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Retinoic acid activates myogenesis in vivo through Fgf8 signalling

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Abstract

Retinoic acid (RA) has been shown to regulate muscle differentiation in vitro. Here, we have investigated the role of RA signalling during embryonic myogenesis in zebrafish. We have altered RA signalling from gastrulation stages onwards by either inhibiting endogenous RA synthesis using an inhibitor of retinaldehyde dehydrogenases (DEAB) or by addition of exogenous RA. DEAB reduces expression of the myogenic markers *myoD* and *myogenin* in somites, whereas RA induces increased expression of these genes and strongly induces premature *myoD* expression in the presomitic mesoderm (psm). The expression dynamics of *myf5* in presomitic and somitic mesoderm suggest that RA promotes muscle differentiation, a role supported by the fact that RA activates expression of fast myosin, while DEAB represses it. We identify Fgf8 as a major relay factor in RA-mediated activation of myogenesis. We show that *fgf8* expression in somites and anterior psm is regulated by RA, and find that in the absence of Fgf8 signalling in the *acerebellar* mutant RA fails to promote *myoD* expression. We propose that, in the developing embryo, localised synthesis of RA by Raldh2 in the anterior psm and in somites activates *fgf8* expression which in turn induces the expression of myogenic genes and fast muscle differentiation.

Keywords: Retinoic acid; Myogenesis; Somites; Presomitic mesoderm; *myoD*; *fgf8*; *acerebellar*; Zebrafish

Introduction

Myogenesis is a complex process initiated during embryonic segmentation and lasting until birth (Buckingham et al., 2003). This process includes the coordination of several steps: the specification of muscle precursors, and the control of their proliferation, migration and differentiation (Buckingham et al., 2003; Parker et al., 2003). During segmentation, the formation of regularly shaped somites associates an oscillating mechanism and the progression of a segmentation boundary (Pourquie, 2003; Stickney et al., 2000; Stockdale et al., 2000). This boundary delimits a posterior domain where somite maturation is repressed and an anterior domain where differentiation begins (Iulianella et al., 2003; Pourquie, 2003).

Myogenesis begins during segmentation and is under the control of several signals arising from structures neighbouring the somites, i.e. surface ectoderm, neural tube and notochord. Hedgehog proteins, particularly Sonic hedgehog (Shh), represent the major signalling pathway in zebrafish myogenesis described until now. Indeed *hh* genes are expressed in axial structures (floor plate and notochord) during myogenesis and are responsible for the maintenance of the early myogenic factors *myf5* and *myoD* in adaxial cells (Blagden et al., 1997; Coutelle et al., 2001; Devoto et al., 1996; Du et al., 1997). Adaxial cells are precursors of slow muscle and muscle pioneers, the remaining myotome producing fast muscle (Devoto et al., 1996). Several mutations affecting the Hh signalling pathways have been characterised including mutations in *shh* (*syu*), *smoothened* (*smu*) and *gli2* (*yot*) (Barresi et al., 2000; Coutelle et al., 2001; Du and Dienhart, 2001; Lewis et al., 1999; Wolff et al., 2003). In mutants lacking functional Hh signalling, there is a diminution or absence of slow muscle depending on the severity of Hh pathway disruption (Coutelle

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et al., 2001; Wolff et al., 2003). Strikingly, fast muscle development appears unaffected (Blagden et al., 1997) and no specific signalling pathway has been unravelled until now that controls development of this muscular subtype.

Recently, an additional control level has been identified in chick, where fibroblast growth factor receptor 4 (Fgfr4) is required for limb muscle differentiation upon activation by Fgf8 (Marics et al., 2002). In addition, over-expression of *fgf8* promotes *fgfr4* and *myoD* expression in somites, while inhibition of Fgfr4 signalling represses limb muscle differentiation (Marics et al., 2002). It has further been shown that Fgf8 controls the differentiation of tendons in mouse somites through activation of Fgfr4 receptors (Brent et al., 2003). Both observations contrast with the previous notion that Fgf signals promote myoblast proliferation and repress their differentiation.

Retinoic acid (RA) has been shown to inhibit proliferation and to promote muscular differentiation of myoblasts by inducing *myoD* expression in vitro (Alric et al., 1998). Although a large number of studies have been performed to analyse the role of RA during development, none of them report a direct implication of RA in the control of myogenesis in vivo. However, several observations suggested that RA plays a role during somitogenesis. Retinaldehyde dehydrogenase 2 (Raldh2), the main RA synthesising enzyme, is expressed during zebrafish development in presomitic mesoderm during early steps of segmentation and in somites, presomitic and lateral plate mesoderm at later stages (Begemann et al., 2001; Grandel et al., 2002). This expression pattern is conserved in ascidian embryos and tetrapods, including mouse, chicken and *Xenopus* (Chen et al., 2001b; Nagatomo and Fujiwara, 2003; Niederreither et al., 1997; Swindell et al., 1999) and is consistent with the detection of RA in chick somites (Maden et al., 1998). In zebrafish, RA and its precursors have been detected in the egg and throughout development (Costaridis et al., 1996), in addition Bcox, an enzyme converting provitamin A to retinal, is expressed during segmentation (Lampert et al., 2003). Finally, RA receptors RAR and RXR are expressed in posterior mesoderm and somites during development of vertebrates including zebrafish (Cui et al., 2003; Jones et al., 1995; Joore et al., 1994; Kawakami et al., 2005; Mangelsdorf et al., 1992).

Both the inactivation of the *raldh2* gene and deprivation in vitamin A lead to defects in somite formation in mouse and quail, respectively (Maden et al., 1996; Maden et al., 2000; Niederreither et al., 1999). In particular, recent work has shown that RA controls segmentation by repressing *fgf8* expression (Diez del Corral et al., 2003; Molotkova et al., 2005; Vermot et al., 2005; Vermot and Pourquie, 2005) or Fgf8 signalling (Moreno and Kintner, 2004) in the tailbud and posterior psm. A down-regulation of *myogenin* has also been reported in vitamin A deficient (VAD) quail embryos (Maden et al., 2000). Taken together, these observations suggest that during embryogenesis, RA signalling could be involved in somitogenesis and muscle development.

In the present report, we investigated the effects of altered RA signalling on myogenesis and identified Fgf8 as an intermediate factor that mediates RA-induced promotion of somitic muscle differentiation in the zebrafish embryo. We propose that during embryonic development, locally synthesised RA activates onset of *fgf8* expression in anterior presomitic mesoderm and somites and by this way controls somitic muscle differentiation.

Material and methods

Fish lines

A local inbreed line of wild-type zebrafish was propagated under standard conditions at 28°C. Stages were determined according to Kimmel et al. (1995). Mutant strains (*smu*^{b641}, *nls*^{i26}, *ace*^{ti282a}) were obtained from the Tübingen stock centre.

Incubations

Embryos were incubated in *all-trans* RA (Sigma) and retinal (Sigma) at a final concentration of 10^{-7} M (except when mentioned otherwise) and DEAB (Sigma, final concentration 3×10^{-5} M) from stocks in DMSO. DMSO was kept to a final concentration of 0.1% for experimental incubations and controls. As a standard procedure, embryos were incubated from 8 hpf until 14 hpf in the dark (timing may vary as indicated; see Fig. 1). All incubations and cycloheximide (Sigma, 10 µg/ml (Shi et al., 2002)) treatments were followed by washes in PTw (PBS 1×, Tween 20 0.1%) and overnight fixation at 4°C in 4% para-formaldehyde.

In situ hybridisation, antibody staining

In situ hybridisation and antibody staining were performed as described in Jowett (1997). Probes were kindly provided by colleagues or obtained after PCR amplification: *cyp26a1* (Dobbs-McAuliffe et al., 2004), *erm1* (Roehl and Nusslein-Volhard, 2001), *fgf8* (Reifers et al., 1998), *fgf17b* (Cao et al., 2004), *krox20* (Oxtoby and Jowett, 1993), *mesp-b* (Sawada et al., 2000), *myoD* (Weinberg et al., 1996), *myogenin* (Eric Weinberg, unpublished data), *myf5* (Chen et al., 2001c), *papc* (Yamamoto et al., 1998), *pax2.1* (Pfeffer et al., 1998), *raldh2* (Begemann et al., 2001), *shh* (Krauss et al., 1993) and *twist* (Halpern et al., 1995). Antisense riboprobes were synthesised using the appropriate RNA polymerase and a DIG-labelling kit (Roche).

Antibody F59 (anti slow-myosin until 24 hpf, then all myosin; used at 1/10 dilution) was kindly provided by Dr. Stockdale. Antibody F310 (fast myosin; 1/300) was purchased at DSHB (Developmental Studies Hybridoma Bank, Iowa City, IA). Embryos were fixed in 4% paraformaldehyde overnight at 4°C or 2 h at RT. Embryos were blocked at least two h in 1× PTw/0.2% BSA at room temperature. Antibodies were added at the appropriate dilution and embryos were incubated overnight at 4°C with gentle shaking. Biotinylated secondary antibodies were used and revealed with an ABC kit (Vectastain).

Embryos were photographed as whole-mounts or flat-mounted, in 80% glycerol and photographed using a Zeiss Axiophot and Kappa camera.

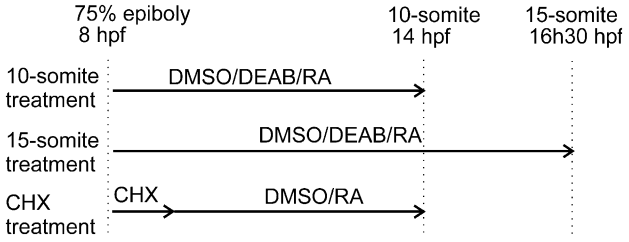

Fig. 1. Treatments timing. In most cases, embryos were incubated in DMSO as a control, DEAB or RA, for 6 h from the 75% epiboly stage (8 hpf) until the 10-somite stage (14 hpf). In some cases, incubation lasts until the 15-somite stage (*twist* and myosins expression). To analyse influence of protein neo-synthesis, we also performed sequential incubations, first in CHX during 30 min followed by incubations in DMSO or RA until the 10-somite stage.