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Effects of retinoic acid signaling on extraocular muscle myogenic precursor cells *in vitro*

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ABSTRACT

One major difference between limb and extraocular muscles (EOM) is the presence of an enriched population of Pitx2-positive myogenic precursor cells in EOM compared to limb muscle. We hypothesize that retinoic acid regulates Pitx2 expression in EOM myogenic precursor cells and that its effects would differ in leg muscle. The two muscle groups expressed differential retinoic acid receptor (RAR) and retinoid X receptor (RXR) levels. RXR co-localized with the Pitx2-positive cells but not with those expressing Pax7. EOM-derived and LEG-derived EECD34 cells were treated with vehicle, retinoic acid, the RXR agonist bexarotene, the RAR inverse agonist BMS493, or the RXR antagonist UVI 3003. *In vitro*, fewer EOM-derived EECD34 cells expressed desmin and fused, while more LEG-derived cells expressed desmin and fused when treated with retinoic acid compared to vehicle. Both EOM and LEG-derived EECD34 cells exposed to retinoic acid showed a higher percentage of cells expressing Pitx2 compared to vehicle, supporting the hypothesis that retinoic acid plays a role in maintaining Pitx2 expression. We hypothesize that retinoic acid signaling aids in the maintenance of large numbers of undifferentiated myogenic precursor cells in the EOM, which would be required to maintain EOM normalcy throughout a lifetime of myonuclear turnover.

1. Introduction

The extraocular muscles (EOMs) are different from limb skeletal muscles developmentally, anatomically, physiologically, and biochemically [1]. One of the more unusual properties of uninjured adult EOMs is their continuous and significant levels of myonuclear remodeling throughout life due to the presence of chronically activated myogenic precursor cells [2–5]. Recent studies using a reporter mouse for Pax7, a transcription factor expressed ubiquitously in satellite cells of skeletal muscle [6], confirmed these earlier studies and showed a significant level of myonuclear turnover in the EOM [7,8]. In contrast, uninjured adult limb skeletal muscles contain largely quiescent Pax7-positive myogenic precursor cells, and only after injury or in disease is there significant activation and proliferation of these cells [6,9–12].

Other significant differences between EOMs and body muscle also exist. For example, distinct transcription factors are required for the early determination, development, and maintenance of EOMs compared to limb muscle [13,14]. Specifically, the transcription factor Pitx2 is required for the development of EOMs, but is not required for the development of limb skeletal muscle [15]. Pitx2 expression is also maintained at high levels in adult EOMs compared to limb skeletal muscle [16,17], and this high level of Pitx2 expression is necessary to maintain characteristic properties of adult EOMs [16–18].

Myogenic precursor cells within adult skeletal muscle are a diverse population, and this diversity is amplified in adult EOMs. Overall, the EOMs have an elevated density of different types of myogenic precursor cells compared to normal uninjured adult limb muscles [19,20]. For example, there is an elevated density of Pax7-positive cells in EOMs compared to limb muscle [21] as well as an elevated population of EECD34 cells, identified using flow cytometry, which are positive for CD34 and negative for Sca1, CD45, and CD31 [19]. In a recent study, we described a significantly elevated population of myogenic precursor cells in EOMs that express Pitx2, which are essentially a non-overlapping population from those cells that express Pax7 [17,20]. The roles

Abbreviations: EOM, extraocular muscles; RAR, retinoic acid receptor; RXR, retinoid X receptor; EECD34 cells, extraocular muscle enriched cells expressing CD34; LEG, tibialis anterior muscle

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played by these heterogeneous myogenic precursor cell populations in the function of the EOMs are unclear. Our recent study showed that high dose gamma irradiation (18 Gy) in a mouse model of muscular dystrophy differentially affected Pitx2 and Pax7 myogenic precursor cells and resulted in the transient appearance of dystrophic changes – centrally located myonuclei – in the irradiated EOMs. This study suggests that these different precursor populations play different temporal and/or functional roles in EOM myofiber remodeling, repair, and regeneration [21].

The factor(s) that maintain high levels of Pitx2 expression in adult EOMs are currently unknown. One candidate factor is retinoic acid, a vitamin A derivative. Retinoic acid regulates Pitx2 expression during ocular and craniofacial development [22,23]. Without proper retinoic acid signaling during development, the EOMs do not form [24,25]. Recent studies have implicated retinoic acid in globally regulating myogenic differentiation [26]. We tested the hypotheses that retinoic acid signaling differs in adult EOM and limb muscle myogenic precursor cells and that retinoic acid controls the maintenance of high levels of Pitx2 expression in adult EOMs and promotes proper function of the Pitx2-positive EOM myogenic precursor cells.

To test this hypothesis, we compared the expression of RARα, RARγ, RXRα, and RXRγ proteins in mouse EOMs and limb muscle. Next, the functional effects of modulating retinoic acid signaling were assessed in myogenic precursor cells *in vitro* isolated from mouse EOMs and limb muscle. Finally, to determine if retinoic acid acts to maintain Pitx2 expression in adult EOM myogenic precursor cells, the effect of retinoic acid signaling on expression of Pitx2 in EECD34 cells *in vitro* was assessed following addition of retinoic acid, RXR agonist, or inhibition of RXR function.

2. Results

2.1. EOMs express different levels of retinoic acid receptor and retinoid X receptor protein than limb skeletal muscle

Retinoic acid receptors (RARs) are nuclear receptors involved in retinoic acid signaling. The canonical retinoic acid signaling pathway involves the dimerization of RAR with retinoid X receptors (RXR) [27]. The RAR-RXR dimers then bind to retinoic acid response elements and stimulate the transcription of retinoic acid responsive genes. However, recent data show that these nuclear receptors can be differentially regulated and can each function in an independent manner in cells [28–31]. To determine if retinoic acid receptors were differentially expressed in EOMs compared to limb muscle, we used western blots to assess the amount of retinoic acid receptor alpha (RARα) and gamma (RARγ) expressed in EOM and LEG whole tissue lysates. RARα protein levels were significantly higher in LEG whole tissue lysates compared to EOM whole tissue lysates, with LEG samples expressing 4-fold more RARα than the EOMs (Fig. 1A, B). However RARγ levels were not statistically significantly different in LEG whole tissue lysates compared to EOM whole tissue lysates (Fig. 1C, D). In contrast, retinoid X receptor alpha (RXRα) and gamma (RXRγ) protein levels were significantly higher in EOM whole tissue lysates, with EOM expressing over almost 91.7% more RXRα than LEG whole tissue lysates (Fig. 1E, F) and 106% more RXRγ (Fig. 1G, H).

2.2. RXRα co-expresses with Pitx2 but not Pax7

The potential myogenic identity of the precursor cells expressing RXRα was examined in tissue sections of EOMs from adult mice. Nuclear expression of RXRα was evident in sections from EOM (Figs. 2 and 3), but was only sparsely found in sections of leg muscle (not shown). The RXRα positive nuclei were seen to co-express Pitx2 (Fig. 2). Using a Pax7 reporter mouse [8], the Pax7-expressing satellite cells did not co-express RXRα (Fig. 3). This suggested a strong interrelationship between RXRα and Pitx2 expression. Note also the RXRα-positive myonuclei in addition to the RXRα-positive cells outside of the sarcolemma (Fig. 3D).

2.3. EOM-derived EECD34 cells maintain Pitx2 expression in vitro

EECD34 cells ($CD34^+/Sca1^-/CD45^-/CD31^-$) are a subpopulation of myogenic precursor cells, the majority of which are positive for Pitx2 [17,19]. To determine if EECD34 cells derived from the EOMs retained their Pitx2 expression *in vitro*, cells were immunostained one, two, three, and four days after plating in proliferation media. At one and two days after the EECD34 cells were plated in proliferation media, essentially 100% of the cells were Pitx2 positive (Fig. 4A, B, D). In these cultures, dividing cells were often present (Fig. 4A), and both daughter cells retained their Pitx2 expression at these time points. By 72 h *in vitro*, 92.3 ± 3.6% of the cells were still Pitx2 positive (Fig. 4C, D), and the vast majority of dividing cells were still both Pitx2 positive (not shown). By 96 h in proliferation medium, 95.0 ± 0.6% % of the cells were Pitx2-positive. This demonstrates that at the time of the proliferation assay, the EECD34 cells retained their Pitx2 expression.

2.4. EECD34 cell proliferation is unchanged by additional retinoic acid or reduced retinoic acid signaling

Knockdown of Pitx2 expression in EECD34 cells *in vitro* significantly reduced their proliferation rate [17]. Given the potential role for retinoic acid to regulate Pitx2 expression in adulthood, as it does during development, and the significant role played by Pitx2 in regulating the proliferation rates of EECD34 cells, EECD34 cell proliferation rates were assessed following the addition of retinoic acid and retinoic acid signaling modulators. The effect of retinoic acid, the pan-RAR inverse agonist BMS493, and the RXR antagonist UVI 3003 on the proliferation rate of EECD34 cells *in vitro* was determined by calculating the percentage of cells that incorporated the thymidine analog EdU following treatment. Addition of retinoic acid, BMS493, or UVI 3003 did not change the proliferation rate of EOM-derived or LEG-derived EECD34 cells compared to control cells (Fig. 5A-E). However, in EOM-derived EECD34 cells there was a significant difference in proliferation rates between cells treated with the RAR inverse agonist compared to those treated with retinoic acid, with RAR inverse agonist treated cells having 29.7% more EdU-positive cells than retinoic acid treated cells (Fig. 5E). Consistent with previous results [17], there were significantly more EdU-positive EOM-derived EECD34 cells compared to LEG-derived EECD34 cells under all conditions except the retinoic acid treatment, with proliferation rates 44.9% higher in control cultures, 30.3% higher in the presence of retinoic acid, 72.1% higher in the presence of the RAR inverse agonist, and 52.5% higher in the presence of the RXR antagonist (Fig. 5E).

2.5. EECD34 cell fusion and desmin expression is changed after modulation of retinoic acid signaling

Knockdown of Pitx2 expression in EECD34 cells *in vitro* significantly reduced their fusion index [17]. Given the potential role for retinoic acid to regulate Pitx2 expression in adulthood as it does during development, and the role of Pitx2 in regulating fusion of EECD34 cells, we investigated if the addition of retinoic acid, BMS493, or UVI 3003 altered the fusion and desmin expression of EECD34 cells *in vitro*. Following 72 h incubation in fusion media containing vehicle, retinoic acid, BMS493, or UVI 3003, the cells were immunostained for desmin, followed by hematoxylin staining (Fig. 6), counted, and the cell fusion index and percent of differentiated cells were calculated. Under all conditions the LEG-derived EECD34 cells had a significantly greater fusion index compared to EOM-derived EECD34 cells (Fig. 7A). For vehicle treated control cells there was a 58.6% difference, retinoic acid treated cells there was a 123.3% difference, BMS493 treated cells there was a 36.3% difference, and UVI 3003 treated cells there was a 65.4%