Contractile function of myofibrils in human stem cell-derived cardiomyocytes *vs*. myofibrils from adult human ventricle

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Given the essentially unlimited availability of cardiomyocytes (CMs) derived from human pluripotent stem cells (hPSC), they hold great potential for the treatment of cardiovascular diseases by cell transplantation or engineered cardiac tissue, for assessing efficiency and toxicity of pharmacological compounds, or to be used as cellular disease models *in vitro*.

Which is the stage of maturation of these hPSC-derived CMs (hPSC-CMs) is not easy to answer, because we have to define from which aspect we address this question: morphology, gene expression, protein isoform composition or CMs' function. We are interested to understand multiple aspects, and the main focus regards the correlations between protein isoform composition (at molecular level) and the contraction-relaxation function of CMs (at cellular and subcellular levels).

The main function of CMs is to generate force and shorten their length periodically determining the pump function of the heart. This process occurs when the subcellular myofibrils contract due to multiple interactions between myosin motors and actin filaments. Myofibrils consist in many sarcomeres arranged in series driving the contraction-relaxation events of CMs upon cyclical variation of intracellular Ca^{2+} concentration ($[Ca^{2+}]$). Isolated single myofibrils (or thin myofibrillar bundles) are an established contractile model used to understand sarcomeric protein-related processes that determine contractile function of cardiomyocytes in the absence of Ca^{2+} handling systems and of upstream signaling.

Myofibrils can be investigated using fast kinetic techniques, because they are small and thus in rapid diffusional equilibrium with their surrounding environment. To study contractile function of myofibrils, a micromechanical setup was built on an inverted microscope. This setup uses an atomic force cantilever as a nN-sensitive force sensor. It allows rapid changes of the solutions to which myofibrils are exposed, and the force kinetic parameters of myofibrillar activation and relaxation at different $[Ca^{2+}]$ can be analyzed with very high time resolution.

Research objective

Our main aim is to understand how the pathomechanism of the hereditary hypertrophic cardiomyopathy (HCM) is initiated and which are the functional primary consequences of HCM-related mutations occurring in some sarcomeric proteins. HCM is the most frequent genetically transmitted cardiac disease with a prevalence of 1:500. It is often characterized by asymmetric hypertrophy of the left ventricular (LV) wall and the interventricular septum and by an increased risk for sudden cardiac death particularly in young adults. Nearly all genotyped HCM cases revealed mutations in sarcomeric proteins, but the underlying mechanisms initiating disease development and adaptational processes of the myocardium are still largely unknown either in developing *in utero* heart or later on in symptomatic adults.

We use as a contractile model the myofibrils either isolated from ventricular tissue of patients carrying HCM-linked mutations in sarcomeric proteins or myofibrils of CMs derived from the same patient-specific induced hPSCs. In particular, we focus on missense HCM-linked mutations in the motor domain of the ventricular isoform of myosin heavy chain (MyHC), such as MyHC-Arg723Gly.

Usually, after typical differentiation protocols, sarcomeres of hPSC-CMs contain a mixture of α (atrial) and β (ventricular) isoforms of the MyHC, while sarcomeres of most ventricular CMs contain only the βMyHC and most known HCM-related myosin-mutations are expressed in the βMyHC rather in the αMyHC isoform.

Outcomes

The first step of our work consisted in generation of hPSC-CMs (either embryonic (hESC-CMs) or induced pluripotent (hiPSC-CMs) cardiomyocytes) expressing in their sarcomeres only the ventricular βMyHC isoform. We have shown that a prolonged (>35 days) cultivation of hESC-CMs plated on a stiff extracellular-type matrix (e.g., laminin) coating an isotropic surface (glass cover-slips) switches expression of MyHC to pure β isoform (Fig.1A; Weber et al., 2016). For identification of the MyHC isoform type, myofibrils of hESC-CMs were immunostained using an anti-α-MyHC antibody designed in our lab and a commercial available anti-β-MyHC antibody (Fig.1A; Weber et al., 2016).

When hPSC-CMs are used, before to understand how a HCM-related mutation impairs the contractile function, the unperturbed wild-type *in vitro*-generated cellular contractile model has to be well defined.

Morphology and ultrastructural aspects of long-term hESC-CMs plated on stiff matrix indicated an improved maturation of these CMs compared to the ones cultivated for a similar time period in floating cardiac bodies (CBs), i.e., on soft conditions (Fig.1B; Weber et al., 2016). Spontaneous contractions and no strict coupling of βMyHC to ventricular-like action potentials suggested that the switch of MyHC isoform did not fully interfere with the hESC-CMs differentiation and maturation process (Weber et al., 2016). Twitch contraction and Ca^{2+} transients of intact hESC-CMs were only little affected by the MyHC-isoform type, although βMyHC-expressing hESC-CMs had much lower ATP-turnover and tension cost than αMyHC-expressing hESC-CMs (Weber et al., 2016).

In the next step, we identified which force parameters of myofibrils within hESC-CMs resembled the corresponding parameters of adult human ventricular myofibrils (hvMFs). Using the micromechanical setup (Fig.2A), we compared the contractile function in isometric condition of myofibrils within hESC-CMs with that of hvMFs (Fig.2B-bottom) isolated from adult donor hearts in identical experimental conditions (Iorga et al., 2018). To access myofibrillar bundles, hESC-CMs (Fig.2B-top) were chemically demembranated (d-hESC-CMs; Fig.2B-middle).

Our experiments showed that although both myofibril types contain only the human ventricular βMyHC, maximum generated force was lower for myofibrils of d-hESC-CMs compared to hvMFs (d-hESC-CMs: 42±10kPa, hvMFs: 94±25kPa; Fig.2C). Importantly, Ca^{2+} sensitivity of force (i.e., [Ca^{2+}] corresponding to half of the maximum force, expressed as pCa_{50}) was higher for myofibrils of hESC-CMs than for hvMFs (ΔpCa_{50}=+0.24; Fig.3A). At saturating [Ca^{2+}] when the accessibility of Ca^{2+}-regulated thin filaments for myosin motors has the highest probability, the rate constant of cycling actin-myosin cross-bridges determining force development (k_{ACT}) or re-development (k_{TR}) was similar between d-hESC-CMs and hvMFs (Table 1), likely because both contractile systems expressed βMyHC isoform. However, at intermediate [Ca^{2+}] as in intact CMs contracting *in vivo*, k_{TR} was faster for d-hESC-CMs than for hvMFs at the same submaximal fractional force level as generated by hvMFs during systole (Fig.3B). Upon Ca^{2+} removal, cross-bridges determined force decay during the first relaxation phase (Fig.2C-left side) with a faster rate constant for d-hESC-CMs than for hvMFs (Table 1), suggesting a potential increase of the tension cost (= ATPase rate / generated force) for myofibrils of hESC-CMs than for hvMFs. Nevertheless, the rate constant (k_{REL}) of the second relaxation phase, which is related to the CMs re-lengthening that contributes to the rapidity of the ventricular pressure decay during diastole, was similar for both myofibril types (Table 1).

Analysis of some sarcomeric proteins involved in force modulation revealed that hESC-CMs had essentially the fetal (slow skeletal) isoform instead of adult (cardiac) troponin-I (TnI) isoform, and partially non-ventricular isoforms of some other sarcomeric proteins summarized in Table 2 (Iorga et al., 2018). This distinct sarcomeric protein isoform pattern may explain the functional differences between myofibrils of hESC-CMs and hvMFs (Iorga et al., 2018). The sarcomeric protein composition of hESC-CMs had features of human CMs at an early developmental stage. Our results indicate that advances in morphological and ultrastructural maturation of hESC-CMs might not necessarily be accompanied by a ventricular-like expression of all sarcomeric proteins.

Conclusions

To further enhance the regenerative potential of hPSC-CMs differentiated *in vitro*, we work in collaboration with the group of Prof. Urlich Martin* and Dr. Robert Zweigerdt* to identify the appropriate chemo-mechanical factors that favor the shift of myofibrillar proteins, like troponin I and T and myosin light chains (MLC-1, MLC-2), towards the isoforms of the adult human ventricle in hPSC-CMs. On the other hand, such hPSC-CMs (hiPSC- or hESC-CMs) with a defined sarcomeric protein isoform composition similar to early stages of the developing human heart provide us useful models for investigating the contractile function of CMs during (pre-natal) development of inherited cardiomyopathies or basic mechanistic aspects of contraction in developing human CMs.

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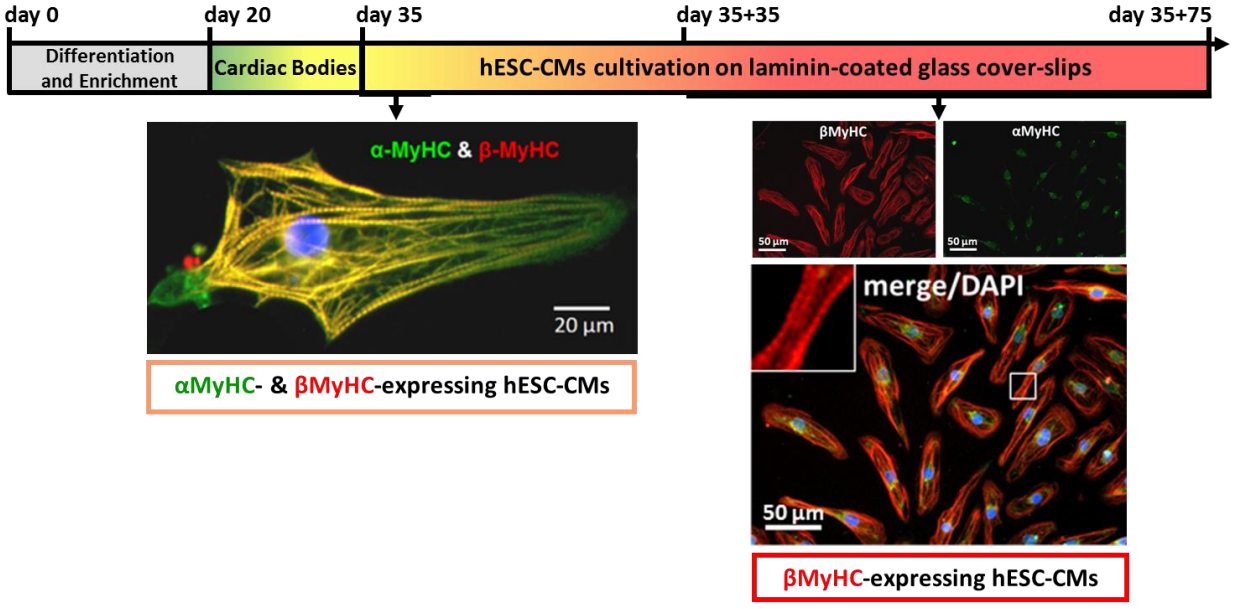
Figure 1.

A. Protocol for h-ESC-CMs differentiation and cultivation. Representative images: immunostaining against αMyHC and βMyHC isoforms shows that at early days after cells were plated, there is a mixture of α and β MyHC isoforms expressed in hESC-CMs, while after long-term cultivation (>35 days) on laminin-coated cover-slips, most hESC-CMs express in their sarcomeres essentially only βMyHC isoform, as usually is found in most CMs of the human ventricle (details in Weber et al., 2016).

B. Ultrastructure by TEM of hESC-CMs. Green marked images: CMs were dissociated from cardiac bodies (CBs) after >70 days post-differentiation; these CMs show myosin filaments, Z-disks or Z-bodies. Red marked images: CMs were dissociated from CBs at day 35 post-differentiation and then plated on laminin-coated cover-slips for >35 days (i.e., >d35+35); these CMs show better sarcomeric organization (H-zone, A- and I-bands, Z-disks, myosin and actin filaments, SR-sarcoplasmic reticulum; details in Weber et al., 2016).

A.



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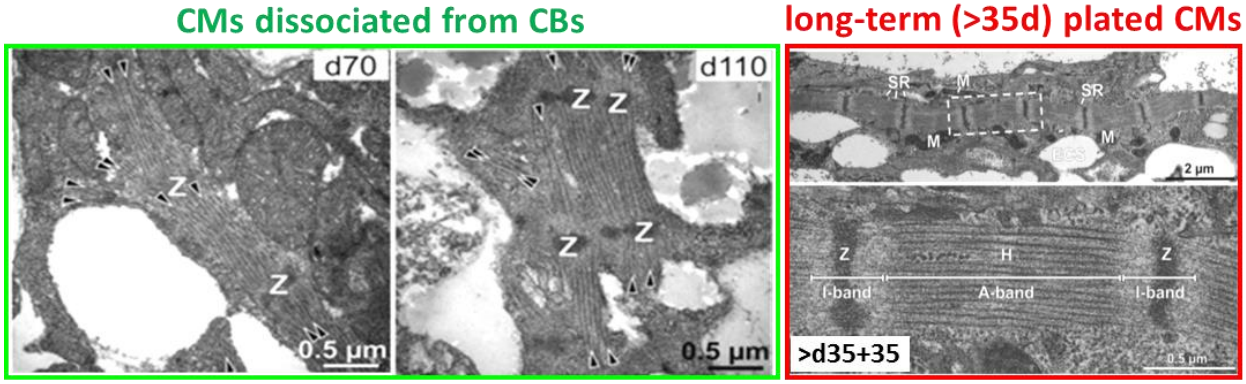

Figure 2.
A. Micromechanical setup used to investigate contractile function of subcellular myofibrils of hPSC-CM or isolated from human ventricles of adult donor hearts. **B**. Intact single hESC-CM in cell culture (top), myofibrillar bundle within single demembranated hESC-CMs (d-hESC-CMs; middle), a bundle of human ventricular myofibrils (hvMFs; bottom). **C**. Force transients recorded with hvMFs and with myofibrils of single d-hESC-CMs in identical experimental conditions (15°C). From these force transients, steady-state (passive F_{pass} and active F_{ACT} force in isometric condition) and kinetic (k_{ACT}, k_{TR}, k_{LIN}, t_{LIN}, k_{REL}) force-related parameters can be determined (Iorga et al., 2018).

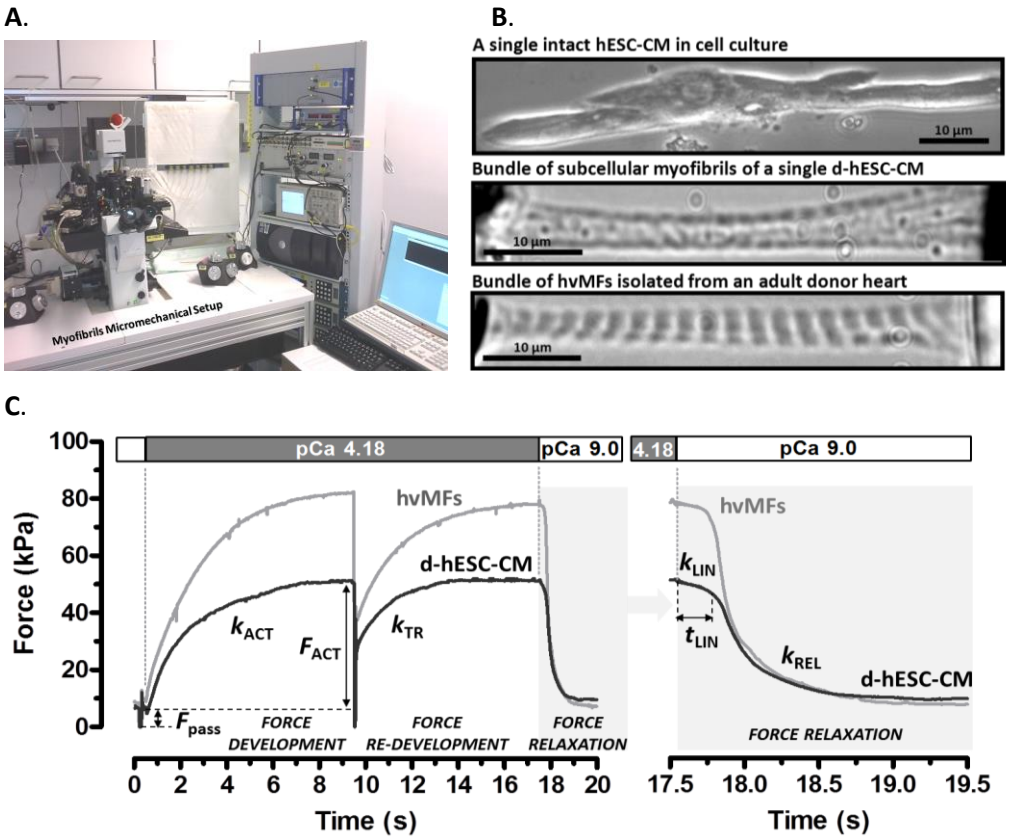

Figure 3.
Force parameters showing differences between subcellular myofibrils determining the contractile function of hESC-CMs and of human ventricular CMs at intermediate Ca^{2+} concentrations ($[Ca^{2+}]$) (Iorga et al., 2018).
A. Fractional (normalized) isometric force ($F_n=F_{ACT}/F_{ACT,max}$) response at different $[Ca^{2+}]$ (given as pCa) of myofibrils within d-hESC-CMs (black circles) compared with hvMFs (grey squares). Steady-state parameters: Ca^{2+}-sensitivity of force, pCa_{50}=6.04±0.08 (d-hESC-CMs), pCa_{50}=5.80±0.05 (hvMFs); steepness, n_H=2.03±0.21 (d-hESC-CMs), n_H=1.91±0.35 (hvMFs); maximum generated force (measured at pCa 4.18), $F_{ACT,max}$=42±10nN/µm^2 (d-hESC-CMs), $F_{ACT,max}$=94±25nN/µm^2 (hvMFs). **B**. Relationships between rate constants k_{TR} of force re-development at different Ca^{2+}-activation levels (following a rapid release-restretch maneuver as shown in Fig.2C) and fractional isometric force (F_n) for d-hESC-CMs compared to hvMFs. All parameters are given and shown as mean±SD; in B, 95%-confidence interval of the non-linear fit is shown.

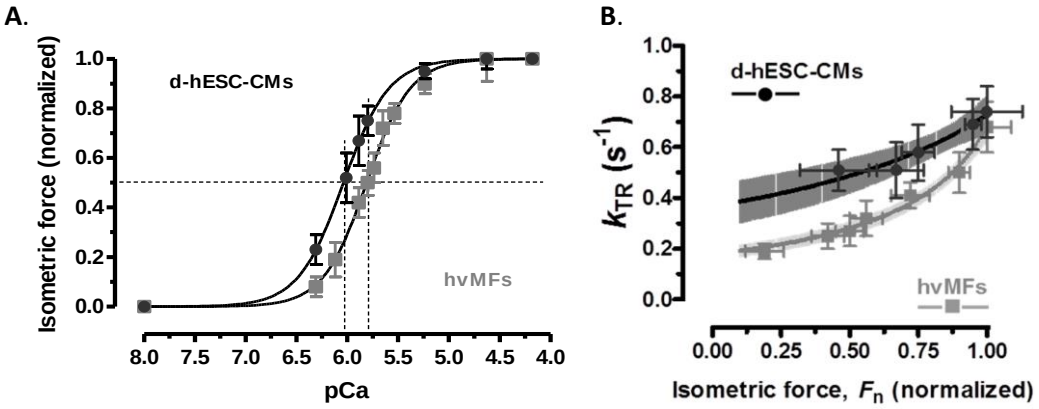

Table 1.
Contractile force kinetic parameters for myofibrils of d-hESC-CMs and hvMFs determined at 15°C following rapid Ca^{2+}-activation to pCa 4.18 and upon rapid relaxation back to pCa > 8.0 (Iorga et al., 2018).

	k_{ACT} (s^{-1})	k_{TR} (s^{-1})	k_{LIN} (s^{-1})	t_{LIN} (ms)	k_{REL} (s^{-1})
d-hESC-CMs	0.66±0.14	0.74±0.10	0.26±0.06*	197±60	4.60±0.60
hvMFs	0.68±0.14	0.68±0.10	0.21±0.07	225±56	5.03±0.89
	Ca^{2+}-induced force development	Mechanically-induced force re-development	Force relaxation upon Ca^{2+} removal		
	force parameters depending on [Ca^{2+}]		*force parameters independent on [Ca^{2+}]*		

* Significantly different (p<0.05; unpaired Student's t-test)

Table 2.
Summary of the main sarcomeric protein isoforms of thick (yellow) and thin (green) filaments that determine and modulate contractile function underlying the differences between myofibrils of d-hESC-CMs and hvMFs. The isoforms of some sarcomeric proteins of hESC-CMs are non-ventricular type resembling to those usually found in developing human cardiomyocytes (Iorga et al., 2018).

	Sarcomeric protein isoforms	
	hESC-CMs	**hvMFs**
Myosin Heavy Chain (MyHC)	slow cardiac (only βMyHC)	slow cardiac (βMyHC predominates)
Myosin Binding Protein C (MyBP-C)	cardiac (only cMyBP-C)	cardiac (only cMyBP-C)
Essential Light Chain (ELC or MLC-1)	atrial (86±3% MLC-1a) and ventricular	ventricular (MLC-1v predominates)
Regulatory Light Chain (RLC or MLC-2)	atrial (82±2% MLC-2a) and ventricular	ventricular (MLC-2v predominates)
Troponin T (TnT)	slow skeletal (~67% ssTnT) and adult cardiac ($cTnT_3$)	adult cardiac (only $cTnT_3$)
Troponin I (TnI)	slow skeletal (only ssTnI)	adult cardiac (only cTnI)
Tropomyosin (Tm)	αTm (predominant), βTm, κTm, and slow skeletal γTm	αTm (predominant), βTm, κTm

Publications

Weber N, Schwanke K, Greten S, Wendland M, Iorga B, Fischer M, Geers-Knör C, Hegerman J, Wrede C, Fiedler J, Kempf H, Franke A, Piep B, Pfanne A, Thum T, Martin U, Brenner B, Zweigerdt R, Kraft T. (2016) - *Stiff matrix induces switch to pure β-cardiac myosin heavy chain expression in human ESC-derived cardiomyocytes*. Basic Res Cardiol. 111:68. doi: 10.1007/s00395-016-0587-9.

Iorga B, Schwanke K, Weber N, Wendland M, Greten S, Piep B, dos Remedios CG, Martin U, Zweigerdt R, Kraft T, Brenner B. (2018) - *Differences in Contractile Function of Myofibrils within Human Embryonic Stem Cell-Derived Cardiomyocytes vs. Adult Ventricular Myofibrils Are Related to Distinct Sarcomeric Protein Isoforms*. Front. Physiol. 8:1111. doi: 10.3389/fphys.2017.01111.