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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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ARTICLE INFO

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).

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

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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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 positive cells per myofiber (minimum of 15–20 fibers were quantified for every trial). MyoD positive myoblasts were counted in 10 random fields per well in 24 well plate at 48 h post induction. Evidence of myogenic differentiation was determined using phalloidin actin filaments staining 1:50 (Sigma) for 30 min. For examination of adult muscle phenotypes and Cx43 distribution, the whole muscle was photographed (N = 3 for each muscle) using the Axio-imager fluorescent microscope linked to Axiovision software (Zeiss, Germany). Individual images were merged using Photoshop (CS6 extended) software. Manual counting was carried out for the determination of total fiber number, percentage of MHCI, IIA, and IIB and Cx43 positive signals. The percentage of MHCIIX was determined by subtracting all the MHC types from the total myofiber number.

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2.6. MTT proliferation assay

Muscle myoblasts were grown in triplicates under basal, myogenic, adipogenic and osteogenic differentiation conditions for up to 2 weeks. MTT assay was performed as previously reported (Janeczek et al., 2013 and Yu et al., 2013). Briefly, the media was removed and the cells were incubated with 1:10 DMEM diluted MTT reagent (thiazolyl blue tetrazolium 0.5% in PBS) at 37 °C for 4 h. MTT reagent was then replaced by 200 μl DMSO and incubated at 37 °C for 10 min. The cells were transferred to a 96 well microplate and the optical densities (OD) was measured at 570 nm using microplate reader (Tecan) loaded with Magellan 6 software.

2.7. Real time-quantitative polymerase chain reaction (RT-qPCR)

RNA extraction was performed using a spin column RNA mini kit (Analytik Jena, Germany), quantified (using a Biophotometer, Eppendorf) and treated with DNase1 (Roche, Germany). Reverse transcription was carried out using master mix and reverse transcriptase (Thermofisher). QRT-PCR was performed in triplicate using SYBER green qPCR-master mix (Promega) with 10 pM primers (see Table 1) for 35 cycles of 5 min at 95 °C, 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C. PCR master mix without cDNA served as a negative control. Gene expression was normalized to gene 18 S expression using ΔCt method (Schmittgen and Livak, 2008).

2.8. Oil red O staining

Adipogenesis was assessed by fixing cells in 4% PFA for 10 min, washing in PBS for 5 min followed by two 3 min washes in water. The cells were incubated with Oil red O for 30 min. Cells were washed in distilled water and mounted with Kaiser's gelatin (Roth, Karlsruhe, Germany). Non-induced cells were used as a negative control (supplementary Fig. 1h).

2.9. Alizarin red staining

Osteogenesis was examined by fixing cells in 4% PFA for 10 min, washing three times in PBS followed by distilled water for 10 min. 1% (w/v) Alizarin red pH 4.2 was added for 15 min. Cells were washed with distilled water and mounted with Kaiser's gelatin. Non-induced cells were used as a negative control (Supplementary Fig. 1i)

Table 1
Primers sequences used for RT-qPCR.

Gene Name	Primer sequence	Product size (bp)
MyoD	Forward: GTGAATGAGGCCTTCGAGAC Reverse: GAGCCTGCAGACCTTCGATG	115
Myogenin	Forward: GAAGAAAAGGGACTGGGGAC Reverse: TCTTGAGCCTGCGCTTCTCC	136
FABP4	Forward: CATCAGCGTAAATGGGGATTTG Reverse: CTTCCTGTCGTCTGCGGTGA	115
PPARγ	Forward: CACTATGGAGTTCATGCTTGTG Reverse: CGACAGTACTGACATTTATTTCTAC	133
Osteocalcin	Forward: CAAGCAGGAGGGCAATAAGG Reverse: TACTGGTTTGATAGCTCGTCAC	135
Osteopontin	Forward: GAATCTGACGAATCTCACCATTC Reverse: CAGTCCGTAAGCCAAGCTATC	134
18S	Forward: GGCGTCCCCCAACTTCTTA Reverse: GGGCATCACAGACCTGTTATTG	76

2.10. Statistical analysis

Two-way ANOVA was performed to examine the impact of myogenic, adipogenic and osteogenic differentiation medium compared to proliferation medium after 48 h, day 7 and day 14 on the myoblasts viability as well as the expression of myogenic (MyoD and myogenin), adipogenic (*PPARγ* and *FABP4*) and osteogenic (*Osteocalcin* and *Osteopontin*) gene expression at day 7, 14 and 21 (the experiments were run in triplicates). One-way ANOVA was performed to assess SC total number, SC clusters and SC per clusters at 48 and 72 h per myofiber (minimum of 15–20 fibers were quantified in triplicates for each condition), number of the MyoD positive myoblasts at 48 h (in 10 random fields per well in 24 well plate at 48 h post induction), Cx43 positive SC up to 120 h, the percentage of MHC type I, IIA, IIX, IIB and Cx43 localization for TA and SOL muscles ($N = 3$). Multiple comparisons and the interactions were examined using Tukey's Post hoc test. All data are presented as means ± SEM. $P < 0.05$ adopted to be significant difference.

3. Results

3.1. The influence of adipogenic and osteogenic promoting conditions on SC cultured on their native substrate

We first determined the robustness of the myogenic program of SC when they were on their native substrate by challenging them with conditions that promote their differentiation into non-muscle lineages. The number of myogenic cells on each fiber was significantly increased by 48 h and 72 h after exposure to myogenic medium ($P < 0.01$ and 0.0001 respectively) compared to control proliferation medium (Fig. 1a–e). However, exposure of fibers to adipogenic medium resulted in a significant decrease in the number of myogenic cells at both times ($P < 0.01$ and 0.05 respectively) compared to control medium (Fig. 1d and e). In contrast, osteogenic medium failed to influence the number of myogenic cells by 48 h (Fig. 1d). However, it did inhibit the development of myogenic cells by 72 h ($P < 0.05$) compared to control (Fig. 1e). SC, once activated, migrate and form clusters which can be used as an indication of their migratory ability. We examined the formation of satellite cell clusters on fibers exposed to different fate-inducing medium. The formation of clusters was promoted by myogenic medium at both 48 h and 72 h ($P < 0.001$ and 0.01 respectively) compared to control (Fig. 1f–g). In contrast, adipogenic medium inhibited the formation of clusters at 48 h ($P < 0.001$) compared to control (Fig. 1 f). Osteogenic medium had no effect in terms of cluster formation compared to control conditions at 48 h or 72 h (Fig. 1f–g). Lastly, we investigated the composition of each cluster and determined the number of myogenic cells in each cluster at the two time points. We found that myogenic medium induced significantly more myogenic cells in each cluster at both 48 h and 72 h ($P < 0.0001$ and $P < 0.05$ respectively) compared to control medium (Fig. 1h–i). Adipogenic medium had no influence in the number of myogenic cells per cluster at 48 h compared to control medium (Fig. 1h) but reduced it by 72 h (Fig. 1i). Osteogenic medium induced a slight increase in the number of muscle cells per cluster at 48 h (Fig. 1h) but this value was significantly lower by 72 h ($P < 0.0001$) compared to control medium (Fig. 1i). These results show that adipogenic and osteogenic medium inhibited the proliferation of SC on their native substrate.

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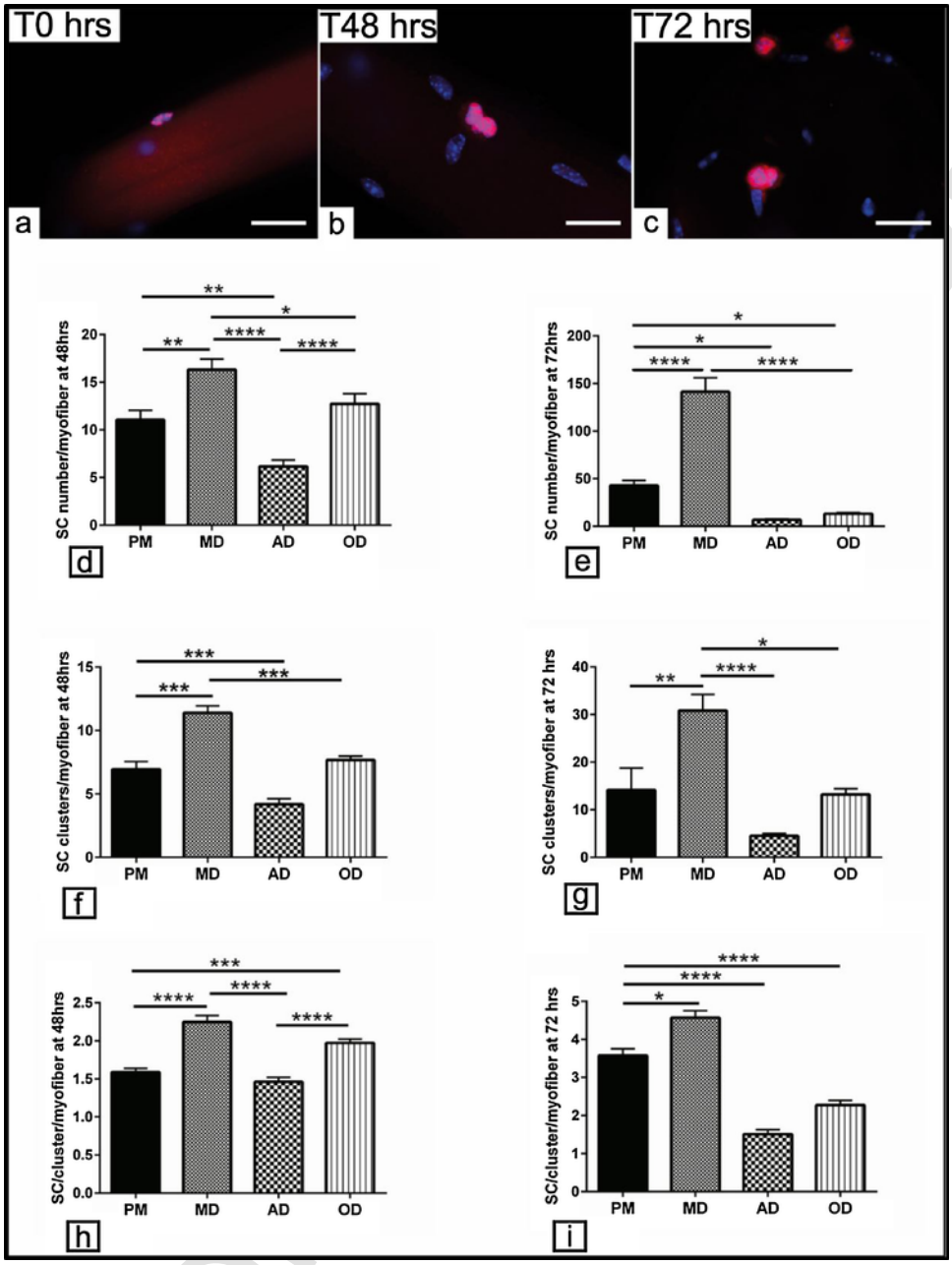

Fig. 1. Adipogenic and osteogenic promoting conditions inhibit the proliferation of satellite cells on their native substrate.(a–c) Representative immunofluorescent staining images of EDL myofibers showing satellite cells (SC) expressing (a) Pax7 (red) at 0 h, (b) MyoD (red) at 48 h, and (c) myogenin (red) at 72 h in the presence of myogenic culture medium. (d) Quantification of the number of Pax7/MyoD positive SC/myofiber at 48 h (minimum of 15–20 fibers) cultured in myogenic differentiation (MD), adipogenic differentiation (AD) and osteogenic differentiation (OD) conditions compared to proliferation media (PM). (e) Average number of myogenin positive cells per myofiber at 72 h. (f) Average number of SC clusters per myofiber at 48 h. (g) Quantification of the average number of myogenin positive SC clusters at 72 h. (h) Average number of Pax7/MyoD positive cells in each cluster per myofiber at 48 h. (i) Average number of myogenin positive SC per cluster per myofiber at 72 h. DAPI was used to visualize the nuclei (blue). The scale bar = 10 μm. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ and **** = $P < 0.0001$. All data are presented as means ± SEM.

3.2. Adipogenic and osteogenic conditions inhibit primary myoblast myogenesis by down regulating myogenic regulator factor gene expression and by inducing lineage specific transcripts

Having shown that adipogenic and osteogenic promoting conditions had a profound effect on myofiber positioned SC, we next determined their influence on primary myoblasts cultured as monolayers. Furthermore, we determined their adipogenic and osteogenic potential as well as underlying mechanisms. Myoblasts counting using immunofluorescence revealed a marked increase in MyoD positive cells after 48 h under myogenic differentiation conditions compared to control ($P < 0.001$), adipogenic and osteogenic induction conditions ($P < 0.0001$) (Fig. 2a and b). Next, we examined the cellular viability using MTT assay, and found at 48 h an increase in the cellular proliferation following myogenic induction ($P < 0.05$) compared to the cells under control or adipogenic medium. After 1 week, the myogenic and adipogenic induced cells showed even greater reductions in cellular proliferation ($P < 0.05$ and 0.01 respectively) compared to control. In contrast, osteogenic medium caused only later reduction in proliferation at 2 weeks ($P < 0.05$) compared to control (Fig. 2c).

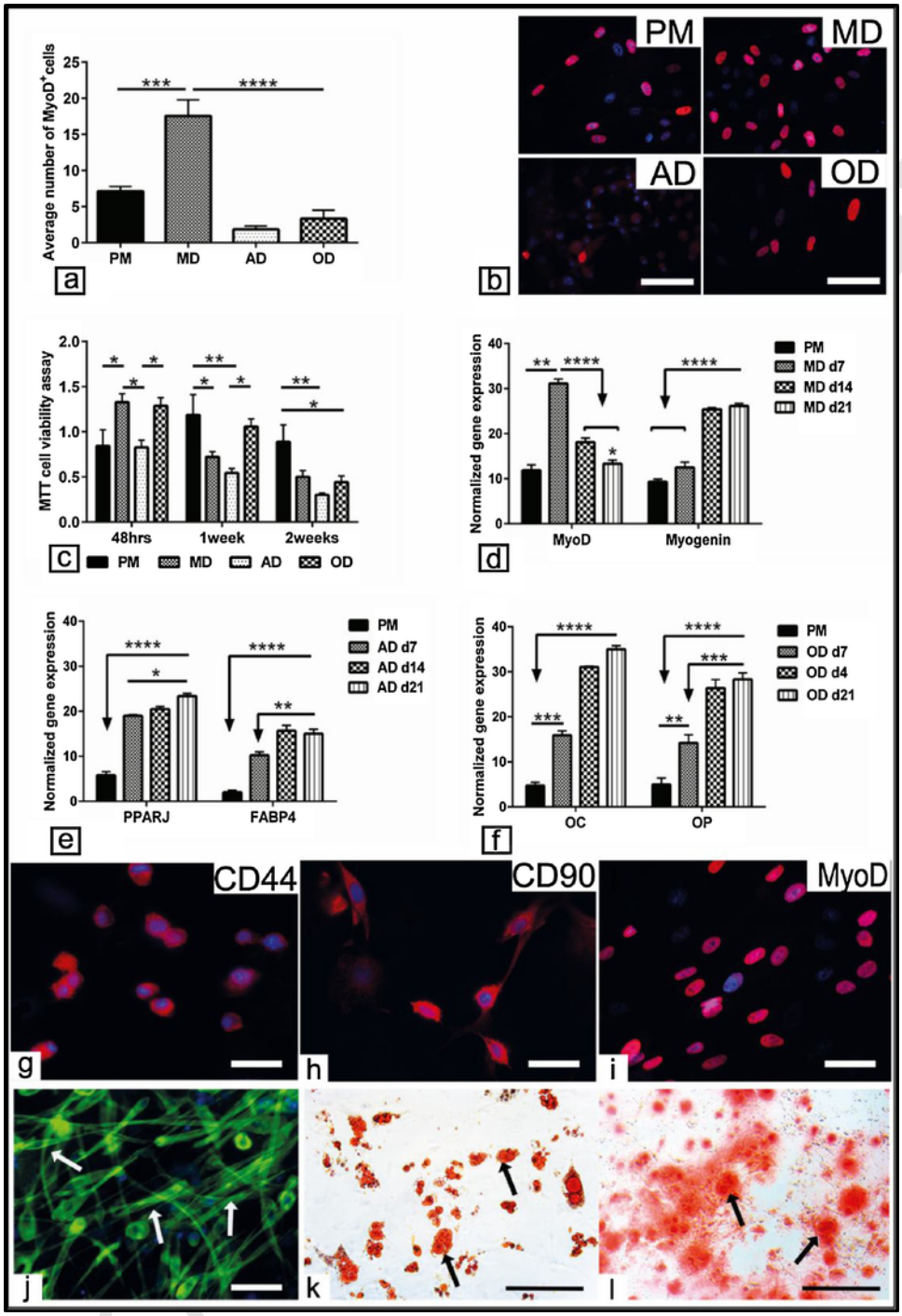

Fig. 2. Adipogenic and osteogenic conditions inhibit primary myoblast myogenesis by down regulating myogenic regulator factor gene expression and by inducing lineage specific transcripts.(a) Quantification of MyoD positive myoblasts in 10 random fields per well in 24-well plate at 48 h in culture media promoting myogenic differentiation (MD), adipogenic differentiation (AD) and osteogenic differentiation (OD) induction compared to proliferation media (PM). (b) Immunofluorescent images of myoblasts grown on a cover slip in a 24-well plate in myogenic differentiation (MD), adipogenic differentiation (AD) and osteogenic differentiation (OD) induction conditions at 48 h compared to proliferation media (PM). (c) MTT cell viability assay demonstrates SC proliferation profile following culture in MD, AD, and OD compared to PM conditions at 48 h, 1 week and 2 weeks. (d-f) Normalized gene expression for SC at day 7, day 14 and day 21 days for *MyoD and myogenin* (d), *PPARγ* and *FABP4* (e), and *OC and OP* (f) following MD, AD and OD inductions respectively compared to PM. The data were analyzed using Δct method using 18S as an endogenous reference. (g-i) Double immunofluorescent staining for extracted myoblasts after 48 h in myogenic medium against CD44 (g), CD90 (h) surface markers and MyoD (i) myogenic marker. (j) Phalloidin actin filaments staining for myoblasts at day 8 post myogenic induction showing myotubes formation (white arrow). (k) Oil Red O staining at day 7 following adipogenic induction exhibits adipocytes with fat vacuoles (black arrow). (l) Alizarin red staining at day 21 following osteogenic induction showing calcium deposition (black arrow). DAPI and Hematoxylin were used as nuclear stains (blue). Scale bar = 10 μm (g, h and i), 20 μm (b and j) and 100 μm (k and l). * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ and **** = $P < 0.0001$. All data are presented as means ± SEM.

Next we examined the impact of ectopic culture conditions on genes that promote muscle, adipose and bone development. In conditions that promote muscle differentiation, we found an initial increase in the expression of *MyoD* which then decreased such that by 21 days it was at the level displayed by cells in proliferation medium (Fig. 2d). The expression of *Myogenin* was almost the opposite of *MyoD* in that it

increased only slightly by 7 days and then amplified in the following two weeks (Fig. 2d). Therefore, myogenic differentiation conditions promoted a biphasic expression of MRF; first a burst of *MyoD* expression which gradually increased and an initial small increase in *Myogenin* which ramped up over time. Next, having shown that both adipogenic and osteogenic conditions inhibited the development of myogenic cells, we determined their influence on their fate. We found that the myoblast expression of two key markers of adipogenesis *PPARγ* (peroxisome proliferator activated receptor gamma) and *FABP4* (fatty acid associated binding protein 4) and two key markers of osteogenesis *Osteocalcin (OC)* and *Osteopontin (OP)* increased over time when cultured in their respective media (Fig. 2e–f). Having shown that adipogenic and osteogenic media promoted gene expression of tissue associated markers, we determined their impact at the cell level. We first established the myogenic fate of primary muscle cells. Evidence for myogenic commitment was evaluated at day 8 using phalloidin staining for the detection of actin filaments in order to evaluate myotube formation. Several myotube-like structures were detected (Fig. 2j and supplementary Fig. 1g). In adipogenic and osteogenic conditions we detected Oil red O and the Alizarin red staining, respectively. Our results showed lipid vacuole formation at day 8 of adipogenic induction and calcium deposition at day 21 of osteogenic induction (Fig. 2k, l and supplementary Fig. 1h, i). In summary, adipogenic and osteogenic conditions inhibited the development of muscle cells and promoted them to adopt adipose and bone fate through the induction of tissue associated genes.

3.3. The influence of tissue specific culture conditions on the expression of Cx43 by SC on their native substrate

Previous reports have postulated a role of Cx43 during myogenic differentiation. Here we determine the impact of culture conditions that inhibit myogenesis on the expression of this protein by SC on muscle fibers. First, we found that Cx43 was not expressed in quiescent SC following fiber isolation. However, Cx43 expression displayed a polar localization at the junction between SC and the myofiber after a 48 h cultivation period (Fig. 3a–a" and supplementary Fig. 1j). At 72–96 h, it was more cytoplasmic in localization (Fig. 3b–b"). At 120 h, Cx43 showed nuclear localization (Fig. 3c–c"). Quantification of Cx43 positive SC at 48 h revealed a significant increase in cell number following myogenic induction ($P < 0.001$) compared to control (Fig. 3d). In contrast, Cx43 expression was markedly reduced following adipogenic induction ($P < 0.001$) and showed a minor decrease after osteogenic stimulation compared to control (Fig. 3d).

We next characterized Cx43 expression in differing condition media over time. Following induction of myogenic differentiation, quantification of Cx43/myogenin positive SC revealed significant increases for up to a 96 h cultivation period ($P < 0.0001$) followed by a decrease at 120 h compared to control (Fig. 3e). In contrast, adipogenic induction never induced an increase in cells of interest and rather caused an early decrease in Cx43/myogenin positive SC at 72 and 120 h compared to time-matched control (Fig. 3f). Osteogenic induction also never promoted the development of Cx43/myogenin positive cells. Instead, it promoted a late decrease in Cx43/myogenin positive cells at 120 h ($P < 0.0001$) compared to time-matched control as well as a remarkable decrease ($P < 0.05$) compared to osteogenic induced cells at 96 h (Fig. 3g). We next examined expression of Cx43 in the clustering process. In myogenic differentiation medium there was an increase in the number of Cx43 positive clusters during the 72–96 time window ($P < 0.0001$) compared to control. Thereafter the numbers of Cx43 clusters were the same as in proliferation medium (Fig. 3h). In contrast, the number of Cx43 positive clusters was never greater than the number found in proliferation medium under adipogenic (Fig. 3i) or osteogenic conditions (Fig. 3j). We then assessed whether adipogenic and osteogenic induction impaired the cluster size. The number of Cx43 positive SC in each cluster was assessed which revealed there was an increase in the size of cluster at 72 h ($P < 0.0001$) in the presence of myogenic differentiation medium (Fig. 3k). The size then dropped to levels statistically similar to those of fibers in proliferation medium ($P < 0.0001$, Fig. 3k). In contrast, adipogenic or osteogenic media never induced an increase in cluster size at any interval examined (Fig. 3k). Immunofluorescent staining showed the cluster size and the colocalization of Cx43 and myogenin following myogenic induction (Fig. 3l–l"). Next, we examined the SC expression for adipogenic and osteogenic markers. Immunofluorescent staining revealed expression of the AP2 adipogenic marker (Fig. 3m–m" and supplementary Fig. 1k) as well as the expression of ALP (Fig. 3n–n" and supplementary Fig. 1l) at 72 h following adipogenic and osteogenic inductions, respectively.

3.4. Cx43 localization in the adult muscle

Our results indicate that Cx43 plays a critical role during myogenesis. We next examined whether Cx43 expression was influenced by the identity of muscle and fiber types. Quantification of the Cx43 immune-reactivity revealed that fibers of the SOL had more Cx43 positive cells than those from the TA muscle (Fig. 4a, b, d, e, f, g and supplementary Fig. 1m, n). Next we correlated Cx43 distribution with muscle fibers type. Therefore, the total myofiber number (Fig. 4h, i and supplementary Fig. 1o), as well as, the percentages of MHC types for TA and SOL muscles were quantified. The TA muscle contained more MHC type IIB and IIX glycolytic fibers ($P < 0.0001$) than the SOL muscle (Fig. 4c, j, k and supplementary Fig. 1p). On the other hand, SOL muscle contained more type IIA and type I oxidative fibers ($P < 0.0001$) compared to TA (Fig. 4c, l, m, n, o and supplementary Fig. 1q and r). These results suggest that slow muscle contains more Cx43 positive cells.

4. Discussion

Investigation of SC will provide mechanistic insights into their ability to promote muscle regeneration as well as their multipotent nature. Although SC have been shown to be multipotent (Asakura et al., 2001), this property has never been investigated when they are on their native substrate. It is well known that the substrate plays a critical role not only in stem cell behavior (e.g. ability to migrate), but also in terms of their fate. The latter was clearly demonstrated in the classic paper by Engler and colleagues who showed that a simple parameter like substrate stiffness determined whether stem cells differentiated into neurons or muscle cells (Engler et al., 2006). Furthermore it has been shown that the gap junction protein Cx43 plays a pivotal role in muscle regeneration following injuries (Araya et al., 2005). However, the expression of Cx43 by SC during the fate making process had never been investigated.

We maintained the environment of the SC niche by culturing them on myofibers which avoided contamination by other cell populations. We detected increases in proliferation, cluster formation and differentiation potential following myogenic induction. However, adipogenic or osteogenic supporting conditions resulted in a delay of myogenesis via reduction of SC proliferation, migration and myogenic differentiation. These results highlight the susceptibility of the myogenic program to environmental factors. Furthermore, we showed that under the conditions used here, we not only inhibited myogenesis but promoted the formation of adipose or osteogenic cells. It is possible that once non-muscle cells have been induced they release factors that further inhibit myogenesis. In keeping with this line of thought are studies that describe adipogenesis inhibiting the proliferation and differentiation of muscle stem cells in diabetes (Fujimaki et al., 2015).

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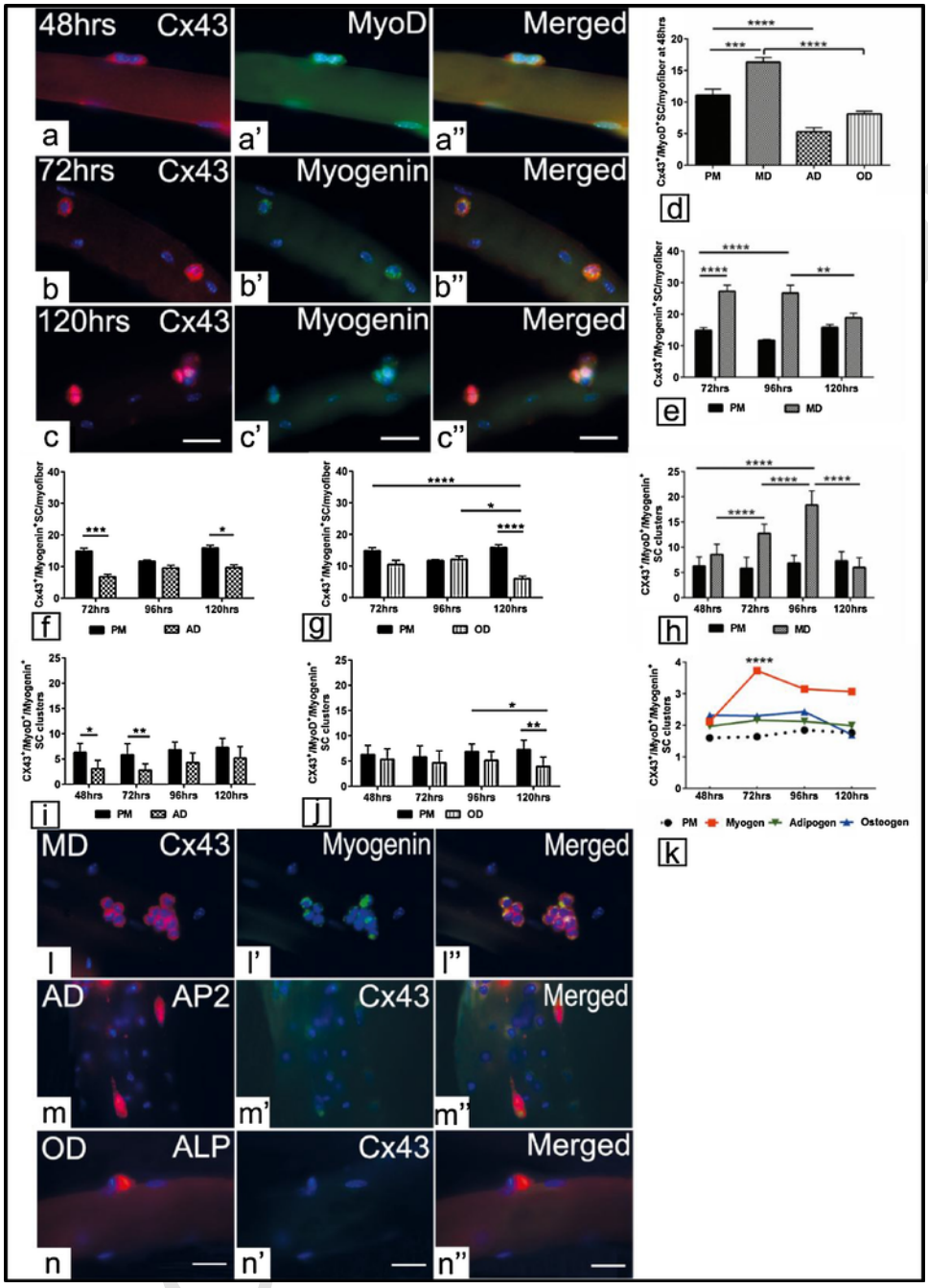

Fig. 3. The influence of tissue specific culture conditions on the expression of Cx43 by satellite cells on their native substrate.(a–c) Double immunofluorescent labelling for SC/myofiber against MyoD (green) and myogenin (green) and Cx43 (red) post myogenic differentiation induction at 48, 72 and 120 h. (a, a', a") Cx43/MyoD positive SC at 48 h. (b, b', b") Cx43/myogenin positive SC at 72 h. (c, c', c") Cx43/myogenin (green) positive SC at 120 h. (d) Quantification of Cx43/MyoD positive SC/myofiber at 48 h following myogenic differentiation (MD), adipogenic differentiation (AD) and osteogenic differentiation (OD) inductions compared to the proliferation media (PM). (e-g) Average number of Cx43/myogenin positive SC at 72, 96 and 120 h post MD, AD and OD induction respectively compared to PM condition. Average number of Cx43 positive SC clusters/myofiber following (h) MD, (i) AD and (j) OD inductions compared to PM at 48, 72, 96 and 120 h. (k) Average number of Cx43 positive SC/cluster/myofiber following MD, AD and OD inductions compared to PM. (l-l") Double immunofluorescent staining of SC cluster show expression of Cx43 (red)/myogenin (green) at 72 h post MD induction. (m-m") AP2 positive SC cluster (red)/Cx43 negative (green) at 72 h post AD induction. (*n*-n") ALP positive SC cluster (red)/Cx43 negative (green) at 72 h post OD induction. DAPI was used to visualize the nuclei. Scale bar = 10 μm. * = $P<0.05$, ** = $P<0.01$, *** = $P < 0.001$ and **** = $P < 0.0001$. All data are presented as means ± SEM.

It is well established that failure of proper regeneration leads to replacement of the muscle tissue by fibrous and adipose tissues like in muscular dystrophic diseases (Lin et al., 1969). Our results demonstrate a reduction of myogenic committed cells together with the expression of AP2 and ALP following adipogenic and osteogenic inductions, respectively. We showed that adipogenic and osteogenic conditions inhibited the development of Cx43 expressing muscle cells. These results are in keeping with reports that Cx43 inhibition with 18 alpha-glycyrrhetinic acid inhibited myogenesis and induced adipogenesis (Yamanouchi et al., 2007), underlining the important role of Cx43 for myogenesis.

Isolated myoblasts from muscle homogenates showed that myogenic induction causes early *MyoD* upregulation at day 7 and late *Myo-*

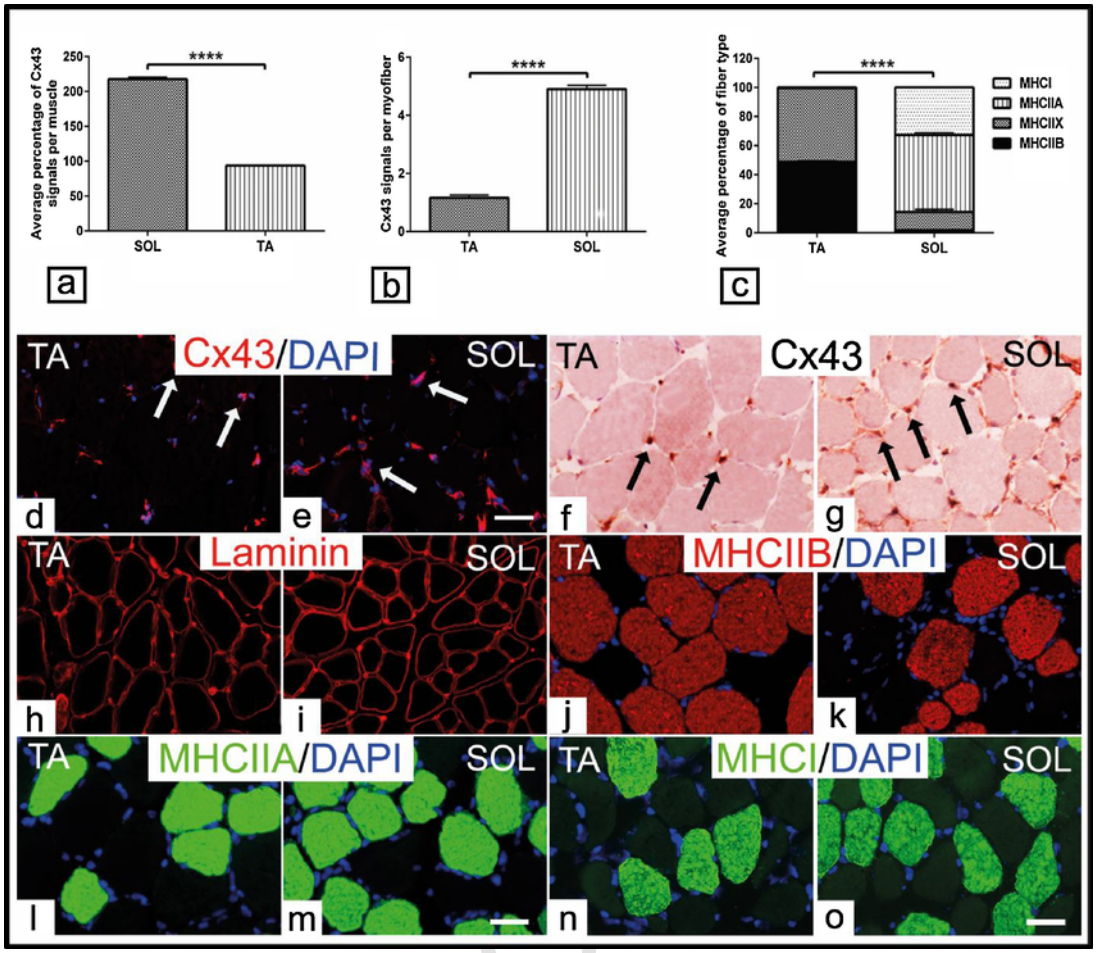

Fig. 4. Cx43 localization in the adult muscle.(a) Average percentage of Cx43 positive signal per/total myofiber number for TA and SOL muscles (n = 3). (b) Average number of Cx43 positive signals per single myofiber for SOL and TA muscles. (c) Average percentage of MHC type IIB, IIX, IIA and I myofibers for TA and SOL muscles. (d-o) Representative immunofluorescent and immunohistochemical staining images for TA and SOL muscles show Cx43 positive signals (d and e, white arrow; f and g, black arrow), MHC type IIB (j and k, red), MHC IIA (l and m, green) and MHC type I (n and o, green). (h and i) Laminin immunostaining (red) for total myofiber counting for each muscle. DAPI and Hematoxylin were used as counterstains. Scale bar = 20 μm. **** = $P < 0.0001$. All data are presented as means ± SEM.

genin expression at day 14 and day 21 post induction suggesting a myogenic commitment. Our data are consistent with previous reports showing that following injury, SC proliferation and differentiation is characterized by an upregulation of MyoD, Myf5, myogenin and MRF4 (Fuchtbauer and Westphal, 1992; Grounds et al., 1992). Our results showed an upregulation for *PPARγ and FABP4* at day 7, 14, and 21 post adipogenic induction as well as increases in the expression level for *OC* and *OP* following osteogenic stimulation. These results clearly show the multipotent nature of SC both on their native substrate and on plastic.

We demonstrated that Cx43 was expressed in activated but not quiescent SC. During periods of proliferation and early differentiation Cx43 was located at the extremities of cells. Thereafter, Cx43 was found in the cytoplasm and then in the nucleus. Investigation of Cx43 expression in oral squamous cell carcinoma revealed that membranous translocation for Cx43 is required to maintain the gap junction, whereas the cytoplasmic localization is important for its storage (Brockmeyer et al., 2014). Based on these observations, we suggest that Cx43 supports signal and molecule transfer in order to support proliferation and differentiation. Thereafter it may be held in the cytoplasm to support these activities as they are induced.

Our study documented an increase in Cx43/MyoD positive SC/myofiber and Cx43/myogenin following myogenic induction suggesting that Cx43 as associated in the myogenic commitment as well as terminal differentiation. Similar reports revealed that Cx43 was expressed during myoblasts proliferation and myotube formation (Balogh et al., 1993; Gorbe et al., 2007; Schmalbruch, 1982). These lines of thought are in keeping with studies showing that Cx43 was upregulated during muscle regeneration following acute injury (Araya et al., 2005; Bakay et al., 2002) and that its deletion perturbs myofiber renewal by decreasing myogenin (Araya et al., 2005).

We show that slow twitch soleus muscle contained a greater number of Cx43 expressing cells than the fast twitch TA muscle. However we have shown that quiescent SC do not express Cx43. Therefore in undamaged muscle, the Cx43 expression must be in cells other than SC. Cx43 expression is likely to be in endothelial and smooth muscle cells. In agreement with our hypothesis, an independent study revealed Cx43 expression within endothelial and smooth muscles cells of microvessels (Little et al., 1995; Yeh et al., 1998). Based on the fact that slow twitch muscle contain higher capillary density compared to fast twitch muscle (Gray et al., 1983), it follows that they have more Cx43 expressing cells, which may impact vasomotor reflexes between muscle types.

In summary, we show that SC on their native substrate can be induced to adopt an adipogenic or osteogenic fate following the exposure to specific chemical cues. These regulate not only cell fate but also impact satellite cell progeny proliferation and migration. Furthermore, we show that the expression of Cx43 is switched on after satellite cell activation. However its expression is labile and can be decreased by cues that promote non-myogenic differentiation.

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Competing interest

All the authors have declared no conflict of interest regarding the publication of this paper.

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