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Systematic transcriptomic and phenotypic characterization of human and murine cardiac myocyte cell lines and primary cardiomyocytes reveals serious limitations and low resemblances to adult cardiac phenotype

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ABSTRACT

Background: Cardiac cell lines and primary cells are widely used in cardiovascular research. Despite increasing number of publications using these models, comparative characterization of these cell lines has not been performed, therefore, their limitations are undetermined. We aimed to compare cardiac cell lines to primary cardiomyocytes and to mature cardiac tissues in a systematic manner.

Methods and results: Cardiac cell lines (H9C2, AC16, HL-1) were differentiated with widely used protocols. Left ventricular tissue, neonatal primary cardiomyocytes, and human induced pluripotent stem cell-derived cardiomyocytes served as reference tissue or cells. RNA expression of cardiac markers (*e.g. Tnnt2*, *Ryr2*) was markedly lower in cell lines compared to references. Differentiation induced increase in cardiac- and decrease in embryonic markers however, the overall transcriptomic profile and annotation to relevant biological processes showed consistently less pronounced cardiac phenotype in all cell lines in comparison to the corresponding references. Immunocytochemistry confirmed low expressions of structural protein sarcomeric alpha-actinin, troponin I and caveolin-3 in cell lines. Susceptibility of cell lines to sI/R injury in terms of viability as well as mitochondrial polarization differed from the primary cells irrespective of their degree of differentiation.

Conclusion: Expression patterns of cardiomyocyte markers and whole transcriptomic profile, as well as response to sI/R, and to hypertrophic stimuli indicate low-to-moderate similarity of cell lines to primary cells/cardiac

Abbreviations: hiPSC-CM, human induced pluripotent stem cell-derived cardiac myocyte; NRCM, neonatal rat cardiac myocyte; NMCM, neonatal murine cardiac myocyte; AC16/H9C2 Diff, differentiated from of AC16/H9C2; ATRA, all-trans retinoic acid; DEG, differentially expressed genes; PCA, principal component analysis; GO, gene ontology; sI/R, simulated ischemia-reperfusion; SV40, simian virus 40; ANGII, angiotensin II; ISOP, isoprenaline; ITS, insulin-transferrin selenium supplement; FBS, fetal bovine serum.

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tissues regardless their differentiation. Low resemblance of cell lines to mature adult cardiac tissue limits their potential use. Low translational value should be taken into account while choosing a particular cell line to model cardiomyocytes.

1. Introduction

The number of publications on pathologies of cardiac cells including myocardial infarction, heart failure or cardiomyopathies are increasing, since these conditions are the leading cause of death in developed countries. The majority of investigations rely on *in vivo* and *in vitro* models due to limited access to human cardiac tissue. Due to ethical reasons, the reduction of laboratory animal use in preclinical experiments is desirable thus, there is an unmet need for reliable *in vitro* cell-based platforms to model cardiac cells and cardiovascular diseases.

Isolated murine adult and neonatal primary cardiomyocytes have been the most widely used models to study cardiac biology *in vitro*, but their use is somewhat limited due to the limited lifespan in culture, the need for high number of animals and the limited possibilities of genetic manipulations [1]. Additionally, the neonatal cardiomyocyte cultures contain mixed cell populations, and after a couple of days in culture, the non-proliferating cardiomyocytes become overgrown by non-myocytes [2,3] rendering the results obtained in these systems irreproducible or questionable. Therefore, there is an emerging need in basic and translational cardiovascular studies for cultured cardiomyocytes.

Immortalized cardiomyocyte lines, which are generated from primary cells, are feasible alternatives of both primary cultures and *in vivo* experiments using laboratory animals (Fig. 1; Table 1). The rat cardiomyoblast cell line H9C2 has been originally described in 1976 by Kimes and Brandt [4]. The mouse atrial myocyte derived HL-1 line was described by Claycomb et al. in 1998 [17] and lately the human AC16 line was established by Davidson and colleagues in 2005 [20]. All these cell lines show quick propagation in culture and homogeneous response to stimuli that allows their use in high throughput drug screening experiment or even in modeling various cardiovascular diseases such as cardiomyopathies, hypertrophic response, cardiac adverse remodeling, metabolic alternations or ischemia-reperfusion injury. Additionally, their culturing protocols are simple, and their maintenance costs are low. However, cardiac cell lines have numerous limitations including their capability of cell division, loss of specific cardiac features and resistance to hypoxic injury [8,36] that may have a significant impact on extrapolating from *in vitro* results to *in vivo* mechanisms (Table 1). To eliminate these disadvantages, specific protocols are available to induce differentiation of these cells towards a more mature cardiac phenotype. Nonetheless, there are only few studies characterizing the phenotype of these cell lines in their differentiated stage [7,20].

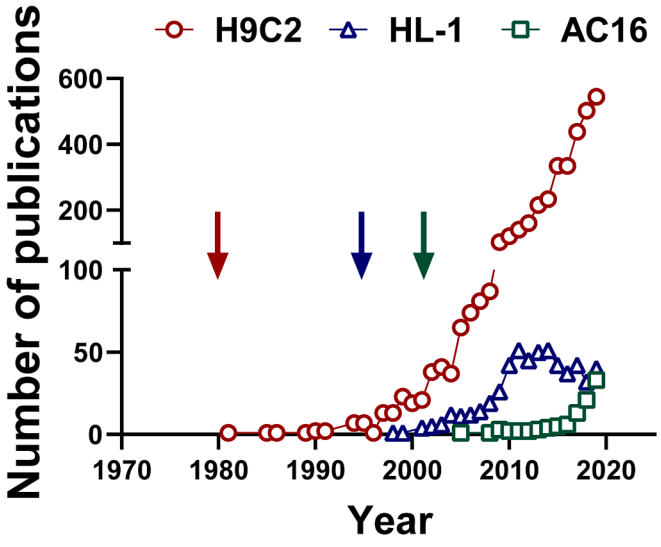

Fig. 1. Trends in the number of publications using AC16, H9C2 or HL-1 cardiac cell lines.
Arrows indicate the year in which H9C2 (red), HL-1 (blue) and AC16 (green) cell lines were first described.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Beside immortalized and primary cardiomyocyte cultures, embryonic or induced pluripotent stem cell-derived cardiomyocytes have been gaining popularity recently. The clear advantages of pluripotent stem cell-derived cardiomyocytes is that these bridge the gap between immortalized cell lines and primary cells, considering their human origin, functional properties, and differentiation stage however, these cells still show some fetal characteristics, and also have limited commercial availability [37]. Although several studies were performed to characterize some of the specific features of these cell lines and primary cardiomyocytes, a systematic comparison of immortalized cell lines, primary cultures or induced pluripotent stem cell-derived cardiomyocytes has not been conducted before.

The aim of our investigation was to compare the most widely used cardiac cell lines with primary neonatal or induced pluripotent stem cell-derived cardiac cell cultures and with adult heart tissues harvested from the corresponding species. We differentiated these cell lines towards a more mature cardiac phenotype, and then we performed a detailed transcriptome analysis, morphological and functional characterization, and tested them at simulated ischemia-reperfusion (sI/R) platforms to explore similarities and cell line-specific features as well.

2. Materials and methods

The extended version of all the materials and experimental methods is found in the Supplementary File.

2.1. Ethical statement

All experimental procedures were performed in accordance with the ethical standards of the responsible institutional and national committee on human experimentation, adhering to the Helsinki Declaration (1975). Written informed consent was obtained from all patients involved in the study according to the protocol approved by the Local Ethics Committees of the Institute of Cardiology, Warszawa, Poland (IK-NP-0021–24/1426/14).

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996), to the EU Directive (2010/63/EU) and was approved by the animal ethics committee of the Semmelweis University, Budapest, Hungary (PE/EA/1784–7/2017).

2.2. Cell line maintenance and differentiation protocols

Rat cardiomyoblast H9C2 cell line obtained from European Collection of Authenticated Cell Culture (passage 29, CRL-1446), while both human cardiac myocyte AC16 cell line (Lot: RD1606008, SCC109) and mouse cardiomyocyte HL-1 cell line were obtained from Merck (Lot: RD1601001, SCC065).

Human induced pluripotent stem cells (hiPSC-CMs) were generated from XCL-1, a male control pluripotent stem cell line reprogrammed from $CD34^{+}$ cord blood cells by episomal vectors (CAXIP-001-1V, XCellScience, Novato, USA).

Neonatal rat and murine cardiac myocytes (NRCM and NMCM,

respectively) were isolated from newborn (1–2 day-old) Wistar rats or C57BL/6 mice as described previously [28]. Cell cultures were used at day 1 or day 3 after isolation.

All lines and cell cultures were maintained and differentiated as described in Supplementary Materials and Fig. 2.

2.3. Statistical analysis

All data are expressed as *mean ± SEM*. Comparisons of two groups were performed using unpaired *Student's t-test*. Experiments with more than two groups were evaluated by *one-way ANOVA* followed by *Tukey's multiple comparisons test*. $p < 0.05$ were considered statistically significant. Statistical analysis was performed with GraphPad Prism 8 (GraphPad Software Inc).

3. Results

3.1. Morphological characterization and mRNA expression of cardiac cell lines and primary cell cultures

One of the unique features of cardiomyocytes is their ability of contraction, thus these cells express contractile proteins of the thick and thin filaments, specific forms of myosins, troponins, tropomyosins and actin. To evidence molecular similarities and differences in cardiac properties of the investigated model systems, we compared expression of major genes involved in cardiac development and myocardial contraction between myocardial samples and cellular models of the given species (Figs. 3A, 4A and 5A). In addition, to assess subcellular localization and structure of the contractile apparatus, and to describe morphological properties of cardiac primary cultures and cell lines, immunofluorescent detection of sarcomere components cardiac α-actinin and cardiac troponin I, (cTnI) and caveolin-3 (Cav3) was performed on human and murine cell lines and primary cultures (Figs. 3B, 4B and 5B).

In cells with human origin, *i.e.*, AC16 and hiPSC-CMs, mRNA expression of elements of the contractile machinery and ryanodine receptor-1 and 2 showed a 50% to 8-fold decrease as compared to the expression levels observed in healthy adult cardiac samples. Expression of myosin heavy chain isoforms 6 and 7 in heart tissue was comparable to hiPSC-CMs, but not to AC16. Ratio of neonatal and adult isoforms was comparable in heart tissue with AC16 and hiPSC-CMs evidencing the fetal gene expression profile of these cell types. Expression of transcription factors *Nkx2.5*, *Gata4*, *Gata6* and *Nr2f2* in hiPSC-CMs was comparable to the expression level in left ventricular samples, however, AC16 cells showed a significantly different expression pattern, which was influenced by differentiation only in case of *Nr2f2* (Fig. 3A). These results further emphasize the fetal characteristics of AC16 cells. The lack of resemblance to adult cardiac tissue was further evidenced by our observation that a similar subcellular localization and organization of sarcomeric α-actinin to that of the cardiac tissue was only found in case of hiPSC-CMs. However, the subcellular distribution of cTnI and α-actinin were not aligned with each other indicating non-functional contractile structures in the investigated human cell types. Interestingly, Cav3 accumulated nearby the nucleus in both hiPSC-CMs and AC16 cells suggesting retention in the Golgi apparatus (Fig. 3B).

Rat H9C2 cells express several genes characteristic of skeletal muscles. *E.g.*, α-actin, ryanodine receptor-1 (*Ryr1*), and the slow-type

Table 1
Summary of cardiac cell cultures used in cardiovascular research.

Cell type		H9C2	HL-1	AC16	Primary neonatal or adult rodent cardiac myocytes	Human iPSC-derived cardiomyocytes
Origin		female embryonic BDIX rat ventricular tissue	SV40 transformed mouse atrial cardiomyocyte (female AT1 cell line)	human ventricular cardiomyocyte fused with SV40 transformed human fibroblast	rodent heart tissue (ventricles) by enzymatic digestion	differentiated from induced pluripotent stem cells
Maintenance	Proliferation	DMEM +10% fetal bovine serum optionally supplemented with glucose or pyruvate	exclusively Claycomb medium + norepinephrine +10% fetal bovine serum	DMEM-F12 + 12.5% fetal bovine serum DMEM +10–15% fetal bovine serum optionally supplemented with glucose or pyruvate	10% fetal bovine serum + DMEM	variable cardiomyocyte differentiation protocols are based on B27 medium supplement and GSK3/Wnt inhibitors in RPMI1640 medium
	Differentiation	1–2% fetal bovine serum supplemented with all-trans retinoic acid, insulin or insulin-like growth factor-1		1× insulin-transferrin-selenium supplement +2% horse serum + T antigen siRNA	1% fetal bovine serum + DMEM	
Utilization		drug screening (cardiotoxicity and cardioprotection), cellular biology, metabolism				
		ischemia-reperfusion injury hypertrophy	electrophysiology: ion channels, atrial fibrillation	ischemia-reperfusion injury	ischemia-reperfusion injury hypertrophy	patient-specific conditions (personalized medicine) myocardial regeneration, transplantation, replacement therapy ischemia-reperfusion injury
Significant features	Morphology and structure	elongated mononuclear cells without contractility	mononuclear beating cells, organized contractile structure	elongated cells with ovoid nuclei without contractility	neonatal: mononuclear cells with contracting colonies adult: rod-shaped cells	variable differentiated and organized contractile structure can be observed
	Metabolism	glycolysis, fatty acid oxidation (differentiated H9C2 > undifferentiated H9C2)	glycolysis and fatty acid oxidation	presumably glycolysis and fatty acid oxidation	fatty acid oxidation (adult >> neonatal) and glycolysis	glycolysis and fatty acid oxidation
Limitations		dedifferentiation after long maintenance	hypoxic resistance expensive maintenance	fibroblast phenotype, dedifferentiation, difficulties with differentiation	high number of animals needed yield and quality depends on personal skills difficulties with standardization	ethical concerns, remained fetal characteristics
References		[4–16]	[8,17–19]	[20–26]	[27–29]	[30–35]

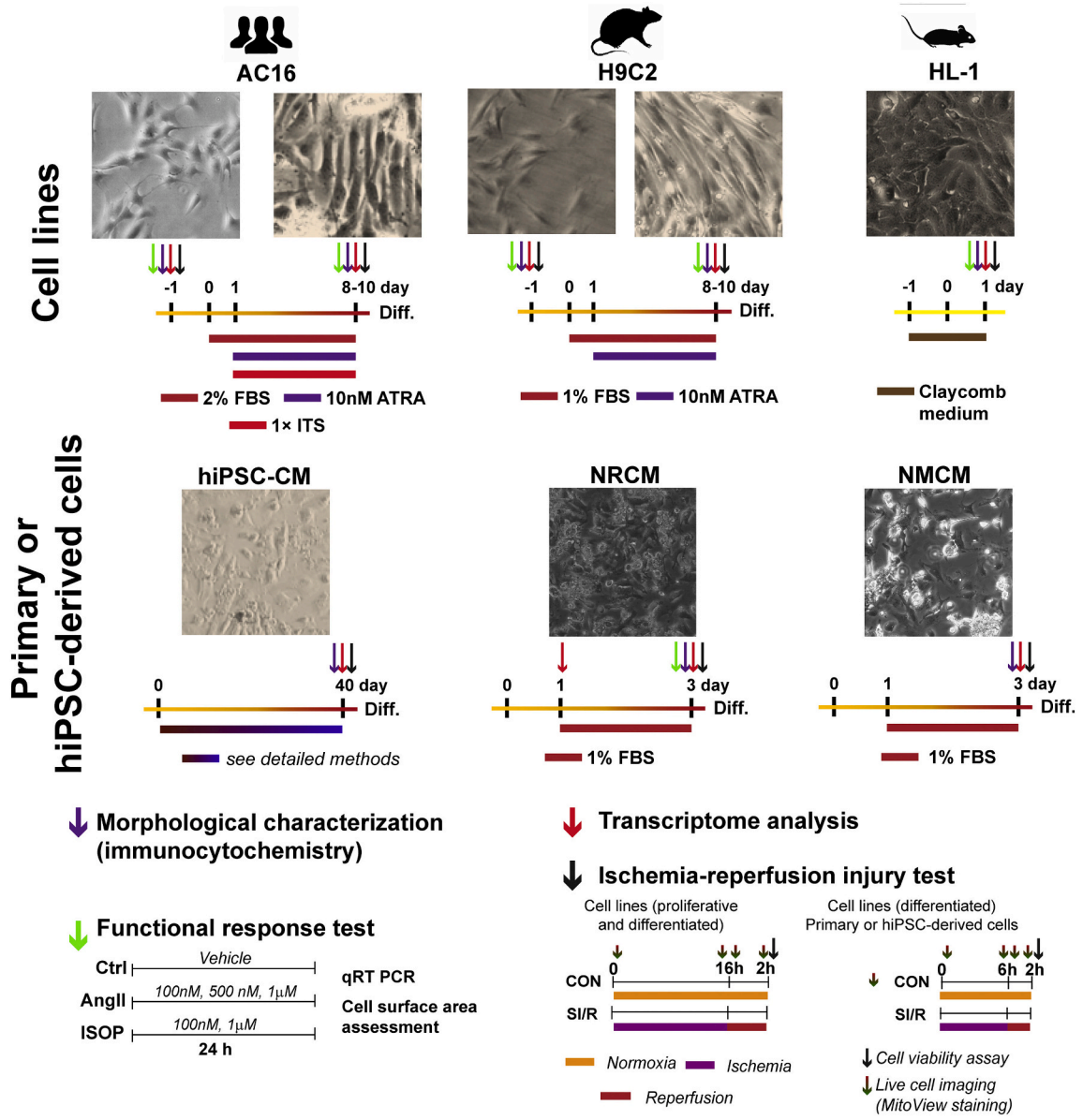

Fig. 2. Protocols of cell maintenance, general differentiation and experimental design.
Abbreviations: FBS – fetal bovine serum, ATRA – all trans retinoic acid, ITS – insulin-transferrin-selenium supplement, Diff – differentiated, hiPSC-CM – human induced pluripotent stem cell derived cardiomyocytes, NRCM – neonatal rat cardiomyocytes, NMCM – neonatal mouse cardiomyocytes, Ctrl/CON – control, AngII – angiotensin II, ISOP – isoprenaline, SI/R – simulated ischemia-reperfusion

troponin I 1 (*Tnni1*) isoforms are expressed in differentiated cells more than 10-fold higher than in the adult heart tissue. Meanwhile, elements of cardiac contractile apparatus are expressed at very low levels in proliferating and in differentiated H9C2 cells as well, and the expression of growth factors by H9C2 cells differ significantly from what was seen in adult cardiac tissue (Fig. 4A). Although, 3-day old NRCMs do not exhibit a skeletal muscle gene expression pattern, level of most cardiac gene products is significantly lower from the adult heart. Primary NRCMs express intact sarcomere structures (Fig. 4B). Cardiac isoform of TnI was under detection level while α-actinin showed a diffuse localization in proliferative H9C2, but differentiation with 10 nM ATRA induced the expression of a more organized structure in case of both TnI and α-actinin (Fig. 4B). Distribution of Cav3 was similar to as seen in NRCMs in both proliferating and differentiated H9C2 cells (Fig. 4B).

The murine HL-1 and NMCMs express cardiac-specific proteins in similar extent, but expression levels both differ from the ones observed in adult mouse heart tissue samples. However, expression of major growth factors shows high similarity with the adult cardiac tissue (Fig. 5A). HL-1 cell immunocytochemistry showed heterogeneous cell population in which only a minority of cells expressed highly organized sarcomere structure. Cav3 was expressed both in HL-1 cells and NMCMs with similar patterns (Fig. 5B).

3.2. Transcriptomic analysis of cell cultures

Gene expression analyses of the differentiated H9C2 cell line was performed previously [38] however, systematic comparison of primary cultures and all the major cell lines have not been performed to date.

Therefore, besides comparing expression levels of mRNAs, we performed analysis of a number of differentially expressed genes (DEGs), principal component analysis (PCA) and hierarchical clustering to estimate overall transcriptome similarity between model systems and the adult cardiac tissue (Fig. 6). Primary cell cultures (NMCM, NRCM D3) and hiPSC-CMs at day 40 generally showed a higher degree of similarity

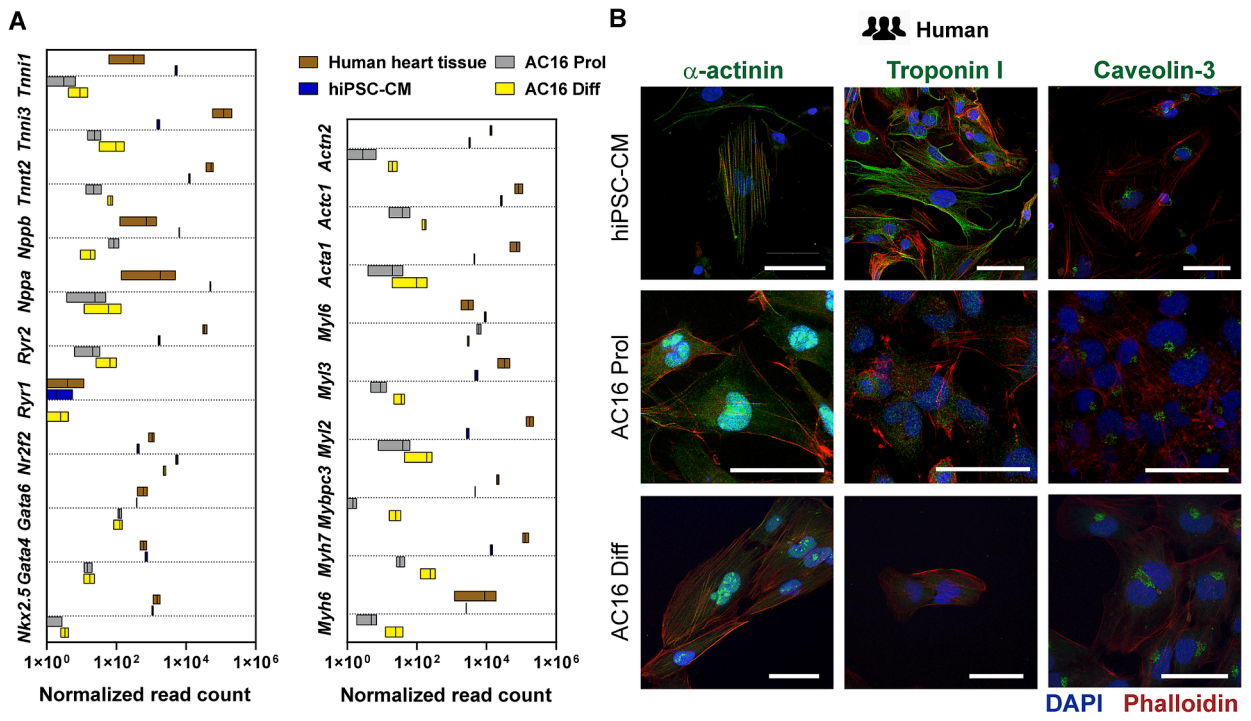

Fig. 3. Morphological characterization and mRNA expression of representative genes of human heart tissue, the proliferative (Prol) and differentiated (Diff) form of human AC16 cardiomyocyte cell line and human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM).
(A) Comparative assessment of mRNA levels of cardiac, embryonic or skeletal muscle-specific genes in different form of cell lines, whole tissue and primary cells. Data is expressed as mean ± SEM; n = 3–4 individual samples. (B) Representative images of immunofluorescent detection of alpha-actinin, troponin I and caveolin-3 proteins. DAPI and phalloidin were used as counterstains. Scale bar: 50 μm.

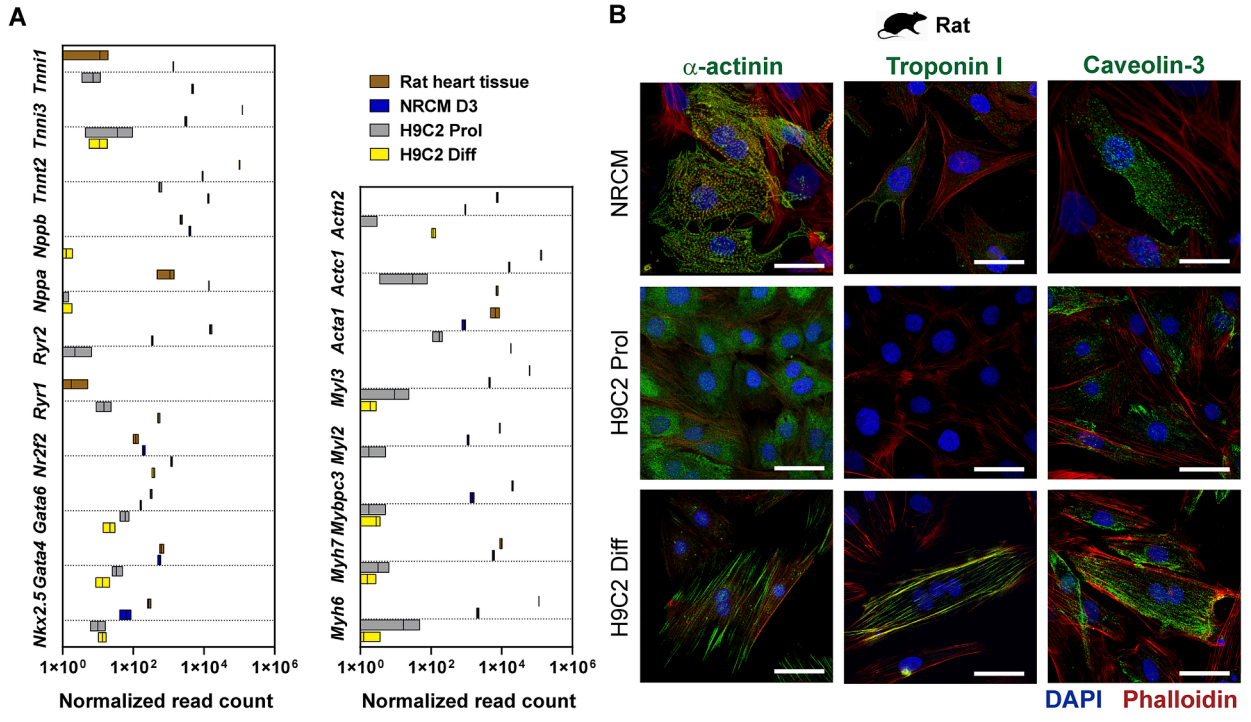

Fig. 4. Morphological characterization and mRNA expression of representative genes of rat heart tissue, the proliferative (Prol) and differentiated (Diff) form of rat H9C2 cardiomyoblast cell line and 3-day old neonatal rat cardiomyocytes (NRCM D3).
(A) Comparative assessment of mRNA levels of cardiac, embryonic or skeletal muscle-specific genes in different form of cell lines, whole tissue and primary cells. Data is expressed as mean ± SEM; n = 3–4 individual samples. (B) Representative images of immunofluorescent detection of alpha-actinin, troponin I and caveolin-3 proteins. DAPI and phalloidin were used as counterstains. Scale bar: 50 μm.

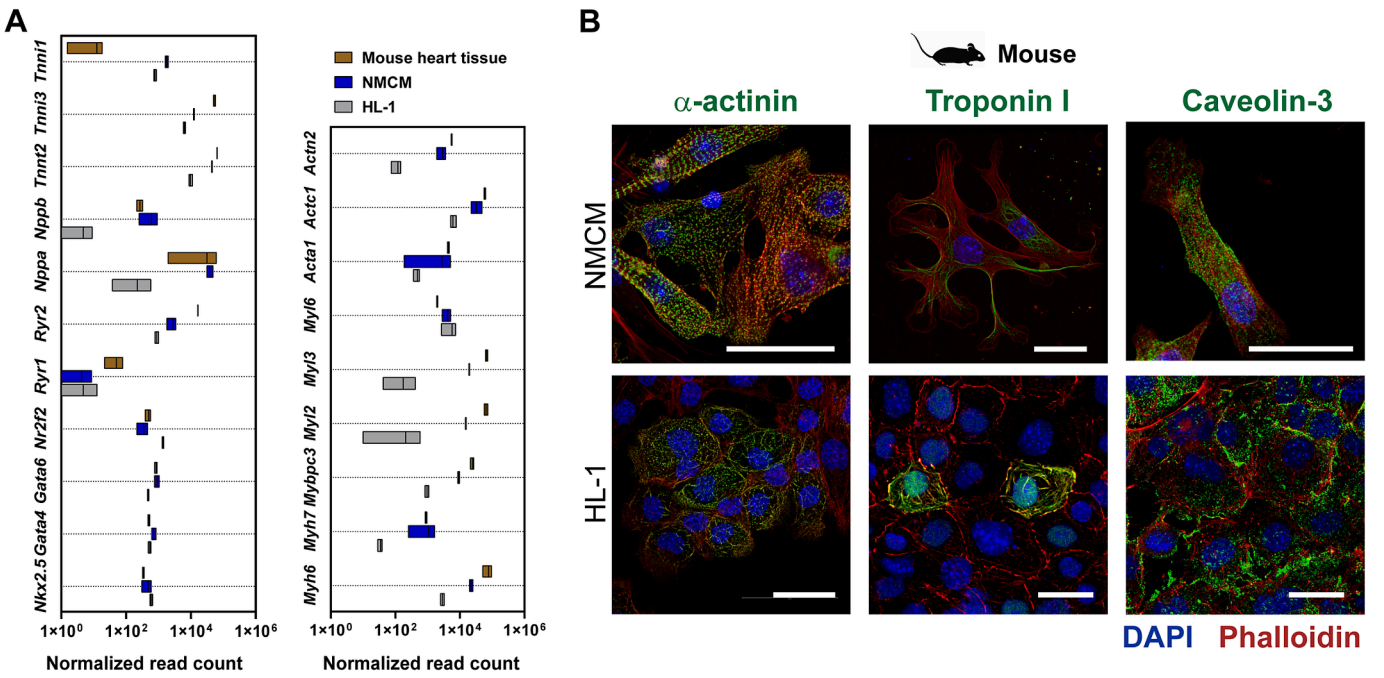

Fig. 5. Morphological characterization and mRNA expression of representative genes of mouse heart tissue, the proliferative form of mouse HL-1 cardiomyocyte cell line (HL−1) and neonatal mouse cardiomyocytes (NMCM).
(A) Comparative assessment of mRNA levels of cardiac, embryonic or skeletal muscle-specific genes in different form of cell lines, whole tissue and primary cells. Data is expressed as mean ± SEM; n = 3–4 individual samples. (B) Representative images of immunofluorescent detection of alpha-actinin, troponin I and caveolin-3 proteins. DAPI and phalloidin were used as counterstains. Scale bar: 30 μm.

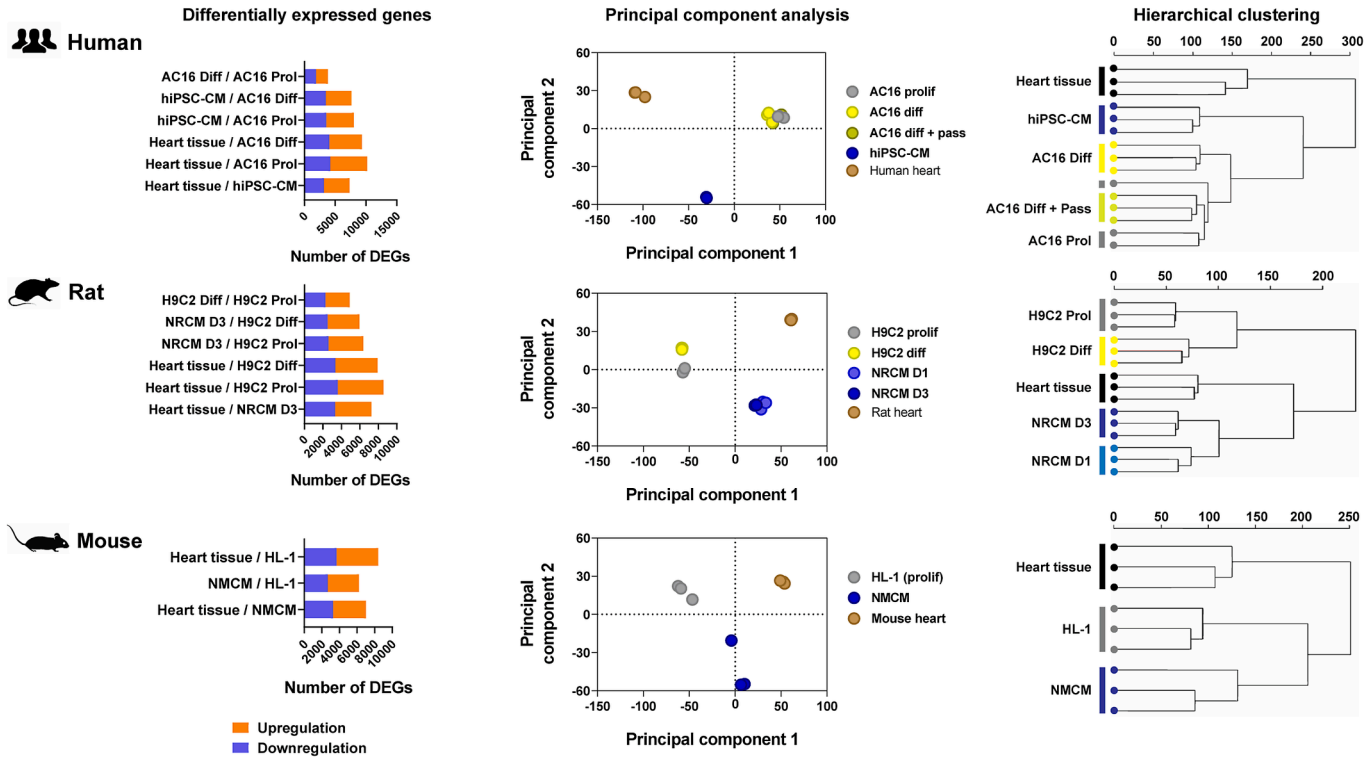

Fig. 6. Transcriptome analysis of samples from human, rat and mouse hearts, cell lines and primary cell cultures.
Number of differentially expressed genes (DEGs), principal component analysis and hierarchical clustering performed on the whole transcriptome. Principal components 1 and 2 are shown. Dots are color-coded to indicate the cell or tissue type from which they were harvested. N = 3 individual samples.

to the reference tissue, whereas cell lines showed less similarity to the adult heart, irrespective of their levels of differentiation (Fig. 6).

In order to get a more comprehensive picture of cell function, comparison of Gene Ontology (GO) term-based functional expression vectors was performed by calculating signed cosine distances for the following fields: cardiac development and differentiation, other cell type

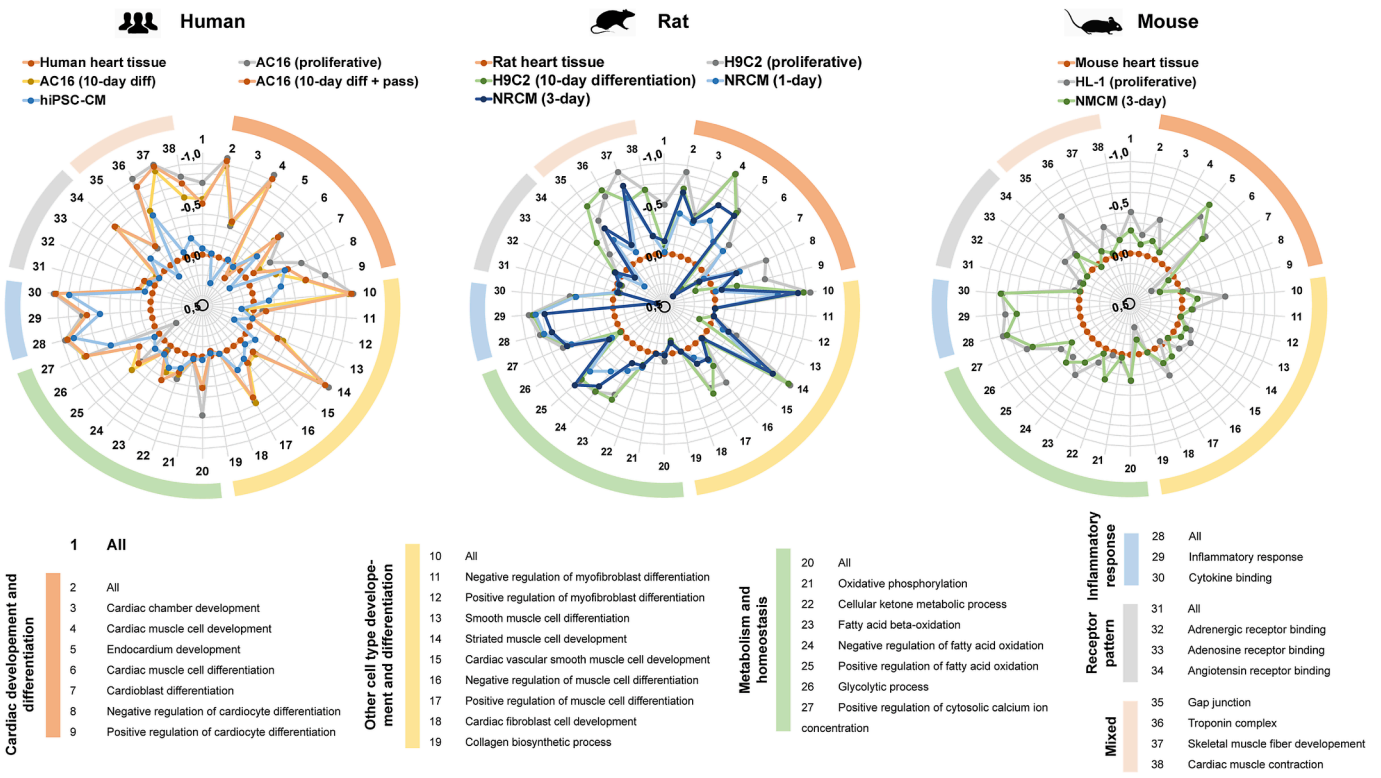

Fig. 7. Radar plot with the dissimilarity of the Gene Ontology term-based functional expression vectors of cardiac cell lines and primary cultures compared to the corresponding whole tissue in human, rat and mouse species.
On the radar plots each concentric ring represents an increase or decrease in signed cosine distance, the measure used to express dissimilarity of functional expression vectors. The outermost ring is the minimum (−1.0) and central point represents +0.5. Negative and positive signed cosine distance values refer to an overall decrease and increase in the expression of the genes annotated to the selected Gene Ontology terms, respectively. The reference (zero) for this analysis is the corresponding heart tissue in each species. $N = 3$ individual samples.

development and differentiation, metabolism and homeostasis, inflammatory response and receptor pattern (Fig. 7). Our analysis indicates that hiPSC-CMs are more similar to cardiac tissue than to AC16 cells in almost all investigated aspects except in inflammatory response. Rat cellular model systems also differ from cardiac tissue in several aspects, and even 3-day old NRCMs show high degree of dissimilarity, *e.g.* in terms of striated muscle cell development or inflammatory response. Both the murine HL-1 and NMCMs, however, showed a better alignment to mouse heart, except in terms of cardiac muscle cell differentiation and inflammatory response (Fig. 7). These results suggest that any of the investigated cardiac cellular model systems may exhibit significant functional differences as compared to *in vivo* conditions.

3.3. Assessment of response to simulated ischemia-reperfusion and hypertrophic stimuli

It is well known that neonatal primary cells or immortalized cell lines, which are less differentiated than adult cardiomyocytes, display more resistance towards hypoxia than adult cells or tissue [8,39]. Therefore, to test the applicability of cardiac cell lines as *in vitro* models of ischemia-reperfusion injury, we exposed both cell lines and primary cells to long or short periods of simulated ischemia, *i.e.* 16 h and 6 h, respectively, followed by 2 h of reperfusion, and then cell viability was assessed (Figs. 2 and 8, Table 2). We calculated the responder ratio defined as the ratio of sI/R experiments with reduced viability to all experiments on the same cell type (Fig. 8, Table 2).

Primary neonatal cultures including murine and rat neonatal cardiomyocytes responded appropriately to the short 6 h/2 h sI/R protocol and had responder ratios 50% and 75%, respectively in all the performed experiments (Fig. 8A). In contrast, the 6 h/2 h sI/R protocol was not able to reduce cell viability in hiPSC-CMs, thus the responder ratio of hiPSC-CMs was 0%; however, the majority of experiments were successful when the long 16 h/2 h sI/R protocol was applied (Fig. 8A). Immortalized cell lines were more resistant to hypoxia. Accordingly, the long sI/R protocol performed on human AC16 or rat H9C2 at a proliferative stage only induced significant cell death in 45.5% and 76.9% of the experiments, respectively (Fig. 8A). Additionally, differentiation of both AC16 and H9C2 increased the hypoxic sensitivity of cells, and in all 16 h/2 h sI/R experiments cell viability was reduced significantly (Fig. 8A). In case of the murine cell line HL-1, the long sI/R protocol reduced cell viability only in 17% of the experiments indicating the highest hypoxic resistance among the three cell lines. Moreover, a slight but significant increase was observed in cell viability (Fig. 8A).

In a separate series of experiments, we examined the morphology of mitochondrial networks of our cellular model systems under normoxic conditions and during simulated ischemia-reperfusion using MitoView Green staining (Fig. 8B–D). Polarization state of mitochondria was assessed with MitoView 633 staining, which is potential-dependent and accumulates in mitochondria proportionally to the electron gradient.

Under normoxic conditions, NRCMs, as well as proliferating and differentiated AC16 cells formed a homogeneous population according to mitochondrial membrane potential. However, differentiated AC16 cells showed less degree of membrane polarization than proliferating AC16 cells. Undifferentiated H9C2, HL-1, and hiPSC-CMs formed several populations with different mitochondrial membrane polarization (Suppl. Fig. 1).

In NRCMs the ratio of polarized mitochondria showed a slight decrease during sI/R (Fig. 8C), however, overall polarization increased at the end of ischemia (Suppl. Fig. 1A, Fig. 8C). In human cell lines (hiPSC-CMs and AC16), a large decrease in mitochondrial polarization was observed at the end of ischemia, which was partially restored at the end of reperfusion in hiPSC-CMs, but not in AC16. Proliferating AC16, as

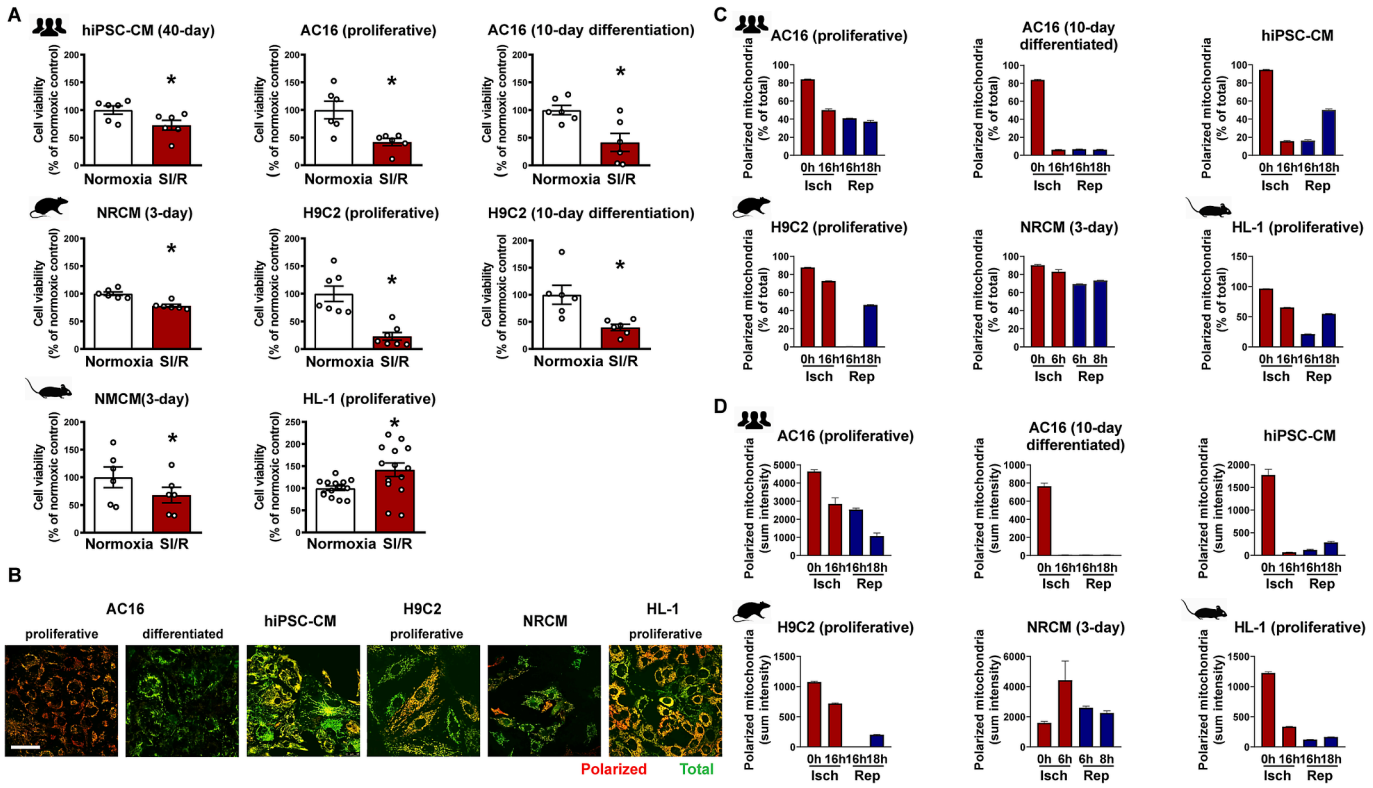

Fig. 8. Response to simulated ischemia-reperfusion stimulus assessed by cell viability assay and live cell imaging.
(A) Cell viability measurement of cell lines and primary cell cultures after 16 h (cell lines and hiPSC-CM) or 6 h (3-day old NRCM and NMCM) simulated ischemia and 2 h reperfusion. Data is normalized to normoxic control and expressed as mean ± SEM; $^*p < 0.05$ *vs* normoxia, Student's *t*-test, $n = 5–8$ independent experiments per cell type. (B) Representative merged images on the staining of polarized (red) and total (green) mitochondria during simulated ischemia-reperfusion. Scale bar: 50 μm. (C) Dynamic changes in the percentage of polarized mitochondria during simulated ischemia (Isch) and reperfusion (Rep). (D) Polarized mitochondrial integrated intensity during simulated ischemia-reperfusion. Data is expressed as mean ± SEM; $n = 8–10$ samples per condition.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Responder ratios of cardiac cell lines and primary cultures.

Immortalized cell line		Responder ratio	Primary cell culture	Responder ratio	
		16H2H		16H2H	6H2R
AC16	Proliferative	45.5%	hiPSC-CM	83.3%	0%
	Differentiated	100%			
H9C2	Proliferative	76.9%	NRCM	–	75%
	Differentiated	100%			
HL-1	Proliferative	17%	NMCM	–	50%

Responder ratio of cell lines and primary cell cultures after simulated ischemia-reperfusion stimulus. Responder ratio was shown as percentage of responder plates/all stimulated plate.

seen in normoxic condition, displayed a higher degree of polarization than differentiated AC16s. Rat H9C2 and mouse HL-1 cells responded to sI/R differently from the cells with human origin: a large decrease in polarization was observed at the beginning of reperfusion, which was followed by a partial regeneration at the end of reperfusion. In rodent cell lines, partial diffusion of MitoView Green was observed at the beginning of reperfusion; however, the staining pattern was restored by the end of reperfusion. Additionally, no significant change was observed in shape and length of mitochondria of the primary cells and cardiac cell lines during simulated ischemia-reperfusion (Suppl. Fig. 1B–C).

Along the same line, the response of cells to cardiac pathological stimuli such as hypertrophy was assessed as well. We treated cell lines or primary NRCMs with increasing doses (100, 500, 1000 nM) of angiotensin II (ANGII) or isoprenaline (ISOP) for 24 h, then, mRNA levels of widely-used cardiac markers, (*i.e.*, *Nppb*, *Ctgf*, *Col1a1* and *Col3a1*) were detected by qRT-PCR (Fig. 2, Suppl. Fig. 2). Surprisingly, neither ANGII nor ISOP treatments altered the levels of the hypertrophy markers assessed, regardless of being in a proliferative or differentiated stage (Suppl. Fig. 2). Alternatively, cell surface area was measured to detect cellular hypertrophy that was undetectable by qRT-PCR analysis. Cell surface area of NRCM was increased by both ANGII and ISOP treatment; however, cell lines did not show significant hypertrophy after the identical treatment (Suppl. Fig. 2).

4. Discussion

Here we have shown with various experimental approaches that in many aspects, immortalized cardiac cell lines such as human AC16, murine HL-1 or rat H9C2 differ significantly from their source tissue or even from the corresponding neonatal primary cardiac cells. We have also demonstrated that all-trans retinoic acid-based differentiation protocols induce only moderate differentiation as assessed by mRNA expression of cardiac markers in both H9C2 and AC16 cell lines. Although, differentiation protocols resulted in favorable changes in the mRNA expression of embryonic genes towards cardiac differentiation markers in H9C2 and AC16 lines, skeletal muscle-specific features became more pronounced as well. In contrast, both the proliferative and differentiated cardiac cell lines failed to respond to short-term hypertrophic or ischemic stimuli suggesting that data obtained with the use of these cells may differ significantly from those of gained *in vivo*.

Current drug screening or toxicity platforms and disease models frequently rely on animal testing. However, *in vivo* studies raise several ethical questions and are expensive. Thus, alternatives for animal

models *e.g.* computer modeling or cell-based platforms may be more applicable for high-throughput screening. Cell platforms might be useful for screening the protective or toxic effects of accepted or novel drugs even in the presence of comorbidities such as hyperglycemia or hyperlipidemia [25,28,40]. Implementing these alternative methods can improve study designing, method optimization and coordination [41,42]. Immortalized cardiac cell lines, primary and stem cell-derived cardiomyocytes particularly with human origin are emerging alternatives for animal models in order to meet 3R (Replacement, Reduction, Refinement) principles [41]. However, results gained on *in vitro* cell platforms might be misleading for the field; numerous targets and drugs such as dipeptidyl peptidase 4 or COX-2 inhibition have been shown to be promising for cardioprotection *in vitro*, but they have no benefits *in vivo* [43–46]. Therefore, the detailed knowledge of strengths as well as limitations of the cardiac cell lines is essential.

By morphological characterization, we confirmed that immortalized cell lines contain immature sarcomeres compared to the primary cell cultures. Organization of contractile machinery is one of the important features of mature cardiac cells that is required for force generation and contraction [47–49]. Organized sarcomeres with ability of force production were not observed in the cardiac cell line H9C2 or AC16 even in differentiated stage, while organized structures were detectable in HL-1 cultures which are able to show contractile activity under certain conditions [50,51]. Ultrastructural characteristics of hiPSC-derived cardiomyocytes were inferior to the structure of primary cardiac cells. This observation is in accordance with previous studies that iPSC-derived cardiomyocytes show various degree of immature sarcomeric organization and remain in fetal stages [49,52].

Systemic analyses and comparisons of other features such as electrophysiological responses, and ion channel expressions, expression of characteristic signaling pathways or expression of other relevant pharmacological targets such as G-protein coupled receptors are still missing from the literature. By comparative transcriptomic analysis, we demonstrated that the immortalized cell lines show fundamental differences in their cardiac and metabolic characteristics compared to the source tissue or to primary cell cultures. The distinct phenotypes and the alterations of gene expression patterns may impact significantly the outcome of the *in vitro* studies conducted on these cardiac cell lines. Studies focusing on adenosine transport are good examples since the equilibrative nucleoside transporters display significantly distinct expression levels in H9C2 as compared to the physiological pattern seen in cardiomyocytes (*i.e.* H9C2 expresses much higher level of ENT-2 as compared to the physiological one). This phenomenon complicates the utility of H9C2 as drug screening platform in the adenosine pharmacology [53]. Our transcriptomic analysis highlights that specific characterization of each cell line before setting up an *in vitro* cardiomyocyte line-based model should be performed.

One of the major findings of this report is that differentiation attempts with widely used protocols from the literature (using all-trans retinoic acid) alter gene expression profile significantly both in case of H9C2 and AC16, although the differentiated cells generally remain heterogeneous [6] and are still markedly distinct from the primary cardiac cells or the original adult cardiac tissue both in morphology and in gene expression patterns. This might be a possible explanation for the fact that the differentiated forms of H9C2 and AC16 lines are generally less popular in cardiovascular research than the undifferentiated ones [5,6,54]. Additionally, differentiation protocols increase the overall duration of cell maintenance by at least 1 week, and there is limited data on the functionality of these differentiated cells. Moreover, we confirmed previous observations [6] that one of the most commonly employed H9C2 cell line undergoes a switch to have skeletal muscle-like characteristics upon application of a commonly used differentiation protocol. Furthermore, differentiated H9C2 has a more mature metabolism which is in line with previous observation [7,55]. No publication is available on differentiated AC16, although Davidson and her colleagues suggest specific protocols generally based on withdrawal of fetal bovine or horse serum and silencing SV40 [20]. In the current study we have used alternative approaches in order to differentiate human AC16 cells, which induced an increased hypoxic sensitivity but a negligible improvement towards cardiac gene expression profile.

Only a few analyses have been conducted so far to provide details on the major characteristic features of cell lines in particular of the most characterized H9C2 regarding metabolism [7,8,56], hypoxic sensitivity [8] or even gene expression profiling [38]. These studies revealed numerous differences between cell lines and primary cell cultures particularly in metabolism and mitochondrial activity that might be a good explanation for the highly diverse hypoxic sensitivity we observed in our sI/R experiments. H9C2 is more sensitive to hypoxia compared to HL-1 therefore, HL-1 line might not be appropriate as cellular platform of ischemia-reperfusion injury [8].

Energy status of mitochondria may influence response to sI/R. This is the first description and comparison of the heterogeneity of mitochondrial membrane potential of cardiac myocytes and related cell lines under normal culturing conditions. Mitochondrial membrane potential of the proliferative AC16 was higher than differentiated AC16s, which may reflect their higher metabolic activity. However, this is in contrast to what has been described in mice and humans, where developing embryonic cardiomyocytes displayed lower mitochondrial activity than mature cardiomyocytes [57,58]. A plausible reason for this contradiction may be that in cell culture, glucose is the primary energy source, which is the primary energy source of immature cardiomyocytes as well [59], while mature cardiomyocytes use predominantly fatty acids as a source of energy [58]. This is further confirmed by the previous report that non-differentiated H9C2 are more reliant on glycolysis while differentiation induces better coupling between glycolytic and oxidative pathways [55]. In contrast, HL-1 was found to be more distinct energetically from primary cardiomyocytes or even from H9C2 as significant differences were observed in mitochondrial biogenesis, morphology and regulation [8].

Here we also report for the first time that mitochondrial energetics shows marked changes after sI/R. While mitochondria of 3-day old NRCMs tolerate sI/R well, other cell lines lose mitochondrial polarization either during hypoxia or at reoxygenation. These results may be explained by molecular differences in the ion channel patterns and/or ischemic defense mechanisms of the different cell types [60]. Thus, direct translation of the results obtained from murine cell lines may not be straightforward. It is interesting to note that those cell types that are more sensitive to sI/R, lost more mitochondrial membrane potential during or after simulated ischemia however, these findings also need further mechanistic investigations.

Responsiveness to hypertrophic and other harmful stimuli might be an important feature of cardiomyocyte cultures particularly when used as drug-screening platforms. We found no clear responsiveness of cardiac cell lines to hypertrophic stimuli by detecting various markers and measuring the cell surface area of them after angiotensin II and isoprenaline stimulation. Our results are in contrast to previous observations with similar doses and time duration [61,62]. Furthermore, controversial reports can be also found since increased cell surface area or mRNA levels of hypertrophy markers are not detectable in every case [14,63]. On the other hand, larger doses (over 1uM) and longer treatments (48 h) are also used [64,65]. Therefore, it is plausible that methodical differences *e.g.* dose, time, pre-treatments and alternative endpoints may have a significant impact on assessing the responsiveness of cell lines and primary cultures. Considering the high variability and lack of broadly accepted protocols in literature, experiments with appropriate group sizes, multiple endpoints and/or confirming results obtained in cellular models in *in vivo* studies are recommended.

Alarmingly, this study reveals that the most frequently used cell lines have limited relevance to model the biology of the adult heart in most of the investigated aspects. Out of the tested cellular models, the ones that more closely resemble myocardial gene expression, such as hiPSC-CMs are used only in a minority of publications. Indeed, human iPSC-

derived cardiomyocyte cells account for only approximately 15–18% of PubMed records searching by keywords including "human", "induced pluripotent stem cell" and "heart" out of the analyzed cell lines including all type of cell lines and primary cultures. Although kits for iPSC-derived cardiomyocyte generation are commercially available, their use still sets numerous challenges. Furthermore, there are variable origins of initiating cells and diverse differentiation protocols when generating hiPSC-CMs that complicates the comparative evaluation of different cell cultures. Apart from the high variability among hiPSC-CMs, they are frequently immature and show distinct characteristic compared to mature cardiomyocytes, which might lead to unsuccessful *in vivo* translation [66,67]. Thus, additional differentiation steps may be necessary to achieve more reliable disease models or drug screening platforms. However, iPSC-derived cardiomyocytes used in our transcriptomic analysis were more similar to the adult cardiac tissue and to primary cells in various aspects even without applying further differentiation procedures as compared to the other *in vitro* systems tested in this study. The immaturity in metabolism was demonstrated in our sI/R experiments as well hiPSC-CMs proved to be more resistant to hypoxia even when compared to neonatal rat or murine cardiomyocytes which can be a consequence of a higher rate of glycolysis in iPSC-derived cardiomyocytes compared to the adult ones [34]. Furthermore, previous studies showed that the metabolism of iPSC-derived cardiomyocytes may be comparable to adult cardiomyocytes after further differentiation steps [68,69]. These results indicate that iPSC-derived cardiomyocytes offer a unique option for setting up cardiac *in vitro* platforms even if further optimization might be needed.

Our study has limitations. We performed our transcriptome analysis on whole heart tissue as reference instead of isolated adult cardiomyocytes, therefore, total RNA samples contained considerable amount of RNA from non-cardiomyocytes (*e.g.* fibroblasts, endothelial or immune cells). Furthermore, we used XCL-1-derived cardiomyocytes to represent hiPSC-CMs therefore, our study is not equipped to show the high variability of hiPSC-derived cardiac cells.

5. Conclusions

In conclusion, cardiac cell lines might be useful and valuable tools in cardiovascular research. However, there are numerous limitations that may impact the results obtained with these *in vitro* cell culture experiments. Therefore, care must be taken when choosing the ideal model, considering the characteristics of these cell lines. In contrast, primary cell cultures, in particular human iPSC-derived cardiomyocytes may be better suited for *in vitro* studies than cell lines due to their higher degree of similarity to adult cardiac tissue. Nevertheless, their limited availability complicates their utilization as an *in vitro* platform for tasks such as high-throughput drug screening. Therefore, further studies should be designed to assure that the *in vitro* conclusions can be extrapolated to *in vivo* processes.

Author contributions

ZO participated in study design and performed *in vitro* experiments, analyzed data, and wrote the manuscript. TV, AK, TK and NN performed *in vitro* experiments and immunohistochemistry, and wrote the manuscript. BK and AM performed primary neonatal cell isolation, simulated ischemia-reperfusion experiments and analyzed data. RNN and TGG performed *in vitro* experiments and cell size measurement. SH performed *in vitro* experiments and live cell imaging, and wrote the manuscript. NV, DR and ÁA generated and handled human induced pluripotent stem cell-derived cardiomyocytes. BÁ and BV analyzed transcriptome data and wrote the manuscript. VET performed *in vitro* experiments. DG participated in *in vitro* experiments. PL collected human heart samples and provided clinical data. ZG, PF, AG and EIB revised the manuscript, the intellectual content and provided professional advice. ZVV designed experiments, wrote manuscript, revised the intellectual content, and provided professional advice. All authors read and approved the final manuscript.

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Data availability

The datasets used and/or analyzed are available from the corresponding author upon request. The mRNA sequencing datasets were deposited in the ArrayExpress database (https://www.ebi.ac.uk/arrayexpress/) under the accession numbers of E-MTAB-10720, E-MTAB-10912 and E-MTAB-10913.

Declaration of competing interest

PF is the founder and CEO, and AG, BÁ and ZG are involved in the management of Pharmahungary Group, a group of R&D companies. EIB is member of the Advisory Board of Sphere Gene Therapeutics Inc. (Boston, US). The remaining authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yjmcc.2021.12.007.

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