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Short report

Substrate and mechanotransduction influence SERCA2a localization in human pluripotent stem cell-derived cardiomyocytes affecting functional performance

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ABSTRACT

Physical cues are major determinants of cellular phenotype and evoke physiological and pathological responses on cell structure and function. Cellular models aim to recapitulate basic functional features of their *in vivo* counterparts or tissues in order to be of use in *in vitro* disease modeling or drug screening and testing. Understanding how culture systems affect *in vitro* development of human pluripotent stem cell (hPSC)-derivatives allows optimization of cellular human models and gives insight in the processes involved in their structural organization and function.

In this work, we show involvement of the mechanotransduction pathway RhoA/ROCK in the structural reorganization of hPSC-derived cardiomyocytes after adhesion plating. These structural changes have a major impact on the intracellular localization of SERCA2 pumps and concurrent improvement in calcium cycling. The process is triggered by cell interaction with the culture substrate, which mechanical cues drive sarcomeric alignment and SERCA2a spreading and relocalization from a perinuclear to a whole-cell distribution. This structural reorganization is mediated by the mechanical properties of the substrate, as shown by the process failure in hPSC-CMs cultured on soft 4 kPa hydrogels as opposed to physiologically stiff 16 kPa hydrogels and glass. Finally, pharmacological inhibition of Rho-associated protein kinase (ROCK) by different compounds identifies this specific signaling pathway as a major player in SERCA2 localization and the associated improvement in hPSC-CMs calcium handling ability *in vitro*.

1. Introduction

Human cardiomyocytes (hCM) can be derived in large quantities by cardiogenic differentiation protocols from human pluripotent stem cells (hPSC) (Mummery et al., 2012). Structural and functional features of these cells are comparable to fetal cardiomyocytes, with similar genetic expression patterns and immature structural organization, largely responsible for poor functional performance (Robertson et al., 2013). The cardiomyocyte function is defined by a finely orchestrated sequence of events strongly relying on the correct genetic program and a strict structural organization (van den Heuvel et al., 2014). In adult hCMs, intracellular spatial distribution of the components of membrane potential regulation, calcium handling and contraction machineries accounts for their painstaking functional performance in the beating heart. hPSC-CMs, on the other hand, often lack either key molecular components or their right spatial intracellular distribution, determining their immaturity (Robertson et al., 2013). Nonetheless, proof of the developmental potential of these early-stage cardiomyocytes stands in the remarkable maturation observed in hPSC-CMs after engraftment in animal myocardia (Laflamme et al., 2007; van Laake et al., 2007; Chong et al., 2014; Cho et al., 2017) and similarly improved cardiac functional features of engineered cardiac tissues in comparison to 2D cultures (Feric and Radisic, 2016). While integration of electro-mechanical stimuli to *in vitro* cultures proved effective in promoting some degree of developmental progression (Hirt et al., 2014; Mihic et al., 2014), the underlying mechanisms and molecular triggers in play remain elusive and poorly understood.

In single cardiomyocytes, calcium handling ability is the centerpiece of their functional performance, with calcium-induced calcium release acting as trigger for contraction, followed by rapid cytosolic Ca^{2+}

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clearance by SERCA2a pumps and NCX (Bers, 2002). As opposite to adult hCMs, hPSC-CMs calcium handling is deeply immature, relying on both RYR2-associated Ca^{2+} release from the SR and trans-sarcolemmal Ca^{2+} influx for contraction triggering (Zhu et al., 2009; Lee et al., 2011; Pillekamp et al., 2012). Moreover, the absence of t-tubule systems (Snir et al., 2003; Lieu et al., 2009) and scarce expression of regulatory components (Liu et al., 2007) decreases considerably the Ca^{2+} release/reuptake rates. Nevertheless, it is possible to promote to some extent functional maturation of hPSC-CMs through either ectopic overexpression of missing adult proteins (Liu et al., 2009) or hormonal treatment (Ribeiro et al., 2015; Kosmidis et al., 2015).

In particular, improvement of functional performance is closely related to the maturation and organization of the sarcoplasmic reticulum (SR), which is the main Ca^{2+} store in mature cells and harbors key elements for calcium cycling, such as RyR channels for release and SERCA2a pumps for reuptake (Germanguz et al., 2011). Recently, a structural link between SERCA2a pumps and integrin structures (through integrin-related kinase, ILK) has been reported (Traister et al., 2014), adding evidence to previous reports of mechanotransduction signaling regulation of SERCA2a transcripts (Vlasblom et al., 2009), highlighting a tight connection between the cardiomyocyte's mechanical environment and SR organization and functional performance.

Here, we show for the first time in hPSC-CMs clear evidence of the involvement of mechanotransduction signaling in SR organization and, in particular, of the SERCA2a localization, with related functional calcium dynamics performance alteration. This mechanism is strongly related to the mechanical properties of the culture substrate sensed after cell adhesion, as expected from an integrin-dependent signaling.

2. Materials and methods

2.1. Cell cultures

The hES cell line HES2 was obtained from WiCell and cultured as described previously (Serena et al., 2012). The hiPS line ADHF#1 was kindly provided by prof. Mitsuo Oshimura and maintained as described previously (Zatti et al., 2014). The hiPS line aSkin119 was kindly provided by prof. Huei-Sheng Vincent Chen (Kim et al., 2013) and maintained in culture in feeder-free conditions, on 50 μg/ml Matrigel Growth Factors Reduced (MGRF) (Corning) in E8 medium (StemCell Technologies). Human cardiomyocytes were derived according to an EB-based protocol (Yang et al., 2008) and maintained constantly in suspension until enzymatic dissociation and single-cell adhesion plating. Detailed description of the cardiac differentiation protocols, dissociation protocol and hPSC-CM plating conditions are available in the Supplementary materials section.

2.2. Hydrogel preparation

Polyacrylamide hydrogels were prepared as previously described by Tse and Engler (Tse and Engler, 2010). In order to tune the hydrogel stiffness different ratios of acrilammide/bisacrilammide in milliQ water were prepared as follows: hydrogels of 3.1 kPa 5%/0.1%, 4 kPa 5%/0.15%, 16 kPa hydrogels 10%/0.15%. The polymerization was induced by addition of 10% ammonium persulfate and 0.1% TEMED and the final height of the gel was set at 90 μm to avoid influence of the supporting glass on the mechanical properties of the hydrogels. All reagents were purchased from Sigma-Aldrich.

2.3. Live imaging

Confocal calcium measurements were performed as reported previously (Martewicz et al., 2012) and are detailed in the Supplementary materials section. Measurements of membrane potential were performed with the same experimental setup as for calcium recording with a line scan speed of 1000 Hz. Cells were loaded for 20 min cells with 0.2 μM VF2.1Cl voltage sensitive dye, kindly provided by Prof. Roger Tsien (Miller et al., 2012).

2.4. Immunofluorescence analysis

A standard immunofluorescence protocol was used. Primary antibodies were: mouse anti-troponin T (NeoMarkers), goat anti-SERCA2a (Santa Cruz), mouse anti-RyR2 (GeneTex). Secondary antibodies used were: Alexa-488 goat anti-mouse (Life Technologies), Cy3 donkey anti-goat (Jackson ImmunoLab). Nuclei were counterstained with DAPI (Sigma-Aldrich) and samples were mounted with Elvanol and viewed under Leica TCS SP5 confocal microscope. Immunofluorescence on EB samples were performed on 20 μm cryosections of OCT (Kaltech) enclosed EBs mounted on poly-L-lysine coated glass microscope slides.

2.5. Data analysis

Detailed description of the quantification for myofibrillar orientation (OCF), SERCA2 distribution and calcium dynamic parameters (decay and TTP) is available in the Supplementary materials section and depicted in Fig. S6. All image analyses were performed with ImageJ software. Data are presented as means ± standard error of means (SEM). Data pairs were compared by non-directional Student's *t*-test, while group data by one-way ANOVA followed by Bonferroni's mean comparison. All data handling and computation was performed with Origin 8.1 software.

3. Results

3.1. Adhesion to substrate promotes structural reorganization

Extension of time in culture has proven multiple times to be a determinant in structural and functional cardiac maturation of hPSC-CMs (Sartiani et al., 2007; Otsuji et al., 2010; Kamakura et al., 2013; Lundy et al., 2013). However, no clear driving mechanism for the observed process was apparent and the contribution of the substrate to intracellular organization was difficult to evaluate in cardiomyocytes maintained in beating clumps with constantly evolving composition and stiffness of the extracellular matrix (Sachlos and Auguste, 2008).

Employing an EB-based cardiac protocol (Yang et al., 2008) to differentiate the hiPSC line ADHF#1 and continuously maintaining the beating EBs in suspension, we aimed at avoiding cell/substrate interactions prior to single-cell dissociation. Beating EBs were then digested with collagenases and trypsin into sparse cultures, in order to reduce CM-to-CM interactions and isolate as much as possible the effect of the adhesion to substrate.

We first tested the effect on intracellular organization of hPSC-CMs-plating on a stiff substrate. Adhesion to substrate allows hPSC-CMs to spread, switching from the round shaped cell packed inside a cellular cluster with high nucleus-to-cytoplasm ratio. The contractile machinery rapidly reorganizes towards clearly defined sarcomeric structures (Fig. 1A) and myofibrils align along the axis of the cardiomyocyte, as shown by the orientation correlation function (OCF) (Fig. 1B). Similarly, the sarcoplasmic reticulum shifts from a perinuclear localization in the EB-enclosed cells and shortly after replating towards a whole-cell distribution (Fig. 1C), increasing considerably the spread area of SERCA2a pumps across the cell (Fig. 1D). With such structural reorganization, we expected calcium handling ability of the cells to vary considerably as well, especially given the increase in amount and spatial distribution of SERCA2a pumps. We observed a dramatic shortening of calcium transient duration between the time-points considered (Fig. 1E), with significant improvement in Ca^{2+} transient kinetics after 1 week (Fig. 1F). The structural reorganization resulted in an improvement in functional performance, with increased Ca^{2+} release and reuptake rates (described by lower Ca^{2+} decay constant (Fig. S7A)). When challenged with 10 mM caffeine, hPSC-CMs displayed characteristic massive Ca^{2+}

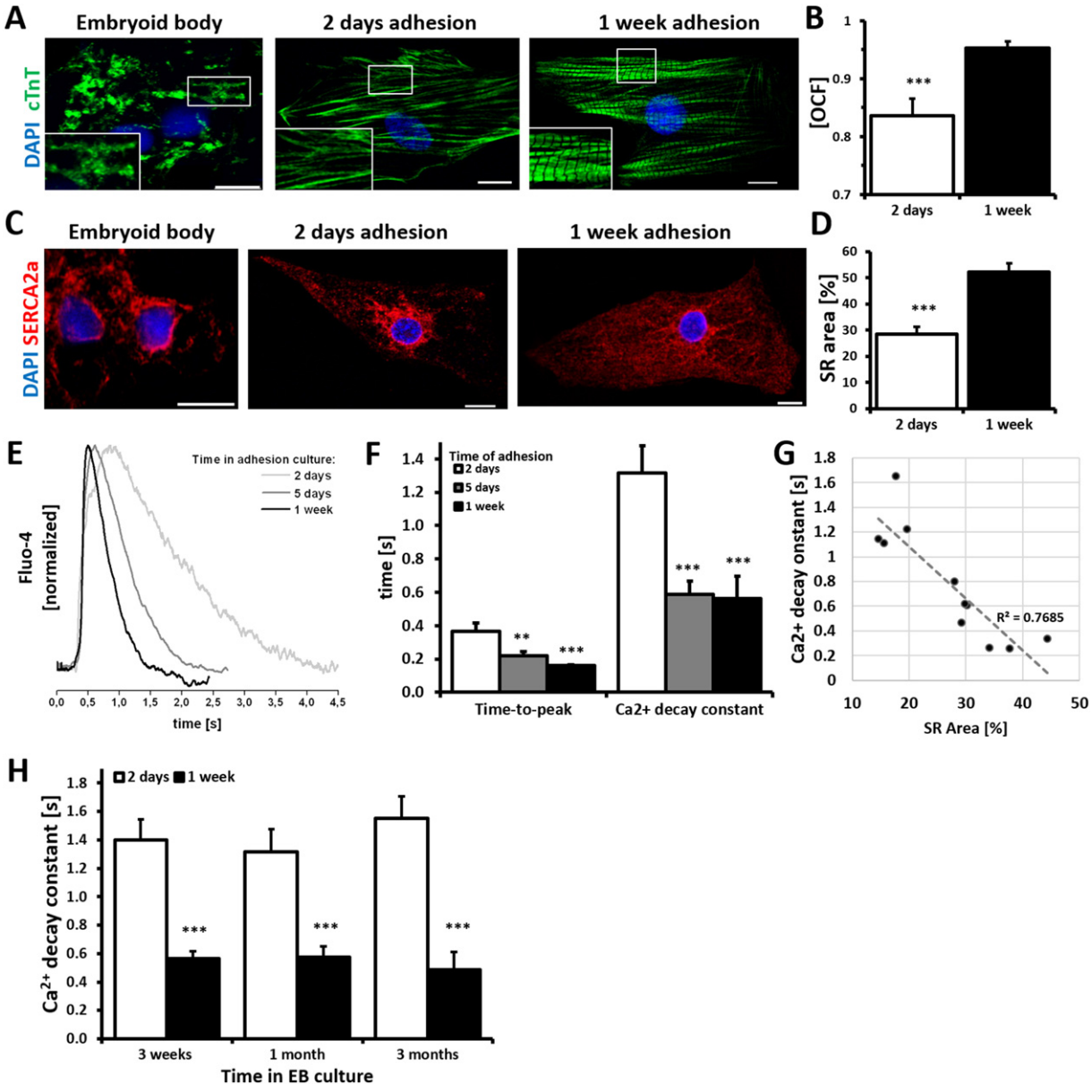

Fig. 1. Effect of adhesion culture on hPSC-CMs. (A) Cardiac troponin T staining (cTnT) at different time-points. Bar = 10 μm. (B) Sarcomeric alignment (OCF) at 2 days (n = 18) and 1 week (n = 24) of adhesion culture. (C) SERCA2a staining at different time points. Bar = 10 μm. (D) Cell projected area coverage of the SERCA2a staining at 2 days (n = 16) and 1 week (n = 13) of adhesion culture. (E) Representative calcium traces at different time-points. (F) Calcium transient kinetic parameters (time-to-peak; n = 20, 20, 22 and Ca2 + decay constant; n = 12, 12, 10). (G) Correlation between SERCA2a spread area and Ca^{2+} decay constant (n = 11). (H) Calcium re-uptake rate as function of time in EB culture at 2 days (n = 7, 12, 6) and 1 week (n = 20, 22, 6) of adhesion culture. *** = $p < 0.001$; ** = $p < 0.01$; n.s. = not significant.

release profiles at both analyzed time-points (Fig. S1A and B), with release and reuptake kinetics significantly improved after 1 week in culture (Fig. S1C and D) and augmented Ca^{2+} stores (Fig. S1E), indicating increased SR volumes. Co-staining of 1 week old hPSC-CMs with anti-SERCA2a and anti-RyR2 antibodies showed that the observed SERCA2a extension through-out the cell volume is concurrent with SR expansion (Fig. S1F and G). Indeed, acquired z-stacks of SERCA2a staining provided estimates of the SR physical volumes at 2 days and 1 week of culture, showing a significant increase in the SR volume (Fig. S1H). Retrieving in immunofluorescence analyses the same cardiomyocytes assayed for Ca^{2+} dynamics, allowed us to assess a good correlation between the SERCA2a staining area and the derived Ca^{2+} decay constant (Fig. 1G). We observed similar trends, with slightly differing absolute values, in the hESC line HES2 (Fig. S2A–F) and in a second hiPSC line αSkin119 (Fig. S2A–J). It is important to note that, for all cell lines considered, there are minimal differences in cell size between the two time-points considered (Fig. S2K) allowing direct comparison between the SR areas and volumes. In order to exclude significant differences in trans-sarcolemmal ion fluxes between the two time-points that could account for the observed calcium transient shortening, we performed experiments on the same hPSC-CMs cultures imaging both calcium dynamics and membrane potential. While the trends in calcium release and reuptake were conserved, we did not observe significant variations in action potential profiles, with action potential duration (APD90) remaining constant (Fig. S2J). The involvement of SERCA2a pumps in the increase of calcium reuptake rate has been tested by exposing hPSC-CMs after 1 week in adhesion culture to tBHQ, a SERCA2a pump reversible inhibitor. After 5 min at a concentration of 1 μM of tBHQ, Ca^{2+} decay constant increased by 28 ± 9% (n = 6) producing longer calcium transients (Fig. S3). In order to exclude major differences in our assays due to extended time in culture, we assayed for Ca^{2+} dynamics hPSC-CMs at different dissociation time-points, from EBs aged 3 weeks (early after the end

of differentiation protocol) up to 3 months. Not excluding any ongoing maturation process in the aging EBs, there was little effect on our read-outs upon extended EB culture, and the structural reorganization due to the adhesion to a stiff substrate resulted in similar improvements in functional performance, in the short-term period of interest (Figs. 1H and S2F).

3.2. Substrate mechanical properties affect structural reorganization

The mechanical properties of the culture substrate are well known for affecting cell behavior (Discher et al., 2009). In particular, the elastic modulus (or stiffness) of the adhesion surface proved to be a determinant in guiding and promoting cell maturation according to tissue-specific parameters (Engler et al., 2006). For muscle tissue, the physiological stiffness has been routinely estimated in the range between 8 and 20 kPa, with the heart stiffness centered around 11–17 kPa (Engler et al., 2007).

We decided to test how the hPSC-CMs structural reorganization would be affected by the change of substrate mechanical properties, by seeding the hPSC-CMs on polyacrylamide hydrogels. We chose to compare hPSC-CMs plated on glass with those plated on a 16 kPa hydrogel, falling inside the estimated range of muscle elastic moduli, and a softer one of 4 kPa, well below the optimal muscle stiffness but still not soft enough to prevent cardiomyocyte spreading (Engler et al., 2008). Hydrogels were prepared according to Engler group protocol in order to maintain the same nominal stiffness referenced for *in vivo* tissues (Tse and Engler, 2010), and coated by protein adsorption to avoid chemical modification that could result in stiffness variability. This coating technique allows only short-term culture up to 5 days, but according to our previous observations, this time span is enough to observe the structural reorganization of hPSC-CMs (Fig. 1F). Hydrogels were fabricated as 90 μm thick disks (Fig. S4A) to avoid any interference from the underlying glass. The elastic moduli were verified by atomic-force microscopy resulting in a 14.5 ± 2.1 kPa and a 4.1 ± 0.4 kPa estimate for the nominal 16 kPa and 4 kPa stiffnesses, respectively (Fig. S4B–D).

By plating hPSC-CMs on hydrogel substrates, we observed the same trend in structural organization on glass and 16 kPa hydrogel, with sarcomeres improving organization (Fig. 2A) and myofibril alignment to the cell long axis (Fig. 2C). Such process was less apparent on the softer substrate of 4 kPa in the short-term taken into account, similarly to other reports (Rodriguez et al., 2011; Young et al., 2014). Furthermore, we observed a striking difference between the 4 kPa hydrogel and the other two conditions tested in the redistribution of the SERCA2a staining, which increased significantly on the stiffer substrates, recapitulating the previous results, but maintained a perinuclear localization (Fig. 2B) and a low spread area on 4 kPa (Fig. 2D). Given this failed relocalization of the SR to a whole-cell distribution, it was not surprising to observe poor performance in Ca^{2+} handling on the soft substrate (Fig. 2E).

3.3. ROCK inhibition prevents SR structural reorganization after plating

Substrate stiffness and changes in cell morphology and geometry are sensed by means of mechanotransduction pathways, with integrin-related signaling and downstream activation of the RhoA/ROCK pathway (Jaalouk and Lammerding, 2009; Jahed et al., 2014). Involvement of RhoA signaling through Rho-associated kinase in cardiac *in vitro* maturation and influence over functional performance has been shown in neonatal rat cardiomyocytes (Jacot et al., 2008). ROCK inhibition by means of 1 μM hydroxyfasudil has proven an effective strategy to counteract the effects of high-stiffness hydrogel substrates (Jacot et al., 2008). Thus, we adopted the same strategy to investigate the effect of ROCK inhibition on human cardiomyocytes, testing the involvement of this mechanotransduction pathway in the structural reorganization after hPSC-CMs adhesion culture. Culturing hPSC-CMs on a stiff glass substrate, the cultures were treated with a low dose of 2 μM Y-27632, a fasudil equipotent inhibitor (Liao et al., 2007), for the whole duration of the experiment. Despite evidence for impairment of myofibrillogenesis in chick embryos by Y-27632 at higher concentrations (Sakata et al., 2007), this inhibitor concentration was not enough to significantly alter the alignment of the contractile machinery or general cell spreading in our cultures (Fig. 3A and C). Nevertheless, the effect on SR spreading (Fig. 3B) and SERCA2a distribution was significant (Fig. 3D) and similar to hPSC-CMs cultures on the soft 4 kPa hydrogel. As already assessed in previous experiments, the perinuclear localization of the SR resulted in poor Ca^{2+} handling even after 1 week of adhesion culture, with the Ca^{2+} reuptake rate not showing significant improvement between our early and late time-points and being far from comparable with the untreated hPSC-CMs (Fig. 3E).

Y-27632 is regarded as a specific ROCK inhibitor even at much higher concentrations as those employed here (Davies et al., 2000), which were reduced to minimize effects over cell shape and myofibrillar reorganization observed in neonatal rat cardiomyocytes (Kuwahara et al., 1999). Nonetheless, in order to verify the correlation of SR reorganization after specific ROCK inhibition, we repeated these experiments on a second hiPS cell line (αSkin119) using alternative compounds H-1152 (Tamura et al., 2005) and GSK429286A (Goodman et al., 2007). These potent and specific ROCK inhibitors are structurally unrelated to Y-27632 and none of these three compounds shared any common off-targets in a large panel of 57 kinases with always a much higher specificity to Rho-associated kinase (Nichols et al., 2009) at the concentration (3 μM) we employed. Again, we observed SR relocalization and SERCA2a spreading in the untreated control (Fig. S4A), as opposed to the treated samples in which all three inhibitors prevented SERCA2a spreading to a whole-cell distribution to a significant degree (Figs. 3F and S5A). Accordingly, there was no improvement from day 2 to 1 week in Ca^{2+} reuptake rates in the treated samples (Fig. 3G), in disregard to the slight but significant increase in SR area in the H-1152 and GSK-429286A treated samples (Fig. S5B).

4. Discussion

During heart development mechanotransduction signaling pathways play a major role in shaping the cardiac tissue and directing its growth and maturation from a rhythmically contracting tube to a fully functional mature organ (Majkut et al., 2014). *In vitro* similar processes could be in place in shaping and prompting maturation of hPSC-CMs, pushing these cells further from their developmentally early stage. In this work, we analyzed the effect of replating into adhesion culture human cardiomyocytes, and specifically providing insight into the molecular triggers involved in the early structural reorganization following adhesion and spreading on a stiff substrate.

The issue of hPSC-CMs maturation *in vitro* has been addressed through a number of strategies, from extension of time in culture spanning several months (Sartiani et al., 2007; Otsuji et al., 2010; Kamakura et al., 2013; Lundy et al., 2013) to genetic engineering (Liu et al., 2009; Lieu et al., 2013), hormonal stimulation (Yang et al., 2014; Kosmidis et al., 2015; Ribeiro et al., 2015) and geometrical patterning (Kuo et al., 2012). All these reports provide evidence for potential improvement of structural and functional cardiac features. A universally recognized determinant of *in vitro* hPSC-CMs maturation is the physical environment provided by the culture substrate. Here, we provide evidence of a rapid reorganization of the contractile machinery (Fig. 1A) and the sarcoplasmic reticulum (Fig. 1C) after plating hPSC-CMs from suspension to single cell adhesion culture. The change in the sensed mechanical cues and reduction of geometrical constrains of the embryoid body cell-aggregate prompted the hPSC-CMs to align myofibrils along cell axis (Fig. 1B), relocalize SERCA2a pumps throughout the whole-cell area (Fig. 1D) and, accordingly, increase Ca^{2+} reuptake rate (Fig. 1F). Regardless of recovery after EB dissociation and maturation events occurring in prolonged culture time up to 3 months (Figs. 1H and S2F),

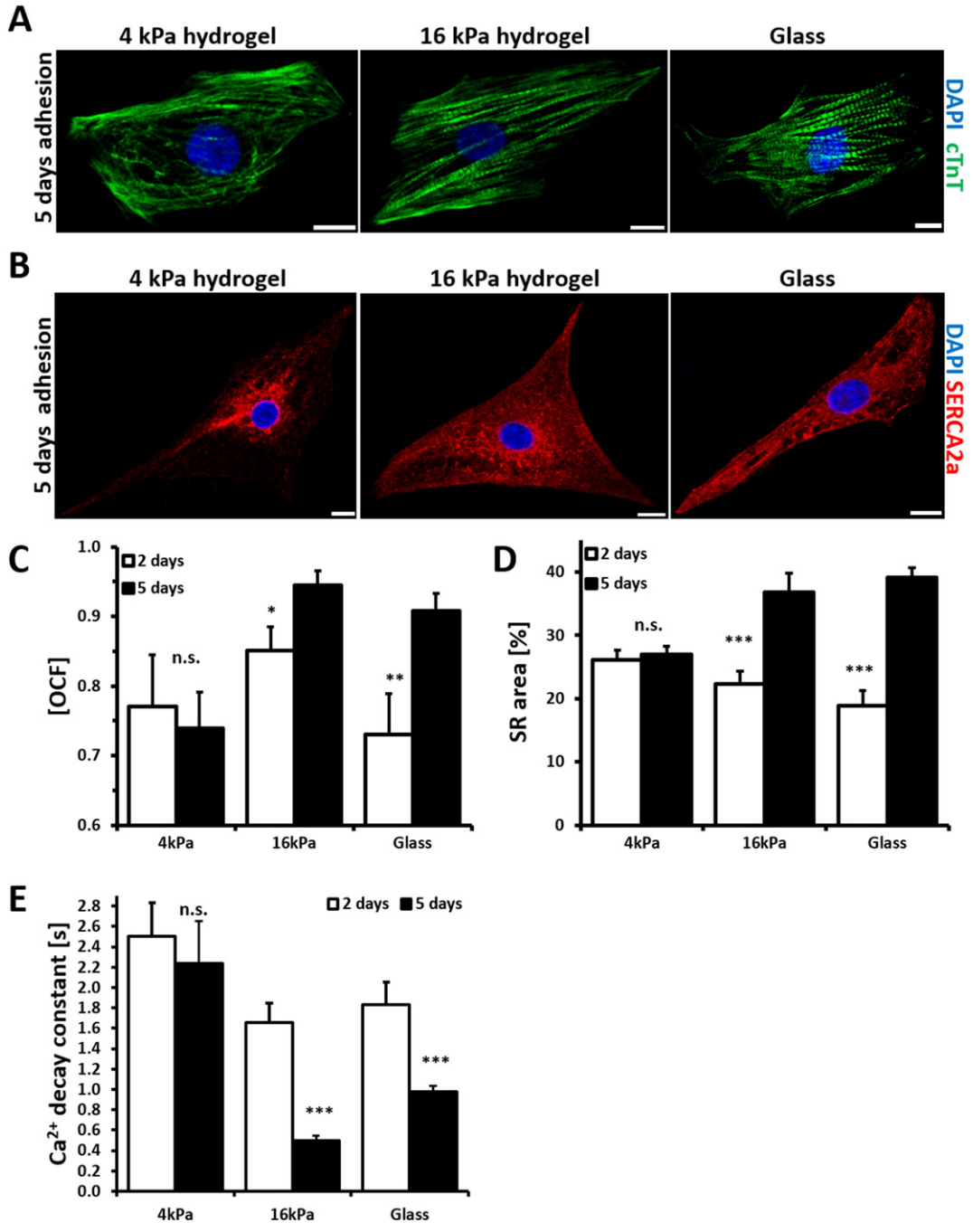

Fig. 2. Substrate mechanical properties influence structural reorganization. (A) Immunostaining for cTnT on different substrates after 5 days in adhesion culture. Bar = 10 μm. (B) Immunostaining for SERCA2a on different substrates after 5 days in adhesion culture. Bar = 10 μm. (C) Sarcomeric alignment on different substrates at 2 days (n = 15, 11, 13) and 1 week (n = 31, 22, 27) of adhesion culture. (D) SERCA2a spreading on different substrates at 2 days (n = 15, 16, 14) and 1 week (n = 31, 21, 30) of adhesion culture. (E) Ca^{2+} decay constant at 2 days (n = 13, 13, 10) and 1 week (n = 9, 6, 5) of adhesion culture. *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$; n.s. = not significant.

this reorganization process was undoubtedly driven by the adhesion substrate.

The mechanical properties of the culture substrate, such as its elastic modulus, have been repeatedly shown to affect *in vitro* primary cardiomyocyte structural features and functional performance. Myofibrillar organization and expression of calcium handling and cardiac structural proteins show dependence on substrate stiffness, with associated changes in functional performance in beating rate, calcium handling and force generation (Engler et al., 2008; Jacot et al., 2008; Rodriguez et al., 2011; Majkut et al., 2013; McCain et al., 2014). In all reports, cardiac structure and performance improve at a faster rate by increasing the substrate stiffness (Dasbiswas et al., 2015) and seem to peak around the physiological muscle elastic modulus, which roughly spans in the range of 8–20 kPa, centering between 11 and 17 kPa for the cardiac muscle (Engler et al., 2007). In order to assess the actual dependence of the structural reorganization on the adhesion substrate, we performed experiments on polyacrylamide hydrogels of 16 kPa and 4 kPa nominal elastic moduli. The comparison between these two moduli was performed similarly to Sniadecki's group 15 kPa *vs.* 3 kPa. The former was chosen as a safe muscle-like stiffness value proven to provide all the necessary mechanical cues for cardiac maturation, while the latter being a value well below the optimal "muscle stiffness", despite still not being soft enough to impair cell spreading (Rodriguez et al., 2011). We mechanically characterized the produced hydrogels by AFM,

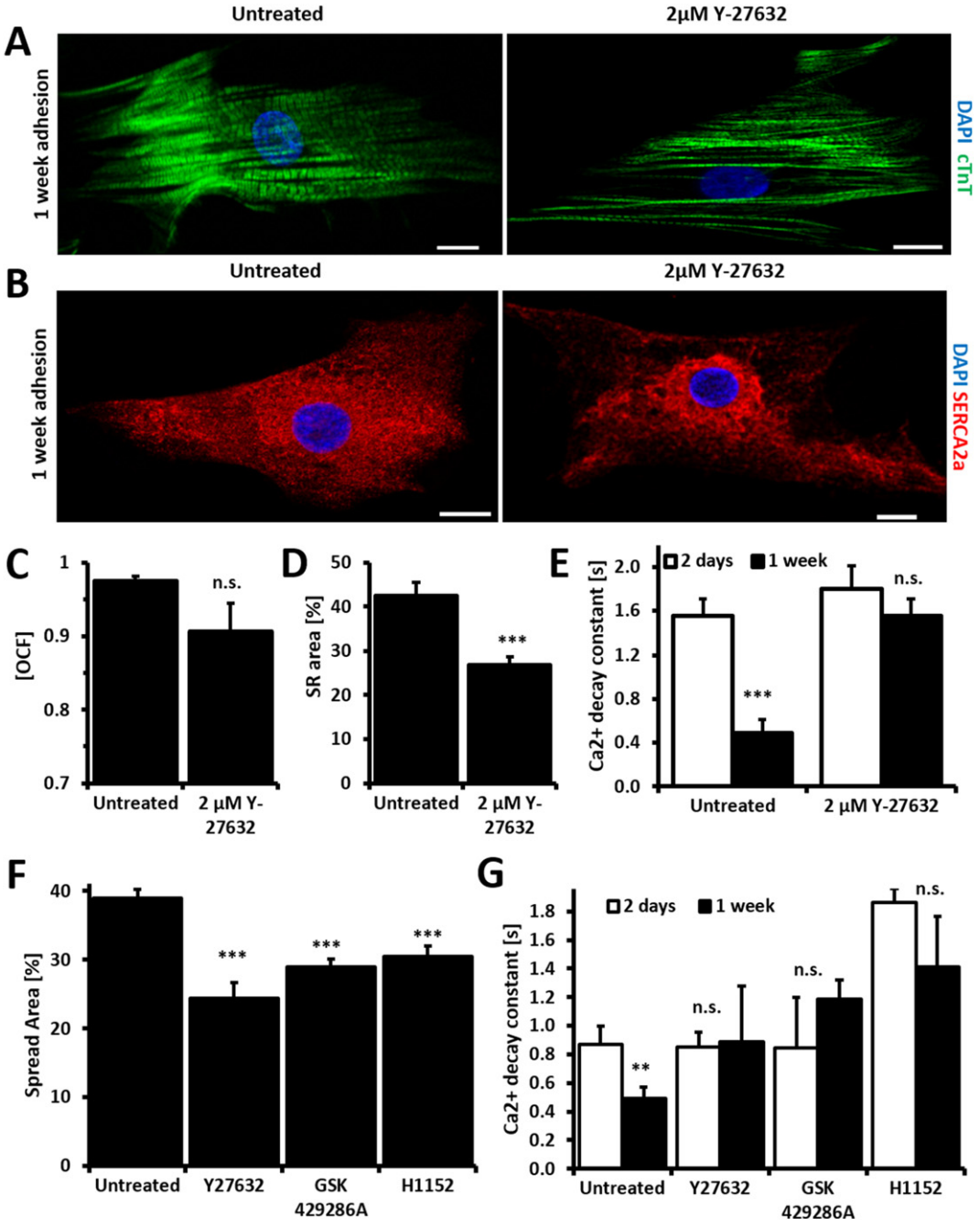

Fig. 3. ROCK inhibition impairs structural reorganization. (A) Immunostaining for cTnT after 1 week of ROCK inhibitor treatment in adhesion culture. Bar = 10 μm. (B) Immunostaining for SERCA2a after 1 week of ROCK inhibitor treatment in adhesion culture. Bar = 10 μm. (C) Sarcomeric alignment in absence (n = 6) and presence of ROCK inhibitor (n = 9). (D) SR area in absence (n = 9) and presence of ROCK inhibitor (n = 8). (E) Calcium re-uptake rate at 2 days (n = 5, 5) and 1 week (n = 5, 5) of adhesion culture. (F) SR area after ROCK inhibition by multiple compounds (n = 11, 11, 8, 13). (G) Ca2 + reuptake rate after ROCK inhibition by multiple compounds at 2 days (n = 18, 7, 7, 5) and 1 week (n = 15, 6, 10, 13) of adhesion culture. *** = $p < 0.001$; ** = $p < 0.01$; n.s. = not significant.

confirming an experimental elastic modulus of 14,5 ± 2,1 kPa and 4,1 ± 0,4 kPa (Fig. S3B–D). Our softer hydrogel did not impact hPSC-CMs activity *per se*, as hPSC-CMs still expressed organized contractile machinery (Fig. 2A) and calcium handling proteins (Fig. 2B), displayed spontaneous beating and cardiac-like calcium transients (Fig. 2E), but it did not promote the structural reorganization observed on the stiffer hydrogel and on glass. As the only difference between the samples being represented by the elastic modulus of the culture substrate, the involvement of the adhesion substrate in the structural reorganization process seems to be confirmed. In literature "soft" substrates such as 5,8 kPa has been reported of being able to sustain hPSC-CMs maturation (Kosmidis et al., 2015; Ribeiro et al., 2015), in apparent conflict with our data. Nonetheless, these studies performed relative comparisons between hormonally treated and control cells, regardless of the contribution of substrate stiffness to the process. Moreover, experimental measurements of elastic moduli show some degree of variability on the absolute stiffness values returned for hydrogels. Comparing literature data for acrylamide/bis-acrylamide ratios, we found that the referenced 5%/0,1% ratio for 5,8 kPa hydrogels (Frey et al., 2007) has been reported to produce as well a slightly softer hydrogel of 3,15 kPa (Tse and Engler, 2010), and in our hands it produced a hydrogel of an experimental elastic modulus of 2,3 ± 0,1 kPa (Fig. S3B and E).

Substrate stiffness, along with other physical properties of the cellular environment, is sensed and translated into cellular responses by a

limited number of signaling pathways (Jaalouk and Lammerding, 2009). The main players in this sensing are integrins and their associated downstream signaling partners such as the RhoA/ROCK pathway (Jahed et al., 2014). In the cardiac muscle, inhibition of this specific pathway produces severe impairment in the developing embryonic structures (Zhao and Rivkees, 2003) and counteracts the effect of hypertrophy-inducing agents in the differentiated cardiomyocytes (Zeidan et al., 2006; Hunter et al., 2009; Ye et al., 2009). Here, we show how structural reorganization early after adhesion plating is promoted by the substrate, and is absent or not efficient on substrates without the necessary physical cues. In particular, the reorganization of the SR, with the associated increase in volume, amount of calcium handling proteins and, most importantly, their localization results in a fast improvement in functional cardiac features. Interestingly, SR in general and SERCA2a pumps in particular have been reported to be mechano-regulated. SERCA2a protein levels have been shown to depend on the substrate stiffness in neonatal rat cardiomyocyte cultures (Jacot et al., 2008), with SERCA2a mRNA transcription regulated by RhoA/ROCK pathway and altered by ROCK inhibition (Vlasblom et al., 2009). More recently, a physical association between integrin-linked kinase (ILK) and SERCA2a/phospholamban complex was observed in the human myocardium, speculating on this complex to be itself a cardiac adhesion-dependent mechanoreceptor (Traister et al., 2014). Disruption of such scaffolding activity by ILK in a pathological setting leads to impairment of cardiac function, while overexpression of ILK and enhancement of the interaction with SERCA2a in hiPS-CMs resulted in increased SR Ca^{2+} storage and improved Ca^{2+} reuptake rates. Moreover, inhibition of ROCK by Y-27632 produces disruption of costameres, the integrin-based cytoskeleton-scaffolding structures to which ILK is associated (Sakata et al., 2007). In this work, we sought to add new evidence on this mechano-regulation of SR and SERCA2a activity, by impairing RhoA/ROCK signaling by ROCK inhibition, applying a similar strategy employed to counteract the effects of stiff substrates on cardiac cultures by means of hydroxyfasudil administration (Jacot et al., 2008). By using 3 potent and specific ROCK inhibitors with unrelated chemical structures and independent off-target activity, we provide evidence of the involvement of this mechanotransduction pathway in the structural organization of the SR and SERCA2a intracellular distribution (Fig. 3B and F) and, by extension, on the functional performance (Fig. 3G) of human cardiomyocytes *in vitro*.

In this study, we focused on the short-term reorganization of structural features of hPSC-CMs after replating in adhesion culture. This process could be associated to either or both improvement of the poor sarcomeric and SR organizations of EB-clustered round hPSC-CMs (Fig. 1A and B) and the recovery from EB dissociation. Nevertheless, we provide evidence it is a substrate-driven process, regulated by RhoA/ROCK pathway. Moreover, our study is limited to hPSC-CMs derived through an EB-based differentiation protocol (Yang et al., 2008), in which we maintained cardiac cultures in suspension until single-cell dissociation to avoid previous cell-substrate interactions. Several monolayer-based differentiation cardiogenic protocols have been developed in recent years and are reported to be more efficient in hPSC-CMs yields (Lian et al., 2012; Burridge et al., 2014). We sought to compare our readouts with the effects of single-cell adhesion replating in hCMs differentiated through a monolayer- small molecule-based protocol (Lian et al., 2012). By comparing the same hiPSC culture differentiated through both methods and analyzed at day 50 of differentiation, we hardly observed a similar reorganization process in the second sample (Fig. S6). While the EB-derived hPSC-CMs displayed structural improvement with sarcomeric alignment and SERCA2a spreading (Fig. S6A), the monolayer-based protocol produced hPSC-CMs with stable structural features (Fig. S6B), especially for SR spreading (Fig. S6C) and calcium handling (Fig. S6D). In the latter case, hPSC-CMs showed a marked hypertrophic response to adhesion culture (Fig. S6E). Overall, differentiation method and differences in culture media could be accountable for the observed differences.

The functional maturation we observed in our hPSC-CMs can be mostly ascribed to the structural reorganization process in progress and it is shown by means of calcium handling ability, as the most directly related feature for a spreading SR (Fig. 1G). In all likelihood, other functional features such as contraction force could be affected by a better performing SR and a uniaxially aligned and organized sarcomeric machinery, but we did not investigate these aspects of cardiac performance.

In conclusion, we provide evidence of a reorganization of structural features and an associated functional improvement in human cardiomyocytes *in vitro*, which is clearly driven by mechanical cues of the adhesion substrate, and mediated by mechanotransduction signaling involving the RhoA/ROCK pathway.

Contributions

S.M. performed most of the experimental work; E.S., S.Z. and S.M. performed pluripotent stem cell cultures, cardiac differentiation and molecular characterization; G.K. provided HES2-CMs and discussed research activity; S.M., E.S. and N.E. designed the research; S.M. and N.E. wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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