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Identification of novel biomarkers for doxorubicin-induced toxicity in human cardiomyocytes derived from pluripotent stem cells

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ABSTRACT

Doxorubicin is a chemotherapeutic agent indicated for the treatment of a variety of cancer types, including leukaemia, lymphomas, and many solid tumours. The use of doxorubicin is, however, associated with severe cardiotoxicity, often resulting in early discontinuation of the treatment. Importantly, the toxic symptoms can occur several years after the termination of the doxorubicin administration. In this study, the toxic effects of doxorubicin exposure have been investigated in cardiomyocytes derived from human embryonic stem cells (hESC). The cells were exposed to different concentrations of doxorubicin for up to 2 days, followed by a 12 day recovery period. Notably, the cell morphology was altered during drug treatment and the cells showed a reduced contractile ability, most prominent at the highest concentration of doxorubicin at the later time points. A general cytotoxic response measured as Lactate dehydrogenase leakage was observed after 2 days' exposure compared to the vehicle control, but this response was absent during the recovery period. A similar dose-dependant pattern was observed for the release of cardiac specific troponin T (cTnT) after 1 day and 2 days of treatment with doxorubicin. Global transcriptional profiles in the cells revealed clusters of genes that were differentially expressed during doxorubicin exposure, a pattern that in some cases was sustained even throughout the recovery period, suggesting that these genes could be used as sensitive biomarkers for doxorubicin-induced toxicity in human cardiomyocytes. The results from this study show that cTnT release can be used as a measurement of acute cardiotoxicity due to doxorubicin. However, for the late onset of doxorubicin-induced cardiomyopathy, cTnT release might not be the most optimal biomarker. As an alternative, some of the genes that we identified as differentially expressed after doxorubicin exposure could serve as more relevant biomarkers, and may also help to explain the cellular mechanisms behind the late onset apoptosis associated with doxorubicin-induced cardiomyopathy.

1. Introduction

Anthracyclines, such as doxorubicin, are amongst the most successful chemotherapy compounds for the treatment of a wide range of cancers, including hematologic malignancies, soft tissue sarcomas, and solid tumours in both children and adults. Doxorubicin binds to DNA associated enzymes such as topoisomerase I and II, responsible for separating the double strands of DNA during replication (Hilmer et al., 2004). The ability of doxorubicin to kill rapidly dividing cells and in turn slowing disease progression has been acknowledged for over 30 years. However, its toxicity on noncancerous cells, with cardiac toxicity being the most

Abbreviations: cTnT, cardiac specific troponin T; hESC, human embryonic stem cells; hiPSC, human induced pluripotent stem cells; hPSC, human pluripotent stem cells; LDH, lactate dehydrogenase; SAM, statistical analysis of microarray.

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prominent, limits its clinical use (Ferreira et al., 2008; Minotti et al., 2004). Anthracycline-induced cardiotoxicity is exponentially dose-dependant (Carvalho et al., 2009) and categorized as acute, early, or late (Zhang et al., 2009). Acute cardiac toxicity occurs during or immediately after initiation of doxorubicin treatment resulting in tachyarrhythmia's, including sinus tachycardia, premature ventricular contractions, and ventricular tachycardia, as well as bradycardia. Early cardiotoxic events develop within one year of exposure and results in dilated cardiomyopathy. The late cardiac toxicity may develop one or several years after initial exposure, leading to a life-threatening form of cardiomyopathy (Wallace 2003; Yeh et al., 2004). Notably, children and adolescents are particularly susceptible to the cardiotoxic effects of anthracycline chemotherapy compared to adult patients (Lipshultz et al., 1991).

Despite intensive research and progress made over the past two decades, the molecular mechanisms responsible for doxorubicin-induced cardiotoxicity remain incompletely understood. Published reports so far have focused mainly on free radical-induced oxidative stress and apoptosis (Childs et al., 2002; Pointon et al., 2010; Zhang et al., 2012). Cardiac mitochondria are the key mediators of anthracycline-induced cardiomyocyte death (Wallace 2007). Additionally to mitochondrial damage, several signalling pathways are triggered by reactive oxygen species and by anthracyclines causing activation of the intrinsic apoptotic pathway. Apart from the intrinsic mitochondrial apoptotic pathway, anthracyclines also activate the extrinsic apoptotic pathway by several mechanisms contributing to cardiomyocyte damage and death (Nakamura et al., 2000; Nitobe et al., 2003; Niu et al., 2009).

Understanding the mechanisms by which doxorubicin induces cardiac injury is crucial not only to inhibit its cardiotoxic action but also to improve the therapeutic use of doxorubicin. To this end, a number of preclinical models, both long-term and short-term, have been developed in order to predict and understand the cardiac toxicity of doxorubicin and other anthracycline analogues (Herman et al., 1985; Jaenke 1974; Maral et al., 1967; Platel et al., 1999; Pouna et al., 1996). Common for these models is that they all are of non-human origin. However, due to species-related variations in general physiology and drug metabolism, studies in laboratory animals are in many cases of limited value for prediction of the potential toxic effects in humans. To address this issue, Licata et al. developed an *in vitro* human heart system in which cytosolic fractions from myocardial samples disposed during coronary artery bypass surgery were used to study doxorubicin metabolism (Licata et al., 2000). However, human heart tissue samples are usually difficult to source and the material that can be made available is usually derived from non-healthy donors. Thus, the establishment of new human myocardium models is essential in order to develop *in vitro* assays that more accurately can predict cardiac toxicity in patients.

Human pluripotent stem cells (hPSC), of either embryonic origin (hESC) or induced by genetic modification (hiPSC), offer a new approach for generating a variety of cells for *in vitro* models. The ability of unlimited propagation and the potential to differentiate into all cell types in the human body makes hPSC very attractive as a source for human specialized cells (Takahashi et al., 2007; Thomson et al., 1998). The protocols for differentiation of hPSCs into cardiomyocytes have improved substantially in recent years, and today cardiomyocytes with high purity can be derived in large scale and in a reproducible fashion (Burridge et al., 2014; Lian et al., 2012). The fact that these cells can be derived robustly from well-characterized hPSCs makes them well suited to use as a toxicity assessment model, especially since the genetic background of the hPSC can be selected to address a specific disease phenotype (Liang et al., 2013).

In the present study, we evaluate the use of hESC-derived cardiomyocytes as a model to study doxorubicin-induced cardiotoxicity. Human cardiomyocyte cultures were exposed to doxorubicin at various concentrations and the toxic responses in the cells were assessed during the acute exposure (up to 2 days) as well as after an additional 12-day recovery period. The release of lactate dehydrogenase (LDH) and cardiac specific troponin T (cTnT) was measured to assess general cytotoxicity and cardiotoxicity, respectively. In addition, global gene expression analysis was performed to investigate the mechanisms and cellular pathways activated in the cells during and after doxorubicin treatment. The results from this analysis identified several novel potential biomarkers, which can be used with high sensitivity to predict doxorubicin-induced cardiotoxicity.

2. Materials and methods

2.1. Cell culture

Human cardiomyocytes, Cellartis® Pure hES-CM, were obtained from Takara Bio Europe AB (former Cellectis AB, Gothenburg, Sweden) and handled according to the instructions from the manufacturer. The cells were thawed and seeded at 200 000 cells/cm^2 in 24- or 96-well plates and medium change was performed every second day.

2.2. Compound exposure and toxicity evaluation

Four days post-thawing, the cells were incubated with or without doxorubicin (D-1515, Sigma–Aldrich, Sweden) at various concentrations (50 nM, 150 nM, and 450 nM) for up to 2 days, followed by a 12-day wash-out period without drug exposure. The experiments were performed in triplicates. The cell morphology as well as the contractile ability was monitored during the entire experiment. Cells and conditioned cell culture medium were harvested at four different time points (1, 2, 7, and 14 days counted from the start of compound treatment) during the exposure and recovery period (see Fig. 1 for an overview of the study outline). LDH was measured in the cell culture medium using Lactate Dehydrogenase Activity Assay Kit (MAK066, Sigma–Aldrich Sweden) according to the providerís instructions. The cTnT levels in the cell culture medium were measured using an automated

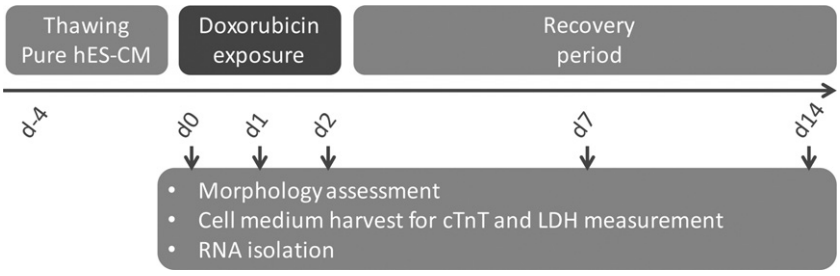

Fig. 1. Study outline. The figure displays a schematic overview of the study outline.

immune-chemiluminescence instrument (Elecsys 2010, Roche) and the Elecsys Troponin T STAT assay reagent (Roche) according to the manufacturer's recommendations (Forest et al., 1998). The data from the toxicity evaluation (cTnT and LDH) are presented as means ± SEM. Statistical significance was determined using one-way independant ANOVA and the Bonferroni post *hoc* test. Differences between two means with $p < 0.05$ were considered as statistically significant.

2.3. RNA extraction and microarray experiments

The cells were harvested in RNAprotect Cell Reagent (QIAGEN, www.qiagen.com) and stored at −20° C until extraction. Total RNA was extracted using QIAGEŃs RNeasy Plus Micro Kit according to the manufacturer's instructions (QIAGEN, www.qiagen.com). Quantification of nucleic acids was performed on NanoDrop ND-1000 (NanoDrop, http://www.nanodrop.com). The quality of the RNA and cDNA, labelled by *in vitro* transcription, was verified using a 2100 Agilent Bioanalyzer. To measure the mRNA levels, fragmented cDNA was hybridized at 45 °C for 16 h to whole transcript HuGene ST 2.0 arrays (Affymetrix, http://www.affymetrix.com) at SCIBLU Genomics (Lund University, Sweden).

2.4. Pre-processing of data

The expression signals were extracted and normalized by means of the Expression Console v.1.1.2 (Affymetrix) applying the robust multichip average (RMA) normalization method. The normalized dataset was further processed to remove low expression probes. Probes with a $\log_2$ expression of 5 or higher in at least one sample were selected for further analysis. The resulting dataset of 21,466 probes was analysed using a hierarchical clustering approach to confirm reproducible replicates.

2.5. Identification of differentially expressed genes

In order to identify differentially expressed genes between the control group and the doxorubicin treated groups, a significance analysis of microarrays (SAM) (Tusher et al., 2001) was performed using the R package *siggenes*. For each time point, the expression values of the control samples were compared to the expression values of the medium (150 nM) and high dose (450 nM) samples combined, in a two class unpaired SAM. A false discovery rate (FDR) of ≤0.05 was considered significant. In parallel to the SAM analysis, an expression profile analysis was performed with the HCE3.5 software (http://www.cs.umd.edu/hcil/hce/). A model-based query was applied and the Pearson correlation was calculated for each gene profile. Using default parameters of the software, genes with $r > 0.9$ with the defined example profile were selected for further analysis. To explore functional properties of the identified genes, the STRING search tool (http://www.string-db.org/) was used to create protein-protein interaction networks and reveal interactivity of these gene products.

2.6. Pathway analysis

To identify overrepresented pathways amongst the significantly up-regulated genes, the meta-database ConsensusPathDB (http://cpdb.molgen.mpg.de/), which integrates the content of 13 different pathway databases, was used. A *p*-value cut-off of 0.01 and a minimum overlap of five genes between the pathways and the gene list were chosen as analysis parameters. The expression levels of all the genes in each of the identified overrepresented pathway

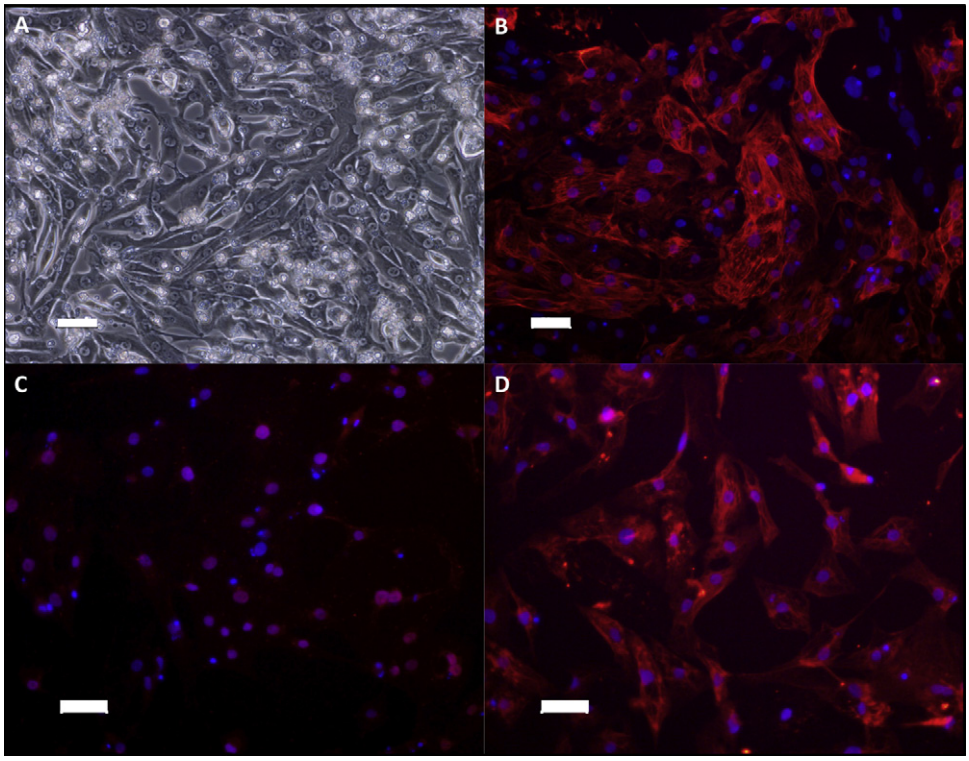

Fig. 2. Characterization of Cellartis® Pure hES-CM at day 4 post-thawing (i.e., start of doxorubicin treatment) (A) light microscopy image (20× magnification) displaying the morphology of the pure hES-CM. (B) Immunocytochemistry staining of cTnT (red) and DAPI (blue). (C) Immunocytochemistry staining of Nkx2.5 (red) and DAPI (blue). (D) Immunocytochemistry staining of MLC2a (red) and DAPI (blue). Scale bar for all images = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were investigated and compared with respect to time points and concentrations using the R statistical software.

3. Results

The human cardiomyocyte cultures were highly homogenous and spontaneous contractions were typically observed already two days post-thawing and seeding. In order to verify the cardiac phenotype, the cells were immunostained using antibodies against several cardiomyocyte specific markers, such as cTnT, MLC2a, and Nkx-2.5 (Fig. 2). Upon doxorubicin exposure the morphology of the cells was altered and the contractile ability was reduced with increasing concentration of doxorubicin. The morphological changes were sustained during the recovery period (Fig. 3).

To assess the general cytotoxic response to doxorubicin, the amount of LDH in the cell culture medium was measured at day 1, 2, 7, and 14. An increase, however not statistically significant, in LDH leakage from the cells was observed after the 2-day doxorubicin exposure at 450 nM compared to the control (0 nM) (Fig. 4A). In addition, a slight but progressive increase in LDH release was detected during the 14-day experimental period also in the control wells (0 nM doxorubicin), indicating a deteriorating effect on some cells during long-term culture. A similar pattern as for the LDH measurement was observed when measuring the cTnT release after 2-days of doxorubicin exposure (Fig. 4B). Notably, cTnT release appears to be a more sensitive read-out, compared to LDH, for doxorubicin induced toxicity in the cells since a significant effect was detectable already at day 1 at the highest doxorubicin concentration. Compared to the vehicle control, the doxorubicin effect disappears during the recovery period and no significant changes in LDH and cTnT release are detectable at day 7 and 14. It should be noted that the concentrations of doxorubicin used in these experiments were specifically selected to be in the low range in order to not induce a substantial general cytotoxicity that would complicate the interpretation of the data. Initial dose-finding experiments indicated that further increasing the doxorubicin concentration to >450 nM caused a substantial cell death already during the time of drug exposure. Thus, since the aim of the present study was to investigate the molecular mechanisms involved in low-dose and long-term doxorubicin-induced cardiotoxicity, we selected doses below those that did induce grossly overt cell deteriorating effects.

In order to interrogate the gene expression profiles of the cells during the course of the experiments we used microarrays. All samples prepared for the microarray analysis passed the quality control and were appointed for further analysis. A global hierarchical clustering of the expression values of all samples showed a similarity between the replicates, as well as a time effect and a treatment effect. To remove background noise, low expressed transcripts ($\log_2$ value ≤ 5) were filtered from the dataset and the remaining 21,466 transcripts were further analysed. Global hierarchical clustering of the mean values of the replicated samples displayed a separation of the samples into six distinct clusters (Fig. 5). The SAM analyses identified differentially expressed genes between exposed samples and the vehicle control, and a family of 36 genes that showed differential expression across all the time points was identified (Table S1). In parallel, the gene profile analysis resulted in 47 annotated genes (Fig. 6A), which to a large extent overlap with the SAM results.

The union of genes from the SAM analysis and the gene profile analysis were investigated for over-represented pathways using the ConsensusPathDB. As summarized in Table 1, 11 significantly overrepresented pathways with at least five genes present in the input list were identified. Most of these pathways involve p53 signalling, DNA damage response, and other cellular defence mechanisms. Investigation of the genes in each pathway showed a general acute response during doxorubicin exposure, with a majority of genes displaying a 2-fold up-regulation. This is contrary to the later time points where a majority of the genes that were initially 2-fold up-regulated genes are instead down-regulated (data not shown).

As indicated in the pathway analysis (Table 1), a majority of the differentially expressed genes (Table S1) are related to cell defence, apoptosis, and p53 signalling pathways. However, genes that are associated with other cellular functions are also present. One such gene is the growth differentiation factor 15 (*GDF15*), previously reported as a potential biomarker for cardiotoxicity related events (Anand et al., 2010; Bonaca et al., 2011; Nickel et al., 2011; Rohatgi et al., 2012; Wallentin et al., 2013; Wang et al., 2012). As illustrated in Fig. 7, *GDF15* seems to be regulated by proteins such as tumour protein p53 (*TP53*), v-akt murine thymoma viral oncogene homolog 1 (*AKT1*), and tumour necrosis factor (*TNF*). *GDF15* itself appears to be a regulator of proteins such as hypoxia inducible factor 1, alpha (*HIFN1A*), hepcidin antimicrobial peptide (HAMP), FBJ murine osteosarcoma viral oncogene homolog (FOS), and carbonic anhydrase II (CA2). The expression of *GDF15* was compared to other known biomarkers for chemotherapy-related cardiotoxicity (Lipshultz et al., 2014) but none of those displayed the dose-response profile detected for *GDF15* (Fig. S1).

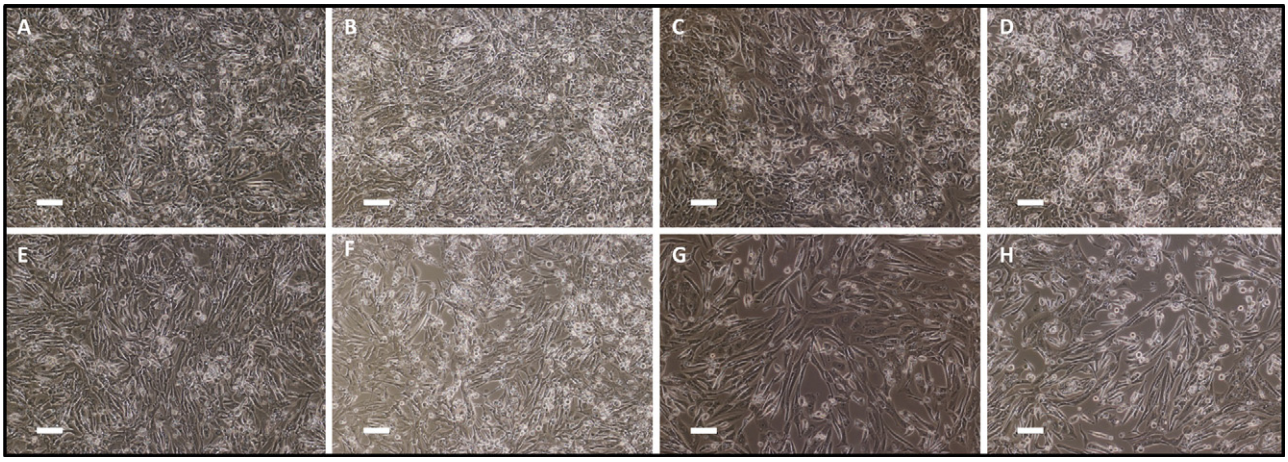

Fig. 3. Morphological images of Cellartis® Pure hES-CM during doxorubicin treatment. The images display the morphological changes seen upon doxorubicin exposure. The upper panel shows untreated cells at day 1 (A), day 2 (B), day 7 (C), and day 14 (D), while the lower panel shows the 450 nM doxorubicin treatment group at day 1 (E), day 2 (F), day 7 (G), and day 14 (H). Scale bar for all images = 50 μm.

GDF15 shows a very interesting profile in this study and responds to doxorubicin with a significant dose dependant up-regulation at day 1, 2, 7, and 14. Based on the gene expression profile analysis of *GDF15* (Fig. 6B), five additional genes from the family of differentially expressed genes (Table S1); neurofilament light polypeptide (NEFL), low density lipoprotein receptor-related protein associated protein 1 (*LRPAP1*), family with sequence similarity 198, member B (*FAM198B*), RAD51 paralog C (*RAD51C*), and growth arrest and DNA-damage-inducible alpha (*GADD45A*), were detected and they all show a highly similar gene expression profile compared with *GDF15* (Fig. 8).

4. Discussion

The use of doxorubicin as a chemotherapeutic drug continues to be limited due to its dose-dependant cardiac toxicity, the molecular mechanism of which remains incompletely understood. In the present study, we used cardiomyocytes derived from hESC to study short- and long-term effects of doxorubicin exposure at low doses, with the aim to interrogate the mechanisms involved in the toxicity as well as to identify potential novel biomarkers for the late onset toxicity. The analysis on the gene expression level reveals several cellular responses affected by doxorubicin treatment, mainly

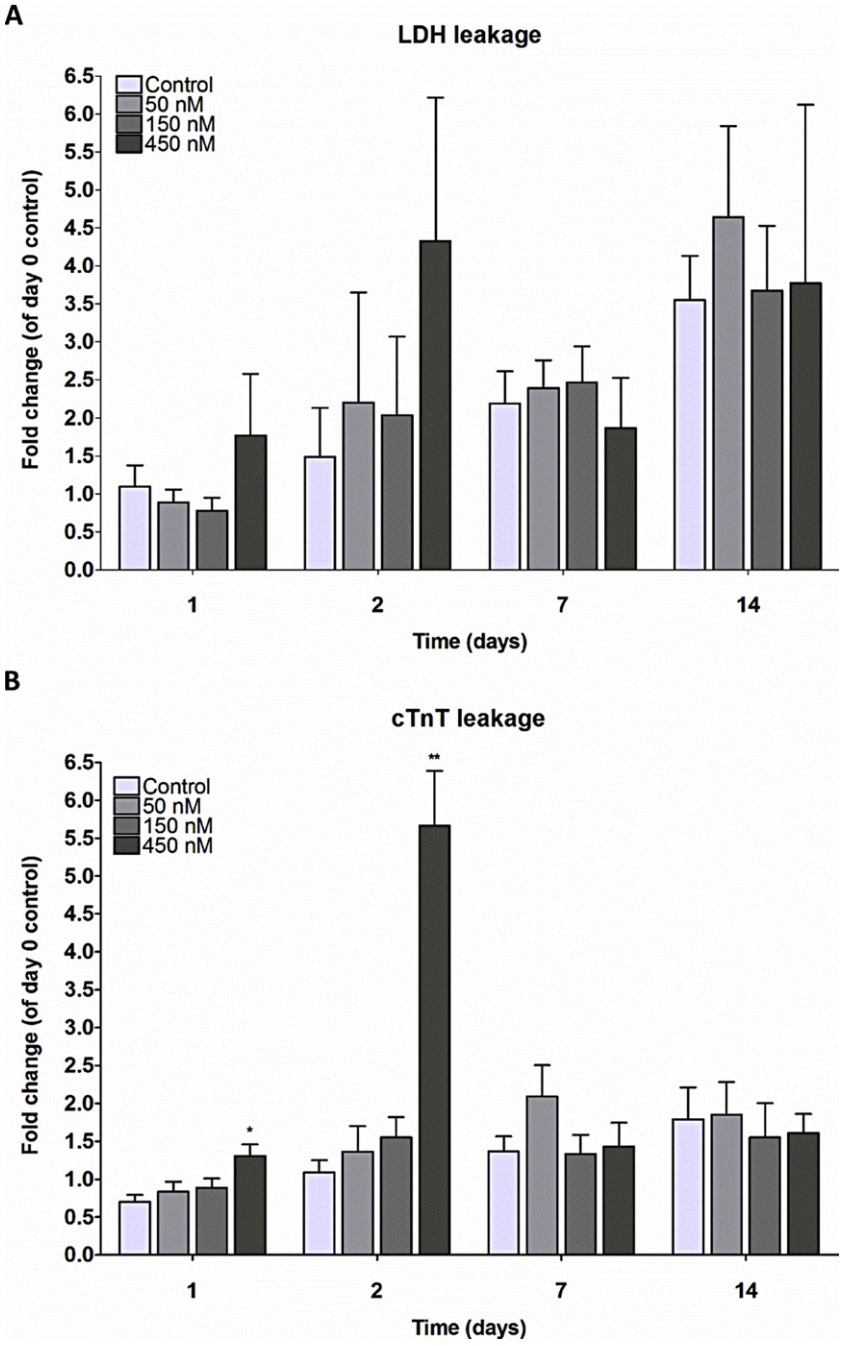

Fig. 4. Toxicity evaluation using cTnT and LDH measurements. Measurements of LDH (A) and cTnT (B) release (see Section 2 for details) from five different batches (assayed in triplicates) of cardiomyocytes exposed to doxorubicin at various concentrations. The bar graph shows the mean values of the fold change related to the baseline values at day 0 and the error bars illustrate the SEM (* = $p < 0.05$, ** = $p < 0.01$).

connected to cellular defence and p53 signalling pathways. Our study also identifies genes that could be employed as biomarkers showing a high association to the doxorubicin exposure, and with a potentially high predictability of the early-, as well as the late onset toxicity.

The cardiomyocytes were exposed to doxorubicin for up to 2 days followed by an additional 12-day wash-out period without drug treatment. The cell morphology and the contractile ability are both affected by the drug exposure, and the morphological changes as well as the reduced contractility were sustained during the recovery period at the highest concentration of doxorubicin used in this study. Measurement of LDH and cTnT in the conditioned cell culture media show an acute toxic response and a dose-dependant increase in the release of these proteins. However, the early response for LDH and cTnT disappears during the recovery period indicating that they might not be suitable as biomarkers for the late onset toxicity.

Global gene expression analysis of the doxorubicin treated cells, in comparison to the untreated controls, reveals several altered genes and signalling pathways. Using SAM we identified a family of genes that shows significant differences between the experimental groups. Interestingly, using a simplified approach applying gene profile analysis, we could identify a number of genes that show high concordance with the results from SAM.

Further analysis of the combined gene lists using the ConsensusPathDB shows that most alterations are, not surprisingly, related to cellular defence and the p53-signaling pathway. The differentially expressed genes represent several downstream targets of p53. For example; *CDKN1A*, a mediator of the p53-dependant cell cycle G1 phase arrest in response to a variety of stress stimuli (el-Deiry et al., 1993); *GADD45A*, involved in growth arrest due to DNA damage, and stimulates DNA excision repair (Cretu et al., 2009); *RRM2B*, which has a pivotal role as a supplier of deoxyribonucleotide during DNA repair (Wang et al., 2009); and *RNF144B*, an apoptosis inducer dependant on p53 but not caspases (Huang et al., 2006). In addition, the list also contained proteins like *MDM2*, which targets suppressor proteins, like p53, for proteasomal degradation (Chao 2014). Furthermore, several histone clusters are also represented, with known important roles in transcriptional regulation, DNA repair and replication, and chromosome stability. Another connection of interest is observed between *PLXNA2*, necessary for semaphorin signalling and remodelling of the cytoskeleton (Tamagnone et al., 1999), and *DPYSL4*, a direct target of p53 signalling (Kimura et al., 2011). Semaphoring signalling has been reported to be required for a proper cardiovascular development (Brown et al., 2001; Gitler et al., 2004). The up-regulation of *PLXNA2* and *DPYSL4* may be involved in the morphological changes detected in the cells following doxorubicin treatment, as well as in the impaired cardiac contractile function. Two cardiac specific genes, *KCNJ2* and *HCN2*, are also up-regulated. These are two potassium channels respectively contributing to the action potential waveform and excitability in muscle tissue as well as the native pacemaker currents in the heart (Munoz et al., 2013; Santoro and Tibbs 1999). An altered potassium current in the cardiomyocytes might contribute to the impaired cardiac function observed after doxorubicin treatment.

Of particular note, in the periphery of the p53-centred network is the growth differentiation factor 15 (*GDF15*), also known as macrophage inhibitory cytokine 1 (MIC-1) and nonsteroidal anti-inflammatory drug-activated gene (NAG-1), which is a distant member of the TGFβ superfamily. The expression of *GDF15* is substantially up-regulated in a dose dependant manner at day 1, 2, 7, and 14, suggesting it can be used as a biomarker for the doxorubicin-induced toxicity. The fact that *GDF15* mRNA expression seems more sensitive to the doxorubicin treatment than cTnT and LDH supports this hypothesis. The comparison of the gene expression profiles between *GDF15* and other genes previously proposed as potential biomarkers for chemotherapy-related cardiotoxicity (Lipshultz et al., 2014) demonstrates that *GDF15* is the only marker in this set of genes that shows a dose-dependant increase upon doxorubicin treatment that is sustained throughout the whole experiment (Fig. S2).

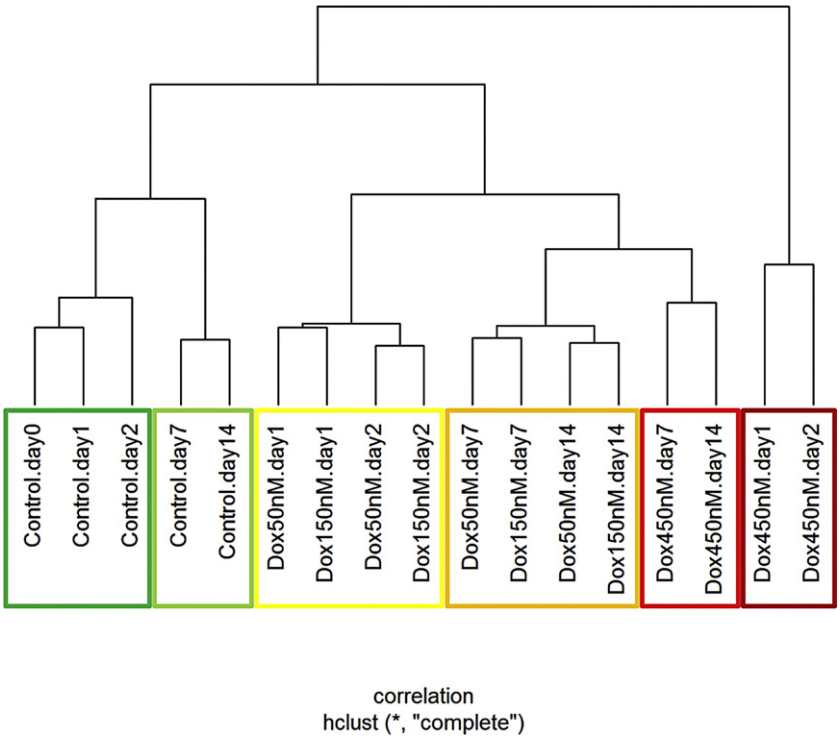

Fig. 5. Global hierarchical clustering of the microarray data. Dendrogram of the mean expression values from three replicated samples, displaying a separation of all samples into six distinct clusters.

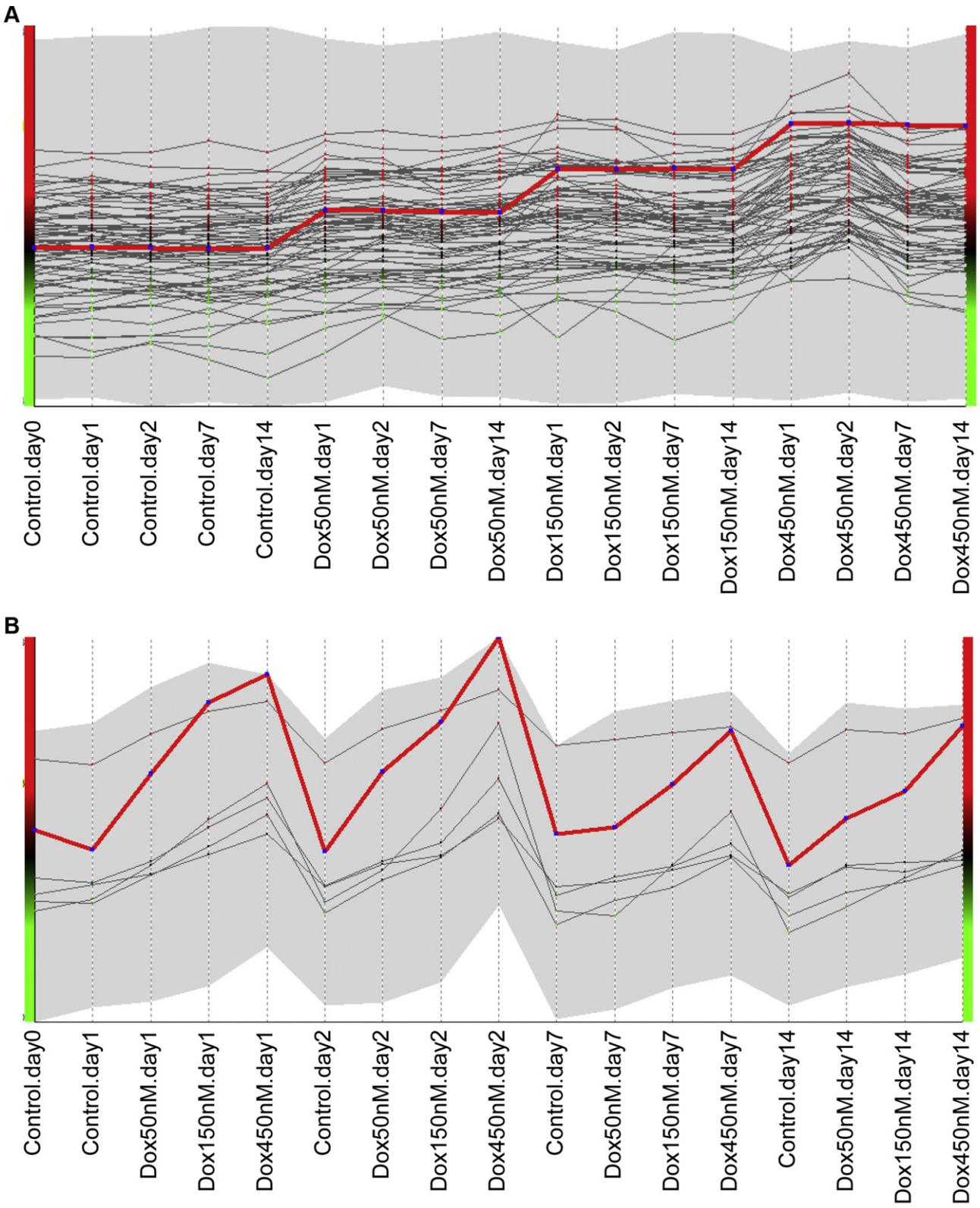

Fig. 6. Analysis of gene expression profiles. (A) Gene expression profile analysis, using the HCE3.5 software, of all transcripts with an FC ≥ 2 between exposed samples and untreated control. The red line represents a target gene profile while the black lines represent the genes with a Pearson correlation coefficient $r > 0.9$. (B) HCE3.5 software image the gene expression profile of *GDF15* (red line) together with the expression profiles of five similarly expressed genes (black lines). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Affected pathways due to doxorubicin exposure. List of over-represented pathways identified using ConsensusPathDB and the differentially expressed genes.

	Genes in pathway	Overlap with candidate list	*q*-value	Pathway source	Differentially expressed genes in pathway
Direct p53 effectors	142	11 (7.7%)	1.30E-10	PID	*GADD45A*, *FAS*, *MDM2*, *RPS27L*, *GDF15*, *TP53I3*, *RRM2B*, *CDKN1A*, *SESN1*, *TNFRSF10C*, *RNF144B*
Validated transcriptional targets of TAp63 isoforms	55	8 (14.5%)	3.75E-10	PID	*GADD45A*, *FAS*, *MDM2*, *SPATA18*, *GDF15*, *TP53I3*, *CDKN1A*, *MFGE8*
p53 Signalling pathway – homo sapiens (human)	68	7 (10.3%)	5.60E-08	KEGG	*GADD45A*, *FAS*, *MDM2*, *TP53I3*, *RRM2B*, *CDKN1A*, *SESN1*
DNA damage response	67	6 (9.0%)	1.23E-06	Wikipathways	*GADD45A*, *FAS*, *MDM2*, *RRM2B*, *CDKN1A*, *SESN1*
miRNA regulation of DNA Damage response	97	6 (6.2%)	8.94E-06	Wikipathways	*GADD45A*, *FAS*, *MDM2*, *RRM2B*, *CDKN1A*, *SESN1*
p73 Transcription factor network	79	5 (6.4%)	4.68E-05	PID	*FAS*, *MDM2*, *GDF15*, *TP53I3*, *CDKN1A*
FoxO signalling pathway – homo sapiens (human)	133	5 (3.8%)	0.00052	KEGG	*GADD45A*, *MDM2*, *FBXO32*, *CDKN1A*, *IRS2*
Cellular senescence	183	5 (2.7%)	0.00196	Reactome	*MDM2*, *HIST1H4H*, *HIST2H2BE*, *CDKN1A*, *HIST1H2BC*
Viral carcinogenesis – homo sapiens (human)	206	5 (2.4%)	0.00296	KEGG	*MDM2*, *HIST1H4H*, *HIST2H2BE*, *CDKN1A*, *HIST1H2BC*
Cellular responses to stress	269	5 (1.9%)	0.00849	Reactome	*MDM2*, *HIST1H4H*, *HIST2H2BE*, *CDKN1A*, *HIST1H2BC*
Neuronal system	277	5 (1.8%)	0.00873	Reactome	*MDM2*, *GABRB2*, *NEFL*, *KCNJ2*, *HCN2*

It has been shown that an enhanced serum level of *GDF15* is an independant risk factor for cardiovascular events (Brown et al., 2002). In recent years, many studies have indicated the role of *GDF15* in heart disease and its potential use as a biomarker (Anand et al., 2010; Bonaca et al., 2011; Nickel et al., 2011; Rohatgi et al., 2012; Wallentin et al., 2013; Wang et al., 2012). For example, Arslan et al. reported that measurement of *GDF15* levels in combination with Tissue Doppler imaging may be a method to detect asymptomatic anthracycline-mediated cardiomyopathy in young cancer survivors (Arslan et al., 2013). Even though more studies are needed to elucidate how the information provided by *GDF15* can be used in a clinical setting, our study strengthens *GDF15*'s emerging role as a biomarker for cardiotoxicity.

Results from the protein-protein interaction analysis illustrated that *GDF15* is regulated by AKT1, TP53, and TNF (Baek et al., 2004; Wilson et al., 2003; Wollmann et al., 2005; Yamaguchi et al., 2004). On the downstream side, *GDF15* seems to regulate HAMP, HIF1A, FOS, CA2, and PDF (Camaschella and Silvestri 2008; Ishaq and Shabbir 2011; Li and Ginzburg 2010; Vanhara et al., 2009). Interestingly, Spagnuolo et al. have previously shown that HIF1A might be involved in the dexarazoxane-mediated protection of cardiomyocytes from doxorubicin-induced toxicity (Spagnuolo et al., 2011). Thus, the activation of HIF might be a mechanism contributing to a protective effect against antracycline-dependant cardiotoxicity. *GDF15* regulates the HIF1A expression via the ErbB2 signalling pathway (Kim et al., 2008). The up-regulation of *GDF15* in the surviving doxorubicin-treated cardiomyocytes could be a cellular defence mechanism involving HIF1A activation. *GDF15* has previously also been shown to have a cardiac protective role during ischaemia/reperfusion injury (Kempf et al., 2006).

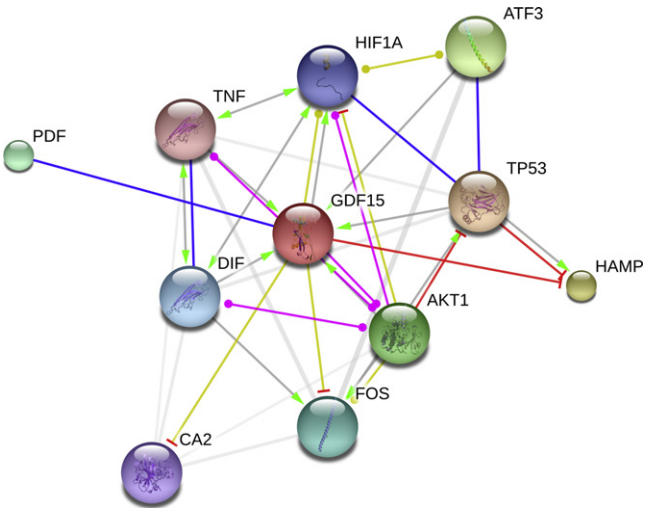

Fig. 7. Protein-protein interaction network of *GDF15*. The STRING search tool was used to generate a protein-protein interaction network using the target gene *GDF15*. Green arrows = activation, red lines = inhibition, blue lines = binding, pink lines = post-translational modification, yellow lines = expression interaction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

From previous work, the involvement of iron in doxorubicin-mediated cardiotoxicity is generally recognised (Minotti et al., 1999; Simunek et al., 2009; Xu et al., 2005). The protein-protein interaction network created in our study shows that *GDF15* is associated with HAMP, which is involved in the maintenance of iron homeostasis (Camaschella and Silvestri 2008; Ishaq and Shabbir 2011; Li and Ginzburg 2010).

Finally, we also identified additional genes presenting an expression profile similar to *GDF15* under the experimental conditions used in this study. For example, *NEFL*, *LRPAP1*, *FAM198B*, *RAD51C*, and *GADD45A* all show a dose-dependant up-regulation upon doxorubicin exposure in a similar pattern as observed for *GDF15*, suggesting that these genes could be interesting to investigate further as potential novel biomarkers for doxorubicin-induced cardiotoxicity. However, additional studies are needed to evaluate these novel findings and validate the usefulness of these candidates as biomarkers for anthracycline-mediated cardiotoxicity.

In the present study, we have demonstrated the utility of hESC-derived cardiomyocytes for the assessment of doxorubicin-induced cardiotoxicity. Importantly, the cells were stable enough in culture to enable a long-term follow-up study of acute anthracycline-mediated toxicity. LDH and cTnT measurements indicate a toxic response in the cells during the acute compound exposure. However, this response disappeared during the 12-day recovery period and the effect of doxorubicin could not be detected using LDH and cTnT measurements as endpoints. On the other hand, global gene expression analysis identified several cellular pathways affected by the doxorubicin treatment. We also demonstrate that the gene expression of *GDF15* is a more sensitive marker compared to cTnT measurement and, as such, might be a more predictive biomarker than the conventional biomarkers used for anthracycline-mediated cardiovascular events. In addition, we identified genes with a similar expression profile as *GDF15* with the potential to be used as novel biomarkers for anthracycline-mediated cardiotoxicity. Taken together, the data presented in this study lend support to the use of

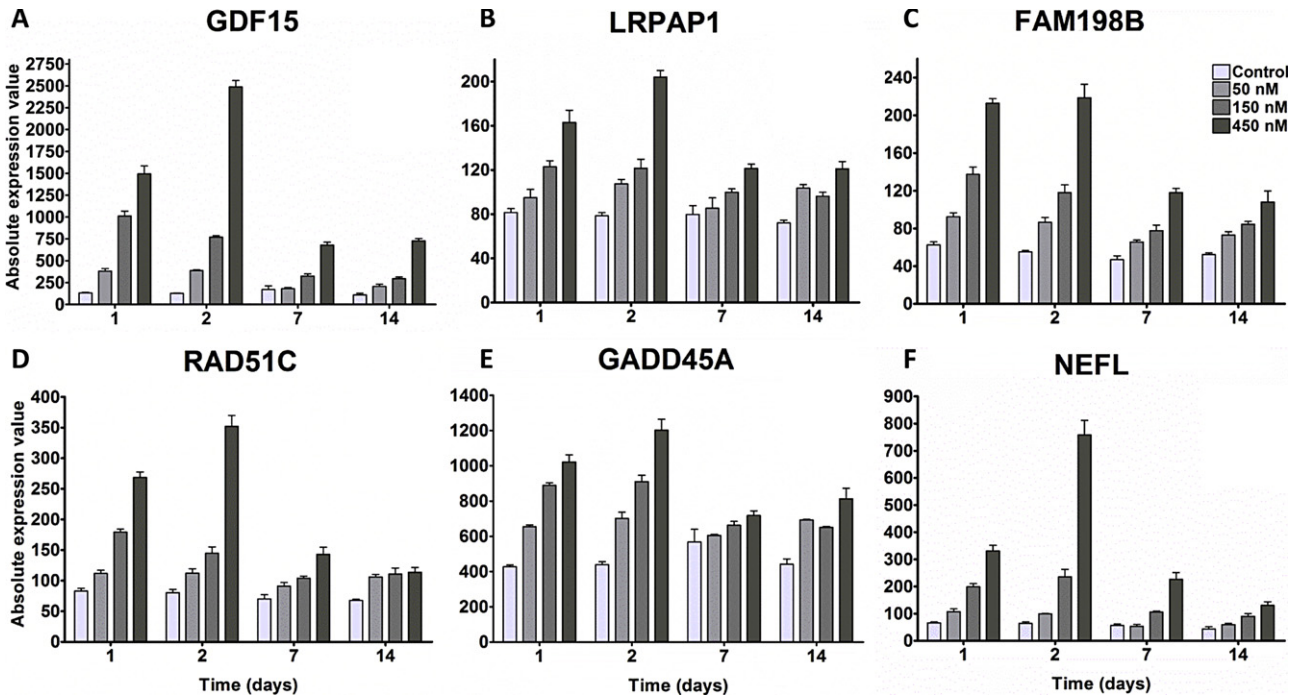

Fig. 8. Gene expression of *GDF15*. The graph shows the expression of *GDF15* and five other genes, identified with the HCE3.5 software, showing similar gene expression as *GDF15*. Results are reported as mean expression values ($n = 3$) from the microarray data, each run in triplicate, with error bars representing the SEM.

hESC-derived cardiomyocytes as a relevant model system to investigate cardiotoxicity *in vitro*.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2014.12.018.

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