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Robust generation and expansion of skeletal muscle progenitors and myocytes from human pluripotent stem cells

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ABSTRACT

Human pluripotent stem cells provide a developmental model to study early embryonic and tissue development, tease apart human disease processes, perform drug screens to identify potential molecular effectors of *in situ* regeneration, and provide a source for cell and tissue based transplantation. Highly efficient differentiation protocols have been established for many cell types and tissues; however, until very recently robust differentiation into skeletal muscle cells had not been possible unless driven by transgenic expression of master regulators of myogenesis. Nevertheless, several breakthrough protocols have been published in the past two years that efficiently generate cells of the skeletal muscle lineage from pluripotent stem cells. Here, we present an updated version of our recently described 50-day protocol in detail, whereby chemically defined media are used to drive and support muscle lineage development from initial CHIR99021-induced mesoderm through to PAX7-expressing skeletal muscle progenitors and mature skeletal myocytes. Furthermore, we report an optional method to passage and expand differentiating skeletal muscle progenitors approximately 3-fold every 2 weeks using Collagenase IV and continued FGF2 supplementation. Both protocols have been optimized using a variety of human pluripotent stem cell lines including patient-derived induced pluripotent stem cells. Taken together, our differentiation and expansion protocols provide sufficient quantities of skeletal muscle progenitors and myocytes that could be used for a variety of studies.

1. Introduction

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) are versatile model systems for studying a multitude of tissues: when given the proper stimuli, hESCs and iPSCs can be differentiated into desired cell types and tissues. In addition to their use in dissecting human developmental pathways and disease, pluripotent cells possess an incredible proliferation capacity amenable to generating large quantities of transplantable material for cell-based therapies. For example, heart disease being a leading cause of death world-wide has arguably motivated the development of numerous protocols to generate cardiac muscle from hESCs [1–3]. In fact, the ability to generate pure populations of functional retinal pigmented epithelial (RPE) cells from pluripotent stem cells (PSCs) has led to several ongoing clinical trials using RPE cells derived from both ESCs and iPSCs to treat age-related macular degeneration and Stargardt disease, with early success being reported in an ESC-derived RPE clinical trial [4,5].

In contrast to cardiac muscle or RPE cells, until very recently, little success has been achieved in terms of skeletal muscle differentiation from human PSCs [6–9]. Thus, while PSC-derived cardiomyocytes have been used to dissect disease mechanisms [10] and provide proof of concept for cardiac regeneration by cardiomyocyte transplantation in non-human primate pre-clinical

Abbreviations: hESCs, human embryonic stem cells; iPSCs, induced pluripotent stem cells; PSCs, pluripotent stem cells; EBs, embryoid bodies; SMPs, skeletal muscle progenitors; TC, tissue culture; MRFs, myogenic regulatory factors; FACS, fluorescence activated cell sorting; BSA, bovine serum albumin; EGF, epidermal growth factor; FGF2, fibroblast growth factor 2; E8, Essential 8 media; RPE, retinal pigmented epithelial; EMT, epithelial to mesenchymal transition; T, Brachyury; MSGN1, Mesogenin 1; GOI, gene of interest; DOI, day of interest.

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studies [11], the skeletal muscle field has not progressed on these fronts.

The development of robust protocols to differentiate human PSCs into a specific cell or tissue type requires a thorough understanding of the developmental networks that drive specification, differentiation and expansion of the desired cell or tissue type to identify factors that can replace the highly organized physical structures and complex signaling networks—both spatial and temporal—naturally found *in vivo* [12]. A detailed account of the embryonic origins of skeletal muscle are succinctly reviewed in [13].

The first generation of non-transgenic strategies to generate skeletal muscle from ESCs utilized cell aggregation into embryoid bodies (EB) to induce differentiation [14–17]. However, cells are exposed to varying concentrations of endogenous and exogenous factors depending on their position within the EB. Furthermore, the first generation of differentiation protocols also relied on media supplemented with undefined serum, which led to variable differentiation efficiencies from one serum lot to the next. As a result, skeletal myocytes typically represented less than 10% of the total differentiated cells [18,19].

Fortunately for the skeletal muscle field, the intensive and productive research performed on cardiac muscle differentiation provides insight into the early stages of skeletal muscle differentiation as both cardiac and skeletal muscle originate from the mesodermal germ layer, allowing well-established PSC cardiac differentiation protocols to serve as a starting point for directing skeletal myogenesis. Within the past couple of years, several remarkable protocols have been established detailing chemically directed approaches to hESC and iPSC skeletal myogenesis, reaching over 50% differentiation efficiency [6,8,9]. Three recent studies, including our own, independently developed a similar approach: stimulating Wnt signaling in monolayer cells by treating with CHIR99021 to induce mesoderm [7,8]. These protocols additionally use fibroblast growth factor 2 (FGF2) to further support mesoderm commitment into the skeletal muscle lineage, although significant differences between the protocols exist including the concentration and time of application for both factors. Additionally, Suzuki and colleagues concurrently reported an efficient protocol using an aggregate-based approach in which hESCs are treated with FGF2 and epidermal growth factor (EGF) [9].

Potentially the most important result in any of the myogenic differentiation protocols—with the purpose of cell therapy in mind—is the persistence of skeletal muscle progenitors (SMPs). This is because terminally differentiated myoblasts or myocytes cannot appreciably proliferate, and therefore, have limited repair potential following transplantation.

Adult SMPs, known as satellite cells, reside beneath the basal lamina in muscle fibers and are defined by their location and expression of the transcription factor PAX7 [20]. Satellite cells are activated to proliferate in response to injury, and these dividing cells contribute to the differentiation of new myocytes and maintenance of the progenitor pool. Satellite cells cannot be identified within *in vitro* differentiation cultures because the structures forming the satellite cell niche simply are not formed. The protocol described here, however, generates proliferative PAX7 expressing cells found interspersed among skeletal myocytes and myotubes.

2. Experimental design

2.1. hESC and iPSC cell culture

Human PSC lines were maintained using Essential 8 (E8) medium as previously described [21]. In this approach, cells are maintained in a serum-free, feeder cell-free environment and cultured in Matrigel coated 6-well tissue culture (TC) plates using E8 medium. We also find routine passage with EDTA solution provides a consistent cell proliferation rate, as well as improved survival compared to enzymatic methods [22]. Another advantage of EDTA-mediated passage is the reduction in spontaneous differentiation of pluripotent colonies. Using EDTA passaging at a split ratio of 1:6, H1 and H9 hESCs as well as a variety of iPSC lines generated in our group routinely take 3–4 days post-passage to become 70–80% confluent with no alterations in karyotype for at least 20 passages: a time point in which maintenance of the cell line is terminated to prevent population drift and a new aliquot of cells is thawed. In contrast to EDTA passaging, collagenase-based passaging is also performed at a split ratio of 1:6 but every 5–7 days depending on cell loss due to spontaneous differentiation. If spontaneous differentiation occurs, we "clean" our cultures under a dissecting microscope by scraping off differentiated colonies marked by irregular, undefined borders (Fig. 1A, 5 DPP) using a pipette tip.

2.2. Differentiation into the myogenic lineage

Prior to differentiation, it is essential to ensure 80–90% of cells are undifferentiated and cultures are 70–80% confluent (Fig. 1A, 3 DPP).

The first step in our differentiation protocol, and a major hurdle of producing an efficient and reproducible protocol, is the dissociation of colonies into single cells while still maintaining their pluripotency and viability. Dissociating colonies into single cells ensures that the small molecules used to induce differentiation act evenly across all cells, and that plating densities can be better controlled for consistent amounts of crucial cell-to-cell contact between experiments.

Prior to dissociation, the pluripotent cells are treated with 10 μM ROCK inhibitor (Y-27632) to reduce dissociation-induced apoptosis [23]. We then utilize TrypLE—a mild form of trypsin—to dissociate the colonies into single cell suspension. Exposure to TrypLE for up to 5 min is sufficient for generating a single cell suspension, and has minimal effect on cell viability.

For the H9 ESC line, re-plating in E8 media supplemented with 10 μM Y-27632 at a density of 1.5×10^5 cells per well of a 12-well tissue culture plate was found to be optimal for cell survival and subsequent mesoderm induction [6]. Optimal re-plating density may vary, however, depending on the cell lines used in the protocol. We have found that optimal seeding of some cell lines—such as the 167-1J Hutchinson–Gilford Progeria Syndrome patient-derived iPSCs (unpublished)—requires up to 3×10^5 cells per well of a 12-well tissue culture plate. Cells are ready for differentiation roughly 24 h later (Fig. 1B).

Cells are then treated with 10 μM CHIR99021 supplemented E6 media—E8 media lacking the pluripotency factors FGF2 and TGF-β1—to simulate Wnt signaling during gastrulation. CHIR99021, a potent GSK3 inhibitor, has been extensively used to drive mesoderm specification in cardiac and other mesoderm-lineage differentiation protocols [1,3,24]. There is a notably high level of cell death—approximately 25% of total cells—24 h after the initial CHIR99021 treatment (Fig. 1B), which we observe to correlate with more efficient mesoderm induction. By 2 days following CHIR99021 treatment, however, the remaining cells appear to recover and their morphology is dramatically altered indicative of epithelial to mesenchymal transition (EMT) (Figs. 1B and 2A). At this point, peak levels of Brachyury (T) and Mesogenin 1 (MSGN1) can be used to confirm the presence of uncommitted and paraxial mesoderm, respectively (Fig. 1C and D). MSGN1 and TBX6, specifically, are key transcription factors which we use as markers of the early stages of skeletal muscle differentiation, as they mark the earliest mesodermal cells that possess skeletal myogenic potential [25,26], rather than cardiac or smooth muscle.