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Tendon-muscle crosstalk controls muscle bellies morphogenesis, which is mediated by cell death and retinoic acid signaling

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Abstract

Vertebrate muscle morphogenesis is a complex developmental process, which remains quite yet unexplored at cellular and molecular level. In this work, we have found that sculpturing programmed cell death is a key morphogenetic process responsible for the formation of individual foot muscles in the developing avian limb. Muscle fibers are produced in excess in the precursor dorsal and ventral muscle masses of the limb bud and myofibers lacking junctions with digital tendons are eliminated via apoptosis. Microsurgical experiments to isolate the developing muscles from their specific tendons are consistent with a role for tendons in regulating survival of myogenic cells. Analysis of the expression of Raldh2 and local treatments with retinoic acid indicate that this signaling pathway mediates apoptosis in myogenic cells, appearing also involved in tendon maturation. Retinoic acid inhibition experiments led to defects in muscle belly segmentation and myotendinous junction formation. It is proposed that heterogeneous local distribution of retinoids controlled through Raldh2 and Cyp26A1 is responsible for matching the fleshy and the tendinous components of each muscle belly.

Keywords: Muscle development; Limb development; Retinoic acid; Apoptotic cell death; Cathepsin D; Caspase 3; Integrin; tcf4

Introduction

The formation of each muscle in the embryo is a complex multi-step process involving guided detachment and migration of muscle precursors, myofiber differentiation and attachment to target tendons (see review by Schnorrer and Dickson, 2004). The analysis of the development of somatic muscles in the *Drosophila* embryo has provided a considerable advance in the knowledge of mechanisms controlling muscle formation and attachment (see reviews by Baylies et al., 1998; Schnorrer and Dickson, 2004). However, the basis of vertebrate muscle morphogenesis is much less understood.

In vertebrates, muscle cells are originated from the somitic mesoderm under the control of the paired homeodomain transcription factor Pax3 and the MyoD family of basic helix–loop–helix transcription factors (see reviews by Buckingham, 2001; Brent and Tabin, 2002). The appendicular muscles are formed by the delamination of muscle precursors from the hypaxial dermomyotome and subsequent migration into the limb field. Muscle cell precursors detachment and proper migration into the limb field seem to be controlled by the homeobox gene Lbx1 (Schafer and Braun, 1999; Brohmann et al., 2000), and the activation of c-Met receptor tyrosine kinase by secreted SF/HGF specifically expressed in the limb mesenchyme (Dietrich et al., 1999). The migration of muscle cells also appears to be critically controlled by the chemokine receptor CXCR4 and its ligand SDF1 (Vasyutina et al., 2005; Odemis et al., 2005) as well as by the EphA4 receptor tyrosine kinase and its ligand, ephrin-A5 (Swartz et al., 2001). This initial step of detachment and migration gives rise to muscle cell precursors grouped in well-defined dorsal and ventral pre-muscle masses. Importantly it has been proposed that the establishment of muscle masses is directed by the distribution of SF/HGF, which is itself under the control of retinoic acid

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signaling (Mic and Duester, 2003). Muscle differentiation is regulated by ectodermal signals including wnt-6 (Geetha-Loganathan et al., 2005).

In the chicken embryo, by day 6.5 of incubation, migrating dorsal and ventral muscle masses establish well-defined boundaries (stylopodial; zeugopodial; and autopodial) along each of the three major joints in the limb in the proximal-distal axis, accompanied by the intercalation of three tendinous laminas (Kardon, 1998). Next in development, each region is segregated into individual muscles of the thigh, shank and foot respectively. The cellular and molecular mechanisms underlying the segregation and independent formation of each muscle belly from the muscle masses remain largely unknown.

Lineage tracing experiments revealed that migrating muscles retain the potential to contribute to any muscle of the limb (Kardon et al., 2002), indicating that local extrinsic signals are critical for the guidance and determination of muscle cell fate. This finding fully agrees with early quail-chick chimera experiments showing that specification of limb muscles is instructed by local signals within the limb bud (Chevallier et al., 1977; Christ et al., 1977). It has been shown that formation of individual muscle bellies is closely coordinated with the formation of tendons (Kardon, 1998) and to some extent with the developing skeleton (Lanser and Fallon, 1987). There is also evidence showing that mesodermal cells expressing the Tcf4 gene, a transcription factor downstream of the Wnt-β-catenin signaling pathway, plays a major role in establishing the zones of muscle differentiation (Kardon et al., 2003).

In spite of the above exposed data, cellular and molecular mechanisms directing the formation of muscle attachments at the correct position or accounting for the cleavage of individual muscles, still await clarification. In this study, we have chosen the formation of chick foot muscles to analyze the mechanism by which the dorsal and ventral muscle masses are segregated into individual muscle bellies. This model system has a double advantage. On the one hand, the position of the muscles in the most distal portion of the limb allows for easy recognition by confocal microscopy and other morphological approaches. On the other hand, these muscles connect with the developing tendons of the autopod, which are formed in an autonomous fashion, independent from the muscles (Hurle et al., 1990; Shellswell and Wolpert, 1977). Moreover, due to the spatial independence of tendons and muscles, it is possible to check for morphogenetic interactions between the tendinous and muscular portion of each muscle belly using surgical approaches. Our findings reveal that the muscle bellies are sculptured from the muscle masses by cell death in close coordination with the developing tendons and myotendinous junction formation. We further propose that local concentrations of retinoids are critical determinants of muscle vs. tendon development.

Material and methods

Animal models

In this work, we employed Rhode Island chicken embryos ranging from 4.5 to 9 days of incubation (stages 24 to 35 of Hamburger and Hamilton, 1951).

Experimental manipulation of the limb

A variety of surgical experiments, including digit amputations and implantation of tungsten barriers, were designed to interfere with the assembly of muscle and tendons. The eggs were fenestrated at the desired stage and operations were performed on the right leg bud. Digit amputations and implantation of tungsten barriers were performed between days 5 and 6.5 of development. In all these surgical experiments, we paid attention to manipulate distally to the domains of MyoD and Pax 3 gene expression to avoid damaging the muscle forming regions of the limb.

Local treatments were performed by implanting AG1X-2 (BioRad) beads incubated in 10 to 100 μg/ml all-*trans*-retinoic acid (Sigma) and SM2 beads (BioRad) incubated in 0.2 g/ml Citral (Fluka). Eggs were windowed at desired stages and beads, previously incubated for 1 h in the corresponding solution, were implanted in the developing limb at the proper position. All the experiments were contrasted by parallel experiments implanting DMSO soaked beads from which no effects were observed. After the period of incubation, samples were processed for immunostaining and imaging. Systemic treatments were performed at day 6 of incubation by injection into the vitelline sac of total doses of Citral ranging between 0.17 and 0.44 mg per egg and/or 1 to 25 μg of all-*trans*-retinoic acid. Proper DMSO control experiments were done in parallel and no effects were observed.

At least 3 independent series of experiment with a minimum of 15 individuals each were performed for every treatment described.

Antibodies and immunolabeling

For tendon or muscle immunostaining, we used the following monoclonal antibodies: MF20 (Hybridoma Bank) or F59 (Hybridoma Bank) to muscle sarcomeric myosin, MF38 (Hybridoma Bank) to collagen I, M1B4 (Hybridoma Bank) and rabbit anti-chick tenascin (Chemicon) to tenascin. Polyclonal antibodies against caspase 3 active (R&D Systems) and cathepsin D (Santa Cruz Biotechnology) has been also used in this study. For confocal microscopy, limbs were dissected and fixed in 80% methanol–20% DMSO O/N at 4°C, washed in TBS and incubated O/N at 4°C with the primary antibody. Specimens were next washed in TBS, incubated O/N in the secondary antibody washed for 2 h in TBS, dehydrated and cleared in Murray's clear (33% benzyl alcohol–66% benzyl benzoate). For double labeling using the terminal deoxynucleotidyl transferase-mediated dUTP-TRIC nick end labeling (TUNEL) assay, limbs were dissected and fixed in 4% PFA. Either longitudinal or transversal 200 μm vibratome sections were taken and bleached in Den't bleach (50% Methanol, 25% H_2O, 15% H_2O_2, 10%DMSO) before incubation O/N in primary antibody at 4°C, washed in PBS and incubated O/N in secondary antibody. TUNEL was performed using the in situ cell death detection kit (Roche) following the manufacturer's instructions.

Confocal microscopy

Samples were examined with a laser confocal microscope (LEICA LSM 510) by using a Plan-Neofluar 10×, 20× or Plan-Apochromat 63× objectives, and argon ion laser (488 nm) to excite FITC fluorescence and a HeNe laser (543 nm) to excite Texas Red. Limbs and vibratome specimens were optically sectioned longitudinally along dorsal–ventral planes at 15 μm intervals. For stacks digitalization and image processing, we used the LSM 5 Image Examiner software on a Windows NT-Based PC. Images shown in this work are the integration of all the Z-stacks taken to cover the whole muscle, except for vibratome sections, which are the integration of three Z-stacks. Every image shown in this study is representative of at least 3 independent experiments.

Light and electron microscopy

The sequential structural changes of the myocytes undergoing cell death were studied by light and transmission electron microscopy (TEM). The autopods were dissected free, fixed in 2% glutaraldehyde, post-fixed in osmium tetroxide and embedded in araldite. For light microscopy, semithin sections were obtained and stained with toluidine blue. For TEM, ultrathin sections were stained with lead citrate and examined with a Philips EM208 electron microscope.