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Circadian networks in human embryonic stem cell-derived cardiomyocytes

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REFEREE REPORTS

Reviewer #1:

In the manuscript entitled "Functional implications of circadian networks in human embryonic stem cell-derived cardiomyocytes" by Dierickx et al., the authors investigate the global gene expression during in vitro differentiation of human embryonic stem (ES) cells into cardiomyocytes. Especially, temporal gene expression profiles are analyzed and they find that undifferentiated human ES cells do not possess a functional circadian clock. On the other hand, day 30 differentiated cardiomyocytes show robust expression rhythms of clock genes. These findings are well compatible with recently reported cellular differentiation-coupled circadian clock developments in mouse ES cells. The authors clearly show that the circadian clock functionality closely correlates with cellular differentiation and cell fate also in humans. Moreover, the authors describe that a stressrelated oscillatory network of genes is established after differentiation into cardiomyocytes, which elicits a time-dependent responsiveness to doxorubicin. Since human ES cell-derived cardiomyocytes are available for disease models and regenerative therapies, the authors propose that the in vitro differentiation culture system using human ES cells can be deployed to test the effectiveness of circadian clock-based medicine to prevent cardiotoxic side effects and timed cell-based therapy. The reviewer thinks that the study includes important findings, especially for cellular differentiation-coupled circadian clock development in human ES cells, and the oscillatory expression network of stress response genes is established cell autonomously.

Moreover, the stress response reaction fluctuates circadianly, suggesting the time-dependent reduction of the cardiotoxic side effects by doxorubicin. In addition, the reviewer thinks that

transition of oscillatory expression network of clock-controlled genes observed during D30 to D45 may recapitulate the dynamic change of cellular physiology such as metabolisms during the perinatal cardiomyocytes in vivo.

Major comments:

1) At first, the authors should describe the origin of the human ES cells used in this study.
2) In Figure 2A, expression data of other core clock genes should be presented as well as BMAL1 and PER2.
3) In Figure 2B, the immunofluorescence results are unclear. The signals are similarly detected in all examined proteins. The authors should present the specificity of the antibodies used in this experiment. At least, the authors should present Western blotting data. For example, PER2 mRNA is dramatically induced during the differentiation culture of human ES cells (Figure 2A). A time-course Western blotting analysis using hES cells, D15, D30 and D45 cells would be very helpful to understand the expression patterns of the core clock proteins.
4) In Figure 3F and G, the phases of bioluminescence rhythms driven by Bmal1-dLuc in D30 and D45 are different despite the fact that the same reporter is used. The authors should explain why D30 and D45 cells show different (nearly anti-phase) Bmal1-dLuc rhythms.
5) In Figure 3F and G, the FACS data should be presented and the gate sorting of the cardiomyocytes should be displayed. The reviewer also recommends for the percentages of cardiomyocytes to be described.
6) In Figure 4, gene expression networks in D30 and D45 cells are dramatically changed (ex, ~90% of rhythmic genes are different between D30 and D45). Does this dramatic change of clock-controlled genes from D30 to D45 affect to any functions of cardiomyocytes? For example, the electrophysiological study would be helpful to understand the implications of dramatic change of clock-controlled gene expression network during the differentiation.
7) Recently, Zhang et al. reported that ~6% of genes showed circadian expression patterns in 7-week-old mice hearts (Zhang et al., PNAS, 2014). The authors described that 786 and 531 genes are rhythmically expressed in D30 and D45 cardiomyocytes, which differentiated from human ES cells, respectively. Are there any common rhythmically expressed clock-controlled genes between mouse hearts and human ES cell-derived cardiomyocytes?

Minor Comments:

1) Figure 1C, the gene names should be indicated in italics.
2) The authors should explain "CEL-seq".
3) The sequences of primers used in qPCR should be described.
4) Which is more physiologically intact between D30 and D45 cardiomyocytes? In Figure 5C, circadian fluctuation of Caspase 3/7 activation in D45 cells is more blunt than D30 cells (see Figure 5B).

Reviewer #2:

In this study, Dierickx et al study the role of circadian clock networks in human ESCderived cardiac differentiation to determine if patterns of cyclic gene expression and cell function recapitulate clock patterns known to occur in vivo. They find that circadian rhythms of key clock genes are not differentially regulated in undifferentiated cells in keeping with prior studies. Circadian patterns of gene expression emerge during differentiation as cardiomyocytes mature. The data suggest that these rhythms impact drug screening and physiological assays of cardiomyocytes.

Major Concerns:

1) The authors suggest in the text that they have thoroughly vetted the cardiac purity and efficiency of cardiomyocytes based on gene expression of NKX2-5, cTnT, FACS, and clear sarcomere structures by IHC. None of this data is provided in the available data sets. In figure 1, please provide FACS plots of cTnT purity from differentiation. IHC in Figure 1 and Supplemental Fig 1 isn't sufficient. Although the use of NKX2-5-GFP cells was used, sorting wasn't performed until day 26 according to the methods. What is the purity prior to sorting since this may substantially impact the interpretation of results for D15 cells vs D30 or D45 cells? The significant batch-batch variation in

differentiation as noted in the RNA-seq data makes this particularly concerning.

2) For IHC, please provide close up images with evidence of sarcomeres and gene expression analysis should include temporal dynamics of well-established genes (NKX2-5, TBX5, GATA4, MYH6, TNNI3) to give a clear indication of temporal dynamics of these known cardiac markers. The authors suggest that clock activity between D15-30-45 is a consequence of increased maturity but this needs to be much more substantially validated through proper gene expression analysis, morphometry (cell size, sarcomere length analysis) to show a substantial change in maturation particularly between D15 and D45. Issues of variation in purity between these time points as noted above need to be addressed as a confounding variable.

3) The authors report that clock activity emerges between D30-D45. Unfortunately, they fail to provide D15 data as an excellent negative control for these studies to show that phenotypes attributed to clock activity are not present at D15 but appear at later stages of maturation. The better comparison in these assays would have been D15 vs D45. This includes the RNA-seq analysis of cycling genes (Figure 4). It also includes experiments performed in Figure 5 where D15 analysis of cardiac toxicity and chronotropy would be an important negative control to attribute these findings specifically to the emergence of clock activity during maturation.

4) The addition NE to elicit an inotropic response seems unnecessary to study the circadian beating rate. Attributing the cycling activity of beating rate to changes in PLN is quite speculative without gain/loss of function studies. It would be more convincing if there was evidence of cycling of PLN at the protein level within this time frame and if the cycling activity of beating rate could be eliminated by shRNA-knockdown or stable overexpression of PLN (or related experiment).

5) The study lacks cohesive mechanistic data. It would be valuable to tie assays in fig 5 (toxicity and beating rate) to the finding in Fig 4 that UBC is a nodal protein in regulating circadian rhythm activity in cardiomyocytes. Is UBC protein abundance similarly regulated in a cyclic manner? Are cardiac toxicity and chronotropic phenotypes lost by stabilized expression of UBC?

Minor Comments:

1) Was an FDR cutoff threshold implement for GO analysis - should only use GO categories for which FDR<0.05
2) Data in Figure 5F have no error bars - provide this assuming the analysis was done more than once.
3) Figure 2 should provide more substantial data on clock activity in pluripotent stem cells using tools already described in the study. Please include data using dLuc clock reporters to clearly show no activity in undifferentiated cells. Since this figure is about undifferentiated cells, I suggest moving Panel A to the beginning of Figure 3.
4) The authors provide no data on patterns of gene expression or reporter activity prior to synchronization. Can you please take D0, D15, D30, and D45 cells with the dLuc reporters and generate examples of clock activity prior to and after synchronization to provide clear evidence that clock activity is dependent on synchronization and only emerges in mature cardiomyocytes.

Reviewer #3:

This manuscript is aimed at charactering the emergence of circadian rhythm during myocardium differentiation using human ES cell differentiated cardiomyocyte model. It also attempted to demonstrate the functional rhythmicity in response to harmacological agents, such as doxorubicin and phenylephrine.

It is known that ES cells do not have circadian rhythmicity but when and how the circadian rhythm gets established during cardiac development is unclear and the impact of this on cardiac diseases or the modeling of cardiac diseases and regeneration was unexplored. The authors showed an induction and oscillation of PER2 expression at RNA level at D30, which precedes the oscillatory expression of BMAL1 at RNA level. This parallels the previously reported observation in mouse ESC during induction with RA. Interestingly, both BMAL1 and PER2 promoter driven luciferase reporter showed antiphasic oscillation at D30 (Figure 3B and 3D). What is the explanation? Likely the

oscillatory transcription in blunted by a slow degradation. Does this occur at prior to Day 30, e.g. at Day 15? How does the cell overcome this? More work is required to elucidate the possible mechanism for the emergence of circadian rhythmicity. And finally, if these cells are depleted of BMAL or Per, is there an effect on cardiac gene expression, differentiation, or the response to doxorubicin and/or norepinephrine? It is commendable that the authors did single cell analysis on Per2-luc, however only 4 cells were observed. It is important to observe more cells on more time points. Does the emergence of rhythmicity reflect heterogeneity in maturation? Or are there pioneer cells that establish rhythms first? What is the process of gaining rhythmicity, a gradual increasing in amplitude? Many more questions can be answered by simply increasing the number of observation.

The CEL-seq at D30 and D45 showed very small overlap in oscillating genes, which may be a true observation that the circadian clock regulates different subsets of genes at different developmental stage or may be an artifact due to low sequencing depth and extensive multiplexing. I agree with focusing on the common 62 genes and discuss the above possibilities if deeper sequencing is impossible in the scope of the current study.

The circadian response to doxorubicin and PE are highly interesting, but need to be further substantiated. Figure 5D (and also Figure 4E) needs to be validated by RT-PCR. It is unclear why D30 and D45 have responses that are in different phases. The NE response in Figure 5F should present cumulative data with statistics.

Minor Comments:

The authors used several different statistical analyses, including Rain, JTK cycle and 2- way ANOVA, to infer circadian rhythmicity. As they each have different sensitivity, it is best to use either Rain or JTK cycle for all analysis. Figure 5B and 5C should be analyzed with Rain or JTK cycle.

Authors' response

We thank the reviewers for the thorough and thoughtful assessment of our manuscript. They raised some important points, which we think we were able to address. Please find below a general outline of key changes that we made to the manuscript and on the subsequent pages a point-by-point outline of how we have addressed the individual questions raised by the reviewers. We feel that the suggested changes and added data have made the manuscript much stronger indeed and hope that you'll be able to consider it for publication.

The most important additions in the revised manuscript include the following items:
The analysis of clock gene mRNA and protein in human ES cells is substantially expanded by comparing gene expression and protein presence between human ES cells and differentiated U2OS cells that are known to harbor a functional clock.

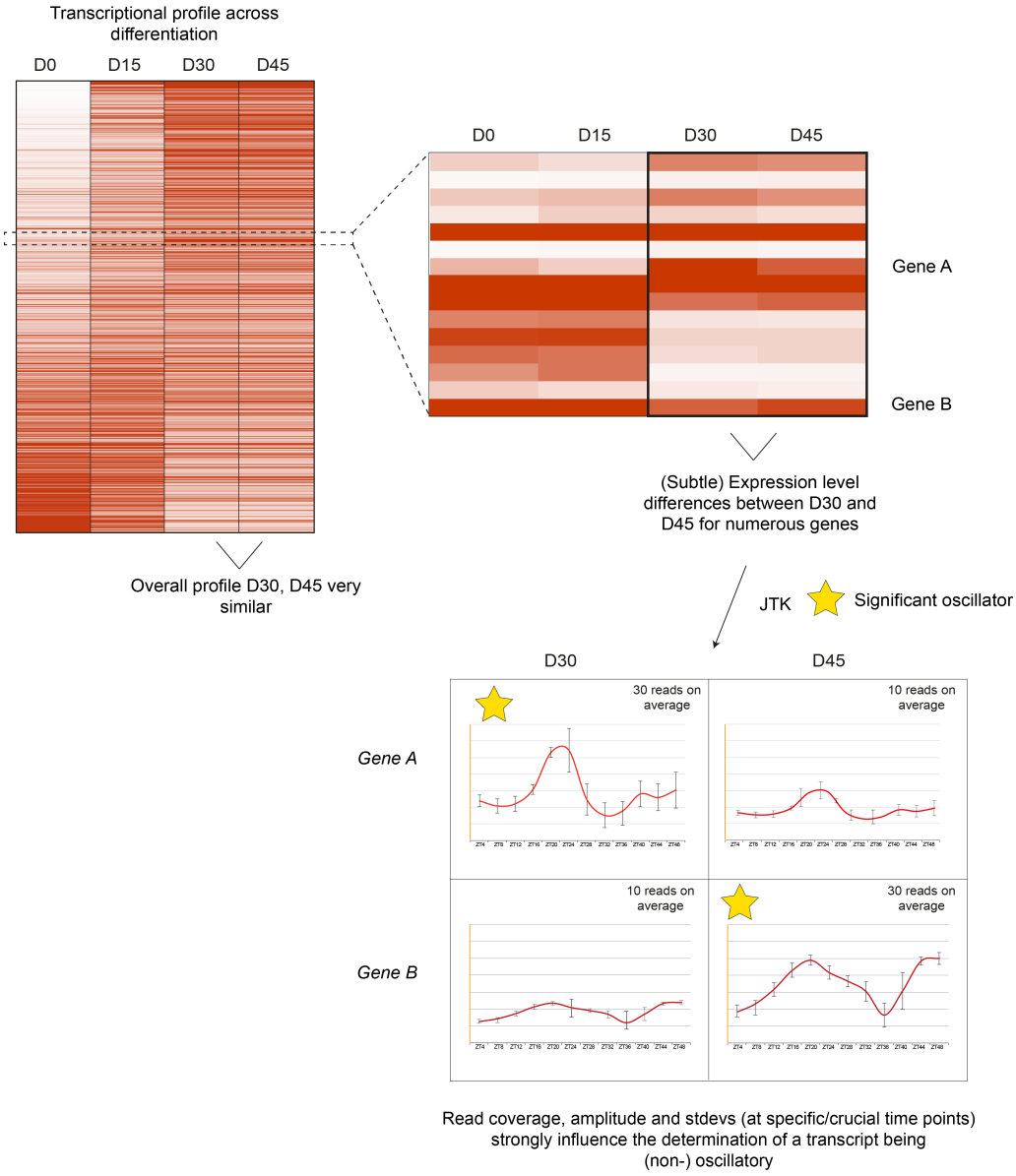
As proposed by all reviewers, differentiation day 15 (D15) during directed cardiomyocyte differentiation is now analyzed. In the previous manuscript we did not perform in depth (sequencing and reporter) assays for D15 human ES cell-derived cardiomyocytes, because of a lack of significant oscillatory clock gene behavior. We thank the referees for pointing this out. Adding these analyses has provided important insights into the origination of circadian rhythmicity as Per2-luciferase reporter experiments revealed upcoming rhythmicity at D15. The presence of an intermediate clock has prompted us to perform CEL-Seq mRNA sequencing across 48 hours at D15, and these additional results are now described in the revised manuscript.

Additionally, we would like to stress that all levels of significance for phasing are calculated across 48 hours (the wave as a whole and not at single time-points). This is now indicated more clearly in all figure panels.

In the first version of the manuscript shared oscillators between D30 and D45 human ES cell-derived cardiomyocytes were found to contain a stress-response network that is centered on UBC. Nonetheless, most of the other oscillators were specific for either D30 or D45. As described by others, the identification of oscillators depends largely on sequencing depth and read coverage per

transcript (Li et al. 2015). While the global transcriptional program between D30 and D45 cardiomyocytes is similar, (subtle) read coverage differences between these stages partially underlie the limited overlap between oscillators (Explanatory Figure-Rebuttal). In the new manuscript, we perform an in depth analysis on read coverage and rhythmicity detection between D15 and D30. Indeed, genes that are more highly expressed are more often detected as significant oscillators. This explains previously observed differences between D30 and D45, but as these differences were a source of confusion (and not the focus of the manuscript), we have decided to focus on D30 in the revised manuscript. Importantly, the stress-response network around *UBC* that is still described in the manuscript for D30, was previously found to be present at both D30 and D45. In addition, we compared our data sets with mouse heart oscillators (as proposed by reviewer 2) and *UBC* is one of the numerous overlapping oscillators between human ES cell-derived cardiomyocytes and mouse hearts, further highlighting the relevance of this network and more general of the data that is described here.

Explanatory Figure: Rebuttal



Reviewer #1

In the manuscript entitled “Functional implications of circadian networks in human embryonic stem cell-derived cardiomyocytes” by Dierickx et al., the authors investigate the global gene expression during in vitro differentiation of human embryonic stem (ES) cells into cardiomyocytes. Especially, temporal gene expression profiles are analyzed and they find that undifferentiated human ES cells do not possess a functional circadian clock. On the other hand, day 30 differentiated cardiomyocytes show robust expression rhythms of clock genes. These findings are well compatible with recently reported cellular differentiation-coupled circadian clock developments in mouse ES cells. The authors clearly show that the circadian clock functionality closely correlates with cellular differentiation and cell fate also in humans. Moreover, the authors describe that a stress-related oscillatory network of genes is established after differentiation into cardiomyocytes, which elicits a time-dependent responsiveness to doxorubicin. Since human ES cell-derived cardiomyocytes are available for disease models and regenerative therapies, the authors propose that the in vitro differentiation culture system using human ES cells can be deployed to test the effectiveness of circadian clock-based medicine to prevent cardiotoxic side effects and timed cell-based therapy.

The reviewer thinks that the study includes important findings, especially for cellular differentiation-coupled circadian clock development in human ES cells, and the oscillatory expression network of stress response genes is established cell-autonomously. Moreover, the stress response reaction fluctuates circadianly, suggesting the time-dependent reduction of the cardiotoxic side effects by doxorubicin. In addition, the reviewer thinks that transition of oscillatory expression network of clock-controlled genes observed during D30 to D45 may recapitulate the dynamic change of cellular physiology such as metabolisms during the perinatal cardiomyocytes in vivo.

We are happy to see that reviewer #1 underwrites the importance of our study, especially for cellular differentiation-coupled circadian clock development in human ES cells, and the oscillatory expression network of stress response genes that is established cell-autonomously. We additionally compared D15 with D30 cardiomyocytes, which represent two transcriptionally different cardiomyocyte stages.

Major comments:

At first, the authors should describe the origin of the human ES cells used in this study.

We have now included the origin of the human embryonic stem cells that were used in the methods section, and refer to the study in which they were first derived in the main text and methods section. The *Nkx2.5-eGFP* reporter line is a homologous recombinant line (Elliott et al. 2011) derived from wild-type HES-3 cells (Reubinoff et al. 2000). In addition, Figure 1A now contains the human ES cell background and includes a timeline depicting the directed differentiation protocol that is used.

In Figure 2A, expression data of other core clock genes should be presented as well as BMAL1 and PER2.

We have expanded our analysis of clock gene expression to also include *CRY1*, *CRY2*, *CLOCK*, and *NR1D1* in Figure 2A. mRNA levels of *BMAL1*, *PER2*, *CRY1*, *CRY2*, *CLOCK*, and *NR1D1* were analyzed in both ES and U2OS cells (a somatic cell type with a well-characterized functional clock), demonstrating clear difference in expression level of key clock genes between these two cell types. To better understand the stoichiometry of clock factors between stem cells and differentiated cells, we have analyzed BMAL1, CRY1 and CLOCK expression at the protein level as well, and have included comparisons between human ES cells and U2OS cells. In addition, we have added analysis of *CLOCK* mRNA levels in the comparison between D0, D15 and D30 human ES cell-derived cardiomyocytes in Figure 3A.

In Figure 2B, the immunofluorescence results are unclear. The signals are similarly detected in all examined proteins. The authors should present the specificity of the antibodies used in this experiment. At least, the authors should present Western blotting data. For example, PER2 mRNA is dramatically induced during the differentiation culture of human ES cells (Figure 2A). A time-course Western blotting analysis using hES cells, D15, D30 and D45 cells would be very helpful to understand the expression patterns of the core clock proteins.

We thank the referee for pointing this out. We agree and have therefore replaced the immunofluorescence images with Western blot analysis, which is more quantitative. Figure 2B now clearly demonstrates comparative analysis of BMAL1, CRY1, and CLOCK protein levels between stem cells (ES cells) and somatic cells (U2OS).

In Figure 3F and G, the phases of bioluminescence rhythms driven by Bmal1-dLuc in D30 and D45 are different despite the fact that the same reporter is used. The authors should explain why D30 and D45 cells show different (nearly anti-phase) Bmal1-dLuc rhythms.

We overlapped both graphs in Figure 3F and G and noted a small difference in phase (~2-3hrs) for single cells experiments in two different cardiac stages. This is probably do to timing of the start of the real-time experiments, and does not represent inherent different phasing. Indeed, as measured on a population scale with LumiCycle we demonstrate the same phasing for both cardiac stages.
Additionaly, to independently confirm the D30 reporter assays in pure populations, we included extra reporter-based experiments in isolated human ESC-derived cardiomyocytes and noted clear anti-phasic oscillations in Bmal1 and Per2-Luc bioluminescence. In Figure 2-figure supplement 1, we show that these phases are similar to the phasing noted in U2OS cells, demonstrating correct response to synchronization, and similar oscillations in reporter gene expression.

In Figure 3F and G, the FACS data should be presented and the gate sorting of the cardiomyocytes should be displayed. The reviewer also recommends for the percentages of cardiomyocytes to be described.

We have now included this FACs data in Figure1-figure supplement 1. A 50% purity, based on cardiac troponin T as a marker for differentiated cardiomyoyctes, is observed, which is in line with previously reported results (Schwach & Passier 2016). For our RNA sequencing experiments, we opted for a strategy that does not interrupt a possible clock. We therefore did not sort for 100% pure cardiomyocytes, and belief this is a better representation of the in vivo situation since human hearts consist of a mixed population of myocytes (~30%) and non-myocytes (~70%) as well (Nag 1980; Zak 1974).

In Figure 4, gene expression networks in D30 and D45 cells are dramatically changed (ex, ~90% of rhythmic genes are different between D30 and D45). Does this dramatic change of clock-controlled genes from D30 to D45 affect to any functions of cardiomyocytes? For example, the electrophysiological study would be helpful to understand the implications of dramatic change of clock-controlled gene expression network during the differentiation.

As stated above and graphically explained by the Explanatory Figure-Rebuttal, we realized that the differences we observed between D30 and D45 were largely a consequence of transcript abundance, assessed via CEL-Seq and may not reflect a true biological difference between these two time points. Indeed, the overall transcriptome between these two time points does not change significantly, as now shown in Figure 4-figure supplement1.
The stress network that is centered on UBC was robustly found in both differentiation time points and we demonstrate the functional circadian effect of doxorubicin on human ESC-derived cardiomyocytes. In addition, as suggested by all three referees, we sequenced D15 human ES cell-derived cardiomyocytes as well and demonstrate that while, at this early differentiation time point, some of the UBC network genes are already expressed, the oscillatory *UBC* centered network is not yet established.

Recently, Zhang et al. reported that ~6% of genes showed circadian expression patterns in 7-week-old mice hearts (Zhang et al., PNAS, 2014). The authors described that 786 and 531 genes are rhythmically expressed in D30 and D45 cardiomyocytes, which differentiated from human ES cells, respectively. Are there any common rhythmically expressed clock-controlled genes between mouse hearts and human ES cell-derived cardiomyocytes?

We thank the referee for this valuable suggestion and retrieved the data from Zhang et al. (Zhang et al. 2014) for comparison with oscillators found in the murine heart, which we have now included in Figure 4. We identified a ~18% overlap between mouse hearts and human D30 cardiomyocytes and, importantly, noticed that *UBC*, the central player of our stress network at D30 follows a circadian expression pattern in murine hearts as well. This suggests that the oscillatory stress network is

conserved between these two species (Figure 4B). In addition, our study identifies several human-specific oscillators with important known roles in cardiac physiology, which highlights the additional insights that can be gained from these data sets, especially in relation to human-related heart processes or phenotypes.

Reviewer #1 (Minor Comments):

Figure 1C, the gene names should be indicated in italics.

We adjusted gene names according to the reviewer's suggestion.

The authors should explain "CEL-seq".

We have now described the CEL-Seq technology in more detail in the main text. It now reads: "To identify CCGs during in vitro cardiomyocyte differentiation, genome-wide mRNA levels were assessed by mRNA sequencing of purified RNA using CEL-Seq, a previously described RNA profiling technique based on sequencing the 3'UTR of mRNAs, generating one read per transcript (Hashimshony et al. 2012)". Additionaly, we described the method in more detail in the methods section.

The sequences of primers used in qPCR should be described.

We have now described all primers in the methods section.

Which is more physiologically intact between D30 and D45 cardiomyocytes? In Figure 5C, circadian fluctuation of Caspase 3/7 activation in D45 cells is more blunt than D30 cells (see Figure 5B).

The blunted reaction in D45 cardiomyocytes is probably due to a higher resistance to doxorubicin at D45 compared to D30 cardiomyocytes.

Reviewer #2:

In this study, Dierickx et al study the role of circadian clock networks in human ESC-derived cardiac differentiation to determine if patterns of cyclic gene expression and cell function recapitulate clock patterns known to occur in vivo. They find that circadian rhythms of key clock genes are not differentially regulated in undifferentiated cells in keeping with prior studies. Circadian patterns of gene expression emerge during differentiation as cardiomyocytes mature. The data suggest that these rhythms impact drug screening and physiological assays of cardiomyocytes.

We thank Reviewer #2 for his valuable input and implemented his suggestions. Most importantly, we included D15 reporter and CEL-Seq data, which has substantially improved our understanding of the emergence of the clock. We have now compared the transcriptionally distinct cardiac stages (D15 versus D30) instead of the D30 versus D45 human ES cell-derived cardiomyocytes.

Major Concerns:

1) The authors suggest in the text that they have thoroughly vetted the cardiac purity and efficiency of cardiomyocytes based on gene expression of NKX2-5, cTnT, FACS, and clear sarcomere structures by IHC. None of this data is provided in the available data sets. In figure 1, please provide FACS plots of cTnT purity from differentiation. IHC in Figure 1 and Supplemental Fig 1 isn't sufficient. Although the use of NKX2-5-GFP cells was used, sorting wasn't performed until day 26 according to the methods. What is the purity prior to sorting since this may substantially impact the interpretation of results for D15 cells vs D30 or D45 cells? The significant batch-batch variation in differentiation as noted in the RNA-seq data make this particularly concerning.

We have added FACS analysis of human ES cell-derived cardiomyocytes via staining for cardiac troponin T, a cardiomyocyte differentiation marker, in Figure1-figure supplement 1. A purity of 50% cardiomyocytes was noted, which is in line previously reported results (Schwach & Passier

2016). Since the human heart consists of ~30% myocytes and ~70% non-myocytes, we opted for a strategy which does not involve FACS sorting before mRNA sequencing, since it is know that the FACS procedure can induce significant cellular stress and would therefore be a confounding factor in our analysis, in particular in light of the oscillatory UBC network. We also include additional sequencing data for D15, and here this stress network was not found. Since *UBC* oscillates in mouse hearts (and skeletal muscle) as well (Figure 4 and 5), we believe this is a cardiomyocyte-specific clock-dependent network.

We think that the differences between D30 and D45 are mostly due to read coverage differences between the two datasets, rather than purity differences, as explained in the Explanatory Figure-Rebuttal. As better addressed in this version of the manuscript, highly expressed genes are more often found to oscillate in a significant manner (Figure 4-figure supplement 1). This highlights that gene expression level differences most likely underlie the limited overlap of oscillators between D30 and D45. Nonetheless, the identified stress network, centered on *UBC*, was found to oscillate at D30 and D45.

2) For IHC, please provide close up images with evidence of sarcomeres and gene expression analysis should include temporal dynamics of well-established genes (NKX2-5, TBX5, GATA4, MYH6, TNNI3) to give a clear indication of temporal dynamics of these known cardiac markers. The authors suggest that clock activity between D15-30-45 is a consequence of increased maturity but this needs to be much more substantially validated through proper gene expression analysis, morphometry (cell size, sarcomere length analysis) to show a substantial change in maturation particularly between D15 and D45. Issues of variation in purity between these time points as noted above needs to be addressed as a confounding variable.

We noticed an increase of *MYH7*/*MYH6* mRNA ratio from D15 to D30, indicating cardiac differentiation/maturation. We have added more immunofluorescent images, showing more detailed sarcomeric structures by stainings for Troponin T and alpha sarcomeric actin, as suggested (Figure 1). In addition, we added stainings for MEF2C, a cardiac transcription factor (Figure 1).

Also, several markers (*GATA4*, *MEF2C*, *MEF2D*, *CACNA1C*, *SIRPA*, *VCAM*, *TNNI3*, and more) were analyzed using the obtained CEL-Seq data for D0, D15 and D30. Figure 4-figure supplement1-2 show the temporal mRNA expression dynamics during directed cardiomyocyte differentiation. A clear difference was noted between D15 and D30, while D45 showed high transcriptional correlation with D30. This highlights that comparison of these 2 stages (D15 versus D30) is more informative than D30 versus D45. Indeed, maturation markers such as *KCNJ2*, *SERCA2A*, *MYH7*/*MYH6* ratio and *CACNA1C*, were higher in D30 compared to D15 cardiomyocytes, demonstrating that cardiomyocyte maturation largely occurs in this timeframe.

3) The authors report that clock activity emerges between D30-D45. Unfortunately, they fail to provide D15 data as an excellent negative control for these studies to show that phenotypes attributed to clock activity are not present at D15 but appear at later stages of maturation. The better comparison in these assays would have been D15 vs D45. This includes the RNA-seq analysis of cycling genes (Figure 4). It also includes experiments performed in Figure 5 where D15 analysis of cardiac toxicity and chronotropy would be an important negative control to attribute these findings specifically to the emergence of clock activity during maturation.

We thank the referee for this suggestion and have now assessed clock gene mRNA expression and circadian rhythmicity of clock-controlled genes (CCGs) in D15 human ES cell-derived cardiomyocytes as well. By the use of a reported-based luciferase assay measured using the LumiCycle, we noted a preliminary circadian clock system that was not sustained over time. Weak *Per2* oscillations were observed and possibly represent the first phases of oscillatory clock gene expression.

While we did not observe a functional oscillatory clock in early D15 ES cell-derived cardiomyocytes, CEL-Seq analysis on D15 human ES cell-derived cardiomyocytes revealed a number of CCGs in this population. The overlap between D15 and D30 was limited as a result of read coverage between highly and lowly expressed genes as demonstrated in Figure 4-figure supplement 1 and Explanatory Figure-Rebuttal. Importantly, at D15 we did not find a UBC-centered stress response network using STRING analysis, which is prominently present in more mature ES

cell-derived cardiomyocytes. The observed oscillators may therefore represent the starting clock, but appear to lack network organization compared to the robust networks observed at D30 cardiomyocytes that do contain a robust clock system.

4) The addition of NE to elicit an inotropic response seems unnecessary to study the circadian beating rate. Attributing the cycling activity of beating rate to changes in PLN is quite speculative without gain/loss of function studies. It would be more convincing if there was evidence of cycling of PLN at the protein level within this time frame and if the cycling activity of beating rate could be eliminated by shRNA-knockdown or stable over-expression of PLN (or related experiment).

We appreciate the reviewer's comment and acknowledge that adding NE to study a circadian response in beating rate is unnecessary. Therefore we did not pursue with this assay and left this panel out of Figure 5.

5) The study lacks cohesive mechanistic data. It would be valuable to tie assays in fig 5 (toxicity and beating rate) to the finding in Fig 4 that UBC is a nodal protein in regulating circadian rhythm activity in cardiomyocytes. Is UBC protein abundance similarly regulated in a cyclic manner? Are cardiac toxicity and chronotropic phenotypes lost by stabilized expression of UBC?

Polyubiquitin C (UBC) is one of the most abundant proteins in a mammalian cell, and one of the main sources of ubiquitin. Polyubiquitin chains can consist in different conformations, and are coupled to numerous proteins targeted for degradation. This, together with the fact that UBC is a very small 8.5kDa protein, makes Western blot assessment of total cellular UBC levels impossible. While UBC is the nodal protein in the UBC network, in isolation its oscillations are not likely to have a major impact. However, we observe that a larger network of UBC-associated genes demonstrates a robust oscillatory pattern, giving support to the notion that the compounded effect of oscillations underlies the observed functional influence on stress response.

Reviewer #2 (Minor Comments):

Was an FDR cutoff threshold implement for GO analysis - should only use GO categories for which FDR<0.05?

We only describe GO-terms with an FDR<0.05 for analysis on D15 and D30 cardiomyocytes. We have adapted the text to clarify this matter and indicated this in Figure 5A-B.

Data in Figure 5F have no error bars - provide this assuming the analysis was done more than once.

As mentioned in major comment 4, we excluded the corresponding panel from Figure 5 in the revised manuscript.

Figure 2 should provide more substantial data on clock activity in pluripotent stem cells using tools already described in the study. Please include data using dLuc clock reporters to clearly show no activity in undifferentiated cells. Since this figure is about undifferentiated cells, I suggest moving Panel A to the beginning of Figure 3.

We have now included *Bmal1*- and *Per2*-dLuc bioluminescent reporter activity in human ES cells, supporting the absence of rhythmicity in pluripotent cells. We followed the suggestion of the reviewer to move panel A to figure 3, and added mRNA quantification for one additional clock gene (*CLOCK*) to this analysis. In figure 2A, we have now compared quantitative mRNA levels of 6 clock genes between ES and a differentiated human cell line (U2OS) known to harbor a robust clock, and noted a clear difference in stoichiometry for *BMAL1*, *CRY1*, and *CLOCK*, that could be confirmed on the protein level via western blot.

The authors provide no data on patterns of gene expression or reporter activity prior to synchronization. Can you please take D0, D15, D30, and D45 cells with the dLuc reporters and generate examples of clock activity prior to and after synchronization to provide clear evidence that clock activity is dependent on synchronization and only emerges in mature cardiomyocytes.

It is known that in vitro cells are not only synchronized with often used synchronization agents, such

as dexamethasone, but a simple medium change can reset the circadian clock in in vitro as well (Guenthner et al. 2014). We confirmed this for U2OS cells, and comparable amplitudes were observed for cells that underwent a medium change, compared to Dexamethasone-synchronized cells. Since human ES cell-derived CMs demand regular medium changes, it is therefore possible that these cells are synchronized daily in the period right before measurement at D30. However, this cannot be measured in the absence of media change, which the cells do require.

Reviewer #3:

This manuscript is aimed at charactering the emergence of circadian rhythm during myocardium differentiation using human ES cell differentiated cardiomyocyte model. It also attempted to demonstrate the functional rhythmicity in response to pharmacological agents, such as doxorubicin and phenylephrine.

It is known that ES cells do not have circadian rhythmicity but when and how the circadian rhythm gets established during cardiac development is unclear and the impact of this on cardiac diseases or the modeling of cardiac diseases and regeneration was unexplored. The authors showed an induction and oscillation of PER2 expression at RNA level at D30, which precedes the oscillatory expression of BMAL1 at RNA level. This parallels the previously reported observation in mouse ESC during induction with RA. Interestingly, both BMAL1 and PER2 promoter driven luciferase reporter showed antiphasic oscillation at D30 (Figure 3B and 3D). What is the explanation? Likely the oscillatory transcription in blunted by a slow degradation. Does this occur at prior to Day 30, e.g. at Day 15? How does the cell overcome this? More work is required to elucidate the possible mechanism for the emergence of circadian rhythmicity. And finally, if these cells are depleted of BMAL or Per, is there an effect on cardiac gene expression, differentiation, or the response to doxorubicin and/or norepinephrine?

We agree and thank the reviewer for pointing this out. We have now added D15 data and observed that D15 human ES cell-derived cardiomyocytes possess an intermediate clock (based on a *Per2*-luciferase reporter) that does not sustain its rhythmicity, which is in line with randomly differentiated murine cells (Yagita et al. 2010). While we acknowledge that slower induction of *BMAL1* rhythmicity may result from slower mRNA degradation, and that CEL-Seq mRNA sequencing for additional days between D15 and D30 may provide interesting insights into how the clock is established, these analyses do not lie in the scope of the current manuscript.

Depletion of BMAL1 or PER2 will most likely affect gene expression. Even though clock knockout studies in human ES cells would be elucidative on the role of these genes during differentiation and/or stem cell physiology, homozygous targeting of human embryonic stem cells is not straightforward and time-consuming. In addition, limited transfection/transduction efficiency for cardiomyocytes hampers a complete clock disturbance, which makes these kinds of analyses challenging in cardiac cultures.

We agree that it will be interesting to further explore the mechanism that controls the start of the circadian clock and the effect of clock gene expression on cardiac differentiation itself. However, we feel that such analyses are outside the scope of the present study, which is focused on the characterization of the emerging circadian clock and the identification of the functional role and consequences of the emerging oscillatory UBC network on the cellular stress response.

It is commendable that the authors did single cell analysis on Per2-luc, however only 4 cells were observed. It is important to observe more cells on more time points. Does the emergence of rhythmicity reflect heterogeneity in maturation? Or are there pioneer cells that establish rhythms first? What is the process of gaining rhythmicity, a gradual increasing in amplitude? Many more questions can be answered by simply increasing the number of observation.

To strengthen our claim that pure cardiomyocytes possess a functional circadian clock, we assessed *Bmal1*- as well as *Per2*-dLuc rhythmicity in purified cells, and noted clear anti-phasic oscillations further solidifying the presence of a clock in cardiomyocytes. We have now included 5 *Per2*-dLuc single cells, which show that most cells oscillate in phase. However, some cells oscillated with a different phase, and other cardiomyocytes did not oscillate robustly. We think that the sorting

procedure may influence the cells (to a differing extend), which is why assessing the presence of pioneer cells would preferentially be performed without sorting. However, while the single cell data obtained from the LV200 is of great value, unraveling the exact molecular mechanism of clock origination during directed human ES cell differentiation lies beyond the scope of this manuscript.

The CEL-seq at D30 and D45 showed very small overlap in oscillating genes, which may be a true observation that the circadian clock regulates different subsets of genes at different developmental stage or may be an artifact due to low sequencing depth and extensive multiplexing. I agree with focusing on the common 62 genes and discuss the above possibilities if deeper sequencing is impossible in the scope of the current study.

We thank the reviewer for pointing this out. In depth analysis of the differences between oscillators at different stages has ben addressed better in the revised manuscript, and show that limited overlap is largely a consequence of transcript abundance. Indeed, the overall transcriptome between D30 and D45 does not change significantly (Explanatory Figure-Rebuttal), which is why the biologically more relevant comparison of D15 and D30 is now described (Figure 4 and 5). The relationship between the expression level and oscillatory behavior of transcripts is addressed in Figure 4-figure supplement 2 of the revised manuscript. Importantly, the oscillatory network centered on UBC was present at both D30 and D45, but not in D15 human ES cell-derived cardiomyocytes.

The circadian response to doxorubicin and PE are highly interesting, but need to be further substantiated. Figure 5D (and also Figure 4E) needs to be validated by RT-PCR. It is unclear why D30 and D45 has response that are in different phase. The NE response in Figure 5F should present cumulative data with statistics.

On request of Reviewer #2, we omitted the norepinephrine assay from our manuscript.

Reviewer #3 (Minor Comments):

The authors used several different statistical analyses, including Rain, JTK cycle and 2-way ANOVA, to infer circadian rhythmicity. As they each have different sensitivity, it is best to use either Rain or JTK cycle for all analysis. Figure 5B and 5C should be analyzed with Rain or JTK cycle.

We acknowledge that different statistical methods have different sensitivities. For this reason, we preferred JTK-cycle to analyze rhythmicity in large datasets such as the once produced for D15 as well as D30 human ES cell-derived cardiomyocytes. For single qRT-PCR experiments however, rhythmic mRNA expression was assessed on a gene-to-gene basis using RAIN, since this algorithm is better suited to assess rhythmic mRNA expression of single candidates. As suggested, we have now used the RAIN algorithm on the doxorubicin-induced apoptosis data to define circadian wave significance in Figure 5F-G.

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1st Editorial Decision 31st January 2017

Thank you for the submission of your revised research manuscript to EMBO reports. We have now received reports from the three referees that were asked to re-evaluate your study, which can be found at the end of this email.

As you will see, all three referees support the publication of your revised paper in EMBO reports. Nevertheless, referees #1 and #2 have some further suggestions to improve the manuscript, which we ask you to address. As the reports are below, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in a point-by-point response. Final acceptance of the manuscript will then depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

I have some further editorial requests:

Please include scale bars in all microscopy images.

For a short report we require to combine the results section and the discussion in one section called "Results & Discussion". Please do that for your manuscript.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The manuscript "Functional implications of circadian networks in human embryonic stem-cell derived cardiomyocytes" by Dierickx et al. aimed to describe the emergence of a functional circadian clock in vitro during the transition of human ES cells to cardiomyocytes. Human embryonic stem cells do not contain a functional circadian clock. As the cells differentiate towards a cardiac fate a circadian clock gradually emerges, by day 30 a fully functional circadian clock is present. The authors support his claim with both functional reporter elements and gene expression

data. The oscillatory nature of the mature cardiomyocytes affects their responsiveness to the cardiotoxic agent doxorubicin. This underscores the importance of considering rhythmicity when using ES-derived cardiomyocytes for drug testing. This study contains novel findings and expands the field of circadian networks from mouse to human-derived cell types. The comparison of day 0, day 15 and day 30 cells convincingly reveals an emerging circadian clock at distinct phases of maturation and sets the stage for future explorations of the relationship between cell rhythmicity and effective cardiac treatment strategies.

Major concerns:

1. Figure 1B claims that there are sarcomeric structures, indicating maturity, however they are not clear in the images provided. Quantification of the IHC images would improve the claims that the cells have matured (sarcomere length and/or density, cell size). Scale bars should also be included.
2. It is claimed in line 131 that CASQ2 is expressed more at day 30 than day 15, but the graph in EV1B shows the opposite. This claim is not substantiated.
3. The data from Supplemental Figure 2 should be incorporated into Figure 2 to better show the differences between stem cells and cells that possess a functional clock.
4. Human ES cells were synchronized with forskolin (line 156) while the cardiomyocytes were synchronized with dexamethasone (line 182). The choice of different reagents for synchronization should be justified.
5. Figure 3 panels C-F appear to show representative plots from experiments. There should also be quantification of the results from several different trials or place this data in supplemental figure and soften any conclusions tied to these data since they appear anecdotal.
6. FACS purity data should be provided for the post-sort cell population. The authors claimed that 100% cardiac cells were not selected for during sorting. With this in mind, it is important to clarify exactly what proportion of the cells were cardiac. As currently written this is misleading. This is a critical point. If the authors claim to be making statements about circadian rhythm during cardiomyocyte differentiation with cultures of less than 50% purity then the entire premise of the paper needs to be reconsidered. Furthermore, analysis of single cell cycling activity is put into question since the identity of those single cells can't be confidently claimed to be a cardiomyocyte.

Minor Comments:

1. Grammar needs to be corrected in abstract, several sentences use incorrect plurality.
2. In line 152 the authors claim that clock gene expression and translation occurs differently in human ES cells and differentiated cells. This is too strong of a conclusion. There are differences between human ES cells and this specific differentiated cell type (U2OS), but not all differentiated cells. The comparison does make it clear that ES cells do not have rhythmicity, but cannot make a conclusion about differentiated cells in general. We recommend softening this assertion to more accurately reflect the conclusions of the data.
3. On line 205 the authors claim that robust oscillations emerge around day 30. This claim should be made less strong, robust oscillations are present by day 30 but could emerge at any time point between day 15 and day 30.
4. Further mention should be made in the methods about whether cells from the same differentiations or cell populations with similar purities were used for experimental replicates.
5. Many of the figure legends do not define the stars correctly. For example, Figure 2A has a result that is *** significant, but this is not defined in the legend.
6. The paper would have better organization if Figure 1 was switched with Figure 2. This would communicate that stem cells do not possess a functional clock then progress to the differentiation of these stem cells.
7. In Figure 3E the results are detrended, are the results also detrended in Figure 3F? The data appears to be centered around 0, similar to the detrended results shown in 3E.
8. Figure 2 shows a western blot with the bands cut from a larger gel. We suggest the inclusion of the entire gel with the ladder.

Referee #2:

In the manuscript entitled "Functional implications of circadian networks in human embryonic stem

cell-derived cardiomyocytes" by Dierickx et al., the authors investigate the global gene expression during in vitro differentiation of human embryonic stem (ES) cells into cardiomyocytes. Especially, temporal gene expression profiles are analyzed and they find that undifferentiated human ES cells do not possess a functional circadian clock. On the other hand, day 30 differentiated cardiomyocytes show robust expression rhythms of clock genes. These findings are well compatible with recently reported cellular differentiation-coupled circadian clock developments in mouse ES cells. The authors clearly show that the circadian clock functionality closely correlates with cellular differentiation and cell fate also in humans. Moreover, the authors describe that a stress-related oscillatory network of genes is established after differentiation into cardiomyocytes, which elicits a time-dependent responsiveness to doxorubicin. Since human ES cell-derived cardiomyocytes are available for disease models and regenerative therapies, the authors propose that the in vitro differentiation culture system using human ES cells can be deployed to test the effectiveness of circadian clock-based medicine to prevent cardiotoxic side effects and timed cell-based therapy. The reviewer feels that the study includes important possibilities examining the mechanisms of cardiovascular diseases. Especially, the authors try to establish the cell-based assay system of differentiation-coupled circadian clock development in human ES cells. Moreover, the stress response reaction fluctuates circadianly after differentiation, suggesting the time-dependent reduction of the cardiotoxic side effects by doxorubicin.

Major comments:

1) In Figure 3C and D, the circadian rhythms of bioluminescence reporters seem to be faint and unstable even in D30. In mouse ES cell-based methods, several reports exhibited that much more robust circadian oscillations were developed after the in vitro differentiation culture of mouse ES cells. For the reliability of this human ES cell-based system, the reviewer strongly recommends that the authors should demonstrate the development of more robust and stable circadian clock oscillation after the differentiation if the authors would like to elucidate the correlation between circadian clock and formation of the stress response network using this model.

2) In Figure 4, similar numbers of genes (643 genes in D15 v.s. 757 genes in D30) showed circadian fluctuation. The authors should clarify whether other clock genes in addition to Per2 and Bmal1 were included or not in these oscillatory genes.

Referee #3: The authors have adequately addressed my key concerns.

Revision - authors' response 20th February 2017

Referee #1 (Report for Author):

The manuscript "Functional implications of circadian networks in human embryonic stem-cell derived cardiomyocytes" by Dierickx et al. aimed to describe the emergence of a functional circadian clock in vitro during the transition of human ES cells to cardiomyocytes. Human embryonic stem cells do not contain a functional circadian clock. As the cells differentiate towards a cardiac fate a circadian clock gradually emerges, by day 30 a fully functional circadian clock is present. The authors support his claim with both functional reporter elements and gene expression data. The oscillatory nature of the mature cardiomyocytes affects their responsiveness to the cardiotoxic agent doxorubicin. This underscores the importance of considering rhythmicity when using ES-derived cardiomyocytes for drug testing. This study contains novel findings and expands the field of circadian networks from mouse to human-derived cell types. The comparison of day 0, day 15 and day 30 cells convincingly reveals an emerging circadian clock at distinct phases of maturation and sets the stage for future explorations of the relationship between cell rhythmicity and effective cardiac treatment strategies.

Major concerns:

1. Figure 1B claims that there are sarcomeric structures, indicating maturity, however they are not

clear in the images provided. Quantification of the IHC images would improve the claims that the cells have matured (sarcomere length and/or density, cell size). Scale bars should also be included.

Response: We thank the reviewer for pointing this out. To clarify, we have now included scale bars for all single immunofluorescent pictures in this figure panel.

The difference in sarcomere length or cell size between D15 and D30 cardiomyocytes is expected to be limited. However, between these two time points, gene expression profiles are profoundly different (Figure EV5). Changes in the expression of key marker genes, observed by qPCR (Fig. 2), and validated by CEL-Seq (Fig. EV5), demonstrate that cardiomyocytes undergo a transcriptional maturation between D15 and D30. These gene expression changes coincide with the emergence of a functional clock and ultimately have a functional effect on the cardiomyocyte stress response. We have now also added D15 doxorubicin-based apoptosis induction data (Figure 5F), which demonstrates that D30 cardiomyocytes are more sensitive to doxorubicin than younger cells, further highlighting the functional changes that take place in the cardiomyocytes between D15 and D30. We have modified the text to clarify that the maturation that occurs between D15 and D30 is at the transcriptome level.

2. It is claimed in line 131 that CASQ2 is expressed more at day 30 than day 15, but the graph in EV1B shows the opposite. This claim is not substantiated.

Response: We acknowledge that there is no substantial increase in CASQ2 compared between D15 and D30 when measured via qPCR. Indeed, using a Student's T-Test there was no significant difference between the time points. To omit confusion, we excluded this figure panel from Figure EV1.

3. The data from Supplemental Figure 2 should be incorporated into Figure 2 to better show the differences between stem cells and cells that possess a functional clock.

Response: We thank the reviewer for this clarifying comment. We have now implemented the data of the previous Figure EV2 into the new Figure 1 (see minor comment 6) to make the difference between non-clock possessing stem cells and U2OS cells, which do have a functional clock, more clear.

4. Human ES cells were synchronized with forskolin (line 156) while the cardiomyocytes were synchronized with dexamethasone (line 182). The choice of different reagents for synchronization should be justified.

Response: Typically, we use dexamethasone for synchronizing cell lines in vitro, but since dexamethasone has been implemented in multiple stem cell differentiation protocols [1-6], we chose forskolin as a synchronizing agent for human ES cells to avoid inducing differentiation of our pluripotent cultures. We have clarified this in the methods section ("Bioluminescent recording and data analysis"). The text now reads: "Human ES cells were cultured in E8 medium and synchronized for 2 hours with Forskolin [37]. Forskolin was chosen as a synchronizing agent for human ES cells, since dexamethasone has been implemented in multiple stem cell differentiation protocols and might therefore induce premature differentiation [73-78]".

5. Figure 3 panels C-F appear to show representative plots from experiments. There should also be quantification of the results from several different trials or place this data in supplemental figure and soften any conclusions tied to these data since they appear anecdotal.

Response: We have now added the quantification of Lumicycle replicates from independent differentiations in a new supplemental Figure EV4 to support the (description of) robust anti-phasic oscillation of Bmal1-dLuc and Per2-dLuc, a hallmark of a functional circadian clock system that we observe at D30. To further strengthen the birth and persistence of circadian rhythmicity we have also added dLuc reporter figures for D45 (Fig. EV4E). We strongly believe these panels are a valuable addition to underscore that the observed clock persists upon longer culturing, which strengthens the claim of robust circadian rhythmicity.

6. FACS purity data should be provided for the post-sort cell population. The authors claimed that

100% cardiac cells were not selected for during sorting. With this in mind, it is important to clarify exactly what proportion of the cells were cardiac. As currently written this is misleading. This is a critical point. If the authors claim to be making statements about circadian rhythm during cardiomyocyte differentiation with cultures of less than 50% purity then the entire premise of the paper needs to be reconsidered. Furthermore, analysis of single cell cycling activity is put into question since the identity of those single cells can't be confidently claimed to be a cardiomyocyte.

Response: For most of our analysis, we have opted to not sort the cardiac cultures in order to prevent the disturbance of clock pathways during the cell dissociation, sorting and replating procedure. The purity of all of our differentiation batches was consistently around 50%, which is comparable to the in vivo heart that on average consist of a mixed population of myocytes (~30%) and non-myocytes (~70%) [7,8]. To emphasize this, we have added an additional FACS plot of a representative differentiation experiment to Figure EV1. To avoid confusion between purified cardiomyocytes and unsorted cardiac cultures, we adjusted the text and now refer to "cardiac cultures" when describing non-sorted cells.

To confirm that cardiomyocytes do in fact contribute to the oscillatory signal in the cardiac cultures, they were sorted and analyzed as a pure population. Purity was confirmed by fluorescent microscopy as shown in the added supplemental figure panels (Figure EV4C and D), which demonstrate that all sorted cells express the Nkx2.5-GFP^{+} cardiomyocyte marker. The images shown are of the cells that were used for subsequent LV200 analysis in Figure 3F-H.

Therefore, we think we have substantial proof of a functional clock in cardiomyocytes. As a whole, the system shows overlap with important, heart-specific oscillators in mouse, which is why we think that the cultures are a good proxy for the heart in vivo.

Minor Comments:

1. Grammar needs to be corrected in abstract, several sentences use incorrect plurality.

Response: We have rechecked the text for grammar and removed errors.

2. In line 152 the authors claim that clock gene expression and translation occurs differently in human ES cells and differentiated cells. This is too strong of a conclusion. There are differences between human ES cells and this specific differentiated cell type (U2OS), but not all differentiated cells. The comparison does make it clear that ES cells do not have rhythmicity, but cannot make a conclusion about differentiated cells in general. We recommend softening this assertion to more accurately reflect the conclusions of the data.

Response: We do acknowledge that our conclusion about differentiated cells in general is too broad. Therefore we softened our assertion and adjusted this in the text that now reads: "We conclude that while core clock genes are expressed and translated into protein in human ES cells, this occurs with a different stoichiometry in comparison to U2OS cells and most likely other differentiated cell types as well".

3. On line 205 the authors claim that robust oscillations emerge around day 30. This claim should be made less strong, robust oscillations are present by day 30 but could emerge at any time point between day 15 and day 30.

Response: We have adjusted this claim and now only state that robust oscillations are present at D30 (which is further solidified in Figure EV4).

4. Further mention should be made in the methods about whether cells from the same differentiations or cell populations with similar purities were used for experimental replicates.

Response: We have added this in the method section ("RNA isolation and CEL-Seq") which now reads: "After synchronization, biological triplicates (independent wells) with comparable cardiac purity were collected every 4 hours over the course of 48 hours (ZT4-ZT48) (Fig EV3)".

5. Many of the figure legends do not define the stars correctly. For example, Figure 2A has a result that is *** significant, but this is not defined in the legend.

Response: We would like to thank the reviewer for pointing this out. We have adjusted significance levels accordingly in Figure 1C, 3A-B, 4D, and 5D.

6. The paper would have better organization if Figure 1 was switched with Figure 2. This would communicate that stem cells do not possess a functional clock then progress to the differentiation of these stem cells.

Response: We thank the referee for suggesting this change in structure. We now start our manuscript with a focus on human ES cells and subsequently elaborate on the emergence of a functional clock system during differentiation.

7. In Figure 3E the results are detrended, are the results also detrended in Figure 3F? The data appears to be centered around 0, similar to the detrended results shown in 3E.

Response: It is correct that the data in Figure 3F is detrended in the exact same manner as in figure 3E. We have now clarified this in the legend of Figure 3.

8. Figure 2 shows a western blot with the bands cut from a larger gel. We suggest the inclusion of the entire gel with the ladder.

Response: As suggested, we have now added the original western blots for BMAL1, CRY1 and CLOCK in the new Figure EV1, including the entire lanes with the ladder.

Referee #2 (Report for Author):

In the manuscript entitled "Functional implications of circadian networks in human embryonic stem cell-derived cardiomyocytes" by Dierickx et al., the authors investigate the global gene expression during in vitro differentiation of human embryonic stem (ES) cells into cardiomyocytes. Especially, temporal gene expression profiles are analyzed and they find that undifferentiated human ES cells do not possess a functional circadian clock. On the other hand, day 30 differentiated cardiomyocytes show robust expression rhythms of clock genes. These findings are well compatible with recently reported cellular differentiation-coupled circadian clock developments in mouse ES cells. The authors clearly show that the circadian clock functionality closely correlates with cellular differentiation and cell fate also in humans. Moreover, the authors describe that a stress-related oscillatory network of genes is established after differentiation into cardiomyocytes, which elicits a time-dependent responsiveness to doxorubicin. Since human ES cell-derived cardiomyocytes are available for disease models and regenerative therapies, the authors propose that the in vitro differentiation culture system using human ES cells can be deployed to test the effectiveness of circadian clock-based medicine to prevent cardiotoxic side effects and timed cell-based therapy.
The reviewer feels that the study includes important possibilities examining the mechanisms of cardiovascular diseases. Especially, the authors try to establish the cell-based assay system of differentiation-coupled circadian clock development in human ES cells. Moreover, the stress response reaction fluctuates circadianly after differentiation, suggesting the time-dependent reduction of the cardiotoxic side effects by doxorubicin.

Major comments:

1) In Figure 3C and D, the circadian rhythms of bioluminescence reporters seem to be faint and unstable even in D30. In mouse ES cell-based methods, several reports exhibited that much more robust circadian oscillations were developed after the in vitro differentiation culture of mouse ES cells. For the reliability of this human ES cell-based system, the reviewer strongly recommends that the authors should demonstrate the development of more robust and stable circadian clock oscillation after the differentiation if the authors would like to elucidate the correlation between circadian clock and formation of the stress response network using this model.

Response: Based on the oscillatory results that we obtained using three independent assays; qPCR, Lumicycle and LV200 (bulk as well as single cell), we are highly confident that human ES

cell-derived cardiomyocytes possess a functional clock. The fact that the reporters seem faint is most likely due to the fact that human ES cell-derived cardiomyocytes are difficult to transduce (with lentivirus), and therefore reporter levels are low in these cells, compared to immortalized cell lines.

To further confirm the circadian nature of the observed oscillations, we have now added an extra supplementary figure with replicates of independent differentiations (Figure EV4) that also demonstrate the anti-phasic character of the Bmal1 and Per2 reporters.

We now also show that the clock is still present at D45 based on anti-phasic Bmal1- and Per2-dLuc reporter data, to further strengthen that the signal is not only robust, but also persists in older cultures.

In addition, to substantiate the functional effect of the clock in differentiating cardiomyocytes, we have now added D15 doxorubicin-based apoptosis induction data (Figure 5F), which shows the functional difference between D15 and D30. Firstly, D15 cardiac cultures are less sensitive to doxorubicin, and while the response is circadian at D30 it is not at D15.

We think that the combination of multiple replicates for D30, our sustained rhythmicity data (D45), as well as our functional data demonstrate beyond doubt the emergence of a stable and functional clock in D30 human ES cell-derived cardiomyocytes.

2) In Figure 4, similar numbers of genes (643 genes in D15 v.s. 757 genes in D30) showed circadian fluctuation. The authors should clarify whether other clock genes in addition to Per2 and Bmal1 were included or not in these oscillatory genes.

Response: One of the downsides of CEL-Seq is that lowly expressed genes are generally not picked up robustly (described in the main text). Most clock genes belong to the class of lower expressed genes (this is depicted in Figure EV5D, in which the average log_2 RPM value of three replicates for ZT48 are plotted). This is why not all classical clock genes are assessed using JTK-cycle. For the ones that are assessed, we find CLOCK to be an oscillator at D30. We have now added to the main text that most clock genes are not picked up by CEL-Seq. We do acknowledge that RNA-Seq with high coverage for all time points is the preferred method to call the most oscillators, as well as the least false positives/negatives. However, deeper sequencing is impossible in the scope of the current study.

Referee #3 (Report for Author): The authors have adequately addressed my key concerns.

Response: We thank the referee for carefully revising our manuscript and are glad to have addressed his comments in a satisfactory manner.

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Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284: 143–147.
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2nd Editorial Decision 13th March 2017

Thank you for the submission of your revised research manuscript to EMBO reports. We have now received reports from the two referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, both referees support the publication of your paper in EMBO reports. Nevertheless, referees #1 has two further comments, which we ask you to address in a final revised version.

I also have some further editorial requests:

For a short report we require to combine the results section and the discussion in one section called "Results & Discussion". The manuscript length (excluding references, materials and methods, and EV legends) should not exceed 29,000 characters with spaces. Please format your manuscript accordingly.

Please update the EV figure names and legends to follow our nomenclature (Figure EV1, Figure EV2 etc. ...).

We noticed some discrepancies comparing the beta-actin panels in Fig. 1D with the source data shown in Fig. EV1. It seems that for the panels in 1D the pictures where horizontally compressed and the contrast was enhanced. Was the quantification done using these modified pictures? Please provide unmodified panels for Figure 1D, and in case, repeat the quantification using the unmodified data. If possible, please provide higher resolution images for the Western blots shown in 1D and EV1

Was the CEL-seq. data deposited? In case yes, please add the accession information in the manuscript text.

Please put both supplemental tables in a single pdf labeled Appendix. The Appendix should also include a table of content on the first page, both tables and their title and legends (remove the legends for both tables from the main text).

Fig. 3G: Could you separate the various images with white frames, indicating that these are single images?

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

Major Comment:

For some of the experiments, the authors sorted the cardiomyocytes to obtain a pure population. The brightfield and fluorescent images of sorted cardiomyocytes in Figure EV4 are not sufficient to claim that a pure population was obtained. The FACS plots from sorting should be provided as well as immunohistochemistry images of sorted cells showing GFP co-stain with a sarcomere gene as in fig 1.

Minor Comment:

The plots of Baml1-dLuc and Per2-dLuc are consistently shown as both relative to T0 and detrended. However, the detrended plots of the data shown in Figures 1F and 3C are not shown. We suggest that the detrended plots also be provided.

Referee #2:

The authors have adequately address my key concerns.

Revision - authors' response 29 March 2017

We received the recent comments on our manuscript describing the emergence of a functional clock in human ES-derived cardiomyocytes (EMBOR-2017-43897V2) and are very pleased to see that both reviewers support the publication of our manuscript in EMBO reports. We have addressed the remaining minor comments of reviewer 1 and have deposited our data in the GEO database and included the accession numbers. With the reviewers suggestions and your editorial comments, I think it has become a very solid piece of work and we hope that with these changes, our manuscript is ready for publication. It has been a pleasure to work with you on this submission.

Point by point response:

Thank you for the submission of your revised research manuscript to EMBO reports. We have now received reports from the two referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, both referees support the publication of your paper in EMBO reports. Nevertheless, referees #1 has two further comments, which we ask you to address in a final revised version.

I also have some further editorial requests:
For a short report we require to combine the results section and the discussion in one section called "Results & Discussion". The manuscript length (excluding references, materials and methods, and EV legends) should not exceed 29,000 characters with spaces. Please format your manuscript accordingly.

We adapted this in our submission and filed the manuscript as a research article.

Please update the EV figure names and legends to follow our nomenclature (Figure EV1, Figure EV2 etc.).

We updated the EV figure names and legends to follow the EMBO Reports nomenclature.

We noticed some discrepancies comparing the beta-actin panels in Fig. 1D with the source data shown in Fig. EV1. It seems that for the panels in 1D the pictures where horizontally compressed and the contrast was enhanced. Was the quantification done using these modified pictures? Please provide unmodified panels for Figure 1D, and in case, repeat the quantification using the unmodified data. If possible, please provide higher resolution images for the Western blots shown in 1D and EV1.

We adapted figure 1 and included the unmodified pictures, on which the quantification was done on, in EV1.

Was the CEL-seq. data deposited? In case yes, please add the accession information in the manuscript text.

We deposited the CEL-Seq data to the GEO database. The accession number is now added to the manuscript and the EP Author checklist.

Please put both supplemental tables in a single pdf labeled Appendix. The Appendix should also include a table of content on the first page, both tables and their title and legends (remove the

legends for both tables from the main text).

Both supplemental tables include gene expression data. Therefore there are too many rows and columns to combine it into a single PDF. Where do we have to put the legends for these two tables with multiple tabs (excel files)?

Fig. 3G: Could you separate the various images with white frames, indicating that these are single images?

We have now separated the various images with white frames to indicate that these are single images.

In addition I would need from you:
- a short, two-sentence summary of the manuscript

The circadian clock is important for proper heart function. This study describes the gradual emergence and the functional implication of a clock in directed cardiac differentiation of human embryonic stem cells.

- two to three bullet points highlighting the key findings of your study

- **Human embryonic stem cells do not have a functional circadian clock**
- **A functional circadian clock emerges during directed cardiac differentiation**
- **Human embryonic stem cell-derived cardiomyocytes possess an oscillatory stress-related transcriptional network**
- **The apoptotic response of human embryonic stem cell-derived cardiomyocytes to doxorubicin shows a time-dependent, circadian pattern**

- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of about 400 pixels) that can be used as part of a visual synopsis on our website.

We uploaded a schematic summary figure that can be used as part of a visual synopsis on the EMBO Reports website.

Referee #1:

Major Comment:

For some of the experiments, the authors sorted the cardiomyocytes to obtain a pure population. The brightfield and fluorescent images of sorted cardiomyocytes in Figure EV4 are not sufficient to claim that a pure population was obtained. The FACS plots from sorting should be provided as well as immunohistochemistry images of sorted cells showing GFP co-stain with a sarcomere gene as in fig 1.

We added a FACS plot of the sorted population GFP$^+$ CMs in Figure EV4D. Yet, we do not have immunostainings from the sorted cells. Elliot et al (Elliott et al., 2011) described and characterized the cardiomyocytes generated from the *Nkx2.5*-eGFP human ES cells. They nicely showed co-expression of the reporter gene (Nkx2.5-eGFP) with the cardiac markers MYL7, ISL1, MYL2, GATA4 and actinin-1. Therefore, we strongly believe that brightfield, fluorescent images in combination with the FACS plot are sufficient to prove that the measured population represents pure cardiomyocytes.

Minor Comment:

The plots of Baml1-dLuc and Per2-dLuc are consistently shown as both relative to T0 and detrended. However, the detrended plots of the data shown in Figures 1F and 3C are not shown. We

suggest that the detrended plots also be provided.

For simplicity we did not include detrended plots, since the original (raw) plots are more informative and prove in an unbiased manner that there is clear circadian rhythmicity at D30, but not D0 and D15. Detrending of non-circadian curves could introduce artificial trends that are not biologically relevant. Therefore, detrended plots are only shown for D30 and D45 to underscore the anti-phasic nature of the Bmal1- and Per2-dLuc rhythms.

Referee #2: The authors have adequately addressed my key concerns.

We thank the referee for carefully revising our manuscript and are glad to have addressed his/her comments in a satisfactory manner.

References

Elliott, D.A., Braam, S.R., Koutsis, K., Ng, E.S., Jenny, R., Lagerqvist, E.L., Biben, C., Hatzistavrou, T., Hirst, C.E., Yu, Q.C., et al. (2011). NKX2-5(eGFP/w) hESCs for isolation of human cardiac progenitors and cardiomyocytes. Nature Methods 8, 1037–1040.

3rd Editorial Decision 10 April 2017

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Linda W. Van Laake and Pieterjan Dierickx
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-43897V2

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- If n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibodypedia
http://1degreebio.org	1DegreeBio
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo	ARRIVE Guidelines
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http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
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http://www.consort-statement.org	CONSORT Flow Diagram
http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSORT Check List
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum	REMARK Reporting Guidelines (marker prognostic studies)
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http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For both qPCR and CEL-Seq experiments biological triplicates were sampled every 4hrs (across 48 hours). This is a widely used sampling depth for circadian studies as the combination of high temporal resolution and number of replicates allows for statistical assessment of circadian rhythmicity. For the doxorubicin-treated apoptosis experiments, we sampled three replicates
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Statistical tests are justified. The statistical methods, results and significance levels are described and indicated in the text, methods, figures as well figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Assessment of rhytmicity using JTK or RAIN is based on non-parametric methods. For qPCR analysis a parametric t-test or ANOVA was used (normal distribution was not tested). Difference in sensitivity to doxorubicin between D15 and D30 were done using the non-parametric Man-Whitney U test .
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Company, catalog numbers as well as the dilutions of the antibodies that were used are described in the Materials and Methods section of our manuscript. For the Immunohistochemistry it reads: "Cells were fixed with 4% paraformaldehyde (PFA) for 15 minutes, blocked for 1 hour in blocking buffer (5% FBS, 0.25% Tx[illegible]
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The human embryonic stem cell line that was used for differentiation towards cardiomyocytes is an NKX2.5-eGFP reporter line generated from wild type HES3 cells (Materials and Methods). Human ES cells as well as U2OS cells were regularly tested for mycoplasm contamination according to the standard operating procedures in the lab.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA

14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	We deposited our CEL-Seq data sets as stated on page X of the manuscript: "The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE97142 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE97142)."
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	CEL-Seq data are deposited in the GEO database as mentioned above.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	We included this in the manuscript under the section Data availability. This section reads: "Primary data Dierickx P, Vermunt MW, Muraro MJ, Creyghton MP, Doevendans PA, van Oudenaarden A, Geijsen N, Van Laake LW (2017) Functional implications of circadian networks in human embryonic stem cell-derived cardiomyocytes. Gene Expression Omnibus GSE97142 Referenced data Zhang R, Lahens NF, Ballance HI, Hughes ME, Hogenesch JB (2014) A circadian gene expression atlas in mammals: Implications for biology and medicine. Gene Expression Omnibus GSE54652"
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No. Our study cannot be directly missaplied to pose a significant threat with negative consequences for broad society.