Derivation, characterization and purification of multipotent cardiovascular progenitor cells derived from human induced pluripotent stem cells

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ABSTRACT: Induced pluripotent stem cells (iPSCs) can differentiate into all types of cells in the human body, including all types of cardiac cells. However, therapies using iPSC derivatives is limited by the tumorigenic potential of iPSCs, which need to be depleted before clinical usage. This work aimed to produce highly pure cardiovascular progenitor cells (CVPCs) from human iPSCs via differentiation and subsequent purification. iPSCs were differentiated as a monolayer in chemically-defined conditions via temporal modulation of the canonical WNT signalling pathway. Human iPSC cardiac differentiation generated on average 50% of $cTnT^+$ cardiomyocytes, with a yield averaging 3.1 cardiomyocytes/iPSC. Pluripotency markers gradually decreased over time, while cardiac markers gradually increased; mesendodermal marker *T/Brachyury* increased until peaking at day 3 and CVPC markers c-KIT and SSEA-1 at days 5 and 12, respectively, then decreasing. CVPCs at day 7 of differentiation were purified via magnetic-activated cell sorting (MACS) and the lactate method. Negative immunomagnetic selection using the TRA-1-60 marker yielded an efficiency of 67.8%, while positive selection using the SSEA-1 marker yielded 10.7%. Purified cells did not reorganise in tissue-like colonies or aggregates like non-purified cells. The lactate method generated over 95% viable $cTnT^+$ cells. MACS compromised cell viability, but with the due optimisation, it has the potential to generate clinical-grade CVPCs for biomedical applications.

KEYWORDS: induced pluripotent stem cell; cardiovascular progenitor cell; cardiac differentiation; canonical WNT signalling pathway; magnetic-activated cell sorting.

INTRODUCTION

Cardiovascular diseases (CVDs) represent a broad range of diseases, involving the heart and circulatory system, such as hypertension, cardiac arrest, pulmonary heart disease, or cerebrovascular disease (stroke). CVDs carry a great socioeconomic burden, as they claim more lives than any other disease in the world[1–3]. Ischaemic heart failure, or myocardial infarction, is the death of cardiomyocytes, caused by oxygen deprivation of these cells. Although the heart features endogenous mechanisms for cardiomyocyte renewal, these are not sufficient to regenerate the lost tissue[1,3,4]. Due to the lack of a suitable response by traditional therapies, stem cells have appeared as an alternative to repair the heart[3,5].

Stem cells are able to self-renew, originating at least one daughter cell identical to themselves, and, by the action of various types of stimuli, differentiate, developing into mature, specialised cells[6,7]. Stem cells may be classified according to their differentiation potential, in decreasing order, as totipotent, pluripotent, multipotent or unipotent[6]. Both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are pluripotent stem cells (PSCs), as they are able to generate all of the terminally differentiated cells arising from the three germ layers. Due to this, they are ideal for various applications, including cell therapy, tissue engineering, drug screening and disease modelling, and are already being used in clinical trials[7–10]. As the name implies, ESCs are derived from the developing embryo, more precisely, from the inner cell mass of the blastocyst[11]. iPSCs are obtained when a somatic, differentiated cell is reprogrammed into a pluripotent state[12,13]. Despite the very distinct derivation methodologies, iPSCs exhibit the same general characteristics as ESCs, such as morphology, surface marker expression, self-renewal capacity and pluripotency. Surface markers stage-specific embryonic antigen (SSEA)-3, SSEA-4, tumour rejection antigen (TRA)-1-60 and TRA-1-81, as well as transcription factors octamer-binding transcription factor (OCT) 4, sex determining region Y-box (SOX) 2 and NANOG, are commonly used for characterisation of both types of cells[12,13]. On the other hand, iPSCs avoid some of the ethical problems associated with ESCs, and can be patient-specific, avoiding rejection by the immune system of the host[7,12–14].

Cardiovascular progenitor cells (CVPCs) are mesoderm-derived cells with a limited lifespan, and which can differentiate into the three types of cardiac cells – cardiomyocytes, smooth muscle cells and endothelial cells. CVPCs occur naturally in the human heart and only allow for very limited regeneration of cardiac cells upon injury, but hold an enormous potential for biomedical applications[1,3]. These cells are not well described in terms of unique markers, as most strategies used to characterise these cells rely on markers expressed by a myriad of other different types of cells[15]. Both SSEA-1 and c-KIT have been proposed for labelling CVPCs, although these markers also label other cell types[1,5,16–18].

Cardiac differentiation requires stimulation of specific cellular mechanisms, such as those observed during embryonic cardiac development[3,19]. Despite the variety of methods for differentiation of human PSCs to cardiomyocytes, these rarely result in high percentages (>90%) of cardiac troponin T $(cTnT)^+$ cells without resorting to a purification method downstream of the differentiation protocol. In order to overcome the low efficiency of existing cardiomyocyte differentiation methods, a monolayer, defined, growth factor-free protocol regulating only elements the canonical wingless-type mouse mammary tumour virus integration site (WNT) signalling pathway was recently developed[20,21]. The WNT signalling pathway synergises with both the bone morphogenic protein (BMP) and activin/NODAL pathways to induce mesendodermal differentiation of human PSCs, similarly to what happens in normal cardiac development[20,22], but prevents cardiomyogenesis in mesoderm-committed cells. Canonical WNT signalling is thus inhibited through the action of mesoderm and CVPC markers, such as

mesoderm posterior transcription factor (MESP) 1 and NKX2-5[20,23], and, along with BMP and activin/NODAL inhibition, can be used to maintain CVPCs at a progenitor state[1]. Cells are exposed to WNT activator CHIR99021 between days 0 and 1, and to inhibitor of WNT production (IWP) 2 or 4 between days 3 and 5. This protocol allows for generation of functional beating cardiomyocytes by day 15[20,21,24].

Purification processes play a crucial role in a product intended for clinical applications. Ideally, differentiating PSCs into only the desired type of cells would be possible. In truth, differentiation protocols result in a heterogeneous mixture of cells, and, at best, the desired type of cell comprises the majority of this mixture. For drug screening or disease modelling purposes, contaminant cells may provoke unexpected reactions, which would not occur *in vivo*, whereas clinical applications require the depletion of dangerous cells, such as undifferentiated PSCs which cause teratomas when transplanted onto a host[1,12,13,16,25]. In this work, magnetic-activated cell sorting (MACS) was used for purification of CVPCs at day 7[5,16], and compared with the lactate method[26] in terms of the generation of cardiac troponin T (cTnT)$^+$ cardiomyocytes by the end of the differentiation protocol.

MATERIALS AND METHODS

Maintenance of human induced pluripotent stem cells. Vector-free human iPSCs reprogrammed from foreskin fibroblasts (DF6-9-9T.B) were cultured on 9.6 cm^2 tissue culture plates (Falcon®) coated with Matrigel® (Corning®), and 1.5 mL/well of mTeSR1™ medium (STEMCELL Technologies™) supplemented with 0.5% penicillin/streptomycin (Gibco®) was changed daily. Cells were kept at 37 °C, 5% CO_2 and 20% O_2 inside a CO_2 incubator (Memmert). For passaging, cells were washed twice with 1 mL/well of ethylenediaminetetraacetic acid (EDTA) dissociation solution (0.5 mM EDTA (Invitrogen™) and 1.8 g/L sodium chloride (Sigma-Aldrich®) in phosphate-buffered saline (PBS) solution (Gibco)), and then left for 5 min also in 1 mL/well of EDTA. After removal of EDTA, cells were flushed twice with 1 mL/well of culture medium and collected in a Falcon tube. mTeSR1 medium was added to the Falcon tube so that the final volume would result, after the passaging, in 1 mL/well. In the new plate, 0.5 mL/well of mTeSR1 medium, and, afterwards, 1 mL/well of cell suspension were added. Passaged cells were kept in a CO_2 incubator.

Cardiomyocyte induction via temporal modulation of the WNT signalling pathway. Differentiation was performed as previously described[20,21,24]. Human iPSCs were plated at a density of 100,000 cells/cm^2 in Matrigel-coated 3.8 cm^2 tissue culture plates. mTeSR1 medium was changed daily, being added at 1 mL/well, until cells achieved 90%-95% confluence. On day 0 of differentiation, 1.5 mL/well of Roswell Park Memorial Institute (RPMI)/B27-insulin medium (RPMI 1640 medium (Gibco) supplemented with 2% B-27® minus insulin (Gibco) and 0.5% penicillin/streptomycin), containing 6 µM of CHIR99021 (Stemgent®) was added to each well. After 24 h, the medium was changed to RPMI/B27-insulin. At day 3, 750 µL/well of medium were removed, and replaced with medium containing IWP4 (Stemgent), to obtain a final concentration of 5 µM in each well. IWP4 was removed in the medium change at day 5. At day 7, the medium was changed to RPMI/B27 (RPMI 1640 medium supplemented with 2% B-27 (Gibco) and 0.5% penicillin/streptomycin) and changed every 3 days until the end of the experiment. Cells were sacrificed for functional analyses at diverse time points throughout the experiment. The performance of the differentiation protocols was assessed by the cellular phenotype, particularly the exhibition of spontaneous contraction (first day of contraction and beating rate measured in at least three different zones of each well, including centre and periphery), and more quantitatively, the expression of cTnT, evaluated by flow cytometry, and the cardiomyocyte yield, calculated as follows:

$$\text{Cardiomyocyte yield} = \frac{(\text{Cells after differentiation}) \times (\%\ \text{of cTnT}^+\ \text{cells})}{(\text{Cells seeded for differentiation})} \quad (1)$$

Single-cell replating of cardiovascular progenitor cells. Cells at day 7 of differentiation were washed with PBS and singularised by incubation in either 500 µL/well of 0.25% of trypsin-EDTA (Gibco) for 5 min at room temperature or 1 mL/well of accutase (Sigma-Aldrich®) for 5 min at 37 °C. Enzymatic digestion was terminated by addition of washing medium (Dulbecco's modified Eagle's medium (DMEM)/F12 containing L-glutamine (Gibco) supplemented with 10% knockout serum replacement (KO-SR; Gibco), 1.0% minimum essential medium amino acids (Gibco) and 1.0% penicillin/streptomycin). Cells were collected in a Falcon tube, placed in a centrifuge (Hermle Labortechnik GmbH, model Z 400 K) and centrifuged for 3 min at 1,500×*g*. The supernatant was discarded, and cells were resuspended in RPMI/B27 medium. Cells were counted and plated at the desired density. Plated cells were kept in a CO_2 incubator. Replated cells were subjected to the remainder of the differentiation protocol.

Magnetic-activated cell sorting. For negative selection, cells were incubated during 1 h at 37 °C in medium supplemented with 10 µM of rho-associated coiled-coil protein kinase (ROCK) inhibitor Y-27632 (STEMCELL Technologies). Afterwards, cells were washed twice with PBS and singularised by incubation in 500 µL/well of 0.25% of trypsin-EDTA for 5 min at room temperature. Enzymatic digestion was terminated by addition of washing medium. Cells were collected in a Falcon tube and counted. According to the desired number of cells, a fraction of the cell suspension volume was collected in another tube. The cells to be separated were centrifuged for 3 min at 1,500×*g*. The supernatant was discarded, and the pellet was resuspended in 100 µL/2×10^6 cells of MACS buffer (0.5% human serum albumin (HSA; Octapharma®) and 2 mM EDTA (Gibco) in PBS), containing 10 µL/2×10^6 cells of phycoerythrin (PE)-conjugated anti-TRA-1-60 antibody (Miltenyi Biotec) and left to incubate for 10 min at 4 °C and in the dark. Cells were washed with 1 mL/2×10^6 cells of MACS buffer. The pellet was then resuspended in 100 µL/2×10^6 cells of MACS buffer containing 20 µL/2×10^6 cells of anti-PE microbeads (Miltenyi Biotec) and left to incubate for 20 min at 4 °C and in the dark. Cells were washed with 1 mL/2×10^6 cells of MACS buffer, and then resuspended in 500 µL of MACS buffer. An LS column (Miltenyi Biotec) was placed in a MidiMACS separator (Miltenyi Biotec) and rinsed with 3 mL of MACS buffer. The cell suspension was applied onto a pre-separation filter (30 µm mesh; Miltenyi Biotec), on top of the column, and the flow-through was collected. The column was washed three times with MACS buffer and unlabelled cells were collected. The column was removed from the separator and placed on a Falcon tube. A volume of 5 mL of buffer was pipetted onto the column, and labelled cells were flushed by pressurising the column with a plunger. Both the non-purified and the purified (unlabelled) fractions were split and cultured on Matrigel-coated 3.8 cm^2 tissue culture plates and subjected to the remainder of the differentiation protocol. For positive selection, cells were washed twice with PBS and singularised by incubation in 1 mL/well of accutase for 5 min at 37 °C. Enzymatic digestion was terminated by addition of washing medium. The protocol until the end of the separation was similar to that of negative selection, except the volumes used were per 10^7 cells, and the antibody used was PE-conjugated anti-SSEA-1 antibody (Miltenyi Biotec). The non-purified, non-purified left in MACS buffer during separation, purified (labelled) and undesired (unlabelled) fractions cultured on Matrigel-coated 3.8 cm^2 tissue culture plates and subjected to the remainder of the differentiation protocol. The purification performance was assessed by calculating the efficiency, efficacy and percentage of lost cells, as follows[25]:

$$\text{Efficiency} = 1 - \frac{\%\text{ contaminant cells in the purified fraction}}{\%\text{ contaminant cells in the non-purified fraction}} \quad (2)$$

$$\text{Efficacy} = \frac{\text{CVPCs in the purified fraction}}{\text{CVPCs in the non-purified fraction}} \quad (3)$$

$$\%\text{ of cells lost} = 1 - \frac{\text{Cells in eluate + Cells in flow-through}}{\text{Cells in the non-purified fraction}} \quad (4)$$

Lactate method. At day 13 of cardiac differentiation, RPMI/B27 medium was changed to RPMI 1640 medium without glucose (Gibco) containing 4 mM of sodium L-lactate (Sigma-Aldrich). At day 15, medium was changed either to RPMI without glucose and with lactate, or RPMI/B27, and at day 17, medium was again changed, to RPMI/B27. Cultures left for 2 or 4 days in lactate were sacrificed for flow cytometry analysis at day 18.

Flow cytometry. Cells in culture were washed with 1 mL/well of PBS and singularised by incubation for 5 min in 0.5 mL/well of 0.25% trypsin-EDTA. Trypsin-EDTA was inactivated by addition of washing medium. Cells were collected in a Falcon tube and centrifuged for 3 min at 1,500×*g*. The supernatant was discarded, and the cells were fixed by resuspension of the pellet in 1 mL of 2% paraformaldehyde (PFA; Sigma-Aldrich) in PBS. Cells were kept in PFA at 4 °C for a maximum of 2 weeks.

Flow cytometry after extracellular staining. Cells in PFA were centrifuged for 3 min at 1,500×*g*. The supernatant was discarded, and the pellet was washed with 2 mL of FACS buffer (4% foetal bovine serum (FBS; Sigma-Aldrich) in PBS). Cells were resuspended in 100 µL of FACS buffer containing primary antibody and left to incubate for 10 min at 4 °C, and, if the antibody was conjugated, in the dark. Following incubation, cells were washed twice with 2 mL of FACS buffer. If the primary antibody was not conjugated, cells were resuspended in 100 µL of FACS buffer containing the secondary antibody, left to incubate for 15 min in the dark and at 4 °C, and washed twice with 2 mL of FACS buffer following incubation. Cells were then resuspended in 300 µL of FACS buffer and transferred to FACS tubes (Falcon) prior to analysis in a FACSCalibur™ flow cytometer (BD Biosciences®). Results were treated with Flowing Software 2.0 (Turku Centre for Biotechnology).

Flow cytometry after intracellular staining. cTnT flow cytometry was performed as previously described[21]. Cells in PFA were centrifuged for 3 min at 1,500×*g*. The supernatant was discarded, and the pellet was resuspended in 1 mL of 90% cold methanol and left to incubate during 15 min at 4 °C. Following incubation, the suspension was centrifuged for 3 min at 1,500×*g*, the supernatant was discarded and cells were washed three times with 2 mL of flow cytometry buffer (FCB) 1 (0.5% bovine serum albumin (BSA; Sigma-Aldrich) in PBS). Cells were then resuspended in 100 µL of FCB2 (0.5% BSA and 0.1% Triton X-100 (Sigma-Aldrich) in PBS) containing 0.4 µL of mouse immunoglobulin (Ig) G anti-cTnT antibody (Thermo Fisher Scientific) and left to incubate for 1 h at room temperature. Following incubation, cells were washed twice with 2 mL of FCB2. Cells were then resuspended in 100 µL of the same buffer containing 0.1 µL of Alexa Fluor® 488-conjugated goat anti-mouse IgG secondary antibody (Invitrogen), and left to incubate for 30 min in the dark and at room temperature. Cells were then washed three times with 2 mL of FCB2, resuspended in 300 µL of FCB1 and transferred to FACS tubes prior to analysis in a FACSCalibur flow cytometer. For OCT4 flow cytometry, cells in PFA were centrifuged for 3 min at 1,500×*g*. The supernatant was discarded, and the cells were washed twice with 1 mL of 1% normal goat serum (NGS; Sigma-Aldrich) in PBS. Cells were resuspended in 500 µL of 3% NGS in PBS and distributed by Eppendorf tubes, previously coated with 1% BSA in PBS during 15 min. At this point, half of the cells were taken from representative samples to function as negative controls. Cells were then incubated in 150 µL of 3% NGS and 150 µL of 1% saponin (Sigma-Aldrich) during 15 min at room temperature. The Eppendorf tubes were placed in a centrifuge (Hermle Labortechnik GmbH, model Z 300 K) and centrifuged for 3 min at 1,250×*g*. Afterwards, cells were resuspended in 300 µL of 3% NGS and left to incubate for 15 min at room temperature. Following centrifugation for 3 min at 1,250×*g*, the pellet was resuspended in 300 µL of 3% NGS containing 1 µL of mouse IgG1 anti-OCT4 antibody (Merck Millipore), and left to incubate for 90 min at room temperature. Cells were washed twice with 500 µL of 1% NGS, resuspended in 300 µL of the same solution containing 1 µL of goat Alexa Fluor® 488-conjugated goat anti-mouse IgG secondary antibody and left to incubate for 45 min at room temperature and in the dark. Cells were washed twice with 1% NGS, resuspended in 300 µL of PBS and transferred to FACS tubes prior to analysis in a FACSCalibur flow cytometer. In both cases, results were treated with Flowing Software 2.0.

Quantitative real-time polymerase chain reaction. Total ribonucleic acid (RNA) was extracted from cells using the PureLink™ RNA Mini Link (Ambion®). RNA amount for each sample was quantified using a NanoVue™ Plus spectrophotometer (GE Healthcare®). Complementary deoxyribonucleic acid (cDNA) was synthesised from 2 µg of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems®). cDNA samples were placed in 48-well plates, along with TaqMan® Gene Expression Master Mix (Applied Biosystems) and the TaqMan probes for each gene (Applied Biosystems). The samples were processed in a StepOne™ Real Time PCR System (Applied Biosystems), the annealing temperature was set to 60 °C and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as an endogenous control. Results were analysed with StepOne Software (Applied Biosystems). Threshold cycles (C_T) for each sample were compared to the endogenous control, resulting in ΔC_T, and ΔC_T values were normalised against the C_T values for day 0, resulting in $\Delta\Delta C_T$. Final results for gene expression were presented as $2^{-\Delta\Delta CT}$.

Immunocytochemistry after intracellular staining. Cells were washed with 1 mL/well of PBS and fixed by incubation in 500 µL/well of 4% PFA in PBS for 20 min at room temperature. Cells were then left in 1 mL/well of PBS for a maximum of 2 weeks at 4 °C. Cells were washed twice with 1 mL/well of PBS blocked by incubation in 400 µL/well of blocking solution (10% NGS and 0.1% Triton X-100 in PBS) during 60 min at room temperature. Cells were then left to incubate for 120 min at room temperature in staining solution (5% NGS and 0.1% Triton X-100 in PBS) containing primary antibody. Following incubation, cells were washed twice with 1 mL/well of PBS and incubated for 60 min at room temperature and in the dark in staining solution containing the corresponding secondary antibody. After being washed 3 times with 1 mL/well of PBS, cells were incubated in 400 µL/well of 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich), diluted 1:100,000 in sodium bicarbonate, for 5 min at room temperature and in the dark. Finally, cells were washed 3 times in 1 mL/well of PBS and observed through a fluorescence microscope (Leica Microsystems CMS GmbH, model DMI3000 B). Still frame images were captured using a digital camera (Nikon, model DXM1200F) and treated with Photoshop Elements 8 (Adobe).

Antibodies. Primary antibodies used for flow cytometry and immunocytochemistry included anti-cTnT (Thermo Fisher Scientific), anti-OCT4 (Merck Millipore); anti-NKX2-5 (Santa Cruz Biotechnology); anti-TRA-1-60 (Stemgent); anti-TRA-1-60-PE, anti-SSEA-1-PE (Miltenyi Biotec), and anti-c-KIT (BioLegend). Secondary antibodies included goat-anti-mouse IgG, goat anti-rabbit IgG and goat-anti-mouse IgM (Invitrogen). Isotype controls included, besides the listed secondary antibodies, recombinant engineered antibody (REA) control (S) (Miltenyi Biotec) and mouse IgG1-PE (BioLegend).

RESULTS AND DISCUSSION

Differentiation of human induced pluripotent stem cells to cardiomyocytes. Human 6-9-9 iPSCs were differentiated through temporal modulation of the WNT signalling pathway, following the protocol optimised in the laboratory for the cell line in question[20,21,24].

The results obtained for the four independent cardiac differentiation experiments conducted are summarised in Table 1.

Table 1 – Summary of the results obtained for four independent cardiac differentiation experiments. All samples were collected at day 15, except for those experiment 3 which were collected at day 24. The first day of spontaneous contraction, the beating rate of cells throughout the wells (measured in at least three different points, including centre and periphery) the percentage of cTnT$^+$ cells as evaluated through flow cytometry and the cardiomyocyte (CM) yield, calculated by Equation 1, are indicated.

Experiment	First day of contraction	Beating rate (bpm)	cTnT$^+$ cells (%)	CM yield (CM/ /iPSC)
1	12	20-30	62.1	3.1
2	–	–	0.14	0.0
3	10	15-35	33.3	3.0
4	11	10-25	53.7	3.1

As it can be observed, experiment 2 did not result in cardiomyocytes, and experiment 3 resulted in a low percentage of cTnT$^+$ cells compared to experiments 1 and 4, even after more days of differentiation. The lower performance of these experiments can be attributed to some spontaneous differentiation of the iPSCs during their routine expansion, which biased them towards a specific lineage, and thus compromised their ability to differentiate into cardiac cells. It is also important to note that cells in experiment 3 had to be seeded at 25% less density than in all other experiments in order to have enough conditions to test the replating process. In experiments which generated cTnT$^+$ cells, the cardiomyocyte yield observed varied less than 4%, indicating some reproducibility of this protocol. Spontaneous contraction was first observed between day 10 and 12 of differentiation. The beating rate of differentiated cells was variable, ranging from 10 to 35 beatings per minute (bpm) in all the experiments. Adult cardiomyocytes generally feature a beating rate between 60 and 90 bpm[27], thus, after 15 days, this protocol generated immature cardiomyocytes, which would require more time in culture or an external stimulus to achieve values similar to adult cells. Another sign of immaturity was the erratic beating of cTnT$^+$ cells, as they sometimes ceased contracting for small periods of time (up to 10 s), before beating again. Despite their immaturity, cardiomyocytes were able to organise into tissues and beat synchronously.

In all experiments, the percentage of cTnT$^+$ cells and the cardiomyocyte yield obtained at day 15 was lower than the average obtained in other experiments with the same cell line and experimental setup in the laboratory (73% and 3.7 cardiomyocytes/iPSC, respectively). The efficiency of the protocol has been shown to depend greatly on factors such the seeding of iPSCs and on the cell confluence when it is started[24]. The protocol would sometimes have to be started before 90% confluence to avoid differentiation by localised confluence. This may then have caused the differentiation efficiencies to be below average, or it may have delayed differentiation, since, as indicated in the flow cytometry characterisation, at day 18, experiment 4 had resulted in 71.6% of cTnT$^+$ cells and a cardiomyocyte yield of 4.1 cardiomyocytes/iPSC. The original protocol[20] claims that values of 87% of cTnT$^+$ cells by day 30 may be achieved, thus, prolonging this differentiation protocol could potentially increase the amount of these cells in culture.

Figure 1 depicts bright field microscopy and immunostaining images of cardiomyocytes following differentiation. Bright field still frames (Figure 1A) reveal the tissue-like organisation of differentiated cardiomyocytes, which can contract synchronously. cTnT staining (Figure 1B) reveals the expression of this marker by cells across the wells, and shows the sarcomeric organisation of cardiomyocytes, although it is disorganised as these cells are still immature. Immunostaining also revealed expression of late cardiac marker NKX2-5 (Figure 1B) by cardiomyocytes. This marker is expressed throughout the life of these cells, and seems to be important for cardiomyocyte growth and proliferation, by activation of structural genes which lead to the organisation observed with cTnT immunostaining[15,28].

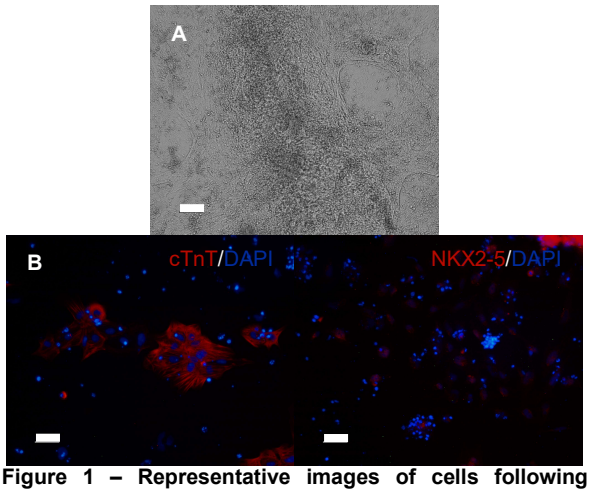

Figure 1 – Representative images of cells following cardiac differentiation. (*A*) Bright field image of cardiomyocytes at day 15 of differentiation (Scale bar – 100 µm). (*B*) Cells were maintained in culture until at least day 15, when they were replated, left to grow for 2 days in RPMI/B27 medium, fixed, and stained for expression of cTnT (*left*) and NKX2-5 (*right*). Total cells were stained with DAPI, and the images obtained with immuno- and DAPI staining were merged together (Scale bars – 50 µm).

Quantitative real-time polymerase chain reaction characterisation. Expression of various genes corresponding to different phases of cardiac commitment of cells were assayed at various time points throughout differentiation by quantitative real-time polymerase chain reaction (qPCR). Figure 2A depicts the relative expression profiles of these genes during cardiac differentiation. Along the differentiation protocol, expression of pluripotency genes *OCT4* and *NANOG* gradually decreased, which is coherent with the loss of naïveté and cardiac commitment. Expression

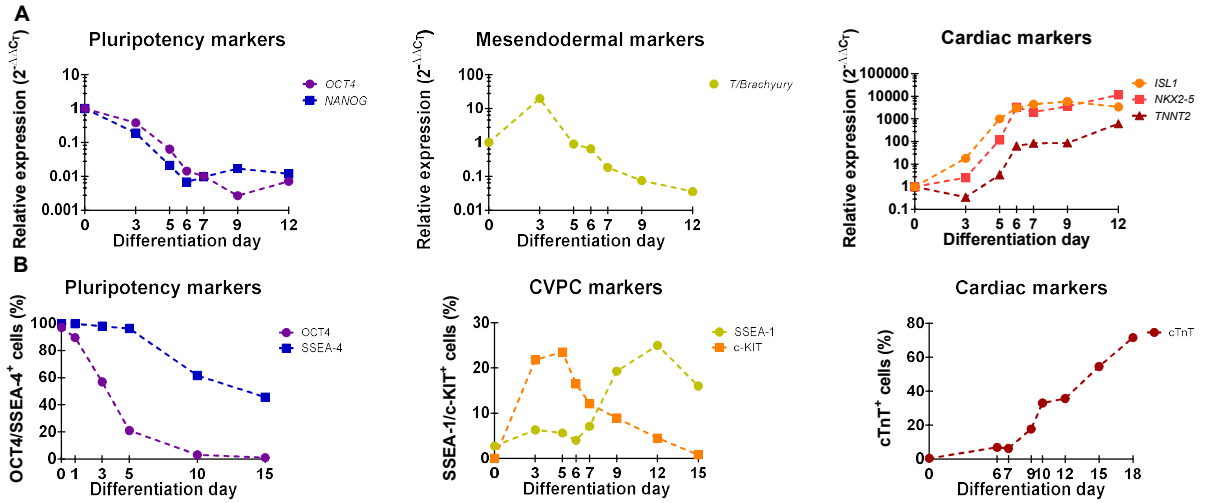

Figure 2 – Expression profiles of pluripotency, mesendodermal and cardiac markers during cardiac differentiation of induced pluripotent stem cells. Samples were collected at various time points of cardiac differentiation for assay of relative gene expression via qPCR (*A*), and for assay of absolute surface and intracellular marker expression via flow cytometry (*B*). qPCR values of gene expression were normalised against expression at day 0 and expression of housekeeping gene GAPDH.

of mesendodermal gene *T/Brachyury* peaked at day 3 of the protocol, when IWP4 was added to the medium, and then gradually decreased until the end of the differentiation protocol. IWP4 was added to the medium when the cells were in an early mesodermal state, thus, at the peak of *T/Brachyury* expression, to inhibit the canonical WNT signalling pathway and direct cells towards a cardiac lineage. At this point, *T/Brachyury* expression started diminishing and did so until the end of the differentiation protocol. Expression of CVPC gene LIM-homeobox transcription factor islet (*ISL*) 1 and late cardiac gene *NKX2-5* gradually increased until day 6-7, reaching a plateau, indicating the peak of CVPCs. At this point, insulin, which would inhibit cardiac differentiation at earlier stages, could be safely added to the medium to protect cells from apoptosis and stimulate proliferation[21,29]. Cardiomyocyte gene troponin T (*TNNT*) 2 showed gradually increased expression over time, starting from day 3. As already mentioned, this was the day when IWP4 was added to the medium, and cells started to become committed to a cardiac lineage. From this day until day 12, the increase in the expression of this gene appears to follow an exponential trend.

Flow cytometry characterisation. Expression of various surface and intracellular markers corresponding to different phases of cardiac commitment of cells were assayed at various time points throughout differentiation by flow cytometry. Figure 2B depicts the absolute expression profiles of these genes during cardiac differentiation. Corroborating the results obtained by qPCR, the expression of pluripotency transcription factor OCT4 reduced over time, labelling 97.0% of cells at day 0, decreasing rapidly over the first 5 days of differentiation and being almost completely depleted starting from day 10 of the protocol. SSEA-4 is also a pluripotency marker, such as OCT4. At the start of the protocol, 99.6% of cells expressed this surface marker, and its expression gradually decreased. Unlike OCT4, however, SSEA-4 still occurred in 45.6% of fully differentiated cells, as it also labels early human progenitor cells, such as CVPCs[30]. Haematopoietic and CVPC marker c-KIT expression increased from 0% at the start of differentiation to a maximum of 23.5% by day 5, gradually decreasing until 0.8% at day 15. c-KIT was, thus, transient, peaking at the appearance of CVPCs and then decreasing as these progenitors develop into cardiomyocytes[31]. SSEA-1, which is a marker for general cellular differentiation, featured a gradual increase in expression from 2.7% at day 0, until a maximum of 25.0% at day 12, decreasing to 16.0% at day 15. SSEA-1 has already been suggested as a marker for CVPCs[1,5,16,17], but in this case, its peak does not correlate with the expected appearance of these progenitors (around day 5, as indicated by qPCR results of *ISL1* and *NKX2-5*). SSEA-1 expression peaking so late into cardiac development would suggest it to be a transient marker, expressed somewhere between the CVPC and the cardiomyocyte state. Cardiomyocyte marker cTnT increased over time during the differentiation, starting at 0.4% at day 0 of expression, and peaking at 71.6% at day 18. Expression of cTnT seems to correlate reasonably well with expression of its gene *TNNT2*, apparently increasing exponentially between days 6 and 10. Afterwards, and until day 18, the increase appears to be linear and more subdued.

Optimisation of the replating process of cardiovascular progenitor cells. The replating process of day 7 CVPCs was optimised as a means of improving their survival and providing the ideal conditions for reorganisation into tissue-like structures following single-cell dissociation and MACS. Cells were dissociated into single cell suspensions using trypsin or accutase, and replated onto new Matrigel-coated 3.8 cm^2 tissue culture plates, at a density of 750,000 or 1,000,000 cells/cm^2. Since cells had to be dissociated into single cells, they required some time before reorganising into tissue-like structures which were able to contract spontaneously. As such, cells were allowed to differentiate for 24 days before analysis. Figure 3 contains the results for flow cytometry analysis of $cTnT^+$ cells in all replating conditions and in the non-replated control. In order to generate the maximum amount of $cTnT^+$ cells, trypsin should be used, and cells should be replated at a density of 750,000 cells/cm^2. However, given that the difference in $cTnT^+$ cells between dissociation with trypsin and accutase is less than 2%, accutase was deemed better for causing less stress on

the cells, and for not being of animal origin. Thus, for the MACS positive selection experiment, cells were dissociated using accutase and replated at a density of 750,000 cells/cm^2.

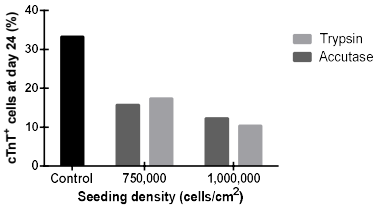

Figure 3 – Effect of enzyme used for dissociation and replating density on expression of cTnT by differentiated cells. Cells at day 7 of cardiac differentiation were dissociated with trypsin or accutase, and replated at densities of 750,000 or 1,000,000 cells/cm^2. Cells were collected at day 24 for flow cytometry analysis. Control represents non-replated cells at the same day of differentiation.

Overall, the replating process had a negative effect on cTnT$^+$ cells, generating at most half of these cells when compared to the control. Given the low number of cells in all conditions when compared to the control, CVPCs probably died upon replating, and likely due to the stress conveyed by enzymatic dissociation and the lack of pro-survival cues when seeded onto new wells. Vascular endothelial growth factor (VEGF) promotes proliferation of these cells, and could have been added to the culture medium[24]. Replated cells did not form the complex tissue-like structures of non-replated cells, even after 24 days of differentiation. Instead, replated cells mostly formed beating tri-dimensional aggregates, mostly gathered throughout the periphery of the wells. The size of the aggregates increased with the increase in replating density. The centre of the wells contained singularised cells, which were stretched and appeared to be more attached to Matrigel, and rarely contracted. Replated cardiomyocytes still presented sarcomeric structures, but more deformed than those of Figure 1.

Although these conclusions were used as a mould to plan the MACS protocol, it is important to note that the starting population (experiment 3 in Table 1) had a low amount of cTnT$^+$ cells compared to what is expected from this protocol. In fact, when replated cells were used as a control for MACS in the optimised conditions, the non-replated population contained 71.6% cTnT$^+$ cells, whereas the replated population contained 72.3% of these cells. Thus, the low efficiency of the differentiation protocol may have caused the CVPCs to be less robust or to be present in smaller quantities, and thus unable to withstand the stress of replating.

Negative immunomagnetic selection. Negative immunomagnetic selection with anti-TRA-1-60 antibody was used to deplete non-differentiated iPSCs from culture. Figure 4A showcases the results of negative immunomagnetic selection of CVPCs undergoing differentiation. The percentage of TRA-1-60$^+$ cells was reduced to a third, from 0.9% to 0.3%, while the eluted fraction contained 25.8%. This selection is likely primed towards recovery of iPSCs from populations following reprogramming, which usually contain less than 2% of these cells[32]. The efficiency (removal of impurities) and efficacy (recovery of CVPCs) of the experiment are given generically by Equations 2 and 3, where the purified fraction is the flow-through. The efficiency and efficacy of this immunomagnetic separation were 67.8% and 86.3%, respectively. Comparing these results to the model determined in the laboratory for negative selection of iPSCs undergoing neural differentiation, the efficiency is lower, whereas the efficacy is slightly higher than the expected (about 85% and 75%, respectively). Equation 4 places the percentage of lost cells at 12.6%, which is lower than the model for neural differentiation[25].

This MACS protocol did not significantly affect the viability of cells, which was nearly 100% both before and after immunomagnetic separation. The high cell viability, which is corroborated by the dot plot for the flow-through (data not shown), can be a proof of higher robustness of CVPCs when compared to iPSCs. However, the dot plot for the eluate reveals a much higher amount of seemingly dead cells, probably due to the stress of the immunomagnetic separation.

Given the low percentage of TRA-1-60$^+$ cells present in culture even prior to immunomagnetic separation, iPSCs are probably not the most substantial impurity which require depletion. However, Figure 2B indicates the presence of more iPSCs in culture at day 7 based on the expression of OCT4. Considering CVPCs do not express OCT4, either the TRA-1-60 antibody was unable to label iPSCs properly, or this experiment in particular generated less iPSCs than the characterisation experiment. The latter may be the case, as this experiment (experiment 2 in Table 1) was unable to generate cardiomyocytes at day 15, indicating there were probably many spontaneously differentiated colonies prior to the cardiac differentiation protocol, and thus, less iPSCs than those to be expected of a regular cardiac differentiation protocol.

Following MACS, purified and non-purified cells were replated at a density of 250,000 cells/cm^2 and subjected to the remainder of the differentiation protocol. This experiment was conducted before the optimisation of the replating protocol, thus, the replating density for differentiating cells was below the density found to be optimal. Cells plated after MACS following 15 days of differentiation were mostly isolated due to the low replating density and substantial cell death, and very adherent to Matrigel. This morphology is very distinct from non-replated cells at the same day (Figure 1A), which are organised in tissue-like structures. Figure 4B showcases the effect on cTnT expression of the immunomagnetic negative selection protocol. Cells which were replated without immunomagnetic purification died, and thus, cTnT$^+$ cells in this fraction could not be quantified. Flow cytometry evidences that the small decrease in TRA-1-60$^+$ cells caused an increase in cTnT$^+$ cells at day 15 of differentiation of 58%. This significant increase can have occurred due to the death of contaminating cells due to the stress of both the immunomagnetic separation and the replating at a low density, causing only CVPCs to survive and proliferate. Despite the flow-through fraction having seemingly no contractile cells, it may have been due to their inability to form colonies. It is important to note that the flow-through fraction contained a very small number of cells after 15 days of differentiation, which resulted in merely 590 gated events in the flow cytometry analysis. Since at least 10,000 gated events are required to label

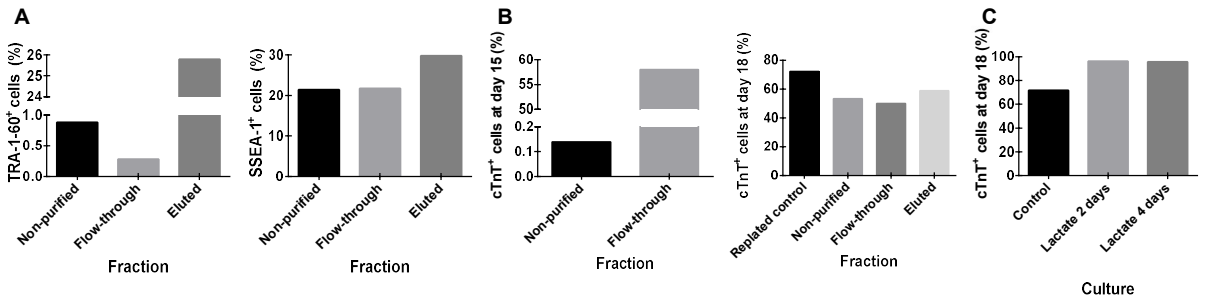

Figure 4 – Results of purification of cardiovascular progenitor cells through MACS and the lactate method. Cells at day 7 of cardiac differentiation were dissociated with trypsin or accutase and depleted of iPSCs by negative immunomagnetic selection based on the expression of the TRA-1-60 surface marker (*A, left*) or positive immunomagnetic selection based on the expression of the SSEA-1 surface marker (*A, right*). These cells were collected and replated, and analysed for expression of cTnT at day 15 for negative selection (*B, left*) or 18, for positive selection (*B, right*). In the latter the non-purified fraction represents cells replated after being left in MACS buffer for the duration of the MACS protocol. Cells were also purified at day 13 by exposure to lactate for 2 and 4 days, and collected at day 18 for analysis of expression of cTnT (*C*).

the analysis as statistically significant, the percentage of $cTnT^+$ cells in the flow-through cannot be fully trusted.

The negative immunomagnetic selection did not generate meaningful conclusions. The differentiation protocol was ineffective, and thus, the population at day 7 cannot be said to replicate the condition that would be expected of regular differentiating cells. Additionally, the replating caused the death of a large number of cells, and thus was optimised for further experiments, as described previously. The negative immunomagnetic selection could not be replicated due to time constraints; although OCT4 expression at day 7 (Figure 2B) indicates some promise in this experimental setup, positive selection using CVPC markers was deemed more important, to test the effect of that type of selection on the end products of differentiation.

Positive immunomagnetic selection. Given the unsatisfactory results of negative immunomagnetic selection, a positive selection was performed. In this case, CVPCs were targeted based on the expression of SSEA-1, based on an experiment with another differentiation protocol[5,16]. Figure 4A showcases the results of positive immunomagnetic selection of CVPCs undergoing differentiation. The percentage of $SSEA\text{-}1^+$ cells increased from 21.4% to 29.8%, whereas it did not change in the flow-through. Given that the percentage of $SSEA\text{-}1^+$ cells in the flow-through is the same as in the non-purified fraction, it was not possible to recover many of these cells. The performance of MACS is widely variable even amongst similar conditions[25], thus, this experiment would require repetition in order to assess an average performance of the positive immunomagnetic separation of CVPCs. Additionally, the MACS products, such as the microbeads and the columns, are proprietary products, generalised for all applications, but they could potentially be optimised for positive selection of CVPCs in order to attempt to increase the performance of the protocol. The efficiency and efficacy of the experiment are given generically by Equations 2 and 3, where the purified fraction is the eluate. The efficiency and the efficacy of this immunomagnetic separation were 10.7% and 11.6%, respectively, which may be due to the capacity of the column having been exceeded. Equation 4 indicates a loss of 58.3% of cells. Regarding these parameters, this immunomagnetic separation setup was the worst, considering many impurities still remained in the purified fraction, and many cells were lost in the column.

Direct counting of cells did not reveal significant variations on cell viability, which was above 90% both before and after immunomagnetic separation. On the other hand, the dot plot for the eluate (data not shown) reveals there was some negative effect of the retention in the column, as there is a higher number of seemingly dead cells, and the dot plot for the flow-through contains less of this type of cells, as this fraction is immediately washed off the column after loading, and may thus be subjected to less stress.

Following MACS, cells from the eluate and the flow-through were replated and subjected to the remainder of the differentiation protocol. Cells from the flow-through were replated at a density of 750,000 cells/cm^2, but the purified fraction had to be replated at a density of 300,000 cells/cm^2, due to a lack of cells. To function as control, henceforth termed "replated control", cells undergoing differentiation were replated at a density of 750,000 cells/cm^2 in the same day as the immunomagnetic separation. Additionally, some cells, henceforth termed "non-purified fraction", were replated, also at a density of 750,000 cells/cm^2, after being left in MACS buffer during the duration of the immunomagnetic separation. All fractions were allowed to grow until day 18 to allow them to reform a tissue-like structure. Replated cells were able to form tissue-like structures which featured spontaneous contraction at a rate of 40-50 bpm, these being more prevalent in the periphery of the wells. In the centre of the wells, cells were more adherent and more isolated. The non-purified fraction also formed tissue-like structures, but they were much smaller, which indicates the MACS buffer alone induces some stress in the cells. These structures had a high beating rate, of about 30-40 bpm. The flow-through behaved similarly to the non-purified fraction, forming cardiac tissues which contracted at about 15-20 bpm; whereas the eluate could not form neither tissue-like structures or aggregates, nor had any apparent beating cell. Besides having been in MACS buffer, these cells may have been compromised due to being plated at 40% the optimal density, due to having been retained in the column, and due to having been labelled with magnetic particles. Although the cell density was not the optimal, these results reveal that positive selection has a negative effect on cells. Thus, this setup still requires considerable optimisation in order to minimise cell stress and to be able to generate viable cardiomyocytes. Figure 4B showcases the effect on cTnT expression of the immunomagnetic positive

selection protocol. Flow cytometry analysis at day 18 of differentiation also reveals the MACS buffer to have had a negative effect on cells, as the non-purified fraction contained 19.0% less $cTnT^+$ cells when compared to the replated control, thus, it would be preferable to run the immunomagnetic separation with medium suitable for cell culture. The flow-through contained 3.2% less $cTnT^+$ cells than the non-purified fraction, whereas the eluted fraction resulted in 5.6% more $cTnT^+$ cells. Although the variations are moderate, they suggest the purification to have had a positive effect on the differentiation protocol. However, the purified cell population, as revealed in the respective dot plot (data not shown), contained a very small amount of viable cells, as it is majorly comprised of seemingly dead cells. For this reason, the analysis for this fraction only contains 253 gated events, which is not statistically significant, thus not allowing for a strong conclusion regarding this experiment. Additionally, despite apparently having more $cTnT^+$ cells than the non-purified fraction, the eluate still falls short of the replated control, by over 13%, suggesting it is preferable to skip the purification step.

The positive immunomagnetic selection, much like the negative selection experiment, did not generate strong conclusions. Selection of CVPCs by SSEA-1 targeting is promising, but cells become too damaged in the process. Thus, this protocol requires a great amount of optimisation, for instance, replacement of MACS buffer by cell culture medium, in order to attempt to generate viable cells, or supplementation of the culture medium with VEGF to improve CVPC proliferation. Additionally, flow cytometry characterisation (Figure 2B) reveals the peak of SSEA-1 expression to be achieved at day 12, thus, this marker may not be the ideal to target CVPCs generated by this differentiation protocol, which, according to *ISL1* and *NKX2-5* expression (Figure 2A), start appearing at day 5. It should be noted that the expression of SSEA-1 obtained in this experiment (21.4%) and in the characterisation experiment (7.1%) were significantly different, but that can be attributed to different sample processing, namely dissociation (accutase and trypsin, respectively) and labelling conditions (live and fixed cells, respectively). c-KIT, whose expression peaks at day 5, could be a suitable marker for CVPC purification, but more immunomagnetic separation experiments would have to be conducted in order to compare the viability of both markers and observe the cell populations they generate.

Lactate method. In order to compare the immunomagnetic selection results with those of a more widespread method, the lactate method was employed. Purified and non-purified cells were able to form tissue-like structures throughout the wells, which featured spontaneous contraction. Non-purified cells contracted at anywhere between 15-50 bpm. Cells subjected to lactate exposure during 4 days would contract generally at a rate of 10-20 bpm, suggesting the prolonged exposure to lactate may have had some inhibitory effect on the cells. Cells which were only 2 days in lactate had widely variable beating rates, from a minimum of 10 bpm to a maximum of 90 bpm. The short exposure to lactate may potentially have matured some cardiomyocyte sub-populations, and caused them to achieve a beating rate which would be expected of adult cells, but this would require confirmation by myosin light chain (MLC) flow cytometry[20]. These cells, in particular, showed more widespread beating across the plate, suggesting a more efficient purification.

Figure 4C showcases the effects on cTnT expression of the lactate method. Flow cytometry reveals this method to have had a very positive effect on cardiomyocytes, improving the percentage of $cTnT^+$ cells from 71.6% to 96.1% (2 days) or 95.5% (4 days). These populations are practically devoid of contaminant cells, and are extremely viable, thus overcoming the limitations of the MACS. It is important to note, though, that the dot plots for this analysis (data not shown) reveal metabolic selection during 4 days generated a larger amount of seemingly dead cells, which was also observed in culture, again indicating this prolonged exposure to lactate had some negative effect. It is possible that some cells detached from the plate, as some $cTnT^-$ cells which supported cardiomyocytes may have been depleted by this selection method, causing cardiac tissue detachment. Previous work in the laboratory[24] describes 2 days as being generally the maximum time of exposure to lactate which avoids cardiomyocyte detachment from Matrigel.

Overall, the lactate method was the separation technique which generated better results, with almost totally pure, viable $cTnT^+$ cell populations. Metabolic selection during 2 days proved to be better than exposure for 4 days, generating more beating tissue-like structures, with higher beating rates, and causing less cell death.

CONCLUSIONS AND FUTURE WORK

Ever since their inception, PSCs have held a great promise in the world of Regenerative Medicine, as a potentially unlimited source of cells to replace those lost through ageing and disease, and also in *in vitro* applications such as drug screening and disease modelling. iPSCs, in particular, can be reprogrammed from adult somatic cells, which avoids some ethical concerns and can overcome immune rejection in the host. The heart is one of the most important targets for iPSC therapies, as CVDs are extremely prevalent throughout the world, and the heart lacks the capacity to regenerate substantially through adulthood and to recover cells lost as a consequence of these diseases. Cardiac differentiation protocols are varied in their execution, but rarely originate cells with the required purity for transplantation. Additionally, these protocols are usually primed to obtain cardiomyocytes, while CVPCs, due to their ability to generate the three types of cells in the heart, have a greater potential for heart regeneration[1,16].

This work aimed to characterise cells undergoing chemically-defined, monolayer, growth factor-free differentiation via temporal modulation of the WNT signalling pathway, which is one of the most efficient differentiation protocols in terms of cardiomyocyte purity, and to purify them through MACS, for both positive and negative selection, due to the efficiency of this method and the possibility to use it for clinical purposes, as it has been approved by the Food and Drug Administration[33]. The main novelty of this work is precisely the usage of MACS as a means of purifying CVPCs obtained from this specific protocol.

The differentiation protocol was heterogeneous in its potential to generate $cTnT^+$ cardiomyocytes derived from the human iPSC line 6-9-9, likely due to spontaneous differentiation in these cells during routine maintenance and to the variable confluence at which differentiation was started, although the cardiomyocyte yield was very similar in all experiments which generated cardiomyocytes. Differentiated cells formed tissue-like structures which featured spontaneous contraction. Cell beating rates were heterogeneous throughout the wells, but were below 35 bpm, corroborating the immaturity of these cells.

Temporal characterisation of differentiating cells revealed the gradual decrease in expression of pluripotency genes *OCT4* and *NANOG* and markers OCT4 and SSEA-4, the peak of mesendodermal gene *T/Brachyury* by day 3, and the gradual increase in cardiac genes *ISL1*, *NKX2-5* and *TNNT2* and marker cTnT until the end of differentiation. c-KIT and SSEA-1 seemed to label different types of progenitors, due to peaking at days 5 and 12, respectively. In particular, c-KIT expression was deemed to be closer to that of CVPCs, while SSEA-1 appeared to mark an intermediate cell population between CVPCs and cardiomyocytes.

The ability of cells to reform their tissue-like structures upon replating was analysed, and the optimal density for replating, in terms of the $cTnT^+$ cell percentage after replating, was placed at 750,000 cells/cm^2, and accutase was selected as the enzyme for dissociation due to causing less cell stress. Replated cells formed spherical aggregates and featured about half the percentage of $cTnT^+$ cells when compared to the non-replated control. A subsequent replating experiment was able to maintain the characteristics of non-replated cells, including the percentage of $cTnT^+$ cells and the colony structure. Given the highly different populations prior to replating, the efficiency of this process was concluded to be dependent on the condition of the initial cell population.

MACS was used as a means of purifying CVPCs at an intermediate stage of differentiation. Negative selection using anti-TRA-1-60 antibodies was employed to deplete the cultures of undifferentiated iPSCs. The results of this selection were inconclusive, since the starting population was already very depleted of iPSCs and was unable to form cardiomyocytes, but the discarded fraction was markedly enriched in iPSCs, indicating some of these were depleted from the initial culture. Positive selection, despite altering the target cells, was deemed to be more suitable for CVPC purification, and was tested using anti-SSEA-1 antibodies. MACS was able to enrich the initial fraction from 21.4% to 29.8% $SSEA\text{-}1^+$ cells, but when purified cells were replated, they were unable to form the tissue-like structures of the starting population and featured less $cTnT^+$ cells at the end of differentiation when compared to the replated control. It was concluded that the MACS process, most notably the exposure to MACS buffer throughout the process, was a source of cell stress which requires optimisation. The lactate method resulted in a marked increase in $cTnT^+$ cells at the end of differentiation, generating over 95% pure cells, with the 2-day exposure generating more contractile cells, probably due to the 4-day exposure having depleted the culture of other cells, such as fibroblasts, which supported cardiomyocyte growth, causing their detachment. Despite the promising results of the lactate method, it cannot be used to purify CVPCs and takes some days to purify cell populations, unlike MACS which can be performed within a few hours.

Considering one of the initial goals of the project, obtaining a very pure population of cardiomyocytes at the end of differentiation, which could potentially have clinical usage, it is evident from the obtained results that there is still a long path to tread before obtaining clinical-grade and scale cells with this protocol.

One major shortcoming of this project as a whole was the lack of replicates, but these were impossible to perform due to time constraints. It was deemed more important to address as much as possible the initial goals of the project, in order to try to achieve the ideal purification condition, before attempting to replicate the results. Temporal characterisation of differentiating cells, in particular, required replicates to strengthen the conclusions, and required further analysis for other markers, namely surface markers signal regulatory protein α (SIRPA) and glial cell line-derived neurotrophic factor receptor α (GFRA) 2, which have been successfully applied in CVPC purification[34,35].

The most important subject to address in future MACS experiments is the stress cells are subjected to. The prolonged exposure to MACS buffer was found to be one such factor of cell stress, and should be replaced with culture medium in subsequent experiments. The separation itself was underwhelming, especially the positive selection experiment, which may be due to the quality of the reagents, namely the microbeads and the columns, which are proprietary products and thus may not be optimised for every potential application, and due to the usage of SSEA-1 to purify CVPCs at day 7, when it was found to peak at day 12. Different separation methods, such as affinity chromatography, or even label-free alternatives, are also interesting options for purification of CVPCs. CVPC survival after replating can be improved by medium supplementation with VEGF[24]. After optimisation of the purification protocol, it is important to assess the safety of purified cells, namely in regards to teratoma formation potential, through tests in immunodeficient mice.

It is also crucial to guarantee the scalability of both the differentiation and purification processes, as large numbers of cells are required for clinical purposes. For differentiation, the most promising alternatives are expansion as aggregates or microcarriers in spinner flasks or bioreactors, which would have to be tested and compared, in order to conclude which is the most efficient setup. For purification purposes, a clinical-scale MACS system, CliniMACS®, already exists[33].

The cardiac differentiation protocol via temporal modulation of the WNT signalling pathway requires animal-derived components, such as B-27 supplement. Additionally, in this work, Matrigel and mTeSR1 medium, which also contain animal components, were used. For clinical purposes, it is important to avoid the usage of such components. As described by Burridge and co-workers[36], and Lin and co-workers[37], this method of differentiation still works even if devoid of animal components, using Essential 8™ medium for cell

expansion on Synthemax, and replacing RPMI/B27 with RPMI 1640 supplemented with ascorbic acid and recombinant albumin, or Essential 8 supplemented with heparin for differentiation. As such, one of these xeno-free protocols should be used in future experiments, in order to minimise exposure of cells to undesired components.

In short, this work attempted to apply MACS to purify CVPCs, but such attempts were unsuccessful, especially when compared to the lactate method, which generated highly pure cultures. Immunomagnetic selection has potential, but it still requires much optimisation in order to generate pure, clinical-grade populations of proliferating CVPCs. The alterations suggested to the established protocol, if successful, will result in a clinical-scale, xeno-free protocol to obtain highly pure, non-tumorigenic populations of CVPCs with potential to generate cardiomyocytes, smooth muscle cells and endothelial cells. These CVPCs would, thus, be able to help restore the cardiac function following substantial cardiomyocyte death, potentially replacing traditional therapies which are as of yet still unable to respond to the prevalence of CVDs in the world.

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