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Real-time functional imaging for monitoring miR-133 during myogenic differentiation

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Abstract

MicroRNAs (miRNAs) are a class of non-coding small RNAs that act as negative regulators of gene expression through sequence-specific interactions with the 3' untranslated regions (UTRs) of target mRNA and play various biological roles. miR-133 was identified as a muscle-specific miRNA that enhanced the proliferation of myoblasts during myogenic differentiation, although its activity in myogenesis has not been fully characterized. Here, we developed a novel retroviral vector system for monitoring muscle-specific miRNA in living cells by using a green fluorescent protein (GFP) that is connected to the target sequence of miR-133 via the UTR and a red fluorescent protein for normalization. We demonstrated that the functional promotion of miR-133 during myogenesis is visualized by the reduction of GFP carrying the miR-133 target sequence, suggesting that miR-133 specifically down-regulates its targets during myogenesis in accordance with its expression. Our cell-based miRNA functional assay monitoring miR-133 activity should be a useful tool in elucidating the role of miRNAs in various biological events.

Keywords

Myogenesis; miR-133; Retroviral vector; Fluorescent protein; Live-cell imaging

1. Introduction

MicroRNAs (miRNAs) are a class of non-coding small RNAs that act as negative regulators of gene expression by promoting mRNA degradation and/or repressing translation through sequence-specific interactions with the 3' untranslated regions (UTRs) of the target mRNA (Bartel, 2004; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Hundreds of miRNAs have been found in various organisms, and many miRNAs are evolutionarily conserved, suggesting their important roles in biological processes (Bartel, 2004). Some miRNAs show localized expression patterns with limited tissue, cell, and spatiotemporal specificities. Moreover, one-third of all mammalian mRNAs seem to be under miRNA regulation (Lewis et al., 2005). Therefore, miRNAs play an essential role in regulating gene expression.

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Recent reports using microarray analyses with bioinformatics have identified miR-1, miR-133, and miR-206 as muscle-specific miRNAs that regulate muscle growth and differentiation (Brennecke et al., 2005; Chen et al., 2006; Rao et al., 2006). Both miR-1 and miR-133 are evolutionarily conserved since they have been found in most animal species, from *Drosophila* to humans. The miR-133 levels increase during the course of myogenic differentiation. However, miR-133 has been reported to enhance myoblast proliferation, despite the fact that miR-1 and miR-206 promote muscle differentiation (Chen et al., 2006). To understand the molecular network involving miRNAs in myogenesis, it is crucial to monitor the dynamics of miRNAs during myogenesis.

To determine the expression levels of miRNAs, Northern blotting and RT-PCR with microarray analysis are often carried out for the direct detection of miRNA. For miRNA visualization, *in situ* hybridization analysis is conventionally performed using specific probes and fixed tissues. Indirect detection of miRNAs entails the use of reporter genes whose UTRs are connected with the target sequence of the miRNAs (Brown et al., 2007, 2006; Mansfield et al., 2004; Zeng et al., 2002). In this system, if the target sequence of miRNAs is located downstream of the reporter genes, including β -galactosidase or luciferase, miRNAs induce a decrease in reporter signaling by reducing protein translation (Mansfield et al., 2004; Zeng et al., 2002). However, there are few reports on monitoring the dynamic function of miRNAs in intact cells or organs among the mixture of closely associated cell state. Recently, Naldini et al. reported that a lentiviral vector encoding green fluorescent protein (GFP) connected to a target sequence allowed them to visualize the activity of miR-142-3p followed by immunostaining of an internal control gene using fixed tissues (Brown et al., 2006, 2007). In this study, we developed a novel retroviral vector to monitor the specific miRNA activity in living cells. Using two fluorescent proteins as reporters, the miRNA activity in living cells can be directly analyzed using fluorescence microscopy. Our functional analysis using a retroviral vector is a useful method to examine the dynamic activity of miRNA in living cells.

2. Materials and methods

2.1. Construction of the retroviral vector

The fragment encoding GFP fused with the blasticidin-resistant gene was amplified by PCR by using the primers 5'-AGGGATCCGCCACCATGGTGAGCAAGGGCGAG-3', 5'-ACTACTCGAGGTAAACGAATTCTAGCCCTCCAC-3', and 5'-GACAAAGGCTTGGCCTGGCCATCGATTTTGTACAGCTCGTCCATGC-3' with pEGFP-C3 and pTracer-EF/bsd as the templates. The resultant product digested with BamHI/XhoI was integrated into pMX-puro to yield pMXGb. The fragment carrying RFP was amplified by PCR with the primers 5'-CGGAAGCTTGCCACCATGGTGAGCAAGGGCGA-3' and 5'-AAAAGTCGACTAATCGATCTTGTACAGCTCGTCCATGCCG-3' by using pRSET-mCherry (Shaner et al., 2004) as a template. The resultant product digested with HindIII/SalI was inserted into pMXGb to yield pMXRGb. The CMV promoter was amplified by PCR with the primers 5'-AACTCGAGTAGTTATTAATAGTAATCAATTACGG-3' and 5'-ACAAGCTTCTAGTGATCTGACGGTTCACTAAA-3', using pEGFP-C3 as a template. The resultant product digested with XhoI/HindIII was inserted into pMXRGb to yield pMXCRGb.

The fragment corresponding to a three tandem repeat of the target sequence that was completely complementary to miR-133 was prepared by annealing two oligonucleotides: 5'-AATTACAGCTGGTTGAAGGGGACCAACAGCTGGTTGAAGGGGACCAACAGCTGGTTGAAGGGGACCAA-3' and 5'-TCGATTGGTCCCCTTCAACCAGCTGTTGGTCCCCTTCAACCAGCTGTTGGTCCCCTTCAACCAGCTGT-3'. The resultant fragment was inserted into pMXCRGb at the EcoRI/XhoI sites to yield pMXCRGb[133].

2.2. Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed according to the previous report (Wilkinson, 1992) with minor modifications. In brief, the embryos (E11.5) were fixed with 4% PFA, 0.2% glutaraldehyde for 20 min at room temperature. Digoxigenin (DIG)-labeled antisense probes (~500 ng/ml) were hybridized for over 14 h at 70 °C. The embryos were treated with an anti-DIG AP Fab fragment antibody (Roche, Mannheim, Germany) with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Roche). A mouse primary miR-133a2 fragment was amplified from mouse cDNA by PCR with the primers 5'-TCTGCCTTCCCAGAGCCATG-3' and 5'-GATCCACTGGGAGGAGAGACTCC-3'. Since the mature region was too short to detect miR-133 under our condition, we used a probe against primary region of miR-133a, although LNA instead of RNA probes are more appropriate for mature miRNA detection (Wienholds et al., 2005). DIG-labeled probes were transcribed with a DIG-RNA labeling kit and T7 RNA polymerase (Roche).

2.3. Induction of myogenesis

Mouse myoblast C2C12 cells were cultured in growth medium (GM) comprising DMEM (Dulbecco's modified Eagle's medium; Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum. They were cultured until they reached a high-confluent density, and the medium was changed to differentiation medium (DM) comprising DMEM supplemented with 2% horse serum to induce differentiation.

2.4. Culture and infection of cells

Retrovirus packaging cell line Plat-E (kindly provided by T. Kita-mura) cells were cultured in DMEM supplemented with 10% fetal bovine serum in the presence of 1.0 µg/ml puromycin and 10 µg/ml blasticidin (Morita et al., 2000). The cells were transfected with a transfection reagent, TransIT-293 (Pan Vera, Madison, WI), according to the manufacturer's protocol. The cells were cultured to a confluence of approximately 80% (1×10^7 cells) and transfected with the retroviral vector. After 18 h, the culture medium was changed to fresh DMEM and further incubated for 30 h, at which point the culture medium was collected and passed through a 0.45-µm filter. C2C12 myoblasts were transduced with the retro-virus from the filtrated medium plus 5 µg/ml polybrene (Sigma). Two days after infection, the cells were selected by 10 µg/ml blasticidin (Sigma). Imaging of transduced cells was done by microscope Eclipse (Nikon, Tokyo, Japan) and 10–20 clones were screened.

2.5. Northern blotting

Total RNA was extracted and purified with the ISOGEN reagent (Wako, Osaka, Japan). An aliquot of total RNA (10 µg per lane) was loaded on a 12% polyacrylamide denaturing gel. After electrophoresis, bands of RNA were electro-transferred to a Hybond-XL membrane (Amersham Biosciences, Piscataway, NJ). The membrane was probed with ³²P-labeled RNA that was complementary to the individual miRNA sequence (Sano et al., 2006).

2.6. Flow cytometry analysis

Synthetic double-stranded RNA (dsRNA) of miR-133 (5'-GCUGGUAAAAUGGAACCAAAU-3' and 5'-UUUGGUCCCCUUAACCAGCUG-3') was transfected with C2C12 myoblasts using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). At 2 days after transfection of miR-133, 10,000 cells from each samples were trypsinized and analyzed using a FACSaria (Becton Dickinson, Sparks, MD). The fluorescent signals from GFP or RFP excited by a laser at 488 nm were monitored by their emissions using 515–545 nm (FL1) or 675–715 nm (FL3) filter, respectively.

3. Results

3.1. Expression of miR-133 during muscle development

The mouse skeletal muscle myogenic progenitor C2C12 cell line possesses pluripotent differentiation potential in mesenchymal cell lineages *in vitro* (Blau et al., 1985; Soulez et al., 1996). The expression of miR-133 is reported to increase during the course of myogenic differentiation of C2C12 myoblasts (Chen et al., 2006). Our Northern blotting analysis revealed that expression of miR-133 was increased approximately 6-fold at 4–7 days after changing the culture medium for C2C12 from GM to DM; this is in agreement with the results of other groups (Chen et al., 2006; Kim et al., 2006; Liu et al., 2007; McCarthy, 2008; McCarthy et al., 2007; Rao et al., 2006) (Fig. 1A). Quantitative RT-PCR analysis revealed that the miR-133 expression during myogenesis increased approximately 5-fold (Fig. 1B). miR-133 correlated with myogenic differentiation leads to a potential marker molecule for myogenic differentiation, similar to myogenin and myoD, both of which are upregulated in response to myogenic differentiation (Arnold and Braun, 1996). Next, we attempted a whole-mount *in situ* hybridization of primary miR-133 (pri-miR-133) to evaluate the developmental role of miR-133. pri-miR-133 was clearly observed in somites in the developing mice (Fig. 1C, visualized as violet); however, *in situ* hybridization with fixed and permeabilized tissues were not suitable for determination of the specific period of cells that are responsible for the expression of miR-133 in the early myogenesis.

3.2. Structure of the retroviral vector encoding green and red fluorescent proteins

To monitor myogenic differentiation in living cells, we constructed a dual-color monitoring system by using a retroviral vector that encodes two different fluorescent proteins. The retroviral vector is based on the Moloney murine leukemia retroviral (M-MLV) vector pMX (Morita et al., 2000), as shown in Fig. 2. Since our retroviral vector encodes two long terminal repeats (LTRs) whose promoters are both active but do not show self-inactivation (SIN), upstream transcription occurs after the integration of the retro-virus in the cellular genome. The provirus yields two independent transcripts: one mRNA encodes the red fluorescent protein (RFP) under the control of the cytomegalovirus (CMV) promoter (transcript 1, Fig. 2B) and the other mRNA encodes GFP under the control of the LTR promoter (transcript 2, Fig. 2B). Transcript 2 possesses the sequences for both RFP and GFP; however, RFP is not translated into protein because eukaryotes do not allow translation of the second open reading frame (ORF) from the 5' cap terminus. The R region of 3' LTR is used for a common polyadenylation signal in both transcripts.

Recent technology on fluorescent proteins allows us to utilize a variety of colors (Shaner et al., 2005) in addition to the conventional colors, such as cyan (CFP), yellow (YFP), green (GFP), and red (DsRed). In particular, RFPs have greatly improved in the brightness and a variety of wavelengths for real-time and multi-color imaging (Shaner et al., 2004). For multi-color imaging without the fluorescent wavelength overlap, green and red are a more favorable combination than the conventional ones: CFP and YFP. To determine a useful RFP for optimal dual-color monitoring with GFP, we transfected plasmids encoding different RFPs. The brightness of the fluorescent proteins was tdTomato > mCherry ~ mOrange > DsRed; however, overexpression caused mOrange, tdTomato, and DsRed to slight overlap with the GFP image in our filter settings for the microscope (Supplementary Fig. 1). Therefore, mCherry was selected as the RFP in the following experiment.

To monitor the expression of miR-133 by using our retroviral system, the complementary sequence against miR-133 was inserted into the pMXCRGb plasmid between the GFP and CMV promoter to yield pMXCRGb[133] (Fig. 2B and C). Since miRNAs suppress the expression of target mRNA with complementarity, the expression of miR-133 can reduce the

level of GFP whose mRNA possesses the target sequence of miR-133, without affecting the level of RFP. A three tandem repeat of the target sequence was used for enhancing the effect of the suppression rather than the single target sequence, as previously reported (Zeng et al., 2002). Expression of the retroviruses that are randomly integrated into the cellular genome following infection may be unexpectedly affected by the surrounding region depending on the integration sites (Cherry et al., 2000; Verma and Somia, 1997). Thus, an RFP cassette was used for normalization.

3.3. Real-time monitoring of miR-133 expression during myogenesis

Recombinant retroviruses were collected from the supernatant medium by transfection of the pMXCRGb or pMXCRGb[133] plasmid into the packaging cell line Plate-E, which is derived from human embryonic kidney 293T cells. Following infection (MOI = 10), we selected the optimal virus-infected C2C12 myoblasts that showed clear visualization using GFP fused with the antibiotic-resistant gene for blasticidin (Supplementary Fig. 2). In these transduced C2C12 myoblasts containing MXCRGb[133] or MXCRGb, both GFP and RFP fluorescence was detected (Fig. 4A, top). miR-133 was not expressed in these GM-cultured cells (Fig. 1A, lane C).

To test our approach for monitoring of miRNA activity in living cells, we quantified the miRNA activity by flow cytometry. When we used C2C12 cells containing MXCRGb[133] in GM, the population was detected at the right-upper region in the plot (Fig. 3A, cyan). Following transfection of a synthetic dsRNA of miR-133, the populations of C2C12 cells containing MXCRGb[133] were shifted from the right-upper region to the center-upper region in the plots (Fig. 3A, yellow). miR-133 activity was then quantified from the means of GFP intensity with RFP normalization as 30.6% and 13.8% after transfection of 10 and 100 nM of miR-133, respectively (Fig. 3B). When we transfected synthetic miR-1 as a negative control, the reduction of GFP was not observed (data not shown).

Next, we monitored endogenous miR-133 during myogenesis. Following 6 days of culturing in DM, the transduced cells successfully differentiated. The induction of differentiation suppressed the expression of GFP in C2C12 myoblasts that had been infected with MXCRGb [133] (Fig. 4A, middle). In particular, GFP fluorescence in myotubes was dramatically reduced (Fig. 4A, middle). RFP fluorescence was unaffected by the same treatment; therefore, the suppression was specific to GFP. In contrast, the activity of GFP due to MXCRGb was unchanged by the induction of differentiation even in myotubes (Fig. 3A, bottom). GFP fluorescence was reduced only in C2C12 myoblasts containing MXCRGb[133], suggesting that the reduction was due to the expression of miR-133 in differentiating myotubes. Since myogenically differentiated cells formed largely fused cell aggregates, we could not apply these cells for quantitative analysis using flow cytometry. Therefore, we quantified fluorescence of differentiated fused cells by densitometry analysis of microscopic images (Supplementary Figure S3). This analysis showed striking changes in GFP compared with RFP signal upon myogenesis. Interestingly, GFP fluorescence in several cells was reduced prior to the myotube formation, indicating that miR-133 expresses and reduces the expression of target genes prior to myotube formation. Our analyses, in agreement with other reports, exhibited the dynamic change of miR-133 expression in living cells during myogenesis.

4. Discussion

4.1. Roles of miR-133 during myogenesis

Multiple factors participate in the step of myogenic differentiation with the regulation of muscle genes. In addition to the transcriptional regulation by transcription factors, post-transcriptional regulation by miRNA plays an important role in myogenic differentiation (Brennecke et al.,

2005; Chen et al., 2006; Liu et al., 2008; Rao et al., 2006). Pairs of miR-1 and miR-133 are transcribed as bicistronic transcripts under the control of E-box, a family of MyoD transcription factors, and MEF2 transcription factor, an essential regulator of muscle development. MyoD and myogenin also modulate the transcription of miR-133 in skeletal muscle.

On the other hand, candidate target genes regulated by miR-133 are reported from a bioinformatics approach. One target is the serum response factor (SRF), which is a transcription factor that directly regulates the expression of miR-133. Transcription factors and miRNAs are suggested to regulate reciprocally in several physiological events (Chen et al., 2006; Hobert, 2008; O'Donnell et al., 2005). Muscle development involving miRNAs is presumed to be integrated in a complicated network since a single miRNA targets multiple mRNAs, and several co-expressed miRNAs may target a single mRNA with a synergetic effect (He et al., 2005; Stark et al., 2005). Our present results related to the role of miR-133 in myogenic differentiation are consistent with previous observations. We further clarified that the expression of miR-133 is induced prior to the formation of myotubes. Further analysis of miRNAs at the single cell level during differentiation will be necessary.

4.2. Retroviral vectors as a tool for sustainable and robust monitoring of miRNAs

In this study, we designed a retroviral vector system that enables real-time monitoring of miR-133 by using fluorescent proteins of different colors. The gene for GFP was connected with the target sequence of miR-133 at the 3' UTR, and the gene for RFP was independently expressed with a promoter other than GFP to ensure the transcriptional activity of the integrated site. Although internal ribosomal entry sites (IRESs) are known to express two or more proteins from a single transcript, IRES-connected transcripts have been reported to be affected by small dsRNA with perfect complementarity (Petersen et al., 2006). Therefore, we did not use IRES here. We observed that miR-133 regulates the expression of GFP; this regulation is not due to differences in the promoters used because the expression of GFP lacking miR-133 target sites was unaffected by the differentiation conditions. In addition to miR-133, we successfully detected other miRNAs, such as endogenous let-7 or adenoviral miRNAs (Kato, unpublished). Moreover, although repeated sequence of miRNA target may be concerned to trap and inactivate the endogenous miRNA, we found that C2C12 transduced with our retroviral vector successfully induced the myogenesis. It has been reported that over-expression of miRNA targets with perfect complementarity do not saturate miRNA regulation due to its catalytic activity of miRNA machinery (Brown et al., 2007).

Since the myogenic differentiation from myoblasts to myotubes takes a week, the reporter genes are required to be expressed sustainably, aside from the plasmid transfection whose expression decreases after 3–4 days. The retroviral vector is integrated into the cellular genome and transgenes from the provirus are passed on to the daughter cells through doubling. However, since integration of the retroviral vector occurs randomly, it is possible that the expression of the transgenes of interest is unexpectedly affected by the control region of the cellular genome near the integration sites. Naldini's group reported that bidirectional expression of two distinct transgenes show excellent correlation regardless of the integration sites, although their vector was larger than ours due to the additional promoter and poly(A) signal. Moreover, the expression of miRNAs was indirectly monitored using a lentiviral vector based on HIV-1 by reducing GFP and a Δ LNGF receptor followed by fixation and fluorescent labeling with an anti- Δ LNGFR antibody (Brown et al., 2006). The lentiviral vector is amphotropic, namely, infectious to humans.

Our retroviral vector lacks such infectivity in humans due to its ecotropic packaging system. However, under biological safety restrictions, the retroviral system is a system of choice. When our retroviral vector is packaged with the vesicular stomatitis virus G (VSV-G) envelope, the virus can infect human cells, excluding non-dividing cells. Our retroviral vector also encodes

two fluorescent proteins in tandem for monitoring miRNA expression by using living cells independent of integration sites of the provirus. The broader infectivity to vertebrates is promising for an extensive study of miRNAs in the *in vivo* tissues as well as *in vitro* cultured cells. Therefore, this retroviral system may be a useful tool for real-time monitoring of not only miR-133 in myogenic differentiation but also other miRNAs in various biological events.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.biocel.2009.04.018.

Fig. 1. Expression patterns of miR-133. (A) Northern blots showing the kinetics of miR-133 expression at various time periods (0, 2, 4, and 7 days) after changing from GM to DM during the myogenesis of C2C12 myoblasts. 5S rRNA was used as a loading control. (B) Expression of miR-133 was determined by quantitative RT-PCR analysis. Results are means of results from three experiments. (C) and (D) Following the fixation of mouse embryos, whole-mount *in situ* hybridization was performed using a DIG-labeled probe against primary miR-133 (violet, allowheads). (Scale bar: 50 μ m) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

Fig. 2.

A schematic of the dual-color retroviral vector system. (A) Proviral form of the retroviral vector based on M-MLV is represented as MXCRGb. Since LTRs are not self-inactivating, the LTR promoters are active after integration into the cellular genome. (B) The three tandem repeats with perfect complementarity to miR-133 were inserted between the region downstream of the GFP and upstream of the RFP cassette. The proviral vector (MXCRGb[133]) is transcribed as two mRNAs: one expresses GFP and the other expresses RFP. In the presence of miR-133, GFP expression is suppressed without affecting RFP expression. (C) Target sequence of miR-133 which is located immediately after UGA stop codon for GFP-coding gene is shown.

Fig. 3.

Quantitative analysis of miRNA by flow cytometry. (A) The fluorescence intensities from two kinds of fluorescent proteins were quantified before and after transfection of synthetic miR-133, indicated by cyan and yellow dots, respectively. Overlapping populations appear as white dots. The fluorescent signals of GFP and RFP were detected using FL1 and FL3 filters, respectively. (B) Means of GFP intensity was normalized by RFP. Relative fluorescence of the value without transfection of miR-133 was set as 100% (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

Fig. 4.

Dual-color imaging of miR-133 expression in living myoblasts. C2C12 myoblasts were transduced with either the MXCRGb[133] or MXCRGb retroviral vector. (A) Strong expression of both GFP and RFP in C2C12 cells cultured in GM indicated that the retroviral vector was efficiently transduced (top). Six days after changing from GM to DM, GFP fluorescence in the differentiated MXCRGb[133] cells was reduced, while RFP fluorescence was retained (center). Fluorescence was unchanged in the case of both probes in MXCRGb cells. (B) Highly fused myotubes were observed 10 days after induction. Myotubes are indicated by arrows. Representative cell image is shown.