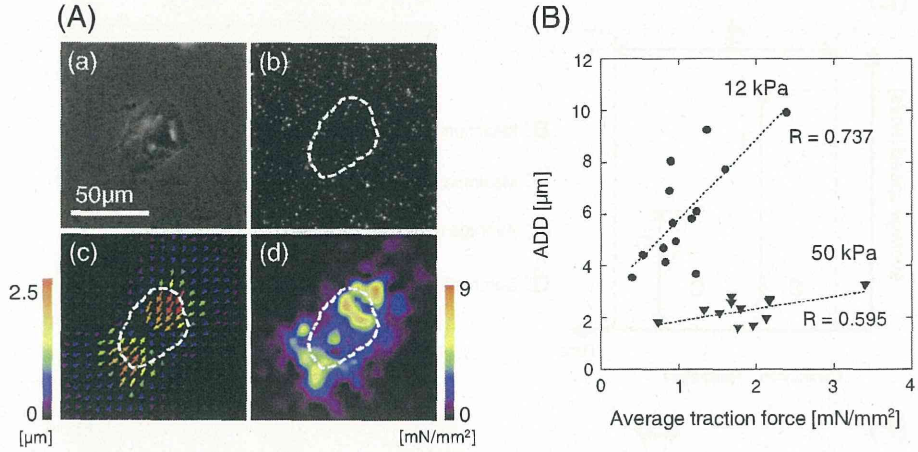

Fig. 2. (A) Parts (a) and (b) show the phase-contrast and fluorescent images of the hiPS-CM and the substrate with fluorescence beads (1 μm diameter), respectively. Part (c) is an example of a displacement field image of fluorescence beads calculated by the particle image velocimetry (PIV) algorithm. Part (d) shows an example of a traction force field image calculated using the PIV result from part (c) by the Fourier transform traction cytometry (FTTC) method. (B) Correlation between ADD and the normalized traction force (at 12 kPa (●) and 50 kPa (▼)) estimated with the FTTC algorithm. Bar in (a) represent 50 μm. The color scales for (c) and (d) are as indicated beside each of the figures.

it was difficult to define the peak point of positive deflection, associated with K^+ current, or FPD in FP waveforms with EAD, we eliminated the data with EAD-like waveform from the evaluation of the CRD–FPD correlation (Fig. 6B). As shown in Fig. 6A and C, the motion profile also exhibited an irregular relaxation pattern that corresponded to the EAD-like FP waveform. This indicates that the speed of the relaxation decreased or almost momentarily stopped at the point of the negative deflection in the FP (see also Supplementary Movies 1 and 2). In the presence of 100 nM E-4031, there was a major decrease in the contractile parameters, MCS, MRS, ADD and the beating rate (Fig. 6E–G).

3.3.3. The effects of a Ca^{2+} channel blocker, verapamil

Fig. 7A shows the alterations in the motion and FP profiles of the hiPS-CMs in accordance with the verapamil concentration (0, 90, 150 nM). Increasing the verapamil concentration caused a progressive decrease in both the FPD and CRD that was well correlated with the correlation coefficient ($R = 0.970$) (Fig. 7B). The slope of the linear regression was found to be 0.633 (FPD/CRD). Since verapamil has an L-type Ca^{2+}-channel inhibiting effect, increasing verapamil concentration led to a decrease in FP_{slow} (Fig. 7A). Addition of verapamil also caused MCS to become smaller. There was a good correlation between the amplitudes of FP_{slow} and MCS, when these parameters were evaluated as a percent of the control, with a correlation coefficient of 0.921 (Fig. 7C). Increasing verapamil concentration also caused the MRS as well as ADD to decrease (Fig. 7C and D), and the beating rate to increase (Fig. 7E).

3.3.4. The effects of the positive inotropic reagent, isoproterenol

Fig. 8A shows the simultaneously measured motion and FP profile at isoproterenol concentrations of 0, 1, and 10 μM. With increasing isoproterenol concentrations, the CRD and FPD progressively shortened (Fig. 8A), with a good correlation observed ($R = 0.943$) (Fig. 8B). The slope of the linear regression was found to be 0.737 (FPD/CRD). For the motion profile, there were increases in the MCS, MRS, ADD as well as the beating rate, all depending on isoproterenol concentrations (Fig. 8C and D). These results suggest that the inotropic, lusitropic and chronotropic effects of isoproterenol can be detected with the motion of hiPS-CM monolayer.

3.4. Variability in contractile data

To test the possibility that contractile parameters of hiPS-CMs detected with the motion vector analysis is critically influenced by the heterogeneity in monolayer preparation, we evaluated the regional variability in MCS and MRS. MCS and MRS were obtained from motion data, and are summarized in Supplementary Fig. 3A and B. The average values of MCS and MRS under control conditions varied from 8 to 15 μm/s and from 4 to 10 μm/s, respectively, and were dependent on the regions of monolayer. These values were altered by the addition of a Ca^{2+} channel blocker, verapamil, which had a negative inotropic effect and similar variability to that of the control. By expressing these values to a percent of the control, we found that each value converged to a similar percentage value (Supplementary Fig. 3A and B). This indicates that the relative values of contractile parameters are significantly less dependent on the region in the preparation.

4. Discussion

The present study aimed to evaluate contractile characteristics and the correlation between contractile motion and electrical properties of hiPS-CM monolayer by using video microscopy, Ca^{2+} transient imaging, traction force microscopy and FP measurement. High resolution motion vector analysis could detect contractile characteristics of hiPS-CMs, i.e., MCS, MRS, ADD and CRD, quantitatively, and demonstrated the correspondence between contractile motion and FP. Motion data further provided complementary information against FP, by detecting the inotropic and lusitropic effects of an experimental drug, isoproterenol. The accessibility to information about relaxation process, or lusitropism, is considered to be one of the advantages of this imaging approach. Recently, there has been increasing attention to the diastolic dysfunction characterized by decreased relaxation velocity and prolonged relaxation and its applicability to common cardiac pathologies, such as ischemic heart diseases and hypertensive heart diseases, and to rare genetic heart diseases, such as DCM [63,64]. The imaging approach could potentially be used to target and analyze hiPS-CMs derived from such diseases.

4.1. Contractile characteristics of hiPS-CMs detected with video microscopy

It has been previously reported that alterations in hiPS-CM area depend on substrate stiffness [26] or cell density [65]. We examined the cell area and the contractile parameters (MCS and MRS) of hiPS-CMs ($n = 40$) and observed no significant dependence of MCS and MRS on cell area. Since in our current study, we sparsely plated hiPS-CMs in order to extract single cell information, average cell area became

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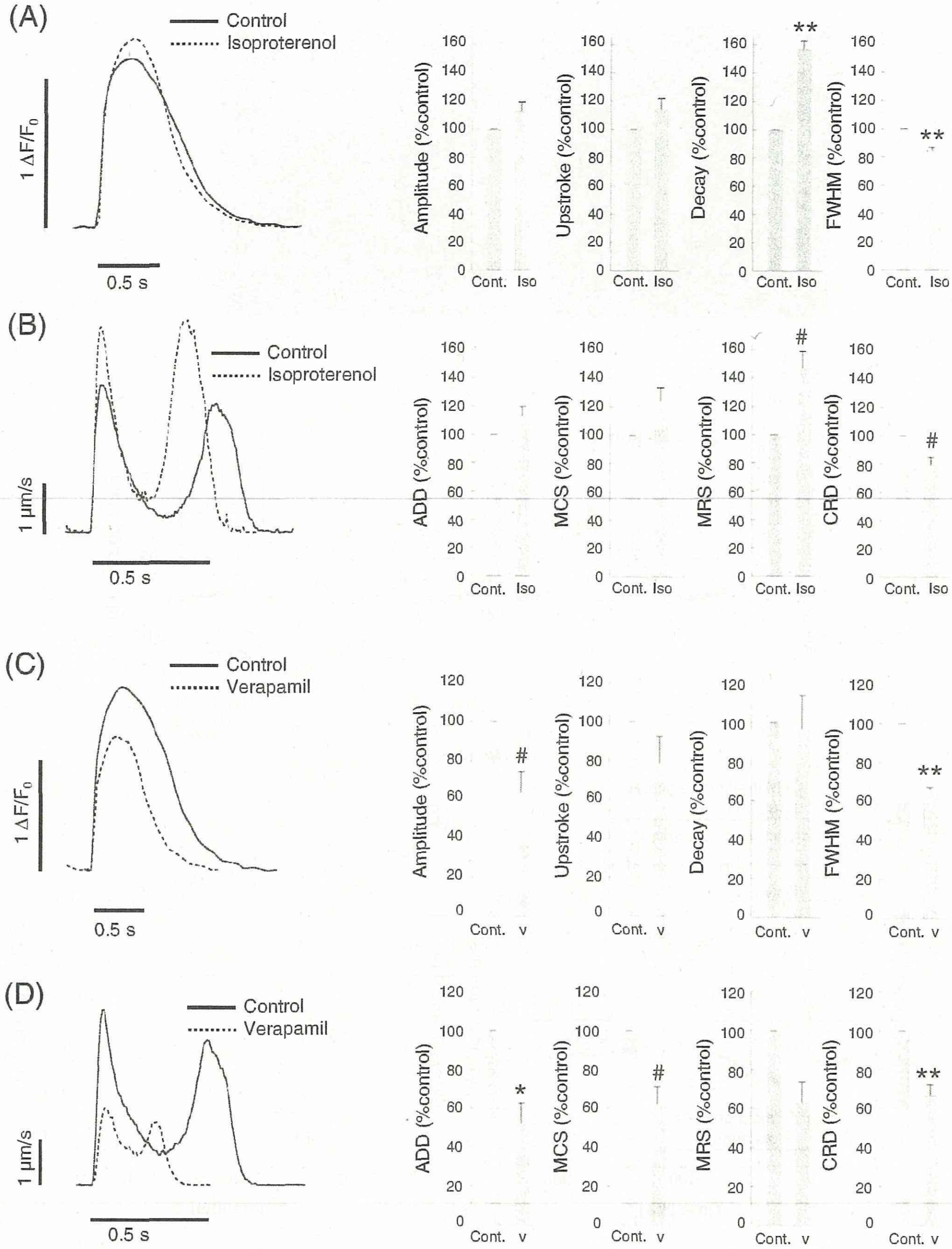

Fig. 3. (A) and (B) show the example profiles of the Ca^{2+} transient and motion waveform of hiPS-CMs, respectively, in the presence of 100 nM isoproterenol. (C) and (D) also show the example profiles of the Ca^{2+} transient and motion waveform of hiPS-CMs, respectively, in the presence of 100 nM verapamil. The bar charts in (A) and (C) represent the drug-induced change in amplitude, maximum upstroke, maximum decay and FWHM of the Ca^{2+}-transient. In (B) and (D), change rate of the contractile parameters, ADD, MCS, MRS and CRD, were also shown in bar charts. The Ca^{2+} transient and motion data were obtained independently. In all the bar charts, values are means ± SE and are expressed as percentage of control.

relatively larger (4244 ± 279 μm^2, $n = 40$) than that recently reported (1654 μm^2, $n = 22$) for hiPS-CMs (iCell CMs) that were plated in a monolayer form with a density of 22,500 cells in the well of a 96-well multiplate [65]. As seen in Fig. 1, the hiPS-CMs attached to the substrate exhibited a heterogeneous shape and their contractile motion often occurred locally in the cell body. Therefore, it should not be surprising that the average velocity of hiPS-CMs would not correlate well with their cell area. It has been reported that hiPS-CMs cultured for prolonged period, e.g., 90 days, exhibited rod-shaped morphology [66] like adult CMs filled with an aligned sarcomere structure [67]. Those morphologically matured hiPS-CMs, which were not tested in this study, could represent area dependence of contractile speed.

The image-based edge-detection technique has been the method of choice for measuring the shortening of the length of the whole cell body or sarcomere of the rod-shaped adult CMs in order to estimate the force development [34,41–43]. In contrast, TFM has been utilized to assess the contractility of cultured CMs that exhibit an amorphous shape [26,68,69]. With TFM, the traction force of the cells can be estimated based on the deformation of the substrate, which is detected by the displacement of fluorescent beads embedded in the substrate, and on the

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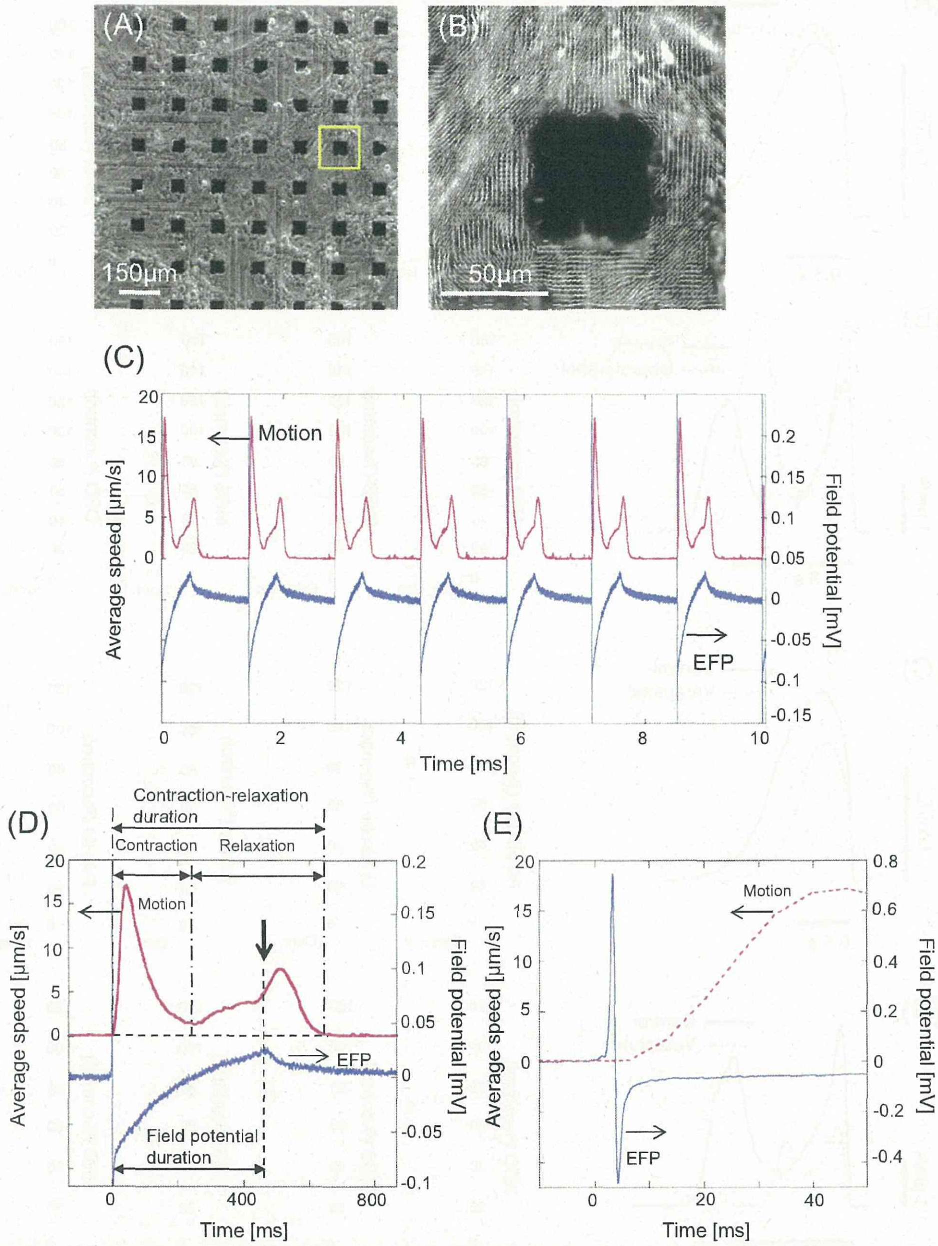

Fig. 4. (A) Phase-contrast image of the hiPS-CM monolayer prepared on the MEA probe. (B) The enlarged velocity field image for the yellow-square region is shown in (A), which shows the motion vectors as fine white lines. (C) Example profile of simultaneously measured hiPS-CM motion and FP. The motion data were evaluated from the region in close vicinity to the electrode (e.g., the yellow-square region in Fig. 4A) that was used for the FP data acquisition. (D) An enlarged single beat profile. The horizontal dashed line represents the baseline of the average velocity (0 μm/s). The vertical dot-dashed lines illustrate the durations of contraction and relaxation of the motion profile. The vertical dashed line with the arrow shows the peak position of positive deflection of the FP. (E) Magnified figure of the onset region of the FP and the motion shown in (D). Time zero corresponds to the onset of the positive FP spike.

elastic modulus of the substrate [70]. In our study, we examined the correlation between the force development and cellular deformation (ADD) of the hiPS-CMs. As shown in Fig. 2B, ADD appeared to be correlated with the force development on the substrates (12 kPa and 50 kPa). Phase-contrast microscopy observes overall deformation/displacement of hiPS-CMs during the contraction–relaxation process, including passively moving cellular boundaries and intracellular compartments or organelle. Our present results suggested that the average cellular deformation, ADD, detected by phase-contrast microscopy and motion vector analysis represents the extent of the force development of the hiPS-CMs on the substrates. As long as intra- and extra-cellular elastic properties (e.g., adhesion between hiPS-CMs and substrate) of hiPS-CMs are not altered during the measurement, ADD can be a surrogate marker for the force development of hiPS-CMs.

Isoproterenol and verapamil have been shown to alter the amplitude of the fluorescence peak of the Ca^{2+} transient in iPS-CMs [19]. In our current study, we examined whether the Ca^{2+} transient of hiPS-CMs was correlated with motion behavior in the presence of isoproterenol and verapamil. Responses of the Ca^{2+} transient in hiPS-CMs observed against isoproterenol included an increase in the amplitude, upstroke and decay (Fig. 3A). Interestingly, the maximum decay of the Ca^{2+} transient in the presence of isoproterenol showed a higher increased rate

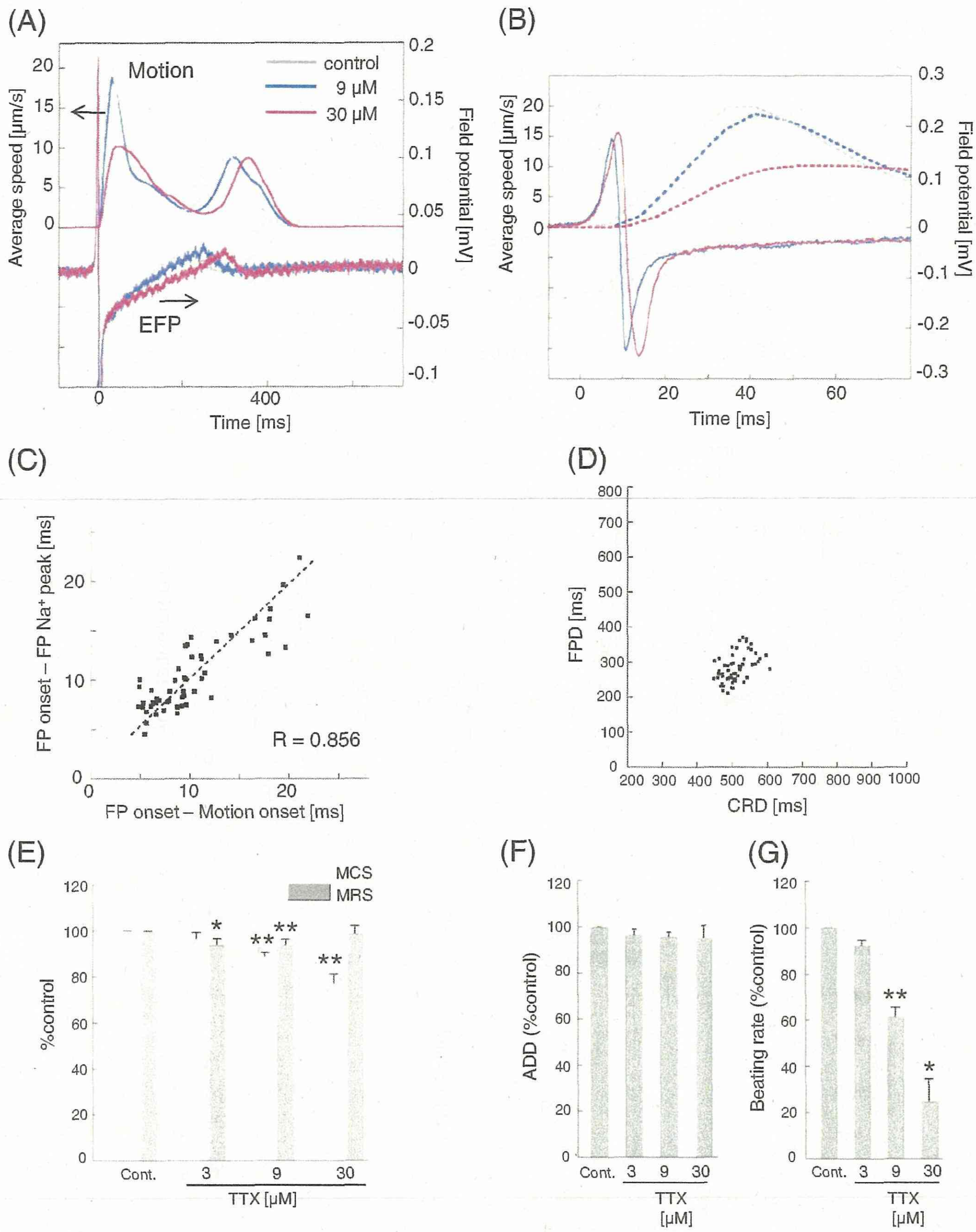

Fig. 5. Contractile and FP responses of the hiPS-CM to tetrodotoxin (TTX). (A) Example of motion and FP profiles of the hiPS-CMs simultaneously measured in the presence of 0, 9, and 30 μM of TTX. (B) Correlation between the CRD and FPD obtained with varied concentrations of TTX (0–30 μM). (C) Enlargement of the onset region of the FP and motion profiles. (D) Correlation of the FP onset-to-FP Na + peak and FP onset-to-motion onset with varied concentrations of TTX (0–30 μM). (E) The normalized change of the MCS and MRS with 0–30 μM of TTX. (F) and (G) show the normalized change of the ADD and beating rate in accordance with the 0–30 μM TTX concentration, respectively. Data were obtained from 15 electrodes with 3 independent preparations and are expressed as means ± SE. $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{\#}p < 0.10$ compared with the control.

(~160% increase from control) compared to that of maximum upstroke, which is consistent with greater increases in MRS than in MCS of motion response. Verapamil decreased all of the parameters of the Ca^{2+} transient in hiPS-CMs (Fig. 3C, D). This is attributed to verapamil's blockage of the L-type Ca^{2+} channel, which is supported by the FP data shown in Fig. 7C. Thus, decreased contraction and relaxation speeds as well as ADD of hiPS-CMs in the presence of verapamil (Fig. 7D and E) can also be attributed to decreased cytoplasmic Ca^{2+} concentration associated with the Ca^{2+}-induced Ca^{2+} release mechanism. Taken together, these data suggest that the cellular deformation in the hiPS-CM monolayer shows a correspondence to the cytoplasmic Ca^{2+} status, observed with a common fluorescence indicator.

4.2. Correlation between the FP and contractile motion of the hiPS-CMs

Our simultaneous measurements of motion and FP confirmed the following correlations under non-arrhythmic conditions: 1) CRD is longer than the FPD; 2) the onset of contraction motion follows the occurrence of the Na^{+} current peak of FP; and 3) the position of the negative broad deflection in FP occurs with the contraction. We also

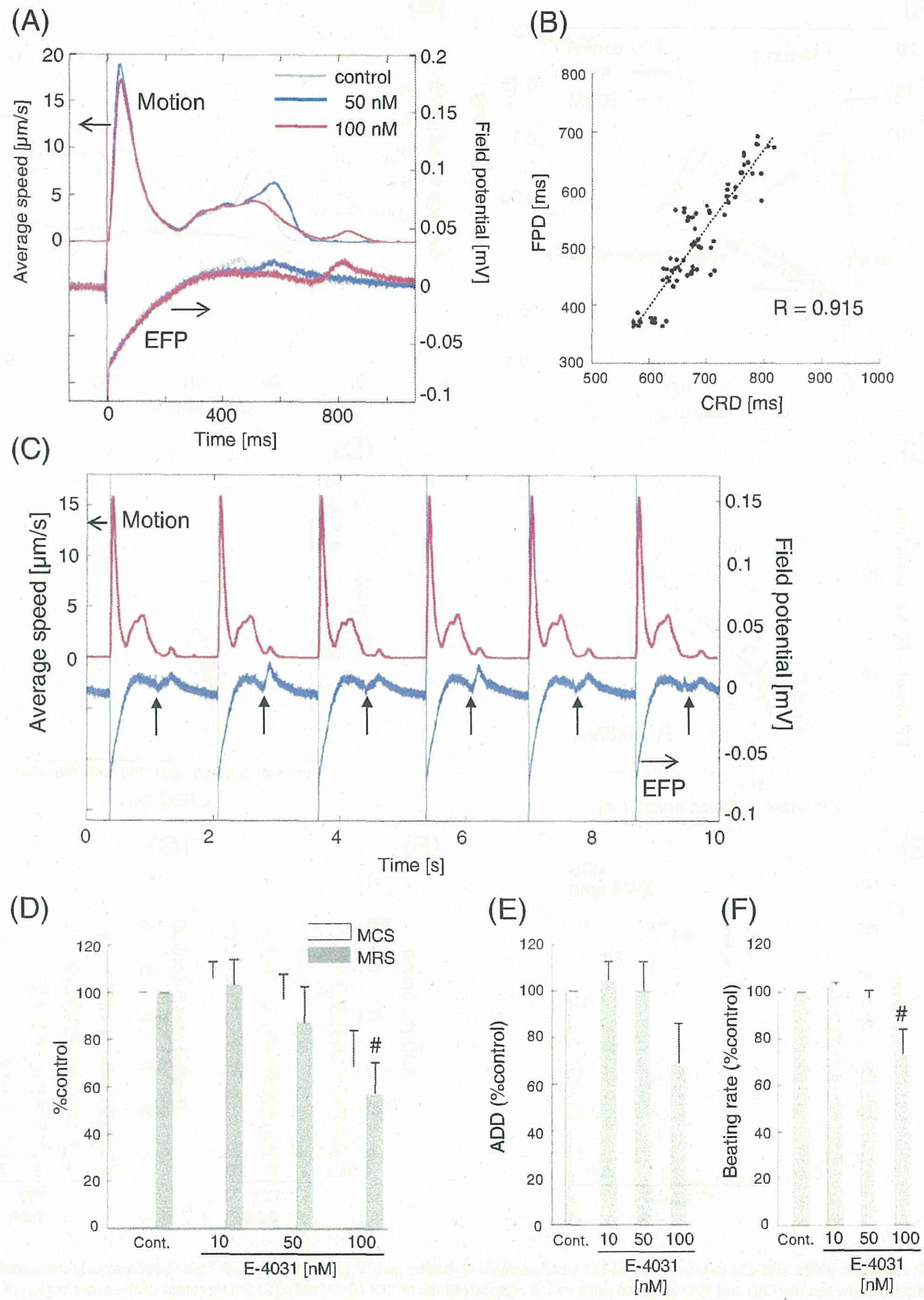

Fig. 6. Contractile and FP responses of the hiPS-CM to E-4031. (A) Example of the motion and FP profiles of the hiPS-CMs simultaneously measured in the presence of 0, 50, and 100 nM of E-4031. (B) Correlation of the CRD and FPD obtained when using varied concentrations of E-4031 (0–50 nM). Dotted line is a linear regression fitted to the data with $R = 0.915$ and the slope = 1.362 (FPD/CRD). (C) Relationship between the motion and FP in the presence of 100 nM E-4031. Arrows in (C) indicate the points of the negative deflection in the FP waveform during the relaxation process. Pauses in the relaxation motion corresponded to the negative FP deflections (see also Supplementary Movie 2). (D) Normalized change of the MCS and MRS with 0–100 nM of E-4031. (E) and (F) show the normalized change of the ADD and beating rate in accordance with the 0–100 nM E-4031 concentration changes. Data were obtained from 7 independent preparations of the hiPS-CM monolayer. In (D)–(F), values are expressed as means ± SE. $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{\#}p < 0.10$ compared with the control.

observed relationships 1) and 2), but not 3), in neonatal rat CMs. It is noteworthy that while we found the motion profile of hiPS-CMs exhibited a certain amount of displacement (velocity) at the minimum point between contraction and relaxation peak (Figs. 1 and 4), neonatal rat CMs showed almost no displacement (velocity) at the same position (Supplementary Fig. 2). While this observation for the hiPS-CMs appeared to be derived from the lack of any synchronized motion at the end of the contraction, the precise mechanism for this phenomenon remains unclear. Differences in the contraction motion between hiPS-CMs and rat CMs may reflect the presence and absence of the plateau phase of their action potential [71–73]. Alternatively, it may be relevant to the immaturity of hiPS-CM sarcoplasmic reticulum, as suggested for hES-CMs [28,29].

We performed a simultaneous measurement of motion and FP from the hiPS-CM monolayer in the presence of TTX, E-4031, verapamil and isoproterenol. The experiments revealed a linear relationship between

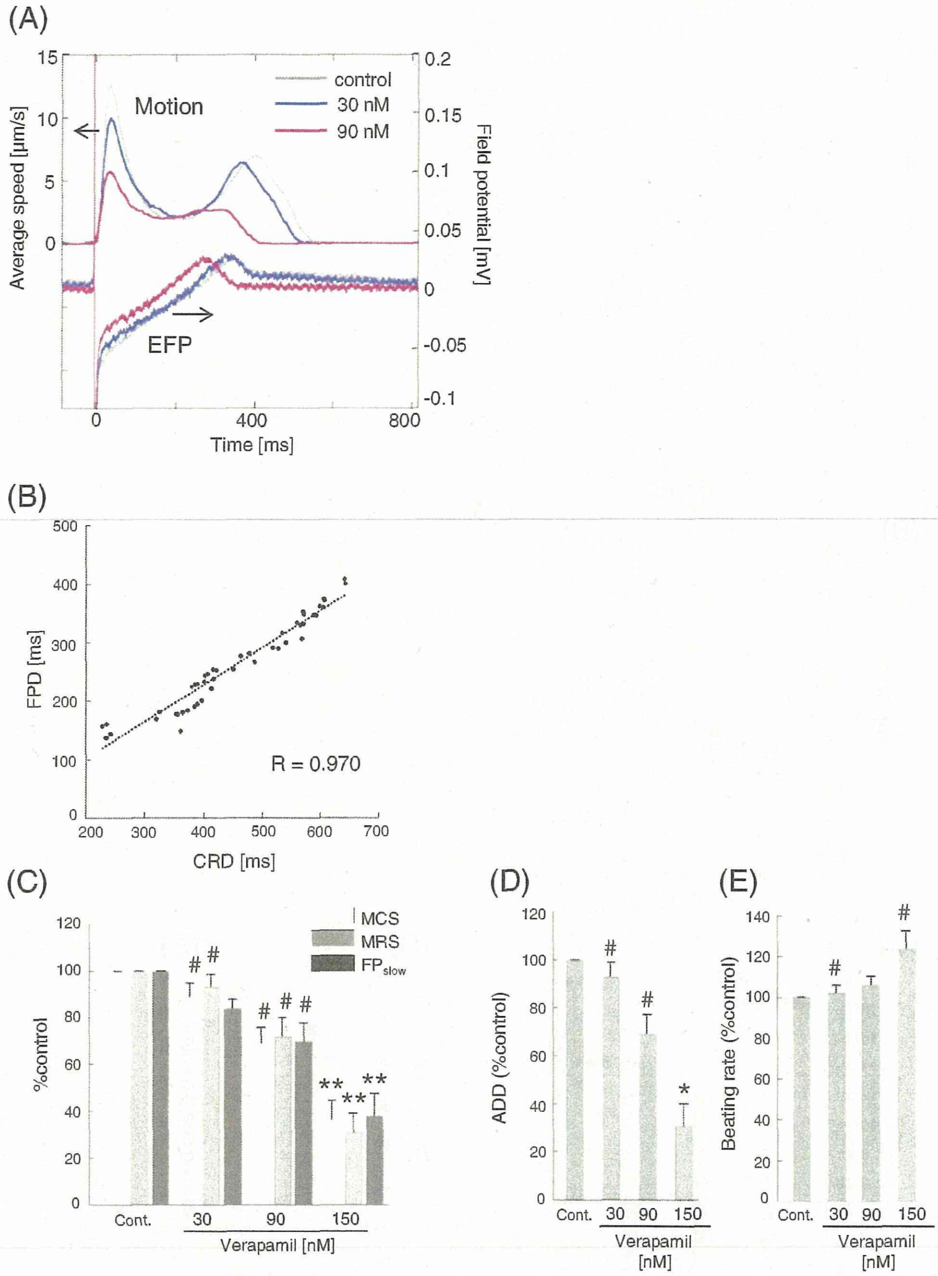

Fig. 7. Contractile and FP responses of the hiPS-CM to verapamil. (A) Example of the motion and FP profiles of the hiPS-CMs simultaneously measured in the presence of 0, 90, and 150 nM of verapamil. (B) Correlation of the CRD and FPD obtained with varied concentrations of verapamil (0–270 nM). Dotted line is a linear regression fitted to the data with R = 0.970 and the slope = 0.633 (FPD/CRD). (C) Change of the rate of the MCS, MRS and the amplitude of FP_{slow}. The amplitude of FP_{slow} was evaluated by averaging the FP value for 6 ms (between 3 ms before and 3 ms after the point of the peak of the contraction motion). (E) and (F) show the normalized change of ADD and beating rate, respectively. Data were obtained from 5 to 8 independent preparations of the hiPS-CM monolayer. In (C)–(E), values are expressed as means ± SE. $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{\#}p < 0.10$ compared with the control.

the CRD and FPD in the presence of E-4031 (10–50 nM), verapamil (30–150 nM) and isoproterenol (0.1–10 μM). Although the slope of the CRD–FPD relationship was suggested to be different in each drug, the present results suggested that the CRD can be a surrogate of the FPD in non-arrhythmic conditions. However, it should be noted that the lower time resolution of motion vector (~6 ms data interval) compared to that of FP (0.05 ms data interval) could be of concern.

Due to the blockage of I_{Kr} with E-4031, it is reasonable to assume that the relaxation speed was decreased at the point where the K^+ current occurred. With regard to the duration, even in the presence of 10–50 nM E-4031, the profile of CRD–FPD correlation appeared to be well correlated with the correlation coefficient of R = 0.915, and the slope of the linear regression was 1.362 (FPD/CRD) (Fig. 6B). This slope value appeared to be significantly larger than the case of verapamil (0.633 (FPD/CRD)) shown in Fig. 7B. This may be relevant to the abnormalities in electro-mechanical relationship reported for the Torsade de Pointes-genic drugs [74–76]. However, to determine the precise relation between FPD and CRD, it is necessary to determine FPD accurately even when the extensive broadening occurred and to consider the beating rate, which is beyond the scope of the present paper and is needed to be examined in a further study. In the presence of 50–100 nM E-4031, the EAD-like negative deflection in the FP

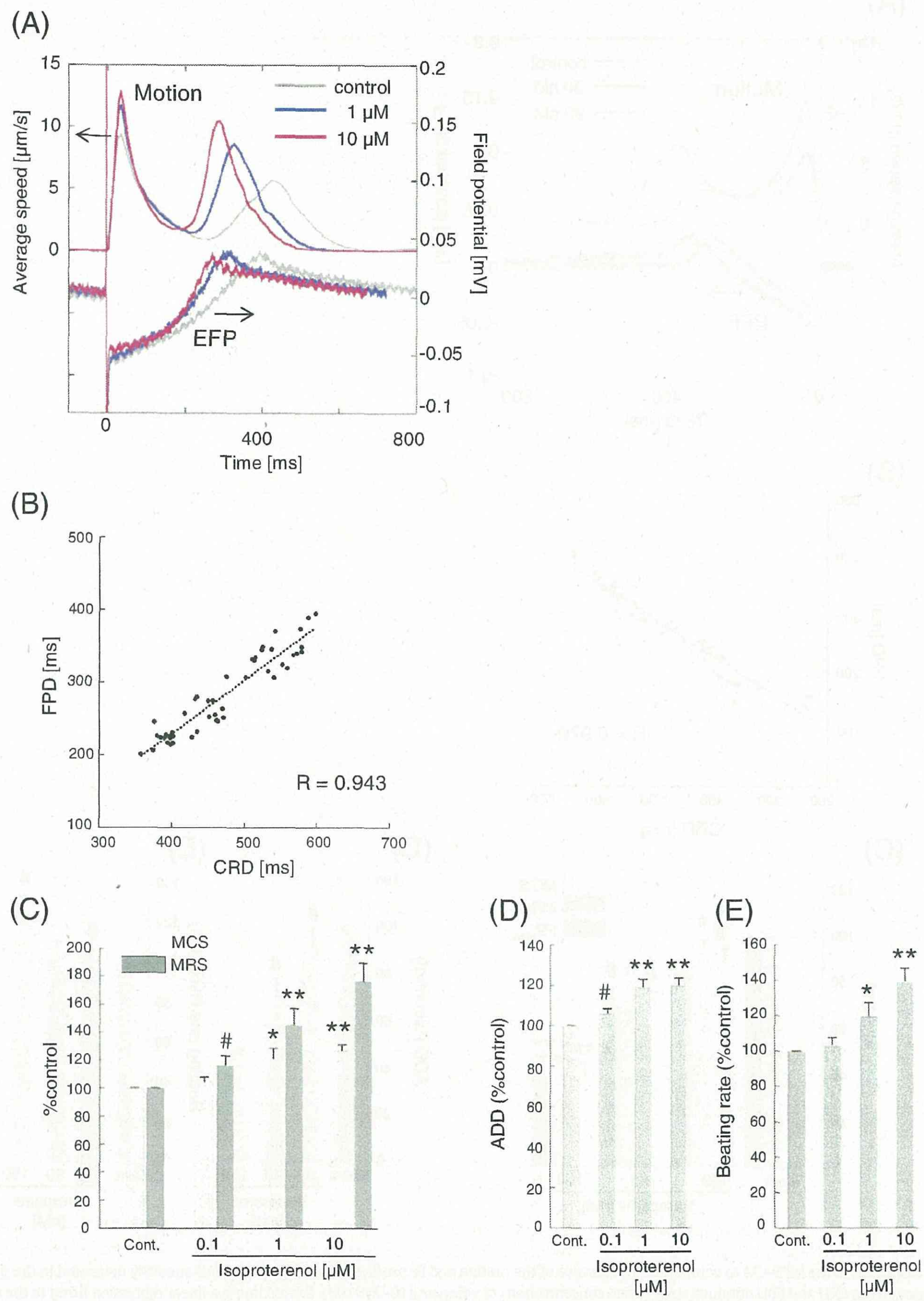

Fig. 8. Contractile and FP responses of the hiPS-CM to isoproterenol. (A) Example of the motion and FP profiles of the hiPS-CMs simultaneously measured in the presence of 0, 1, and 10 μM of isoproterenol. (B) Correlation of the CRD and FPD obtained when using various concentrations of isoproterenol (0–10 μM). Dotted line is a linear regression fitted to the data with R = 0.943 and the slope = 0.738 (FPD/CRD). (C) Normalized change of the MCS and MRS with 0–10 μM of isoproterenol. (D) and (E) show the normalized change of the ADD and beating rate, respectively. Data were evaluated from 7 independent preparations of the hiPS-CM monolayer. In (C)–(E), values are expressed as means ± SE. *$p < 0.05$; **$p < 0.01$ and #$p < 0.10$ compared with the control.

waveform was observed. EADs are caused by the re-activation of the inactivated L-type Ca^{2+} current or the inactivated voltage-dependent Na^{+} current, with the latter associated with the activation of the forward cycle of the Na^{+}/Ca^{2+} exchanger and the resultant Ca^{2+} influx. Thus, it is conceivable that the EAD is associated with the transient increase in the intracellular Ca^{2+} concentration, which leads to a reduction of the relaxation motion speed before completion of the relaxation process, thereby resulting in the appearance of another motion peak at the end of relaxation (Fig. 6C). As recognized in the video images of hiPS-CMs in the presence of 100 nM E-4031 (Supplementary video 2), however, such additional single peaks were a part of relaxation motion, not of an independent contraction–relaxation motion. After the occurrence of this type of two-step relaxation motion, triggered activity followed by arrhythmic beating were often observed (data not shown). EAD-induced contraction, or triggered activity, was also reported using a video edge-detection system for hiPS-CMs in the presence of E-4031 [77]. The occurrence of another motion peak at the end of relaxation could be a potential marker for the early detection of EAD.

Verapamil increased the beating rate of hiPS-CM (Fig. 7E). Although this effect would not be expected to occur based on verapamil's mechanism of action and previous clinical findings [78–80], verapamil has been reported to have a positive chronotropic effect on hES-CMs [54]. In accordance with the concentration of verapamil used in the current study, decreases were observed in MCS and in the amplitude of FP_{slow}. FP_{slow} was also decreased under Ca^{2+}-free condition in embryonic mouse CMs and was suggested to reflect the current of L-type Ca^{2+} channel [55]. Although FP_{slow} does not solely represent the extent of the Ca^{2+} current, their relative values (% of control) were in good agreement with those of the MCS.

Isoproterenol was also observed to increase beating rate, MCS, MRS and ADD. The increasing rate of maximum velocity was greater during relaxation (176% at 10 μM, $n = 7$) versus that during contraction (126% at 10 μM, $n = 7$) (Fig. 8C). Although the precise reason for these findings is currently unknown, Turnbull et al. described the negligible inotropic response of hES-CMs against isoproterenol and pointed out the immaturity of the sarcoplasmic reticulum of the hES-CMs [29]. Pillekampt et al. also reported that isoproterenol significantly induced positive chronotropy and lusitropy but not inotropy in early hES-CMs [28]. The mechanism underlying the hES-CMs findings in their study could be relevant to our current hiPS-CM observations. On the other hand, the FP profile showed no major alterations by the addition of isoproterenol with the exception of the shortening in FPD. Although the L-type Ca^{2+} channel is one of the targets of the isoproterenol action, alterations in FP_{slow} were not clearly detected with isoproterenol. This could be partly due to that negative deflection in FP does not solely reflect the L-type Ca^{2+} current, since the FP is an extracellular potential and not a cell membrane potential.

4.3. Variability in the contractile data

To some extent, the absolute values of MCS and MRS of hiPS-CMs depend on the monolayer region (Supplementary Fig. 3A). The reasons for this regional heterogeneity can be considered to be as follows: 1) the cell density may not be thoroughly homogeneous in the well, 2) the cell size and contractile characteristics have some variability, 3) the hiPS-CM monolayer contains a certain amount of non-cardiac (non-contracting) cells (~2%), and 4) the monolayer preparations contain a variety of shapes and types (atrial-, ventricular- and nodal-type) of hiPS-CMs. However, as long as we evaluate the contractile parameters from the same field of view in the monolayer and express the parameters using a relative value (i.e., % of the control), the inter-region variability of the contractile parameters should be fairly small (Supplementary Fig. 3B). It is possible that non-cardiac cells may have affected the contractile properties of our cultures because those cells move passively with lower motion speed than that of contracting hiPS-CMs. However, we assumed that they were present to a similar extent in all regions in the monolayer and hence should not have affected the validity of our results.

In conclusion, this study demonstrated that the contractile motion of 2D cultured hiPS-CMs, detected by a high-speed camera and motion vector analysis, quantitatively corresponded to their electrophysiological and functional behaviors under non-arrhythmic condition. Although the relationship between hiPS-CM motion and FP during excitation–contraction decoupling or proarrhythmic conditions is of great interest, it is not within the scope of this current paper and will need to be examined in a further study. The results of the present study will open up the possibilities of detecting cellular-level information on the electric and mechanical relationship of cultured CMs and will contribute to expand the applicability of hiPS-CMs in the field of cellular cardiology, drug screening and cardiac therapeutics.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2014.09.010.

Disclosure Statement

T.H., T.K., S.K., E.M. and H.Y. are employed by Sony Corporation.

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