Miltenyi Biotec

Cardiomyocyte purification from pluripotent stem cells

Todd Herron, Andre Monteiro da Rocha, and Katherine Campbell
University of Michigan, Ann Arbor, MI, USA

Background

Pluripotent stem cells (PSCs) offer an unlimited source of human cardiovascular cells for research and the development of cardiac regenerative therapies.
The development of highly efficient cardiac-directed differentiation methods makes it possible to generate large numbers of cardiomyocytes (CMs).[1, 2] However, due to varying differentiation efficiencies, further enrichment of CM populations for downstream applications is essential. Non-genetic purification techniques have been described based on mitochondrial dyes and flow sorting[3], but this technique requires sophisticated instrumentation and results in the loss of significant numbers of CMs. Another non-genetic approach utilized metabolic starvation of non-myocytes using glucose-depleted and lactate-enriched media to select for CMs.[4] Although this metabolic flow approach yields up to 99% pure CMs, the non-myocytes are not available for further study and the CMs facing metabolic challenge are compromised. Recently, a CM-specific cell surface marker called SIRPα (signal-regulatory protein alpha, also termed CD172a) was reported to be a useful tool for flow sorting of human stem cell–derived CMs.[5] Dubois *et al.* also showed the effectiveness of using magnetic bead–based sorting in combination with SIRPα antibodies, but reported significant loss of CMs with this technology.[5] Recently, in our laboratories we have optimized a MACS® Technology–based protocol to enrich human iPSC/ESC-derived CMs by SIRPα expression.[6-8] However, our expression analysis revealed that SIRPα only labels a subpopulation of CMs indicated by cardiac Troponin T (cTnT) expression. Moreover, SIRPα is also expressed on a subpopulation of non-CMs, hence making SIRPα an inadequate marker to enrich PSC-derived CMs. To circumvent the disadvantages of SIRPα for CM enrichment, we further evaluated the novel PSC-Derived Cardiomyocyte Isolation Kit, human, which utilizes different surface markers than SIRPα for CM enrichment. The kit provides two different strategies for CM enrichment which can be applied depending on the initial differentiation efficiency. For differentiation cultures with a CM content above 50% a single separation step involving depletion of non-CMs is sufficient to achieve purities >90%. For low differentiation efficiencies below 50% a second enrichment step, utilizing a novel, highly specific CM marker, can be added to further increase purity. To assess and compare the kit performance to SIRPα-dependent enrichment, the CMs were analyzed phenotypically and functionally after the cell separation step.

Materials and methods

Materials

Antibodies

- Anti-Cardiac Troponin T-FITC, human, mouse, rat; clone REA400 (# 130-106-687)
- Anti-α-Actinin (Sarcomeric)-FITC, human, mouse, rat; clone REA402 (# 130-106-997)
- Anti-MLC2a-PE, human, mouse, rat; clone REA398 (# 130-106-142)
- Anti-MLC2v-APC, human, mouse, rat; clone REA401 (# 130-112-759)
- CD172a (SIRPα)-Biotin, human; clone REA144 (# 130-099-768)

Buffers and reagents

- PSC-Derived Cardiomyocyte Isolation Kit, human (# 130-110-188)
- Anti-Biotin MicroBeads (# 130-090-485)
- autoMACS® Running Buffer – MACS Separation Buffer (# 130-091-221)
- MACS SmartStrainers (70 µm) (# 130-098-462)
- Multi Tissue Dissociation Kit 3 (# 130-110-204)

Media

- StemMACS™ iPS-Brew XF (# 130-104-368)
- RPMI media +B27

Cells

- Human iPSC lines derived from skin fibroblasts

Methods

Differentiation of CMs from human iPSCs

CMs were differentiated from human iPSC lines according to published protocols[1,2].

Cell preparation and harvest before magnetic enrichment of CMs

For both enrichment methods, the differentiated CMs from monolayer cultures were dissociated into single-cell suspensions using the Multi Tissue Dissociation Kit 3 according to the corresponding **protocol**. To download the PDF simply click on the hyperlink in boldface.

CM isolation based on positive selection of SIRPα-positive cells

Up to 3×10^6 cells of a single-cell suspension were stained with 20 µL CD172a (SIRPα)-Biotin antibody in a total volume of 100 µL of MACS Separation Buffer. After a 5-min incubation at 4 °C, the cells were washed with MACS Separation Buffer and magnetically labeled with 20 µL Anti-Biotin MicroBeads in a final volume of 100 µL of MACS Separation Buffer. The SIRPα$^+$ cells were then isolated according to the **Anti-Biotin MicroBead data sheet**.

CM isolation utilizing the PSC-Derived Cardiomyocyte Isolation Kit, human

With the PSC-Derived Cardiomyocyte Isolation Kit, human, CMs were isolated either in a single-step or two-step isolation protocol, depending on the initial differentiation efficiency (fig. 1). As a general guideline, we recommend performing the second (positive selection) step if the culture contains <50% of CMs. Isolation of the CMs was performed as described in the **protocol provided with the kit**. The standard protocol enables enrichment of CMs from up to 5×10^6 total cells, and up-scaling is possible for up to 1×10^7 total cells.

Results

Characterization of CMs isolated based on SIRPα expression

In this study we compared two different methods based on MACS Technology to isolate CMs differentiated from human PSC cultures. Initially, CMs were isolated based on SIRPα expression of the cells. This method involves indirect magnetic labeling using a biotin-conjugated CD172a (SIRPα) antibody followed by Anti-Biotin MicroBeads to enrich the PSC-derived CMs by positive selection. This approach appeared to be highly reproducible, resulting in enriched CM populations as indicated by the expression of cTnT (fig. 2). The isolated cells could be cultured and started beating again after a lag phase. Western blot analysis of all different cell populations further confirmed the CM phenotype of the enriched cells (fig. 3). Moreover, a strong increase of the CM-specific sarcomeric myosin was observed in the enriched cells compared to the original non-purified, heterogeneous cell fraction. However, as depicted in figure 4, it became apparent that SIRPα is not an ideal CM marker, since it is not expressed on all cTnT-positive cells and also labels cTnT-negative cells.

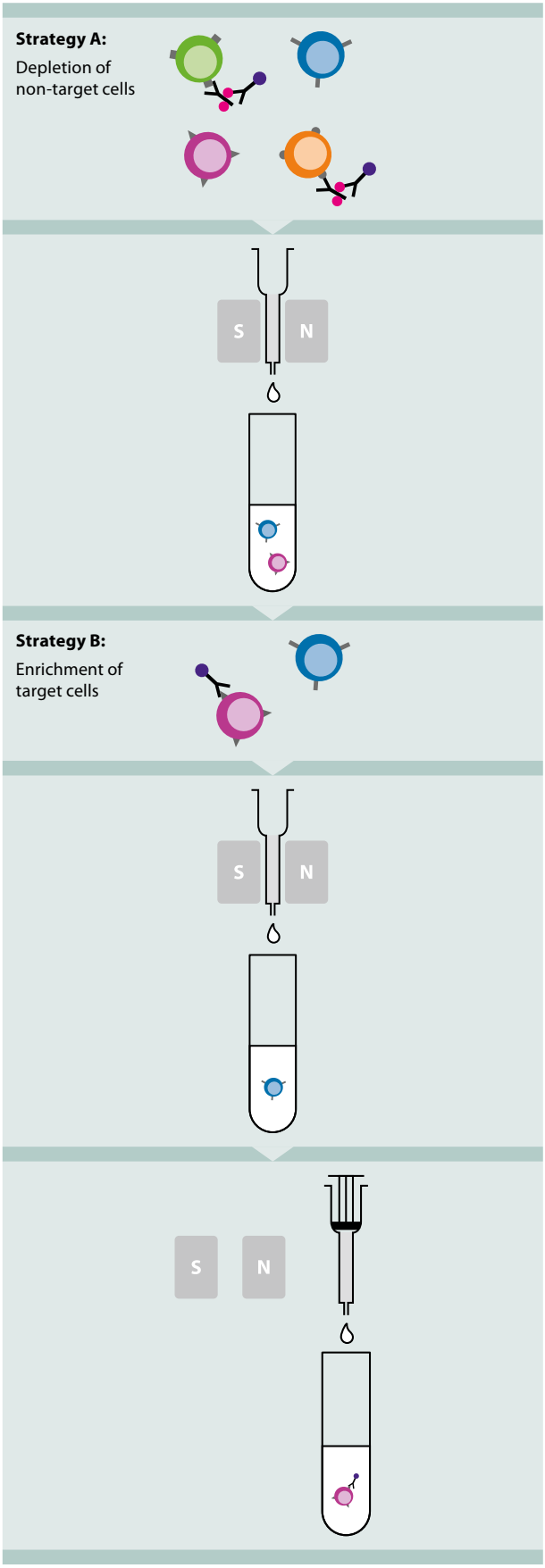

Figure 1: Strategies for the isolation of hPSC-derived CMs based on MACS Technology. Depending on the hPSC differentiation efficiency different strategies for the magnetic enrichment of hPSC-derived CMs may be applicable. For cell populations with low differentiation efficiencies, i.e., at CM ratios of <50%, it is recommended to combine strategy A (depletion of non-myocytes) with strategy B (positive selection with CM-specific MicroBeads). At higher differentiation efficiencies (>50%) strategy A is sufficient to achieve high CM purities.

We compared the SIRPα-based enrichment to the now commercially available PSC-Derived Cardiomyocyte Isolation Kit, human. This kit includes a proprietary cocktail of MACS MicroBeads to deplete the non-CM cells. This single depletion step often results in sufficiently high CM purities and recoveries. However, further enrichment of the PSC-derived CM population may be achieved by an optional second separation step using the Cardiomyocyte Enrichment Cocktail included in the kit. CMs isolated with this kit could also be cultured and started beating 24 h after plating. Flow cytometric analysis revealed very high purities based on cTnT expression of the cells in the CM population (fig. 5A, right bar). In contrast, the non-CM fraction did not express any cTnT and showed no beating activity after re-plating (not shown). These data indicate that the depletion of non-CMs was very effective. CMs separated with the kit (strategy A only) showed a higher purity of cTnT-positive cells and contained higher percentages of MLC2a- and MLC2v-positive cells than CMs isolated based on SIRPα expression (fig. 5A). Immunofluorescence microscopy demonstrated that CMs isolated with the kit displayed high expression levels of the CM-specific markers, N-Cadherin and cTnT (fig. 5B). Electrophysiological analysis revealed that the re-plated CMs showed normal action potentials (fig. 6). These data suggest that CMs isolated by depletion of non-CMs are functional and possess the appropriate phenotype.

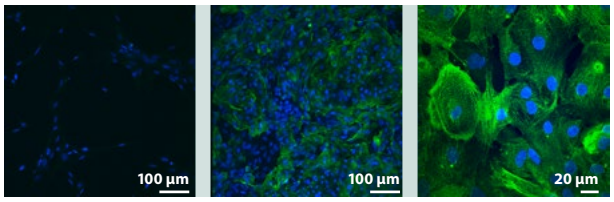

Figure 2: Confocal microscopy analysis of cTnT in CMs isolated based on SIRPα expression. CMs were isolated by MACS Technology based on SIRPα. The resulting SIRPα-negative and –positive fractions were cultured for 5 days, stained for cTnT (green), and analyzed by confocal microscopy. The negative fraction did not contain cTnT⁺ cells (left panel). The positive fraction contained ~95% cTnT⁺ cells (center, 20× objective; right, 60× objective). Nuclei were stained with DAPI (blue).

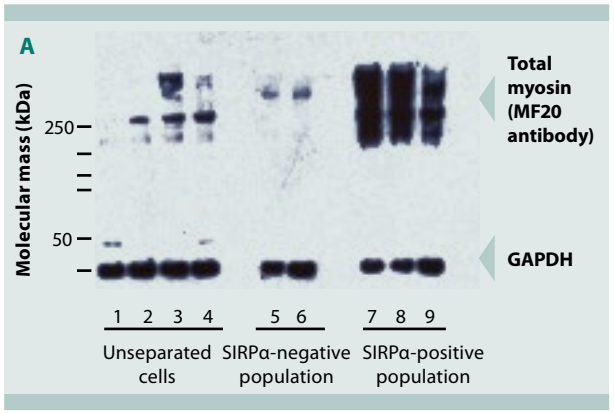

Figure 3: Western blot analysis of total sarcomeric myosin in CMs isolated based on SIRPα expression. A) Western blot probing for total sarcomeric myosin in the unseparated fraction and the SIRPα-negative and -positive fractions.

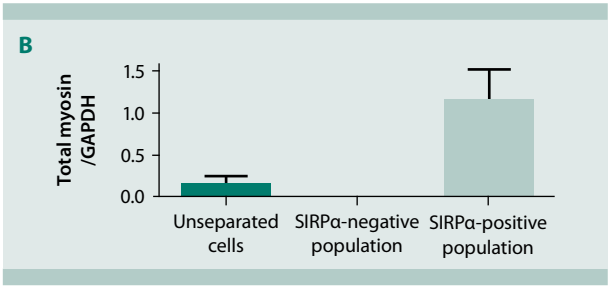

Figure 3 (cont.): Western blot analysis of total sarcomeric myosin in CMs isolated based on SIRPα expression. B) Quantification of the Western blot signals. The myosin signal was normalized to the ubiquitous protein GAPDH.

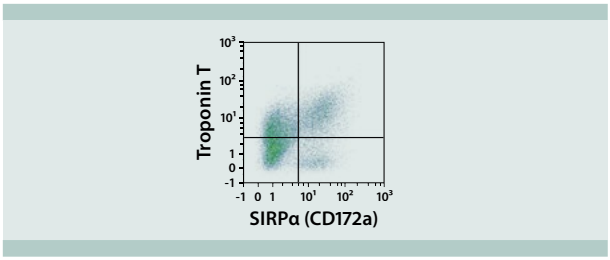

Figure 4: Expression of SIRPα (CD172a) in relation to cTnT on PSC-derived CMs. SIRPα is expressed on both cTnT-positive and -negative cells, indicating that SIRPα is not sufficient as a marker for the isolation of CMs.

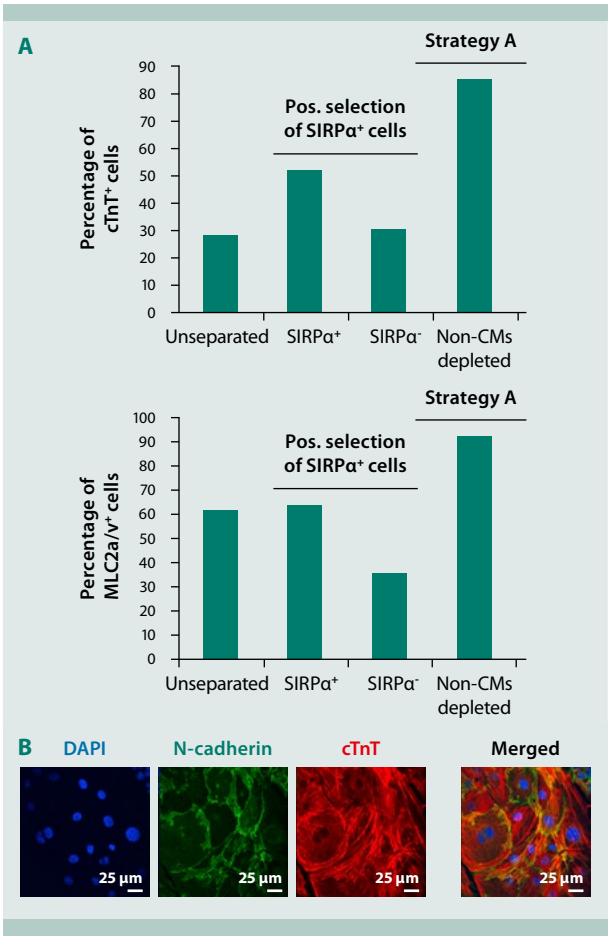

Figure 5: Enrichment of CMs by depletion of non-CMs using the PSC-Derived Cardiomyocyte Isolation Kit, human. A) Quantification of cTnT⁺ and MLC2a/v⁺ cells in the unseparated population (left bar), enriched CMs obtained by positive selection of SIRPα⁺ cells (second from left) and the corresponding SIRPα⁻ fraction (second from right), as well as enriched CMs obtained by using the kit for depletion of non-CMs (right). Cells were stained with antibodies for cTnT, MLC2a, and MLC2v and quantified by flow cytometry. B) Immunofluorescence microscopy of CMs enriched by depletion of non-CMs, using the kit.

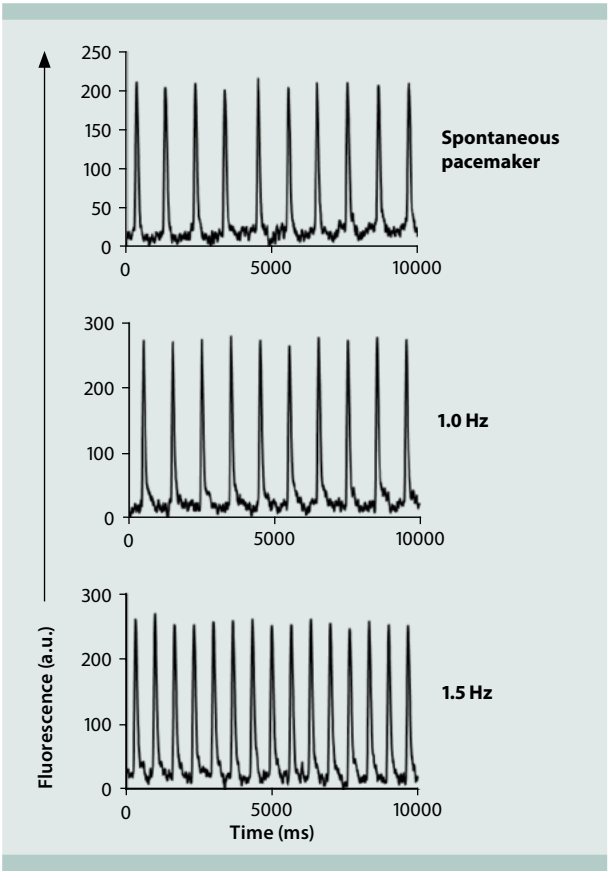

Figure 6: Functional characterization of CM monolayers derived from BJ-iPSCs after purification using the new kit. Following purification of hiPSC-CMs using the new kit, CMs were re-plated as confluent monolayers and loaded with an action potential dye (FluoVolt™). In addition to spontaneous activations (top), monolayers were also paced at 1 Hz (center) and 1.5 Hz (bottom).

Conclusion

In this study we compared two different approaches to enrich PSC-derived CMs based on MACS Technology. We could show that principally both methods were useful for CM enrichment. However, detailed analysis of the enriched cell populations revealed limitations of SIRPα as a marker for CM isolation. First of all, SIRPα expression levels varied with regard to the cells' differentiation status and time point of differentiation, thus hampering an easy standardization for CM enrichment (data not shown). Furthermore, we showed that not all SIRPα-positive cells also expressed cTnT. Moreover, we repeatedly observed that a number of cTnT-positive cells, i.e., CMs, did not express SIRPα at all, indicating that a substantial number of CMs might be lost when SIRPα is used for enrichment. Taken together, these data indicate that SIRPα is not an ideal marker for CM detection and cell separation.

By depleting the non-CM cell population using the PSC-Derived Cardiomyocyte Isolation Kit, human, we could circumvent the limitations of SIRPα and achieved a superior recovery of cTnT-positive cells. In addition, these cell populations showed a higher content of MLC2a- and MLC2v–positive cells compared to the CMs obtained by using SIRPα, indicating that the SIRPα-based CM enrichment left substantial impurities. Strikingly, the CMs enriched by depletion of non-CMs could be re-plated and showed normal action potential pacing. We conclude that the PSC-Derived Cardiomyocyte Isolation Kit, human is a fast and cost-effective approach to purify CMs derived from human PSCs. The PSC-derived CMs are highly functional and display the appropriate structural characteristics. Furthermore, the process is gentle on the cells, resulting in high viability. Magnetic separation methods for enriching CMs from PSCs is a highly efficient alternative to currently used methods.

References

1. Lian, X. *et al.* (2012) Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. Proc. Natl. Acad. Sci. USA 109: E1848–1857.
2. Zhang, J. *et al.* (2012) Extracellular matrix promotes highly efficient cardiac differentiation of human pluripotent stem cells: The matrix sandwich method. Circ. Res. 111: 1125–1136.
3. Hattori, F. *et al.* (2010) Nongenetic method for purifying stem cell-derived cardiomyocytes. Nat. Meth. 7: 61–66.
4. Tohyama, S. *et al.* (2013) Distinct metabolic flow enables large-scale purification of mouse and human pluripotent stem cell-derived cardiomyocytes. Cell Stem Cell 12: 127–137.
5. Dubois, N.C. *et al.* (2011) SIRPA is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells. Nat. Biotechnol. 29: 1011–1018.
6. Jiang, G. *et al.* (2016) Human cardiomyocytes prior to birth by integration-free reprogramming of amniotic fluid cells. Stem Cells Transl. Med. 5: 1595–1606.
7. Monteiro da Rocha, A. *et al.* (2016) Deficient cMyBP-C protein expression during cardiomyocyte differentiation underlies human hypertrophic cardiomyopathy cellular phenotypes in disease specific human ES cell derived cardiomyocytes. J. Mol. Cell. Cardiol. 99: 197–206.
8. Herron, T.J. *et al.* (2016) Extracellular matrix–mediated maturation of human pluripotent stem cell–derived cardiac monolayer structure and electrophysiological function. Circ. Arrhythm. Electrophysiol. 9: e003638.

Miltenyi Biotec