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Multipotency of skeletal muscle stem cells on their native substrate and the expression of Connexin 43 during adoption of adipogenic and osteogenic fate

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ABSTRACT

Muscle regeneration is performed by resident muscle stem cells called satellite cells (SC). However they are multipotent, being able to adopt adipogenic and osteogenic fate under the correct stimuli. Since SC behavior can be regulated by the extra-cellular matrix, we examined the robustness of the myogenic programme of SC on their native substrate-the surface of a myofiber. We show that the native substrate supports myogenic differentiation judged by the expression of members of the Myogenic Determination Factor (MRF) family. However SC even on their native substrate can be induced into adopting adipogenic or osteogenic fate. Furthermore conditions that support adipose or bone formation inhibit the proliferation of SC progeny as well as their migration. We show that Connexin43 (Cx43), a gap junction complex protein, is only expressed by activated and not quiescent SC. Furthermore, it is not expressed by SC that are in the process of changing their fate. Lastly we show that intact adult mouse muscle contains numerous cells expressing Cx43 and that the density of these cells seems to be related to capillary density. We suggest the Cx43 expression is localized to angioblasts and is more prominent in oxidative slow muscle compared to glycolytic fast muscle.

1. Introduction

Skeletal muscle contains a resident stem cell population called Satellite cells (SC) which have a huge capacity to regenerate damaged tissue. Transplantation of a single fiber consisting of less than ten cells is able to generate tens of thousands of myonuclei within a matter of a few weeks (Collins et al., 2005). SC take their name from their peripheral position relative to the muscle fiber (Mauro, 1961). They are located under the basal lamina, in direct contact with the sarcolemma of muscle fibers. In undamaged muscle, they are relatively metabolically inactive as indicated by their low cytoplasmic content and they exist in a quiescent state. However, they express certain markers including Pax7 that aid their identification (Zammit et al., 2006). Upon muscle damage, SC become activated by inducing a number of genes including *MyoD* that encodes a member of the Myogenic Determination Factor family (MRF) of transcriptions factors (Zammit et al., 2006). Activation of SC permits cell division as well as migration. Initially, SC migrate under the basal lamina but then take up a supra-basal position by remodeling their overlying extra-cellular matrix (Otto et al., 2011). Activated SC can either revert to their quiescent state by down-regulating MyoD while maintaining Pax7 or can commit to myogenic differentiation by shutting off Pax7 expression and inducing the expression of Myogenin, another member of the MRF family (Zammit et al., 2006).

Following acute muscle injury, SC progeny only form muscle cells (Collins et al., 2005). However, they are multipotent and under appropriate conditions can adopt an adipogenic or osteogenic fates (Katagiri et al., 1994; Yamaguchi et al., 1991). A number of diseases including Duchenne Muscular Dystrophy result in the formation of adipose deposits in skeletal muscle (Li et al., 2015). The origin of the adipose deposits is much debated and some have postulated that they are derived from SC. Understanding the mechanisms that control SC fate change away from the myogenic lineage is of considerable clinical value since adipose tissue is believed to hinder muscle regeneration (Cordani et al., 2014).

Abbreviation: SC, satellite cells; Cx43, connexin43; PPARγ, peroxisome proliferator-activated receptor gamma; FABP4, fatty acid binding protein 4; OC, osteocalcin; OP, osteopontin; MHC, myosin heavy chain; AP2, adipocytes protein2; ALP, alkaline phosphatase; BMP-2, bone morphogenetic protein-2

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A number of studies highlight the importance of the surface matrix in almost all aspects of cell behavior. The matrix governs the mechanisms by which cells migrate. For example, SC on their native substrate, the muscle fiber, move through an amoeboid-based mechanism, yet when the same cells are placed on plastic they develop lamellipodia (Morash et al., 2017; Otto et al., 2011) Surface matrix governs not only how cells migrate but ultimately can control what they become by regulating their fate. Engler and colleagues have elegantly demonstrated that hydrogel stiffness over two orders of magnitude directs fate decisions of human mesenchymal stem cells, and ultimately determines whether they form bone, muscle or neurons (Engler et al., 2006).

We have previously shown that seeding non-muscle stem cells onto the surface of muscle fibers induces them to adopt a SC mode of migration (Morash et al., 2017). More remarkable, we showed that adipose derived stem cells undergo a myogenic conversion following culture on the myofiber surface (Morash et al., 2017). These and other studies show that the native substrate of SC supports myogenic characteristics.

Here we examined the impact of adipogenic and osteogenic culture conditions on SC located on muscle fibers. Furthermore, we investigated the expression of Connexin 43 on muscle SC and how it was influenced by adipogenic and osteogenic culture conditions. Connexin43 (Cx43) is a gap junction protein that regulates intercellular transfer of signals and molecules less than 1 kD (Kumar and Gilula, 1996). Cx43 plays a pivotal role during development and maintenance of the myocardium via modulation of cell polarity and the migration potential of cardiomyocytes (Rhee et al., 2009). Furthermore, Cx43 is expressed during muscle regeneration following cardiotoxin and $BaCl_2$ injection implying a role in this process (Araya et al., 2005).

2. Materials and methods

2.1. Animals

All experimental procedures were approved by the institutional ethics committee and the Regierungspräsidium Gießen GI 20/10 Nr. 105/2014. 6–8 week old C57/Black 6 male mice were kept in conventional housing at the animal facility of Justus Liebig University of Giessen. Mice were provided water and chow diet *ad libitum*.

2.2. Muscle dissection

Hind limb *M. tibialis anterior* (TA) and *M. soleus* (SOL) were dissected and snap frozen in Tissue-Tec OCT (Sakura, Germany) using isopentane precooled with liquid nitrogen. 10 μm mid-belly cryostat sections were placed on poly-L-lysine coated slides (VWR Germany) and stored in −80 °C for immunohistochemistry.

2.3. Isolation of SC/Myoblasts with or without the myofibers

M. extensor digitorum longus (EDL) was used due to its amenability to produce single muscle fibers (Keire et al., 2013; Pasut et al., 2013). EDL muscles were dissected and extra-cellular matrix partially digested using 0.2% collagenase IV (Biochrome, Germany) in DMEM (Gibco, Life technologies, Germany) at 37 °C with 5% CO_2 for 1.5–2 h. Hind limb muscles were dissected into ice-cold PBS containing 1% penicillin/streptomycin (Gibco Fisher Scientific) for myoblasts extraction. All tendons, nerves and blood vessels were removed under sterile conditions. Muscles were minced, centrifuged at 2000*g* for 5 min and digested in 0.1% collagenase IV in DMEM with shaking at 37 °C for 40 min. Digested samples were triturated using an 18 gauge needle. Equal volume of 2% FCS (fetal calf serum, Biocell) DMEM was added and the mixture filtered through a 70 μm cell strain before centrifuging at 2000 g for 10 min. Cell pellets were resuspended in fresh media and cells counted using a hemocytometer. Surface markers CD44 and CD90 as well as preplating for 48 h was used for SC selection as previously described (Chirieleison et al., 2012; Scimeca et al., 2015). Briefly, 10 μl of goat anti mouse IgG magnetic bead solution was incubated with 5–10 μg of anti-mouse CD90 and CD44 (1:50, DSHB) primary antibodies in 5% FCS/PBS with agitation at 4C° for 1 h. After washing three times in PBS, 1×10^7 cells were incubated with bead-coated primary antibodies at 4 °C for 30 min. Bead coated cells were magnetically separated and washed in PBS. To reduce non-satellite cells contamination, harvested cells were preplated in 20% FCS/Hamś F-10 (Gibco Fisher Scientific) supplemented with 1% penicillin and streptomycin for 48 h. Non-attached cells were centrifuged at 800*g* for 5 min, the pellet suspended in 10% FCS/DMEM and seeded on collagen coated flasks (0.4% bovine calf skin/acetic acid, Biochrom, Germany) until 50–70% confluency. The expression of surface marker CD44, CD90 and specific myoblasts marker MyoD were assessed using the immunofluorescent staining (Fig. 2g–I and supplementary Fig. 1 d, e and f). The cells at passage 1–3 showing greater than 80% positivity for MyoD were used in all experiments.

2.4. SC culture and differentiation induction

SC with intact fibers (15–20 fibers) were cultivated in triplicate in 24 well plates. Alternatively 1×10^4 cells/well were grown in 24 well plates in 10% FCS/DMEM as a monolayer. After 48 h, the basal medium was removed and replaced by one of the following: Proliferation medium (PM), 1 mg/ml glucose DMEM containing 5% FCS. Myogenic differentiation medium (MD) 1 mg/ml glucose DMEM supplemented with 5% horse serum (Millipore, Darmstadt, Germany), 2.5 ng/ml human Fibroblast Growth Factor (Invitrogen), 1% Sodium pyruvate (Sigma). Adipogenic differentiation medium (AD) 4.5 mg/ml glucose DMEM supplemented with 5% FCS, 1 μM dexamethasone (Sigma), 5 μg/ml Insulin-transferrin-selenium and 5 μM Rosiglitazone (Sigma). Osteogenic differentiation medium (OD) 1 mg/ml glucose DMEM supplemented with 5% FCS, 0.1 μM dexamethasone, 2.5 μM retinoic acid (Sigma), 250 μM ascorbic acid (Sigma), 100 ng/ml BMP-2 (Peprotech, Germany) and 10 mM β-glycerophosphate (Fluka, Germany). Myofibers were cultured for up to 120 h whereas myoblasts were maintained for up to 3 weeks.

2.5. Immunohistochemical staining

The cells and muscle sections were fixed in 4% paraformaldehyde (PFA) for 10 min, washed twice with PBS for 10 min, permeabilized with buffer containing 20 mM Hepes, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$ and 0.5% Triton-X100 (pH 7) for 15 min, blocked in 5% FCS with 0.05% Triton X-100 in PBS for 30 min. They were then incubated with primary antibodies overnight at 4 °C. All the primary antibodies were purchased from DSHB, USA unless otherwise stated. The following primary antibodies were used; anti-mouse CD90 1:20, CD44 1:20, Pax7 1:30, MyoD 1:100 (B & D Pharmingen), myogenin 1:30, adipocytes protein 2 1:30 (AP2), alkaline phosphatase 1:30 (ALP) and Cx43 1:100 (Invitrogen). Expression of myosin heavy chain (MHC) type I, IIA and IIB and total muscle fibers was determined using anti mouse A.4840 1:50, A.474 1:50, BFF3 1:50 and Lam BS 1:30 respectively. Incubation with secondary antibodies: goat anti-mouse fluorescent isothiocyanate (FITC) and anti-mouse IgG Cy3 was carried out in the dark at room temperature for 1 h. Immunohistochemical staining for Cx43 was performed using anti-mouse horseradish peroxidase (HRP 1:500, Dianova, Germany) for 30 min at 37 °C. HRP was developed by dissolving 4 mg/ml dimethylformamide (AEC) 3-Amino-9-ethylcarbazole and 2.5 μl H_2O_2 in 50 mM sodium acetate (pH 5.2) for 30 min. Incubation omitting primary antibodies served as a negative control (Supplementary Fig. 1). The slides were mounted using aqua Polymount (Polysciences, Germany). Nuclei were visualized using DAPI (ThermoFisher) or a hematoxylin (Sigma). Quantification of SC was performed by counting the number of Pax7, MyoD, myogenin and Cx43