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

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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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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.