluating miRNAs could be a useful marker in various steps of the myocyte differentiation pathway [22]. As a result of this, some researchers have reported the utilization of micro RNAs as an inducer of transforming myoblasts to myocytes such as miR-148a. Zhang et al stated that miR-148a is up-regulated during the differentiation process. They have explained this overexpression due to inhibiting ROCK-1 in protein levels [29]. Hence, based on our results, it seems that miR-202 may be an effective regulator of myogenesis through direct suppression of ROCK-1 and following indirect activation of PI3K signaling (**Figure 4**).

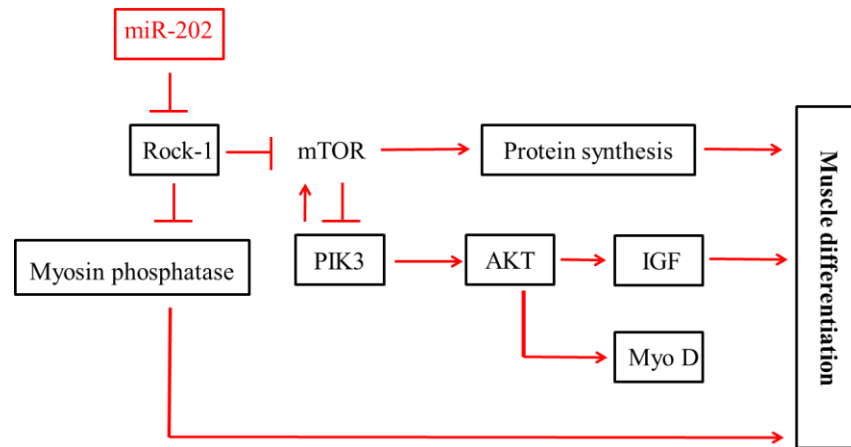


Figure 4. miR-202/ Rock-1/ PI3K relationship in the muscle differentiation process

However, our data has revealed that the other target of miR-202, Akt2, is deregulated during the differentiation process and it seems that miR-202 has failed to enforce its inhibitory effect on Akt2 expression. Although our bioinformatics prediction showed targeting of Akt2 mRNA by miR-202, the unexpected result is also justifiable.

As mentioned before, several studies have confirmed the positive role of PI3K signaling in myoblast differentiation, and the downstream genes of this pathway such as Akt2 were surveyed in various studies to differentiate their functions [31-33]. Recent studies have defined the specific expression of Akt2 in insulin-responsive tissues specifically skeletal muscle which creates the role of Akt2 in the muscle-cell differentiation process [34]. Furthermore, other published data has firmed the increased level of Akt2 in 10T1/2-MyoD cells, C2C12 myoblast, and Sol8

myoblast, respectively [35-37]. Additionally, Sumitani et al reported Akt2 function due to its serine/threonine kinase activity stimulating myogenin [6, 38]. It is also notable that Akt2 could induce IGF-II which is an activator for differentiating myoblasts to myotubes [39]. Moreover, Rotwein et al results have confirmed that inhibition of Akt2 in myoblasts causes myotube cells with the incomplete size of nuclei and the content of them [40]. Furthermore, Vandromme et al have suppressed the myogenesis process using Akt2 antibody [41].

Therefore, Akt-2 is a fundamental part of myogenesis and the cellular configuration is such that the expression of this gene increases [42]. Subsequently, do not mind if our examined microRNA could not inhibit Akt2 expression, while in other processes or cell types, it may be down-regulated by mentioned miRNA.

Since elucidating the molecular settings of cellular signaling helps to better understanding myogenesis, non-coding RNA such as miRNA could be a useful marker to identify the controlling mechanisms of muscle differentiation [43-45].

Our results may determine that Rock-1 inhibition through miR-202 could be one of the capable procedures involving in myogenesis. Accordingly, down-regulation of miR-202 expression may be considered as an aspect of producing induced pluripotent stem cells (iPSCs) from myocytes which have therapeutic advantages.

5. Conclusion

In this study, we demonstrated that miR-202 is up-regulated during the differentiation process which is responsible for ROCK-1 down-regulation. However, there should be multiple examinations due to confirmation of miR-202 function. Likewise, this study along with other researches could introduce the function and application of miRNAs in muscle development. Incorporation of several aspects via miRNAs' role in signaling pathways and possible treatments should be future study approaches.

Conflict of interest

The authors state that they have no conflict of interest.

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