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Original Article

Improving cardiac differentiation of human pluripotent stem cells by targeting ferroptosis

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ABSTRACT

Generation of cardiomyocytes from human pluripotent stem cells (hPSCs) is of high interest for disease modelling and regenerative medicine. hPSCs can provide an unlimited source of patient-specific cardiomyocytes that are otherwise difficult to obtain from individuals. Moreover, the low proliferation rate of adult cardiomyocytes and low viability *ex vivo* limits the quantity of study material. Most protocols for the differentiation of cardiomyocytes from hPSCs are based on the temporal modulation of the Wnt pathway. However, during the initial stage of GSK-3 inhibition, a substantial number of cells are lost due to detachment. In this study, we aimed to increase the efficiency of generating cardiomyocytes from hPSCs. We identified cell death as a detrimental factor during this initial stage of *in vitro* cardiomyocyte differentiation. Through pharmacological targeting of different types of cell death, we discovered that ferroptosis was the main cell death type during the first 48 h of the *in vitro* differentiation procedure. Inhibiting ferroptosis using ferrostatin-1 during cardiomyocyte differentiation resulted in increased robustness and cell yield.

1. Introduction

Human pluripotent stem cells (hPSCs) including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) hold great potential for *in vitro* disease modelling and for regenerative medicine applications [1,2]. An important feature of hPSCs is their ability to differentiate into virtually any cell type, including cardiomyocytes.

Human material to study cardiomyopathy is scarce. Obtaining cells from a myocardial biopsy poses significant risks for the patient, while offering only a limited amount of material. These cells are unable to survive long-term in *in vitro* culture [3]. hPSCs provide an attractive alternative strategy, as they can be used to generate patient specific cells. Currently, there are various well-described protocols for the differentiation of hPSCs to cardiomyocytes [4–7]. These protocols are mainly based on modulating the Wnt pathway. First, hPSCs are directed to mesodermal progenitors by blocking GSK-3, resulting in an upregulation of Wnt. Subsequently, these progenitors are pushed further to cardiac mesodermal progenitors by inhibiting Wnt. By day 8–10, the cardiomyocytes start contracting, developing into a synchronized contracting layer by day 14.

Numerous research efforts are currently directed at improving the maturation of hPSC-derived cardiomyocytes, which generally display a relatively immature phenotype compared to adult cardiomyocytes, hampering the translational value [8]. Maturation of

Abbreviations: BMP4, bone morphogenic protein 4; FBS, fetal bovine serum; FER-1, Ferrostatin-1; hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells; hPSCs, human pluripotent stem cells; PBS, phosphate buffered saline; PUFA-ePLs, polyunsaturated ether phospholipids.

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cardiomyocytes can be improved by prolonged culturing, co-culture, 3D culture such as engineered heart tissue, metabolic cues and electrical stimulation.

The *in vitro* cardiac differentiation protocol manipulating the Wnt signaling pathway is robustly generating cardiomyocytes. However, substantial cell death occurs during the differentiation process [9] without an intrinsic selection bias for specific progenitor cells [10]. Especially in the first 48 h of differentiation with upregulated Wnt, significant numbers of detaching cells are observed. GSK-3 inhibition is commonly achieved through the application of the small molecule CHIR99021. Besides targeted Wnt modulation, this small molecule has also been shown to display toxic effects [11].

Different cell death modalities have been described [12]. Caspase activity causes nuclear disintegration, inter-nucleosomal fragmentation of DNA and blebbing of the plasma membrane while the apoptotic cell loses contact with neighboring cells. The activation of caspase-1 does not result in apoptosis but leads to lytic cell death, called pyroptosis. Besides caspase-mediated cell death, it is becoming evident that necroptosis and ferroptosis, two other forms of regulated cell death, play important roles in various pathologies [13]. Ferroptosis is regulated by excessive and iron-dependent lipid peroxidation.

Several drugs that specifically target these different cell death modalities are available or currently under development. For instance, zVAD, a pan-caspase inhibitor, inhibits apoptosis and pyroptosis, while being a potential trigger of necroptosis. DEVD is a potent inhibitor of several caspases, especially caspase-3 activity, resulting in blockade of apoptosis. Ferrostatin-1 (FER-1) is a synthetic lipophilic radical trapping anti-oxidant, which blocks lipid peroxidation in cellular membranes, thus a specific inhibitor for ferroptosis. In this study we investigated if the cell death observed in the early stage of *in vitro* cardiac differentiation, during CHIR99021 treatment, could be reduced by the application of cell death inhibitors (zVADfmk, DEVDfmk, FER-1). Our data highlights the potential of FER-1 for cardiac disease modelling and regenerative medicine as inhibition of ferroptosis by this compound significantly reduces cell death and improves efficiency and robustness of cardiomyocyte differentiation.

2. Materials and methods

All products used are purchased from Thermo Fisher unless mentioned otherwise.

2.1. Stem cell cultures

The H9 hESC line (WA09) was obtained via WiCell. hiPSCs (UGENTi001-A-1 [14] and IPSC-001) were in-house established lines generated previously using the CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Cat No. A16517). hPSCs were maintained at 37 °C, 5% CO_2 and 5% O_2. Cultures were kept in feeder-free conditions using Geltrex coating (Cat No. A1413302) and cultured in Essential 8 medium (Cat No. A1517001) supplemented with 100 u/ml Penicillin and 100 μg/ml Streptomycin (Cat No. 15140-122). For routine culturing, hPSCs were single-cell passaged using 3–5 min of TrypLE Select (Cat No. 12563011). Cells were dissociated by gently pipetting up and down and TrypLE was inactivated by diluting in Essential 8 medium. Cell suspension was centrifuged for 5 min at 200×*g*, the cell pellet was dissolved in Essential 8 medium supplemented with 1:100 RevitaCell (Cat No. A2644501) and cells were seeded with a density of 2.1 × 10^4 cells/cm^2. Phase-contrast images of cell cultures were made using EVOS™ XL Core Cell Imaging System.

2.2. Directed differentiation towards cardiomyocytes

For the derivation of cardiomyocytes from hPSCs, a directed differentiation protocol was used based on the modulation of the Wnt pathway [4]. At the start of the protocol, when the culture confluency reached 70%, or mentioned otherwise for specific experiments, hPSCs were washed with phosphate buffered saline (PBS) and the medium was changed to differentiation medium supplemented with 4 μM CHIR99021 (Merck, Cat No. 361559). The differentiation medium consisted of RPMI 1640 with HEPES (5958 mg/l) and GlutaMAX (L-Alanyl-Glutamine 446 mg/l) (Cat No. 72400-021) supplemented with 0.125 mg/ml Albumin (Sigma–Aldrich, Cat No. A9731) and 0.05 mg/ml L-Ascorbic Acid 2-Phosphate (Sigma–Aldrich, Cat No. A8960). Exactly 48 h after the start of the differentiation, the cells were washed with PBS and medium was changed to differentiation medium supplemented with 5 μM IWP2 (Merck, Cat No. 681671). On day 4 and day 6, the medium was changed to differentiation medium. On day 8 and every other day, the medium was refreshed with maintenance medium which consisted of RPMI 1640 with HEPES and GlutaMAX supplemented with 1:100 B27 supplement with insulin (Cat No. 17504-044). Cardiomyocytes and cells during differentiation were kept at 37 °C, 5% CO_2 and 19% O_2.

2.3. Passaging of cardiomyocytes

The Multi Tissue Dissociation Kit 3 (Miltenyi Biotec, Cat No. 130-110-204) was used to transfer the cardiomyocytes to glass coverslips for immunocytochemical analysis. The cells were washed three times with PBS and subsequently incubated for 10 min at 37 °C with the dissociation mix. Cells were dissociated by gently pipetting up and down and the dissociation enzymes were inactivated by diluting with maintenance medium, which was supplemented with 20% fetal bovine serum (FBS). The cell suspension was centrifuged for 5 min at 200×*g*, the pellet was dissolved in maintenance medium supplemented with 20% FBS and 1:100 RevitaCell and cells were seeded with a density of 2 × 10^5 cells/cm^2.

2.4. Cell death inhibitors and ferroptosis inducer

The following concentrations of cell death inhibitors were used in the cell cultures from day 0–2 during the cardiac differentiation: caspase peptide inhibitor zVADfmk (10 μM, Bachem, Cat No. N-1510), DEVDfmk (10 μM, APExBIO, Cat No. A1920) and FER-1 (500 nM, Xcess Biosciences, Cat No. 053224).

2.5. Sytox Green assay

The cell membrane-impermeable dye Sytox Green (1 μM, Cat No. S7020) was added to the differentiation medium that was used to initiate the cardiomyocyte differentiation. IncuCyte ZOOM Live-Cell Analysis system (Sartorius) was used to image the Sytox Green uptake of the cells with different cell death inhibitors for the first 48 h of cardiac differentiation in a 24-well plate at 37 °C and 5% CO_2. Cells were imaged with a 10× objective. The Sytox Green positive cell region was calculated by dividing the area of Sytox Green by the total area of the cells as was determined by phase-contrast images at each time point.

2.6. Immunocytochemical analysis

Cardiomyocytes or hPSCs on coverslips were fixed with 4% paraformaldehyde diluted in PBS at RT for 20 min. The cells were permeabilized with 0.1% Triton X-100 diluted in PBS for 8 min. Subsequently, the cells were blocked by incubation with blocking

solution consisting of PBS containing 0.05% Tween20 and 1% bovine serum albumin (BSA) for 60 min. The cardiomyocytes were incubated overnight at 4 °C with primary antibody for α-actinin (1/800 mouse mAb (IgG1), Merck, Cat No. A7811) or TNNT2 (1/250 mouse mAb, Cat No. MA5-12960) and NKX2.5 (1/100 rabbit mAb, Cat No. 701622) diluted in the blocking solution. The hPSCs were incubated overnight at 4 °C with primary antibody NANOG (1/600 rabbit mAb, Cat No. PA1-097), OCT4 (1/600 mouse mAb, Santa Cruz, Cat No. SC-5279) and SOX2 (1/800 rabbit mAb, Cat No. PA1-094) diluted in blocking solution. The next day, the cells were incubated for 60 min at RT with secondary antibody goat-anti-mouse IgG dylight 488 (1/500, Cat No. 35503), goat-anti-rabbit IgG dylight 594 (1/500, Cat No. 35561) and 0.1% Hoechst solution (Cat No. H3570) diluted in blocking solution. Microscopy images for cardiomyocytes were made using a ZEISS LSM900 confocal microscope, images from hPSCs were made using EVOS™ FL cell imaging system.

2.7. Lipid ROS kinetics assay

hPSCs were seeded in 96-well plates (CellCarrier-96 ultra microplates, PerkinElmer, Waltham, MA, USA). At the start of the experiment, the differentiation of hPSCs was initiated using differentiation medium supplemented with 4 μM CHIR99021. C11-BODIPY (1 μM, Cat No. D-3861) and DRAQ7 (0.5 μM, Biostatus Cat No. DR71000) were added to each well 30 min before measurements. Also, ferroptosis inducer ML162 (0.5 μM, Aobious, Cat No. AOB1514) and FER-1 (500 nM) were included as control conditions in a subset of the wells in the experiment. Images were taken every 105 min for 17 h and a final measurement was performed at 24 h using the OPERA Phenix high content imaging instrument (PerkinElmer) with 20× water immersion objective. Using DRAQ7, dead cells could be identified from live cells. C11-BODIPY is used to detect lipid peroxidation events as oxidation of the probe results in a shift of the fluorescence emission peak from 590 nm to 510 nm when exited by 488 nm. This allowed the acquisition of the reduced and oxidized probe fluorescence in separate image channels. Visualization was done using the TIBCO Spotfire software package. Image analysis included single-cell segmentation and subsequent analysis of the percentage of dead cells (DRAQ7 positive cells) and/or calculations for the percentage of cells positive for lipid peroxidation (oxidized C11-BODIPY positive cells).

2.8. Statistical analysis

Statistical analysis was performed using SPSS statistics version 23.0 for Windows (IBM Corp., Armonk, NY). Means were compared using unpaired t-test and reported as two tailed p-value for comparison of two independent means with a significance level of 0.05. GraphPad Prism software for Windows was used to generate graphs. ImageJ 1.53v was used to determine cell culture confluency in phase-contrast images [15]. Zen 3.1 blue edition microscopy software (Zeiss) was employed to measure sarcomere lengths.

3. Results

3.1. Induction of cardiac differentiation initiates cell death in the first 24 h

A short overview of the methodology is depicted in Fig. 1a. In this study, we investigated if we could increase the cardiomyocyte differentiation efficiency from stem cells. More specifically, by intervening in the first 48 h of the differentiation protocol where excessive release of dead cells into suspension was observed. The main experiments as depicted in the figures are performed using the hESC line H9. To show reproducibility, two hiPSC lines (UGENTi001-A-1 and IPSC-001) were used, these results are displayed in supplementary figures. If the cell membrane is impaired, Sytox Green can enter the cell and bind to gDNA, thus indicating cell death. Analysis of the mentioned control differentiation using the Sytox Green assay revealed the expected excessive cell death within the first 24 h of *in vitro* cardiac differentiation (Fig. 1b–d) as indicated by a Sytox Green positive region of approximately 80%.

3.2. FER-1 blocks cell death during cardiac differentiation of human pluripotent stem cells

We used one hESC line (H9) and two hiPSC lines (UGENTi001-A-1 and IPSC-001) to determine if excessive loss of cells in the early stages of *in vitro* cardiomyocyte differentiation could be reduced. Three different cell death inhibitors were tested (zVAD: a pan-caspase inhibitor, inhibiting apoptosis and pyroptosis; DEVD: inhibitor of executioner apoptotic caspases; FER-1: inhibitor for ferroptosis; or a combination) in the first 24 h of the differentiation of hESCs/hiPSCs. The confluency of the cell culture could be significantly increased by applying zVAD or FER-1 inhibitor (Fig. 1b, Supplementary Vid 1-6), whereas FER-1 performed best and was able to increase culture confluency from approximately 32% ± 1.5% (control differentiation) to 55% ± 4.5%. Combining FER-1 together with zVAD or DEVD did not result in a higher confluency compared to only FER-1 application. Application of FER-1 significantly reduced the Sytox Green positive cell region from 80% ± 5.1% (control differentiation) to 4% ± 3.1% (Fig. 1c). FER-1 showed strong inhibition of cell death in the early stages of *in vitro* differentiation of hPSCs to cardiomyocytes.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.reth.2024.02.007

3.3. Cardiomyocyte differentiation leads to accumulation of lipid ROS that can be blocked by FER-1

Lipid ROS, a hallmark of ferroptosis, is measured to investigate if ferroptosis is the major type of cell death occurring in early *in vitro* cardiomyocyte differentiation. The C11-BODIPY assay has two fluorescent readouts, the reduced and the oxidized form, whereas the oxidized form represents the lipid ROS levels. DRAQ7, a cell impermeable fluorescent dye, was used to determine if lipid ROS accumulation progresses in cell death. ML162, a ferroptosis inducer, was used as a positive control in the C11-BODIPY assay. The optimal dose was determined to be 0.5 μM for a time window of 24 h (Supplementary Fig 1). Fig. 2 and Supplementary Vid 7-9 illustrate representative images and videos of fluorescent signals for oxidized C11-BODIPY and DRAQ7 for control differentiation and FER-1 or ML162 supplemented cardiac differentiation after 24 h.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.reth.2024.02.007

In the C11-BODIPY analysis, a minimal threshold for fluorescence intensity was set to identify cells positive or negative for lipid peroxidation. An increase in the percentage of oxidized C11-BODIPY positive cells was observed after initiating the control differentiation, reaching 40% in 24 h (Fig. 3a). FER-1 was able to completely block lipid ROS accumulation. In the ML162 condition, all cells were positive for oxidized C11-BODIPY. The lipid ROS formation resulted in cell death as pointed out by increasing percentages of DRAQ7 positive cells, reaching 100%, 16% and 1% for respectively ML162, control differentiation and FER-1 differentiation (Fig. 3b). Inhibition of lipid ROS formation by FER-1 resulted in

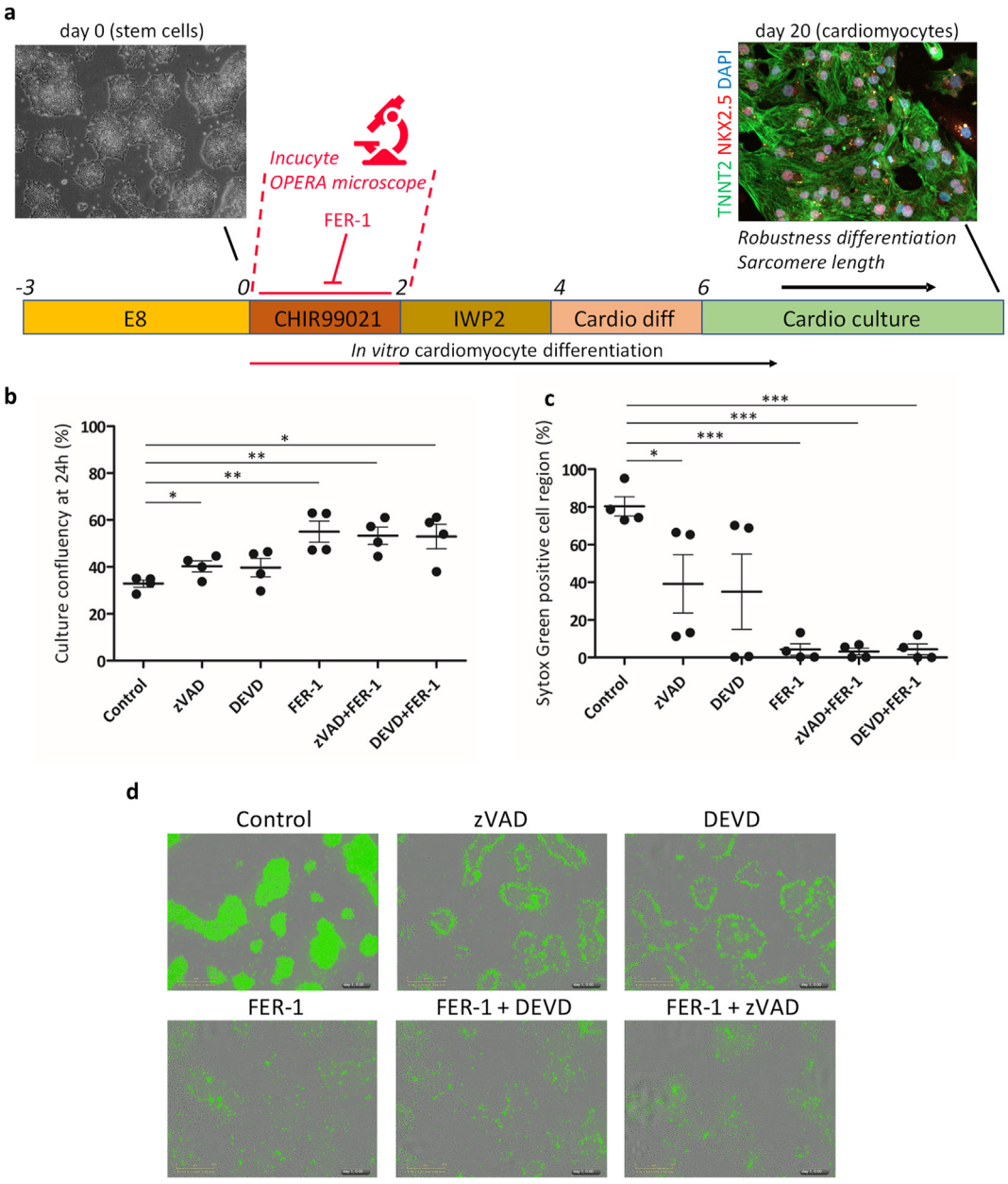


Fig. 1. Overview of the *in vitro* cardiomyocyte differentiation protocol and assessing the effect of different cell death inhibitors on cardiac differentiation. At day −3, 18.500 hPSCs/cm^2 are seeded. Directed differentiation is initiated at day 0 with CHIR99021 (upregulation of Wnt), after 48 h, differentiation medium is changed and supplemented with IWP2 (downregulation of Wnt). Sytox Green assay and C11-BODIPY assay are performed using Incucyte and OPERA microscope, respectively, in the early phase of differentiation. Robustness of the differentiation and the sarcomere length of generated cardiomyocytes is analysed after completion of the differentiation protocol **(a)**. Sytox Green assay was used to follow the first 24 h of *in vitro* cardiomyocyte differentiation (control differentiation) of H9 human embryonic stem cells in a 24-well plate. Three different cell death inhibitors are evaluated: zVAD, DEVD and FER-1 or a combination thereof. The average of 4 wells per condition is evaluated. Culture confluency is assessed for all the conditions after 24 h of differentiation and is determined as the percentage of area that is covered by the cells **(b)**. Combining the results from cell culture confluency and the cell region positive for Sytox Green fluorescent signal allows to determine the percentage of Sytox Green positive cells **(c)**. Images of the time-lapse at the 24 h timepoint shows the abundance and distribution of the Sytox Green fluorescent signal in the cell cultures **(d)**. Error bars indicate standard error. Asterisk indicates significance level, * <0.05, **<0.01, ***<0.001. Scalebar, 300 μm.

decreased cell death compared to control differentiation, which further confirms that ferroptosis is the acting player causing cell death in this initial stage of *in vitro* differentiation.

Lastly, the total number of cells (Fig. 3c) and cell region area (Fig. 3d) show a similar pattern for control differentiation and FER-1 supplemented differentiation within the first 24 h. However, in the ML162 condition, which resulted in strong responses of oxidized C11-BODIPY and DRAQ7 fluorescent signal, the number of cells and cell region area decreased compared to the control differentiation. These experiments were replicated with hiPSCs (Supplementary Fig 2) and resulted in similar findings.

3.4. FER-1 has no effect on the routine pluripotent stem cell cultures

To rule out that FER-1 impacts hPSCs cultures, the pluripotent cells were treated daily with FER-1 and imaged using phase-contrast microscopy. During routine culture of hPSCs, only minimal cell death was observed, therefore no beneficial effect of FER-1 is to be expected. On the other hand, FER-1 might negatively impact hPSC cultures. The results show that the addition of FER-1 in the culture medium did not affect morphology, culture confluency or viability in hPSCs compared to control, non-treated cultures (Fig. 4a). Also, evaluation of pluripotency markers NANOG, OCT4

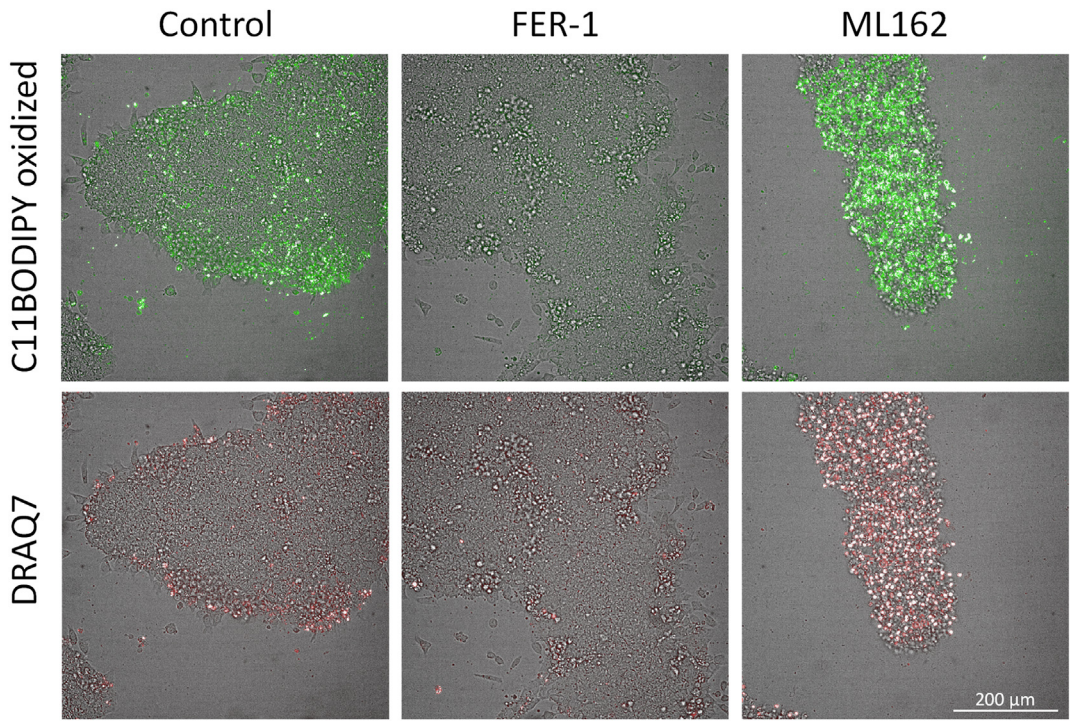


Fig. 2. Analysis of ferroptosis during cardiac differentiation using lipid ROS assay. Confocal for control differentiation, FER-1 (0.5 µM) and ML162 (0.5 µM) after 24 h of cardiac differentiation of H9 cells. Green fluorescent signal indicates oxidation of C11-BODIPY, red fluorescent signal presents DRAQ7, overlayed with brightfield image of the cell culture. Scalebar, 200 µm.

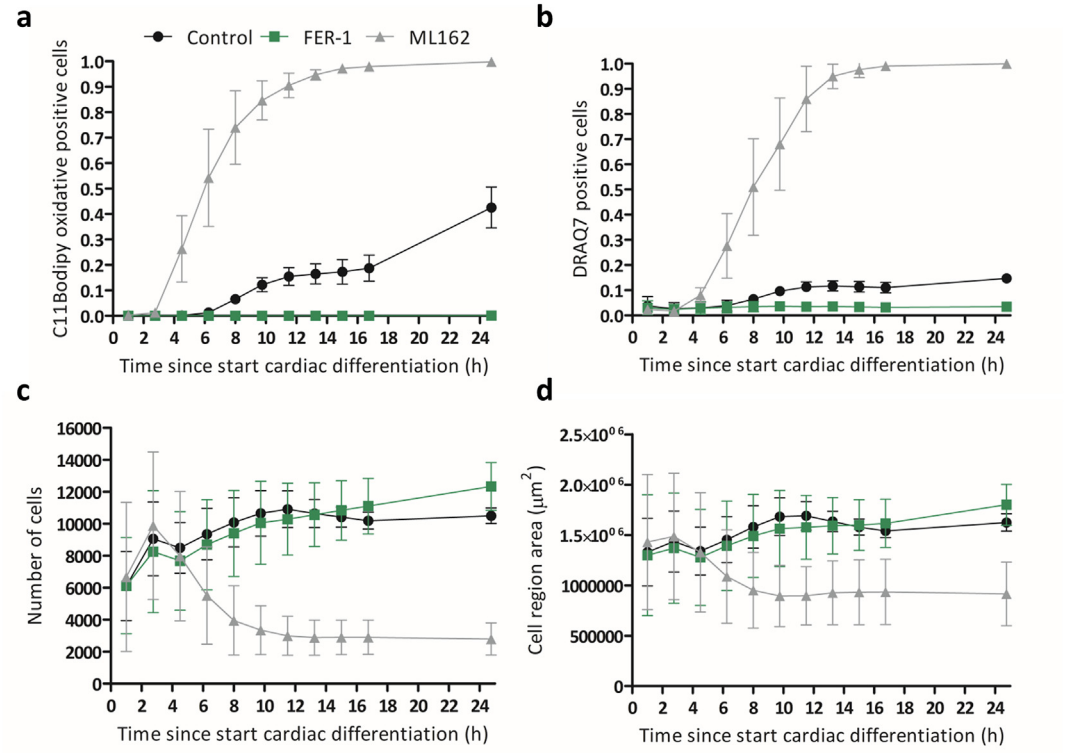
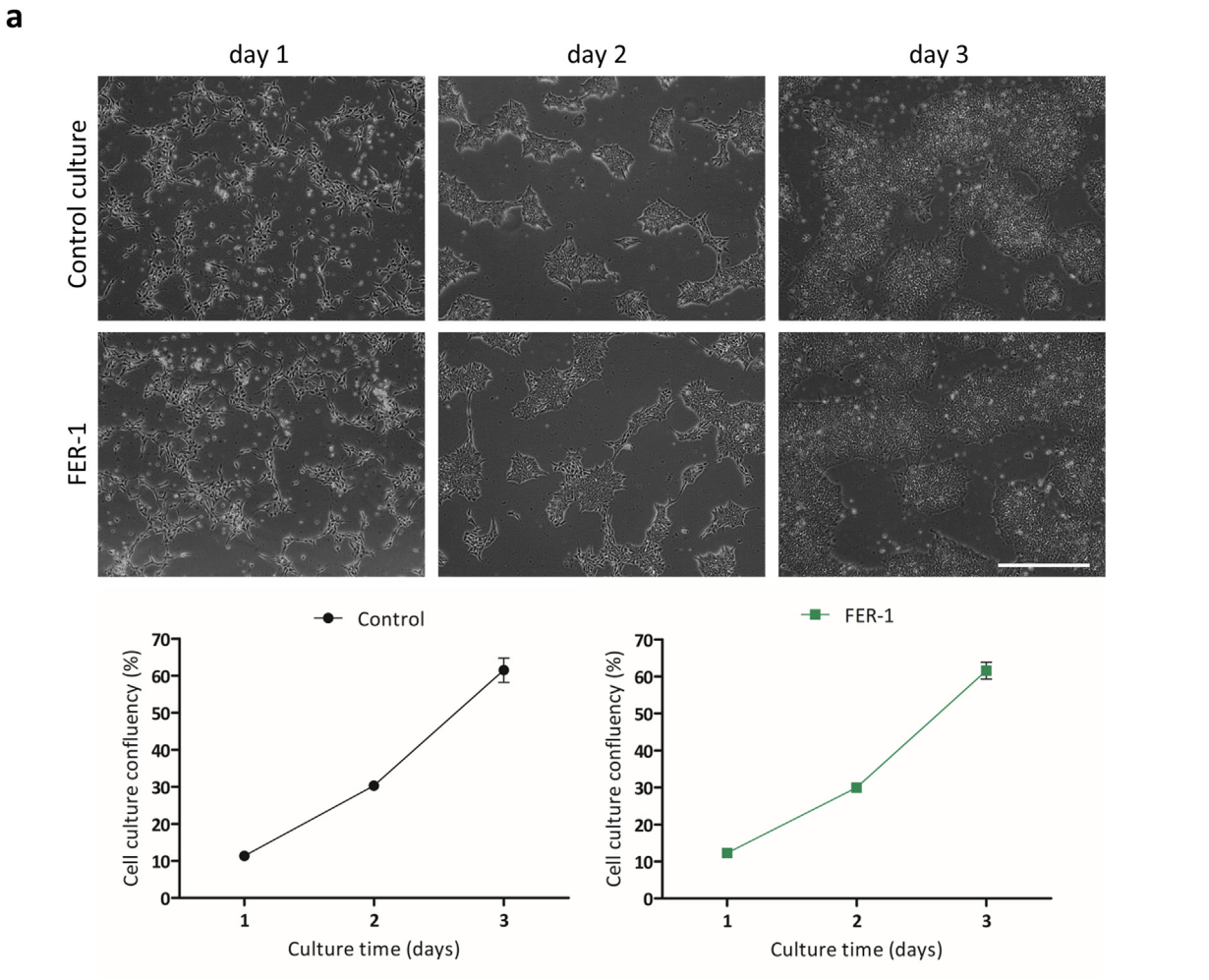


Fig. 3. Results of C11-BODIPY assay. Lipid ROS is evaluated using C11-BODIPY assay during the first 24 h of *in vitro* cardiomyocyte differentiation of H9 cells for control differentiation (black line, round), 0.5 µM FER-1 supplementation (green line, square) or 0.5 µM ML162 supplementation (grey line, triangle), 6 replicates per condition. Single-cell segmentation was used to calculate the percentage of single-cells positive for oxidation of C11-BODIPY **(a)** and positive for DRAQ7 **(b)**. The number of cells during the differentiation is also monitored **(c)**. Similarly, cell region area of the cells is determined **(d).** Error bars indicate standard deviation.


a
day 1
day 2
day 3
Control culture
FER-1
Control
FER-1
Cell culture confluency (%)
Culture time (days)
b
Control
FER-1
NANOG
Overlay
Day 1
Day 2
Day 3

and SOX2 revealed no differences (Fig. 4b and Supplementary Fig 3). These results imply that ferroptosis does not play a role during hPSC culturing.

3.5. Cardiomyocyte differentiation efficiency is enhanced by FER-1 in terms of yield

To evaluate the long-term effect of FER-1 on the *in vitro* cardiomyocyte differentiation, the hESC differentiation was monitored daily by phase-contrast microscopy for 14 days (Fig. 5). It was noted that the confluency reached a peak at day 2 for the FER-1 condition, while the control differentiation took an additional day (day 3) to reach peak confluency. This reflects the benefit of FER-1 supplementation in the first phase of *in vitro* cardiomyocyte differentiation. To assess the effect of FER-1 in the longer term, cardiomyocytes were followed up until day 15. Both conditions resulted in beating cardiomyocytes from day 7 on (Supplementary Vid 10-11). The confluency of the control differentiation decreased over time. In contrast, FER-1 supplementation in the first 48 h of the differentiation maintained high culture confluency over time, demonstrating the increased yield by using FER-1. No additional benefit was observed when FER-1 supplementation was prolonged to the first 96 h, compared to 48 h (data not shown). Also, FER-1 supplementation during only the 48–96 h time window did not increase the efficiency compared to control differentiation. This implies that the effective supplementation window for FER-1, to have a beneficial effect and increase the differentiation efficiency in terms of yield, is situated in the first 48 h of *in vitro* cardiomyocyte differentiation.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.reth.2024.02.007

3.6. FER-1 addition during cardiomyocyte differentiation does not lead to changes in sarcomere length, an intrinsic cardiomyocyte property

The generated cells by the control differentiation and FER-1 supported differentiation were confirmed to be cardiomyocytes based on positive staining for TNNT2 and NKX2.5 (Fig. 1a). The sarcomere length was measured to evaluate if the resulting cardiomyocytes from control differentiation and FER-1 supported differentiation are similar. Sarcomere length is an intrinsic property of cardiomyocyte with an important role for the contractile output of the cells. The resulting H9 cardiomyocytes were passaged to coverslips and stained for α-actinin on day 20 (Fig. 6). Based on the α-actinin staining the sarcomere length was determined, revealing that both cardiomyocyte culture conditions resulted in similar sarcomere length (approximately 1800 nm). This finding was replicated with hiPSCs (Supplementary Fig 4) which also did not show a significant difference in sarcomere length. This indicates that FER-1 does not impact the functionality of the generated cardiomyocytes.

3.7. FER-1 increases robustness of in vitro cardiomyocyte differentiation with low cell seeding densities

Regenerative applications of *in vitro* derived cardiomyocytes are limited by the large number of cells that are required. Increasing a higher functional cell yield during cardiac differentiation from fewer starting material could help to overcome this hurdle. Here, we tested if FER-1 could support differentiation of H9 stem cells with a lower seeding density, thus requiring less starting material. The standard differentiation protocol in 24-well plates requires 35.000 cells (18.500 cells/cm^2). Seeding densities of 3.500 (1.850 cells/cm^2) and 7.000 (3.700 cells/cm^2) cells were evaluated, representing respectively ten and five times less starting material, reflected in the starting confluency of 4, 10 and 70% respectively for 3.500, 7.000 and 35.000 cells at day 0 (Fig. 7). Control differentiations with 3.500 and 7.000 seeded cells failed to generate cardiomyocytes. When supplementing FER-1 in the first 48 h, successful differentiation of contracting cardiomyocytes was achieved with 7.000 seeded cells. FER-1 was not able to create a sufficient protective environment for the cardiomyocyte differentiation in the 3.500 cell seeded condition, leading to a failed differentiation. These results show that FER-1 enables successful differentiation with less starting material, increasing robustness of *in vitro* cardiomyocyte differentiation.

4. Discussion

hPSC-derived cardiomyocytes hold great potential in regenerative cardiology [16], however clinical translation is hindered by the high number of cardiomyocytes required. The aim of this study was to increase the efficiency and robustness of *in vitro* cardiomyocyte differentiation from hPSCs by identifying and blocking the cell death modality that occurs during the early stage of the differentiation. This phenomenon is commonly observed with various well-described and established directed differentiation protocols for the generation of cardiomyocytes. During the early stage of the differentiation, GSK-3 is inhibited by the small molecule CHIR99021, which is presumed to impact the cell viability. On the other hand, the occurring cell death might be intrinsic to the cardiomyocyte differentiation process itself, possibly indicating strong selective pressure. In this study, we were able to demonstrate that ferroptosis is the predominant cell death modality, responsible for excessive cell death in the early phase of cardiac differentiation (Fig. 1). Importantly, this study reports only on *in vitro* cultures with the aim to increase the efficiency of cardiac differentiation. To the best of our knowledge, ferroptosis is not yet described to play a role in the myocardial development *in vivo*.

Cell death was evaluated using the Sytox Green assay (Fig. 1) and DRAQ7 (Fig. 3). From these experiments, it can be concluded that FER-1 is able to significantly decrease cell death in the first 24 h of *in vitro* cardiomyocyte differentiation. However, Sytox Green indicates a higher percentage of cell death (80%) compared to DRAQ7 (16%) in the control differentiation. This discrepancy can be explained by the higher intensity of the fluorescent signal for Sytox Green dye compared to DRAQ7, resulting in a possible overestimation of cell death. On the other hand, DRAQ7 has been described to be less reliable, impacted by a low signal-to-noise ratio [17]. The percentage of cells positive for oxidized C11-BODIPY, an indicator of ferroptosis, in the control differentiation reached 50% (Fig. 3a) which further underlines the potential overestimation and underestimation of Sytox Green and DRAQ7, respectively.

The cell density at the start of the cardiac differentiation protocol is an important parameter. The density could dictate the cell–cycle profile of the hPSCs, which in turn impacts the optimal induction concentration of CHIR99021 [18]. Increased cell death was reported when a higher percentage of cells were in the G1 phase, associating with denser starting cultures (>90%). Also, lower

Fig. 4. Effect of FER-1 on hPSC culture. Phase-contrast images of H9 hPSC culture at three timepoints (1, 2 and 3 days after passaging of hPSCs) comparing control cultures and 0.5 μM FER-1 treatment. Scalebar indicates 500 μm. Graphs shows the culture confluency of H9 hPSC culture for control (black line, round) and FER-1 (green line, square) for 3 days of culture **(a)**. Pluripotency marker NANOG to evaluate the effect of FER-1 supplementation on H9 stem cells at day 1, 2 and 3. Control culture is compared with FER-1 (0.5 μM) supplementation. Pluripotency marker NANOG is depicted in red, nuclei are visualized with HOECHST (blue) and displayed in the overlay. Scalebar indicates 400 μm **(b)**. Error bars indicate standard deviation.

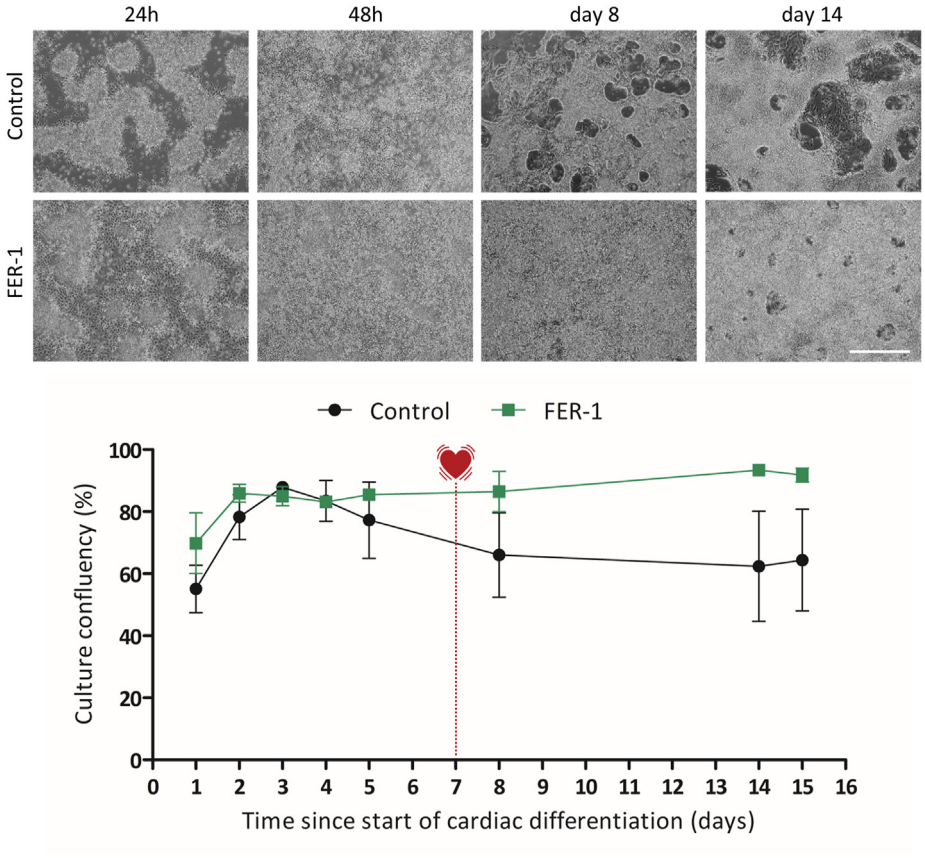


Fig. 5. Morphology of the H9 cell culture during *in vitro* cardiomyocyte differentiation. Phase-contrast microscopy images show the cell culture at different time points (24 h, 48 h, day 8, day 14) during the differentiation process for control differentiation and FER-1 supplementation (only during the first 48 h) differentiation. Cardiomyocyte contraction is observed from day 8 onward for both differentiation conditions. Scalebar, 300 μm. Graph shows the culture confluency of control and FER-1 differentiation for the first 15 days of cardiac differentiation. Error bars indicate standard deviation.

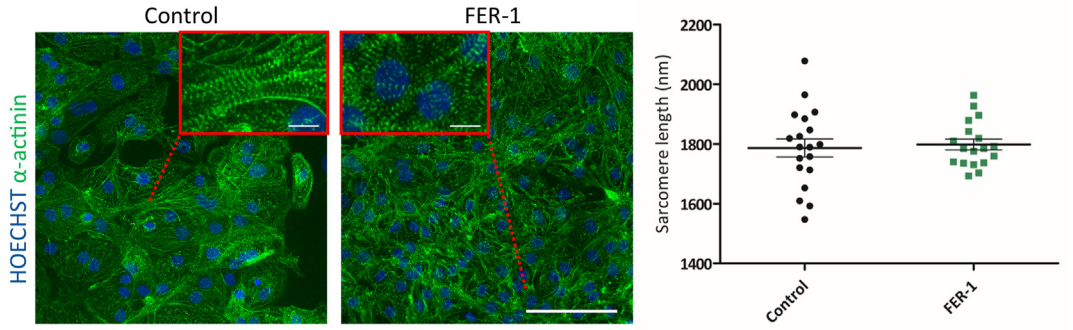


Fig. 6. Effect of FER-1 on sarcomere length of cardiomyocytes. Confocal images showing staining for α-actinin in green and the nucleus in blue for 20-day old H9 cardiomyocytes derived from the control differentiation and FER-1 supplemented differentiation. Sarcomere length is determined by α-actinin staining and shows no significant difference between the two conditions. Scalebar 100 μm, and 10 μm for the magnified inserts. Error bars indicate standard error.

concentrations of CHIR99021 were required for successful differentiation of denser starting cultures. Starting cardiac differentiation with low seeding densities has been proven challenging, resulting in low yields of cardiomyocytes [19]. We showed that by supplementing FER-1, it was possible to successfully differentiate cultures with lower seeding densities to cardiomyocytes compared to control differentiation (Fig. 7). Also, the long-term effects of FER-1 treatment in the first 48 h on cardiac differentiation show maintenance of high culture confluency, in contrast to declining culture confluency over time in the control differentiation (Fig. 5). This has especially great benefit in the field of regenerative medicine, where high numbers of cardiomyocytes are required. By minimizing the amount of failed cardiac differentiations and increasing the differentiation robustness, FER-1 could accelerate *in vitro* disease modelling. In this current study, all differentiations were performed in 2D. It still remains to be elucidated if FER-1 also has a similar impact in 3D cultures.

Mesodermal progenitors with good proliferative capacity are formed from day 0 to 2 of the cardiac differentiation protocol, during which GSK-3 is inhibited by CHIR99021. The proliferative

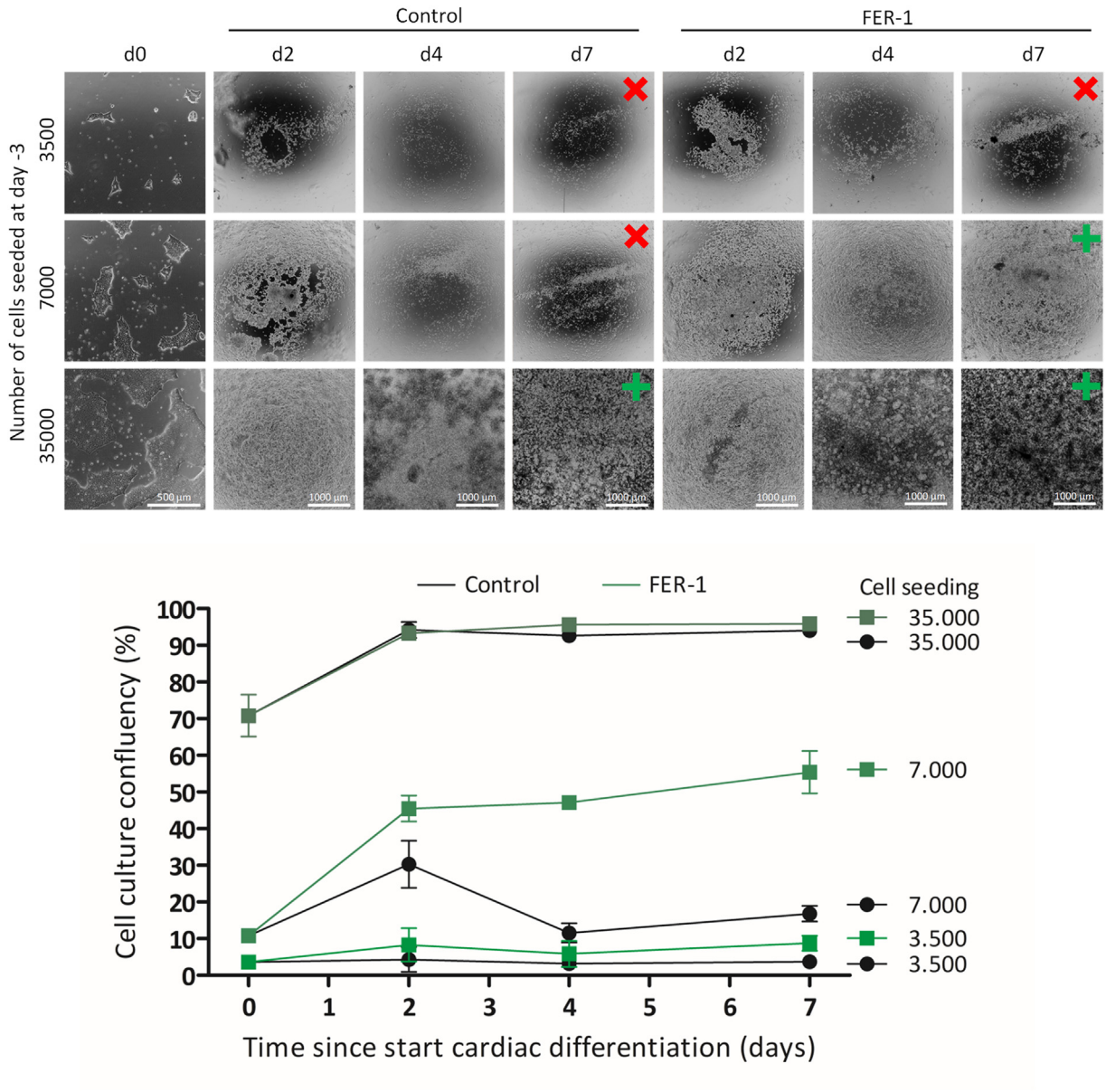


Fig. 7. Robustness of *in vitro* cardiomyocyte differentiation with low cell seeding numbers. 3.500, 7.000 and 35.000 H9 hPSCs were seeded at day −3. Phase-contrast images were taken at day 0, day 2, day 4 and day 7 of the differentiation for both control differentiation and FER-1 supplementation. Contracting cardiomyocytes were observed on day 7 for control differentiation condition with 35.000 seeded cells and FER-1 with 35.000 and 7.000 seeded cells. Successful differentiation is indicated with a green +, failed differentiation is indicated with a red x. Graph shows the culture confluency of control differentiation and FER-1 differentiation with different cell seedings (3.500, 7.000 and 35.000 cells) for the first 7 days of cardiac differentiation. Error bars indicate standard deviation.

capacity of these progenitors is in contrast with the limited proliferation of terminally differentiated cardiomyocytes. The propagation of these progenitors is more pronounced in the cultures treated with FER-1 (Fig. 5). This could point to increase of viability of this cell type specifically. The mesodermal progenitors subsequently transition into cardiac mesodermal progenitors by downregulation of Wnt via the Wnt inhibitor IWP2. In our experiments we observed that FER-1 is only effective in the specific 48 h window during CHIR99021 application. FER-1 has no effect on hPSCs (Fig. 4) or in a later phase of the differentiation (>48 h). Together, this implies that FER-1 is beneficial for cardiac progenitors specifically. On the other hand, it could be argued that the observed cell death originates from cells that did not progress into mesodermal progenitors yet and that FER-1 targets an earlier subpopulation or hPSCs that become sensitive for ferroptosis after CHIR99021 exposure. It remains to be elucidated if FER-1 supplementation could also increase the efficiency of other mesodermal and endodermal differentiation protocols, as it might have beneficial roles in early differentiation of hPSCs in general.

Other modes of cell death besides ferroptosis have been described in cardiac differentiation previously. It was reported that rapamycin was able to increase the efficiency and robustness of *in vitro* cardiomyocyte differentiation by preventing P53-mediated apoptosis and reducing mitochondrial reactive oxygen species production [9]. Without the use of rapamycin the authors only achieved low differentiation efficiencies with H9 hPSCs (less than 20% as measured by % cTnT). Another study revealed that the medium switch at induction of the cardiac differentiation induces apoptosis, rather than the WNT activator CHIR99021 [20]. The lack of bFGF in the differentiation medium, that helps hPSCs to retain

their pluripotency, was found to be responsible. Interestingly, they showed that inhibition of apoptosis by pan-caspase inhibitor Q-VD-OPH overcomes the observed cell death, however, it also caused failing of the cardiac differentiation. In our study, apoptosis was not the main cell death modality, as pan-caspase inhibitor zVAD did not decrease cell death to the extent of FER-1. We used a lower concentration of CHIR99021 for activation of WNT (4 μM compared to 7.5 μM) which may explain this discrepancy. Also, it could be that the medium is not supportive for hPSCs when deprived of bFGF and that directed cues are necessary to facilitate prompt cell transitioning. Thus, apoptosis may play an important role in cardiomyocyte differentiation when differentiation conditions are not optimal. Here, we provide evidence that mainly ferroptosis is responsible for the cell death that is observed in the early stages of an optimized differentiation protocol.

Atomic force microscopy has been employed to distinguish different cell death modalities in murine cancer cells, revealing cell shrinking and formation of circular protrusions on the membrane, pointing to destabilization of the membrane due to buildup of peroxidized lipids [21]. Another group found that the peroxisome plays a role in ferroptosis by the synthesis of polyunsaturated ether phospholipids (PUFA-ePLs) [22]. This pro-ferroptotic contribution was also found for cardiomyocytes. Interestingly, they showed that fully differentiated cardiomyocytes were more sensitive to GPX4 inhibition than cardiac progenitors, coinciding with up-regulated PUFA-ePLs compared to cardiac progenitors. In our study, we investigated the cells that give rise to cardiac progenitors, the mesodermal progenitors. In contrast to previously reported [22], inducing ferroptosis by GPX4 inhibition was not necessary in our experiments, since activation of ferroptosis occurred spontaneously as a result of the differentiation process.

5. Conclusions

Our data reveals ferroptosis as a specific cell death modality during the early stage of *in vitro* cardiomyocyte differentiation from hPSCs. Supplementation of FER-1 in the first 48 h of the differentiation protocol reduces cell death by specifically blocking ferroptosis. The supplementation of FER-1 would be an appropriate adjustment of the cardiac differentiation protocol for laboratories that aim to generate large numbers of cardiomyocytes or use cardiomyocytes in disease modelling. FER-1 increases the efficiency and robustness of the cardiac differentiation without changing intrinsic cardiomyocyte properties.

Author contributions

Author contributions: J.A., T.V.B. and J.v.H.: Study conception and design. J.A., L.L., B.H. and V.G.: Performed the experiments and analyzed the data. J.A.: Writing of the manuscript. All the authors contributed to the manuscript preparation and writing. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Experiments with hPSCs were approved by the local ethical committee of Ghent University Hospital. Project title: Cardiomyocyte models for Marfan and AVC. Approval number: UZG 2017/0855. Date of approval: 02/08/2017.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

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