Cardiomyocyte differentiation: Experience and observations from 2 laboratories

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INTRODUCTION

The affliction of cardiovascular disease (CVD) is escalating rapidly throughout the world, with the majority of sufferers residing in developing countries.[1] According to the World Health Organisation (WHO), CVD is the leading cause of death globally accounting for an estimated 31% of all deaths worldwide.[2] Cardiovascular disease also poses a threat to the African continent, with several studies showing the prominence of a variety of cardiovascular afflictions in the African context, including heart failure and systolic dysfunction,[3] ischaemic heart disease (IHD),[4] hypertension and hypertensive heart disease[5] with associated heart failure.[6] Available data from the Medical Research Council of South Africa suggests that the predominance and treatment of common heart conditions in the population such as heart failure, cardiac hypertrophy and diseases of the valves and heart muscle are to a large extent, poorly managed.[7] Duly, it has become a crucial goal for researchers to develop and implement new and improved treatment strategies to protect the heart against disease and injury.

In previous research to date, a number of in vitro cell based models have been used to investigate heart physiology and pathology, with the advantage that cell models provide an ethically acceptable research tool, enabling the targeting of specific cellular processes with relative consistency and reproducibility.[8] In 1976, Kimes and Brandt established an undifferentiated clonal cell line, H9c2, derived from embryonic

ABSTRACT

The undifferentiated clonal cell line, H9c2, derived from left ventricular rat heart tissue, has been extensively used in cardiovascular research. In the present study, 2 independent laboratories aimed to investigate the cells' capacity to differentiate into distinct cardiac-like cells. Undifferentiated H9c2 cells were supplemented daily for a period of 6 - 12 days, with varying concentrations of retinoic acid (RA) (10nM, 30nM and 1μM), in standard cell culture medium containing either 1% foetal bovine, or horse serum, in order to stimulate differentiation of the cells into a more cardiac-specific phenotype. Light microscopy confirmed some degree of morphological change associated with differentiation, and a significant increase in oxidative phosphorylation following RA treatment was observed. However, Western blot probing for the cardiac-specific markers Cardiac Troponin T (cTnT) and Myosin Light Chain-2v (MLC2v) indicated little to no differentiation, although immunocytochemistry indicated the presence of cTnT expression. Thus, it was found that the differentiation protocol induced differentiation in some, but not all cells, thereby generating a heterogeneous cell population. Our findings suggest that the H9c2 cell line may display some degree of resistance to differentiation. This should be kept in mind when considering to use this model for cardiovascular research. SAHeart 2017;14:96-107

left ventricular rat heart tissue.[9] While the cells do not beat and differ morphologically from isolated adult cardiomyocytes, similarities with primary cardiomyocytes have been reported, specifically in terms of the presence and dynamics of L-type calcium channels, a similar cell membrane lectin binding pattern and comparable G-protein composition associated with ligand stimulated intracellular signalling.[10,11] As such, the H9c2 cell line has been used extensively in research to study the mechanisms of cardiac hypertrophy,[12] oxidative stress,[13,14] ischaemia/reperfusion (I/R),[15,16] and cardiotoxicity.[17] These cells, however, also have the capacity to adopt features of differentiated skeletal or cardiac muscle upon long term culture with low serum levels,[9,18] and in the case of cardiac specific differentiation, daily treatment with retinoic acid (RA).[19,20]

It has previously been reported that H9c2 cells maintained at confluency for an extended period of time (7 - 10 days), in the presence of relatively low concentrations of serum (1%), as well

as daily supplementation with RA, exhibit features indicative of cardiac specific differentiation.[19,20] These include an increased expression of cardiac specific proteins such as Myosin Light chain-2v (MLC-2v) and Cardiac Troponin T (cTnT),[19,20] as well as a shift in metabolic preference from primarily glycolytic to oxidative metabolism.[20] Lopashuk, et al. (2010) identified that a switch from glycolysis to oxidative phosphorylation is a fundamental characteristic of differentiated cardiac cells, since cardiomyotubes prefer fatty acids to glucose as a source of fuel.[21]

It could be argued that when differentiated, the H9c2 cells would offer a more closely matched representation of adult cardiomyocytes than when undifferentiated. Menard, et al. (1999) stated that the H9c2 cells are easy to manipulate, and when differentiated, provide a suitable cardiac cellular model with a maintained cardiac phenotype for a range of investigations commonly challenging to perform on primary cardiomyocytes.[19] It is therefore tempting to pursue differentiation of these cells as a more relevant experimental model than the undifferentiated cardiomyoblast cell line.

This paper will describe the efforts of 2 laboratories, working independently from one another, to differentiate H9c2 cells. Both laboratories are affiliated with the University of Stellenbosch (South Africa). "Lab 1" is situated at the department of Physiological Sciences in the Faculty of Natural Sciences, while "Lab 2" is part of the division of Medical Physiology in the Faculty of Medicine and Health Sciences. Each laboratory independently investigated the capacity of the H9c2 cell line to differentiate into a more cardiac-specific cell line upon daily treatment with RA and/or reduced serum conditions as described by Pereira, et al. and Menard, et al. respectively.[19,20]

Lab 1 made use of bright field microscopy to investigate morphology, Western blotting to investigate the expression of the cardiac specific marker MLC-2v, and immunocytochemistry to assess the expression of a second cardiac specific marker, cTnT. Oxidative phosphorylation analysis was carried out to establish whether any changes in oxygen consumption had occurred following differentiation. Similarly, following daily RA treatment, Lab 2 attempted to confirm differentiation of the H9c2 cell line using Western blotting techniques to probe for the expression of the cardiac specific markers MLC and cTnT.

Although it was hypothesised that H9c2 cells would easily differentiate to offer a good representation of cardiac physiology, both laboratories generated data suggesting that the ability of these cells to fully differentiate is somewhat limited. This implies that the H9c2 cell line may not be as easy to manipulate as previously thought, and their use as a cardiac model in future research should be thoroughly assessed due to their apparent resistance to differentiation.

METHODS AND MATERIALS

Lab 1
Cell culture and general cell maintenance

An H9c2 (2-1) cell line, originally derived from embryonic left ventricular rat heart tissue through the method of selective serial passaging,[9] was purchased through Sigma Aldrich from the European Collection of Authenticated Cell Cultures (ECACC), Salisbury, UK. These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, 41965-039) supplemented with 10% foetal bovine serum (FBS) (Biocom Biotech, FBS-G1-12A) and 1% penicillin/streptomycin solution (ThermoFisher Scientific, 15140-122), at 37°C under 95% O_2 and 5% CO_2. H9c2 cells adhered well to the surface of culture dishes and no adhesion factor was necessary. Culture medium was refreshed every second day and the cells were maintained until they reached 70% confluency. Once confluent, culture medium was discarded and trypsin (Trypsin-EDTA, Gibco, 25200-012) was then used to lift the cells from the base of the culture plate. Cells were counted to determine the appropriate seeding densities for each experiment. Experiments were done on cells between passage 7 and 10.

Differentiation of H9c2 cells

H9c2 cardiomyoblast differentiation was induced according to a protocol described by Pereira, et al. (2011), in which serum was reduced to 1% and cells were supplemented daily with 10nM of RA (Sigma, R2625) for 11 days.[20] For comparison, some cells were maintained in either 10% FBS (control), or in 1% FBS for the same duration, but without RA.

Morphological assessment

Morphological changes associated with differentiation of the treated cells were observed using bright field microscopy on days 6 and 11 of RA treatment. As described by Kimes and Brandt (1976), undifferentiated H9c2 cells appear typically spindle shaped and mononucleated.[9] Upon differentiation however, the cells have been reported to appear as long and thin branched cardiomyotubes.[20]

Immunocytochemistry

H9c2 cells were seeded onto coverslips and grown and treated as previously described. At the end of the treatment protocol, culture medium was removed, cells were washed with ice cold PBS and fixed in cold 4% paraformaldehyde (Sigma, 158127) for 10 mins. Cells were washed in cold PBS, blocked in 1% BSA, permeated with 0.1% triton solution (Sigma, X-100) and incubated at 4°C with cardiac specific troponin T (cTnT) antibody (Abcam, 11C11) overnight. The cells on the coverslip were again washed with cold PBS and incubated with an Alexa Fluor anti-mouse secondary antibody (ThermoFisher Scientific, A11029) for 1 hour in the dark. This was followed by staining with Hoechst (ThermoFisher Scientific, H1399), where after

the slide was left to air dry. Once dry, the coverslip was mounted on a glass slide using Dako fluorescent mounting medium (Diagnostech, S302380) and images were acquired using a Nikon eclipse E400 fluorescent microscope equipped with a DMX1200 CCD Camera 200. Images were acquired using the Nikon plan Flour 10x and 20x objective lens.

Preparation of cell extracts

Cell extracts were prepared as follows: lysis buffer (pH 7.4) containing: Tris–HCl (50mM), NP-40 (1%), Na-Deoxycholate (0.25%), EDTA (1mM), NaF (1mM), SBTI (4mg/ml), PMSF (1mM), Benzamidine (1mM), leupeptin (1mg/ml) and Triton X-100 (1ml) was added to the cells and the cell solution was then transferred to new tubes. Cells were sonicated, centrifuged (8 000rpm at 4°C for 10 mins), and the supernatant transferred to new tubes. Protein concentrations were determined with the Bradford method,[22] while others were diluted in Laemmli buffer and stored for Western blot analysis.

Western Blot analysis

Protein separation was performed on a 12% stain free, fast cast gel (Bio-rad, 161-0184) which was prepared according to the manufacturer's instructions. The gel was run to completion at 180mV and then activated on the Chemi Doc™ XRS system (Bio-rad). The proteins were then transferred onto nitrocellulose membranes (Bio-rad, 170-4156) for 10 mins at 25V and 1.3A. Following transfer, the membrane was blocked for 2 hours in Tris-buffered saline and Tween-20 (TBST, pH7.6) containing 5% non-fat dry milk and incubated overnight at 4°C with the Myosin Light Chain (Cell Signaling, D511C) primary antibody solution. The membranes were incubated with the appropriate secondary antibody (Cell Signaling, 70745) at room temperature for 1 hour followed by washes with TBST. The Pierce ECL Western blotting substrate (Thermo Scientific, 32106) was used to detect protein bands using the ChemiDoc™ XRS+ System with Image Lab™ Software (Bio-Rad). The bands generated were expressed using the volume analysis tool and represented as a percentage (%) of the control sample. GAPDH (Cell Signaling, 1745) was used for the standardisation of protein loading.

Oxygraph analysis

The assessment of respiration rate is an ideal measure of the oxidative phosphorylation capacity in cells. The Hansatech oxygraph system was used to determine respiration rate in differentiated cells. Assessment was only performed at day 6 for this experiment. The oxygraph was calibrated beforehand according to the manufacturer's protocol. Following treatment, the cells were trypsinized and counted. Then 1×10^6 cells were transferred into the oxygraph respiration chamber containing Hedly's intracellular buffer. Saponin (1µM) (Sigma, 47036) was added to the cells to permeate them where after, mitochondrial complex 1 substrates, malate (5µM) (Sigma, M1000) and glutamate (10µM) (Sigma, G1251) and the mitochondrial com-

plex II substrate succinic acid (100µM) (Sigma, S3674) were added. Active respiration was induced by the addition of adenosine dinucleotide (ADP) (Sigma, 01905). Respiration rate was then measured for 60 mins. The average respiration rate during this period was determined and compared between groups. Carbonyl cyanide-p-trifluoro-methoxy-phenyl-hydra-zone (FCCP) (Sigma, C2920), a mitochondrial un-coupler was used to confirm that mitochondria were actively respiring at the end of the experiment.

Statistical analysis

All data are presented as the mean ± SEM. Comparisons between the different groups were performed by one-way ANOVA followed by Bonferroni's post hoc test conducted with the statistical programme GraphPad Prism, version 5.0 (GraphPad Inc.). A value of $p<0.05$ was considered statistically significant.

Lab 2

Cell culture and general cell maintenance

The H9c2 (2-1) cell line purchased from ECACC through Sigma Aldrich was maintained in a proliferative state through culture in high (4.5g/L) glucose DMEM (Lonza, BE12-604F) supplemented with 10% FBS (Sigma, F6178) and 1% penicillin/streptomycin (Sigma, P4333) at 37°C and 5% CO_2, as described above. No attachment factor was necessary.

Growth medium of the cells was changed every 2 - 3 days and the cells were split in a ratio of 1:3 once they reached 70 - 80% confluency. Warm phosphate buffered saline (PBS) was used for all washing steps. A combination of trypsinization and cell scraping was used to lift cells from the base of the culture plates for the purpose of cell division, or collecting cells for Western blotting. Centrifugation at 700rpm for 4 mins at 4°C was used when pelleting of cells was necessary. Cells between passage 18 and 25 were used for differentiation experiments.

Differentiation of H9c2 cells

Once the cells were 70 - 80% confluent, differentiation was initiated by switching to DMEM growth medium supplemented with either 1% FBS or 1% Horse Serum (HS) (Biochrome AG, S9135), as well as daily administration of RA (Sigma Aldrich, R2625). Several different concentrations of RA were tested (10nM, 30nM and 1µM). Initially distilled water (d.H_2O) and phosphate buffered saline (PBS) were used for the serial-dilution preparation of the RA, however, in subsequent protocol repeats, dimethylsulphoxide (DMSO) (Merck, 102931) was used as RA was found to be inadequately dissolved in the d.H_2O and PBS. DMSO alone was therefore also used as a vehicle control. Cells were cultured under these conditions for a period of 8 - 12 days with growth medium changed every second day. Control cultures received DMEM growth medium supplemented with 10% FBS, and medium was also changed every 2 - 3 days.

Western Blot analysis for markers of cardiac differentiation

Following treatment, cell lysates were prepared using a bullet blender homogeniser (Next Advance Bullet Blender 24) and lysis buffer (200mM Tris+ EGTA, 10mM EDTA, 1M NaCl, 1mM ß-glycerophosphate, 2.5mM tetra-Na-pirophosphate, 10mM Na3VO4, 10µg/ml Leupeptin, 10µg/ml Aprotinin, 1% Triton x-100 and 50µg/ml PMSF). Protein determination was carried out using the Bradford method.[22] Following 5 mins of boiling, lysates were stored at -80°C in Laemmli sample buffer. Cardiac tissue samples were also prepared via pulverising and homogenising frozen heart tissue in a lysis buffer solution. These lysates were used as positive controls for cardiac specific markers.

Depending on the protein yield, between 15 - 18µg of protein was loaded per sample and resolved according to the molecular weight on an SDS-polyacrylamide gel electrophoresis (SDS-PAGE) system, using a 12% acrylamide gel. This was followed by transfer of the proteins to an inert polyvinyl fluoride (PVDF) membrane via a wet tank-transfer or semi-wet TurboBlot system. Protein samples were fixed onto the membrane and 5% milk was used as a blocking agent to minimise non-specific binding of antibodies. The specific proteins of interest were selected by overnight incubation of membranes at 4°C with primary antibodies directed against cardiac specific markers, myosin-Light chain 2v (MLC2v) (Cell Signaling, 1297) and Cardiac Troponin T (cTnT) (Cell Signaling, 5593).[20] Membranes were then further incubated at room temperature with anti-rabbit secondary antibody (Cell Signaling, 7074), and visualised using a chemiluminescence interaction between the detection reagent and the anti-rabbit horseradish peroxidase conjugated secondary antibody. Proteins were detected using either a Chemi-Doc system in association with stain-free technology,[23] or exposure in a traditional dark room. Stain-free technology was used to confirm equal loading (supplementary data).

This procedure was repeated 3 times with between 2 - 4 cell plates per condition (10nM RA, 30nM RA, 1µM RA, control, vehicle control and positive heart sample controls).

RESULTS

Lab 1

Morphological assessment

Differentiation of H9c2 cells was induced through serum reduction and daily supplementation with RA for 11 days, as described above. Undifferentiated H9c2 cardiomyoblasts are typically mononucleated and spindle shaped,[9] whereas once differentiated, the cells have been reported to form long, thin, branched multinucleated cardiomyotubes.[20]

After 6 days of daily RA treatment and/or serum reduction, only very few cells had begun to display the anticipated cardiomyotube morphology. According to Yang and colleagues

(2014), immature cardiomyocytes present themselves in a circular (rounded) shape while mature cells are rod (elongated) shaped.[24] When compared to control cells (Figure 1E), no obvious differences were observed between the serum starved group (Figure 1A) and the serum starved plus RA treatment group (Figure 1B). Differentiation treatment was continued and by day 11 more cells appeared to have undergone differentiation in both groups when compared to control (Figures 1C and 1D). These cells presented with elongation and shrunken width indicative of differentiation (supplementary Figures 1 and 2).

Cardiac troponin T (cTnT) expression

Immunocytochemistry was used to assess the expression of cTnT indicative of differentiation. In addition, the number of nuclei per cell were also noted. Cells expressing cTnT, as well as presenting more than 2 nuclei, were regarded as differentiated cardiomyotubes. Figure 2A indicates that after 6 days, serum reduction alone did not result in substantial cTnT expression. However, Figure 2B shows that the combination of serum reduction with RA supplementation did increase the expression of cTnT, when compared to control cells (Figure 2E) after 6 days. By day 11, the expression of cTnT in the serum starved group (Figure 2C) had increased in comparison to day 6 (Figure 2A), with more cardiomyotubes containing multiple nuclei. Similarly, the combination of serum reduction and RA resulted in a greater expression of cTnT after 11 days (Figure 2D), and a greater capacity to induce the formation of multinucleated cardiomyotubes. Supplementary Figure 2 shows the morphology of these cells in conjunction with the fluorescent signal.

Myosin Light Chain 2V (MLC-2v) expression

The expression of a second cardiac specific marker, MLC, was investigated using Western blotting in an effort to further distinguish between myoblasts and differentiated cardiomyotubes. MLC has been used as an indicator of myoblast differentiation into cardiomyotubes.[25]

Figure 3 shows that the combination of serum starvation with RA supplementation significantly increased the expression of the marker (209.5 ± 7.4%, p<0.001) when compared to the serum only (118.4 ± 9.9%) and control (100.0 ± 9.8%) group on day 6. A substantial increase in the expression of MLC was further observed in both the serum only (236.9 ± 16.6%, p<0.001) and combination group (244.4 ± 9.2%, p<0.001) compared to the control by day 11 of treatment. Large differences were also observed between the reduced serum groups on days 6 and 11. The results obtained here suggest that under conditions of reduced serum only, the expression of cardiac specific markers takes much longer than when serum reduction is combined with RA supplementation. However, it was also noted that RA appears to lose its effect on the expression of MLC when the treatment period is extended.
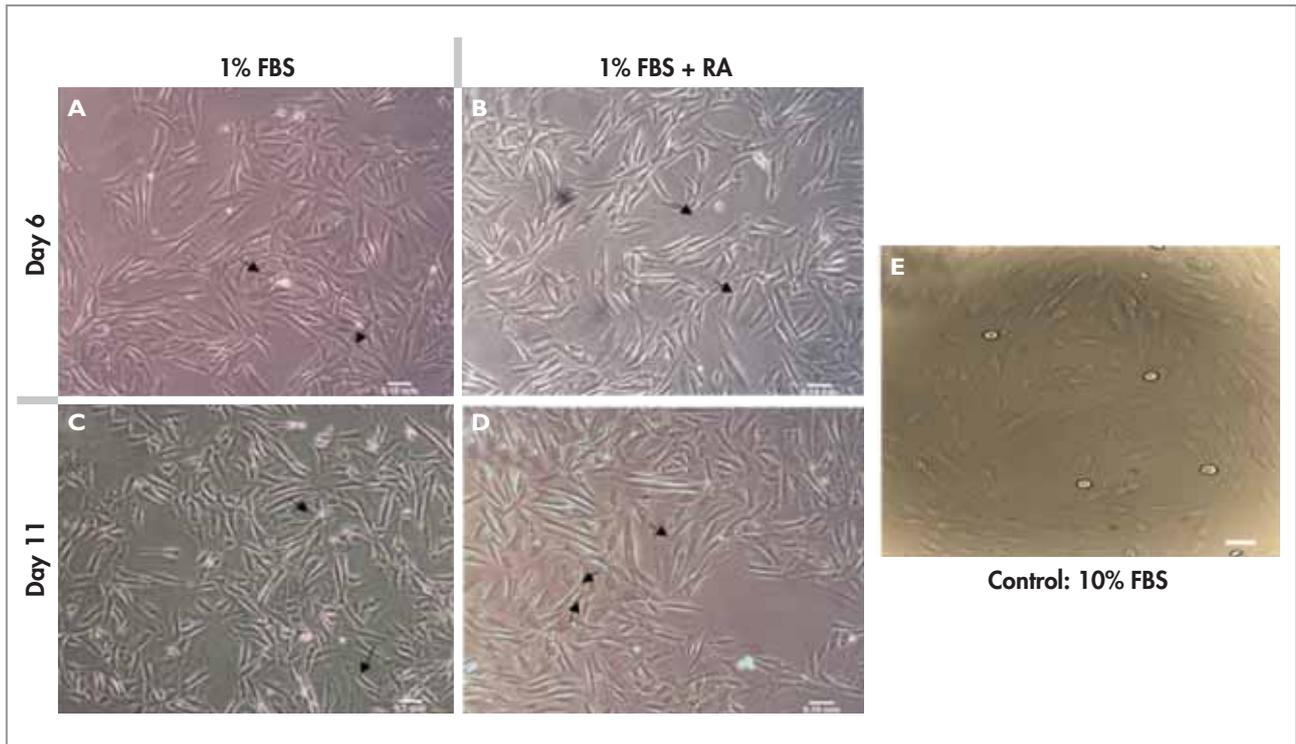
FIGURE 1: Morphological assessment of H9c2 differentiation, analysed using bright field microscopy on days 6 and 11. (n=3). A: 1% FBS after 6 days; B: 1% FBS plus RA at 6 days; C: 1% FBS at 11 days; D: 1% FBS plus RA at 11 days, and E: control (10% FBS). Arrows indicate possible cardiomyotube formation. Magnification = 5x. Scale bar = 0.1 mm.
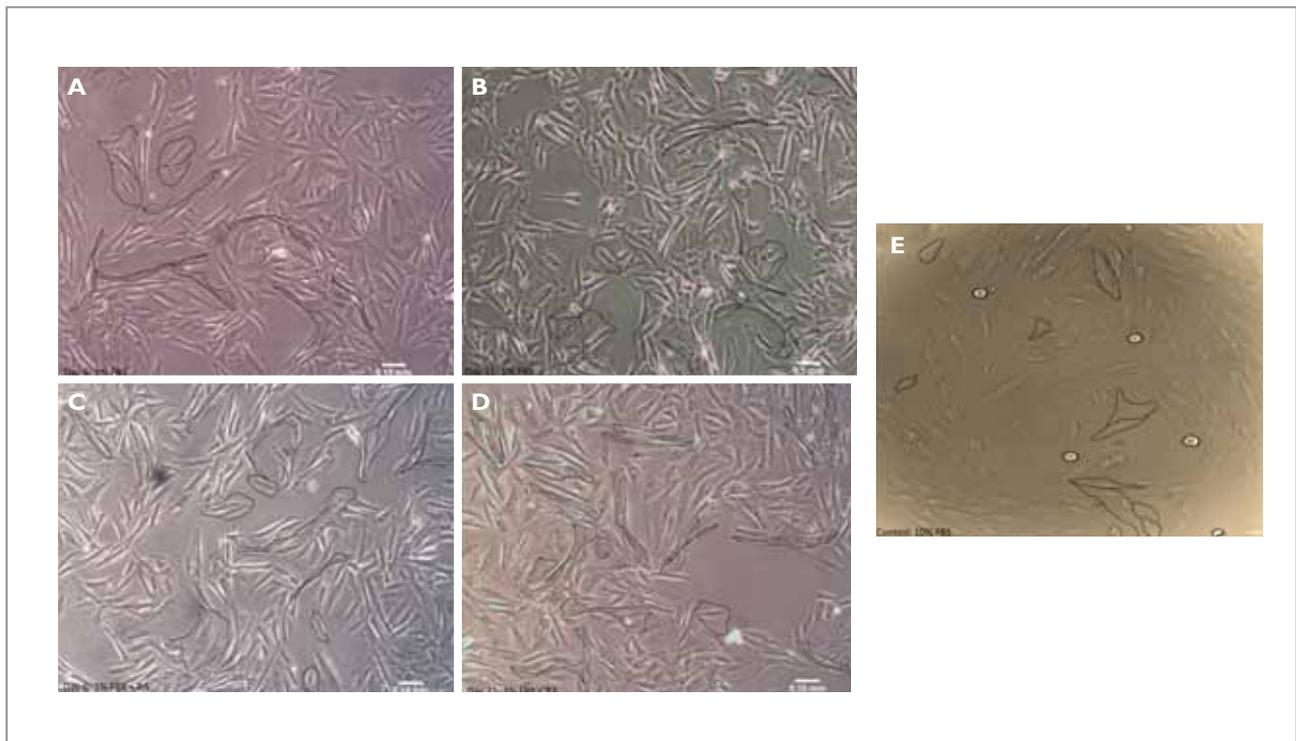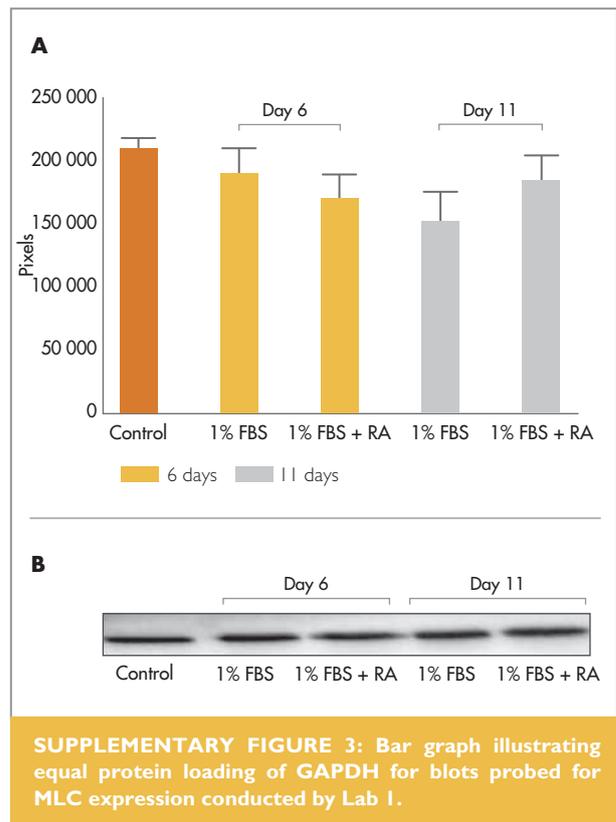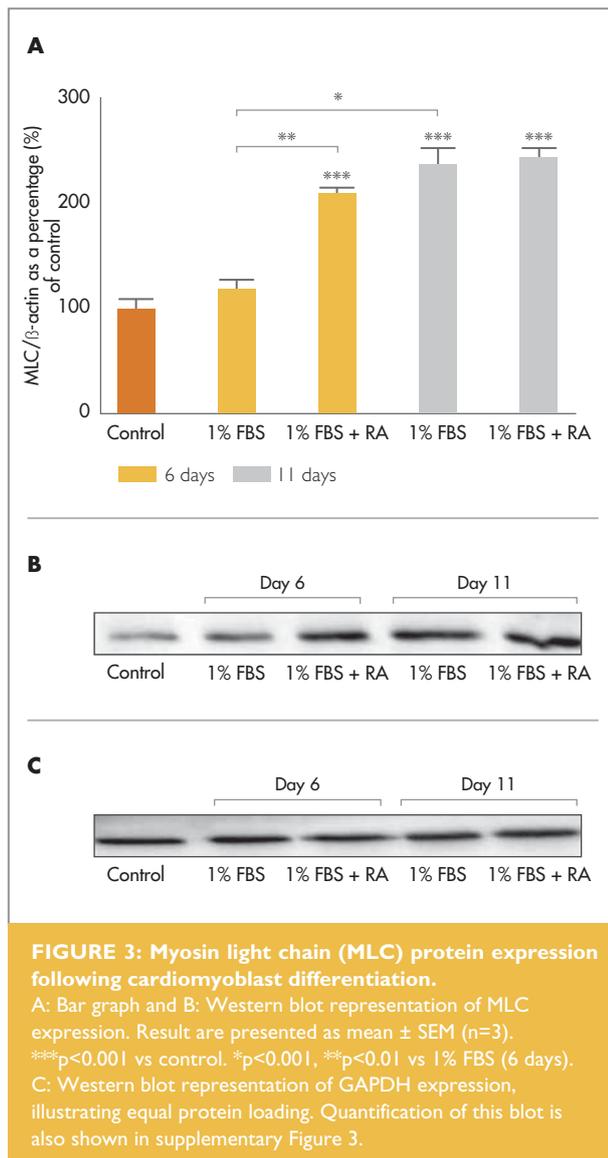

SUPPLEMENTARY FIGURE 1: Morphological assessment of H9c2 differentiation, analysed using bright field microscopy on days 6 and 11. (n=3). A: 1% FBS after 6 days; B: 1% FBS at 11 days; C: 1% FBS plus RA at 6 days; D: 1% FBS plus RA at 11 days, and E: control (10% FBS). Cell outlines indicate differences in morphology attributable to the degree of maturity or differentiation of the cells, where a round appearance is associated with immature cells, while the rod shaped cells are more mature. Magnification = 5x. Scale bar = 0.1 mm.
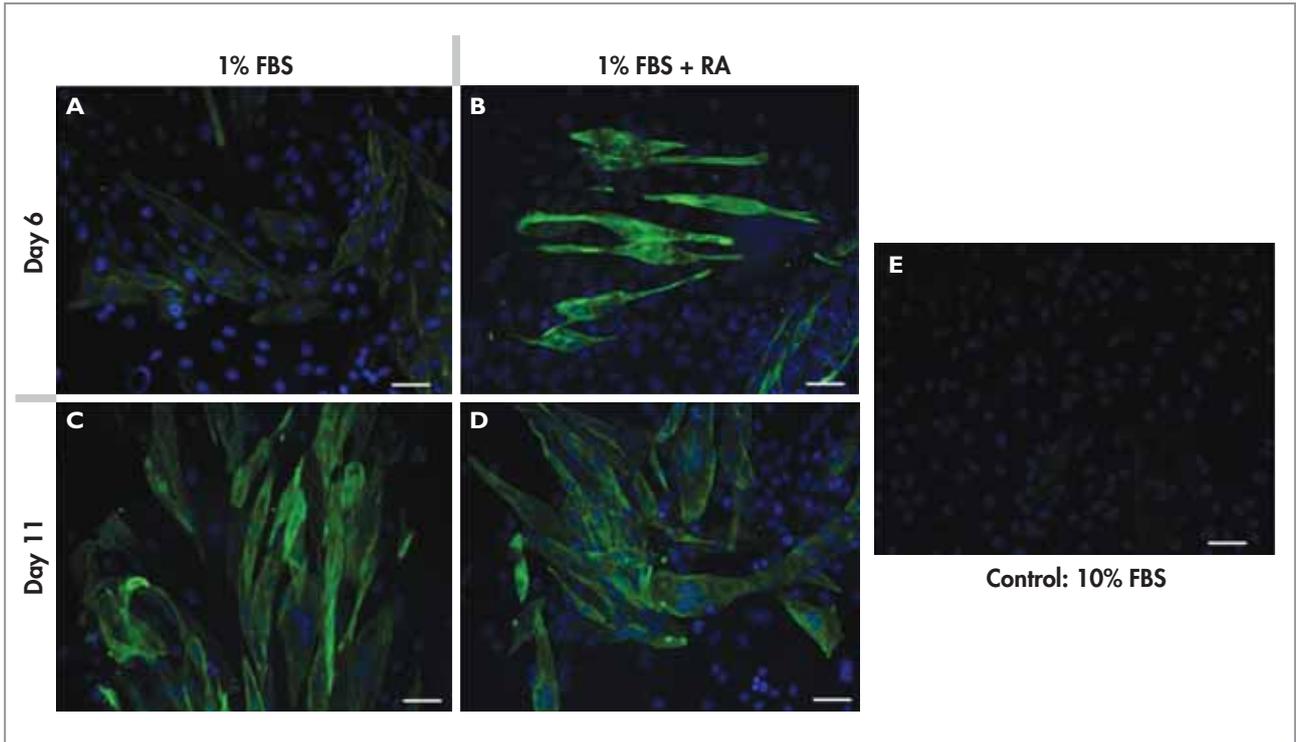
FIGURE 2: Analysis of cardiac troponin T (cTnT) expression following H9c2 differentiation for days 6 and 11. (n=3). A: 1% FBS (6 days), B: 1% FBS plus RA (6 days), C: 1% FBS (11 days), D: 1% FBS plus RA (11 days) and E: control. Green: cardiac troponin expression. Blue: nuclei. Magnification = 20x. Scale bar = 0.05 mm.
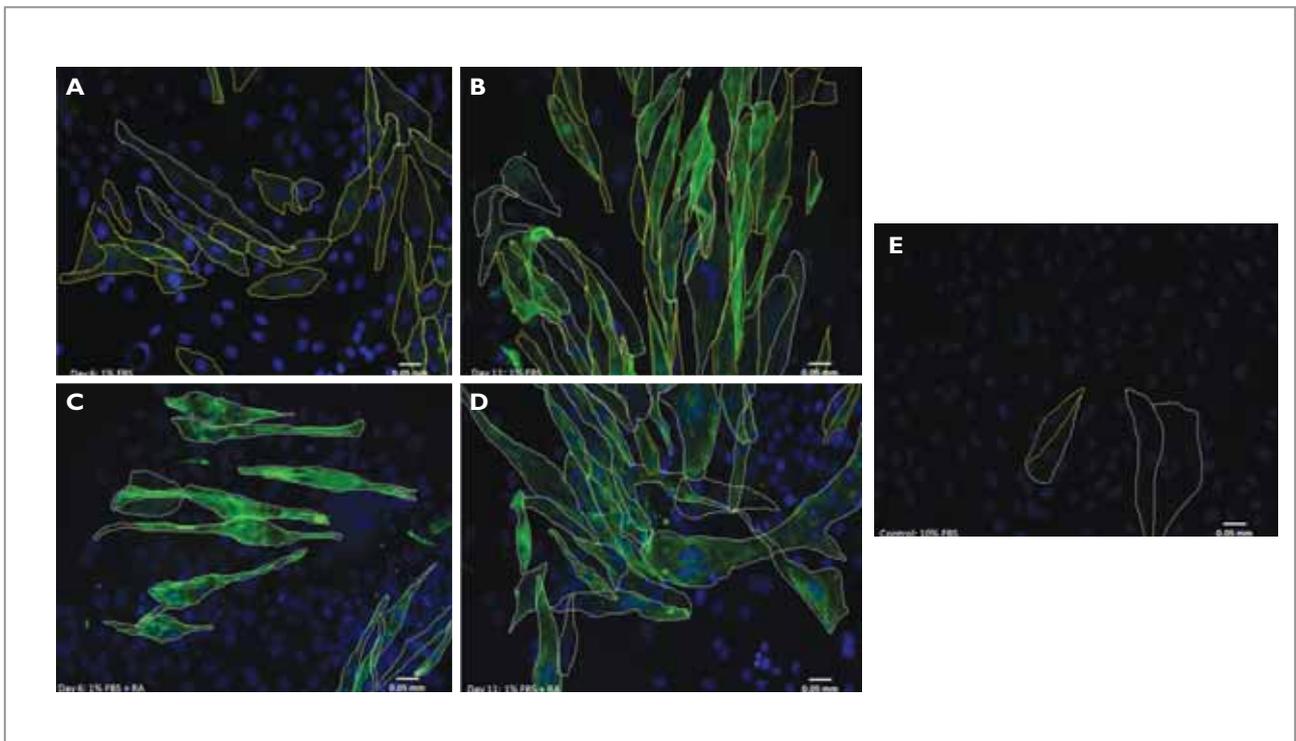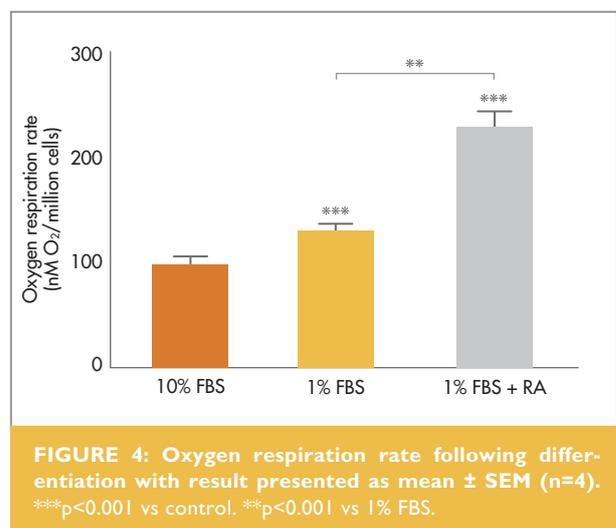

SUPPLEMENTARY FIGURE 2: Analysis of cardiac troponin T (cTnT) expression following H9c2 differentiation for days 6 and 11. Cell outlines indicate the morphology of these cells. (n=3). A: 1% FBS (6 days), B: 1% FBS (11 days), C: 1% FBS plus RA (6 days), D: 1% FBS plus RA (11 days) and E: control. Green: cardiac troponin expression. Blue: nuclei. Yellow outline: immature cells. White outline: differentiated mature cells. Magnification = 20x. Scale bar = 0.05 mm.

A

B

C

FIGURE 3: Myosin light chain (MLC) protein expression following cardiomyoblast differentiation.
A: Bar graph and B: Western blot representation of MLC expression. Result are presented as mean ± SEM (n=3). ***p<0.001 vs control. *p<0.001, **p<0.01 vs 1% FBS (6 days). C: Western blot representation of GAPDH expression, illustrating equal protein loading. Quantification of this blot is also shown in supplementary Figure 3.

Oxidative phosphorylation analysis

The switch in preferential energy metabolism, from glycolysis to oxidative phosphorylation, is a fundamental characteristic observed in differentiated cardiac cells.[21] Thus determining the rate of oxygen respiration would provide an ideal measure of oxidative phosphorylation in differentiated H9c2 cells. It should be noted that this variable was only investigated in cells differentiated for 6 days and no fatty acids were included as the researchers merely aimed to establish if a change in oxygen consumption was evident following differentiation. A significant increase in respiration rate was observed in both serum starved, and serum starved plus RA groups versus the control (100.0 ± 6.1nM O_2/million cells), indicative of differentiation (Figure 4). Large differences were also observed between the reduced serum group (131.9 ± 5.2nM O_2/million cells, p<0.05) compared to the serum starved plus RA group (230.7 ± 15.8nM O_2/million cells, p<0.001).

A

B

SUPPLEMENTARY FIGURE 3: Bar graph illustrating equal protein loading of GAPDH for blots probed for MLC expression conducted by Lab 1.

From the above results it can be seen that serum starvation, combined with daily RA supplementation, presents a positive influence on cardiomyoblast differentiation and the expression of cardiac specific proteins. It was also evident that supplementation with RA mediated the switch in substrate utilisation during differentiation to oxidative phosphorylation. Although a reduction in serum concentration was seen to produce similar characteristics, the effects were far less pronounced. This study, however, was not able to establish a definitively homogenous

FIGURE 4: Oxygen respiration rate following differentiation with result presented as mean ± SEM (n=4). ***p<0.001 vs control. **p<0.001 vs 1% FBS.

cell population, and even though many cells did undergo differentiation, the majority seemed to remain undifferentiated as was evident from light microscopy.

Lab 2

In this laboratory, cells were treated daily with RA (10nM, 30nM and 1μM) and reduced serum (1% FBS or HS), and cultured over a period of 8 - 12 days, followed by Western blot analysis probing for the cardiac specific markers cTnT and MLC-2v. Figure 5, however, clearly indicates that in our investigations no presence of either of these 2 cardiac markers was detected via Western blotting methods. The only visible bands appeared under the positive heart control samples, suggesting no fault with the primary or secondary antibodies used, or the blotting techniques, and rather indicating that no significant differentiation of the H9c2 cells had taken place.

This lack of meaningful differentiation was confirmed by initial light microscopy observations (supplementary Figure 5), which revealed a cell population similar to the undifferentiated cell type, with only a few cells showing changes in cell morphology – echoing the results obtained in Lab 1.

DISCUSSION

The H9c2 cell line is a versatile cell line, which due to its cardiac origin, has been used extensively in cardiac research. The cells are, however, fundamentally undifferentiated myoblasts. It has previously been reported that culturing these cells over a period of days in relatively low concentrations of FBS, as well as in the presence of RA will stimulate differentiation into a more cardiac-like phenotype. We were, however, unable to elicit suitable differentiation as defined by attaining a relatively homogenous cell population. These results were obtained in 2 laboratories working independently of each other, thereby confirming the resistance of these cells to differentiation.

Three different parameters were used to assess the level of differentiation, namely: metabolic profile, expression of cardiac
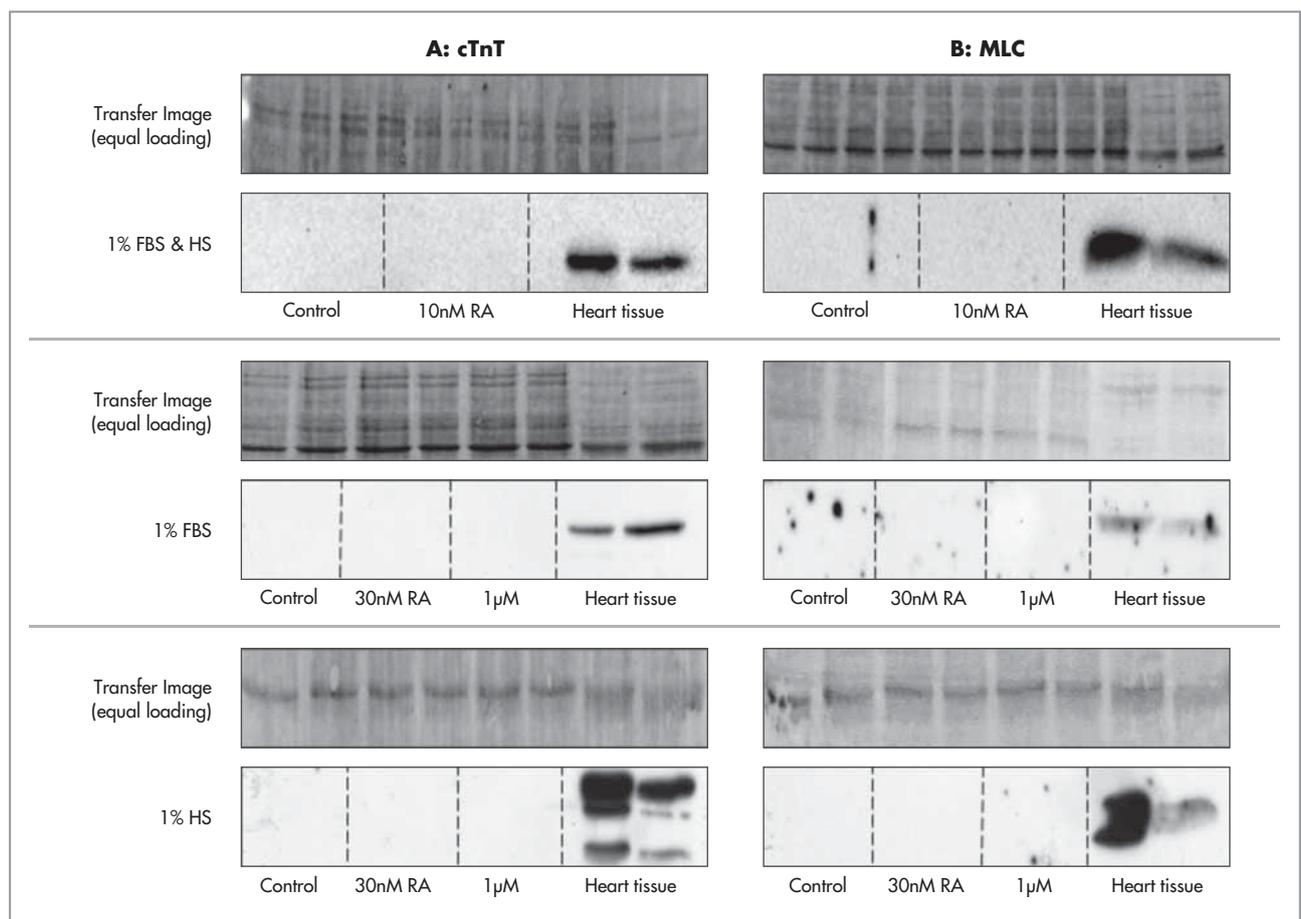
FIGURE 5: Western blots probed for cardiac specific markers. A: Cardiac Troponin T and B: Myosin light chain 2v, following the differentiation protocol with RA and reduced serum. Bands are only visible for the 2 positive control heart tissue samples loaded. No cardiac markers were detected in either the undifferentiated control cells, or the cells that have undergone differentiation culturing. Equal protein loading is demonstrated through the corresponding Western blot transfer images above each blot. This data is also quantified and depicted in Supplementary Figure 4.
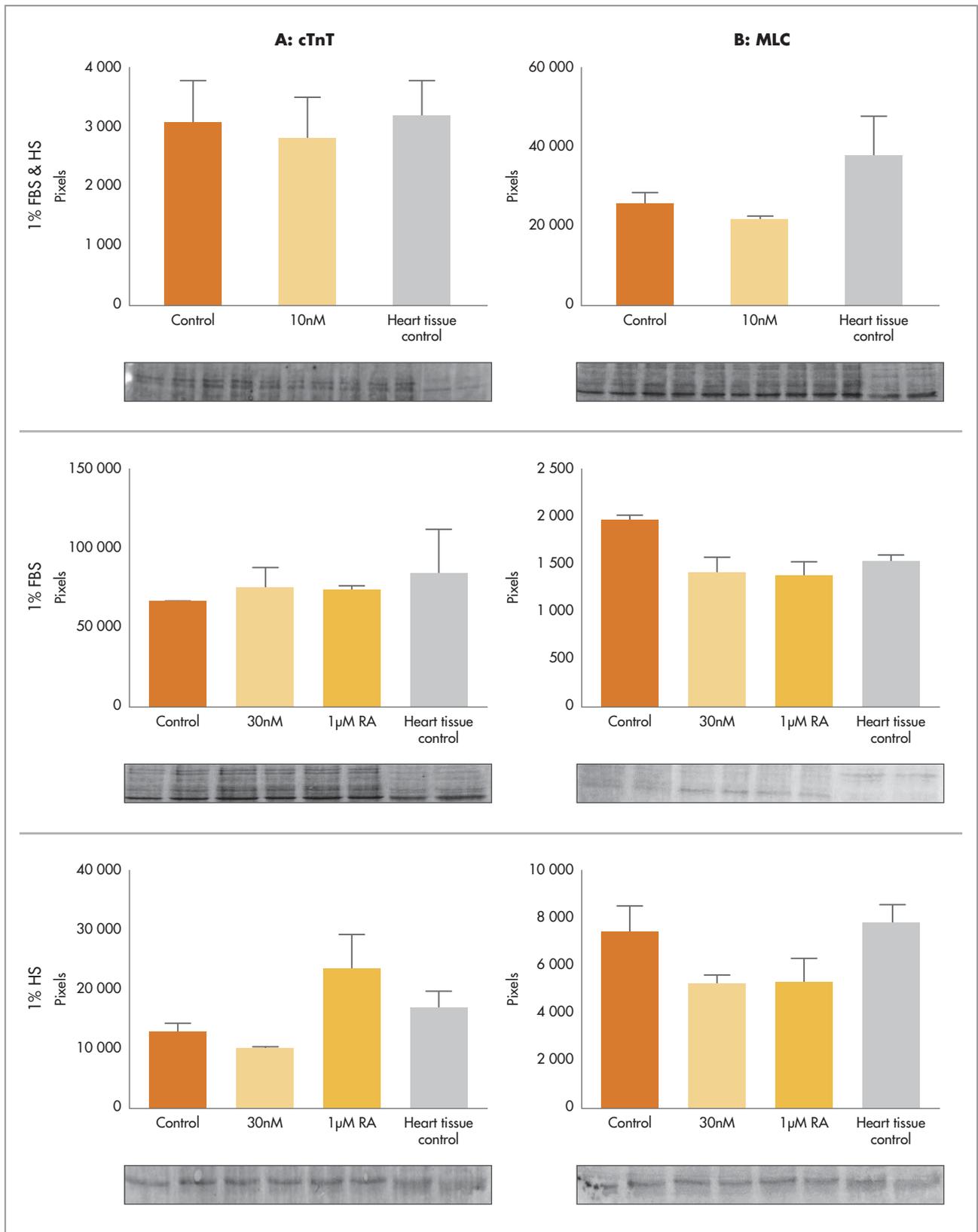
SUPPLEMENTARY FIGURE 4: Bar graphs representing equal protein loading of Western blot transfers probed for cardiac specific markers. A: Cardiac Troponin T and B: Myosin light chain 2v, following the differentiation protocol with RA and reduced serum. Comparisons were done using either an unpaired T-test, or a one-way ANOVA followed by Bonferroni's post hoc test. Since the protein profile of the heart tissue control was visibly different than the protein profiles from the H9c2 cells, this group was not included in the statistical analysis.
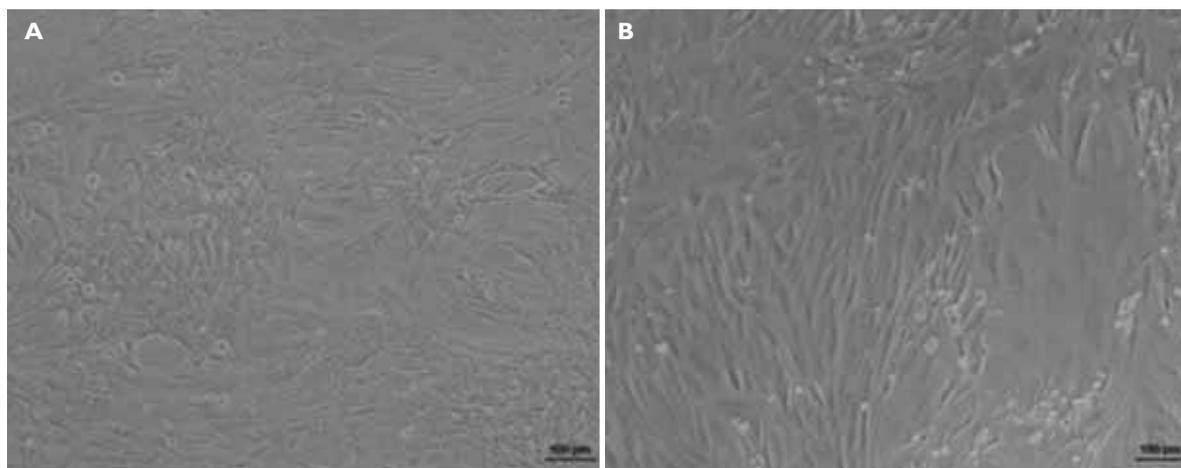
specific proteins and cell morphology. Concerning metabolic preference, it is known that heart tissue is predominantly dependent on oxidative phosphorylation, while the H9c2 myoblasts are mostly dependent on glycolytic metabolism. Using the described differentiation protocol, Pereira and colleagues[20] demonstrated a shift from glycolytic metabolism to oxidative phosphorylation in the differentiated cells. We also observed such a shift in terms of an increased capacity for the differentiated cells to utilise oxygen. This was especially true for cells treated with RA. This observation confirms that although long-term culture in 1% FBS can induce differentiation,[9,18] the addition of RA is necessary to optimally drive the cells in the direction of cardiomyotubes.[20] Investigations into the expression of cardiac specific proteins, specifically cTnT and MCL, revealed a less robust degree of differentiation. Lab 1 showed an increase in the expression of both these proteins, again dependent on the inclusion of RA in the culturing media, while Lab 2 failed to demonstrate any expression of these proteins, despite testing several concentrations of RA, as well as comparing FBS with horse serum. Finally, cell morphology also indicated a less than optimal result, in that a relatively small proportion of the cell population exposed to the differentiation media demonstrated the expected morphology. As with the other measurements, the inclusion of RA was associated with a greater increase in the degree of the total cell population showing multi-nucleation.

It is interesting that Lab 1 found quite an impressive degree of differentiation in terms of protein expression and oxygen-dependent metabolism, which was not necessarily reflected in the degree of morphological change. This could be due to differences in the stage of differentiation, where it could be argued that intracellular changes precede morphological differentiation. Our criteria for the experimental use of differentiated H9c2 cells was that the cells should be homogenous and differentiation complete, as determined by several end-points. The fact that many of the cells did not have the morphology of mature heart cells therefore led to doubt concerning the exact nature of these cells and if they were completely differentiated. Although the use of several end-points is the optimal way to assess differentiation, it is a short-coming in the field that there are no consensus characteristics and criteria to define differentiated H9c2 cells. This lack could impede effective comparison and replication in science.

In summary, our combined results indicate that incubation of H9c2 cells for between 6 - 12 days with 1% FBS and 10nM RA does stimulate differentiation of the cells into a more cardiac-like cell, although this only occurs in a portion of the cells. The protocol is therefore unsuccessful in stimulating robust differentiation capable of generating a homogenous cell population. This therefore limits the potential utilisation of this approach for general research purposes. Potential reasons for the discrepancy between our laboratories and the work published by Menard, et al.[19] and Pereira, et al. [20] are unknown. Two potential confounding variables must be kept in mind, namely (i) the passage number and history of the cells used in the differentiation experiments and (ii) the handling of RA.

Concerning the passage number, there does not seem to be consensus regarding the optimal passage number for experimenting with H9c2 cells and, in fact, information concerning the effects of aging cell culture on the cells seems sparse. Recently, Witek and colleagues reported that passage number influences the response of H9c2 cells to cytotoxic agents.[26] In the context of differentiation, passage number is an unknown, but probably relevant, variable.

Regarding the handling of RA there are 2 important factors to consider: (i) RA is light sensitive and (ii) it is also extremely hydrophobic. The combination of these make it virtually impossible to be absolutely confident that enough RA is truly dissolved in the growth medium to interact with the H9c2 cells. It has been reported that RA is stable under standard cell culturing conditions and, at least in the context of stem cells, is readily taken up by cells in culture[27-29] with a half-life ranging between 3.5[30] and 6[31] hours, although 24 hours seems to be an acceptable interval for the replenishment of RA. Both Tsukuda, et al. and Sharrow, et al. reported on the necessity of working with RA in the dark, or under yellow light illumination.[28,29] We took great care to protect the RA from light, while also dissolving it in sequential dilutions of DMSO before adding it to an aqueous medium. The dynamics of RA uptake in H9c2 cells is however unknown. This is a shortcoming in the existing literature on the use of RA to mediate the differentiation of H9c2 cells.

Despite our negative results, the benefit of differentiating the H9c2 cells into a more cardiac-like cell type is without doubt. Potential alternative variations on the current protocol include investigating the addition of other growth factors, in addition to RA, in order to drive a more robust differentiation, or finding alternative ways to effectively administer RA. These could include the generation of micelles, or the use of nanoparticles to ensure effective delivery of RA to the cells, or even just the inclusion of albumin in the growth medium in order to stabilise the RA.[28] A third variable to investigate is the time allowed for differentiation. Although both Menard, et al.[19] and Pereira, et al.[20] cultured their cells for 5 - 7 days, it is noteworthy that for the original description of the H9c2 cells by Kimes and Brandt[9] cells started showing pronounced myotube formation after 15 days of culturing and much less so after 7 days. It is therefore conceivable that an extended period of culturing, in the range of 15 - 20 days, might produce a more pronounced degree of differentiation. Yet a fourth variable, which has received some attention, is the surface on which the cells are cultured and differentiated. In this regard it has been reported that the degree of differentiation is influenced by interventions such as growing cells on an extracellular matrix[32] or growing cells on a surface with micro grooves.[33]

The most significant limitation of this study is that it was done in a retrospective manner, in other words the research efforts of the 2 laboratories were not planned or coordinated in advance. Effective collaboration would have entailed measuring the same end-points, making use of the same protocols and exchanging cells between laboratories at different points in the protocol. This limitation is however also a strength, since despite the absence of collaboration, we still reached similar conclusions.

In conclusion, long-term culture of H9c2 cells with 1% FBS and RA could induce a degree of differentiation, although this seemed limited to certain cells. The absence of a homogenous differentiated cell population, however, complicates the use of these cells for research. Investigators thus need to be aware of this when selecting this as an experimental model for their research.

Conflict of Interest: The authors declare no conflict of interest and all authors have approved the final manuscript submitted.

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