Developmental Biology ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Specific pattern of cell cycle during limb fetal myogenesis

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A R T I C L E I N F O

Article history:
Received 25 October 2013
Received in revised form
24 March 2014
Accepted 21 May 2014

Keywords:
Pax7
MyoD
Fucci
Cell cycle
Fetal myogenesis

A B S T R A C T

Tight regulation of cell proliferation and differentiation is required to ensure proper growth during development and post-natal life. The source and nature of signals regulating cell proliferation are not well identified in vivo. We investigated the specific pattern of proliferating cells in mouse limbs, using the Fluorescent ubiquitylation-based cell-cycle indicator (Fucci) system, which allowed the visualization of the G1, G1/S transition and S/G2/M phases of the cell cycle in red, yellow or green fluorescent colors, respectively. We also used the retroviral RCAS system to express a Fucci cassette in chick embryos. We performed a comprehensive analysis of the cell cycle state of myogenic cells in fetal limb muscles, adult myoblast primary cultures and isolated muscle fiber cultures using the Fucci transgenic mice. We found that myonuclei of terminally differentiated muscle fibers displayed Fucci red fluorescence during mouse and chick fetal development, in adult isolated muscle fiber (ex vivo) and adult myoblast (in vitro) mouse cultures. This indicated that myonuclei exited from the cell cycle in the G1 phase and are maintained in a blocked G1-like state. We also found that cycling muscle progenitors and myoblasts in G1 phase were not completely covered by the Fucci system. During mouse fetal myogenesis, Pax7+ cells labeled with the Fucci system were observed mostly in S/G2/M phases. Proliferating cells in S/G2/M phases displayed a specific pattern in mouse fetal limbs, delineating individualized muscles. In addition, we observed more Pax7+ cells in S/G2/M phases at muscle tips, compared to the middle of muscles. These results highlight a specific spatial regionalization of cycling cells at the muscle borders and muscle–tendon interface during fetal development.

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Introduction

An open question is to understand how the balance between proliferation and differentiation is maintained over time in order to ensure progressive tissue growth during development. The spatial and temporal organization of cell cycle is not characterized in vivo.

Embryonic, fetal and peri-natal myogenesis occur in successive and overlapping phases. The myogenesis processes involve different muscle progenitor populations, which are defined throughout development by the expression of the *paired-box* transcription factors, *Pax3* and *Pax7* (Hutcheson et al., 2009; Kassar-Duchossoy et al., 2005; Relaix et al., 2005; Schienda et al., 2006). Pax3+ cells define the embryonic muscle progenitors, while Pax7+ cells identify fetal muscle progenitors and satellite cells (Hutcheson et al., 2009; Kassar-Duchossoy et al., 2005; Relaix et al., 2005). Once specified to a muscle fate, muscle progenitors use a common muscle program, which involves the myogenic regulatory factors (MRFs). The four members of this family of DNA binding proteins (Myf5, MyoD, Mrf4 and Myogenin) induce the expression of a variety of genes involved in the contractile properties of mature skeletal muscle cells. While embryonic myogenesis involves specification and determination processes, fetal myogenesis is based on muscle growth (Messina et al., 2010). Moreover, it is by the end of fetal myogenesis that the satellite cells are generated and acquire their specific location underneath the basal lamina (Brohl et al., 2012). Perinatal muscle growth is due to an increase in the number of myonuclei and to muscle hypertrophy (White et al., 2010). In the adult, satellite cells have a stem cell potential (Seale et al., 2000). Under resting conditions satellite cells are quiescent, while after damage they become activated, proliferate

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http://dx.doi.org/10.1016/j.ydbio.2014.05.015
0012-1606/© 2014 Published by Elsevier Inc.

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and reactivate the myogenic program via the MRFs (reviewed in Buckingham and Relaix, 2007).

A tight control of cell proliferation is crucial for proper skeletal muscle formation, particularly during fetal myogenesis. Different signaling pathways regulate fetal muscle progenitors, including Notch, Wnt and Bmp pathways (Biressi et al., 2007; Hutcheson et al., 2009; Vasyutina et al., 2007; Wang et al., 2010). Recently, a transcription factor, Nfix, has been identified as a major regulator of the switch between embryonic and fetal myogenesis, by regulating directly the transcription of several fetal specific genes and concomitantly inhibiting embryonic specific genes (Messina et al., 2010). Several arguments exist to say that fetal muscle progenitors are not all equivalent (Hauschka, 1974; Schafer et al., 1987; Biressi et al., 1997). It has been observed that a subpopulation of fetal muscle progenitors responding to Bmp signaling are preferentially located at the tips of muscles, close to the tendons in chick embryos (Wang et al., 2010). Lastly, two distinct muscle progenitor populations have been described during fetal development, which display different rates of cycling in chick and mouse embryos (Picard and Marcelle, 2013), although there is no indication of spatial regionalization for these two cell populations. All together these data suggest a heterogeneity of proliferating fetal muscle progenitors.

Adult satellite cells are a heterogeneous population as observed through multiple parameters. Cre/LoxP lineage tracing studies identified a sub-population of satellite cells that had never expressed *Myf5* and functioned as a stem cell reservoir (Kuang et al., 2007). These Pax7+/Myf5-satellite cells give rise to Pax7+/Myf5+ committed cells through apical–basal oriented divisions, which asymmetrically generate a basal Pax7+/Myf5-cell and an apical Pax7+/Myf5+ cell (Le Grand et al., 2009; Kuang et al., 2007). ex vivo work on FACS-sorted satellite cells demonstrated that while the vast majority of activated satellite cells were fast-dividing cells, slow-dividing cells were observed as a minority population (Ono et al., 2012). In addition, clusters of activated satellite cells are more concentrated at the extremities of isolated adult muscle fibers (Wang et al., 2010).

in vitro studies have highlighted the role of cell cycle components during the muscle differentiation process (reviewed in Ciemerych et al., 2011). In particular, pRb, (retinoblastoma protein), which is associated with cell cycle exit and terminal differentiation, has been shown to up-regulate *MyoD* transcriptional activity and to induce the expression of late muscle differentiation markers (Gu et al., 1993). Moreover, MyoD promotes the transcriptional activation of the Cdk inhibitors (CKI) from the CIP/KIP family, p21, p27 and p57, in differentiating myoblasts, contributing to cell cycle arrest (Cenciarelli et al., 1999; Figliola and Maione, 2004; Otten et al., 1997). MyoD activity is higher during G1, while it starts to be degraded as the cells enter S phase and proceed with the cell cycle (Kitzmann et al., 1998). While MyoD is associated with G1 phase and cell cycle withdrawal, Myf5 expression starts in late G1 phase and is higher throughout the S/G2/M phases and in the quiescent G0 state (Kitzmann et al., 1998; Lindon et al., 1998). However, the organization of cell cycle is poorly characterized in vivo. It has been shown that the number of proliferating Pax7+ cells decreases over development in both mouse and chick embryos (He et al., 2005; Picard and Marcelle, 2013).

The Fucci (Fluorescent ubiquitination-based cell cycle indicator) system has been developed based on the fact that several proteins oscillate through the different phases of the cell cycle, due to the activity of ubiquitin ligase complexes (Sakaue-Sawano et al., 2008). Both Cdt1 and Geminin proteins are involved in the licensing of the DNA replication and therefore their activities are tightly regulated (McGarry and Kirschner, 1998; Nishitani et al., 2000; Wohlschlegel et al., 2000). Geminin is ubiquitinated during G1 phase by the APC^{Cdh1} complex, while Cdt1 is tagged to degradation by the SCF^{Skp2} ubiquitin ligase complex at the S phase (Li et al., 2003; McGarry and Kirschner, 1998), allowing a non-co-localization of these proteins except at the G1/S transition phase. By fusing human Geminin to the red fluorescent protein monomeric Kosabira Orange 2 (mKO2) and Cdt1 to the green fluorescent protein monomeric Azami-Green (mAG), it has been established a color system that covers the different cell cycle phases (Sakaue-Sawano et al., 2008). Two transgenic mouse lines expressing ubiquitously mKO2-hCdt1 (Fucci red) or mAG-hGem (Fucci green) have been established (Sakaue-Sawano et al., 2008).

In this paper, we analyzed the pattern of cell cycle in limbs during mouse and chick fetal myogenesis using the Fucci system. We also analyzed the Fucci state of adult myoblasts and isolated fibers.

Materials and methods

Mouse lines and chick embryos

The Fucci mice expressing either Cdt1-KO2 (FucciTG mouse #596, Fucci red) or Gem-AG (FucciTG mouse #504, Fucci green) were obtained from the RIKEN Brain Science Institute, (Japan). Mouse embryos were collected after natural overnight matings. For staging, fertilization was considered to take place at midnight. Fertilized White Leghorn chick eggs (HAAS, Kaltenhouse) were incubated at 38.5 °C. Chick embryos were staged according to days in ovo.

RCAS-Fucci-2A plasmid construction and grafting RCAS-Fucci-2A-expressing cells

The Fucci-2A cassette containing mKO2-hCdt1 and eGFP-hGem sequences was established by Patrick Martin and Franck Delaunay (IBV, Nice, France) using the 2A peptide, which allows the production of two proteins under the same promoter (Szymczak et al., 2004). The Fucci-2A cassette was excised from the pPRIHy Fucci-2A vector by performing a double digestion with ClaI and AccI restriction enzymes, and inserted into the RCAS-BP(A) vector, digested with ClaI and further dephosphorilated with the Antartic Phosphatase enzyme, using the T4 DNA Ligase.

Chicken embryonic fibroblasts (CEFs) obtained from E10 chick embryos were transfected with RCAS-Fucci-2A at a confluence of 50% using the Calcium Phosphate Transfection Kit (Invitrogen), overnight at 37 °C and 5% CO_2. Pellets of approximately 50–100 μm in diameter were grafted into limb buds at E3.5. The embryos were harvested 5 days after grafting at E8.5, and processed for immunohistochemistry to tissue sections.

Adult primary myoblast culture

Skeletal muscles of hindlimbs of 2 month-old Fucci green and red mice were dissected, transferred to a sterile 6 cm Petri dish on ice, mulched into a smooth pulp and incubated in CollagenaseB/DispaseII/$CaCl_2$ solution (1.5 U/ml, 2.4 U/ml, and 2 M, respectively, in DMEM; Roche). After a 15 min incubation at 37 °C in the culture incubator, the muscle pulp was triturated with heat-polished glass Pasteur pipettes, and this incubation/trituration step was repeated. The tissue digestion was stopped with the addition of FBS, cells were filtered and washed twice with PBS, re-suspended in growth medium consisting of Ham's F10 supplemented with 20% FBS and 2.5 ng/μl of bFGF, and let to adhere onto a non-coated 10 cm plate for 2 h. At the end of the preplating procedure, the media was transferred onto collagen-coated Petri dishes. Cultures were maintained in growth medium until cells reached 80% confluence.

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Please cite this article as: de Lima, J.E., et al., Specific pattern of cell cycle during limb fetal myogenesis. Dev. Biol. (2014), http://dx.doi.org/10.1016/j.ydbio.2014.05.015

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