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Developmental restriction of the jaw skeletal muscle cardiogenic potential

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Cardiovascular diseases are a leading cause of mortality worldwide and heart failure remains an end-stage disease with no other treatment than transplantation. A promising field to tackle heart failure is cardiomyocyte implantation. Whereas cardiomyocyte generation from pluripotent cells has shown promising results, direct derivation of cardiomyocytes from somatic stem cells is still challenging. Skeletal muscle progenitors have been extensively studied as a source of cardiac cells, due to their cellular and functional similarities. During development, the right ventricular myocardium and jaw skeletal muscles derive from a common pool of myogenic mesodermal progenitors. Previous studies have reported the derivation of cardiomyocyte-like cells specifically from adult mouse jaw muscle stem cell populations, but not from other skeletal muscles, suggesting that developmental origin endows cardiogenic potential. Here we evaluated the cardiogenic potential of satellite cells and mesenchymal progenitor cells from the masticatory muscle by reproducing, optimizing and diversifying previously described protocols. We consistently found that stem cells from masticatory muscles are unable to differentiate into cardiac cells under any tested cardiogenic environment. Furthermore, pharmacological erasing of repressive epigenetic marks did not activate cardiogenic potential, suggesting that barriers restraining lineage plasticity are profound and unlikely to be reversed by simple exposure to cardiogenic environments.

Heart failure represents one of the leading causes of morbidity and mortality in humans. During the last decades many attempts have been made to improve patient prognosis, both by promoting the replacement of the lost myocardial tissue and by activating cardiac regeneration and repair. Several different cell types, such as bone-marrow stem cells, dermal fibroblasts, fetal or neonatal cardiomyocytes (CMs), human induced pluripotent stem cells (hiPSCs)-derived CMs or skeletal myoblasts, have been extensively tested in clinical trials with varying degrees of success[1]. Skeletal and cardiac myocytes partially share regulatory networks, structure and contractile function. Therefore, skeletal myoblasts showed some advantages as donor cells in previous engraftment strategies. In fact, in the early 1990s, the first attempt using cell therapy for heart repair was conducted with skeletal myoblasts[2]. Unfortunately, although some beneficial effects were observed after transplantation[3–5], the cells did not differentiate into CMs but into skeletal myocytes (SMs)[6]. Nevertheless, even though several clinical studies using skeletal myoblasts suggested cardiac function improvement, other studies failed to reproduce these promising findings[7].

Given that skeletal myoblasts exhibit low cellular plasticity and are mostly committed to myogenesis, several studies have also explored the cardiac repair potential of adult skeletal stem cells, or satellite cells (SCs), by direct cell implantation. Skeletal muscle SCs reside between the basal lamina and the sarcolemma and have the capacity to self-renew, expand and differentiate for generating new muscle fibers during skeletal muscle regeneration[8]. SCs were the first adult stem cell population to be tested for cardiac regeneration in canine models of myocardial injury. In this context, SCs engraft and differentiate into cardiac-like cells, presumably instructed by the cardiac environment. However, functional improvement associated to SC implantation was not evaluated[9,10].

Besides direct transplantation of SCs into the infarcted heart, and given the limited regenerative capacity of the adult heart, the scientific community has intensively searched for new sources of CMs that could be used in therapy. Cardiac repair requires the replenishment of large amounts of cardiomyocytes, and several strategies have been explored for the induction in vitro of cardiac differentiation from SCs of various origins. Although these cells are canonically committed to myogenesis, several studies have shown that, under specific conditions, undifferentiated skeletal muscle-derived cells acquire cardiac features, including the expression of

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cardiomyocyte- specific markers and the acquisition of functional properties, such as spontaneous beating and electromechanical coupling[11–14].

The mammalian heart is made up of a diversified set of muscle and non-muscle cell types that originate from multipotent progenitor cells located in the first heart field (FHF) and second heart field (SHF)[15]. Islet-1 has been identified as a predominantly marker of progenitor cells in the SHF[16,17]. From an ontogenic perspective, craniofacial muscles originate from embryonic populations that share a common expression of Islet-1 and therefore a close lineage relationship with right ventricle and outflow tract cardiac precursors. This common origin suggests a potential developmental link between craniofacial muscle stem cells and cardiac lineages, thereby endowing their resident SCs with a latent cardiogenic potential[18–20]. Moreover, SCs in craniofacial muscles exhibit distinct regenerative behaviors different from those in limb muscles, likely reflecting their specific embryonic origins[21].

Building on these developmental insights, different protocols have been designed to test whether the cardiac potential of skeletal muscle cells can be recalled in adult tissues, particularly in the masseter muscle[12,13]. However, the evidence remains controversial, because canonical SCs or related muscle-derived stem cells predominantly form skeletal myofibers and only a very small subset of cells seems to exhibit cardiac features[12,13].

SCs are considered as the primary progenitors in adult skeletal muscle. However, besides these cells, other mesenchymal stem cells (MSCs) coexist with SCs and contribute to muscle regeneration and homeostasis[22]. This population can be propagated as free-floating myospheres[23]. Previous works have shown that cells differentiated from MSC-derived myospheres display several electrophysiological properties similar to those of cardiomyocytes[24] or skeletal myocytes, depending on the culture conditions. Moreover, myospheres have been shown to exhibit heterogeneity in developmental origin and are composed by two types of cells that can be characterized by the differential expression of several markers, such as α7-integrin, Myf5, MyoD and Stem Cell Antigen 1 (Sca1), among others[12,25]. Whereas a majority of cells in myospheres are mesenchymal cells characterized by the expression of platelet-derived growth factor receptor alpha (PDGFα) and Sca1, there is a subset of cells that are Sca1- negative and express MyoD and Pax7, suggesting myogenic capacity[25]. Other authors corroborated the skeletal myogenic fate of Sca1-negative cells and found that Sca1-positive cells could be induced to differentiate into cells with cardiomyocyte-like characteristics, displaying structural features such as organized sarcomeres, expression of contractile proteins and functional electrophysiological activity[12].

In the present study we sought to assess and enhance the cardiogenic potential of SCs and MSCs from the masseter muscle, by refining and improving the described protocols. Despite thoroughly applying the published protocols and extensively exploring culture condition variants, we observed no ability of masseter muscle stem cells to acquire cardiac features. A main reason for the discrepant results between our observations and those previously reported is the unreliability of cardiac versus skeletal muscle markers when immature muscle fibers are characterized. These findings suggest that exposing SCs or MSCs to a cardiogenic differentiation regime is not sufficient to induce cardiomyocyte-like properties. To further explore whether epigenetic de-programming could unlock the ability of skeletal muscle cells to acquire cardiac identity, we applied pharmacological treatments that erase the main known repressive epigenetic modifiers. Such treatments failed again to show any reprogramming of skeletal muscle cells to a cardiac fate, indicating that despite their common embryological origin, the masseter and cardiac muscle cells do not retain common features that would allow fate switching. Our results will help other researchers to build around this topic on realistic grounds.

Results

In order to study the cardiogenic potential of SCs from jaw muscle versus other muscles, we followed a previously described method in which SCs were exposed to cardiogenic signals[13]. Briefly, SCs were isolated from both the masseter and the tibialis muscles of adult mice by sequential FACs. Negative selection using CD45, CD31 and Sca1 was followed by positive selection with CD34 and α7-Integrin (Fig. S1A).

To ensure the purity of the satellite cell population, we performed this sequential sorting isolation protocol using muscle from Pax7-GFP reporter mice[26], in which SCs express the fluorescent green protein as a reporter. The analysis by FACS showed that 97% of the marker-selected population was positive for Pax7-GFP, indicating that the sorting method based on marker detection with antibodies allowed isolation of a highly pure population of SCs (Fig. S1B).

Isolated cells were first cultured in HAM's F10 medium containing 20% fetal bovine serum (Fig. 1A). For the specification step, the medium was supplemented with bone morphogenetic protein 4 (BMP4) plus basic fibroblast growth factor (bFGF) during four days followed by supplementation with bFGF, dickkopf 1 (DKK1) and vascular endothelial growth factor (VEGF) for two more days. Then, the growth factors were removed, and cells were differentiated in low serum (5% FBS) Dulbecco´s minimal essential medium (DMEM) for at least 2 weeks (Fig. 1A).

Freshly isolated SCs from tibialis muscle displayed small size and heterogeneous shape (from round to elongated) (Fig. 1B). To further verify that these cells were SCs, we took again advantage of the Pax7-GFP reporter. Confocal imaging of isolated cells 2 days post-isolation showed expression of GFP in most of the cells (Fig. 1C). During the first 6 days, cells proliferated and, when confluency was reached, underwent cell fusion (Fig. 1B). Myotubes were observed from day 7 onwards and constituted the majority of the population. SCs isolated from the masticatory muscles showed the same morphological, proliferative and fusion dynamics as those coming from tibialis muscle. At endpoint, both cultures were mostly formed by myotubes (Fig. 1B).

Previous works have shown that SCs from masticatory muscle at an early stage of culture start to express cardiac progenitor cell markers[12,13]. To assess this aspect in our cultures, we analyzed the expression of Gata 4 in masseter-derived SCs exposed to cardiogenic factors in comparison with their absence (only bFGF added). We observed that Gata 4 detection is slightly elevated when cells were exposed to BMP4 (Fig. 1D, E). To evaluate whether this early transcriptional change had an effect on the differentiation towards the cardiac lineage in later stages, we checked the expression of cTNT as a marker of mature cardiomyocytes. Interestingly, we found that 80% of masseter satellite cell derived cells were labeled with cTNT (Fig. 2A). However, the same results were obtained in cultures derived from the tibialis muscle (Fig. 2A). Furthermore, we also detected cTNT staining in control conditions (only adding bFGF) (Fig. 2A). We then re-examined the specificity of the anti-cTNT antibody by immunofluorescence in both adult heart and skeletal muscles. While cTNT is highly expressed in adult CMs, adult SMs do not show any expression, which is in agreement with the described cardiac specificity of cTNT (Fig. 2B). Nonetheless, unlike adult muscles, the myoblast cells and myotubes obtained in vitro are immature muscle cells more similar to those in embryonic muscles[27,28]. To assess whether the cTNT antibody could discriminate embryonic CMs from SMs, we evaluated the expression of cTNT during skeletal muscle development and found that in fetuses of 15.5 days of development (E15.5) hind limb muscles show specific labeling with the anti-cTNT antibody (Fig. 2C). This finding agrees with previous works that showed cTNT detection in embryonic/fetal skeletal muscle and regenerating muscles fibers in some avian and mammalian species[29–31]. Therefore, cTnT is not a reliable cardiac marker in this context as it is also detected in immature skeletal muscle cells.

To further investigate the identity of the cultured cells we studied the expression of Myogenin. Myogenin (MyoG) activates the skeletal myogenic differentiation program which results in myoblasts exiting the cell cycle and fusing to form multinucleated myotubes[32]. Both, developing skeletal muscle cells and SC-derived cultures exhibited co-expression of MyoG and cTNT (Fig. 2D). Furthermore, striated pattern reflecting sarcomere structure is noticed in large myotubes (Fig. 2D) comparable to cells in the developing hind limb (Fig. 2C, D). These observations corroborate that cTNT expression is a feature of immature skeletal muscle and cannot be used as a reliable marker of cardiac identity.

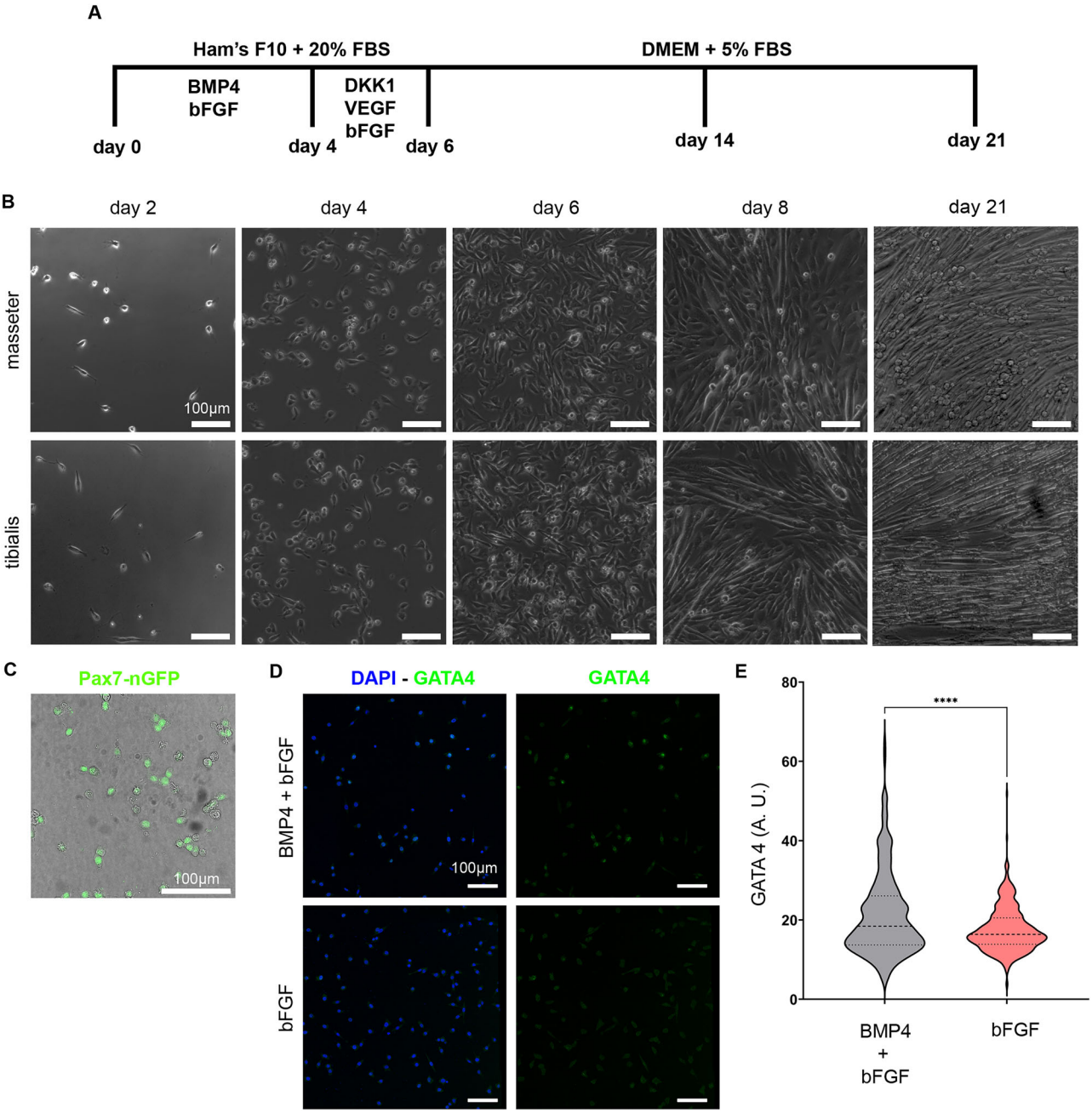


Fig. 1 | Differentiation protocol and early characterization of SCs. A Scheme of the protocol to expand and differentiate SCs[13]. **B** Bright field images showing temporal evolution of the isolated SCs derived from the masseter or tibialis muscle. Scale bars, 100 µm. **C** GFP signal reporting Pax7 expression in SCs at 2 days of culture. Scale bar, 100 µm. **D** Confocal images showing immunostaining against GATA4 and DAPI in SC cultures with or without BMP4 at 4 days of culture. Scale bars, 100 µm. **E** Quantification of GATA 4 levels comparing both conditions ($n = 445$ cells for BMP4 + bFGF, $n = 522$ cells for bFGF). Violin plots represent median (dash lines) + quartile (dot lines). Mann–Whitney test was used for statistical analysis. p-val $p < 0.0001$ (two-tailed, approximate).

We then looked for alternative markers specific for cardiac muscle cells. We tested antibodies recognizing 6 markers presumably specific of cardiomyocytes vs skeletal myocytes: Myh6, ßAR, MLC2V, TNNI3, Nav1.5 and Cav1.2. Surprisingly, all the antibodies tested equally stained adult cardiac and adult tibialis skeletal myocytes (Fig. 3A). Given that at least for some of these markers mRNA expression data support their specificity, we suggest that low specificity of epitope recognition, due to similar proteins being produced in skeletal and cardiac myocytes, underlies these results.

We then used *Myh6*$^{IRES\text{-}Cre}$[33], as a genetic tracer that allows to identify cardiomyocytes by Cre recombinase activity. To permanently label all cells that activate *Myh6* expression at any time, we combined *Myh6*$^{IRES\text{-}Cre}$ with the Cre-reporter allele *ROSA26R*$^{CAG\text{–}GFP}$[34]. With this line we observed 100% labeling of cardiomyocytes with GFP in adult mice and no skeletal muscle myocyte labeled (Fig. 4A). These results show the high sensitivity and specificity of the marker, given that any transient expression of the Cre recombinase during skeletal muscle development would have permanently labeled the skeletal myocyte lineage. Nonetheless, occasionally we observed animals in the *Myh6*$^{IRES\text{-}Cre}$; *ROSA26R*$^{CAG\text{–}GFP}$ stock with GFP expression in all cells of the animal (data not shown). Such events are due to spurious recombination of the reporter in the germline and, although rare, required regular checking of the mouse colonies to avoid non-specific outcomes.

We then performed masseter SCs isolation from *Myh6*$^{IRES\text{-}Cre}$; *ROSA26R*$^{CAG\text{–}GFP}$ mice following the differentiation protocol described above (Fig. 1A) and we analyzed the presence of GFP positive cells by FACs.

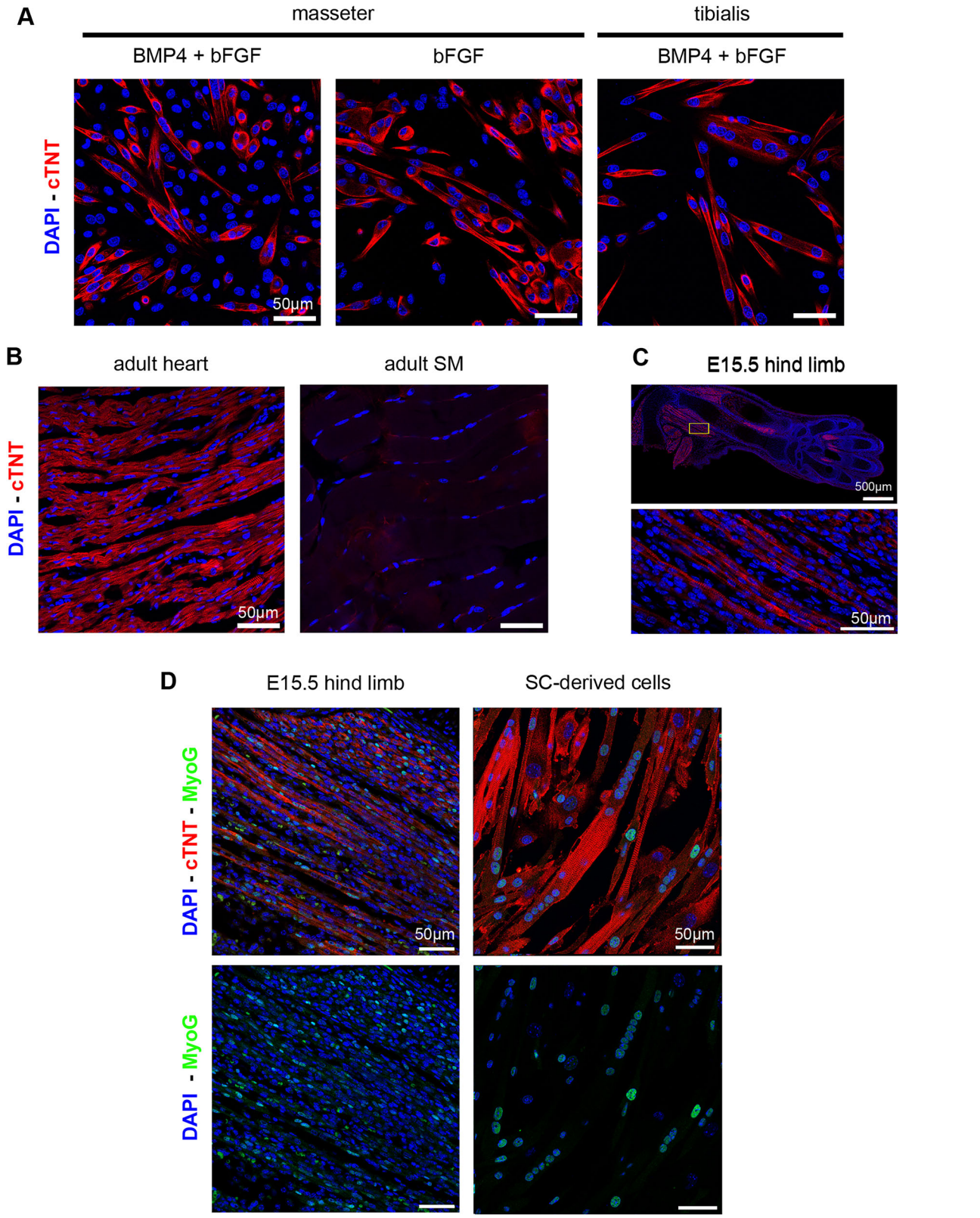


Fig. 2 | Characterization of SC-derived cells and assessment of cTNT antibody. **A** cTNT immunofluorescent signal in SC-derived cells from masseter and tibialis muscle. Scale bars, 50 µm. **B** Confocal images showing cTNT expression in heart and skeletal muscle adult tissues. Scale bars, 50 µm. **C** Detection of cTNT signal in E15.5 hind limb. Scale bar, 500 µm; high magnification scale bar, 50 µm. **D** Immunofluorescence of MyoG and cTNT showing cells co-expressing both proteins in developing hind limb and SC-derived cells. cTNT staining shows sarcomere structure in both large myotubes from SC-derived cultures and developing skeletal muscle cells. Scale bars, 50 µm.

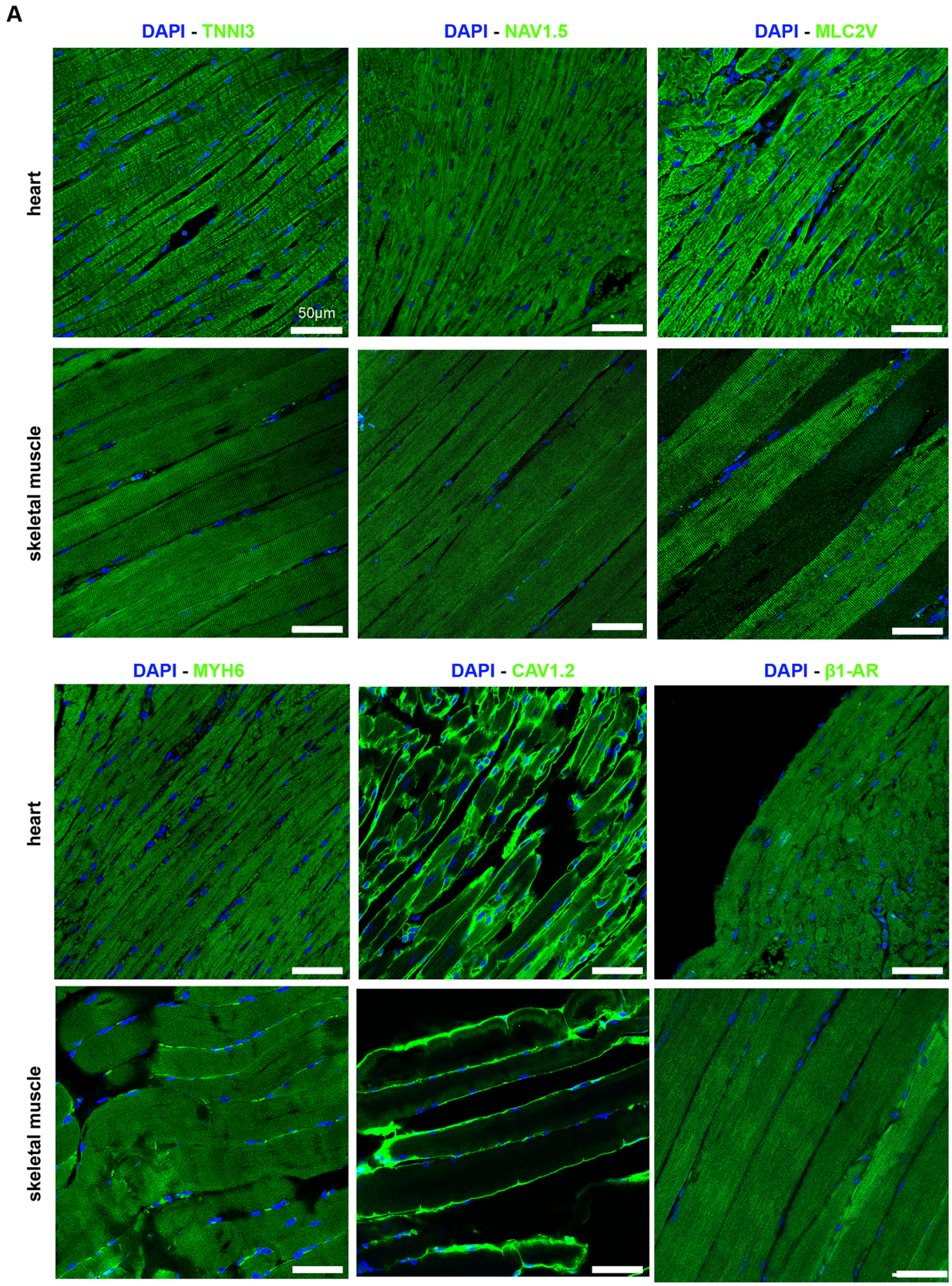


Fig. 3 | Evaluation of different cardiomyocyte markers. A Immunofluorescence against different markers for cardiac-like cells in adult heart and adult skeletal muscle (Myh6, ßAR, MLC2V, TNNI3, Nav1.5 and Cav1.2). Scale bars, 50 µm.

Notably, we did not observe cells positive for GFP when we added the cardiogenic factors (Fig. 4B). This result suggests that, although a cardiogenic environment seems to slightly activate the expression of some early cardiac progenitor cell markers in SCs, these transcriptional changes are not sufficient to allow redirection towards the cardiac lineage.

During the last years, researchers have developed robust, reproducible and cost-effective protocols that allow direct reprogramming to generate

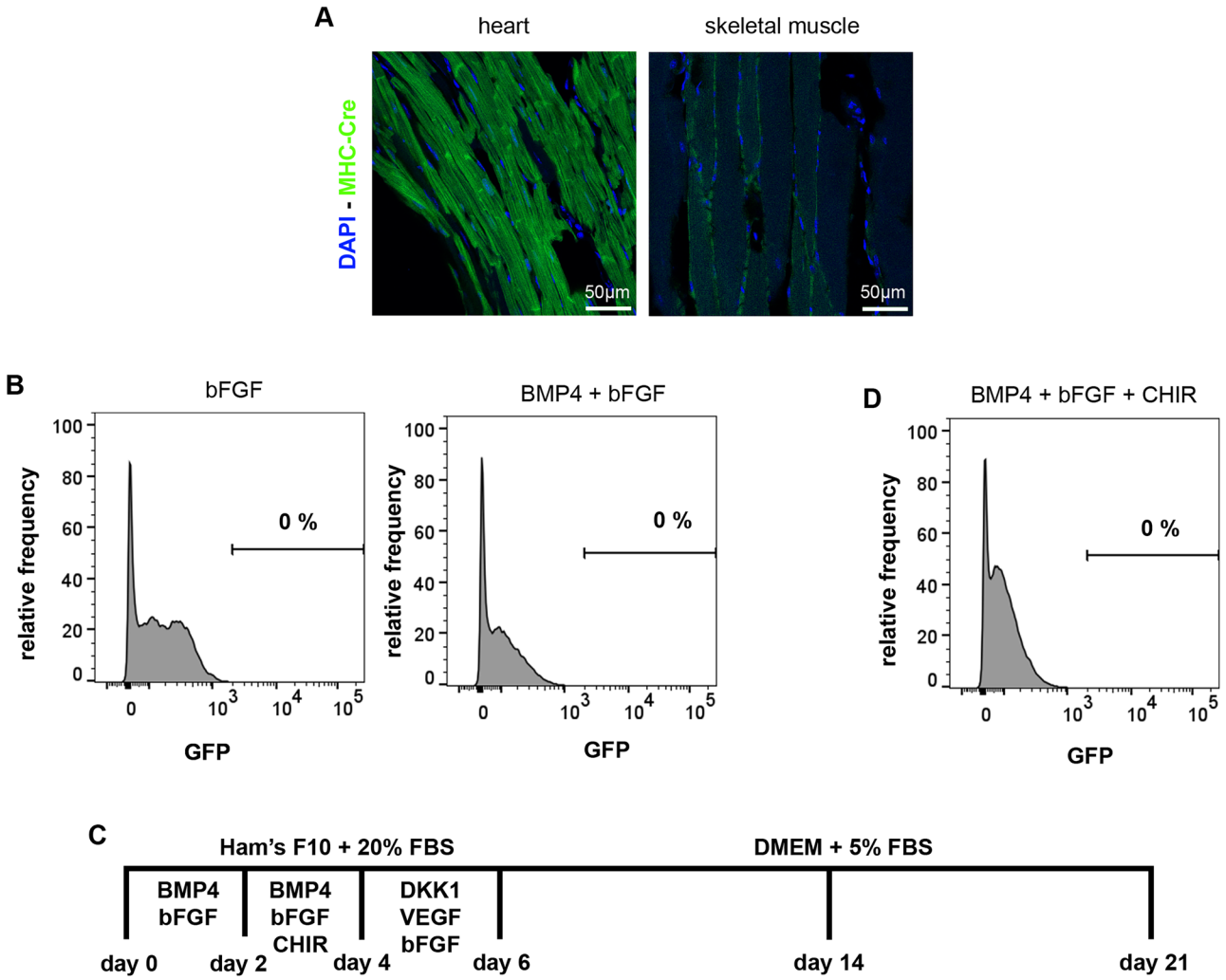


Fig. 4 | Analysis of the SCs differentiation efficiency and optimization of the protocol using Myh6$^{IRES\text{-}Cre}$; ROSA26R$^{CAG\text{–}GFP}$ reporter mice. A Confocal images showing heart and skeletal muscle sections of *Myh6*$^{IRES\text{-}Cre}$; *ROSA26R*$^{CAG\text{–}GFP}$ reporter mice. Scale bars, 50 µm. **B** Histogram representing the distribution of GFP signal in SC-derived cells from masseter at 21 days of culture with and without BMP4. The proportion of GFP positive cells is indicated in the plots. **C** Scheme of the modified protocol for the proliferation and differentiation of SCs in which CHIR was added from day 2 to 4. **D** Distribution of GFP signal in SC-derived cells from masticatory muscle at 21 days of culture following a modified protocol (adding CHIR).

mature cardiac cells. The cross-talk between the BMP4/activin A and Wnt/β-catenin pathways was identified as the essential element for cardiac differentiation[35]. With the purpose of improving the differentiation scheme to obtain cardiac-like cells from SCs, we conducted several experiments modifying the timing and/or doses of supplementation with BMP4 and DKK1 (Supplementary Table 1). Additionally, we added new molecules such as CHIR and activin A in different steps of the protocol and used GATA4 expression level as a readout of early cardiac differentiation at day 6 to 8 of the protocol (Supplementary Table 1) (Fig. S2). We observed that adding CHIR (5 µM) during 48 h (day 3 to 4) significantly increased GATA4 expression levels (Supplementary Table 1, Fig. S2). Therefore, we performed the entire differentiation protocol following this new approach and using the genetic reporter for the cardiomyocyte lineage (Fig. 4C). We did not obtain any GFP expressing cells at day 21 (Fig. 4D). Furthermore, although some cells exhibit spontaneous beating, these contractions are not rhythmic, but irregular and uncoordinated (Movies S1–S2)[36,37], a feature that we also detected in skeletal myotubes from control cultures where cardiogenic factors were not added (Movie S3). Furthermore, we also observed spontaneous contractions in SC-derived cell cultures from tibialis muscle (Movie S4). These results indicate that despite we were able to increase GATA4 expression in the initial steps, this was not sufficient to redirect masticatory muscle SCs towards the cardiac lineage.

Earlier research has shown that myospheres derived from MSCs display several electrophysiological characteristics comparable to cardiomyocytes[24] and that Sca1-positive cells can differentiate into cardiomyocyte-like cells with organized sarcomeres, contractile proteins, and functional electrophysiological properties[12]. Therefore, we assessed whether this subset of MSCs possessed the latent potential for cardiac differentiation when removed from their native niche and provided with specific signals. To do this, MSCs from jaw muscles were isolated according to a modified myosphere isolation protocol[12] (Fig. 5A) using the *Myh6*$^{IRES\text{-}Cre}$; *ROSA26R*$^{CAG\text{–}GFP}$ reporter mice as a reliable readout of CM identity. The isolated cells were grown on low attachment plates for suspension cultures in expansion medium (see methods) and they formed clusters within the first days (Fig. 5A, B). On day 3–4 the myospheres were seeded on regular plates where they adhered (Fig. 5B). On day 7 cells were detached and moved again to low attachment plates for re-sphering. Cells were maintained following these cycles of suspension-adhesion culture for up to two weeks (Fig. 5A, B).

We analyzed the expression of Pax 7 among the myospheres, and we found that 4,9% of the isolated cells were positive for Pax7 (Fig. 5C) which is in accordance with previous publications[25]. This indicates that MSC-derived myospheres contain a small population of SCs.

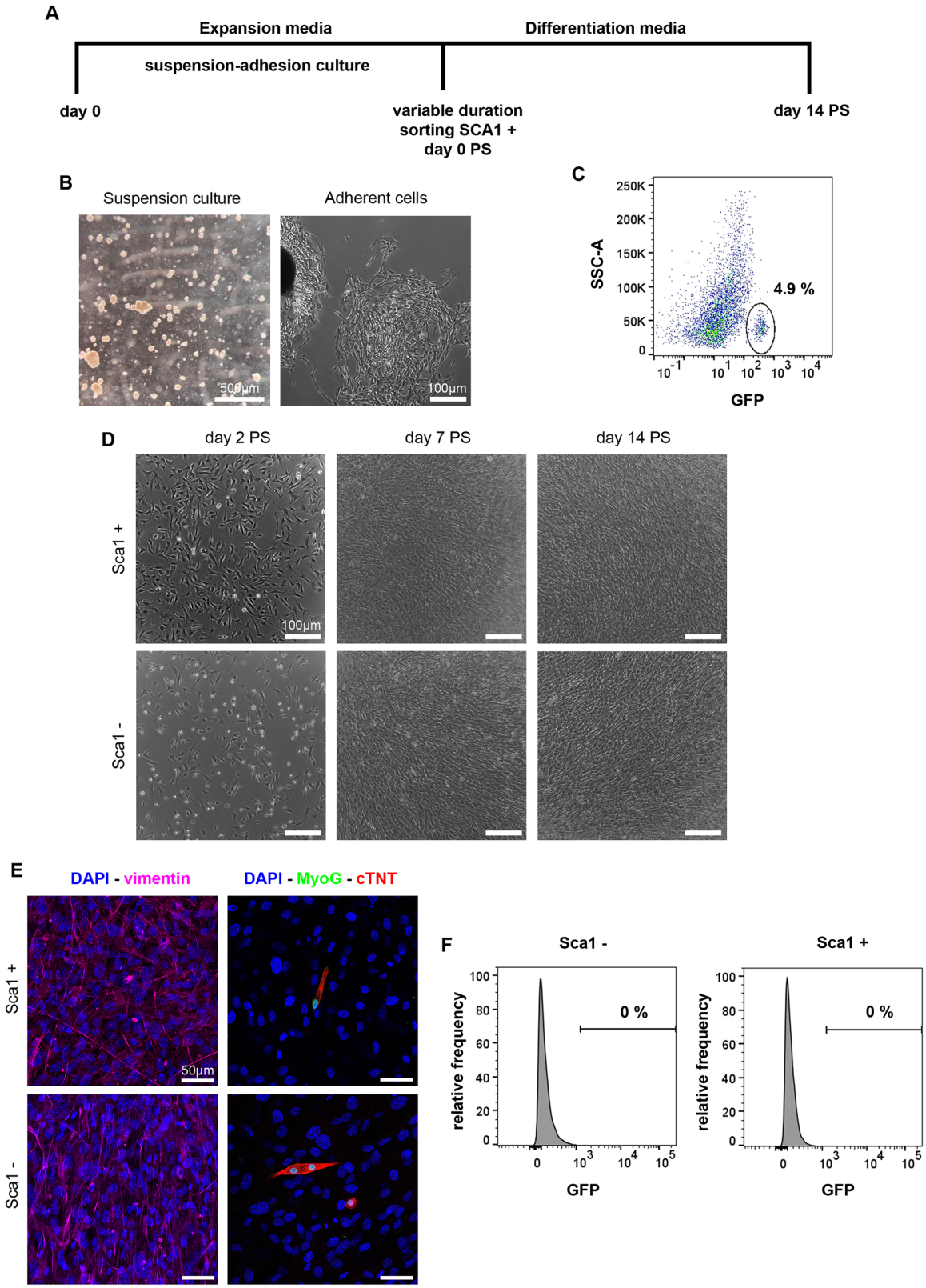


Fig. 5 | Myosphere expansion and differentiation protocol and characterization of MSCs. A Scheme of the modified myosphere protocol to expand MSCs followed by a differentiation process[12] after Sca1 MAC sorting. **B** Bright field images showing cultures of MSCs in suspension (scale bar, 500 µm) and adhered to the plate (scale bar, 100 µm) during the myosphere expansion protocol. **C** Flow cytometry analysis (pseudocolor) of single MSCs obtained from masseter muscle. Percentage of Pax7-nGFP positive population is indicated in the plot. SSC-A means side scatter (cell complexity). **D** Bright field images at different days after Sca1 MAC sorting (post-sorting, PS) of Sca1 positive and negative populations. Scale bars, 100 µm. **E** Immunofluorescence against vimentin, MyoG and cTNT of Sca1 positive and negative MSC derived cultures 14 days after sorting. Scale bars, 50 µm. **F** Distribution of GFP levels of Sca1 positive and negative populations 2 weeks PS indicating the percentage of GFP positive cells.

After serial passaging in alternating suspension-adhesion culture, cells were sorted using MACs by Sca1 expression and allowed to differentiate in medium containing bFGF and EGF growth factors (see methods) (Fig. 5A). After Sca1 MACs sorting we found that both Sca1+ and Sca1- cell populations presented similar morphologies during 14-day culture follow-up (Fig. 5D). After two weeks of culture, we performed immunofluorescence against Vimentin, a classical marker for mesenchymal cells, and observed that most cells were expressing this protein (Fig. 5E). Very few isolated cells expressed cTNT, but also MyoG, reflecting their skeletal muscle identity (Fig. 5E). Additionally, when we analyzed the presence of MHC-GFP cells in both populations at day 14 we found no positive cells (Fig. 5F). These results indicate that MSCs do not have the ability to transdifferentiate into CMs, irrespective of whether they belong to the Sca1+ or Sca1- subpopulations. The high prevalence of Vimentin+ cells suggests that most of them belong to mesenchymal lineage, with only few cells showing myogenic potential.

Collectively, all these results indicate that MSCs are committed to skeletal myogenic or mesenchymal lineages and that this fate is stable and unsensitive to cardiogenic stimuli.

Next, we explored new strategies to try to activate the cardiogenic potential of cell populations from the masseter muscle. Genome-wide changes in chromatin modifications have been shown to heavily influence cell reprogramming. Indeed, somatic cell nuclear transfer (SCNT) indicates that epigenetic regulation is critical for the differentiation processes during development[38].

Epigenetic regulators are sensitive to treatments with specific small molecule compounds and histone deacetylase (HDAC) inhibitors increase reprograming efficiency during SCNT strategy and in reprogramming protocols by defined factors[39]. Among HDAC inhibitors, Valproic acid (VPA), suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA) have been found to greatly improve reprogramming efficiency, being VPA the most potent of the three.

On the other hand, Polycomb Repressive Complexes 1 and 2 (PRC1 and PRC2) are essential for stable gene expression repression during development and homeostasis, and they are relevant in processes of cell reprogramming. In somatic cells, PRC1 and PRC2 are principal epigenetic regulators that maintain cell identity by establishing a repressive chromatin environment through histone modifications. In the context of reprogramming, the sustained activity of these complexes constitutes a major barrier. Inhibition of these complexes is emerging as a promising strategy to enhance reprogramming efficiency by alleviating epigenetic repression and enabling the activation of pluripotency genes[40,41].

With the aim of modifying the chromatin status to make it more prone to fate reprogramming, we added VPA, TSA or PRC1/PRC2 inhibitors during the differentiation protocol. The drugs were added during 72 h before Sca1 MACs sorting (Fig. 6A). The treatments did not change cell morphology during culture in any condition (data not shown). Moreover, MHC-GFP analysis after 14 days in differentiation did not show GFP positive cells in any condition (Fig. 6B). We tried similar treatments on the SC isolation and culture protocols, but in this case, all treatments led to massive death in the cultures.

These observations suggests that skeletal muscle cell exposure to epigenetic modulators is not enough to provoke any effect in the re-direction capacity of MSCs towards the cardiac lineage.

Discussion

In this study, we investigated whether SCs or skeletal muscle-associated MSCs isolated from masticatory muscles retain a latent cardiogenic potential associated with their developmental origin, building on previous observations of a developmental link between head muscle stem cells and cardiac lineages[18–20]. Despite carefully optimizing differentiation protocols, our data consistently demonstrate that neither SCs nor MSCs can reliably acquire cardiomyocyte properties in vitro.

One notable finding concerns the limitations observed in widely used cardiac markers. We observed cTnT expression in immature myotubes and its co-expression with myogenic proteins such as Myogenin, indicating that cTNT is unreliable as a specific marker of cardiomyocyte identity. This lack of specificity during early skeletal muscle development may have contributed to potentially misleading interpretations in previous studies. Using genetic lineage reporter *Myh6*$^{IRES\text{-}Cre}$; *ROSA26R*$^{CAG\text{–}GFP}$ we confirmed that no bona fide cardiac cells emerged from SCs or MSCs cultured under any tested conditions. In this respect, the comparison of our study with previous reports has the limitation that during the first steps of the protocol high serum conditions are used and therefore we cannot exclude the possibility that different sera may pre-condition the cells in different ways. Nonetheless, these first steps are only used for the expansion of the SC population, whereas during differentiation, low serum conditions are used.

The inability of SCs and Sca1-positive MSC population to adopt a cardiomyocyte fate, even when exposed to modified culture conditions and, in the case of MSCs, chromatin remodeling treatments, suggests that lineage commitment in adult skeletal muscle progenitors is highly stable. Our results indicate the presence of strong epigenetic barriers that restrain lineage plasticity in these populations and that the addition of extrinsic factors is not sufficient to modify their fate.

Our findings align with the broader challenge in regenerative medicine to generate functionally mature cardiomyocytes from non-cardiac somatic sources. While protocols for reprogramming fibroblasts to cardiomyocytes using defined transcription factors have shown promising results[42,43], here we showed that direct conversion from committed myogenic lineages towards cardiac-like cells remains inefficient and poorly reproducible.

Overall, this work contributes to clarifying the limitations of skeletal muscle progenitors as a potential source for cardiac cell replacement therapies and highlights the necessity of setting realistic expectations and thoroughly assessed methodologies in the ongoing pursuit of effective heart regeneration strategies.

Material and methods

Mouse strains

All animal procedures were approved by the CNIC Animal Experimentation Ethics Committee, by the Community of Madrid (Ref. PROEX 144.1/21), and conformed to EU Directive 2010/63EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 1201/2005. Mice have access to food and water ad libitum, and their availability is checked daily to ensure that sufficient quantities are provided. Similarly, environmental conditions of temperature and humidity comply with the requirements established by the relevant regulations for rodents (temperature: 20–24 °C; relative humidity: 45–65%). In this study, mice were maintained on a mixed genetic background. Mice were euthanized by CO2 inhalation according to the European Commission recommendations for the euthanasia of experimental animals.

Wild type mice used were of the CD1 strain. The Pax7-nGFP mouse line has been described previously[26]. *Myh6*$^{IRES\text{-}Cre}$; *ROSA26R*$^{CAG\text{–}GFP}$ mice were generated by crossing *Myh6*$^{IRES\text{-}Cre}$[33] with *ROSA26R*$^{CAG\text{–}GFP}$[34] mice.

Isolation and culture of satellite cells

Masseter or tibialis anterior muscle tissue from 8–10 week-old mice were removed, and minced. For each independent experiment, satellite cells were isolated from the tibialis anterior and masseter muscles of six mice. Muscles were collected in 1.5 mL ice-cold digestion medium containing Liberase (5 mg/ml) (Roche, 5401127001), Dispase (0.1 g/ml) (Sigma-Aldrich, D4693-1G), $CaCl_2$ (0.4 μM) and $MgCl_2$ (5 μM) in DMEM (41965-039, Gibco). Tissue was incubated at 37 °C for 90 min and enzymes were inactivated by adding FBS (F7524, Sigma Aldrich). The digested mix was filtered through 70 μm cell strainers and then through 40 μm cell strainers and centrifuged for 10 min at 1100 rpm. Cell pellets were incubated with Red Blood Buffer Lysis for 4 min and centrifuged again. SCs were sorted by sequential fluorescence activated cell sorting (FACs). Cells obtained from the six animals were pooled separately according to muscle type and subsequently plated into multiple wells for the in vitro experiments.

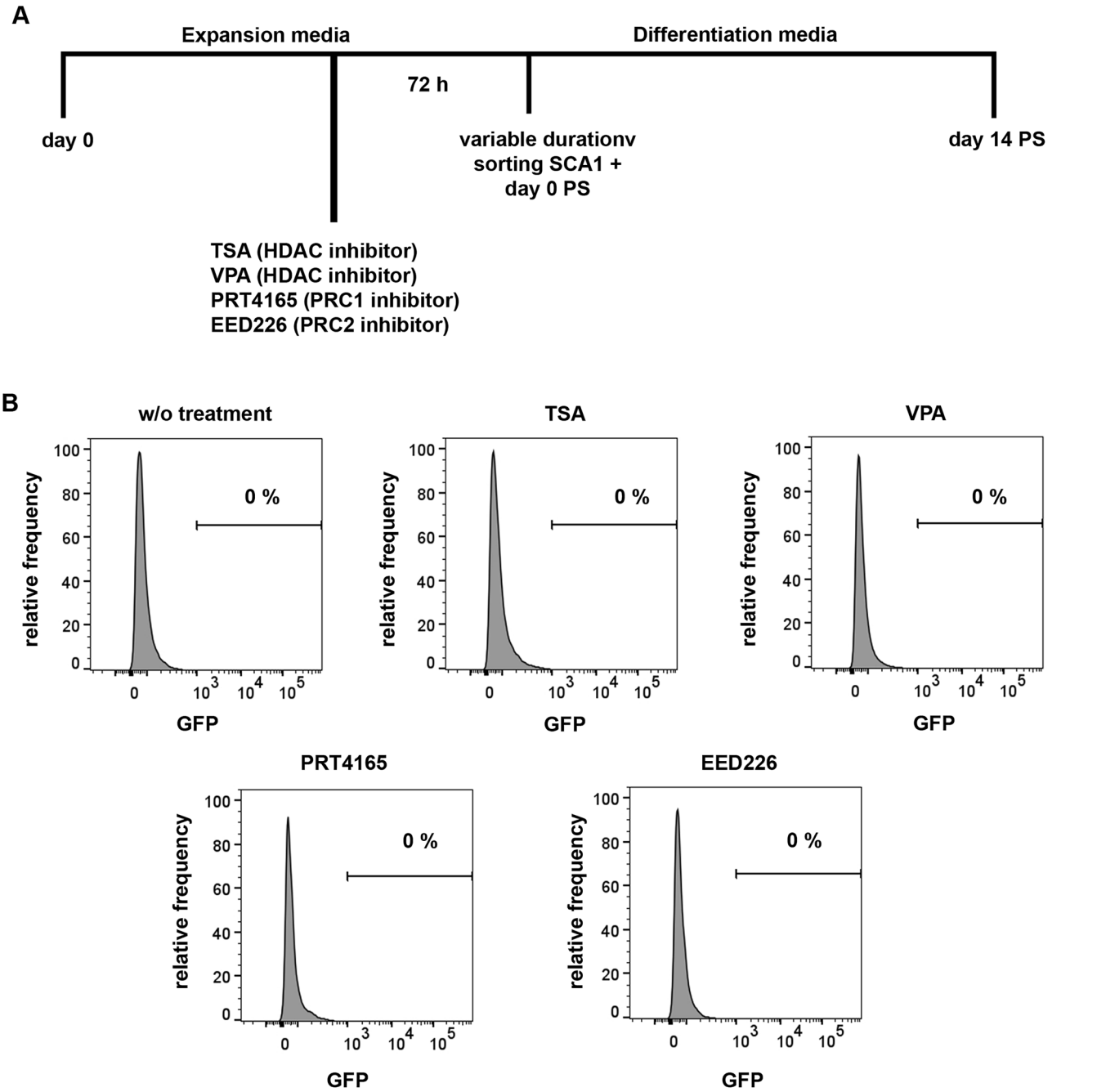


Fig. 6 | Modified differentiation protocol of MSCs using small molecule inhibitors. A Scheme of the modified myosphere protocol in which HDAC and PRC inhibitors were added 72 h before Sca1 MAC sorting. **B** Histograms showing GFP intensity levels distribution of Sca1+ cells in control conditions and under treatment with inhibitors. The proportion of GFP expressing cells is indicated in the plots.

Sorted SCs were seeded on Matrigel coated plates and cultured in HAM's F10 (L0140-500, Biowest) medium containing 20% FBS. The culture protocol is based on a previously described procedure[13] and is shown in (Fig. 1A). Medium was initially supplemented with 100 ng/ml BMP4 (314-BP-010, R&D Systems) and 10 ng/ml bFGF (233-FB-025, R&D Systems) for 4 days and then with 100 ng/ml DKK (5439-DK-010, R&D Systems), 5 ng/ml VEGF (293-VE-010, R&D Systems) and 10 ng/ml bFGF for another two days. In the modified protocol, CHIR (130-106-539, Miltenyi Biotec) was added from day 2 to day 4. In one of the protocols, Activin-A (338-AC-010, R&D Systems) was added for the first four days. Modifications of this protocol are all shown in Supplementary Table 1. Cells were then cultured in DMEM plus 5% FBS up to 2 weeks.

Isolation and myosphere culture of MSCs

MSCs were isolated following a myosphere culture protocol[12]. For each independent experiment, masseter muscle tissue was collected from 8–10-week-old six mice, washed, finely minced, and enzymatically dissociated in 0.1% collagenase II (LS004176, Worthington-biochem) in DMEM for 2 h at 37 °C. The tissue suspension was then further digested with 0.125% trypsin (15090-046, Gibco) for 45 min. Enzymatic activity was quenched by adding 10% FBS. The resulting cell mixture was passed through a 70 µm strainer and centrifuged at 2000 rpm for 5 min. Cell pellets were washed in PBS and resuspended in 10 mL of growth medium, consisting of high-glucose DMEM supplemented with 10% FBS, 0.1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol, 1000 IU/Ml LIF, and 10 ng/mL BMP-4. The suspension was filtered again through a 40 µm strainer and plated in low-

attachment dishes for suspension culture. After 3 days the myospheres were seeded on regular plates and adhered. On day 7 cells were detached by using Tryple select 10x (A1217701, Life Technologies) and moved again to low attachment plates for re-sphering. Myosphere culture protocol allows to maintain and expand the cells up to several weeks. Following repeated passaging through alternating suspension and adhesion cultures, cells were dissociated using Tryple select 10x and sorted by MACS® based on Sca1 expression (Anti-Sca-1 (non-HSC) MicroBeads, mouse, 130-106-641, Miltenyi Biotec). Sca1 positive and negative MSCs were cultured in differentiation medium containing 20 ng/mL bFGF, 10 ng/mL EGF (236-EG-200, R&D Systems), 1 mM L-glutamine, 0.1 mM nonessential amino acids, 2% B-27 supplement (17504044, Fisher Scientific), 10% FBS in DMEM:M199 (M4530, Merck) (4:1).

Treatments with inhibitors of MSCs

For the modification of the chromatic status of MSCs, HDAC or PRC inhibitors were added for 72 h before Sca1 based MACs sorting. Inhibitors were added at the following concentrations: 20 nM or 10 nM TSA (S1045, Selleck Chem), 2 mM or 1 mM VPA (P4543, Sigma Aldrich), 10 µM or 5 µM PRT4165 (HY-19817, MedChemexpress) and 10 µM or 5 µM EED226 (HY-101117, MedChemexpress).

Flow cytometry

SCs from masseter and anterior tibialis muscle were isolated by FACs based on Pax7-nGFP signal or by sequential sorting for CD45 (103114, BioLegend), CD31 (102418 BioLegend), SCA1 (108114, BioLegend) negative followed by CD34 (560230, BD Pharmigen) and alpha7-integrin (53-0010-05, AbLab).

In order to analyze the presence of GFP when using $Myh6^{IRES\text{-}Cre}$; $ROSA26R^{CAG\text{–}GFP}$ mice, SC or MSC derived cells were dissociated with TrypleX, resuspended in PBS containing 1% FBS and 1% P/S and stained with DAPI (1:1000) for dead cell exclusion.

All the sortings and analyses were conducted using BD FACSAria™ Fusion CellSorter.

Immunofluorescence

For cell immunostaining assays, cells were fixed overnight in 2% paraformaldehyde (PFA) in PBS at 4 °C, permeabilized with PBT (PBS with 0.5% Triton X-100) and blocked in Tris/NaCl/blocking reagent (TNB) (FP1012, Perkin Elmer). Samples were incubated with both the primary and secondary antibodies in blocking solution for 1 h at room temperature.

Hearts, adult anterior tibialis and E15.5 hind limb were dissected and fixed overnight at 4 °C in 2% PFA in phosphate buffered saline (PBS) and embedded in OCT or paraffin for sectioning. Cryosections or paraffin sections were permeabilized with PBT (PBS with 0.5% Triton X-100) and blocked with universal TNB reagent. Primary antibodies were incubated at 4 °C overnight and secondary antibodies for 1 h at room temperature.

Primary antibodies used were anti-GATA4 (1:100) (sc-25310, Lot# B0824, Santa Cruz), anti-cTNT (1:50) (CT3, DSHB), anti-MyoG (1:100) (MA5-11486, Lot# 2758025, clone F5D, Invitrogen), anti-TNNI3 (1:100) (TI-4, DSHB), anti Nav1.5 (1:100) (ASC-005, Lot# ASC005AN4250, Alomone Labs), anti-MLC-2V (1:100) (10906-1-AP, Lot# 00200297, Proteintech), anti-MYH6 (1:100) (22281-1-AP, Lot# 00133062, Thermo Fisher), anti-Cav1.2 (1:100) (L57/46, DSHB), anti β1-AR (1:100) (AAR-023, Lot# AAR023AN0302, Alomone Labs), anti-Vimentin (1:100) (ab45939, Abcam).

Microscopy

Immunofluorescences of fixed cells or tissue sections were imaged with the Leica TCS SP8 coupled to a DMi8 inverted confocal microscope Navigator module equipped with light laser and fitted with 40x/1.3 oil objective or 20x/1.0 immersion objective. 1024×1024 pixel and A.U. set to 1 were commonly used in imaging. Image J (https://imagej.nih.gov/ij/) was used for image analysis.

Brightfield images were obtained with The Olympus Microscope IX2-ILL30 fitted with 10x objective.

Statistics and reproducibility

For each independent experiment, cells were isolated from six animals and pooled prior to plating. The pooled cells were subsequently distributed among different wells as technical replicates. The experiments were independently repeated at least three times using different pools of animals. Statistical analyses were performed using Prism v5.0 and have been included in Figure legends.

Data availability

Raw data for this article are available at "Mendeley Data" with 10.17632/zmvvv96zzx.1. The dataset contains the underlying data used to generate the figures and reported results in this article. The data are currently stored and publicly accessible through Mendeley Data under the corresponding DOI. No restrictions apply to access or reuse of the deposited data. The source data underlying the graph presented in Fig. 1 are provided in Supplementary Data 1.

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Author contributions

C.D. conceptualization, methodology, formal analysis, investigation, visualization, writing-original draft, writing-review and editing. O.O. conceptualization, methodology, investigation. J.I. and P.M. conceptualization, methodology, materials; M.T. conceptualization, writing-review and editing, supervision, funding acquisition.

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Competing interests

The authors declare no competing interests.

Additional information

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