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RESEARCH LETTER

A reporter system for live cell tracking of human cardiomyocyte proliferation

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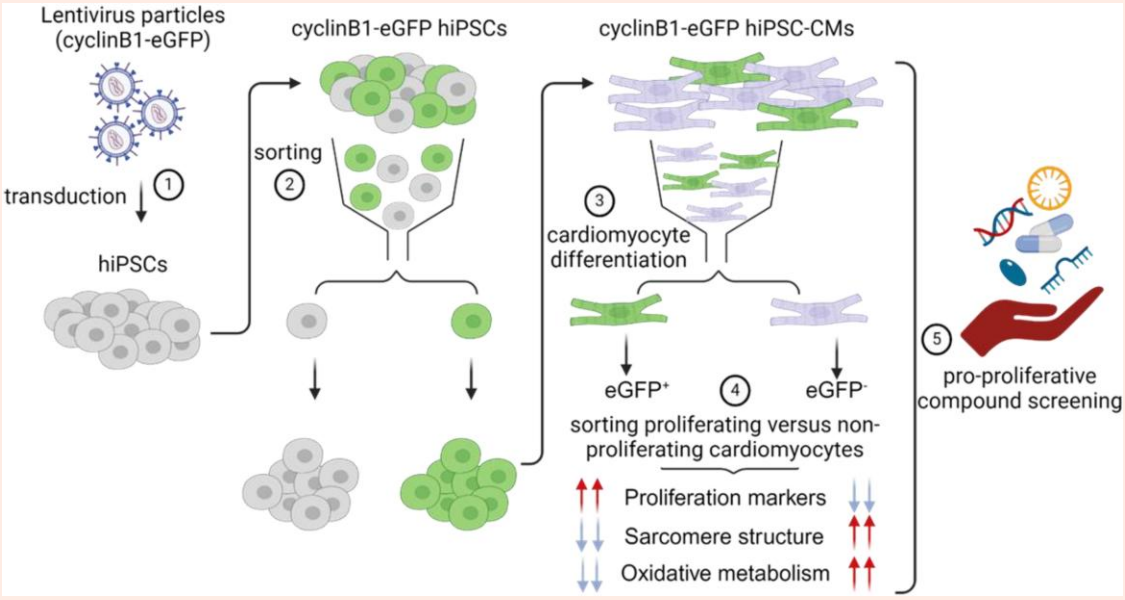
Graphical abstract



1. Introduction

As the heart develops, the majority of cardiomyocytes exits the cell cycle becoming terminally differentiated that renders the adult heart without significant regenerative capacity in response to stress and injury. Novel strategies in regenerative medicine aim to activate the very limited, but evident, regenerative potential of the heart through the stimulation of cardiomyocyte proliferation. While promising approaches have been conducted in animals, a suitable human *in vitro* model system to screen for conditions permissive for cardiomyocyte proliferation is urgently needed. In this study, we generated a human induced pluripotent stem cell (hiPSC) fluorescent reporter line that can be differentiated into cardiomyocytes to specifically

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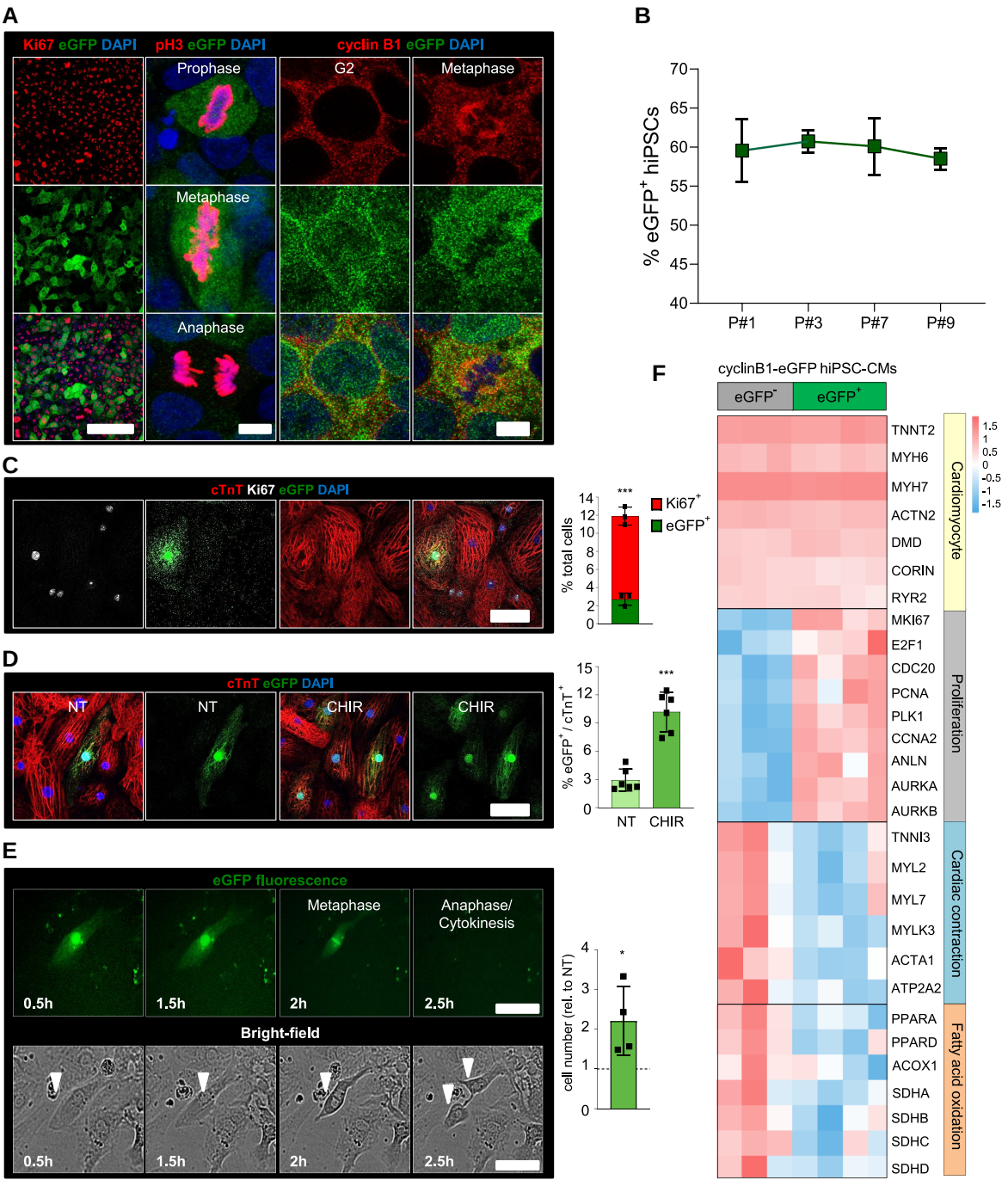

Figure 1 Generation and functional validation of a cyclinB1-eGFP reporter allowing live-cell detection of human cardiomyocyte proliferation. (*A*) Representative images of cyclinB1-eGFP hiPSCs immunostained for Ki67, or pH3 indicating different mitotic phases (prophase, metaphase, and anaphase), or endogenous cyclin B1, co-stained with eGFP and DAPI. Scale bars, 200 µm (Ki67) and 10 µm (pH3 and cyclin B1). Cyclin B1 immunostaining co-localizes with eGFP indicating overlap between the endogenous cyclin B1 and cyclinB1-eGFP construct expression profile. (*B*) Percentage of eGFP$^+$ hiPSCs determined by flow cytometry analysis at passages P#1, P#3, P#7, P#9 ($n = 1$–3; $N = 2$–5). (*C*) Purified hiPSC-CMs were stained for cTnT, Ki67, eGFP, and DAPI. Scale bar, 50 µm. Percentage of Ki67$^+$ and eGFP$^+$ cells were analysed relative to total cell count ($n = 1$; $N = 3$, 500 cells or more per condition). (*D*) eGFP and cTnT immunostaining in cyclinB1-eGFP hiPSC-CMs after treatment with 5 µM CHIR99021 for 24 h ($n = 3$; $N = 6$, 1500 cells or more per condition). Scale bar, 100 µm. (*E*) Representative time-lapse imaging of a cyclinB1-eGFP$^+$ positive, dividing cardiomyocyte and corresponding bright-field images (arrows). Scale bar, 50 µm. Fold change of total cell count relative (rel.) to non-treated (NT) cells (set to 1, dotted line) 24 h after 5 µM CHIR99021 stimulation in cyclinB1-eGFP hiPSC-CMs ($n = 4$; independent differentiations). (*F*) Heatmap of marker gene expression for cardiomyocytes, proliferation, cardiac contraction, and fatty acid oxidation between cyclinB1-eGFP$^+$ hiPSC-CMs ($n = 4$; independent differentiations) and cyclinB1-eGFP$^-$ hiPSC-CMs ($n = 3$ independent differentiations). *N* denotes technical replicates and *n* denotes biological replicates. Data are presented as mean ± SD and were analysed by using unpaired, two-tailed Student's *t*-test. *$P < 0.05$; ***$P < 0.0001$.

track and sort replicating living cells. Indeed, quantifying proliferating cells remains a bottleneck for high-throughput screenings, as mainly colorimetric or immunofluorescence based assays are currently available.[1] These methods are labour-intensive and require cell fixation, thus precluding the isolation of live replicating cells for further studies. Live-cell labelling using endogenous proteins that are specifically expressed during the cell cycle, fused to a fluorescent marker protein, would immensely facilitate screening approaches and would allow sorting of proliferating cells for downstream mechanistic studies (e.g. OMICS approaches). Therefore, we developed a hiPSC reporter cell line that can be used to derive any cell-type of choice, including cardiomyocytes. This tool can be utilized as an *in vitro* screening platform for potential pro-regenerative drugs or conditions aiming at stimulating endogenous cardiomyocyte proliferation and ultimately heart regeneration.

2. Methods and results

To generate a reporter cell line that allows live assessment of proliferating cardiomyocytes, we used a previously published reporter construct consisting of a constitutive human phosphoglycerate kinase promoter driving an eGFP, fused with a regulatory fragment of the cyclinB1 that is detected by the APC/C E3 ubiquitin ligase.[2] Therefore, the cyclinB1-eGFP is specifically expressed during the S/G2 and M phases of the cell cycle and proteasomally degraded during the transition from metaphase to anaphase when the APC/C complex is activated. An hiPSC line from a healthy donor (MHHi001-A)[3] was transduced with cyclinB1-eGFP lentiviral particles at the MOI of 15. Fifteen days after transduction, eGFP positive ($eGFP^+$) cells were isolated by fluorescence-activated cell sorting (Aria Fusion System; BD Bioscience), re-sorted, and replated, generating the polyclonal cyclinB1-eGFP hiPSCs reporter line (four integration events). Since lentiviral transgene silencing is a common feature of hiPSCs, cyclinB1-eGFP expression was monitored over several passages by flow cytometry that revealed a constant proportion of $eGFP^+$ cells for a minimum of nine passages, confirming a stable expression of the reporter construct (*Figure 1B*). Immunofluorescence staining, flow cytometry, and RT-qPCR were used to demonstrate that lentiviral transduction and extended passaging did not compromise the pluripotency and trilineage differentiation potential of the reporter hiPSC line. The specificity of the cyclinB1-eGFP reporter to accurately label proliferating cells was confirmed by co-staining with common markers of proliferation such as Ki67, pH3, as well as endogenous cyclinB1 (*Figure 1A*). Next, a standardized protocol[4] was applied to differentiate the hiPSC line into cardiomyocytes (cyclinB1-eGFP hiPSC-CMs). To obtain a pure population, these cardiomyocytes were subjected to metabolic selection and maturation (≥60 days). Confocal microscopy further confirmed cardiomyocyte purity and maturity indicated by organized sarcomeric structures as shown by cardiac troponin T (cTnT) immunostainings (*Figure 1C*). Importantly, all $eGFP^+$ cardiomyocytes were additionally $Ki67^+$, while the percentage of $Ki67^+$ cardiomyocytes ($11.89\% \pm 1.0$) strongly exceeded the number of $eGFP^+$ cells ($2.69\% \pm 0.67$), concluding that cyclinB1-eGFP has a superior specificity in labelling proliferating cardiomyocytes (*Figure 1C*). To demonstrate that the cyclinB1-eGFP reporter can be utilized to reliably screen cardiomyocyte proliferation-inducing agents, the proliferative capacity was assessed in response to the well-known pro-proliferative Wnt pathway activator, CHIR99021.[5] Mature cardiomyocytes (differentiation day ≥ 60) were treated with 5 μM CHIR99021 for 24 h that led to a significant increase in the number of proliferating cardiomyocytes, analysed by $eGFP^+$ and $cTnT^+$ immunofluorescence staining (*Figure 1D*), combined with increased mRNA expression of eGFP and cell cycle markers (not shown). To further verify true cardiomyocyte division, in contrast to potential bi-/polynucleation, live-cell time-lapse imaging was performed. CyclinB1-eGFP cardiomyocytes were pre-treated with CHIR99021 for 8 h and then monitored for 16 h with images captured every 40 min. Potential dividing cardiomyocytes expressed eGFP in the first phases of mitosis until metaphase, and then lost eGFP expression during anaphase as expected according to the regulation of cyclinB1 (*Figure 1E*). Parallel bright-field images confirmed that these cells gave rise to two daughter cardiomyocytes, which is further confirmed by increased cardiomyocyte cell count (*Figure 1E*). Finally, to gain insight into the transcriptional changes underlying human cardiomyocyte proliferation, whole transcriptome sequencing was performed after large scale cardiomyocyte differentiations and sorting into proliferating cyclinB1-$eGFP^+$ hiPSC-CMs ($2.2\% \pm 1.2$) and non-dividing cyclinB1-$eGFP^-$ hiPSC-CMs. Gene expression analysis showed that both cell populations ($eGFP^+$ and $eGFP^-$) abundantly express cardiomyocyte marker genes (*Figure 1F*). Gene set enrichment analysis (GSEA; v3.0 Broad Institute, Massachusetts Institute of Technology) revealed a significant up-regulation of genes involved in cell cycle progression and mitosis while simultaneously showing a significant down-regulation of genes involved in cardiac muscle contraction, oxidative phosphorylation, and fatty acid metabolism for the cyclinB1-$eGFP^+$, proliferating cardiomyocytes (*Figure 1F*).

3. Discussion

The drastic decrease of heart function after a myocardial infarction is mainly attributable to the massive loss of cardiomyocytes which the adult mammalian heart is incapable of regenerating. Aside from replacing lost contractile tissue through cell and tissue therapy approaches,[6] an alternative strategy for heart regeneration is to stimulate the low but existing intrinsic proliferative capacity of cardiomyocytes.[7] Our study proposes a reliable strategy to identify and isolate living cardiomyocytes undergoing mitotic activity and cell division. To date, the majority of studies focusing on cardiomyocyte proliferation has used animal models mainly in combination with static immunostainings of proliferation markers. However, the development of defined protocols for cellular reprogramming, genetic manipulation, and directed differentiation of hiPSCs heralded a new era in regenerative medicine enabling the production of human cardiomyocytes with ease.[8] These cardiomyocytes are a highly versatile tool for developing new strategies and identifying new targets for cardiac regeneration.

Here, we generated and characterized a cyclinB1-eGFP reporter cell line that represents a powerful tool to track and/or isolate proliferating live cells. Of note, there are other proliferation reporters, e.g. the dual FUCCI system using which cycling and non-cycling cells can be distinguished.[9] Nevertheless, the ubiquitous PGK promoter in our reporter allows its use in hiPSCs and any cell-type derived thereof. In addition, the eGFP expression is regulated precisely as endogenous cyclin B1, specifically allowing to monitor the transition from S to G2 to M phase of the cell cycle. Here, we differentiated the reporter hiPSCs into cardiomyocytes and showed that the cyclinB1 regulated eGFP efficiently labels proliferating cells far more specifically compared to the commonly used Ki67 marker. Ki67, which is used as a classical proliferation marker, is expressed in all the phases of the cell cycle, both in interphase (G0/1, S, and G2) and mitosis. Thus, Ki67 has limited specificity in terms of detecting mitotic/dividing cardiomyocytes because expression in the S phase does not necessarily predict completion of cell division, especially as polynucleation is frequently found in maturing cardiomyocytes. Interestingly, transcriptome profiling revealed that cardiomyocyte proliferation requires metabolic reprogramming and structural changes, i.e. a certain degree of de-differentiation that is in agreement with previous reports on regenerating cardiomyocytes in zebrafish and mice.[10] Importantly, we demonstrate the utility of the reporter line for live-cell drug screening approaches that specifically target cardiomyocyte proliferation, highlighting the potential of this platform to identify novel drugs for cardiac regenerative strategies (*Graphical abstract* Created with Biorender).

Conflict of interest: T.T. is founder and CSO/CMO of Cardior Pharmaceuticals GmbH, a wholly-owned subsidiary of Novo Nordisk Europe A/S (outside this manuscript). All other authors declared no conflict of interest.

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Data availability

RNA sequencing data and the reporter cell line will be available to the scientific community upon reasonable request.

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