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Expressional studies of the aldehyde oxidase (AOX1) gene during myogenic differentiation in C2C12 cells

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ABSTRACT

Aldehyde oxidases (AOXs), which catalyze the hydroxylation of heterocycles and oxidation of a wide variety of aldehydic compounds, have been present throughout evolution from bacteria to humans. While humans have only a single functional aldehyde oxidase (*AOX1*) gene, rodents are endowed with four AOXs; AOX1 and three aldehyde oxidase homologs (*AOH1*, *AOH2* and *AOH3*). In continuation of our previous study conducted to identify genes differentially expressed during myogenesis using a microarray approach, we investigated AOX1 with respect to its role in myogenesis to conceptualize how it is regulated in C2C12 cells. The results obtained were validated by silencing of the *AOX1* gene. Analysis of their fusion index revealed that formation of myotubes showed a marked reduction of up to 40% in $AOX1_{kd}$ cells. Expression of *myogenin* (*MYOG*), one of the marker genes used to study myogenesis, was also found to be reduced in $AOX1_{kd}$ cells. AOX1 is an enzyme of pharmacological and toxicological importance that metabolizes numerous xenobiotics to their respective carboxylic acids. Hydrogen peroxide (H_2O_2) produced as a by-product in this reaction is considered to be involved as a part of the signaling mechanism during differentiation. An observed reduction in the level of H_2O_2 among $AOX1_{kd}$ cells confirmed production of H_2O_2 in the reaction catalyzed by AOX1. Taken together, these findings suggest that *AOX1* acts as a contributor to the process of myogenesis by influencing the level of H_2O_2.

1. Introduction

Muscle fibers (myofibers) are syncytia established during embryogenesis by fusion of myogenic precursor cells (myoblasts). Despite being mitotically inactive, the addition of new myonuclei or the formation of new myofibers during later stages of life depends on satellite cells, which are myogenic stem cells located underneath the myofiber basal lamina [1–3]. The myogenic potential of satellite cells in postnatal muscle is attributed to its property of self-renewal and potential to produce differentiating progeny. Upon receiving activation signals, myogenic satellite cells (MSCs) in the metabolically quiescent state results in rapid proliferation and differentiation into myoblasts that fuse with each other to form nascent myotubes or with damaged myotubes to repair them [4]. As MSCs are required for repair and maintenance of myofibers, myogenesis of MSCs follows a highly orchestrated genetic program to ensure stringent regulatory control over the induction and repression of specific genes involved in maintaining a balance between quiescence, proliferation, differentiation and self-renewal [5,6]. Maintaining a balance between activation, proliferation and differentiation of quiescent MSCs within the muscle requires the involvement of various myogenic regulatory factors (MRFs), including several transcription factors such as *MYF5*, *MYOD*, *MRF4* and *myogenin* (*MYOG*), which regulate the expression of target genes during myogensis [7]. In previous functional studies of MSCs, we found several genes that were up-regulated, including the gene encoding *aldehyde oxidase 1* (*AOX1*), which has no known function in myogenesis [8,9].

AOX1, an enzyme belonging to the molybdoflavoenzyme (MOFEs) family, which require flavin adenine dinucleotide (FAD) and molybdopterin cofactor for their catalytic activity, shows a high degree of conservation throughout the animal kingdom [10]. AOX1 catalyzes the hydroxylation of heterocycles and oxidation of a wide variety of aldehydic compounds into the corresponding carboxylic acids. Although its physiological substrates have not been identified, AOX1 is considered one of the principal drug metabolizing enzymes in the liver. AOX1 has largely been studied with respect to its role in metabolizing anticancer drugs (e.g., methotrexate), as an immunosuppressant agent (e.g., 6-mercaptopurine) and for use in antiviral drugs (e.g., famciclovir) [11,12]. Another important aspect of AOX1 is that it contributes to endogenous production of

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reactive oxygen species (ROS), thereby regulating intracellular signaling pathways determining cell proliferation, apoptosis, migration and differentiation [13,14]. However, its role with respect to regulation of muscle development via signaling pathways has not previously been reported.

To date, AOX1 has been investigated for its role in cellular detoxification processes and its contribution to endogenous ROS production. However, its role in the myogenesis process remains unclear. In this study, we examined the role of AOX1 in the differentiation of mouse-derived C2C12 myoblast cells. Consistent with the previous study, *AOX1* was found to be significantly up-regulated during differentiation of C2C12 cells into myotubes. During the course of our study, we observed that knock-down of *AOX1* significantly reduced myotube formation, resulting in suppression of the expression of MRFs, particularly *MYOG*. To our surprise, we found that knock-down of *MYOG* does not suppress *AOX1* expression, indicating that it might act upstream of *MYOG*. Intracellular ROS production was also found to increase during the differentiation of C2C12 cells, while it showed a significant decline in the case of *AOX1* knock-down. Taken together, these results indicate that *AOX1* plays a regulatory role in myogenesis, probably via its contribution to endogenous ROS production.

2. Materials and methods

2.1. Cell culture and differentiation of C2C12 cells

The mouse-derived C2C12 myoblast cell line was provided by the Korean Cell Line Bank, Republic of Korea. C2C12 cells were grown in DMEM medium (Dulbecco's Modified Eagle's Medium; HyClone Laboratories, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, USA) by incubation at 37 °C in a humidified atmosphere of 5% CO_2. The myogenic differentiation of C2C12 cells was induced at 60–70% confluence by decreasing the FBS content from 10% to 2% in growth medium followed by incubation for 6 days by changing the differentiation medium every 2 days. Cells were then harvested at the indicated days of incubation for experiments.

2.2. Quantitative real time PCR

Total RNA was extracted from the cells using Trizol™ reagent and stored in diethyl pyrocarbonate-treated water (DEPC) at −80 °C until use. The cDNA was synthesized from 1 μg of RNA using reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Real time PCR was performed with 2 μl of cDNA, 10 pmol of each gene-specific primer and Power SYBR® Green PCR Master Mix (Applied Biosystems, USA) on a 7500 real-time PCR system (Applied Biosystems). Detailed information describing the primer sequences for PCR is provided in the Supplementary Table 1.

2.3. Gene knock-down

C2C12 cells were transfected with either 100 μM siRNA or 1 ng of shRNA.*si-RNA transfection:* Cells were allowed to grow in antibiotic free complete media and then transfected with either 100 μM control or *AOX-1* siRNA (Thermo Scientific Dharmacon, USA) complexed with 5 μl of lipofectamine (Invitrogen) in Opti MEM media (Gibco, USA). Cells were then allowed to recover, after which they were switched to differentiation media.*sh-RNA transfection:* Cells allowed to grow in 6-well plates to 30–50% confluence were transfected with either Vector containing GFP (control) or *MYOG* shRNA using transfection reagent and transfection medium (Santa Cruz Biotechnology, CA, USA). Cells were allowed to recover in 10% FBS culture media for 24–48 h, followed by selection with 2 μg/mL Puromycin (Santa Cruz Biotechnology). Selected cells were grown to 70% confluence before switching to differentiation media.

2.4. Western blot

Cells were washed in ice cold PBS and then lysed in RIPA buffer with protease inhibitor cocktail (Thermo Scientific, USA) for Western blot analysis. Total protein isolated by centrifugation at 13,000 rpm for 10 min at 4 °C was quantified by the Bradford assay [15]. After estimation, protein (60 μg) was separated by 8% SDS–PAGE and transferred to PVDF membrane. Blots were blocked with 5% skimmed milk in TBST for 1 h and then incubated overnight with either AOX-1 (1:1000) (Proteintech, USA) or β-actin (1:1000) antibody (Santa Cruz Biotechnology) at 4 °C. Following this, blots were washed in TBST and incubated with horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology) for one hour at room temperature. After wash with TBST, the blots were developed using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific).

2.5. Immunocytochemistry

On the indicated days after differentiation, cells were washed three times in PBS and then fixed in 4% formaldehyde for 10 min. Cells were subsequently permeabilized in 0.2% Triton X, treated with signal enhancer and then blocked using 5% normal goat serum diluted in PBS. Cells were incubated at 4 °C with primary antibody AOX1 (1:100) and MYOG (1:100) (Santa Cruz Biotechnology) for 12 h. Following three washes with PBS (5 min each), cells were incubated with secondary antibodies for 1 h. The cells were then rinsed with PBS and nuclei were counterstained with DAPI (Sigma Aldrich, USA). Finally, cells were washed with PBS and pictures were taken using a fluorescent microscope equipped with a digital camera (Nikon, Japan).

2.6. Determination of fusion index for myotube formation

The fusion index was calculated as previously described by Lee et al. [16]. Briefly, C2C12 cells grown to 70% confluence were switched to differentiation media and incubated for 5 days. Cells fixed in methanol were stained with Giemsa G250 (Sigma Aldrich), after which pictures were captured randomly at three different spots. The number of nuclei in each myotubes and the total number of nuclei in cells were counted in each field, and the fusion index was calculated as the percentage of total nuclei incorporated into myotubes vs. the total number of nuclei.

2.7. Determination of intracellular ROS during myogenesis

C2C12 cells were allowed to differentiate in differentiation media (DMEM + 2% FBS + 1% P/S). When the cells reached 60–70% confluence, the levels of ROS were measured at 0 h, Day2, Day4 and Day6, respectively. Briefly, media was aspirated and cells were washed twice with DMEM only (serum-free medium). Cells were then incubated with 10 uM 2′-7′-Dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma Aldrich) in DMEM for 30 min at 37 °C. Further, cells were washed twice with PBS, after which the fluorescence intensity in DCFDA treated cells was determined by fluorescence microscopy. ROS activity was also measured in $AOX1_{kd}$ and vector transfected cells after 5 days of differentiation.

2.8. Statistical analysis

Data values are expressed as the means ± SEM. Differences between sets of experiments were analyzed by an unpaired Student's *t*-test. *P* values <0.05 were considered statistically significant.

3. Results

3.1. Up-regulation of AOX1 in the differentiation of C2C12 cells

AOX1 expression was examined during the differentiation of mouse-derived C2C12 cells. Characteristic morphological changes in the form of myotubes were observed during the differentiation of C2C12 cells (Fig. 1A). The mRNA levels of *AOX1* determined by quantitative RT-PCR were found to have increased by about 12-fold and 7-fold on days 3 and 5 after the induction of differentiation process, respectively (Fig. 1B). The expression profile of *AOX1* at the protein level showed consistent increase during the differentiation of C2C12 cells, thereby demonstrating its role in the differentiation process during myogenesis (Fig. 1C). Localization of AOX1 protein in differentiated C2C12 cells was confirmed by immunocytochemistry (Fig. 1D).

3.2. AOX1 knock-down partially inhibits the differentiation of C2C12 cells

To determine the role of *AOX1* in the differentiation of C2C12 cells, expression of *AOX1* was inhibited using siRNA, which resulted in about 50% reduction in the mRNA level and consistent reduction at the protein level (Fig. 2A and B). During the differentiation process, *AOX1* knock-down cells showed morphological differences from control cells. When compared to control cells, alignment of cells for the myotube formation was lower in *AOX1* knock-down cells during the mid-differentiation process (Fig. 2C). In addition, the fusion index for myotube formation in the differentiated cells was found to be reduced by about 30% in *AOX1* knock-down cells (Fig. 2D). The expression of myogenic marker genes was also found to be reduced in *AOX1* knock-down cells. The mRNA levels of *MYF5* and *MYOD* were about 2-fold lower in *AOX1* knock-down cells than in control cells (Fig. 2E), confirming the inhibition of differentiation by *AOX1* knock-down. In addition, shRNA knockdown study also showed similar effects with that of siRNA on *AOX1* knock-down. (Supplementary Fig. 1). Sequence information on siRNA and shRNA are provided as a Supplementary Table 2.

3.3. AOX1 acts upstream of MYOG

The expression of *MYOG* that remains up-regulated during differentiation of C2C12 cells was found to be significantly reduced by *AOX1* knock-down. The mRNA level of *MYOG* was found to be about 2-fold lower in *AOX1* knock-down cells than in control cells (Fig. 3A). Consistently, expression of MYOG protein determined by immunoblotting and immunocytochemistry was found to be significantly reduced by *AOX1* knock-down (Fig. 3B and C). In another set of experiments, inhibition of *MYOG* expression using shRNA resulted in about 60% reduction in the mRNA level (Fig. 3D) and consistent reduction at the protein level. When compared to what we observed in the *AOX1* knock-down cells, the mRNA level of

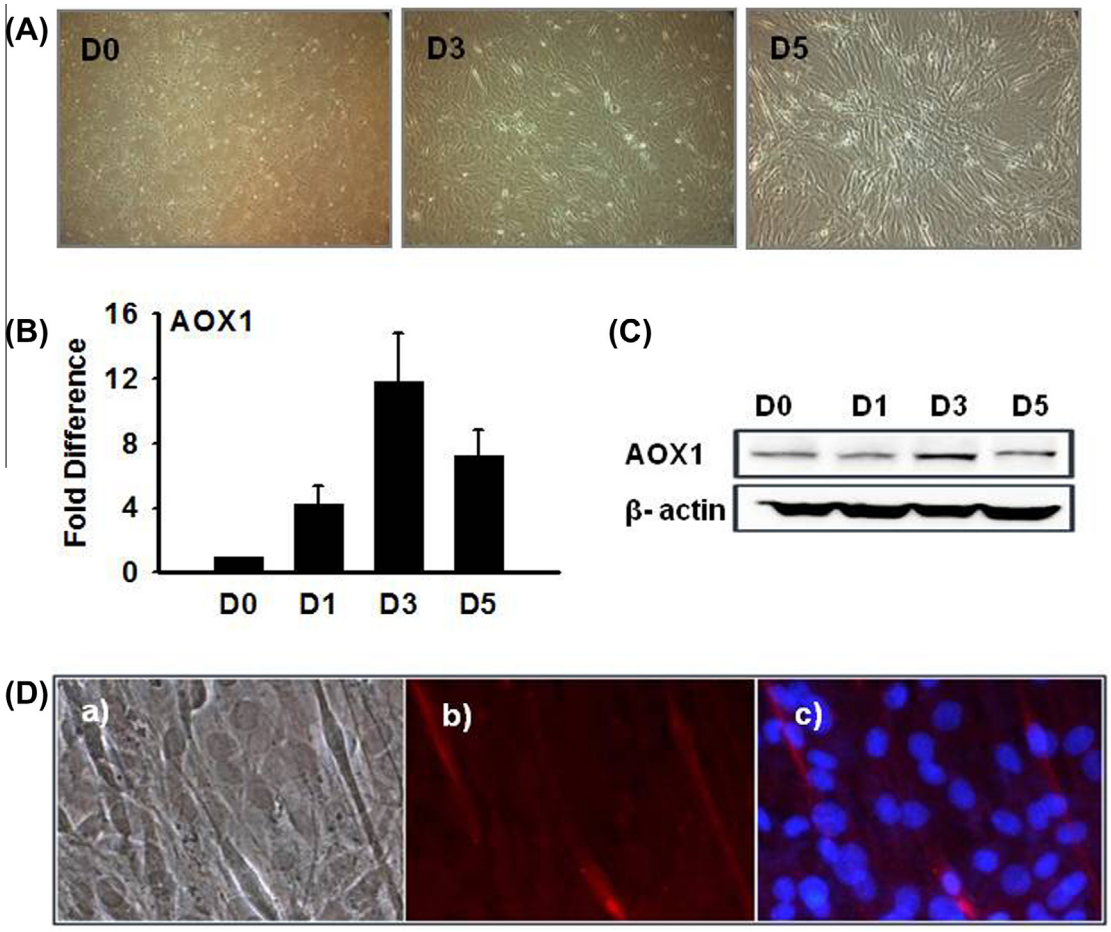

Fig. 1. Cells were grown in culture media (DMEM + 10% of FBS + 1% P/S) to 70% confluence. For differentiation, the FBS concentration was reduced to 2% and cells were harvested at different time intervals to check the expression of *AOX1* during myogenesis. (A) Representative cell picture showing C2C12 myogenesis. (B) mRNA expression of *AOX1*. (C) Western blot of AOX1 during myogenesis. (D) Immunocytochemistry of AOX1 during myogenesis. (a) Cell pictures (b) localization of AOX1, (c) merged image of DAPI-stained nuclei and AOX1 protein at 72 h.

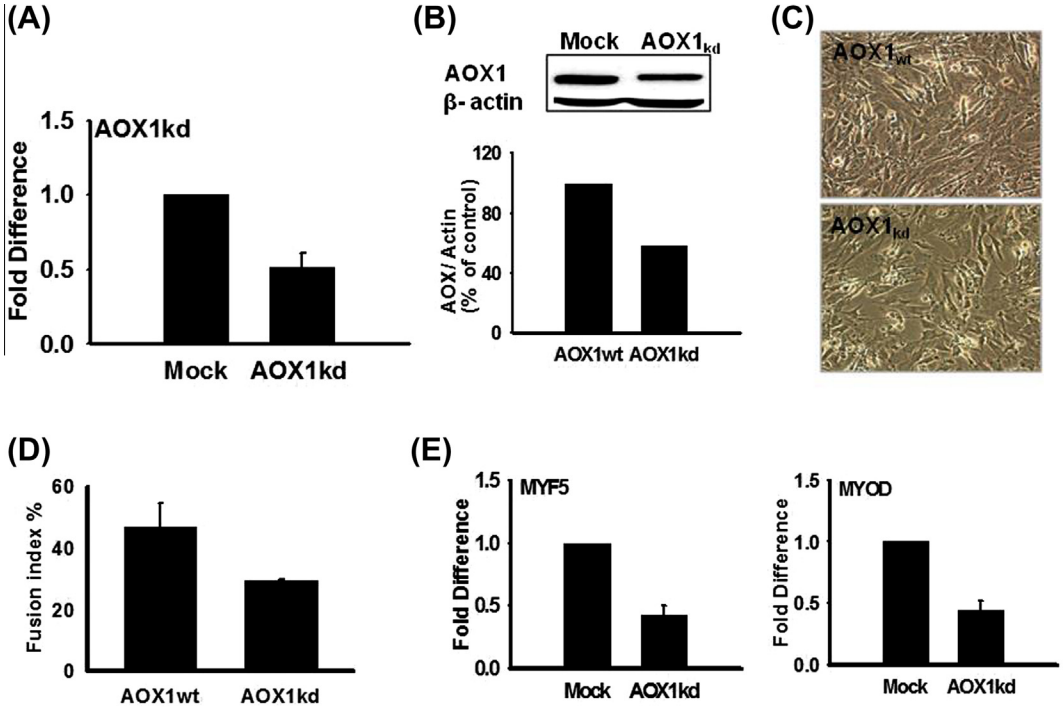

Fig. 2. Effect of *AOX1* knock-down ($AOX1_{kd}$) on mRNA and protein expression in C2C12 cells during myogenesis. (A) mRNA expression of $AOX1_{wt}$ and $AOX1_{kd}$ during differentiation in C2C12 at Day3. (B) Reduced protein expression of AOX1 after knockdown as analyzed by Western blotting and its Quantification. (C) Cell pictures of $AOX1_{wt}$ and $AOX1_{kd}$ showing cell morphology change in $AOX1_{kd}$. (D) A fusion index was performed with $AOX1_{wt}$ and $AOX1_{kd}$, which showed a reduction in myotube formation at Day5. (E) Effect of $AOX1_{kd}$ on mRNA expression of different myogenic marker genes.

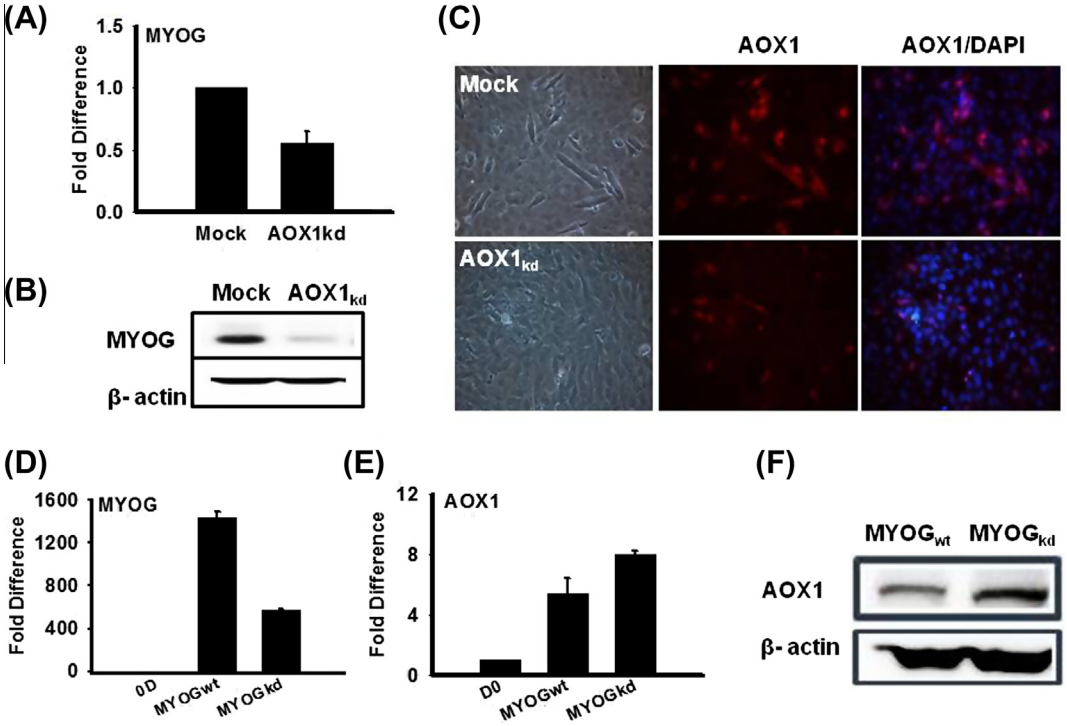

Fig. 3. C2C12 cells were grown to 70% confluence and then transfected with shRNA for *MYOG* knock-down ($MYOG_{kd}$). (A) Effect of $AOX1_{kd}$ on *MYOG* expression on Day3 in C2C12 cells. (B) Protein expression of *MYOG* in $AOX1_{kd}$ at 72 h by immunoblot. (C) Protein expression of *MYOG* in $AOX1_{kd}$ at 72 h by immunostaining. (D) Expression of *MYOG* was reduced almost to 60% relative to the control vector after *MYOG* knock-down. (E and F) $MYOG_{kd}$ showed no down regulation in *AOX1* during myogenesis with respect to mRNA and protein expression.

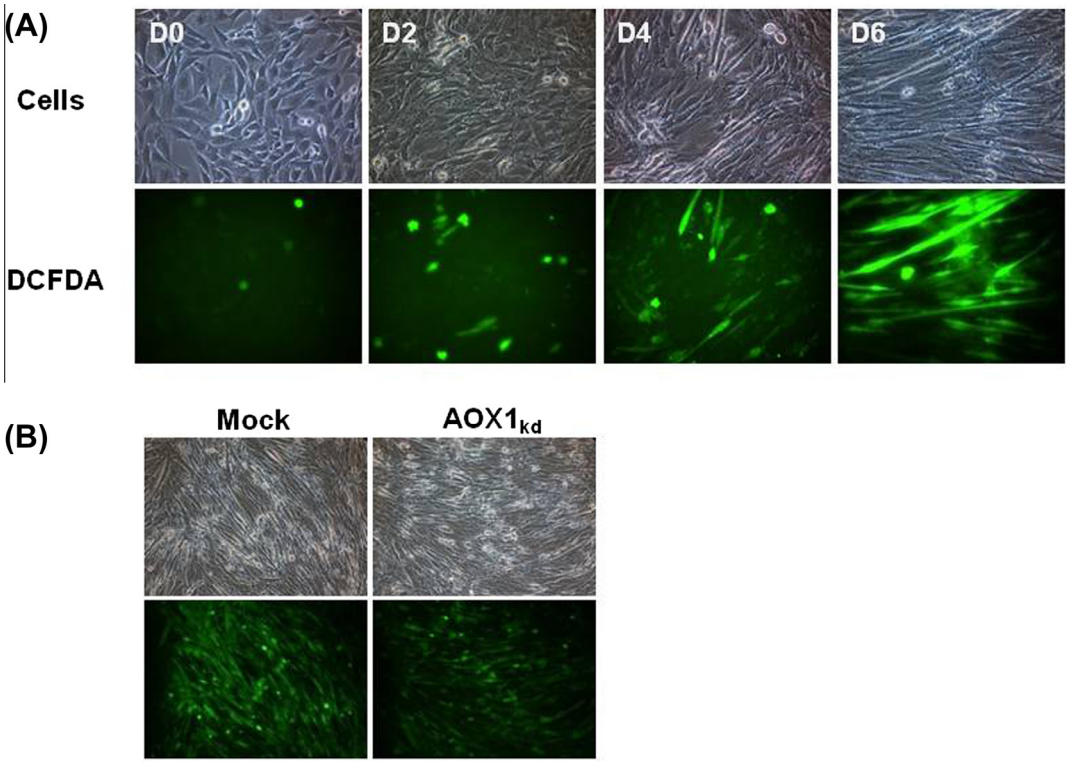

Fig. 4. Cells were seeded in 12 well plates at a density of 2×10^4 cells per well. When cell confluence reached 60%, media was replaced with differentiation media (DMEM + 2% FBS + 1% P/S). (A) ROS activity was measured by florescence microscopy to determine the involvement of H_2O_2 at 0 h, Day2, Day4, and Day6 during myogenesis. (B) Difference in effect of $AOX1_{wt}$ and $AOX1_{kd}$ on H_2O_2 production on Day5.

AOX1 was found to be higher in *MYOG* knock-down cells than in control cells (Fig. 3E), indicating that *AOX1* expression is increased by *MYOG* knock-down. Subsequently, AOX1 protein was found to be consistently increased by *MYOG* knock-down (Fig. 3F).

3.4. AOX1 knock-down suppresses intracellular ROS accumulation

Intracellular ROS was determined during the differentiation of C2C12 cells (Fig. 4A). Before induction of differentiation, the level of ROS was found to be insignificant in cells. However, it showed a gradual increase following differentiation, resulting in its accumulation in the differentiated myotubes. Moreover, level of ROS was significantly lower in *AOX1* knock-down cells than control cells upon myotube formation (Fig. 4B).

4. Discussion

Consistent with our previous study investigating up-regulation of *AOX1* during the myogenic differentiation of bovine satellite cells [8,9], we report here significant up-regulation of *AOX1* during the differentiation of mouse-derived C2C12 cells that has been widely used to study myogenesis [17]. This study revealed that *AOX1* knock-down significantly reduces myotube formation and suppresses the expression of all tested MRFs, thereby clearly indicating that *AOX1* is, at least in part, involved in the regulation of myogenesis, although its physiological role and action mechanism are yet to be elucidated. As part of a study that was directed toward elucidating the mechanism of action of *AOX1*, expression of *MYOG*, involved in the terminal phase of myogenesis [7], was found to be significantly suppressed by *AOX1* knock-down. Two different approaches (siRNA and shRNA) were used for the knock-down study. It increases the reliability of data, further ruling out the possibility of siRNA off target effect. Conversely, *AOX1* expression was found increased slightly, indicating that *AOX1* likely acts upstream of *MYOG* in myogenesis. Moreover, it is believed that *AOX1* acts upstream of *MYF5* and *MYOD*, although this has not been experimentally examined.

AOX1 is an evolutionary conserved enzyme, catalyzing oxidation of aldehydes with subsequent generation of hydroxylation products and hydrogen peroxide [10]. In this study, we observed that, in addition to hydroxylation products and hydrogen peroxide, oxidation of aldehyde by AOX1 results in the production of superoxide, suggesting its contribution to endogenous ROS production [13]. Previous reports have established that ROS play a role not only as cytotoxic molecules, but also as signaling molecules regulating cell proliferation, apoptosis, migration and differentiation [14]. Recent investigations of myogenesis have indicated that muscle development is sensitively regulated by environmental and endogenous ROS [18,19]. It is now believed that high concentrations of ROS inhibit the differentiation of C2C12 cells [20,21] and induce the apoptosis of differentiated myotubes [22], while low concentrations of ROS generated endogenously by NADPH oxidase enhance the proliferation and differentiation of muscle precursor cells [23,24]. Endogenous ROS are primarily generated as by-products in mitochondrial oxidative phosphorylation. It was recently reported that AOX1 accepts NADH as a good substrate and efficiently generates superoxide [25]. In continuation of this study, we report here an increase in intracellular ROS, followed by differentiation of C2C12 with *AOX1* up-regulation and a subsequent reduction in the ROS level in differentiated myotubes by *AOX1* knock-down. Although the roles of ROS in myogenesis are currently unclear, the regulation of myogenesis by *AOX1* may be mediated by ROS functioning as signaling molecules. These results indicate the contribution of *AOX1* to endogenous ROS production, which enhances myogenesis via an unknown signaling pathway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.126.

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