

Regulation of f-Channels by Caveolin-3 in Early Human Heart Development

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Abstract

Rationale: Maturation of human embryonic stem cell-derived cardiomyocytes (hESC-CM) is accompanied by changes in ion channel expression, with relevant electrophysiological consequence. In rodent cardiomyocytes, the properties of HCN4, a major f-channel isoform, depends on association with caveolin-3 (Cav3). To date, no information exists on changes in HCN4-Cav3 expression and its associative relationship upon hESC-CM maturation.

Objective: To test the hypothesis that compartmentalization of Cav3 with HCN channels during hESC-CM maturation may account for progression of f-current properties toward adult phenotypes.

Methods and Results: hESC (H1 line) were differentiated into spontaneously beating CM and examined at ~30, ~60 and ~110 days of differentiation. Human adult and fetal CM served as reference. HCN and Cav3 expression and localization were analyzed by qRT-PCR and immunocytochemistry. f-current was measured in patch-clamped single cells. HCN4 and Cav3 co-localize in adult human atrial and ventricular CM, but not in fetal CM. Protein and mRNA for Cav3 were not detected in undifferentiated hESC, but expression increased during hESC-CM maturation. At 110 days, HCN4 appeared to be co-localized with Cav3. Voltage-dependent activation of the f-current was significantly more positive in fetal CM and 60-day hESC-CM (midpoint activation, V_h , ~ -82mV) than in 110-day hESC-CM or adult CM (V_h ~ -100mV). In the latter cells, caveolae disruption reversed voltage-dependence toward the immature phenotype, with V_h at -75mV.

Conclusions: Our data show, for the first time, a developmental change in HCN4-Cav3 association in hESC-CM. Cav3 expression and its association with ionic channels likely represent a crucial step of cardiac maturation.

Introduction

Human embryonic stem cell-derived cardiomyocytes (hESC-CM) represent a suitable model for studying the developmental changes in the electrophysiological properties of cardiac cells. We have previously demonstrated that the molecular and functional expression of several channels in hESC-CM change during *in vitro* differentiation and maturation over a long period of time¹. An interesting paradigm is held by the Hyperpolarization-activated Cyclic Nucleotide-gated (HCN) channels, i.e., a family of non-selective cation channels comprised of four different isoforms, HCN1-4^{2,3}. In the heart, HCN accounts for the so-called f-current (I_f) which is involved in the generation of spontaneous rhythm in sinus node cells⁴; however, an I_f-like current is constitutively present also in human adult atrial and ventricular cardiomyocytes^{5,6}, although its amplitude is much smaller, and it activates at negative (non-physiological) potentials. These differences in I_f properties (and consequently in its physiological roles) are likely due to the relative differences in the quantitative and qualitative expression of HCN isoforms, and are the result of an early divergence of pacemaker vs. non pacemaker cells occurring during cardiac morphogenesis and development⁷. Interestingly, our previous work demonstrated that the biophysical features of f-channels vary during *in vitro* maturation of hESC-CM¹. We speculated that changes in molecular attributes, such as the relative amount of HCN isoforms, and/or modulation by other membrane proteins co-assembling with the HCN channels may contribute to this phenomenon through a process resembling that which occurs during *in vivo* maturation toward the atrial or ventricular phenotype.

Recently, HCN4 - one of the prominent cardiac HCN isoforms - has been shown to co-localize with caveolin-3 in cardiomyocytes from rabbit sinoatrial node (SAN)^{8,9}. Caveolin-3 (Cav3) is a constituent of the sarcolemma, typical of cardiac and skeletal muscles, is abundantly expressed in caveolae (a subset of morphologically distinct membrane lipid rafts) where it is co-localized with several membrane proteins including ion channels¹⁰. In rabbit SAN cells, the Cav3-HCN4 association affects channel biophysical properties, especially its activation-dependence. It is unknown if such an association is present in human cardiomyocytes nor if such an association has consequences in terms of electrophysiological characteristics of the channel.

We hypothesized that changes in the relative expression and compartmentalization of Cav3 vs. HCN channels during hESC-CM maturation may account for the progression of I_f properties toward the adult atrial/ventricular phenotype. Therefore, in the present study, we have examined the expression/localization of cardiac HCN isoforms (namely, HCN4 and

HCN2) and Cav3 in undifferentiated hESC and hESC-CM at different stages, to ascertain the differences in expression of the f-channels according to developmental age and maturity; at the same time, we performed electrophysiological measurements to determine If properties. Human adult atrial and ventricular cardiomyocytes and human fetal cardiomyocytes were used as reference.

Methods

Cell Culture

All chemicals were obtained from Gibco BRL (Grand Island, NJ, USA) unless otherwise indicated. All cultures were kept in a humidified incubator at 37 °C and maintained with a 5% CO₂ atmosphere. Feeder cells were commercially available human foreskin fibroblasts (HFF) (ATCC, Manassas, VA, USA). HFF cultures were mitotically inactivated using gamma irradiation (45 Gy), and plated onto 6-well culture dishes (Becton Dickinson AG, Basel, Switzerland) at a density of 2.5x10⁴ cells/cm². Feeder cell culture medium was comprised of high glucose DMEM + Glutamax with the addition of 50U/mL penicillin and 50mg/mL streptomycin and 10% FCS.

The hESC line H1 from WiCell Research Institute (Madison, WI) was cultured as described previously¹. Briefly, cells were cultured in stem cell medium consisting of DMEM/F12 with 2mM glutamine, 50U/mL penicillin and 50mg/mL streptomycin, 1x MEM amino acids, 0.1mM β-mercaptoethanol (Sigma-Aldrich GmbH, SG, Switzerland), 20% Knock-out Serum Replacement (KSR) and 4ng/mL bFGF (R&D Systems, Minneapolis, USA) on irradiated HFF. After collagenase IV treatment for 10–15 minutes at 37°C followed by mechanical dissociation, colonies of hESC were passaged once a week at a ratio of 1:3 to 1:5 onto mitotically inactivated HFF.

Human fetal heart cells were isolated from fetal hearts obtained from aborted material (gestational weeks 9-12), upon validated informed consent procedure and prior approval by the regional ethical committee in Geneva (protocol authorization #02-088, Gyn-Ob 02-007) and the local ethical committee in Florence (protocol #6783-04, Azienda Ospedaliera Careggi, 1-02-2002). The investigation conforms to the principles outlined in the Declaration of Helsinki.

For tissue culture, the cardiac material was minced into small pieces and digested in 3–5mL a buffer solution (containing in mmol/L: NaCl 116; HEPES 20; NaH₂PO₄ 1; glucose 5.5; KCl 5.4; MgSO₄ 0.8; pH 7.35) containing collagenase (Collagenase type IV, Worthington 1mg/mL) and pancreatin (0.15mg/mL, Life Technologies), during continuous stirring for 15 minutes at 37 °C in an incubator at humidified atmosphere containing 5% CO₂. The supernatant fraction of cells was collected while the remaining tissue fragments in the

supernatant were repeatedly digested in fresh enzyme solution. The supernatant was saved every 15 minutes until all pieces were mostly dissociated. The obtained cells were washed by centrifugation at 140 g for 5 minutes, resuspended in culture medium composed of KO-DMEM with 2mM glutamine, 50U/mL penicillin and 50mg/mL streptomycin, 1x MEM amino acids, 0.1mM β -mercaptoethanol (Sigma-Aldrich GmbH, SG, Switzerland), 20% FBS (Hyclone, Utah, USA) and seeded onto gelatin-coated glass coverslips.

Embryoid Body Formation and Culture

Differentiation media 1 (DM1) was comprised of KO-DMEM with 2mM glutamine, 50U/mL penicillin and 50mg/mL streptomycin, 1x MEM amino acids, 0.1mM β -mercaptoethanol (Sigma-Aldrich GmbH, SG, Switzerland), 20% KSR. Differentiation media 2 (DM2) was comprised of KO-DMEM with 2mM glutamine, 50U/mL penicillin and 50mg/mL streptomycin, 1x MEM amino acids, 0.1mM β -mercaptoethanol (Sigma-Aldrich GmbH, SG, Switzerland), 20% FBS (Hyclone, Utah, USA). EB were prepared as previously described¹¹. Briefly, 60%–80% confluent hESC cultures at passages 40–70 were treated with collagenase type IV for 15–20 minutes at 37°C and rinsed once with PBS (Ca^{2+} and Mg^{2+} free). Colonies were scraped gently in DM1, transferred to 15mL polystyrene tubes, and allowed to sediment by gravity for 5–10 minutes. Supernatant containing most of the irradiated HFFs was discarded. Cell clumps were then transferred to Costar Ultra-Low attachment (ULA) 6-well plates (Corning, Schiphol-Rijk, the Netherlands) at a 2:1 ratio. After 2 days medium was changed with DM1, then at day 4 approximately three EB were transferred to each well of a gelatin-coated 24-well plate (Falcon, BD Biosciences, Basel, Switzerland). Medium was changed every 2-3 days with DM2. The appearance of beating areas was monitored using an inverted microscope (Eclipse TE300, Nikon, Egg, ZH, Switzerland) that was equipped with a Coolpix 995 camera (Nikon) to take phase-contrast pictures and movies.

Cell isolation from EBs and fetal ventricles used for electrophysiological recordings

Beating EBs were dissected using a microscalpel under an inverted microscope and rinsed with PBS containing 0.9mM CaCl_2 . Human fetal ventricles were minced into small pieces in PBS supplemented with 0.9mM CaCl_2 . Digestion was performed with collagenase B (Roche Applied Science) in PBS (1-2mg/mL) supplemented with 30 μ M CaCl_2 for 15–20 minutes at 37°C. Dissociated cells were plated on gelatin-coated dishes in DM2 medium and used within 2 days.

Isolation of single human atrial or ventricular myocytes

For all experiments involving human tissue, each investigation conforms to the principles outlined in the Declaration of Helsinki and had been approved by the local ethical committee

in Florence (No. 2006/0023797). Atrial cardiomyocytes were isolated from human atrial appendages routinely excised from the heart of patients undergoing coronary bypass surgery. Ventricular cells were isolated from septal tissue of patient affected by hypertrophic cardiomyopathy and undergoing septal myectomy. The specimens were transported to the laboratory in cold transport saline solution, cut into small blocks and then digested in a tissue dissociation vessel at 37°C, using collagenase type IV (Sigma) and protease type XXIV (Sigma)¹². After isolation, the cells were collected in tubes with Kraft- Bruhe solution (see *Solutions and chemicals*) for ½ hour. Cells were centrifuged at 700 rpm for 5 minutes and the pellet was resuspended in Tyrode solution (see *Solutions and chemicals*) for electrophysiological recordings or in new Kraft-Bruhe solution for immunocytochemistry.

Immunocytochemistry

Beating clusters derived from hESC and human fetal heart cells were grown on gelatin-coated coverslips. Adult atrial and ventricular cardiomyocytes were seeded on laminin-coated dishes in Kraft-Bruhe solution. Before staining, coverslips were rinsed once with PBS, fixed for 15 minutes with 4% paraformaldehyde (PFA), rinsed three times with PBS and permeabilized with 0.3% Triton X-100 in PBS for 10 minutes. Cells were then rinsed 3 times with PBS for 5 minutes and blocked with 1% bovine serum albumin (BSA) in PBS for 10 minutes. Primary antibodies at appropriate dilutions were incubated for 1 hour at 37°C in PBS containing 0.1% Tween-20 and 1% BSA. Before incubation with secondary antibodies, cells were blocked again for 10 minutes with 10% BSA in PBS. Secondary antibodies at appropriate dilutions were incubated for 1 hour at 37°C in PBS containing 0.1% Tween-20 and 1% BSA. Cells were then washed with 0.1% Tween-20 in PBS containing DAPI for 10 minutes. Finally, cells were washed two more times with 0.1% Tween 20 in PBS for 10 min, then mounted onto glass slides with Mowiol and left overnight in the dark at room temperature before observation under the microscope. Atrial and ventricular cardiomyocytes were mounted with HardSet Mounting Medium containing DAPI (Vectashield) The following primary antibodies were used: mouse monoclonal anti-Oct-4 (Santa Cruz Biotechnology); mouse monoclonal anti- α -actinin (Sigma); mouse anti-Caveolin-3 (BD Biosciences); rabbit anti-HCN-4 (Alomone); goat anti-Nkx-2.5 (Santa Cruz). The following secondary antibodies were used: goat anti-mouse Alexa 568 (Molecular Probes); goat anti-rabbit Alexa 568 (Molecular Probes); rabbit anti-goat IgG-FITC (Sigma); goat anti-mouse Alexa Fluor 594 (Invitrogen) and goat anti-rat Alexa Fluor 488 (Invitrogen) for atrial cells; anti-mouse Fi2000 490 (Vector Laboratories) and goat anti-rat Alexa Fluor 594 (Invitrogen) for ventricular cells. Images were visualized with a Zeiss axiophot microscope (Carl Zeiss, Oberkochen, Germany) equipped with an Axiocam camera (Carl Zeiss); confocal images were obtained using the LSM 510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

For atrial and ventricular cells images were obtained with a Leica TCSSP5 and a Leica SP2-AOBS confocal microscope. 3D images were created using the image analysis program, Imaris.

Immunohistochemistry

Beating clusters derived from hESC and human fetal heart (~12 weeks) were embedded in paraffin and sectioned into 3µM slices and placed on glass slides, as reported elsewhere. Slices were deparaffinised then rehydrated and unmasking of the epitopes was performed using a pressure cooker in citrate buffer. After blocking with 10% BSA in PBS for 1 hour, primary antibodies at appropriate dilutions were incubated for 1 hour at room temperature, then stained as per the Dako EnVision™ Detection Systems Peroxidase/DAB Kit (see manufacturer's instructions).

RNA extraction, RT-PCR and real-time PCR (qPCR)

Total RNA was extracted from cells with TRIZOL reagent (Invitrogen AG, Basel, Switzerland) according to the manufacturer's instructions. 1µg of total RNA was converted to cDNA with M-MLV Reverse Transcriptase (Invitrogen) using random primers in a 20µL reaction incubated at 25°C for 10 minutes followed by 50 minutes at 37°C and 15 minutes at 70°C. Volume was adjusted to 50µL. RT-PCR was performed with 1µL of cDNA in a total volume of 25µL. The amplification program included an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing for 30 seconds at the appropriate temperature according to primer properties (Table 1) and extension for 1 minute at 72°C. A final extension step at 72°C for 7 minutes was performed. Results of reactions were visualized by conventional 2% agarose gel electrophoresis. qPCR was performed using SYBR green with the ABI Prism 7900 HT detection system (Applied Biosystems, Foster City, CA). Primers sequences used were as follows:

GusB - F: CCACCAGGGACCATCCAAT; R: AGTCAAATATGTGTTCTGGACAAAGTAA

HCN1 - F: TCATTCAACACGGTGTGCTG; R: AGCAGGCAAATCTCTCCAAAGT

HCN2 - F: TCAACGAGGTGCTGGAGGA; R: CCTTGTGCAGGAGGATGGAA

HCN4 - F: GACTGCTGGGTGTCCATCAA; R: AGAGCGCGTAGGAGTACTGCTT

Cav3 - F: GCCCAGATCGTCAAGGAT; R: AGCAGCGTGGACAACAGA

Patch-clamp recordings

The experimental set-up for patch-clamp recordings and data acquisition was similar to that described previously^{1,13}. Isolated cells were superfused by means of a temperature controlled (37°C) micro-superfusor, allowing rapid changes of the solution. Patch-clamp pipettes, prepared from glass capillary tubes (Harvard Apparatus Ltd, Kent, U.K.,

<http://www.harvardapparatus.com>) by means of a two-stage horizontal puller (P-87; Sutter Instrument, Novato, CA) had a resistance of 2–3M Ω when filled with the internal solution (see *Solutions and chemicals*). Series resistance (R_s) and membrane capacitance (C_m) were compensated to minimize the capacitive transient and routinely checked during the experiment. Only cells showing a stable C_m and R_s were included in the analysis. I_f current was measured in the whole-cell configuration by using appropriate external and pipette solutions (see *Solutions and chemicals*). From a holding potential of -40mV, I_f was elicited by depolarizing steps to -60/-140mV and current amplitude was measured as difference between the peak inward current at the beginning of the step and the steady-state current at the end of the step.

Specific conductance was determined following the equation:

$$G_f = I / V_m - V_{rev}$$

where G_f (pS pF⁻¹) is the conductance calculated at the membrane potential V_m (mV), I (pA pF⁻¹) the current density and V_{rev} (mV), the reversal potential. I_f specific conductance was normalized with respect to maximal conductance and values, expressed as mean \pm SEM, were plotted versus membrane potential. Activation data were fitted by a Boltzmann function expressed by the equation:

$$Y = 1 / 1 + \exp((V_m - V_h)/k)$$

where V_m (mV) is the membrane test potential, V_h (mV) the fitted potential for half-maximal activation and k (mV) the slope factor of the activation curve.

Solutions and chemicals

Kraft-Bruhe solution (mM): glucose 20, creatine 5, taurine 5, EGTA 0.5, succinic acid 5, K₂-ATP 2, pyruvic acid 5, β -hydroxy-butyric acid 5, KCl 85, K₂HPO₄ 3H₂O 30, MgSO₄ 7H₂O 5, pH 7,1 with KOH.

Normal Tyrode solution (mM): NaCl 140; KCl 5.4; CaCl₂ 1.8; MgCl₂ 1.2; D-glucose 10; Hepes 5, pH 7.35 with NaOH.

Modified Tyrode solution for I_f current was obtained from normal Tyrode solution supplemented with (mM): MnCl₂ 2, BaCl₂ 2, 4-aminopyridine 0.5 to eliminate Ca²⁺ current (T- and L-type), inward rectifier K⁺ current, I_{K1} and transient outward K⁺ current, I_{to} , respectively. KCl was increased to 25 mM to amplify I_f

Pipette solution (mM): K⁺ aspartate 130; Na₂GTP 0.1; Na₂ATP 5; MgCl₂ 2; EGTA 11; CaCl₂ 5 (pCa 6.9); Hepes 10, pH 7.2 with KOH.

To deplete cholesterol, freshly isolated human atrial cardiomyocytes were resuspended in Tyrode solution containing 200 μ M CaCl₂ and incubated with 2mM methyl- β -cyclodextrin (Sigma) for 90 minutes^{14,15}.

Results

HCN4 and Cav3 co-localize in human adult ventricular and atrial cardiomyocytes but not in human fetal cardiomyocytes

As shown in Figure 1(A, B), HCN4 and Cav3 appear to be expressed across the whole cell surface membrane of human adult atrial and ventricular cardiomyocytes (hAA- and hAV-CM); co-localization can be inferred by the merging of the green and red channels in the same focal plane as shown by the yellow marking (right panels of A and B). HCN4 and Cav3 expression patterns in human fetal heart cells (hF-CM, approximately 12 weeks of age), was characterized by a spotted appearance across the cell membrane of both HCN4 and Cav3 (Fig.1C; n=5 independent experiments). Simultaneous expression of Cav3 and HCN4 in the same cells was inconsistent and co-localization was not observed in >99% of the cells examined, either when studied in individual cells (Fig. 1C), or in CM clusters (not shown).

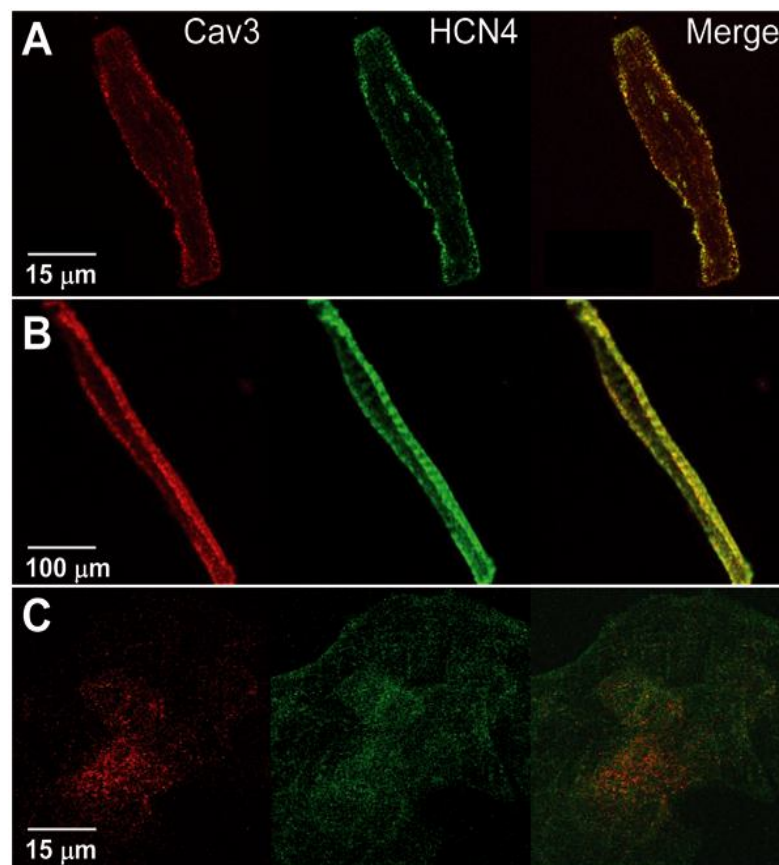


Figure 1. Expression of HCN4 and Cav3 in human adult and fetal cardiomyocytes. Human adult ventricular [A] and atrial [B] cardiomyocytes expressing HCN4 (green) and Cav3 (red). Co-localization is observed upon merging (yellow). [C] Human fetal cardiomyocytes expressing HCN4 (green) and Cav3 (red). No co-localization is observed upon merging.

Immunocytochemical (ICC) analysis of hESC, hESC-CM and HCN4-Cav3 co-localization

As expected from the previous demonstration of their functional presence¹, expression of HCN4 protein was clearly detected in undifferentiated hESC; expression is restricted to the cell surface membrane and characterized by a low density pattern of expression with a spotted appearance across the membrane of the whole cell (Fig.2A). Expression of HCN4 in hESC was further examined using 3D image reconstruction, clearly showing that the channels were localized only in the cell surface (Fig.2B). Cav3 was absent in undifferentiated hESC.

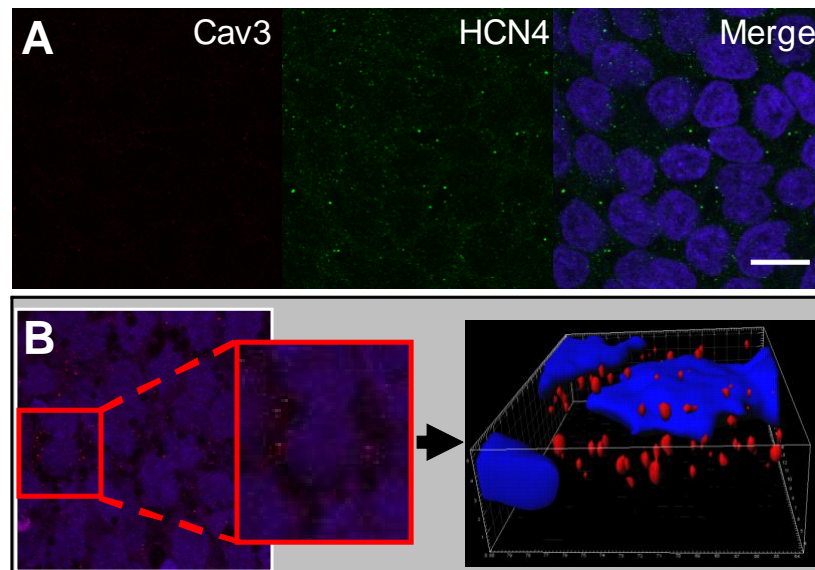


Figure 2. Expression of HCN4 and Cav3 in undifferentiated hESC. [A] Undifferentiated hESC expressing HCN4 (green) but not Cav3. [B] 3D reconstruction of a stack of confocal images showing HCN4 expression (red) in a single undifferentiated hES cell, showing clearly the cell surface (sarcolemmal) localization of the ion channel. Scale bar = 10 μ m.

Since both HCN4 and Cav3 both appear to be expressed and co-localized across the whole cell sarcolemma only in hAA- and hAV-CM (Figure 1A, B), but not in undifferentiated hESC nor hF-CM, we reasoned that Cav3 expression and co-localization with f-channels should occur at a certain point during maturation. Thus, we sought the time-course of this process in hESC-CM at different stages of maturation. To examine the expression and localization of HCN4 and Cav3 at the single cardiomyocyte level, whole beating hESC-CM clusters were fixed and stained immunocytochemically. At d30, HCN4 had a low level of expression with a spotted appearance in hESC-CM (Fig.3A, white arrow), similar to what was observed in undifferentiated hESC (Fig.2A); although approximately 20% of the cells exhibited a higher density of expression, as can be seen in Fig3A, yellow arrow. Low expression of Cav3 was seen in approximately 20% of the cells of the cluster, localized also at the cell surface membrane (n=3 independent experiments). HCN4 and Cav3 were

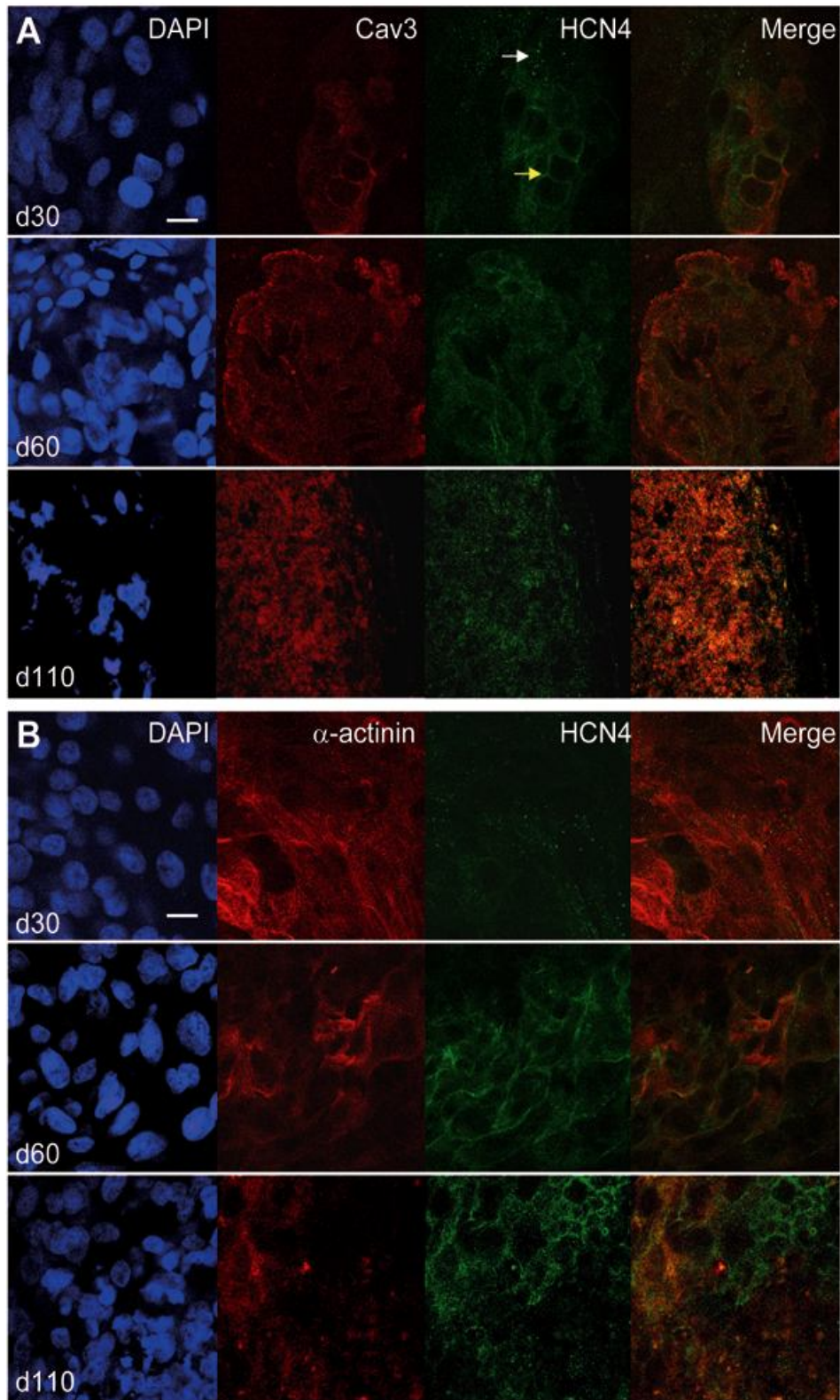
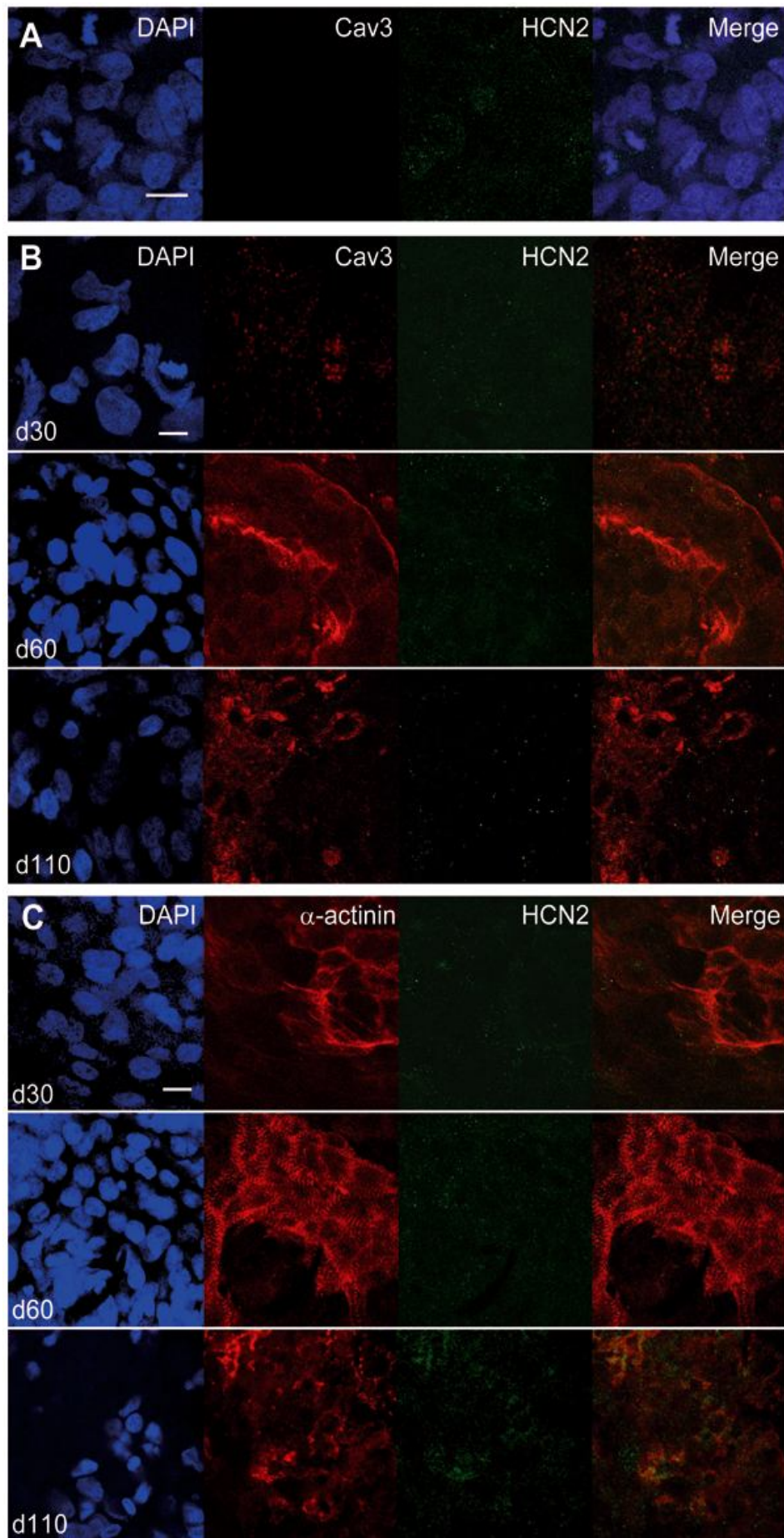


Figure 3. Expression of Cav3, HCN4 and α -actinin in d30, d60 and d110 hESC-CM. [A] Cav3 and HCN4 double staining (d30: white arrow = low density; yellow arrow = high density) [B] α -actinin and HCN4 double staining to verify expression of HCN4 in cardiac specific cells. Scale bar = 10 μ m.

Figure 4.(Following page) Expression of Cav3 and HCN2 in undifferentiated hESC and Cav3, HCN2 and α -actinin in d30, d60 and d110 hESC-CM. [A] Undifferentiated hESC expressing HCN2 (green) but not Cav3 [B] Cav3 and HCN2 double staining in hESC-CM, [C] α -actinin and HCN4 double staining in hESC-CM. Scale bar = 10 μ m.



expressed in the same cells (Fig.3A), but co-localization was absent, as suggested by the lack of yellow upon merging. A similar staining pattern was seen in hESC-CM at d60; however Cav3 was expressed in approximately 50% of cells and density was greater (n=3 independent experiments). Again, HCN4 and Cav3 were observed to be expressed in the same cells. Finally and more interestingly, HCN4 and Cav3 were not only expressed in the same cells, but also co-localized in hESC-CM at d110; co-localization was never detected at earlier stages of maturation. A similar pattern was observed in n=3 independent experiments. Expression of α -actinin was concurrently examined in HCN4-positive cells to confirm the cardiomyocytic nature of these cells (Fig.3B).

As the status of expression of HCN2 is not known in hESC and hESC-CM, we examined the expression of HCN2 in undifferentiated hESC and in hESC-CM. During maturation a consistent “spotted”, low density expression pattern was detected for HCN2 (Fig.4B), which did not change from the undifferentiated hESC (Fig.4A) thus ruling out a developmental interplay between HCN2 and Cav3.

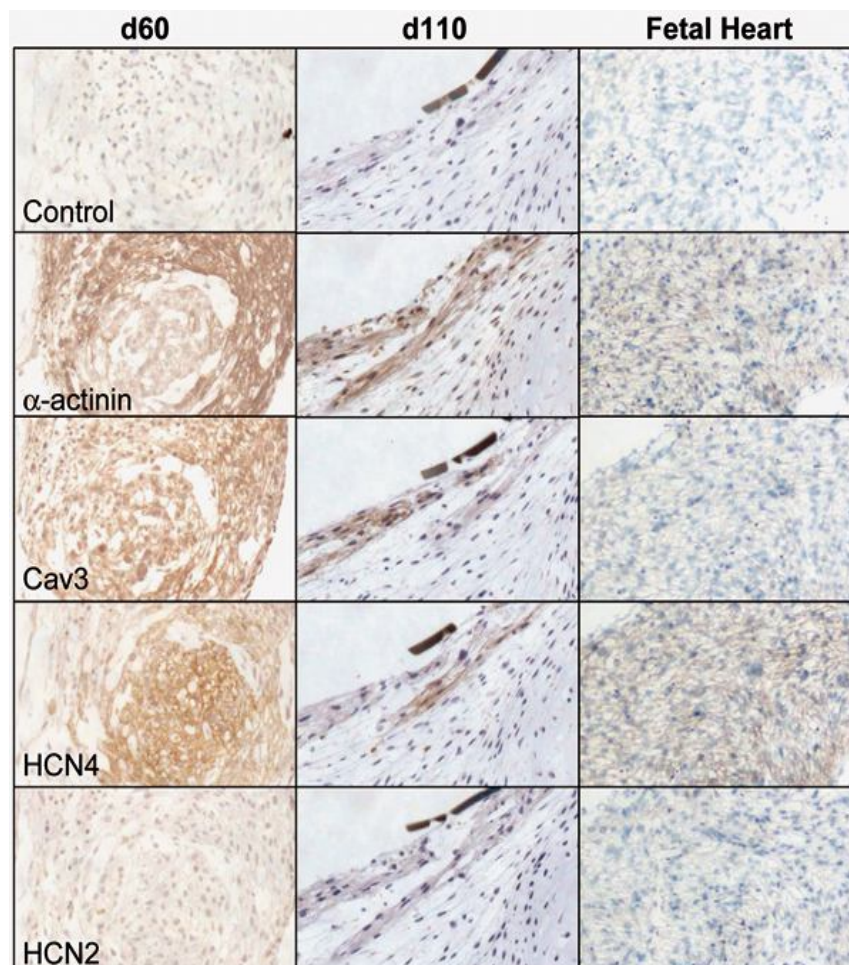


Figure 5. Immunohistochemical (IHC) staining of hESC-CM clusters and whole fetal heart. IHC staining of hESC-CM at d60 and d110, and in human fetal heart (~12 weeks) with HCN4, HCN2, Cav3 and α -actinin.

Immunohistochemical (IHC) analysis of HCN4-Cav3 expression in hESC-CM and fetal heart

To preserve tissue structural integrity and spatial relationship among cells, immunohistochemical staining of HCN4, HCN2 and Cav3, as well as α -actinin was performed at d60 and d110. α -Actinin was expressed in beating clusters at both d60 and d110, albeit at varying expression levels within the beating clusters, as clearly seen in Figure 5. Cav3 was also expressed at d60 and d110 as expected, but with an even expression through the cardiomyocyte portions of the clusters. Similarly, we could detect differential HCN4 expression within a single beating cluster, with some areas appearing to be more highly enriched than others, as clearly shown in Figure 5 (d60). Lastly, when examining HCN2 expression, we observed stable expression throughout the different areas of the beating (cardiac) cluster. Expression was compared to that found in fetal heart slices, where a clear-cut expression of each protein examined was observed.

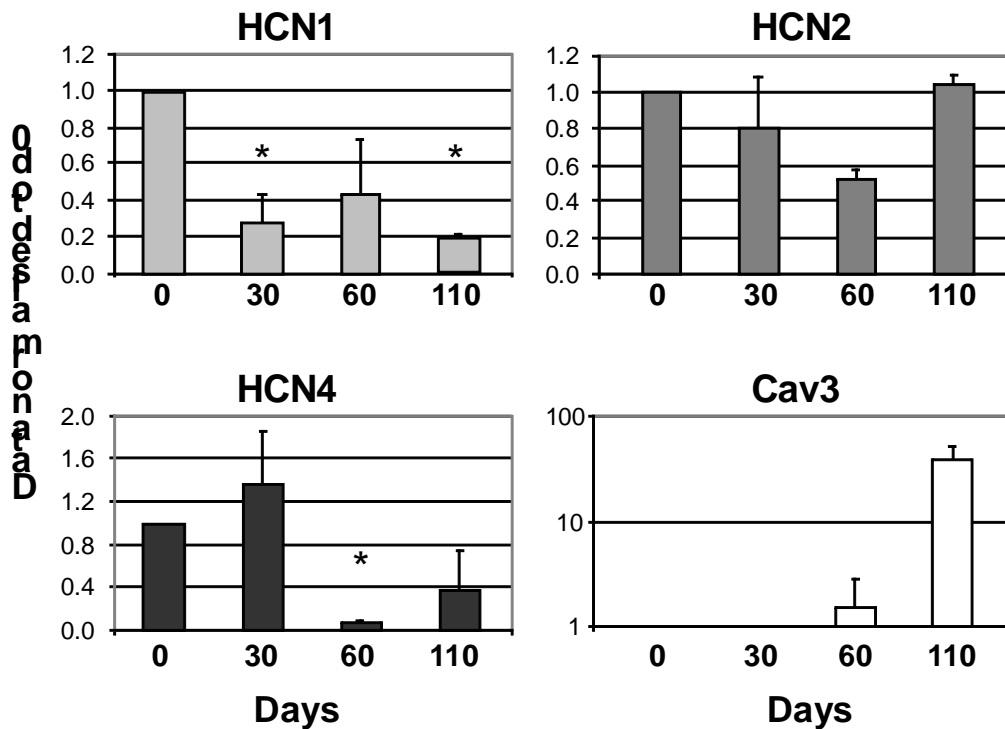


Figure 6. Transcriptional regulation of HCN isoforms and Cav3 in undifferentiated hESC and hESC-CM qRT-PCR data for the expression of different HCN isoforms and Cav3 in hESC-CM over time (* $p < 0.05$).

Transcriptional regulation of HCN isoforms and Cav3 in undifferentiated hESC and hESC-CM

As protein expression is regulated by different mechanisms in the cell, we assessed whether HCN isoforms and Cav3 are regulated at transcriptional level during maturation. We have shown previously¹ that hESC and hESC-CM express mRNA of different HCN isoforms, however, the level of expression of each HCN isoform over extended time periods had not been examined in detail. Thus, we manually isolated beating clusters of hESC-CM from

hESC-derived embryoid bodies (EB), at three time points (d30, d60, d110) and measured the mRNA expression of different HCN isoforms in these cells. We observed that HCN4 mRNA was highly expressed in undifferentiated hESC cells and in early CM (d30), but decreased over time (d60 and d110) (Fig.6). Beside HCN4 expression, HCN1 and HCN2 were also expressed in undifferentiated hESC (d0) cells; HCN1 decreased over time upon maturation, whereas HCN2 expression remained relatively constant. Cav3 on the other hand, was not expressed in undifferentiated hESC (d0); it was clearly expressed at d60 and dramatically increased at d110. Overall, mRNA expression of different HCN isoforms and Cav3 in these cells mirrors that of proteins, suggesting major transcriptional regulation.

Functional consequences of developmental changes in HCN4/Cav3 expression and localization

Figure 7 (A-E) shows typical I_f recordings obtained from hESC-CM at d60 and d110, hF, hAA- and hAV-CM. Currents exhibited different activation kinetics likely reflecting diverse isoform distribution, as previously reported by our and others' laboratories^{1,16}. When examining the voltage dependence (Fig.7F), the activation voltages for hAA-CM and hAV-CM were more negative (-101.5 ± 4.1 mV, $n=3$ and -102.9 ± 0.3 mV, $n=6$ respectively) with respect to hF-CM (-82.5 ± 2 mV, $n=9$). Interestingly enough, a similar difference of I_f voltage dependence was detected between hESC-CM at d100 (-96.8 ± 5.9 mV, $n=6$) and hESC-CM at d60 (-83.2 ± 6.9 mV, $n=8$). This modification has a functional consequence at physiological resting potentials, (e.g. -90 mV, for ventricular cells). In fact I_f fractional activation differs significantly in hESC-CM at d100 (0.30 ± 0.14 , $n=6$) with respect to hESC-CM at d60 (0.65 ± 0.12 , $n=8$) ($p < 0.05$), thus suggesting that I_f contribution to the diastolic phase decreases during maturation, to values similar to those observed in hAA-CM (0.34 ± 0.07 , $n=3$) and hAV-CM (0.26 ± 0.04 , $n=6$).

Caveolae disruption changes the properties of I_f current expressed in human adult atrial cells

Previous data suggest that HCN4-Cav3 co-localization modulates voltage-dependent activation in rodent sinoatrial node cells⁸. We reasoned that, if a similar Cav3 function also holds true for human cardiomyocytes, disruption of caveolar localization of the f-channel should have resulted in regression of voltage-dependence of I_f toward the immature phenotype. Therefore, we disrupted the compartment by depletion of membrane cholesterol with methyl- β -cyclodextrin (M β CD). Figure 7F shows I_f activation curves detected from hAA-CM incubated with or without M β CD, which resulted in a positive shift of I_f V_h (from -101.5 ± 4.1 to -75.9 ± 2.7 mV, $n=3$, $p < 0.001$), that is, toward values measured in immature cardiomyocytes such as hESC-CM at d60 and hF-CM.

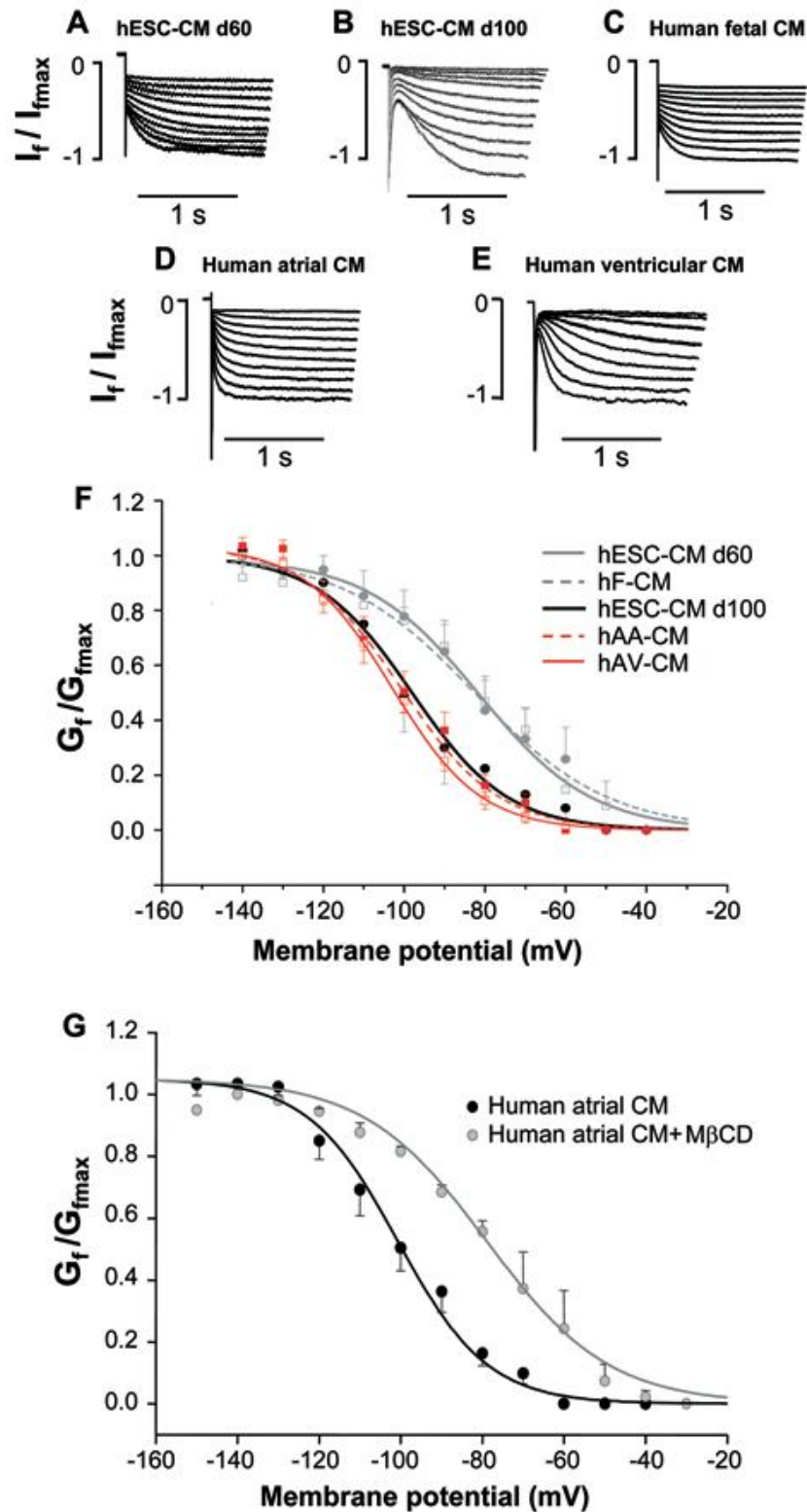


Figure 7. Electrophysiological recordings of cardiomyocytes from different cell sources. [A-E] Typical examples of I_f current traces recorded by single cell patch-clamp technique in hESC-CM at 60 and 100 days, human fetal cardiomyocytes (hF-CM) and human adult atrial and ventricular cardiomyocytes (hAA- and hAV-CM). [F] Activation curves of I_f current calculated for hESC-CM at 60 and 100 days, hF-CM and hAA- and hAV-CM. Plot reports I_f current conductance (G_f) normalized with respect to I_f maximal conductance (G_{fmax}) versus tested membrane potential (mV, * $p < 0.05$). Activation curves are obtained by fitting conductance values with a Boltzman function. [G] Effects of membrane cholesterol depletion on the activation properties of f-channels in hAA-CM using 2mM of methyl- β -cyclodextrin (M β CD, $n=3$).

Discussion

In the present study, we have investigated the expression of the different HCN isoforms and Cav3 in cardiomyocytes derived from human adult atrial and ventricular heart, human fetal heart, as well as in hESC-CM at different stages of maturation. Here we demonstrate that the presence, and more specifically the co-localization, of Cav3 in cells highly expressing HCN4 represents a crucial step of cardiac maturation, which modifies channel activation properties. Due to the role of I_f , i.e. the HCN-mediated ion current, on diastolic membrane potential, developmental changes in channel compartmentalization and association to Cav3 may have functional relevance on basal cell physiology and contribute to the progression toward the adult cardiac phenotype.

Membranous lipid rafts enriched in caveolae are known to influence the function of many different cardiac ion channels, which has been well reviewed in ¹⁷. It has been previously shown that HCN4 associates with Cav3 in mouse and rabbit sinoatrial node cells^{8,18}, where the association regulates channel activation properties and responsiveness to adrenergic modulation⁹. That HCN4-Cav3 association also occurs for human proteins has been previously inferred on the basis of co-immunoprecipitation in human HEK293 cells stably expressing HCN4 channels and Cav3¹⁹. Here, we directly demonstrate that indeed HCN4 and Cav3 are present and co-localize in native human adult, but not fetal, cardiomyocytes, with a cell surface membrane pattern of expression (Fig.1). These different patterns in fetal vs. adult myocytes suggest that acquisition of a precise sarcolemmal organization of ion channels occurs in a development-dependent fashion: indeed, in undifferentiated hESC, HCN4 - but not Cav3 – is present and functionally active. We have found that in hESC-CM, HCN4-Cav3 association is dependent on the stage of cardiomyocyte maturation (Figs.1, 3 and 5), a clear co-localization being consistently present only in late hESC-CM (d110) but not before. We suggest that this developmental event may represent a crucial step in cardiac maturation leading to caveolar localization of HCN4 channel, similar to that present in native human atrial and ventricular cells.

Functional consequences of this phenomenon have been assessed in human adult atrial cardiomyocytes and evidenced a positive shift of voltage-dependence of HCN-mediated current, I_f , upon caveolar disruption, in accordance with data obtained from rabbit sinoatrial node cells^{8,9}. On the other hand, midpoint activation voltage of I_f lies around -95 mV in adult atrial and ventricular cardiomyocytes (when caveolae are intact) and in late hESC-CM (d100), where, immunocytochemical and histochemical analysis reveals high Cav3 expression and co-localization with HCN4 compared to d60 hESC-CM. At earlier stages (d60

hESC-CM) and in fetal cardiomyocytes, the lack of HCN4-Cav3 co-localization and a more positive activation voltage of I_f go *hand-in-hand*. Altogether, these data support the hypothesis that during maturation, HCN4 - a major isoform coding for f-channels in the human heart- is recruited into caveolar compartments and interact with Cav3 protein. By contrast, HCN2 maintains the similar and constant expression found in undifferentiated cells, with no co-localization with Cav3 protein upon maturation.

At variance with what is known about native sinoatrial node cells^{8,9,18}, the functional significance of HCN4-Cav3 co-localization in hESC-CM in terms of modulation exerted by autonomic stimuli is unknown and remains to be established. However, it is worth noting that beta-adrenergic stimulation positively modulates I_f activation only in late but not in early hESC-CM¹, suggesting that channel responsiveness to adrenergic signalling is acquired when channel is localized into the caveolar compartment. Similarly to adult cardiac myocytes²⁰, we hypothesize that in late hESC-CM, adrenergic receptors and HCN4 channels likely cluster in close proximity, such as in the caveolar compartment, thereby allowing an effective functional coupling.

Cardiomyocytes derived from hESC are known to spontaneously start beating in culture upon differentiation through embryoid body formation. Within these cell clusters there is a mixed population of cells which individually show either an immature action potential or a more mature action potential, resembling nodal, ventricular or atrial cardiomyocytes^{13,21}. The relative abundance of these cell populations is known to change and mature over time; along this period, the differential expression of ion channels is fundamentally responsible for the modifications of action potential profile¹. Within the native myocardium, the presence, location and distribution of ion channels in cardiomyocytes differentiate the types of cells and determine both the specific shape of the action potential^{22,23} and its function within the myocardium. In particular, I_f current is associated with the diastolic depolarization phase of the action potential typically present in the pacemaker cells, but also in human adult atrial and ventricular cardiomyocytes^{5,6}. Due to this characteristic, it is of particular significance that the association of HCN4 with Cav3 in human atrial cells shifts the activation properties of the channel (Fig.7), which thereby became less relevant at physiological atrial potentials. In this line, the hypothesis of a progressive clustering of HCN4 channels into caveolae and the interaction with Cav3 protein during maturation of hESC-CM fully agrees with our previous data demonstrating that the frequency of spontaneous action potential and the rate of diastolic depolarization phase slow-down during hESC-CM maturation¹.

Besides the widely known function of I_f current as a major component of the spontaneous diastolic depolarization phase in sinoatrial node cells and its role in cardiac primary and secondary pacemaker centres^{2,4}, I_f is detectable in spontaneously active cells, such as fetal and neonatal ventricular myocytes of different mammals^{24,25}. Our data provide evidence that I_f current is functionally expressed in native human fetal cardiomyocytes, supporting the concept that I_f current is a conserved marker of fetal cardiac phenotype. Over time, the majority of cells lose or lessen, depending on species, their ability to generate spontaneous action potentials. This evidence fully agrees with our observation of a decreased HCN4 expression over time, or upon maturation, as seen in the hESC-CM (Fig.6). In the beating clusters we hypothesize that those cells which maintain high levels of HCN4 at later time points are likely to have a “pacemaker-like” phenotype and to become a “pacemaking centre” (Fig.3).

The functional relevance of HCN channels in undifferentiated hESC, where I_f current is active¹, is currently unknown. However, further research is needed to understand the possible significance of this channel in early human development. Interestingly, a recent study²⁶ suggests a role of HCN channel on proliferation and cell cycle progression of mouse embryonic stem cells. Whether a similar function takes place in human embryonic stem cells is an intriguing speculation that deserves further investigation.

The present study may have possible implications for cardiac pathology, such as hypertrophy and failure. Indeed, it is well established that several modifications occur at functional and molecular levels in cardiac myocytes, a phenomenon known as cardiac cellular remodelling that may underlie the increased propensity of cardiac tissue to develop arrhythmias. Mounting data demonstrate that cardiac remodelling originates from a fetal pattern of genes which are re-expressed during the diseased state. I_f current gain-of-function joined to HCN over-expression are integral part of this pattern, as assessed in animal and human diseased ventricular myocytes^{5,6}. In this context I_f current has been proposed as a mechanism contributing to the increased propensity to develop arrhythmias²⁷. The regression toward a fetal phenotype in this disease state opens the question of whether a disruption of HCN4-Cav3 protein interaction takes place in this context. Indeed, a positive shift of I_f activation curve has been observed in cardiomyocytes from diseased human hearts²⁸. Finally, perturbations in the expression and/or function of HCN4 in humans have been shown to elicit arrhythmias and chronotropic disruptions²⁹⁻³², indicating the importance of the correct expression and function of this ion channel in the heart

In conclusion, our work shows for the first time that caveolin-3 expression, a crucial component of cardiac sarcolemma, increases during development *in vitro* in hESC-CM. At the same time, co-localization with HCN4, whose functional and molecular expression is detected in hESC before commitment and differentiation toward the cardiac phenotype, contributes to attainment of the adult biophysical properties of the HCN-mediated current, I_f .

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