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Human embryonic stem cell-derived cardiomyocytes as an *in vitro* model to study cardiac insulin resistance☆

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ABSTRACT

Patients with type 2 diabetes (T2D) and/or insulin resistance (IR) have an increased risk for the development of heart failure (HF). Evidence indicates that this increased risk is linked to an altered cardiac substrate preference of the insulin resistant heart, which shifts from a balanced utilization of glucose and long-chain fatty acids (FAs) towards an almost complete reliance on FAs as main fuel source. This shift leads to a loss of endosomal proton pump activity and increased cardiac fat accumulation, which eventually triggers cardiac dysfunction. In this review, we describe the advantages and disadvantages of currently used *in vitro* models to study the underlying mechanism of IR-induced HF and provide insight into a human *in vitro* model: human embryonic stem cell-derived cardiomyocytes (hESC-CMs). Using functional metabolic assays we demonstrate that, similar to rodent studies, hESC-CMs subjected to 16 h of high palmitate (HP) treatment develop the main features of IR, *i.e.*, decreased insulin-stimulated glucose and FA uptake, as well as loss of endosomal acidification and insulin signaling. Taken together, these data propose that HP-treated hESC-CMs are a promising *in vitro* model of lipid overload-induced IR for further research into the underlying mechanism of cardiac IR and for identifying new pharmacological agents and therapeutic strategies. This article is part of a Special issue entitled Cardiac adaptations to obesity, diabetes and insulin resistance, edited by Professors Jan F.C. Glatz, Jason R.B. Dyck and Christine Des Rosiers.

1. Introduction

Type 2 diabetes (T2D) is a growing health problem worldwide. Fueled by life style changes, such as increased intake of energy-rich food and sedentary lifestyle, the diabetes epidemic has grown parallel with the obesity epidemic [1]. The global prevalence of diabetes among adults has increased dramatically in recent decades, with 4.7% of adults reported with T2D in 1980 and 8.5% in 2014 [2]. This major increase in T2D is associated with an increased risk for the development of heart failure (HF) and other cardiovascular diseases, emphasizing the serious need for research in this particular field [3–6]. Insulin resistance (IR), a hallmark of T2D [7,8], is a complex metabolic disorder that occurs upon persistent exposure of the respective organ or tissue to fat. The IR heart has lost the ability to respond adequately to normal plasma levels of insulin, *i.e.*, the rate of insulin-stimulated (GLUT4-mediated) glucose uptake is reduced [9,10]. The consequent alterations in substrate metabolism eventually lead to impaired cardiac function. Despite improvements in primary and secondary prevention over the past years, patients with T2D or IR still have a 2–4 times higher risk of developing HF [11,12]. This highlights the importance of further elucidating the pathophysiological mechanism behind IR-induced HF. Development of representative model systems to investigate the molecular mechanism will facilitate the search for new targets to replace or support the current treatment strategies.

In recent decades, several animal models have been developed to investigate the underlying mechanism of IR-induced HF. For further in

Abbreviations: T2D, type 2 diabetes; IR, insulin resistance; HF, heart failure; FA, fatty acid; hESC-CMs, human embryonic stem cell-derived cardiomyocytes; HP, high palmitate; GLUT, glucose transporter; v-ATPase, vacuolar-type H^+-ATPase; hSC-CMs, human stem cell-derived cardiomyocytes; hiPSC-CMs, human induced pluripotent stem cell-derived cardiomyocytes; hiPSCs, human induced pluripotent stem cells; hESCs, human embryonic stem cells; AP, action potential; LV, left ventricle

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depth unraveling of this molecular mechanism, cellular models of the hearts of these animals would be preferred, since they have the additional advantage of allowing precise control of the experimental environment, including pharmacological manipulation [13,14]. However, the use of cellular models obtained from experimental animals also comes with limitations, as they do not perfectly correlate to normal human cardiac physiology. To closer reflect the human situation, human *in vitro* models would be particularly beneficial in exploring the underlying mechanisms of cardiac disease. However, primary human cardiac cells or human heart tissue cannot be obtained easily. Therefore, the emerging human stem cell-derived cardiomyocytes could be a useful tool for drug discovery and the development of personalized treatment strategies.

In this review, we provide an overview of the current knowledge and use of human stem cells to study IR and the potential application of human stem cell-derived cardiomyocytes (hSC-CMs) for studying cardiac IR. First, we summarize the available human cardiomyocyte sources after which we provide more details about the basic characteristics of stem cell-derived cardiomyocytes (including human induced pluripotent and embryonic stem cell-derived cardiomyocytes (hiPSC-CMs and hESC-CMs, respectively)) and the use of cell models to study IR. Finally, we present novel metabolic data on hESC-CMs as a promising human *in vitro* model for the study of cardiac IR.

2. Sources of human cardiomyocytes

2.1. Primary cardiac tissue

Cardiac tissue biopsies, obtained during cardiac surgery, can be used for the isolation of cardiomyocytes. These primary sources directly represent the situation in the human heart. However, when isolating cardiomyocytes, tissues need to be immersed into a collagenase-containing bath as quickly as possible, since snap-frozen or fixated tissue is unsuitable for cardiomyocyte isolation [15]. A major drawback of this collagenase treatment to digest the heart tissue is the low yield. Moreover, cardiomyocytes are terminally differentiated cells, making it difficult to obtain sufficient cells for metabolic measurements. Additionally, biopsies are usually taken at a localized area of the heart, during surgery or with catheters, and might not be representative for the characteristics and function of the left ventricle (LV). Removing cardiac tissue from healthy individuals is not ethically approved and there is a limited availability of unused donor hearts, which prevents us from comparing healthy individuals to patients with specific cardiac disease. Moreover, cardiac biopsies are mainly obtained from patients with end-stage HF and are therefore not representative of the early pathological changes in the heart [16].

2.2. Commercially available cell lines

Alternatives to these cardiac biopsies are the commercially available proliferating human cell lines, such as AC16 and SV40 cells. The AC16 cell line was gained by fusing primary cultures of adult ventricular heart tissue with SV40 transformed human fibroblasts [17]. This cell line is stable, expresses cardiomyocyte specific markers (α-MHC, β-MHC, α-Actin, troponin I), and displays electrophysiological properties, but detailed information on its metabolic characteristics is not available. A number of other immortalized human SV40 cardiomyocyte lines are also commercially recommended. However, literature on their cardiomyocyte specific characteristics is lacking. Before these cell lines can be deemed suitable for the study of cardiac metabolism, more research into their cardiac and metabolic properties has to be conducted [18,19].

2.3. Human stem cell-derived cardiomyocytes

In the last decade, great efforts have been made to identify less-invasive and more representative sources of human cardiomyocytes for various applications, especially for drug cardiotoxicity screening, myocardial regeneration, and drug discovery [20–23]. The human pluripotent stem cell appears to be a suitable candidate, because of its potential to proliferate and differentiate into various cell types including cardiomyocytes [24–26]. Human stem cell-derived cardiomyocytes, such as hiPSC-CMs and hESC-CMs have attracted considerable attention. Specifically, based on their myogenic gene profile and contractile properties they appear to be functional cardiomyocytes [27–29]. hiPSCs are generated by overexpressing embryonic transcription factors in somatic cells, such as fibroblasts [30]. The major advantage of hiPSCs is that they can be obtained *via* minimally invasive procedures; they can usually be isolated from skin biopsies. Besides the skin biopsies, new methods have become available to obtain hiPSCs, namely from urine and blood [31–33]. These methods appear to be non-invasive, relatively simple, cost-effective, and a reasonably quick process compared to isolation from skin biopsies and hold great promise [33,34]. During fibroblast reprogramming and differentiation of fibroblast-derived hiPSCs, the patient-specific genetic profile is retained. In this way, cardiomyocytes from patients with specific metabolic or cardiac disease related mutations can be produced, thus opening a window for personalized, patient-specific drug discovery [33,35]. However, patient specific epigenetic markers are also maintained during cellular reprograming to hiPSCs, which might influence the differentiation process. Conversely, hESCs are derived from human healthy blastocysts that are surplus to the requirements of *in vitro* fertilization, without the influence of genetic mutations and epigenetic differences. They are commercially available, thus a standardized cell line can be obtained on a regular basis. Although hESCs are less suitable for a personalized-medicine approach, as they are obtained from a healthy donor, they have proven useful as *in vitro* model for testing the safety and efficacy of human drugs and for studying underlying mechanisms in the development of cardiac disease, independent of patient-specific genetic abnormalities.

3. Basic characteristics of human stem cell-derived cardiomyocytes

3.1. Structural, molecular and electrophysiological characteristics

hSC-CMs differ from adult human cardiomyocytes with respect to shape, sarcomeric organization and receptor expression. Adult human cardiomyocytes have a large and cylindric shape, aligned sarcomeres and large numbers of mitochondria, whereas hSC-CMs have a smaller and oblong morphology, sarcomeres are short and disorganized and less mitochondria are present. It has however been pointed out that maturity can be achieved by prolonged culture of these hSC-CMs (up to 100 days), thereby enhancing structural organization and functional performance over time [36–38]. Additionally, the transcriptional or molecular profile of hSC-CMs differs from undifferentiated stem cells. Important differences include the loss of pluripotency markers (OCT4, NANOG, klf4, POU5F1, SOX2) and the upregulation of mesodermal and cardiac myofilament markers [29,36,39–41]. During the differentiation process, an expression pattern can be distinguished of early and late cardiomyocyte markers. After 5–6 days of differentiation the early markers (GATA-4, ISL1 and KDR) become highly expressed. The expression level of late markers (NKX2–5, TBX5, MEF2C, HAND1/2) peaks around day 8–9, followed by an upregulation of myofilament genes (TNNT2 and MYH6) at day 8–10 [42,43]. Twenty to 30 days differentiated cardiomyocytes showed homogeneity among cells based on expression levels of the above-mentioned markers. Furthermore, hSC-CMs are generally characterized as spontaneously, synchronic beating cells derived from human stem cells. The capability of a cell to contract depends on its capability to generate action potentials [44,45]. Whether action potential (AP) phenotype, ion channels and calcium handling in hSC-CMs are similar to the adult heart has been

investigated extensively. Similar to human adult cardiomyocytes, a heterogeneous population of atrial-, nodal-, or ventricular-like cells can be distinguished in hSC-CMs based on AP phenotypes [26,46]. However, these AP characteristics seem to vary between culture conditions and culture time, possibly undermining the correctness of this classification [38,47,48]. To what extent hSC-CMs represent the human adult heart in terms of structural, molecular and physiological characteristics remains debatable.

3.2. Metabolic properties

In terms of metabolism, adult cardiomyocytes are highly active, and for the synthesis of ATP, these cells are mainly (50–70%) dependent on oxidative FA metabolism. In comparison, embryonic and fetal cardiomyocytes mainly rely on glycolysis. For the uptake of glucose the class 1 facilitative transporters (GLUT1, GLUT2, GLUT3 and GLUT4) are the most abundant. Throughout the embryonic period, GLUT1 is expressed ubiquitously and is considered responsible for basal glucose uptake. In all forms of hESCs as well as in fibroblasts glucose uptake is mainly facilitated by the constitutively expressed GLUT1 transporter [49]. The human adult heart, conversely, abundantly expresses GLUT4, a high-affinity insulin sensitive glucose transporter, next to GLUT1 [50]. FA uptake in the adult heart is mainly facilitated by fatty acid translocase (CD36) [51]. Whether these transporters are present in hSC-CMs and to what extent they contribute to substrate uptake in hSC-CMs has not been studied so far, but will be further discussed in Section 6. These changes in transporter content (at least as studied so far for the GLUTs) are expected to occur in concordance with changes in metabolism. Whereas stem cells are mainly dependent on glycolysis, a switch towards mitochondrial oxidative metabolism occurs upon differentiation into cardiomyocytes. Compared to hSCs, hSC-CMs have been shown to have increased mitochondrial oxygen consumption and reduced glycolytic reliance [28,52,53]. Moreover, this switch is accompanied by a substantial increase in mitochondrial biogenesis, higher expression of genes and proteins related to oxidative phosphorylation, FA oxidation and lipolysis, which changes continue during the maturation of cardiomyocytes in culture. Furthermore, these unique metabolic properties of cardiomyocytes can be used as a selection method to generate purified cardiomyocytes. For instance, treatment of 20 day-differentiated hiPSC-CMs with lactate, which can only be metabolized *via* oxidative phosphorylation, was shown to result in 99% purification of hSC cultures [53]. These data suggest that hSC-CMs have the potential to use oxidative metabolic pathways, like adult cardiomyocytes [54–57], and therefore could potentially be useful to study the underlying molecular mechanisms of human metabolic diseases.

4. *In vitro* and *ex vivo* rodent models of lipid-induced insulin resistance

In recent decades, several animal models have been developed to investigate the underlying mechanism of HF in T2D. Many of these models are based on lipid overload. For instance, the chronic exposure of rodents to a HP diet is often used to achieve lipid overload-induced IR in skeletal muscle [58,59] and heart [60–63]. These animal models allow the metabolic changes to be studied in relation to cardiac structural and functional changes. Additionally, *ex vivo* primary cell culture models, such as neonatal rat cardiomyocytes, isolated adult rat and mouse cardiomyocytes, as well as *in vitro* immortal mouse atrial cardiomyocyte cell lines, such as HL-1 cells, are commonly used to study cardiac metabolism [14]. Together, these *in vitro*, *ex vivo* and *in vivo* animal models were instrumental in unraveling the mechanism of the onset of lipid-induced IR. This mechanism includes (i) a rapid CD36 translocation to the sarcolemma and chronically increased FA uptake, (ii) subsequent accumulation of toxic lipid intermediates (*e.g.*, ceramides), (iii) inhibition of insulin signaling (as determined by decreased phosphorylation of protein kinases in the canonical insulin signaling cascade, such as Akt, upon insulin treatment), (iv) decreased insulin-stimulated glucose and FA uptake, ultimately leading to cardiac dysfunction.

The use of these cellular models allows full control of the experimental environment including pharmacological manipulation [13,14]. Though, isolated cells lack the inter-cellular and intra-organ contacts, are not subjected to a preload or afterload and hormonal/neuronal changes, and therefore do not perfectly represent the situation on the whole organ. Furthermore, cardiac cells from animal models are genetically not equivalent to their human counterparts, and subcellular/organellar differences in these cells occur between species [16,64]. Primary human cardiac cells and human heart tissue can, however, not be obtained easily. Therefore, the emerging hSC-CMs could be a useful new tool for drug discovery and the development of personalized treatment strategies.

5. Insulin resistance in human induced pluripotent stem cells

As described earlier, considerable research has been conducted on hSCs and their derived cardiomyocytes. However, detailed information on their functional metabolic properties and whether IR can modulate metabolism in these cells is scarce. As recently shown [65,66], iPSCs and iPSC-derived skeletal myotubes possess the intrinsic ability to acquire IR, including the expected metabolic changes. In iPSCs from patients with a genetic insulin receptor defect, IR can lead to reduced mitochondrial oxidative capacity, decreased expression of glucose metabolism related genes, and increased reliance on anaerobic metabolism. Interestingly, after differentiation, the skeletal myotubes, derived from these patient iPSCs still showed impaired insulin signaling and insulin-stimulated glucose uptake, decreased mitochondrial function, impaired glycogen synthase activity, and glycogen accumulation when compared to myotubes of control subjects. These data indicate that the insulin resistant phenotype is conserved after reprogramming and re-differentiation into human skeletal myotubes. Moreover, a decrease in gene expression of insulin-regulated genes (RAD1, HK2 and GLUT4) was found [65–67]. Whether these findings on hiPSC-derived skeletal myotubes also apply to hiPSC-CMs or hESC-CMs remains unknown.

Another study [68] aimed at developing an *in vitro* model of diabetic cardiomyopathy, using hiPSC-CMs from healthy donors. hiPSC-CMs were cultured without glucose to force the cells towards FA utilization. The investigators hypothesized that additional exposure to a diabetic environment would promote features similar to diabetic cardiomyopathy. A diabetic environment was mimicked through culture in a maturation medium including 10 mM glucose and relevant concentrations of endothelin-1 and cortisol. Compared to cells on standard maturation medium, cells exposed to this diabetic environment showed disorganized sarcomere structures, altered calcium transients, cellular hypertrophy, intracellular lipid accumulation and oxidative stress. Further, an upregulation was shown of genes involved in the Krebs cycle, mitochondrial electron transport chain, and glucose metabolism, whereas a downregulation was observed of genes controlling protein synthesis and the cellular response to dysfunctional protein production. These data indicate that hiPSC-CMs exposed to a diabetic milieu recapitulate a diabetic cardiomyopathic phenotype *in vitro* [68].

The above findings [65,66,68], each in different hiPSC models of IR and with distinct outcomes, may relate to differences in genetic background between the individual patients and maintained residual epigenetic differences during cellular reprogramming. Consequently, the establishment of an *in vitro* model without genetic variation would allow us to study the underlying mechanism of IR independent of genetic mutations.

6. Insulin resistance in human embryonic stem cell-derived cardiomyocytes

To our knowledge, it has not been investigated whether hESC-CMs

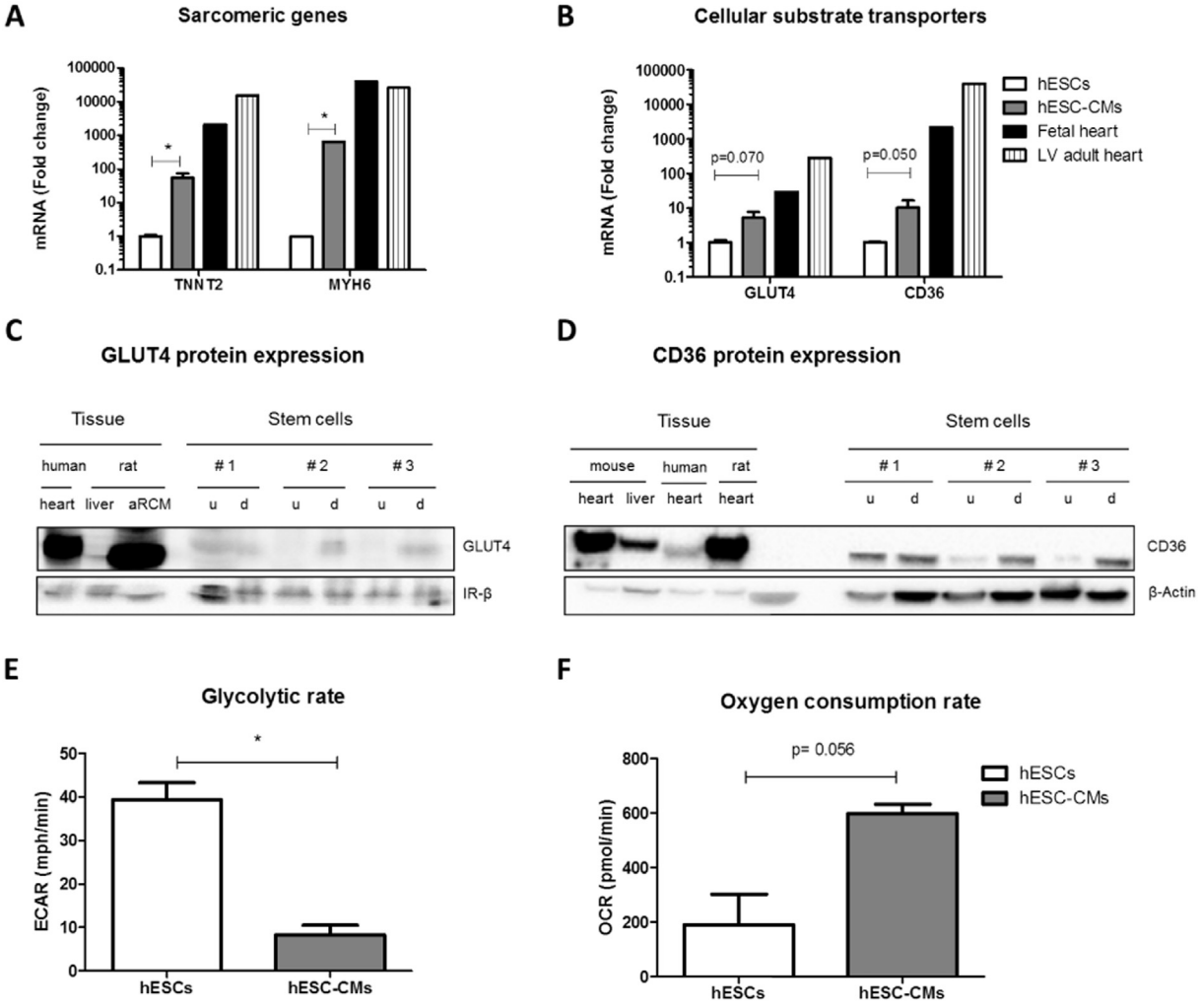

Fig. 1. Differentiation of human embryonic stem cells into cardiomyocytes. Human embryonic stem cells (hESCs) were kindly provided by the Wicell Research Institute (Madison, WI) and subsequently maintained and differentiated for 30 days into hESC-CMs according to manufacturer's protocol (Gibco) as described previously [81]. Quantitative RT-PCR analysis of cardiomyocyte specific genes (A) and substrate transporter genes (B) was performed upon differentiation towards hESC-CMs. TATA box binding protein was used as housekeeping gene and data were normalized against undifferentiated cells, whereas human fetal whole heart (pooled from 4 hearts) and adult left ventricle (LV) tissue (pooled from 3 hearts) were used as positive controls. Immunoblot images of GLUT4 (C) and CD36 (D) are shown for hESCs (u) and hESC-CMs (d). Human, mouse and rat heart tissues and adult rat cardiomyocytes (aRCM) were used as positive controls whereas mouse and rat liver tissues served as negative controls. Cellular acidification rates (ECAR) (E), as a measure for glycolytic rate, and oxygen consumption rates (OCR) (F) were determined with a Seahorse device (Agilent; Seahorse, North Billerica, MA, US), according to the manufacturer's XF Glycolysis Stress protocol, using glucose (10 mM), oligomycin (1 μM), and 2-deoxy-D-glucose (50 mM). Values are displayed as mean ± S.E.M. (n = 3). Data were analyzed by using two-sided Student *t*-test and considered statistically significant at $p < 0.05$. *$p < 0.05$ compared to hESCs.

can function as a suitable *in vitro* model to study cardiac IR. In previous *in vitro* studies on rodent cell lines, prolonged HP treatment triggered IR with defects in insulin signaling, substrate metabolism, and also diminished contractile function [60,61,63]. In the current experimental study, we differentiated hESCs into functional cardiomyocytes to study the impact of HP-induced IR on insulin signaling and cardiac metabolism in hESC-CMs. We aimed at establishing a human *in vitro* model to study the underlying mechanism of cardiac IR. Promising data on this new human *in vitro* model are described in more detail in the next paragraphs.

6.1. Differentiation of hESCs towards hESC-CMs

hESCs were differentiated into cardiomyocytes for 30 days. It has been described that hSC-CMs at this point already express various features specific for human cardiomyocytes even though they might not be fully matured yet [42,43,69]. After 30 days of differentiation, > 30% of the cells showed spontaneous contraction and a robust induction (50 to 600-fold) of the expression of key cardiac sarcomeric markers (Fig. 1A), thus confirming differentiation of hESCs towards functional cardiomyocytes. Although these results are in agreement with reports from other groups [29,36,42,43], it should be noted that the observed levels are not closely comparable to the levels measured in human fetal or LV heart tissue. Nevertheless, these data indicate that we were able to differentiate hESCs into hESC-CMs, keeping in mind that the contractile properties still depict an incompletely matured phenotype.

To obtain information about the specific metabolic properties of the 30 days differentiated hESC-CMs, we examined gene and protein expression levels of the specific glucose- and fatty acid transporters (GLUT4 and CD36, respectively), glycolytic capacity, and oxygen consumption rate in biological replicate samples. GLUT4 and CD36 mRNA levels were 5.1-fold and 10.1-fold increased, respectively, in hESC-CMs as compared to hESCs, suggesting that the differentiating cells are switching towards a more mature phenotype (Fig. 1B). These findings on mRNA level were confirmed by western blot analysis where hESC-CMs showed increased expression of both GLUT4 (Fig. 1C) and CD36 (Fig. 1D) compared to hESCs, although it should be noted that GLUT4

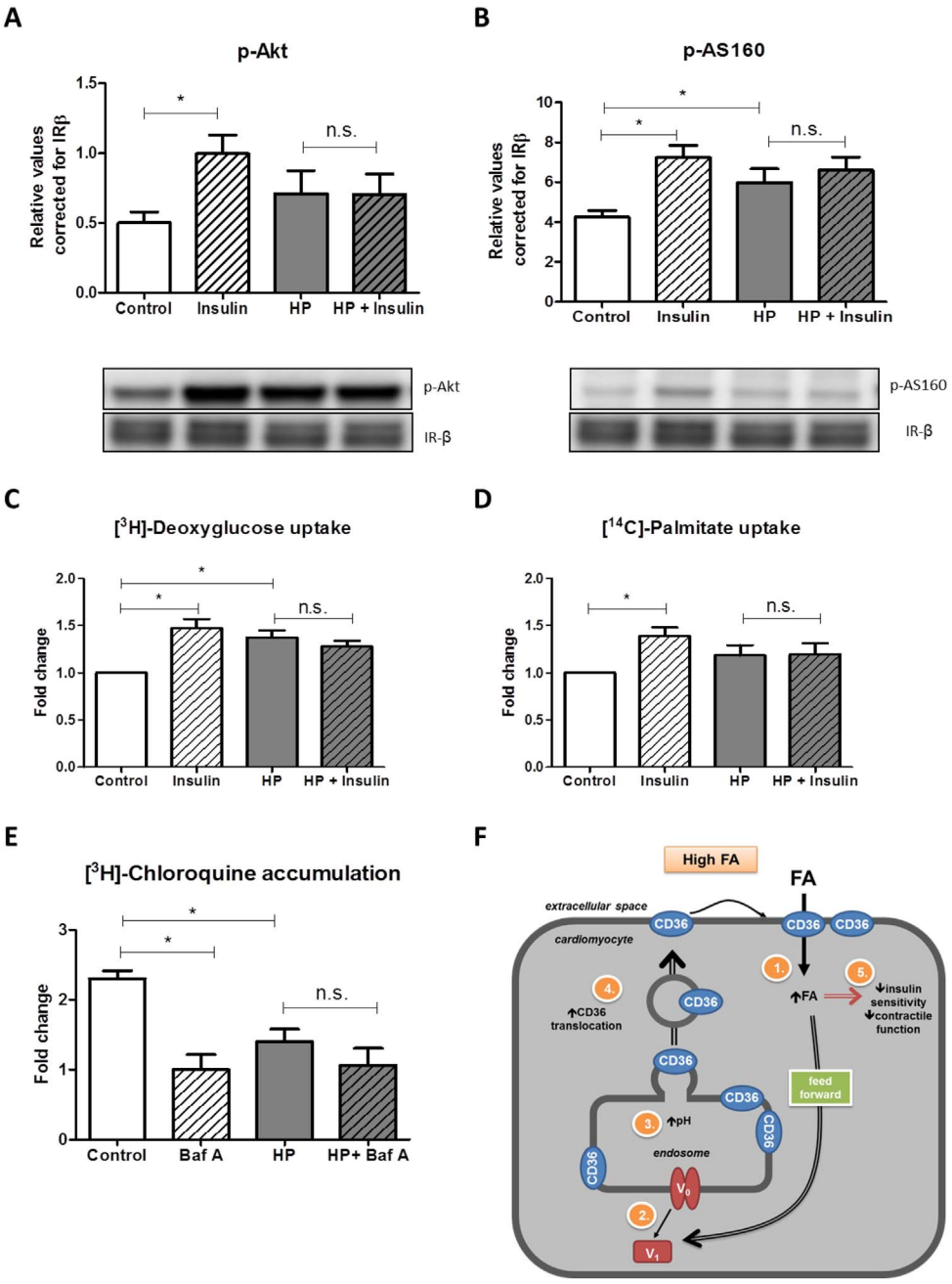

Fig. 2. Lipid overload leads to insulin resistance (IR) and impairment of vacuolar-type H^+-ATPase (vATPase) function in human embryonic stem cell-derived cardiomyocytes (hESC-CMs). hESC-CMs were cultured under control or high palmitate conditions (250 μM palmitate; 50 nM insulin) for 16 h to induce IR, followed by short-term (30 min) insulin stimulation (100 nM) on day 31 for measurements of insulin sensitivity. A–B) Protein expression levels of insulin signaling related proteins, phospho-Akt-Ser473 (p-Akt) and phospho-AS160-Thr642 (p-AS160). Insulin receptor beta (IRβ) was used as loading control. C–D) Glucose- and fatty acid uptake in hESC-CMs, determined by means of [^{3}H]-deoxyglucose and [^{14}C]-palmitate, respectively (n = 4). E) v-ATPase function is measured by means of cellular chloroquine accumulation [60]. hESC-CMs were treated with [^{3}H]-chloroquine with/without 100 nM Bafilomycin A (Baf A, a potent v-ATPase inhibitor) for 25 min. The Baf A sensitive component of the total cellular chloroquine accumulation reflects v-ATPase activity (n = 3). F) Schematic presentation of lipid-induced v-ATPase inhibition and its consequences for insulin sensitivity and contractile function (adapted with permission from [60]). When long-chain fatty acid (FA) supply is low, the FA transporter CD36 is primarily found in endosomes. The v-ATPase V_0 subcomplex, which is integral to the endosomal membrane, is assembled with the cytosolic V_1 sub-complex allowing for acidification of the endosomal lumen. Upon chronic lipid oversupply, when FA uptake surpasses the cellular metabolic needs, a series of events is triggered: [1] Increased CD36-mediated FA uptake results in elevated intramyocellular FA levels [2]. FAs cause the V_1 and V_0 sub-complexes to dissociate. Therefore, V_1 is shifted into the soluble cytoplasm [3]. The disassembly of v-ATPase leads to endosomal alkalinization [4]. Increased endosomal pH triggers the translocation of CD36 vesicles to the cell surface [5]. Palmitate overload eventually culminates into loss of insulin sensitivity and contractile function. Data are expressed as mean ± S.E.M. Data were analyzed by the Mann-Whitney *U* test and considered statistically significant at *$p < 0.05$. Data were normalized to control (A–D) or Baf A (E).

and CD36 levels are several orders of magnitude lower than human fetal and adult cardiac tissue levels. Mitochondrial respiration measurements (Seahorse analyser) showed a 73% lower glycolytic rate (ECAR activity) in the hESC-CMs as compared to hESCs (Fig. 1E) and an increased oxygen consumption rate (Fig. 1F). In this respect, our 30 days differentiated hESC-CMs more closely resemble the (oxidation-dependent) human adult heart compared to the (glycolysis-dependent) fetal heart. Taken together, the presence of substrate transporters in hESC-CMs and the switch to aerobic metabolism offers potential for studying insulin signaling and other metabolic pathways in these cells.

6.2. Lipid overload induces IR in hESC-CMs

To examine the onset of lipid overload-induced IR in hESC-CMs, these cells were cultured in media with HP and then assessed on parameters of insulin sensitivity in biological replicate samples. Using a similar protocol as we have previously established to induce IR in HL-1 cells [63], we exposed 30 days differentiated hESC-CMs to 250 μM HP and 50 nM insulin for 16 h (further referred to as HP treatment). Additionally, a short-term 100 nM insulin stimulus was given to evaluate whether the IR state has fully developed within these cells by assessing established metabolic and subcellular parameters of IR. Similar to rodent cardiomyocytes, hESC-CMs showed increased Akt and AS160 phosphorylation (2.0-fold and 1.7-fold, respectively) upon insulin stimulation (Fig. 2A–B). Additionally, uptake of [^{3}H]-deoxyglucose and of [^{14}C]-palmitate was significantly increased upon insulin stimulation. These observations indicate that 30 days differentiated hESC-CMs are insulin sensitive under basal conditions. When cells were pre-treated with HP, both the activity of insulin signaling related proteins as well as cellular substrate uptake did not change upon insulin stimulation (Fig. 2C–D), suggesting that lipid overload induces IR in hESC-CMs. Furthermore, glycogen content was 58% lower in HP-treated hESC-CMs as compared to untreated cells (4.1 ± 0.4 and 1.7 ± 0.2 μg/10^6 cells in hESCs *versus* hESC-CMs, respectively; $p < 0.01$), suggesting that

glycogen synthesis is also decreased. This is in line with other studies showing decreased glycogen synthesis in T2D patients and first degree-relatives [70,71].

Our group has recently unveiled a novel mechanism underlying lipid overload-induced IR. This includes inhibition of vacuolar H^+-ATPase (v-ATPase), a resident protein in membranes of acidic organelles, such as endosomes, which are the storage compartments for CD36 [60]. V-ATPase is responsible for maintaining endosomal acidification by means of its proton pumping activity. We have shown that lipid overload directly impairs v-ATPase function, causing loss of endosomal acidification. Since proper endosomal acidification is necessary for intracellular CD36 retention [72], the alkalinized endosomes cannot harbor CD36 anymore, so that it translocates to the cell surface to increase fatty acid uptake, the key step in lipid overload-induced IR. The degree of endosomal acidification can be assessed by cell-associated radioactivity upon incubation of cells with tracer amounts of the divalent weak base [^{3}H]chloroquine, as previously established [72]. Fig. 2E shows that hESC-CMs exposed to HP display a reduction of cell-associated [^{3}H]chloroquine accumulation compared to untreated control cells, indicating impairment of v-ATPase function (Fig. 2E). This novel mechanism underlying lipid overload-induced IR is depicted in (Fig. 2F). In conclusion, all tested hallmarks of lipid-induced IR in these cells are in close agreement with the described mechanism of lipid-induced IR (see Section 4), thereby establishing the suitability of these cells for cardiac IR-related studies.

There are some limitations to the presented IR-model. In particular, the 30 days differentiated hESC-CMs do not fully represent the phenotype of the human adult cardiomyocyte. It has been pointed out that structural and functional maturity can be achieved by adjusted culture conditions (such as oxygen concentration) [73], alternate medium composition (such as galactose and FA-rich medium) [74], electrical stimulation [75], and prolonged culture [36–38,76]. Furthermore, we used a 100 nM insulin concentration, as it is known that cells in culture are less responsive to insulin stimulation than *in vivo* animal or *ex vivo* isolated heart models. Perhaps, further maturation of hESC-CMs may lead to a higher insulin response, as neonatal cardiomyocytes compared to adult, have high basal glucose uptake levels due to a relatively high GLUT1 expression, which makes them less responsive to insulin-stimulated glucose uptake [77]. Nevertheless, the 30 days-differentiated hESC-CMs show signs of metabolic differentiation and the ability to become insulin resistant thereby providing promising future outlook for the use of hESC-CMs as a model to study metabolic diseases.

7. Concluding remarks

In summary, human *in vitro* cell models appear useful to obtain metabolic insight into human disease pathogenesis, especially in case of IR in the heart. On a large scale the molecular mechanisms in these human models seem to be mostly comparable to the currently used animal models, although it remains necessary to exclude whether fundamental differences, due to genetic, structural and physiological interspecies variation, are present. Currently, several human *in vitro* cell models are available, with hSC-CMs showing a good potential for application in future research. The ability to generate cardiomyocytes from human stem cells provides an unlimited source of cells without the need of invasive procedures. In our present study, we have developed a human *in vitro* model to study lipid-induced IR in human cardiomyocytes. We observed that upon 16 h HP treatment hESC-CMs acquire impairment in v-ATPase function and lose their sensitivity to insulin, suggesting that hallmark features of IR in the heart are conserved between rodents and humans. Although these 30 days differentiated cells might not fully display the cardiomyocyte-specific morphology and sarcomere organization [43,78–80], their metabolic characteristics show a maturing metabolic phenotype as seen in the adult human heart. Therefore, HP treated hESC-CMs provide a promising, valuable human *in vitro* cell model to study cardiac IR. Moreover, functional measurements such as cardiac contractility and electrophysiological properties could be of additional value to confirm that the observed IR phenotype upon HP treatment also affects contractile function in this model. Finally, this model may be important for identifying novel therapeutic targets to combat T2D-induced heart failure.

Transparency document

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