Differentiation 78 (2009) 195–204

Contents lists available at ScienceDirect

Differentiation

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

Activation of RXR and RAR signaling promotes myogenic differentiation of myoblastic C2C12 cells

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ARTICLE INFO

Article history:
Received 28 January 2009
Received in revised form
25 May 2009
Accepted 6 June 2009

Keywords:
Myogenic progenitor cells
Myogenic differentiation
Retinoid signaling
Myoblast cells
Nuclear receptors

ABSTRACT

Differentiation of embryonic and adult myogenic progenitors undergoes a complex series of cell rearrangements and specification events which are controlled by distinct gene regulatory networks. Delineation of the molecular mechanisms that regulate skeletal muscle specification and formation should be important for understanding congenital myopathies and muscular degenerative diseases. Retinoic acid (RA) signaling plays an important role in development. However, the role of RA signaling in adult myogenic progenitors is poorly understood. Here, we investigate the role of RA signaling in regulating myogenic differentiation of myoblastic progenitor cells. Using the mouse myoblast progenitor C2C12 line as a model, we have found that the endogenous expression of most RAR and RXR isotypes is readily detected. While the nuclear receptor co-repressors are highly expressed, two of the three nuclear receptor co-activators and the enzymes involved in RA synthesis are expressed at low level or undetectable, suggesting that the RA signaling pathway may be repressed in myogenic progenitors. Using the α-myosin heavy chain promoter-driven reporter (MyHC-GLuc), we have demonstrated that either ATRA or 9CRA is able to effectively induce myogenic differentiation, which can be synergistically enhanced when both ATRA and 9CRA are used. Upon ATRA and 9CRA treatment of C2C12 cells the expression of late myogenic markers significantly increases. We have further shown that adenovirus-mediated exogenous expression of RARα and/or RXRα is able to effectively induce myogenic differentiation in a ligand-independent fashion. Morphologically, ATRA- and 9CRA-treated C2C12 cells exhibit elongated cell body and become multi-nucleated myoblasts, and even form myoblast fusion. Ultrastructural analysis under transmission electron microscope reveals that RA-treated myogenic progenitor cells exhibit an abundant presence of muscle fibers. Therefore, our results strongly suggest that RA signaling may play an important role in regulating myogenic differentiation.

1. Introduction

During embryonic myogenesis, myogenic progenitors undergo a complex series of cell rearrangements and specification events in different regions of the body, all of which are controlled by distinct gene regulatory networks (Bryson-Richardson and Currie, 2008; Shih et al., 2008; Pownall et al., 2002). These progenitors reveal their myogenic nature by the subsequent onset of expression of the master switch genes MyoD and/or Myf5. Once initiated, the myogenic progression ultimately forms mature muscle (Shih et al., 2008). Regulatory functions of the myogenic regulatory factors, MyoD, Myf5, Myogenin, and MRF4, and the transcriptional and signaling mechanisms control their expression during the specification and differentiation of muscle progenitors. Myf5 and MyoD genes have genetically redundant, but developmentally distinct regulatory functions in the specification and the differentiation of somite and head muscle progenitor lineages (Pownall et al., 2002). Myogenin and MRF4 have later functions in

Abbreviations: ATRA, all-trans retinoic acid; 9CRA, 9-cis retinoic acid; RA, retinoic acid; RAR, retinoic acid receptor; RT-PCR, reverse transcriptase-polymerase chain reaction; RXR, retinoid X receptor

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doi:10.1016/j.diff.2009.06.001

muscle differentiation, and Pax and Hox genes coordinate the migration and specification of somite progenitors at sites of hypaxial and limb muscle formation in the embryo body. Pax3 and a number of other homeobox transcription factors are essential in specifying pre-myogenic progenitors in the dermomyotome. Developmental signals and their signal transduction effectors function both interactively and independently to control Myf5 and MyoD activation in muscle progenitor lineages, likely through direct regulation of their transcription enhancers (Bryson-Richardson and Currie, 2008; Shih et al., 2008; Pownall et al., 2002).

It has been well documented that bone marrow-derived stromal cells (MSCs) can differentiate into multiple lineages, including myocytes, osteocytes, chondrocytes, and adipocytes (Deng et al., 2008; Luo J., et al., 2005; Luu et al., 2007). The satellite cells (or, the skeletal muscle stem cells) are considered the main source of muscle cells for postnatal growth and regeneration (Seale et al., 2001; Dezawa et al., 2005; Zammit et al., 2006; Negroni et al., 2006). Understanding the molecular mechanisms that regulate skeletal muscle specification and formation should provide a foundation for understanding congenital myopathies and muscular degenerative diseases. We have demonstrated that several major signaling pathways, such as BMP and Wnt signaling, may play an important role in regulating osteogenic and adipogenic differentiation of MSCs (Deng et al., 2008; Luo J., et al., 2005, 2007a; Luu et al., 2007; Tang et al., 2009; Kang et al., 2009). However, the molecular mechanisms that direct MSCs to myogenic differentiation have not been well understood.

Retinoic acid (RA) plays an important role in development and functional maintenance of vital organs in adult (Duester, 2008; Niederreither and Dolle, 2008). Retinoic acid is formed solely from retinaldehyde (Rald), which is derived from vitamin A. The metabolism of vitamin A and the diverse effects of its metabolites are tightly controlled by distinct retinoid-generating enzymes, retinoid-binding proteins, and retinoid-activated nuclear receptors (Duester, 2008; Ziouzenkova et al., 2007). RA regulates differentiation and metabolism by serving as a ligand for two families of nuclear receptors, the RA receptors (RARα, RARβ, and RARγ) that bind to all-trans-RA (ATRA) and the retinoid X receptors (RXRα, RXRβ, and RXRγ) that bind to 9-cis RA (9CRA) (Mark et al., 2006; Chawla et al., 2001). 9CRA is normally undetectable except when vitamin A is present in excess (Mic et al., 2002). RXR forms heterodimers with RAR and several other nuclear receptors when bound to DNA, suggesting that RXR may function as a scaffold protein to facilitate DNA binding for several types of nuclear receptors (Mark et al., 2006; Chawla et al., 2001).

In vivo studies have demonstrated that ligand binding to the RAR portion of RAR/RXR heterodimers is sufficient and necessary to rescue a lethal defect in RA synthesis, whereas ligand binding to RXR does not rescue the defect and is unnecessary (Mic et al., 2002). RA binding to RAR/RXR heterodimers bound to a regulatory DNA element leads to a cascade of events resulting in recruitment of transcriptional co-activators and initiation of transcription (Germain et al., 2002; Mark et al., 2006). As for other members of the nuclear receptor superfamily, RA-induced transcriptional activity is tightly regulated by nuclear co-repressors (NCORs) and nuclear receptor co-activators (NCOAs) (Collingwood et al., 1999). Genetic manipulations in animals have revealed that RA signaling is important for the development of the forebrain and the segmented hindbrain, and for the elongation of the body axis (Mark et al., 2006; Duester, 2008; Niederreither and Dolle, 2008). RA signaling has also been implicated in early heart patterning, forelimb induction, pancreas induction, lung induction, eye formation, and some aspects of genitourinary tract development (Duester, 2008; Niederreither and Dolle, 2008). However, our current understanding of the potential role of RA in adult stem cells and tissue-specific progenitors has been relatively limited.

In this study, we investigate the role of RA signaling in regulating myogenic differentiation of myoblastic progenitor cells. Using mouse myoblast progenitor cell C2C12 line as a model, we have found that the expression of most RAR and RXR isotypes is readily detected in C2C12 cells. While the NCORs are highly expressed, two of the three NCOAs and the enzymes involved RA synthesis are expressed at low level or undetectable, suggesting that the RA signaling pathway may be repressed in myogenic progenitors. Using the α-myosin heavy chain promoter-driven Gaussia luciferase (MyHC-GLuc) reporter, we have demonstrated that either ATRA or 9CRA is able to effectively induce myogenic differentiation, which can be synergistically enhanced when both ATRA and 9CRA are used. We have also demonstrated that upon ATRA and 9CRA treatment of C2C12 cells the expression of late myogenic markers (such as troponin T and α-MyHC) significantly increases. Exogenous expression of RARα and/or RXRα is able to effectively induce myogenic differentiation in a ligand-independent fashion. Morphologically, ATRA- and 9CRA-treated C2C12 cells exhibit elongated cell body and became multi-nucleated myoblasts, and even form myoblast fusion. Ultrastructural analysis under transmission electron microscope reveals that RA-treated C2C12 cells show an abundant presence of muscle fibers. Thus, our results have demonstrated that RA signaling may play an important role in regulating myogenic differentiation.

2. Material and methods

2.1. Cell culture and chemicals

HEK293 and C2C12 lines were obtained from the ATCC (Manassas, VA), and maintained in complete Dulbecco's modified Eagle's medium (DMEM). Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich or Fisher Scientific.

2.2. Construction of α-myosin heavy chain (MyHC) promoter-driven Gaussia luciferase (MyHC-GLuc) reporter

The 5.5 kb genomic DNA fragment upstream exon 4 of mouse α-myosin heavy chain gene was isolated from α-5.5 vector (Subramaniam et al., 1991), and subcloned into the *BamH* I/*Xho* I sites of our homemade retroviral reporter vector pBGLuc to drive the expression of Gaussia luciferase, resulting in pMyHC-GLuc. The reporter vector was used for transient transfection, as well as for making stable lines via retroviral infection. The cloning junctions were verified by DNA sequencing. Cloning and construction details are available upon request.

2.3. Construction of adenoviral vectors expressing RARα and RXRα

Recombinant adenoviruses expressing human RARα and RXRα were generated using the AdEasy technology as previously described (He et al., 1998b; Cheng et al., 2003; Kang et al., 2004; Luo et al., 2007b). Briefly, the coding regions containing human RARα and RXRα were PCR amplified, and subcloned into pAdTrace-TO4 and subsequently used to generate adenoviral recombinants. Recombinant adenoviruses (i.e., AdR-RARα and AdR-RXRα) were produced and amplified in packaging HEK293 cells as described (He et al., 1998b; Luo et al., 2007b). The AdR-RARα and AdR-RXRα also co-express RFP. An analogous adenovirus expressing only RFP (AdRFP) was used as a control (He et al., 1998a, 1998b, 1999; Luo J., et al., 2007b; Luo X., et al., 2008; Sharff et al., 2009; Tang et al., 2009). All PCR-amplified fragments and cloning junctions were verified by DNA sequencing. Details about the vector construction are available upon request.

2.4. Isolation of total RNA, reverse transcription, quantitative real-time PCR (qPCR) and semi-quantitative RT-PCR analysis

Subconfluent cells were seeded in 75-cm^2 cell culture flasks in a medium supplemented with 1% fetal bovine serum (FBS) with or without adenovirus infection. Total RNA was isolated using TRIZOL Reagents (Invitrogen), according to the manufacturer's instructions. Reverse transcriptase-PCR was carried out as described (Luo Q., et al., 2004; Luo X., et al., 2008; Peng et al., 2003, 2004; Si et al., 2006; Tang et al., 2009; Sharff et al., 2009). Briefly, 10 μg of total RNA were used to generate cDNA templates by reverse transcription with hexamer and Superscript II reverse transcriptase (Invitrogen). The first strand cDNA products were further diluted 5–10-fold and used as PCR templates. The PCR primers were 18–20mers, designed by using the *Primer3* program, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, to amplify the 3′-end (approximately 120–150 bps) of the gene of interest (Supplemental Table 1).

The qPCR was carried out as described (Luo Q., et al., 2004; Peng et al., 2004; Si et al., 2006). SYBR Green-based qPCR analysis was carried out using the Opticon DNA Engine (Bio-Rad Laboartories). The specificity of each qPCR reaction was verified by melting curve analysis and further confirmed by resolving the PCR products on 1.5% agarose gels. Four-fold serially diluted pUC19 was used as a standard. Duplicate reactions were carried out for each sample. All samples were normalized by endogenous level of GAPDH.

Semi-quantitative RT-PCR reactions were carried out using a touchdown protocol: 94 °C × 20″, 68 °C × 30″, 70 °C × 20″ for 12 cycles, with 1 °C decrease per cycle, followed by 25–30 cycles at 94 °C × 20″, 56 °C × 30″, 70 °C × 20″. PCR products were resolved on 1.5% agarose gels. All samples were normalized by endogenous level of GAPDH.

2.5. Transfection and Gaussia luciferase reporter assay

Exponentially growing cells were seeded in 25 cm^2 cell culture flasks and transfected with 2 μg per flask of pMyHC-GLuc or pTOP-Luc using LipofectAMINE (Invitrogen). At 16 h after transfection, cells were replated to 24-well plates and treated with all-trans RA, 9-cis RA, or DMSO. Gaussia luciferase possesses a natural secretory signal and upon expression is secreted into the cell medium. Thus, at the indicated time points medium from the treated cells was collected for Gaussia luciferase assays using the Gaussia Luciferase Assay Kit (New England Biolabs). Firefly luciferase activity was assayed as described (Sharff et al., 2009; Tang et al., 2009). Each assay condition was performed in triplicate. Reporter activity was expressed as mean ± S.D.

2.6. Immunofluorescence staining

Immunofluorescence staining was carried out as described (Luo Q., et al., 2004; Peng et al., 2003, 2004; Si et al., 2006; Tang et al., 2009). Briefly, cells were fixed with methanol at 4 °C for 15 min and washed with PBS. The fixed cells were permeabilized with 1% NP-40 and blocked with 10% bovine serum albumin, followed by incubation with Troponin T (TnT) antibody (Santa Cruz Biotechnology) for 60 min. After being washed, cells were incubated with DyLight 594-labeled secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min. The presence of proteins of interest was examined under a fluorescence microscope. Staining without the primary antibody, or with control IgG, was used as negative controls.

2.7. Western blotting analysis

Western blotting was carried out as described previously (Peng et al., 2004; Luo X., et al., 2008; Sharff et al., 2009; Tang et al., 2009). Briefly, C2C12 cells were treated with ATRA and 9CRA. The treated cells were lysed in 1 × Laemmli sample buffer and loaded onto 4–20% gradient SDS-PAG gels. After being resolved by electrophoresis, proteins were transferred onto an Immobilon-P membrane (Millipore) via electroblotting. The membranes were blocked with SuperBlock DryBlend blocking buffer in Tris-buffered saline (Pierce) at room temperature for 1 h and probed with Troponin T antibody for 1 h, followed by a 30-min incubation with a secondary antibody conjugated with horseradish peroxidase (Pierce). The presence of TnT was detected using the SuperSignal West Pico or West Femto chemiluminescent substrate kit (Pierce). Anti-β-actin was used as a loading control.

2.8. Histological evaluation

For Giemsa staining, cultured cells were fixed in methanol for 30 s, and then stained with a freshly prepared 5% Giemsa stain solution for 20–30 min. The stained cells were rinsed in tap water and images were taken immediately.

2.9. Transmission electron microscopy (TEM) analysis

TEM was conducted as described (Kang et al., 2009). Briefly, C2C12 cells were treated with ATRA, 9CRA or DMSO for 7 days, and fixed with 4% glutaraldehyde (in 0.1 M sodium phosphate buffer, pH 7.4) at 4 °C for 30 min. The fixed cells were collected using cell scrappers followed by a brief centrifugation. The cell pellets were fixed for additional 2 h in 2.5% glutaraldehyde, and 2 h with 1% osmium tetroxide. The cell pellets were dehydrated in ascending ethanol serial washes and embedded in Epon 812. Serial ultrathin sections were examined using Zeiss 900 electron microscope. Magnifications, 7,000 × to 30,000 ×.

2.10. Statistical analysis

We used the Microsoft Excel program to calculate standard deviations (SD) and statistically significant differences between samples using the two-tailed Student's *t* test.

3. Results

3.1. Endogenous expression of myogenic regulatory genes in myoblast C2C12 cells

Specification and differentiation of muscle progenitors are controlled by a network of regulatory factors, such as MyoD, Myf5, and Myogenin (Pownall et al., 2002; Bryson-Richardson and Currie, 2008). We first analyzed the endogenous expression level of myogenic regulatory genes. As shown in Fig. 1A, Myf5 had the highest expression level, and MyoD and Pax3 had a modest level of expression, whereas expression of MEF2C and myogenin genes was relatively low. Interestingly, the two factors that are primarily involved in cardiomyocyte differentiation, Nkx2.5 and GATA4, were also highly expressed in C2C12 cells. However, most of these proteins were expressed at a relatively low level as detected by Western blotting (data not shown). Nonetheless, these results indicate that C2C12 cells retain the properties of muscle progenitor cells.

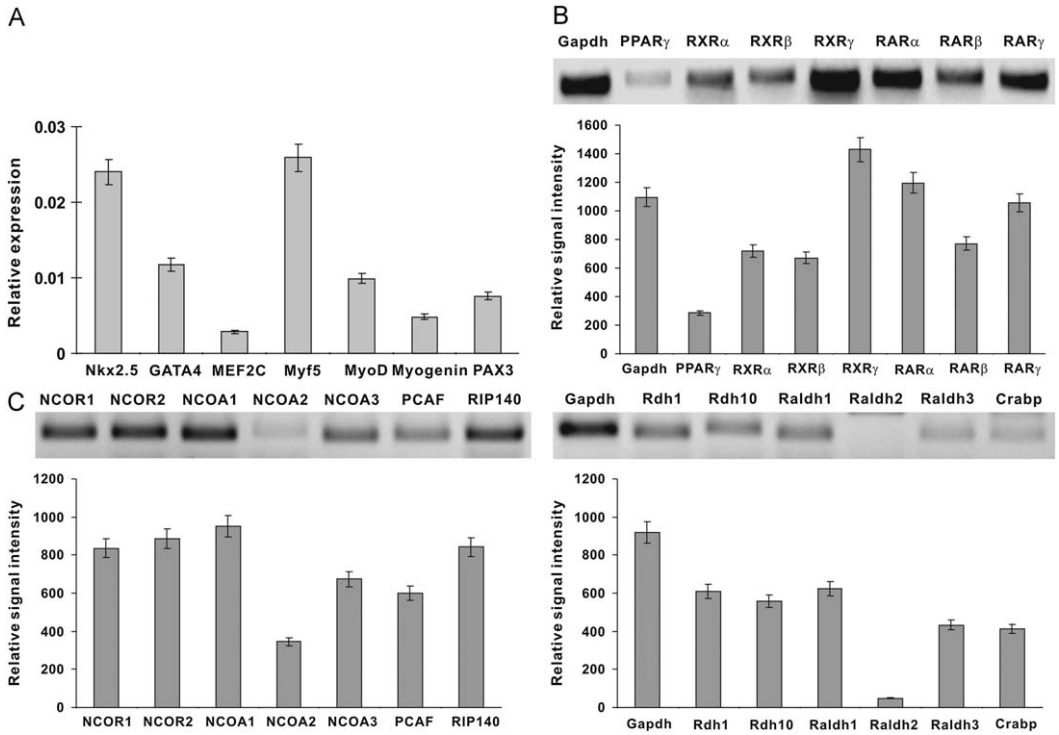

Fig. 1. Endogenous expression of myogenic genes, nuclear receptors, nuclear receptor co-regulators, and RA metabolizing enzymes in myoblastic C2C12 cells. (A) Quantitative analysis of myogenic gene expression in C2C12 cells. Total RNA was isolated from C2C12 cells and subjected to reverse transcriptase-PCR, followed by quantitative real-time PCR analysis using gene-specific primers. (B) Endogenous expression of RAR and RXR isotypes in C2C12 cells. The same set of samples as that described in Fig. 1A was used for semi-quantitative RT-PCR analysis. PCR results were confirmed in at least three batches of independent experiments. (C) Endogenous expression of nuclear receptor co-repressors (NCOR1 and 2), co-activators (NCOA1, 2, and 3), PCAF, RIP140, Rdh1/10, Crabp, and Raldhs. The same set of samples as that described in Fig. 1A was used for semi-quantitative RT-PCR analysis. PCR results were confirmed in at least three batches of independent experiments. The results reported in Figs. 1B and C were further quantitatively analyzed via densitometry.

3.2. *Expression of retinoid acid receptors, nuclear receptor co-activators, co-repressors, and RA synthesis enzymes in myoblastic C2C12 cells*

We next determined the endogenous expression levels of the RAR and RXR isotypes in C2C12 cells. Using semi-quantitative RT-PCR analysis, we demonstrated that expression of the α and γ isotypes of both RAR and RXR was readily detectable, whereas RARγ and RXRγ expressed at a slightly lower level (Fig. 1B). All RAR and RXR isotypes were expressed at a much higher level than that of the PPARγ2's. These results indicate that RAR and RXR receptors are widely expressed in C2C12 cells.

As for other members of the nuclear receptor superfamily, RAR and RXR activities are regulated by a network of nuclear receptor co-repressors (e.g., NCOR1 and NCOR2), nuclear receptor co-activators (e.g., NCOA1, 2, and 3), receptor-interacting protein 140 (RIP140), and/or histone acetyltransferase (HAT) PCAF. We examined the endogenous expression of these factors in C2C12 cells. As shown in Fig. 1C, a high level of expression of NCOR1, NCOR2, NCOA1, and RIP140 was readily detected in C2C12 cells, while NCOA3, PCAF, and NCOA2 expressed at a low or marginally detectable level.

Retinoic acid is produced solely from vitamin A derivative retinaldehyde by retinol dehydrogenases (Rdh) and retinal dehydrogenases (Raldh) (Duester, 2008; Niederreither and Dolle, 2008). We examined the expression of the two Rdh isoforms and three Raldh isoforms. As shown in Fig. 1C, expression of Rdh1, Rdh10, and Raldh1 was modest, while expression of Radh3 and Raldh2 was low or marginally detectable in C2C12 cells. The cellular retinoic acid binding protein II (Crabp) also expressed at a relatively low level. These results indicate that although RAR and RXR receptors are abundantly expressed in myoblast C2C12 cells the RA signaling activity may be repressed due to the lower expression level of NCOAs and RA synthesis enzymes.

3.3. *The α-myosin heavy chain gene promoter-driven reporter was used to monitor myogenic differentiation*

Myosin is a major constituent of muscle contractile apparatus, and it contains two heavy chain and four light chains (Subramaniam et al., 1991). The α-heavy chain of myosin was shown to be expressed in a tissue-specific manner in transgenic mice (Subramaniam et al., 1991). Here, we constructed an α-myosin heavy chain gene promoter-driven Gaussia luciferase reporter, pMyHC-GLuc, using the 5.5 kb genomic fragment of mouse MyHC promoter that also contains the first three exons as reported (Subramaniam et al., 1991) (Fig. 2A). There are several advantages of this reporter. It contains a large portion of the MyHC promoter sequence. The reporter Gaussia luciferase is a secreted protein so it is convenient to monitor the promoter activities longitudinally. Finally, pMyHC-GLuc is a retroviral-based vector so it can be used for either transient transfection or making stable reporter lines.

As previously reported (Subramaniam et al., 1991), the MyHC-GLuc reporter was shown to be myocyte-specific as its reporter activity, but not the control reporter or the irrelevant reporter pTOP-Luc, increased during the basal level of spontaneous differentiation in C2C12 cells (Fig. 2B). It has been well documented that confluent C2C12 cell (especially cultured in 2% horse serum) can undergo myogenic differentiation (Portier et al., 1999). Furthermore, dexamethasone (Dex) was shown to effectively induce MyHC-GLuc reporter activity in C2C12 cells in a

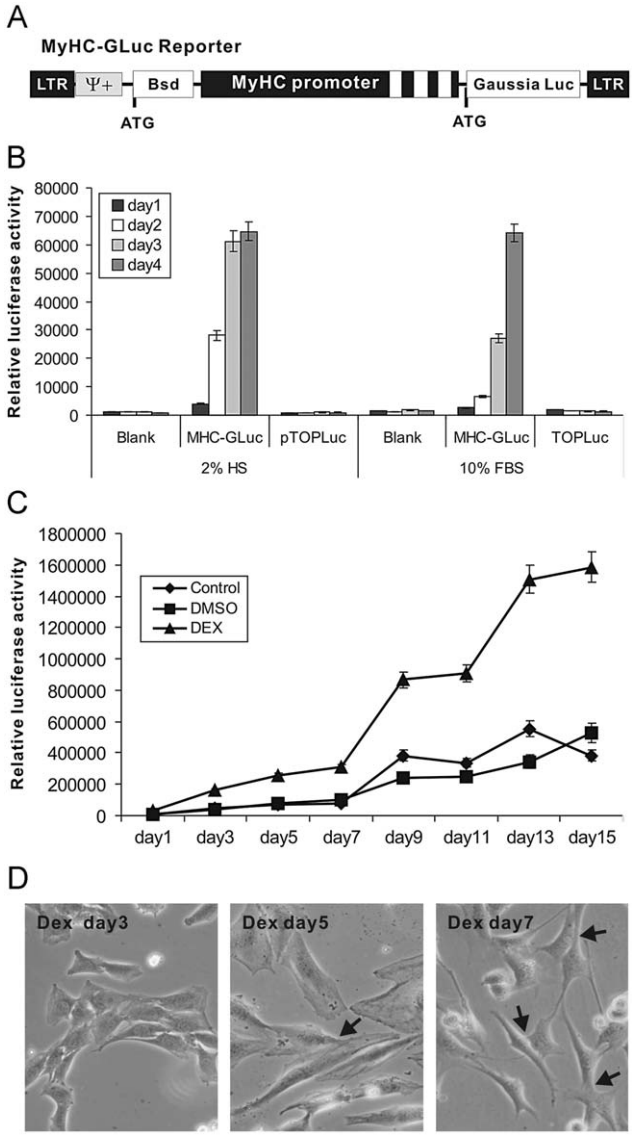

Fig. 2. RA induction of α-myosin heavy chain (MyHC) promoter-driven Gaussia luciferase reporter activity in C2C12 cells. (A) Construction of the MyHC-GLuc reporter. A 5.5 kb α-myosin heavy chain promoter-driven Gaussia luciferase reporter (MyHC-GLuc) is schematically shown. The promoter region also contains the first three exons (white boxes). (B) Specificity of MyHC-GLuc reporter. C2C12 cells were transfected with pMyHC-GLuc, vector control (blank), or β-catenin/Tcf4 reporter pTOP-Luc and cultured in 2% horse serum or 10% fetal bovine serum medium to induce spontaneous differentiation. Luciferase activity (mean ± SD) was determined at the indicated time points. (C) Dex induction of MyHC-GLuc activity in C2C12 cells. Cells were transfected with MyHC-GLuc reporter plasmid, and treated with DMSO (1%), Dex (1.0 μM), or untreated. Relative Gaussia luciferase activity (mean ± SD) was assayed at the indicated time points. Differences between DMSO- and Dex-treated cells were statistically significant after day 5 ($p < 0.001$). Each assay condition was carried out in triplicate. (D) Dex-induced morphological changes. C2C12 cells were treated with Dex (1.0 μM) and cell morphological changes were recorded under a bright field microscope at days 3, 5, and 7. Elongated cells and/or potential cell fusions were indicated by arrows.

time-dependent fashion (Fig. 2C). Dexamethasone has been shown to induce progenitor cell differentiation, including myoblastic progenitors (Nishimura et al., 2008). Accordingly, Dex-induced increase in MyHC-GLuc reporter activity was correlated well with Dex-induced myogenic differentiation phenotype, as the treated C2C12 cells exhibited an elongated cell bodies and/or myoblast fusion (Fig. 2D). Taken together, the above results strongly indicate that MyHC-GLuc reporter is myoblast specific and highly responsive to myogenic differentiation inducers.

3.4. ATRA and 9CRA acted synergistically on inducing myogenic differentiation

We sought to determine if ATRA and 9CRA were able to effectively induce myogenic differentiation. We first tested the pMyHC-GLuc reporter activity in C2C12 cells induced by various concentrations of ATRA or 9CRA, and found that ATRA (1.0 μM) or 9CRA (1.0 μM) was sufficient to induce MyHC-GLuc activity (data not shown). In fact, ATRA (1.0 μM) or 9CRA (1.0 μM) was shown to activate MyHC-GLuc activity more effectively than Dex when the reporter activity was determined at different time points (Fig. 3A). Under the same conditions, ATRA was seemingly more potent

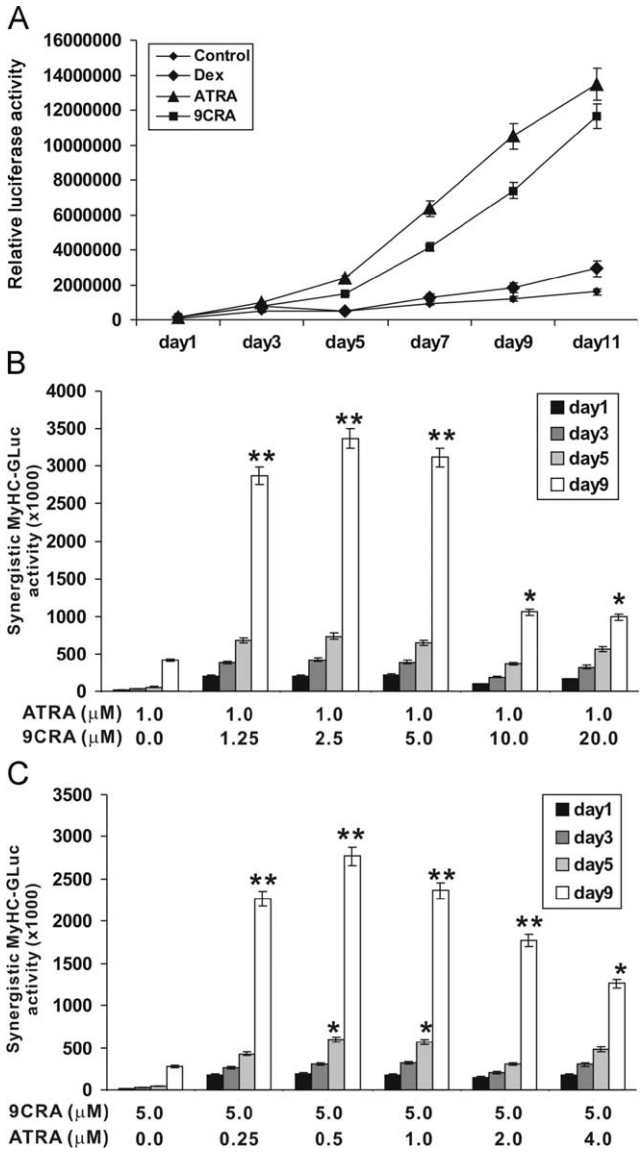

Fig. 3. Synergistic effect of ATRA and 9CRA on myogenic differentiation. (A) C2C12 cells were transfected with pMyHC-GLuc and treated with DMSO (1%), Dex (1.0 μM), ATRA (1.0 μM), or 9CRA (1.0 μM). GLuc activity (mean ± SD) was assayed at the indicated time points. (B) C2C12 cells were transfected with MyHC-GLuc and treated with 1.0 μM ATRA and various concentrations of 9CRA (ranging from 1.25 to 20 μM). GLuc activity (mean ± SD) was assayed at the indicated time points. (C) C2C12 cells were transfected with MyHC-GLuc and treated with 5.0 μM 9CRA and various concentrations of ATRA (ranging from 0.25 μM to 4.0 μM). GLuc activity (mean ± SD) was assayed at the indicated time points. Synergistic GLuc activity was calculated by subtracting the GLuc activity from respective ATRA and 9CRA-treated samples. Each assay condition was carried out in triplicate. Statistical significance was calculated by comparing the synergistic luciferase activity over the respective sum of ATRA and 9CRA-treated samples. "*", $p < 0.05$; "**", $p < 0.001$.

than 9CRA after 7 days ($p<0.05$). These results also indicate that the MyHC-GLuc activity significantly increased at 5 days after ATRA or 9CRA treatment. Thus, we used the day 6–day 9 as a timeframe for terminal differentiation of C2C12 cells upon ATRA and/or 9CRA stimulation.

It is known that ATRA binds to RAR receptors and 9CRA interacts with RXR receptors (Duester, 2008; Niederreither and Dolle, 2008). Since RARs and RXRs can form heterodimers, we tested whether ATRA and 9CRA act synergistically on inducing myogenic differentiation. We introduced the MyHC-GLuc reporter into C2C12 cells, and treated the cells with 1.0 μM of ATRA, various concentrations of 9CRA (1.25–20 μM), or a combination of ATRA (1.0 μM) and 9CRA (1.25–20 μM). The reporter activities were assessed at days 1, 3, 5, and 9. The synergistic reporter activities were significantly increased at day 5 ($p<0.05$), and became more pronounced at day 9 ($p<0.001$), especially at the lower concentration ranges of 9CRA (Fig. 3B). The decreased synergic activity at higher concentrations of 9CRA may be due to the fact that the cell proliferation was inhibited. A similar synergistic effect on MyHC-GLuc reporter was observed when C2C12 cells were treated with a fixed concentration of 9CRA (5 μM) and various concentrations (0.25–4 μM) of ATRA (Fig. 3C). Thus, these findings strongly suggest that ATRA and 9CRA alone or in combination may effectively induce myogenic differentiation.

3.5. RA effectively induced late markers of myogenic differentiation

We further analyzed the expression of myogenesis-related genes upon RA stimulation. Using semi-quantitative RT-PCR analysis, we demonstrated that expression of Myf5 was decreased ($p<0.05$) while there was a slight increase in MyoD expression upon ATRA and 9CRA stimulation ($p<0.01$) (Fig. 4A). Interestingly, Pax3 expression was significantly induced by 9CRA ($p<0.001$) but not by ATRA ($p=0.23$) (Fig. 4A), although the mechanism behind this phenomenon is currently unknown.

Myogenic late marker Troponin T is one of the three subunits of Troponin (Tn) which together with tropomyosin is responsible for the regulation of striated muscle contraction. When C2C12 cells were treated with ATRA and 9CRA, TnT expression was significantly induced at protein level ($p<0.0001$) (Fig. 4B). In fact, most of the ATRA- or 9CRA-treated cells exhibited a significantly elevated level of TnT expression as demonstrated in immunofluorescence staining (Fig. 4C). These results indicate that RA signaling can induce terminal myogenic differentiation.

3.6. Exogenous expression of RARα- and RXRα-induced myogenic differentiation in a ligand-independent fashion

ATRA and 9CRA were prepared in DMSO, which also has a weak ability to induce cell differentiation (Jacob and Herschler, 1986; Marks and Breslow, 2007). We constructed recombinant adenoviruses expressing human RARα and RXRα (designated as AdR-RARα and AdR-RXRα), which co-express monomeric RFP (Campbell et al., 2002). Using the same virus titer, we demonstrated that C2C12 cells were effectively infected by these adenoviral vectors (AdRFP was used as a control) (Fig. 5A). The adenoviral vector-mediated expression of exogenous RARα and RXRα was readily detected by RT-PCR using human gene-specific primers, although the human RARα primers exhibited a weak non-specific amplification of human RXRα gene (Fig. 5B).

We next analyzed the effect of AdR-RARα and AdR-RXRα-mediated transgene expression on myogenic differentiation. C2C12 cells were transfected with MyHC-GLuc reporter plasmid and then infected with AdR-RARα, AdR-RXRα, and/or AdRFP alone or in combinations. The Gaussia luciferase activity was determined at the indicated time points. The infection of AdR-RARα or

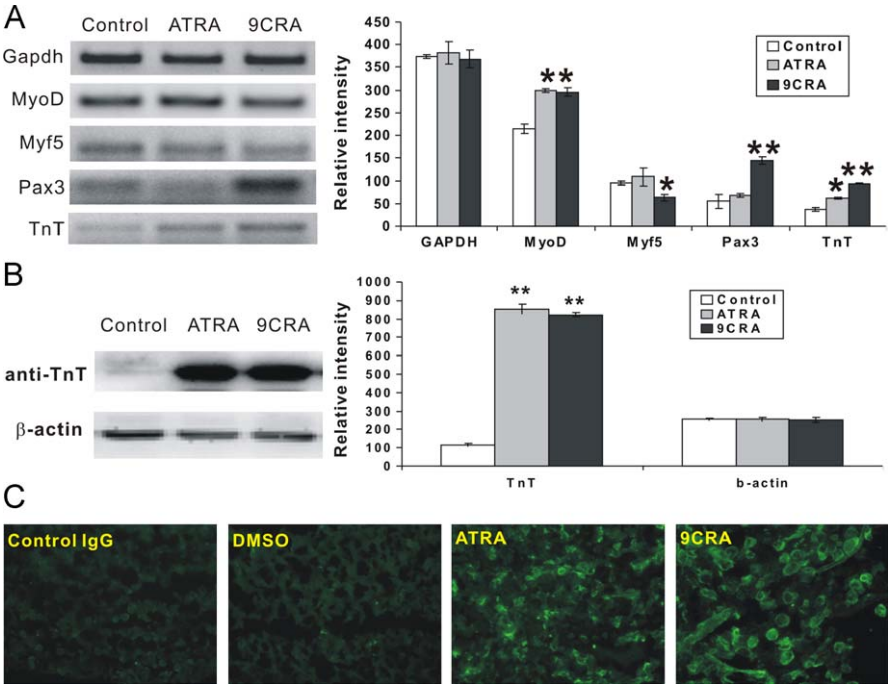

Fig. 4. RA-induced terminal myogenic differentiation of C2C12 cells. (A) RA-induced expression of myogenic genes. C2C12 cells were treated with ATRA (1 μM), 9CRA (1 μM), or DMSO (control) for 3 days. RNA was isolated from the treated cells and subjected to RT-PCR analysis using primers for MyoD, Myf5, Pax3, and TnT. All samples were normalized with GAPDH. (B) C2C12 cells were treated with ATRA (1 μM), 9CRA (1 μM), or DMSO (1%, control) for 3 days. Cells were lysed and subjected to SDS-PAGE and Western blotting using antibody against Troponin T (TnT). Anti-β-actin Western blotting was used to check equal loading. The results reported in Figs. 4A and B were further quantitatively analyzed via densitometry. "*", $p<0.05$; "**", $p<0.001$. (C) RA-induced terminal differentiation of C2C12. C2C12 cells were treated with ATRA (1 μM), 9CRA (1 μM), or DMSO (control) for 5 days. Cells were fixed and stained with anti-TnT antibody or control IgG, followed by staining with DyLight 594-labeled secondary antibody.

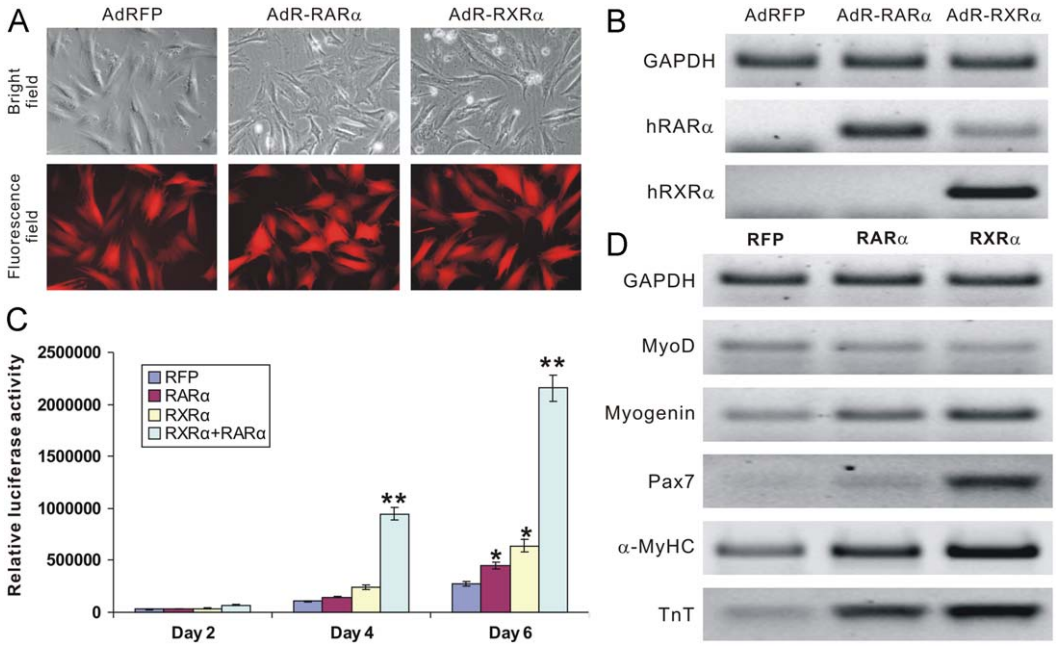

Fig. 5. Exogenous expression of RARα and RXRα-induced myogenic differentiation in a ligand-independent fashion. (A) Efficient transduction of C2C12 cells by RARα and RXRα adenoviruses. Subconfluent C2C12 cells were effectively infected by recombinant adenoviruses AdR-RARα, AdR-RXRα, and AdRFP control. Red fluorescence signal was recorded at 40 h after infection. (B) RT-PCR analysis of transgene expression mediated by AdR-RARα and AdR-RXRα. C2C12 cells were infected with AdR-RARα, AdR-RXRα, or AdRFP. At 36 hrs, total RNA was isolated and subjected to RT-PCR analysis using primers for human RARα and human RXRα. (C) Synergistic induction of myogenic differentiation of C2C12 cells by AdR–RARα and AdR–RXRα. C2C12 cells were transfected with MyHC-GLuc reporter plasmids, replated and infected with AdR–RARα, AdR–RXRα, and/or AdRFP alone or in combination. Gaussia luciferase activity (mean ± SD) was measured at the indicated time. The luciferase activity was confirmed by at least three batches of independent experiments. "*", $p<0.05$; "**", $p<0.001$. (D) AdR–RARα and AdR–RXRα-induced expression of myogenic regulators and differentiation markers. C2C12 cells were infected with AdR–RARα, AdR–RXRα, or AdRFP. At day 5, total RNA was isolated and subjected to RT-PCR, as described in Fig. 4B.

AdR-RXRα alone was shown to induce MyHC-GLuc reporter activity at day 6 ($p<0.05$) (Fig. 5C). Moreover, co-expression of RARα and RXRα acted synergistically on inducing myogenic differentiation of C2C12 cells at as early as 4 days after infection (Fig. 5C). We further analyzed the AdR-RARα and AdR-RXRα-induced expression of myogenic regulators and differentiation markers in C2C12 cells. We found that MyoD expression was slightly decreased, while myogenin expression increased (Fig. 5D). The Pax7 expression was increased significantly in AdR-RXRα-transduced cells, while the late markers α-MyHC and TnT were induced in the cells transduced by either AdR-RARα or AdR-RXRα (Fig. 5D, and Supplemental Fig. 1). Taken these results together, our results have demonstrated that exogenous expression of RARα or RXRα alone can effectively induce myogenic differentiation, and that co-expression of RARα and RXRα acts synergistically on inducing myogenic differentiation, both in a ligand-independent fashion.

3.7. *RA-induced myogenic differentiation was correlated with differentiated morphological changes*

We further examined the morphological changes in RA-treated C2C12 cells. Under the bright field, ATRA- and 9CRA-treated myoblastic C2C12 cells exhibited elongated cell shape and became multi-nucleated myoblasts, and even formed myoblast fusion (Fig. 6A). The RA-induced elongated cell bodies and multi-nucleated myoblasts were more apparent when the C2C12 cells were tagged by AdRFP infection (Fig. 6B) or visualized with Giemsa staining (Fig. 6C). Lastly, we examined the ultrastructures of RA-induced C2C12 cells using transmission electron microscope. The 9CRA-treated C2C12 cells exhibited an abundant presence of muscle fibers in the longitudinal sections (Fig. 7A) and the characteristic arrangement of muscle fibers in the transverse sections (Fig. 7B). Similar ultrastructure features were observed in ATRA-treated C2C12 cells (data not shown). Therefore, our findings strongly indicate that RA signaling is capable of inducing myogenic differentiation.

4. Discussion

Here, we have investigated the role of RA signaling in regulating myogenic differentiation using the mouse myoblast progenitor C2C12 cells, as the role of RA signaling in adult myogenic progenitor cells is not well understood. RA plays an important role in tissue patterning and morphogenesis during development (Mark et al., 2006; Duester, 2008; Niederreither and Dolle, 2008). It has been shown that RA may alleviate the inhibition of myogenic differentiation by activating MyoD protein and myogenin gene transcription (Arnold et al., 1992). 9CRA has been shown about 10 times more efficient than ATRA in repressing Myf5 expression in myoblasts (Carnac et al., 1993). The expression of dominant negative RARs or dominant negative RXRs in myoblasts delays RA-induced growth arrest and differentiation (Alric et al., 1998). However, RA treatment of primary cultures of embryonic somites, limb buds, and neonatal limbs inhibited myogenic differentiation, suggesting that the pleiotropic effects of RA on myogenesis may be affected by extracellular signals during development (Xiao et al., 1995). Thus, the molecular basis of RA action in myogenesis requires to be thoroughly investigated.

In this report, we have found that the expression of most RAR and RXR isotypes are readily detected in C2C12 cells. While the nuclear receptor co-repressors are highly expressed, two of the three nuclear receptor co-activators and the enzymes involved in RA synthesis are expressed at low level or undetectable (Fig. 1). These results suggest that the RA signaling pathway may be

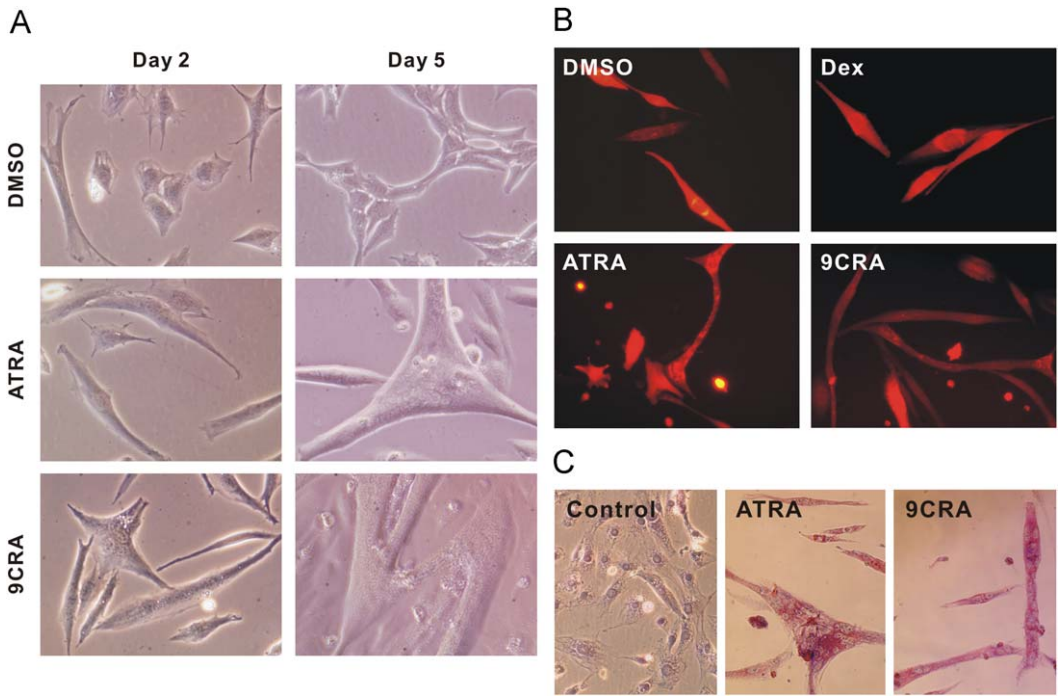

Fig. 6. Morphologic changes of the RA-induced terminal differentiation of C2C12 progenitor cells. (A) C2C12 cells were treated with ATRA (1 μM), 9CRA (1 μM), or DMSO (1%). Bright field images were taken at the indicated time points. Magnification, 300 ×. (B) C2C12 cells were labeled via AdRFP infection, and treated with ATRA (1 μM), 9CRA (1 μM), Dex (1 μM), or DMSO (1%). At 5 days after treatment, cells were subjected to imaging using a fluorescence microscope. Magnification, 200 ×. (C) Giemsa staining of RA-induced myogenic differentiation of C2C12 cells. C2C12 cells were treated ATRA or 9CRA (1.0 μM) or DMSO for 5 days. Cells were fixed and subjected to Giemsa staining. Magnification, 200 ×.

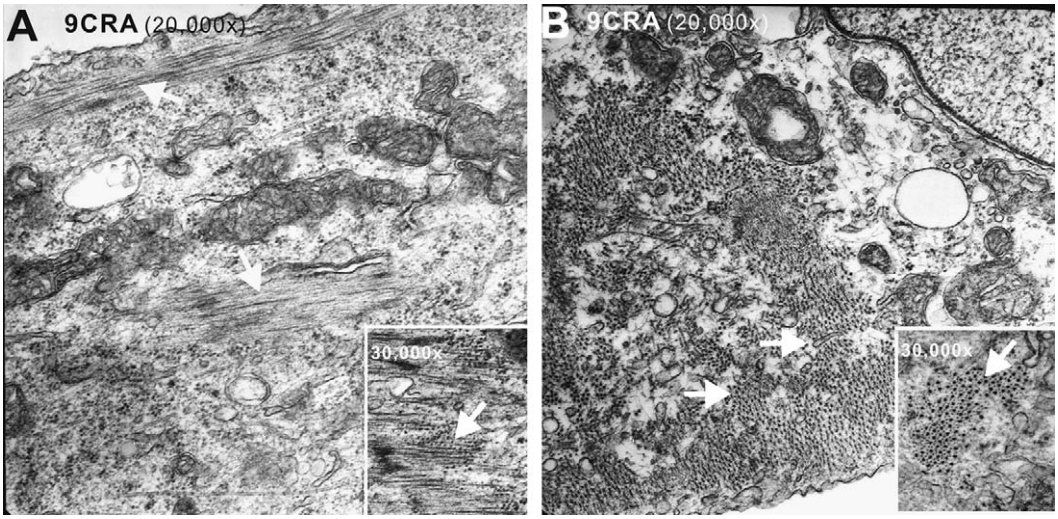

Fig. 7. Transmission electron microscope (TEM) analysis of terminal differentiation of myogenic progenitors. C2C12 cells were treated with RA for 7 days. The 9CRA treated cell pellets were collected, fixed, and subjected to TEM analysis. Muscle fibers were seen in longitudinal sections (A) and cross sections (B). Magnifications, 20,000 × (inset 30,000 ×).

repressed in myogenic progenitors in the absence of RA-initiated differentiation cues. Using the α-myosin heavy chain gene promoter-driven reporter (MyHC-GLuc), we have demonstrated that either ATRA and/or 9CRA are able to induce myogenic differentiation alone or synergistically (Fig. 3). Upon ATRA and 9CRA treatment of C2C12 cells, the expression of late/mature myogenic markers, such as TnT and Pax3, significantly increases, while the expression of MyoD and Myf5 does not change or slightly decreases (Fig. 3). We have further demonstrated that adenovirus-mediated exogenous expression of RARα and/or RXRα is able to induce myogenic differentiation in a ligand-independent fashion (Fig. 5). These results have demonstrated that RA signaling plays an important role in regulating myogenic differentiation.

Satellite cells are dormant progenitors located at the periphery of skeletal myofibers that can be triggered to proliferate for both self-renewal and differentiation into myogenic cells (Peault et al., 2007). Satellite cells are typified by markers such as M-cadherin, Pax7, Myf5, and neural cell adhesion molecule-1. The Pax3 and Pax7 transcription factors play essential roles in the early specification, migration, and myogenic differentiation of satellite

cells (Peault et al., 2007). A new cell population that expresses Pax3 and Pax7 but no skeletal muscle-specific markers has been identified (Relaix et al., 2005). These cells have been shown to constitute resident Pax3/Pax7-dependent muscle progenitor cells that subsequently become myogenic and form skeletal muscle (Relaix et al., 2006). Our results have indicated that 9CRA and RXRα overexpression can induce Pax3 (Fig. 4A) and Pax7 (Fig. 5D), respectively.

The fusion of postmitotic mononucleated myoblasts to form syncytial myofibers is a critical step in the formation of skeletal muscle (Bryson-Richardson and Currie, 2008; Pownall et al., 2002). Myoblast fusion occurs both during development and throughout adulthood, as skeletal muscle growth and regeneration require the accumulation of additional nuclei within myofibers (Bryson-Richardson and Currie, 2008; Pownall et al., 2002). Although many factors regulating myoblast fusion have been identified, the precise mechanism by which these molecules act in concert to control fusion remains to be elucidated. We have shown that morphologically ATRA- and 9CRA-treated C2C12 cells exhibit elongated cell bodies and become multi-nucleated myoblasts, and form muscle fibers (Figs. 6 and 7). Nonetheless, a better understanding of how myoblast fusion is controlled should contribute to the treatment of various disorders associated with loss of muscle mass (Peault et al., 2007; Kuang and Rudnicki, 2008).

In summary, we have demonstrated that RA signaling pathway plays an important role in regulating myogenic differentiation. One practical use of RA-induced skeletal muscle differentiation is the potential use of RAs as differentiation therapeutic agents for cancers. ATRA and 9CRA have been shown to promote differentiation of rhabdomyosarcoma (RMS) cells by inducing the expression of myogenic proteins and the mature muscle marker Troponin T (Barlow et al., 2006; Palomares et al., 2006; Ricaud et al., 2005; Gee et al., 2005). While RA has been shown to induce differentiation of cancer cells (Okuno et al., 2004; Hansen et al., 2000; Haydon et al., 2002, 2007; Park et al., 2001; Barlow et al., 2006; Palomares et al., 2006; Ricaud et al., 2005; Gee et al., 2005), the best example is the therapeutic application of ATRA to treat acute promyelocytic leukemia (APL) and the preventive approach to hepatocellular carcinoma by a synthetic retinoid analog, acyclic retinoid.

Future investigations, through both in vitro and in vivo approaches, should be directed towards delineating the molecular mechanisms through which RA signaling regulates myogenic differentiation. *Rara*-, *Rarb*-, and *Rarg*-null mutant mice are viable, but they display some aspects of fetal and postnatal vitamin A deficiency (VAD) syndromes (Mark et al., 2006). Mutants lacking two RAR isotypes (*Rara/b*-, *Rara/g*-, and *Rarb/g*-null mutants) die in utero or at birth from severe developmental defects that include the complete spectrum of malformations belonging to the fetal VAD-induced syndrome (Mark et al., 2006). Compound mutants between RXRs, RARs, and RA synthesizing enzymes (Raldh1, Raldh2, and Raldh3) have revealed an extensive functional redundancy within the members of each family (RARs or RXRs), although each of these members appears to individually exert at least one specific physiological function (Mark et al., 2006). Thus, muscle-specific conditional mutants of RARs and/or RXRs need to be investigated.

Acknowledgements

We thank Dr. Jeffrey Robbins of the University of Cincinnati and the Children's Hospital Medical Center, Cincinnati, OH, for generous provision of α-5.5 vector (α-MyHC reporter plasmid). The reported work was supported in part by research grants from the American Cancer Society (TCH and HHL), The Brinson Foundation (TCH), the Orthopaedic Research and Education Foundation (HHL), the Natural Science Foundation of China and the Ministry of Science and Technology of China (BCH, JL, YW, QK, LC, QS, and TCH), and the National Institutes of Health (RCH, TCH and HHL).

Appendix A. Supporting Information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.diff.2009.06.001.

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