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CXCR4 and CXCR7 play distinct roles in cardiac lineage specification and pharmacologic β-adrenergic response

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Abstract

CXCR4 and CXCR7 are prominent G protein-coupled receptors (GPCRs) for chemokine stromal cell-derived factor-1 (SDF-1/CXCL12). This study demonstrates that CXCR4 and CXCR7 induce differential effects during cardiac lineage differentiation and β-adrenergic response in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). Using lentiviral vectors to ablate CXCR4 and/or CXCR7 expression, hiPSC-CMs were tested for phenotypic and functional properties due to gene knockdown. Gene expression and flow cytometry confirmed the pluripotent and cardiomyocyte phenotype of undifferentiated and differentiated hiPSCs, respectively. Although reduction of CXCR4 and CXCR7 expression resulted in a delayed cardiac phenotype, only knockdown of CXCR4 delayed the spontaneous beating of hiPSC-CMs. Knockdown of CXCR4 and CXCR7 differentially altered calcium transients and β-adrenergic response in hiPSC-CMs. In engineered cardiac tissues, depletion of CXCR4 or CXCR7 had opposing effects on developed force and chronotropic response to β-agonists. This work demonstrates distinct roles for

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the SDF-1/CXCR4 or CXCR7 network in hiPSC-derived ventricular cardiomyocyte specification, maturation and function.

Keywords

CXCR4; CXCR7; Cardiogenesis; hiPSC; Engineered tissue

1. Introduction

Pluripotent stem cells hold great promise in the field of regenerative medicine because of their ability to grow indefinitely and give rise to all cells of the body (Takahashi and Yamanaka, 2006). Both embryonic and induced pluripotent stem cells (ESCs and iPSCs, respectively) have been invaluable tools in the investigation of in vitro disease modeling, drug testing, and in vivo cell replacement therapies, as human primary cells are nearly impossible to obtain and survivability in culture is low. Human iPSCs (hiPSCs) have now been generated from several human tissues using a variety of approaches (Takahashi and Yamanaka, 2006; Karakikes et al., 2014). Clinicians are exploring the use of stem cell therapy for many diseases, including neurodegenerative disease, diabetes, rheumatologies and hematological disease (Karussis et al., 2010; Matsumoto, 2010; Persons, 2010; Szodoray et al., 2010; Trounson et al., 2011), but since stem cells run the risk of forming teratomas in vivo, progenitor cells are being explored as a more realistic therapy option (Le and Chong, 2016). Recently, the stromal cell-derived factor-1 (SDF-1)/C-X-C chemokine receptor type 4 (CXCR4) axis has been proposed as a potential therapeutic target for ischemic heart disease. This led to the recent clinical trial (JVS-100) in which mobilizing heart failure patients' own stem cells was considered using SDF-1 gene therapy as a homing signal (Penn et al., 2013). SDF-1/CXCR4 signaling is important to a variety of fundamental processes, including organogenesis, embryogenesis, cell mobilization, and trafficking (Busillo and Benovic, 2007). Recent work has demonstrated that SDF-1 also binds to the chemokine receptor CXCR7 (SDF-1 alternative receptor) (Balabanian et al., 2005); however, the roles of CXCR7 are poorly characterized, confounding the understanding of this signaling pathway.

CXCR4 is a widely expressed chemokine receptor that is essential for development, hematopoiesis, organogenesis, and vascularization (Busillo and Benovic, 2007). The CXCR4/SDF-1 axis is important in cardiac development as evidenced by the observed cardiac phenotype in knockout transgenic strains (Nagasawa et al., 1996; Ma et al., 1998), and CXCR4 and/or SDF-1 deficiency has been shown to cause perinatal lethality in mice, partly due to defective cardiac septum formation (Zou et al., 1998). Cardiac-specific deletion of CXCR4 in mice resulted in increased susceptibility to isoproterenol-induced heart failure, which was accompanied by an upregulation of apoptotic markers and reduced mitochondrial function (Wang et al., 2014). This pathogenic phenotype was alleviated by the delivery of CXCR4 by adeno-associated viral-9 vectors. A physical association between CXCR4 and the β2-adrenergic receptor was found to lead to G-protein signal modulation, suggesting a novel mechanism for regulating cardiac myocyte contractility (LaRocca et al., 2010). Supporting its role in cardiogenesis, several studies have suggested that the CXCR4/SDF-1

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axis is required for cardiogenic induction of ESCs (Nelson et al., 2008; Chiriac et al., 2010). SDF-1 has been used in gene therapy to promote the migration of myocardial progenitor cells to injured myocardium to repair and prevent the progression to heart failure (Clinical Trials.gov – NCT01082094) (Penn et al., 2013). These beneficial effects of SDF-1 were directly attributed to its monogamous receptor CXCR4.

The evidence that SDF-1 binds to CXCR7 raises a concern as to how to distinguish the potential contributions of the SDF-1/CXCR7 pathway from the SDF-1/CXCR4 pathway in all processes that were previously attributed solely to SDF-1/CXCR4. CXCR7, previously considered an orphan receptor, is now known to bind to SDF-1, which triggers G-protein dependent (Lipfert et al., 2013) and/or independent β-arrestin-dependent signaling (Rajagopal et al., 2010). Similar to CXCR4, deletion of CXCR7 in mice is lethal soon after birth due to cardiovascular defects, implying its role in cardiogenic lineage differentiation (Gerrits et al., 2008). CXCR7 binds SDF-1 with ten-fold higher affinity than CXCR4 but has been hypothesized to act as a decoy receptor, scavenging SDF-1 and positively influencing CXCR4-mediated migration by preventing the downregulation of its surface expression (Sanchez-Alcaniz et al., 2011). However, another study showed that a CXCR7 agonist can result in downregulation of CXCR4 protein levels, acting as a negative regulator of the CXCR4/SDF-1 axis (Uto-Konomi et al., 2013). In primary lymphocytes, CXCR7 induced conformational rearrangements in preassembled CXCR4/$G_{\alpha i}$ protein complexes, impairing CXCR4-mediated $G_{\alpha i}$-protein activation and calcium responses (Levoye et al., 2009). In order to provide additional insight into the SDF-1/CXCR4/CXCR7 network in human iPSC-derived cardiomyocytes (hiPSC-CMs), we set these studies to disseminate the specific roles of CXCR4 and CXCR7 in cardiogenic differentiation of hiPSCs and their resultant effects on hiPSC-CM function with the premise that it will provide new insights into hiPSC-derived ventricular cardiomyocyte specification, maturation, and function, potentially translating into therapeutic benefits.

2. Material and methods

2.1. Cell culture, viral infection and cardiac differentiation

HEK293T cells were cultured in DMEM with 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were plated at 50% confluency in a 6-well plate and treated with 4 μM polybrene for 30 min at 37 °C after which 20 μL virus (lenti-shscramble (sc-108080), -shCXCR4 (sc-35421-V), -shCXCR7 (sc-94573-V), or -shCXCR4 and shCXCR7) was added (Santa Cruz Biotechnology, Dallas, Texas). These viruses are a pool of concentrated, transduction-ready viral particles containing 3–5 target-specific constructs. After 48 h, the media was changed and 3 μg/mL puromycin was added for selection of infected cells. The hiPSC line (SKiPS-31.3) was derived by the reprogramming of human dermal fibroblast obtained from a skin biopsy of a 45-year-old volunteer with informed consent (Staten Island University Hospital, Staten Island, NY), as described (Galende et al., 2010; Karakikes et al., 2014). All lines were propagated under feeder-independent conditions, as described (Ludwig et al., 2006). HiPSCs were cultured in mTeSR1 media (STEMCELL Technologies, Vancouver, Canada) and passaged according to published protocols. Infection of hiPSCs with

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lentiviruses was performed identically to HEK293T cells, and a minimum of 3 clones or biological replicates (separate infection and differentiation) were used for each condition. All procedures involving recombinant DNA were practiced according to the National Institutes of Health guidelines. Cardiac differentiation of hiPSCs was performed by two methods: embryoid bodies (EBs) (Karakikes et al., 2014) and monolayer (Bhattacharya et al., 2014; Burridge et al., 2014). These methods have previously been described in detail and have been shown to produce ventricular cardiomyocytes that are genetically and histologically identical.

2.2. Counting of beating EBs

Using the embryoid bodies method, cardiosphere clusters would begin to beat approximately 6–8 days after the start of differentiation. At 5, 7, 8, 9, 10, 15 and 20 days after the start of differentiation, 30 randomly selected embryoid bodies were counted in each condition (Control, shScramble, shCXCR4, shCXCR7, and shCXCR4 and 7) and beating activity was verified by eye, as previously described (Chiriac et al., 2010).

2.3. Quantitative RT-PCR

Total RNA was extracted from hiPSC-CMs and hECTs using the Quick-RNA MiniPrep kit (Zymo Research, Irvine, CA) and quantified using a NanoDrop 2000 (ThermoFisher, Rockville, MD). Reverse transcription was performed using the iScript cDNA synthesis kit (Biorad Laboratories, Hercules, CA). Quantitative PCR (10 ng cDNA/reaction) was performed using a two-step system with SYBR Advantage qPCR Premix (Clontech Laboratories, Mountain View, CA) on the ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). Primers used are shown in Supplementary Table 1. Fold changes in gene expression were determined using the comparative Ct method (Ct) with normalization to the housekeeping gene β2-microglobulin (β2M).

2.4. Flow cytometry

Cells were collected, dissociated into single cell suspension with 0.025% trypsin (PromoCell Detach 2 kit, Heidelberg, Germany), and counted to obtain between 500,000 and 1 million live cells (after staining with Trypan blue). Cells were resuspended in flow buffer (0.5% BSA, 2 mM EDTA, 1× PBS no cations). Extracellular marker antibodies were added (CXCR4/APC-Cy7 (10 μL/500,000 cells; Biolegend 306528), CXCR7/PE-Cy7 (10 μL/500,000 cells; Biolegend 331116), Zombie Aqua (1:500/500,000 cells; Biolegend 423102)) and incubated on ice for 1 h in the dark. After washing with flow buffer, cells were fixed in 1% paraformaldehyde in flow buffer for 5 min at room temperature. After a gentle spin (3000 ×*g*), cells were permeabilized (flow buffer plus 0.01% Triton X-100) and stained with intracellular markers (Oct-3/4/AlexaFluor647 (20 μL/500,000 cells; BD Biosciences 560329), Sox2/AlexaFluor488 (10 μL/500,000 cells; Biolegend 656110), and cardiac troponin T/Blue violet 421 (5 μL/500,000 cells; BD Biosciences 565618) on ice for 1 h in the dark. After washing with flow buffer, cells were resuspended in fresh flow buffer and pipetted into polystyrene tubes through a cell strainer. Compensation was performed using the AbC anti-mouse bead kit (Invitrogen, Carlsbad, CA). Cells were analyzed on a LSR II flow cytometer (BD Biosciences) at the Flow Cytometry Core (Icahn School of Medicine at Mount Sinai, New York, NY, USA) and analysis was performed using FCS Express 6.0

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(DeNovo Software, Glen-dale, CA, USA). Live cells were gated for using the Zombie Aqua dye and, from this population, percent positive for each group was calculated (Supplementary Fig. 1, upper panels). CXCR4 and CXCR7 gates were set using a sample with all antibodies except CXCR4 and CXCR7 (FMO control, Supplementary Fig. 1, lower panels).

2.5. Calcium transient analysis

At 8 or 18 days post-differentiation, hiPSC-CMs were dissociated with 0.025% trypsin (PromoCell Detach 2 kit), plated on matrigel-coated coverslips, and cultured in basal media for 48 h. Ten or twenty days post-differentiation, the CMs were loaded with a calcium-sensitive fluorescent dye (Fura-2 AM, cell permeant; ThermoFisher, Rockville, MD) and the ratios of fluorescence intensities (excitation ratio of 340/380 nm) were recorded using the IonOptix system (Ionoptix, Milton, MA). The electrically-induced calcium transients were triggered by pulses from a MyoPacer (IonOptix, Milton, MA) at 40 V and 0.5 Hz and measurements were obtained at room temperature. Calcium traces were analyzed using IonWizard software (IonOptix) to calculate the baseline, amplitude (peak height), and tau (time of relaxation). For measurements with isoproterenol (DL isoproterenol hydrochloride; Sigma-Aldrich, St. Louis, MO), basal calcium transients were recorded, isoproterenol (final concentration of 500 nM) was added to the cells, and then calcium transients were measured again.

2.6. Western blot of lysate from hiPSC-CMs

HiPSC-CMs were collected and resuspended in RIPA buffer (ThermoFisher, USA) containing protease inhibitors and PMSF. After sonication, the mixture was centrifuged and the supernatant was collected. Protein concentration was measured using the 2D Quant Kit (GE HealthCare Life Sciences, USA). 100 μg total protein was loaded on a 10% SDS-PAGE gel and transferred to nitrocellulose membrane. The membrane was blocked in 1% BSA and probed with rabbit polyclonal SERCA2a (custom-made) and mouse monoclonal GAPDH (G8795; Sigma Aldrich) primary antibodies followed by corresponding secondary antibodies. The blot was visualized using a BioRad imager.

2.7. Generation of human engineered cardiac tissues (hECTs)

On day 15 post-differentiation, hiPSC-CMs were washed with 1× PBS (no cations) and enzymatically dissociated with 0.025% trypsin (PromoCell Detach 2 kit) for 5 min at 37 °C. Cells were collected by centrifugation (5 min at 300 ×*g*). Cell pellets were resuspended in RPMI + B27 culture media (ThermoFisher) and combined with ice cold collagen type I (Sigma-Aldrich) and Matrigel hESC-qualified Matrix (BD Biosciences, San Jose, CA), at a ratio of 1:8:1 (v/v/v) as previously described (Serrao et al., 2012). Briefly, 100 μL of the combined cell-matrix suspension was pipetted into a custom-made polydimethylsiloxane (PDMS) elastomer mold with integrated flexible end-posts within a rectangular well and removable inserts. After a 2 h incubation at 37 °C and 5% CO_2, to allow the collagen to polymerize, the hECTs were bathed with RPMI + B27 culture media followed by daily half-media exchanges until day five when culture media was changed to high glucose DMEM supplemented with 10% newborn bovine serum (Atlanta Biologicals, Lawrenceville, GA), 1% penicillin/streptomycin (GIBCO, Carlsbad, CA), and 0.2% amphotericin B (Sigma-

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Aldrich). HECTs were generated from five different groups of hiPSC-CMs that underwent lentivirus infection with either shCXCR4, shCXCR7, shCXCR4 and shCXCR7, shScramble, or no infection (Control).

2.8. Characterization of hECT contractile function

Characterization of contractile function of the hECTs was performed at 21–25 days after differentiation (6–10 days after tissue creation) using the integrated flexible endposts as force sensors for real-time noninvasive measurement of cardiac muscle contractile function. The hECT within the PDMS mold was transferred to a laminar airflow hood while submerged in culture media at 37 °C. The post deflection was captured in real-time with a high-speed camera (100 frames/s) and a LabVIEW program (National Instruments, Austin, TX). The contractility of hECTs was evaluated with and without electrical stimulation. The post deflection was used to calculate the twitch force by applying a beam-bending equation from elasticity theory as previously described (Serrao et al., 2012). To apply electrical stimulation, carbon rods were placed along each end of the hECT and connected to a S88X Grass Stimulator (Astro-Med, Inc., West Warwick, RI) at rates of 0.5 Hz–2.75 Hz with 0.25 Hz increments at 12 Volts.

2.9. Statistical analysis

All statistics were performed using Prism (GraphPad, La Jolla, CA) and analysis was done by one-way ANOVA followed by the Bonferroni post hoc test or two-way ANOVA followed by Tukey's test (*$P < 0.05$, **$P < 0.01$, $^{\#}P < 0.001$, and 'ns' is not significant). The statistical test used for each experiment is specified in the figure legend.

3. Results

3.1. Knockdown of CXCR4 and CXCR7 in hiPSC-CMs by lentiviral-shRNA

HEK293T cells were infected with lentivirus particles encoding shRNAs against CXCR4, CXCR7 or a Scramble control (lenti-shCXCR4, -shCXCR7, or -shScramble) to test the efficiency of gene knockdown. Gene expression was determined using qPCR, and Supplementary Fig. 2 shows that lenti-shCXCR4 resulted in a 67% decrease in CXCR4 expression and lenti-shCXCR7 resulted in a 45% decrease in CXCR7 expression, with neither having a significant effect on expression of the other CXCR. Lenti-shScramble did not significantly alter expression of CXCR4 or CXCR7.

Undifferentiated hiPSCs were then infected with shScramble or shCXCR4 and/or shCXCR7 lentivirus particles. After infection, the hiPSCs were differentiated into cardiomyocytes using previously reported and well-established embryoid body and monolayer differentiation methods (Bhattacharya et al., 2014; Karakikes et al., 2014). Previous electrophysiological and molecular characterization has shown that the hiPSC-CMs are highly enriched (50–70%) in ventricular CMs and recapitulate cardiac gene expression and calcium handling observed in vivo. While more similar to neonatal cardiomyocytes, these hiPSC-CMs are an important source of human primary cells for basic and translational research. Gene expression of CXCR4 and CXCR7 was measured by qPCR 0, 1, 3, 4, 5, 10, 15 and 20 days after differentiation. As shown in Fig. 1A, expression of CXCR4 began to increase 3 days

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after the start of differentiation, peaked at day 5 and then began to decrease. Infection with lenti-shCXCR4 resulted in an approximate 50% decrease in CXCR4 expression at days 4, 5 and 10. Infection with shCXCR7 or shScramble had no effect on CXCR4 expression. Expression of CXCR7 began to increase 3 days after the start of differentiation, slightly subsided at day 4 and was sustained from day 5 (Fig. 1B). Infection with lenti-shCXCR7 resulted in a 40–50% decrease in CXCR7 expression starting at 3 days post-differentiation and this was sustained until day 20. Infection with shCXCR4 or shScramble had no effect on CXCR7 expression.

We then performed flow cytometry 0, 1, 3, 4, 5, 10, 15 and 20 days after differentiation to verify that gene knockdown of CXCR4 and CXCR7 also resulted in a concomitant reduction of functional protein levels. Functional CXCR4 and CXCR7 protein is defined as having surface expression on the plasma membrane, and labeling was performed before fixation and permeabilization, as multiple factors can affect internalization of the CXCRs. For CXCR4, peak expression occurred 4–5 days after the start of differentiation and infection with lenti-shCXCR4 resulted in a 60–70% decrease in cells positive for surface CXCR4 protein on those days (Fig. 1C). For CXCR7, peak expression occurred at 3 days post-differentiation and infection with lenti-CXCR7 resulted in a ~50% decrease in cells positive for surface CXCR7 protein on that day (Fig. 1D). Functional levels of CXCR4 and CXCR7 were significantly decreased compared to Control and lenti-shScramble-infected cells starting at day 1 and 3 of differentiation, respectively, and continued to be decreased until day 20 (Figs. 1C and D). In addition, at days 10, 15 and 20 post-differentiation (when terminal differentiation to ventricular cardiomyocytes is complete), we gated for CXCR4 or CXCR7 positive cells from cardiac Troponin T (cTnT) positive cells. We found that in the cardiomyocyte population (cTnT positive), infection with lenti-shCXCR4 and/or lenti-shCXCR7 resulted in an approximate 30–50% reduction in cells positive for surface CXCR4 and CXCR7 protein (Fig. 1E and F).

Interestingly, we found that while infection with lenti-shCXCR4 didn't affect gene expression of CXCR7, it did significantly reduce its protein expression at the plasma membrane (Fig. 1G, top panels). We performed flow cytometry on total CXCR4 and CXCR7 (after fixation and permeabilization) in both Control and lenti-shCXCR4 treated cells and found that CXCR7 levels were comparable between the two conditions (Fig. 1G, middle and lower panels). Considering recent work, which has identified heterodimers of CXCR4 and CXCR7 and their reciprocal effects on the other's functionality and expression (Levoye et al., 2009; Decaillot et al., 2011), we conclude that knockdown of CXCR4 also appears to prevent the proper trafficking or retention of functional CXCR7 in the plasma membrane of hiPSC-CMs.

3.2. Knockdown of CXCR4 and CXCR7 delays cardiogenesis in hiPSC-CMs

It has been previously demonstrated that knockdown or deletion of CXCR4 or CXCR7 is detrimental to cardiogenesis and causes perinatal lethality in mice partly due to cardiovascular defects (Gerrits et al., 2008; Wang et al., 2014). To further evaluate cardiomyocyte differentiation efficiency and to confirm that knockdown of CXCR4 or CXCR7 delays cardiogenesis, we examined the effects of infection with shCXCR4 or

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shCXCR7 lentivirus on the expression of pluripotent (*OCT4* and *SOX2*) and late cardiac (*TNNT2*) markers (Fig. 2A–C). *OCT4* and *SOX2* are transcription factors critically involved in the maintenance of stem cell self-renewal. They are commonly used as pluripotency markers in stem cells and their expression is high at the beginning of cardiac differentiation, dramatically decreasing one day after the start of differentiation. Infection with shCXCR4 and/or shCXCR7 resulted in extended high expression of *OCT4* and *SOX2* on day 1 of differentiation (Fig. 2A and B). This is corroborated by flow cytometry experiments showing an increased number of Oct3/4 and Sox2 double positive cells at day 1 of differentiation after knockdown of CXCR4 and/or CXCR7 compared to Control (Fig. 2D). *TNNT2* encodes for cardiac troponin T, which is part of the troponin complex and regulates muscle contraction. This gene is highly expressed in terminally differentiated ventricular cardiomyocytes and is an ideal marker for late cardiac development. In Control and shScramble-infected hiPSC-CMs, the gene expression of *TNNT2* starts at day 5 and peaks at day 10, after which its expression is sustained (Fig. 2C). Following knockdown of CXCR4 and/or CXCR7, gene expression of *TNNT2* also begins at day 5 but does not peak until 15 days after the start of differentiation. Flow cytometry analysis also demonstrated this delay in cardiac Troponin T (cTnT) protein expression, with repression extending into day 15 and only catching up with Control at day 20 (Fig. 2E). These data demonstrate that knockdown of CXCR4 and/or CXCR7 delays the cardiogenic lineage progression but does not affect terminal differentiation of hiPSCs to ventricular cardiomyocytes.

3.3. Knockdown of CXCR4 inhibits spontaneous beating of hiPSC-CMs

We examined the effect of CXCR4 and/or CXCR7 gene knockdown on cardiac lineage inhibition by analysis of spontaneously beating cardiac clusters. The cardiomyocyte differentiation efficiency was assessed based on spontaneous contractile activity. It has been previously demonstrated that inhibition of CXCR4 expression by siRNA in hiPSC-CMs results in a disruption of spontaneous beating (Nelson et al., 2008). We also found that knockdown of CXCR4 by lenti-shCXCR4 resulted in a delay in spontaneous beating of hiPSC-CMs (Fig. 3). At day 9, approximately 50% of the Control and shScramble-infected hiPS-CMs were beating but <20% of the shCXCR4-infected hiPS-CMs were beating. This phenomenon was also seen with the dual infection of shCXCR4 and shCXCR7 but not with shCXCR7 alone. Twenty days after the start of differentiation, the same number of hiPSC-CMs were spontaneously beating across all conditions.

3.4. Knockdown of CXCR4 and CXCR7 differentially alters calcium transients and β-adrenergic response in hiPSC-CMs

To assess the importance of CXCR4 and CXCR7 in hiPSC-CMs, it is important to examine their functional properties beyond the expression of cardiomyocyte-specific molecular markers. Thus, we assessed their Ca^{2+} handling properties and β-adrenergic receptor signaling responses. We previously published that CXCR4 negatively regulates the β2-adrenergic receptor and cardiomyocyte contractility (LaRocca et al., 2010; Wang et al., 2014). Here, using the calcium-sensitive fluorescent dye Fura-2, we were able to measure calcium transients 10 and 20 days after the start of differentiation. The hiPSC-CMs were paced at 0.5 Hz (40 V) and calcium transients were measured either in the absence or presence of 500 nM isoproterenol, a mixed β1- and β2-adrenoceptor agonist. Typical

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calcium transients of hiPSC-CMs, both basal and in the presence of 500 nM isoproterenol, are shown in Fig. 4A. Three parameters were determined using the IonOptix System and Analysis software: baseline, amplitude (peak height as a percentage of baseline), and tau (time for relaxation). These results are shown in Fig. 4B and C and Supplementary Fig. 3. The baseline is the pre-stimulation value of the recorded signal, and, while the baseline has no real value to itself, is an indication of cell health and can be used to determine the reliability of the data. Baseline values were consistent in all hiPSC-CMs and all conditions measured (Supplementary Fig. 3). Under basal conditions, the amplitudes of hiPSC-CMs infected with shCXCR4 or shCXCR4 and shCXCR7 were significantly higher than Control (Fig. 4B). After stimulation with isoproterenol, all amplitudes were significantly higher in all conditions compared to their corresponding basal values except for hiPSC-CMs infected with shCXCR7. At day 20, the amplitudes of hiPSC-CMs infected with shCXCR4 or shCXCR4 and shCXCR7 were significantly higher after treatment with isoproterenol compared to Control after treatment with isoproterenol. Interestingly, the amplitude of hiPSC-CMs infected with shCXCR7 was significantly lower than Control after treatment with isoproterenol at day 10 and 20, revealing an abolishment of typical inotropic stimulation and implying CXCR7's involvement in β-adrenergic stimulation. Moreover, the myocardial relaxation time (tau) significantly decreased after isoproterenol stimulation in all conditions except shCXCR7 at day 20, further suggesting that CXCR7 might be important in regulating β-adrenergic-mediated inotropic responses (Fig. 4C). The trends observed in calcium transients for each condition were similar between 10 and 20 days after differentiation, but were more pronounced at day 20 when cardiac Troponin T levels were identical between all conditions and the hiPSC-CMs have more mature signaling and calcium handling pathways (Robertson et al., 2013). All hiPSC-CMs showed similar levels of SERCA2a expression at day 20, implying that these changes in calcium parameters are due to changes in expression of CXCR4 and/or CXCR7 (Fig. 4D). SERCA2a is a Ca^{2+}-ATPase that transfers Ca^{2+} from the cytosol of the cell to the lumen of the SR at the expense of ATP hydrolysis during muscle relaxation and is an important regulator of contractility.

3.5. CXCR7 depletion results in increased developed force and CXCR4 depletion results in increased chronotropy in hECTs

To further assess the role of CXCR4 and CXCR7 in hiPSC-CM contractility, we generated 3-dimensional (3D) hECTs made of hiPSC-CMs and collagen. Physical forces generated by cells drive morphological changes during development, which can feedback to regulate cellular phenotypes (Beloussov et al., 1975; Keller et al., 2003). Under basal conditions at 1 Hz pacing frequency, the developed force of the hECTs infected with shCXCR7 or shCXCR4 and shCXCR7 showed a trend to be higher than Control and shScramble (Fig. 5A). When stimulated by a single dose of 500 μM isoproterenol, a β-adrenergic receptor agonist, under spontaneous beating conditions to assess their chronotropic response, shCXCR4-infected hECTs resulted in a statistically significant increase in chronotropy as compared to shScramble and Control hECTs (Fig. 5B). This is in accordance with our previously published data indicating that CXCR4 is a negative modulator of β-adrenergic receptors (LaRocca et al., 2010). Knockdown of CXCR7 alone had no significant effect on chronotropic response in hECTs and a similar result was obtained from shCXCR4 and shCXCR7-infected hECTs (Fig. 5B). Together, this finding supports our spontaneous

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beating data (Fig. 3) indicating that CXCR4 is a major axis involved in regulating the contractility. However, the role of CXCR7 in chronotropic response and regulation of β-adrenergic receptor signaling seems to be more complex and needs to be clarified further to distinguish the potential contributions of the SDF-1/CXCR7 pathway from SDF-1/CXCR4.

3.6. CXCR4 and CXCR7 depletion result in increased expression of hypertrophic markers

The growth and differentiation of hiPSC-CMs in an organ-specific manner were further assessed by measuring hypertrophic genes such atrial natriuretic factor (*ANF*) and the α/β myosin heavy chain ratio (α/β-*MYHC*) in hECTs. *ANF* expression is marker of cardiomyocyte differentiation and hypertrophy, and a decrease in the *α/β-MYHC* ratio is a common indicator of cardiac hypertrophy, as a shift occurs from the α to β isoform during cardiac disease. There was a significant increase in *ANF* gene expression in tissue generated from hiPSC-CMs infected with shCXCR4 or shCXCR4 and shCXCR7 but not with shCXCR7 alone (Fig. 5C), further implying a critical role for CXCR7 in regulating β-adrenergic receptor signaling. The α/β-*MYHC* ratio was reduced in all three groups: shCXCR4, shCXCR7, and shCXCR4 and shCXCR7 (Fig. 5D).

4. Discussion

CXCR4 and CXCR7 are prominent G protein-coupled receptors (GPCRs) for chemokine stromal cell-derived factor-1 (SDF-1/CXCL12) involved in cardiogenesis. Both CXCR4 and CXCR7 can differentially contribute to SDF-1-mediated responses, but the relative contributions of each receptor to cardiac development and calcium handling need further clarification. For a long time CXCR4 was thought to be the only receptor for SDF-1; therefore, most of the effects of SDF-1 were contributed to CXCR4. The fact that SDF-1 has an alternative receptor, CXCR7, that has even higher affinity for SDF-1 than CXCR4 raises concerns (Balabanian et al., 2005). Further complicating matters, CXCR4 and CXCR7 have been shown to form heterodimers and have concomitant effects on the other's function and expression pattern (Levoye et al., 2009).

In this study, lentiviral vectors were used to ablate CXCR4 and/or CXCR7 expression using shRNAs, and the derived hiPSC-CMs were tested for phenotypic and functional properties due to gene knockdown. Gene expression and flow cytometry studies confirmed the cardiomyocyte phenotype of the differentiated hiPSCs, although reduction of CXCR4 and CXCR7 expression resulted in a delayed cardiac phenotype. Knockdown of CXCR7 had no effect on spontaneous beating but CXCR4 knockdown significantly reduced spontaneous beating. Ca^{2+} homeostasis is crucial for excitation-contraction coupling and, subsequently, regulates the contractile properties of functional cardiomyocytes (Bers, 2000). The β-adrenergic signaling cascade is also an important regulator of myocardial function, which serves as the most powerful regulatory mechanism to enhance myocardial performance in response to stress. A positive chronotropic and/or inotropic response to β-adrenergic stimulation requires appropriate surface membrane receptors coupled to a signaling pathway that stimulates the appropriate ion channels, receptors, and myofilament proteins. Indeed, functional characterization by way of intracellular calcium movement of hiPSC-CMs showed that typical inotropic responses to β-adrenergic stimulation were impaired in the

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absence of CXCR7 and were significantly enhanced in the absence of CXCR4. The hECTs also showed similar results, where CXCR4 depletion resulted in increased chronotropy and CXCR7 depletion resulted in diminished chronotropy in response to β-adrenergic agonist stimulation. This is in agreement with our previously published data that CXCR4 is a negative modulator of the β-adrenergic receptor (LaRocca et al., 2010) and that cardiac-specific deletion of CXCR4 in mice results in increased susceptibility to isoproterenol-induced heart failure (Wang et al., 2014). These experiments further demonstrate that in the absence of CXCR4 there is an exacerbated response to β-adrenergic stimulation. Interestingly, knockdown of both CXCR4 and CXCR7 maintained characteristics of both individual knockdown conditions after treatment with a β-agonist: the hiPSC-CMs demonstrated an increase in inotropy and lusitropy (similar to shCXCR4) and the hECTs showed no chronotropic effect (similar to shCXCR7). The reason for this isn't known but warrants further exploration. It should be noted that measurement of chronotropic responses in hiPSC-CMs are extremely difficult in the IonOptix system due to the sensitivity of the cells (they do not beat unless stimulated and paced). Additionally, inotropic responses to β-agonists in hECTs has not been demonstrated using our current methodology; the reason for this is currently being investigated.

Although knockdown of CXCR4 didn't affect gene expression of CXCR7, it significantly reduced the amount of surface CXCR7 protein at the plasma membrane. We confirmed that total CXCR7 levels (surface and internalized) were similar between Control and shCXCR4-treated conditions, implying that CXCR4 is necessary for proper CXCR7 trafficking to or retention in the plasma membrane. The mechanisms by which CXCR4 and CXCR7 reach the plasma membrane are unknown but, as with other chemokine receptors, internalized CXCR4 traffics through endosomal compartments either to lysosomes for degradation or to be recycled back to the plasma membrane (Neel et al., 2005). Previously, it was shown that CXCR4 and CXCR7 form both homo- and hetero-dimers, and CXCR7 alters CXCR4-mediated $G_{\alpha i}$ protein activation and calcium responses in primary T cells (Levoye et al., 2009; Decaillot et al., 2011). It has also been observed that heterodimeric chemokine receptors undergo functional modulations regulated through an allosteric mechanism, affecting signaling or ligand binding differentially once engaged in heterodimers (Springael et al., 2005). Therefore, this surprising result in our study warrants further exploration.

Collectively, these results indicate that both CXCR4 and CXCR7 are necessary for proper differentiation and function of hiPSC-CMs and hECTs, but they might act by separate mechanisms. There have been multiple reports suggesting differential signaling and activity of SDF-1/CXCR4 and SDF-1/CXCR7 pathways in different cell types and tissues but the relative contributions to SDF-1-mediated responses in cardiomyocytes are not clear. Moreover, there is evidence suggesting that CXCR7 modulates CXCR4 by heterodimerization (Levoye et al., 2009; Decaillot et al., 2011), and that CXCR4 modulates the β2-adrenergic receptor by direct interaction (Larocca et al., 2012). Collectively, these reports and others may explain why some but not all of the CXCR7-mediated effects might be CXCR4-dependent. The functions of CXCR7 and its molecular interactions in cells after binding with SDF-1 remain poorly defined and need to be further studied.

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5. Conclusions

This study clearly indicates that CXCR7 has a distinct role from CXCR4 in hiPSC-CM cardiogenesis and functional responses to β-adrenergic stimulation. Our data strongly suggest both CXCR4 and CXCR7 are necessary for proper cardiac development and subsequent calcium handling in hiPSC-CMs and hECTs, particularly in response to pharmacologic β-agonists. Additionally, our data also demonstrate that proper trafficking/targeting of CXCR7 to the plasma membrane may be disrupted by gene knockdown of CXCR4. These data provide important insights into the molecular mechanisms that coordinate cardiac lineage specification and calcium handling in hiPSC-CMs.

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Abbreviations

hiPSC human induced pluripotent stem cell

hiPSC-CM human induced pluripotent stem cell-derived cardiomyocyte

hECT human engineered cardiac tissue

GPCR G-protein coupled receptor

SDF-1 stromal cell-derived factor-1

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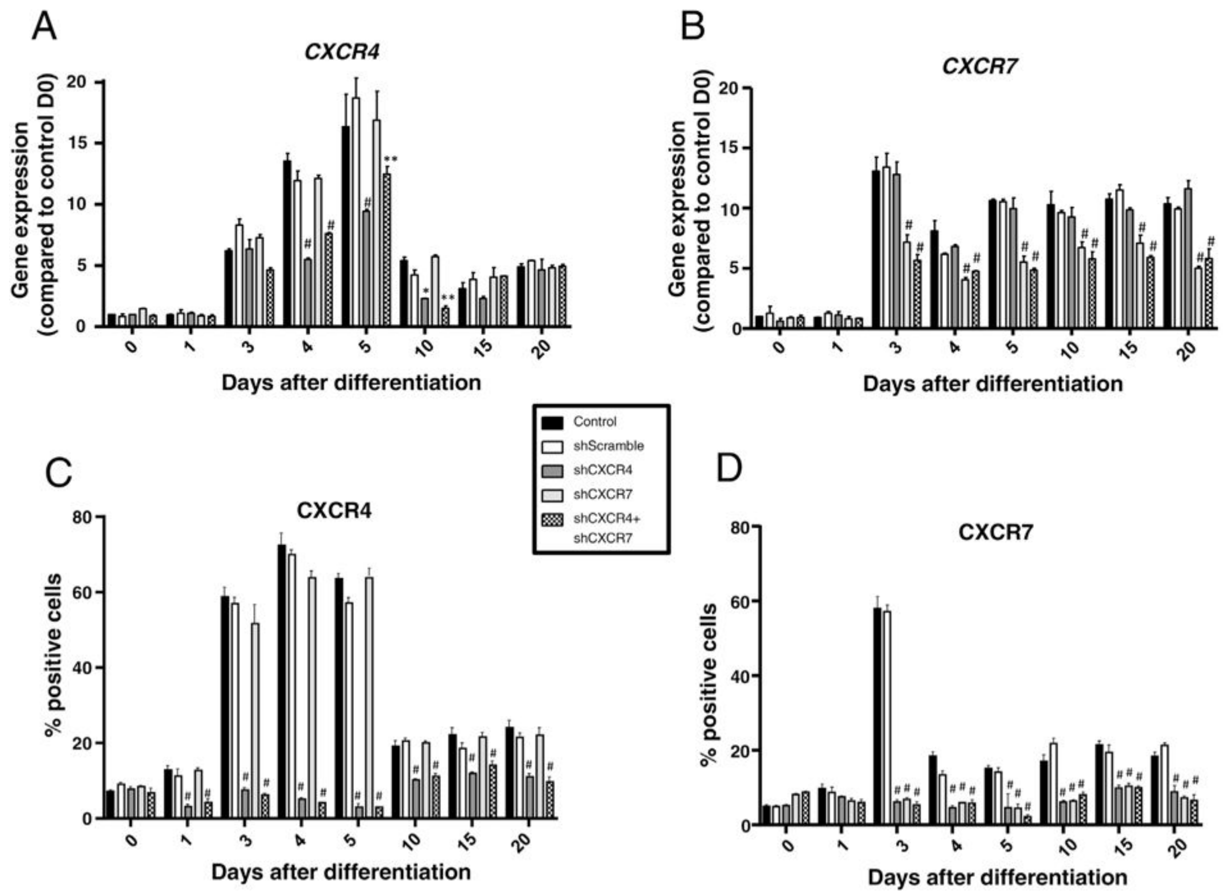

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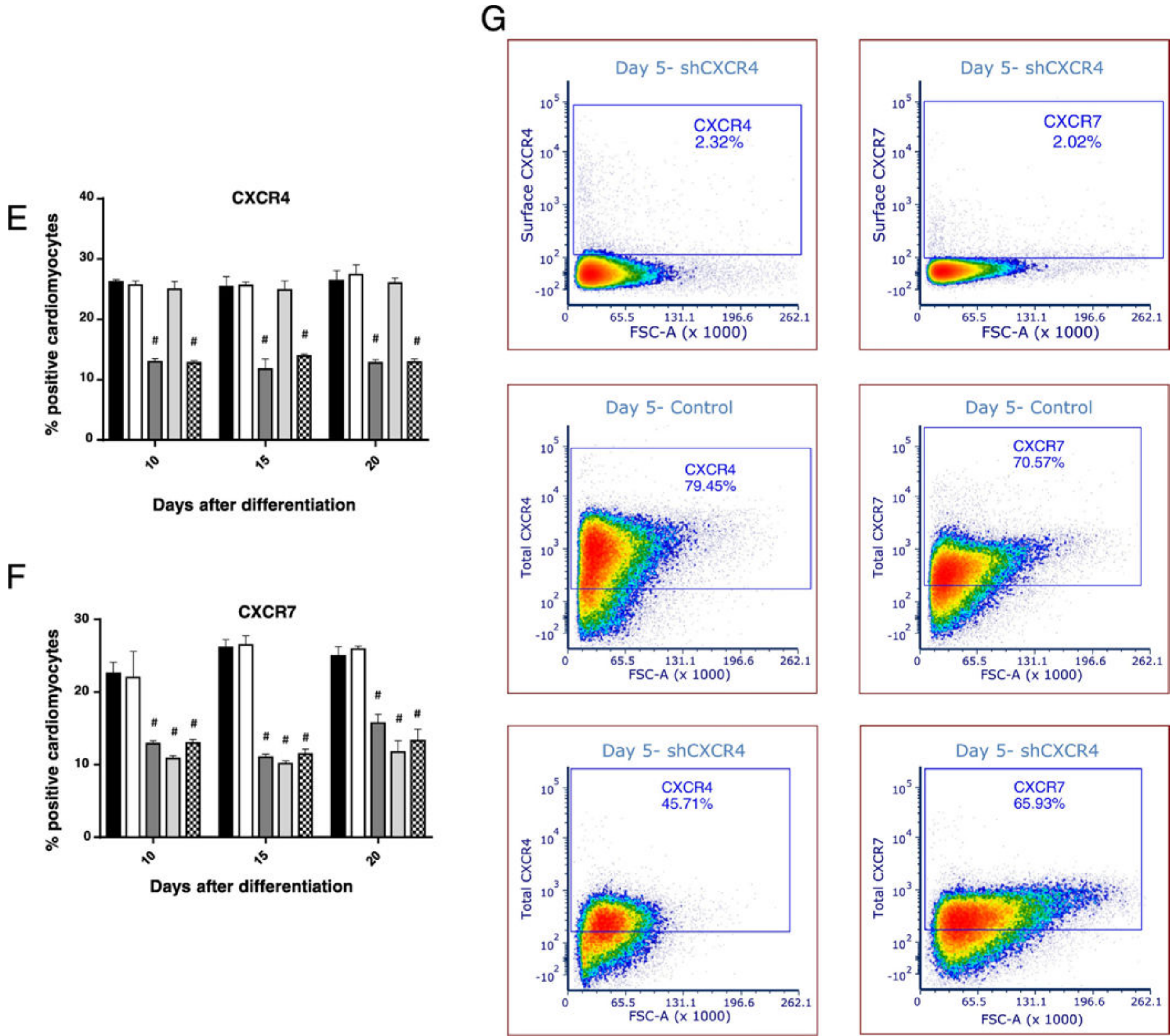

Fig. 1.

Efficient knockdown of CXCR4 and CXCR7 in hiPSC-CMs. Knockdown of CXCR4 and/or CXCR7 by lentivirus shRNAs in hiPSCs/hiPSC-CMs was evaluated by gene expression (A and B) and flow cytometry (C–G) over the course of differentiation (0, 1, 3, 4, 5, 10, 15 and 20 days after the start of differentiation). (C and D) Surface levels of CXCR4 and CXCR7 protein. (E and F) Surface levels of CXCR4 and CXCR7 protein in cardiomyocytes only (cardiac Troponin T positive cells). (G) Surface protein levels (top panels; shCXCR4) compared to total CXCR4 and CXCR7 protein levels at day 5 of differentiation (middle and lower panels show Control and shCXCR4, respectively). Surface and total protein levels were obtained by labeling CXCR4 and CXCR7 before and after fixation/permeabilization, respectively. Gene expression was normalized to β2-microglobulin (β2M) and compared to control day 0. Flow cytometry is shown as percent positive cells after gating for live, single

cells. Data is shown as mean ± SEM and statistical analysis was done by two-way ANOVA followed by Tukey's test (n = 3 biological replicates for gene expression, n = 2 biological replicates for flow cytometry; *P < 0.05, **P < 0.01, #P < 0.001 vs. control).

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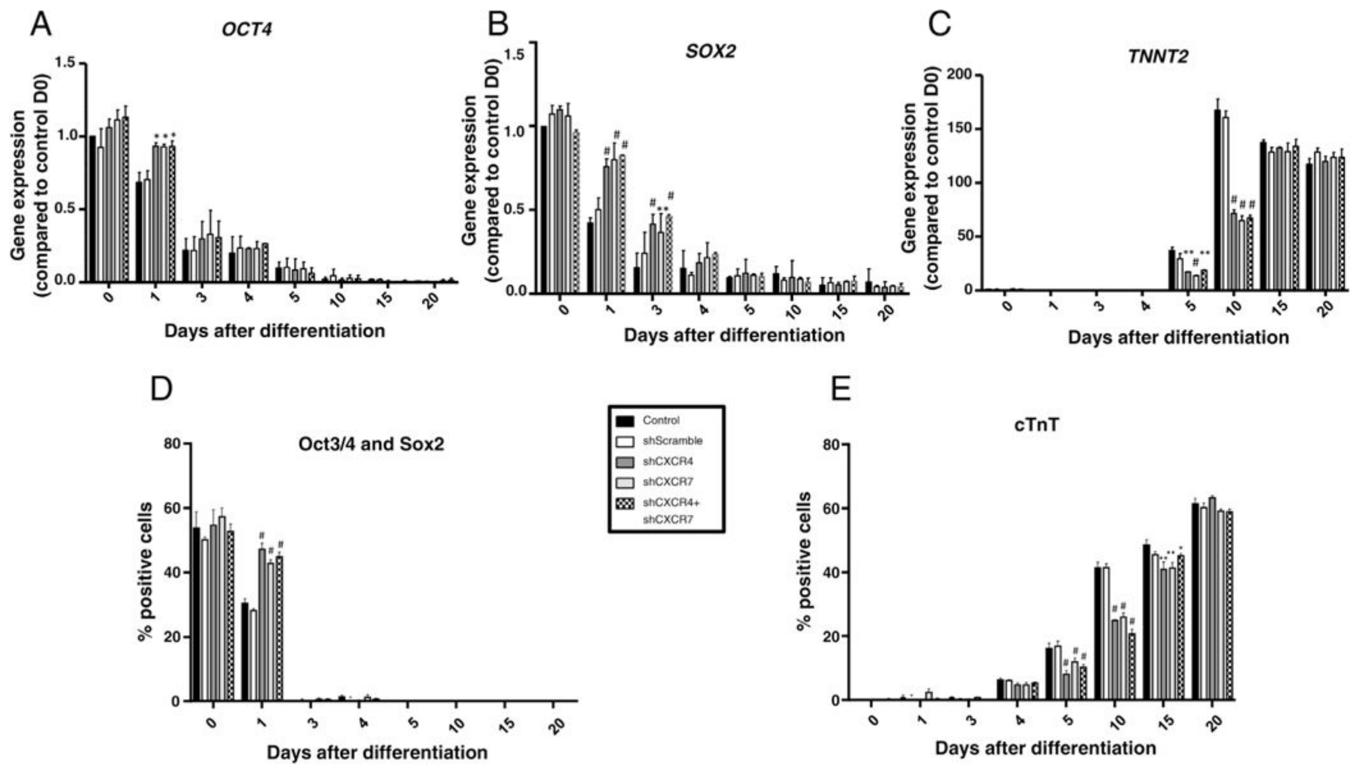

Fig. 2.

Gene expression and protein levels of pluripotent and cardiomyocyte markers in hiPSC-CMs. Gene expression was determined by qPCR using primers specific for *OCT4* (A), *SOX2* (B) and *TNNT2* (C). *OCT4* and *SOX2* are markers of pluripotency, and TNNT2 is a late cardiac marker; all values were normalized to β2-microglobulin (β2M) and compared to control day 0. Protein levels were determined by flow cytometry: (D) percent cells positive for both Oct3/4 and Sox2 and (E) percent cells positive for cardiac TnT. Flow cytometry is shown as percent positive cells after gating for live, single cells. Data is shown as mean ± SEM and statistical analysis was done by two-way ANOVA followed by Tukey's test (n = 3 biological replicates for gene expression, n = 2 biological replicates for flow cytometry; *P < 0.05, **P < 0.01, $^{\#}$P < 0.001 vs. control).

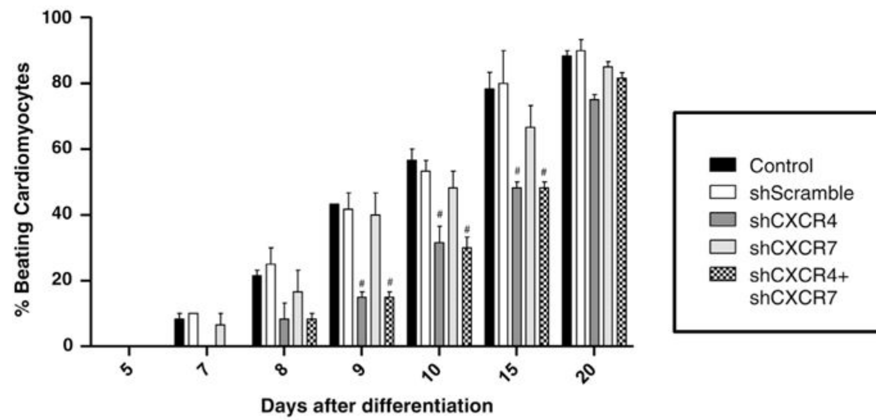

Fig. 3.

Effect of CXCR4 and CXCR7 knockdown on spontaneous beating of hiPSC-CMs. Spontaneously beating hiPSC-CMs (embryoid bodies) were counted starting at 5 days after differentiation. Data is shown as mean ± SEM and statistical analysis was done by two-way ANOVA followed by Tukey's post-test (n = 3; *P < 0.05, **P < 0.01, #P < 0.001 vs. control).

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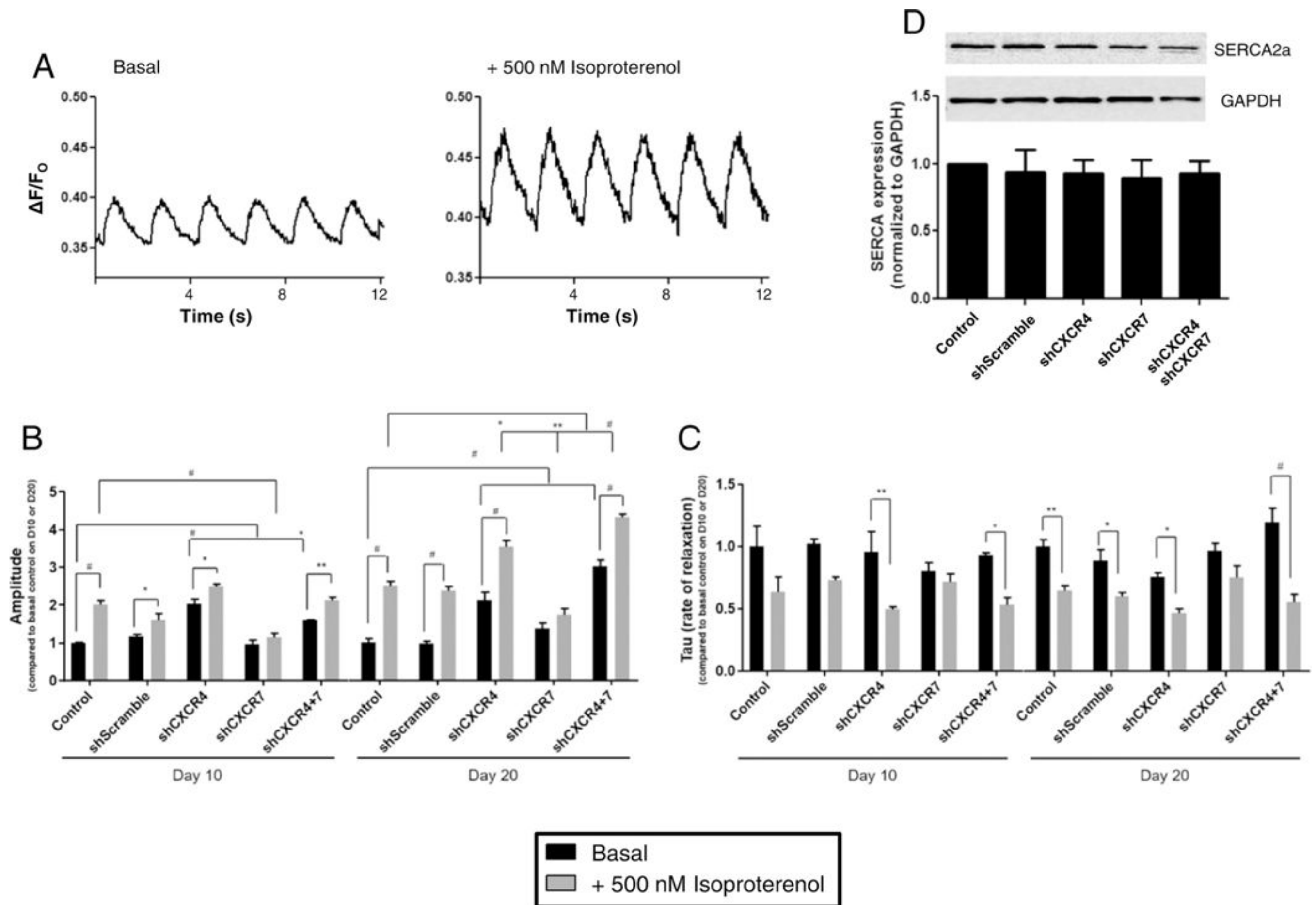

Fig. 4.

Calcium transient parameters of hiPSC-CMs. Calcium transients of hiPSC-CMs were measured using the calcium-sensitive fluorophore Fura-2 at 10 and 20 days after differentiation (40 V, paced at 0.5 Hz). Typical calcium transients obtained are shown in (A). Transients were measured either at basal levels (black bars) or in the presence of 500 nM isoproterenol (grey bars), a β-agonist. Values for amplitude (B) and tau (C) are shown. (D) Protein levels of SERCA2a were similar in all conditions in hiPSC-CMs at day 20 following normalization to GAPDH. Data is shown as mean ± SEM and statistical analysis was done by two-way ANOVA followed by Tukey's post-test (n = 5–8; *P < 0.05, **P < 0.01, $^{\#}$P < 0.001 vs. control).

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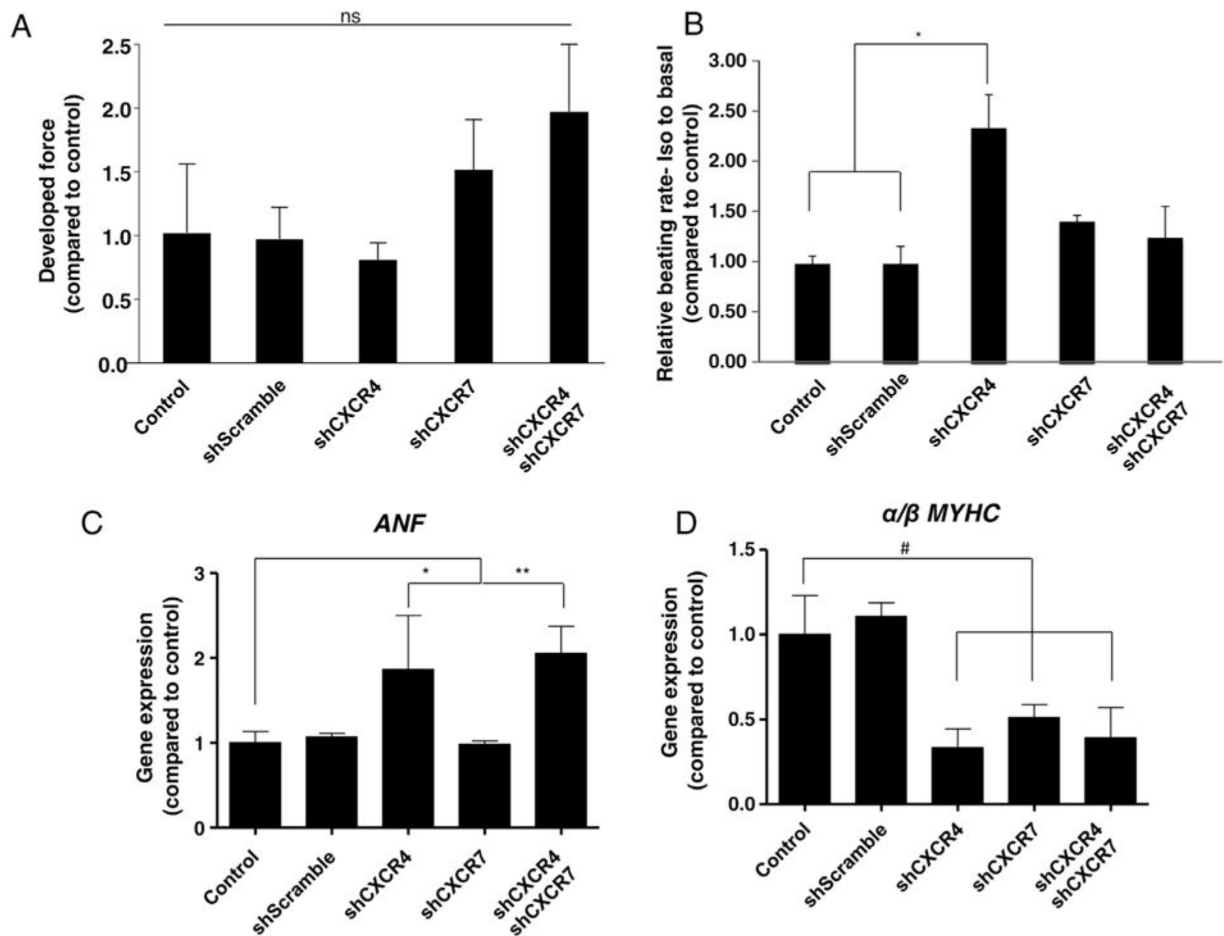

Fig. 5.

Developed force, relative beating rate, and hypertrophic gene expression in hECTs. Developed force (mN) was measured in hECTs 21–25 days post-differentiation and normalized to control values (n = 4,5,4,6 and 2 per group, respectively; error bars represent SEM). (A) Relative beating rate (bpm) was measured following addition of 500 μM isoproterenol and normalized to control values; one-way ANOVA with Bonferroni post hoc test, *P < 0.05 vs. control; n = 2 per group; error bars represent SD. (B) Gene expression was determined by qPCR using primers specific for *ANF*, α*MYHC*, and β*MYHC*. Gene expression normalized to β2-microglobulin (β2M). Data is shown as mean ± SEM and statistical analysis was done by one-way ANOVA followed by the Bonferroni test (*P < 0.05, **P < 0.01, $^{\#}$P < 0.001 vs. control).