Elucidating the role of integrin *alpha-6* in cardiomyocytes differentiated from human embryonic stem cells

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Abstract

Integrins are heterodimer transmembrane protein receptors that are composed of an α and a β subunit, which are connected by covalent interactions forming an extra-cellular head, two multi-domain legs, two single pass transmembrane helices and two short cytoplasmic tails. The two primary functions of integrins are attachment to the extracellular matrix and signal transduction from the extracellular matrix to the cell. Signaling also operates in the opposite direction: signals generated inside the cell can either enhance or inhibit the ability of integrins to bind to their ligand outside the cell (Alberts, et al., 2002). Integrin alpha-6 (*ITGA6*) is a particular isoform of the integrin family, which is expressed across stem cell populations and has been shown to play an integral role in human embryonic stem cell (hESC) self-renewal (Villa-Diaz 2016). During the differentiation process of hESCs to cardiomyocytes, there is a population of cells found to be Isl-1 positive and *ITGA6* positive, which have been shown to produce all three cardiac cell types: endothelial, smooth muscle and cardiomyocytes. This suggests that this sub-population might be human cardiac stem cells (hCaSCs). Determining and creating a cardiac stem cell line would be beneficial in regenerative medicine, to create heart models and treatments for damaged hearts in the future. We develop the hypothesis that *ITGA6* is a key protein in the formation of hCaSCs, which in turn lead to cardiomyocytes.

To test our hypothesis, we knocked out *ITGA6* in hESCs using three constructs of doxycycline (DOX) inducible CRISPR-Cas9 single-guided (sg) RNAs that target specific sequences of nucleic acids in *ITGA6*, and we utilized a CRISPR-Cas9 as a control. We found through flow cytometry analysis that the construct denominated as CRISPR-Cas9 sg10 had the greatest effect on *ITGA6 expression*. Three days into our cardiomyocyte differentiation

protocol, we activated the knockout with DOX. This allowed the knockout of *ITGA6* by the time when it was detected as its highest expression in Isl-1^{+}/ITGA6^{+} cells. This experiment was done in triplicate. Evaluation of cultures was done at day six (144 hours) and day eight (192 hours) after initiation of differentiation, by determining accumulation/sphere growth of Isl-1^{+}/ITGA6^{+} cells and beating of cardiomyocytes, and compared to control groups, as an indicator of successful differentiation. Our data proved that the hypothesis was correct, as knockout of ITGA6 resulted in no large accumulations or spheres formed, and cardiomyocytes did not mature, or beat. The cultures fixed at day 6 and day 8 to perform immunocytochemistry (ICC) to test for the expression of *ITGA6*, Isl-1 and Oct4. The CRISPR cas9 sg10 was shown to have no expression of any of these proteins. This supports that *ITGA6* is necessary for the formation of cardiomyocytes and also cardiac stem cells.

Introduction

Human pluripotent stem cells (PSC) have the property of pluripotency, or the ability to develop into any cell type within the human body. Pluripotent stem cells temporally develop in the inner cell mass (ICM) of the blastocyst stage during embryonic development, and cells from the ICM can be isolated and propagated *in vitro* as embryonic stem cells (ESC). These cells can then be treated with growth factors, cytokines and inhibitors to differentiate into a desired cell type. PSCs are considered "immortal", as they have the capacity to continuously divide to make a new cell type or another stem cell, also known as self-renewal. This ability of differentiation and self-renewal theoretically might be supported by integrin alpha-6 (*ITGA6).* In hESCs knockdown of integrin α6 and activation of β1 integrin lead to FAK phosphorylation and reduction of pluripotent-related transcription factors Nanog, Oct4, and Sox2; suggesting that integrin α6 functions in inactivation of integrin β1 and FAK signaling and prevention of hPSC differentiation (Villa-Diaz 2016). Integrins are heterodimer transmembrane protein receptors that are composed of an α and a β subunit, which are connected by covalent interactions forming an extra-cellular head, two multi-domain legs, two single pass transmembrane helices and two short cytoplasmic tails. The two primary functions of integrins are cell attachment to the extracellular matrix and signal transduction from the extracellular matrix to the cell, and are involved in cell differentiation, migration and survival. *ITGA6* is present in 35 somatic stem cell populations, and it is used to identify true stem cell populations (Krebsbach & Villa-Diaz, 2017).

Currently, a true cardiac stem cell population has not yet been differentiated from embryonic stem cells. However, through an invasive biopsy of the human heart, a population resembling cardiac stem cells had been isolated. Isolation and expansions of these spherical cell

aggregates, coined as “cardiospheres”, showed clonogenic and self-renewal capabilities, as wells as multipotent differentiation into cardiomyocytes, endothelial cells and smooth muscle cells (Smith, et al., 2007). Because of the invasive procedure and extraction of limited cells of the heart, this practice has not been adopted as standard procedure. In our differentiation from undifferentiated hESCs to cardiomyocytes, we are able to find an Isl-1+ population, which is a marker for putative cardiac stem cells, that are able to differentiate into the three major cell types in the heart: cardiomyocytes, endothelial cells and smooth muscle cells. These cells are also *ITGA6*+, which as discussed earlier plays a vital role in stem cells ability to perform self-renewal and differentiation. To test if *ITGA6* plays a role in their differentiation, we knocked it out using CRISPR Cas9 during the process of differentiation from hESCs to cardiomyocytes. We hypothesized that *ITGA6* plays a role in the differentiation pathway and, without it, cardiomyocytes will not be able to form as cardiac stem cells themselves will not be able to be developed.

Materials and Methods

Replicates

Each experiment and analytic procedures were performed in triplicates.

Cell Culture

Human embryonic stem cells (hESCs) from the cell lines H1 and H9, were grown on synthetic polymer-coated plates named PMEDSAH (Villa-Diaz, et al., 2010) in a cell culture incubator set up at 37˚C with 5.0% CO_2 and high humidity. Human cell conditioned medium (hCCM; GlobalStem, Gaithersburg, MD, http://www. globalstem.com) supplemented with 4 ng/ml human recombinant basic fibroblast growth factor (FGF2, GlobalStem), 1X streptomycin and penicillin (Hyclone), and 10μm/mL rock inhibitor (Y27632; Stem Cell Technologies, Vancouver, Canada) was used to maintain hESCs in undifferentiated state. The media was changed every other day and hESCs were grown until mature colonies and a high confluence was reached. Then, hESCs were then passaged to 35mm culture plates coated with Matrigel (Invitrogen) and left to seed for 48 hours. After this period of time, if the cells reached 90-100% confluency, initiation of the cardiac differentiation protocol began and was considered as Day 0. The confluent cells were then washed twice with filtered phosphate buffered saline (PBS, Gibco) and given Day 0 media after 48 hours, which consisted of 1640 RPMI (Gibco), 2X of B-27 without insulin, 12 μM of CHIR inhibitor (99021, Tocris) and 1X of streptomycin and penicillin (Hyclone). After 24 hours, this media was changed and replaced with Day 1 media, which consisted of 1640 RPMI (Gibco), 2X of B-27 without insulin and 1X of streptomycin and penicillin (Hyclone). After 48 hours, half of the spent media was aspirated and doubled with Day 3 media containing 1640 RPMI (Gibco), 2X of B-27 without insulin and 1X of streptomycin and

penicillin (Hyclone) and 5 µM of IWP-4 (Tocris). After another 48 hours, the media is changed to Day 1 media again. Finally, after another 48 hours, the media was replaced to Day 7 media, which contained 1640 RPMI (Gibco), 2X of B-27 with insulin and 1X of streptomycin and penicillin (Hyclone). The cultures were then fed every other day with this media cocktail up to 90+ days to obtain fully differentiated cardiomyocytes. Our experiment finished normally on day 8 when we detected the first cardiomyocytes beating.

Cardiomyocyte Characterization

Pre-cardiomyocyte colonies were identified by large grouped accumulations that grew into 3-D cellular structures. Many of these groups formed large, spheroid like structures, and some formed anchor like points with long extensions, all which eventually would show beating cells. The cells were also defined by their expression of Oct4 and Isl-1 by analysis of their protein translation by immunocytochemistry (ICC).

Immunocytochemistry (ICC)

Cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature. The cells were maintained in PBS, sealed with parafilm, and stored at 4˚C until ICC was performed. Cells were treated with unmasking, quenching, and permeabilization solutions for ten minutes each at room temperature. Cells were then treated with blocking solution for one hour at room temperature. The first protein specific antibody was incubated at 4˚C for 12 hours according to the manufacturer's recommended dilutions (Table 1). After washing with PBS 3 times for 10 minutes each under rotation conditions, the cells were incubated in secondary fluorescent-labeled antibody at room temperature for 1 hour in dark conditions. The cells were then washed with PBS for 10 minutes in dark conditions. The cells were incubated with DAPI diluted in PBS

for 10 minutes to detect the cell's chromatin and then washed with PBS for 10 minutes in dark conditions. A 1% paraformaldehyde solution was utilized for 5 minutes to stabilize the cell-antibodies bound and then stored at 4˚C in dark conditions. A control omitting the first antibody and contained only secondary antibody was utilized. The cells were visualized and captured utilizing an EVOS FL microscope (Life Technologies).

Table 1 Antibodies utilized in immunocytochemistry

Antibody	**Source**	**Concentration**	**Company**
Integrin alpha-6	Mouse	1:100	Santa Cruz
Oct4	Goat	1:100	Santa Cruz
NANOG	Mouse	1:200	Santa Cruz
Isl-1	Rabbit	1:250	Santa Cruz

CRISPR-Cas9 Knockout

To study the role of *ITGA6* expression during the derivation of cardiomyocytes, hESCs with Cas9 were generated utilizing the GE Healthcare Dharmacon Edit-R Doxycycline-inducible CRISPR-Cas9 Gene Engineering with Lentiviral Cas9 and sgRNA system (GE, Boston, MA, ge.com). The multiplicity of infection of lentiviral particles was calculated to be 0.3. To perform the infection, 0.67 µL of Cas9 lentivirus were suspended in 500µL of OptiMEM medium (Gibco). H9 hESCs were cultured pre- and post-infection as indicated above. On the third day post-infection, selection of Cas9 positive cells was initiated with selection medium supplemented with 10µm/mL rock inhibitor (Y27632; Stem Cell Technologies, Vancouver, Canada) and 0.6 µg/mL blasticidin (InvivoGen, San Diego, CA), as the Cas9 construct contains

a blasticidin-resistant cassette. The selection process continued for 12 days, in which the medium with blasticidin was changed every other day and rock inhibitor was removed. The cells were expanded until approximately 90-100% were obtained, then cells were passaged in 35mm tissue culture plates or frozen until further use. We then utilized three independent single guided (sg) RNA constructs targeting different nucleotide sequences specific for *ITGA6* and one control sg that contained random nucleotides that did not target any gene in particular. The sgRNA constructs targeting *ITGA6* were identified following that last two digits of the ID provided by supplier, as CRISPR-Cas9/10, 14, and 15 (Fig 1). The volume of sgRNA utilized was 1μL for each sgRNA utilized based on an approximate MOI of 0.3. The 1μL of sgRNA lentivirus was added to 500 μL of OptiMEM (Gibco) for each respective *ITGA6* knockout sgRNA (control, 10, 14, and 15) to create the transduction medium without antibiotics and antimitotic. The H9 Cas9 were then infected with the transduction medium and left to incubate for 12 hours. After 12 hours, 1mL of DMEM/F12 media was added. Three days after infection, the media was changed to selection medium with 0.3 μg/mL blasticidin (InvivoGen), and 0.4 μg/mL puromycin (Gibco), to select the sgRNA positive cells utilizing a puromycin-resistant cassette. The cells were then expanded or frozen for further experiments. Once grown to Day 3 in the cardiomyocyte differentiation protocol the media was supplemented with 1μg/mL doxycycline (Alfa Aesar, Haverhill, MA) to induce the recombination of the CRISPR/CAS9 system targeting *ITGA6*. Through flow cytometry, it was found that CRISPR Cas9 sg10 had the greatest effect on *ITGA6*, thus this was the cell line of choice for subsequent experiments, and therefore data shown in results was obtained with this cell line.

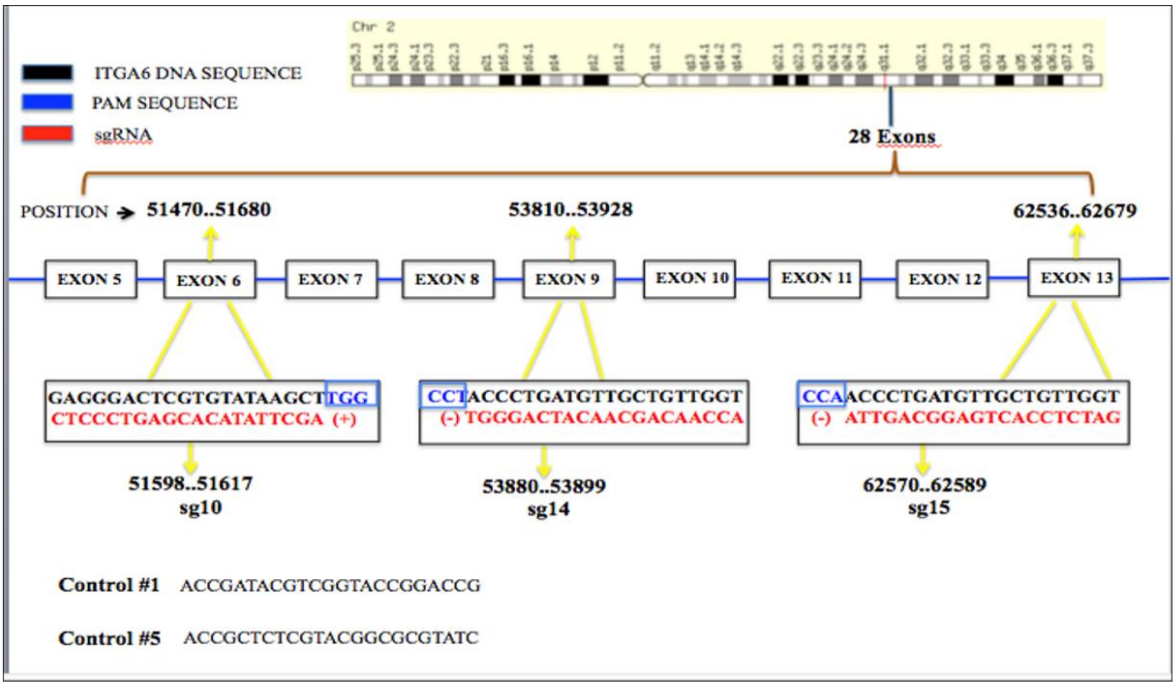

Fig. 1 Illustration indicating nucleotide sequences and their localization in the gene of *ITGA6* to induce its knockdown. Three single RNAs were selected to carry out mutations of ITGA6 in H9 hESCs cells using an inducible CRISPR cas9 system. Representative random nucleotide sequences are indicated as well as the controls.

Flow Cytometry

CRISPR cas9 sg10 cells were collected using trypsin 0.25% EDTA, and single cell suspensions were washed in cold bovine serum albumin (BSA) 0.5% (wt/vol) in Dulbecco's phosphate-buffered saline (DPBS) and incubated at a concentration of 1 x 10^6 cells per milliliter in 1 g/ml unconjugated goat anti-human IgG (Invitrogen) on ice for 15 minutes, to block nonspecific protein binding. Samples were incubated on ice with optimal dilution of fluorochrome-conjugated monoclonal antibodies (mAbs) in dark, and to further control for nonspecific detection, control samples were incubated with phycoerythrin (PE) mouse immunoglobulin G1 (IgG1) j isotype control and FITC mouse IgG1 isotype control. All mAbs and isotype controls

were of the IgG1 isotype and from BD Biosciences. The conjugated antibody fluorescein isothiocyanate (FITC)-conjugated against CD49f (ITGA6) was used. After 30 minutes incubation, cells were washed twice with ice cold 0.5% BSA/DPBS. At least 10,000 events were acquired for each sample using a FACSCalibur instrument (BD Biosciences) and cell flow cytometry data were analyzed using FlowJo software (BD Biosciences). Human embryonic stem cells were analyzed by flow cytometry as controls.

Results

Derivation of cardiac related cell types

Figure 2 shows that the cardiac differentiation protocol is producing all three cell types that are present in the heart. This particularly highlights that we are able to obtain cardiomyocytes, as we observed beating cells (Video can be observed in Luis Villa-Diaz's lab webpage). We characterized these cells with Pecam-1, which marks for epithelial cells, Smooth Muscle Actin (SMA), which marks for smooth muscle, and Troponin, which marks for cardiomyocytes.

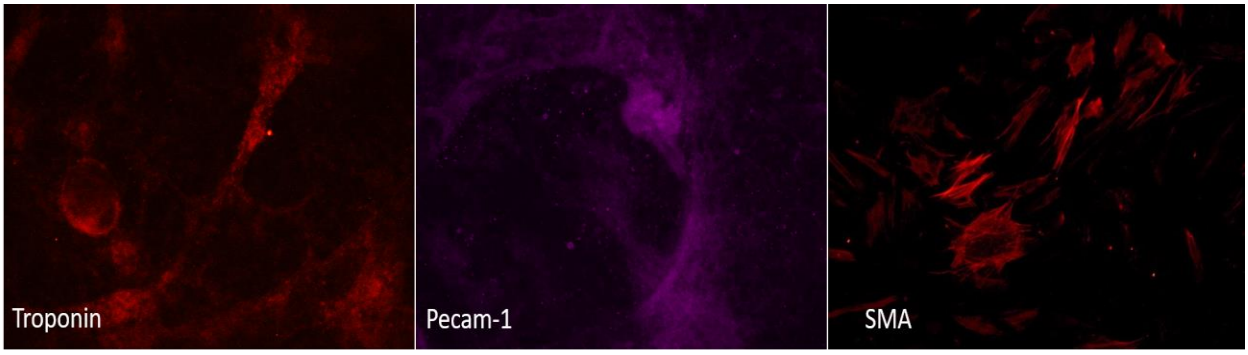

Fig. 2 Differentiation of hESCs into cardiac related cell lines. Representative micrographs showing positive expression of Troponin, Pecam-1 and Smooth Muscle Actin indicating the presence of cardiomyocytes, endothelial cells and smooth muscle cells respectively.

Further evidence of Cardiomyocytes

Cardiomyocytes are characterized by the expression of several markers. Here we show further evidence that cardiomyocytes were reached with early and mature cardiomyocyte markers in figures 3A to 3F.

Early:

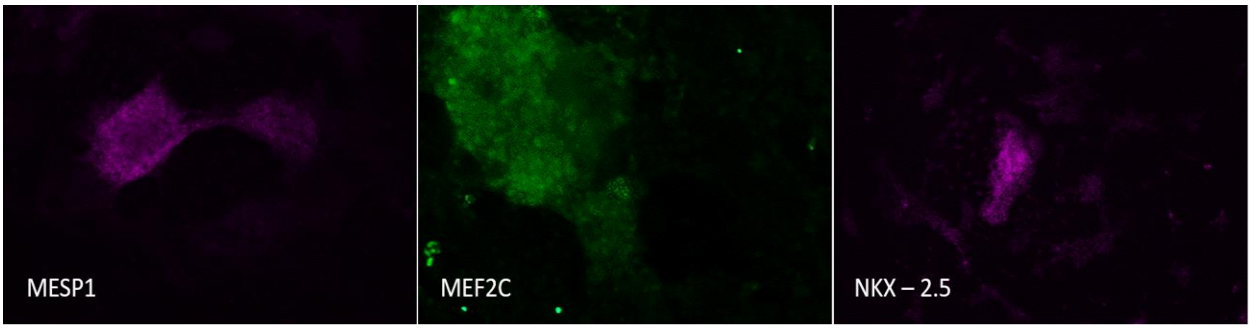

Fig. 3A-C Early markers of cardiomyocyte development. Representative micrographs showing positive expression of: **(A)** MESP1 in hESCs on day 3. This transcription factor is the earliest determined marker in cardiac development. **(B)** MEF2C in hESCs on day 3. This is another important transcription factor in cardiac development. **(C)** Nkx -2.5 in hESCs on day 3. This is a significant transcription factor in development of ventricular cardiomyocytes.

Mature:

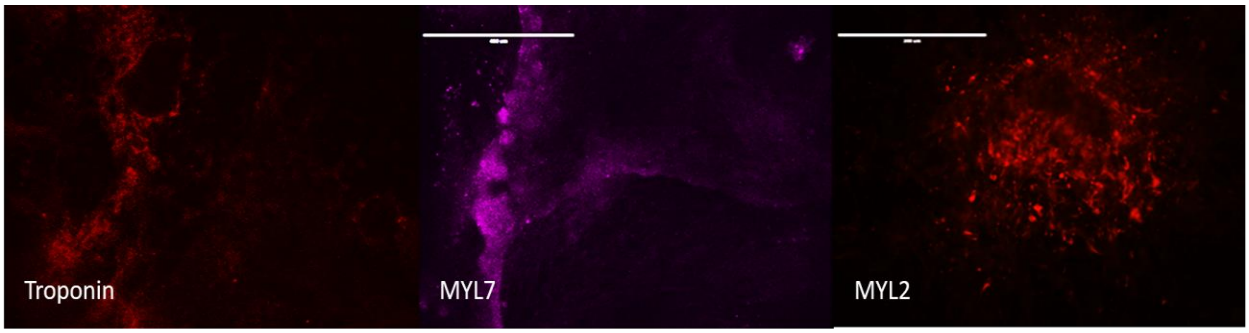

Fig. 3D-F Late markers of cardiomyocyte development. Representative micrographs showing positive expression of: **(A)** Troponin in hESCs on day 11. This protein marks for mature cardiomyocyte formation. **(B)** MYL7 in hESCs on day 14. This protein marks for mature atrial cardiomyocyte formation. **(C)** MYL2 in hESCs on day 14. This protein marks for mature ventricle cardiomyocyte formation.

Effect on ITGA6 knock-out on cardiomyocytes differentiation

Normally in the differentiation protocol, it has become more apparent where these cardiomyocytes tend to form. There are large accumulations of cells with a 3-D dome like appearance. Later, these areas become smoother and more spheroid, where beating of the cells has been observed (Fig. 4).

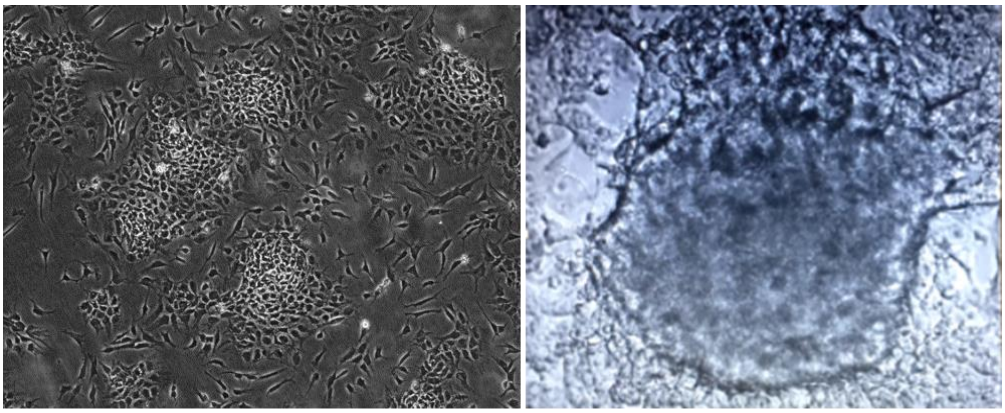

Fig. 4 Formation of a monolayer to cardiomyocytes. Representative bright field image showing the general morphology of the accumulations we observed in the control trial of cardiomyocytes. Beating was often observed in this sort of formation.

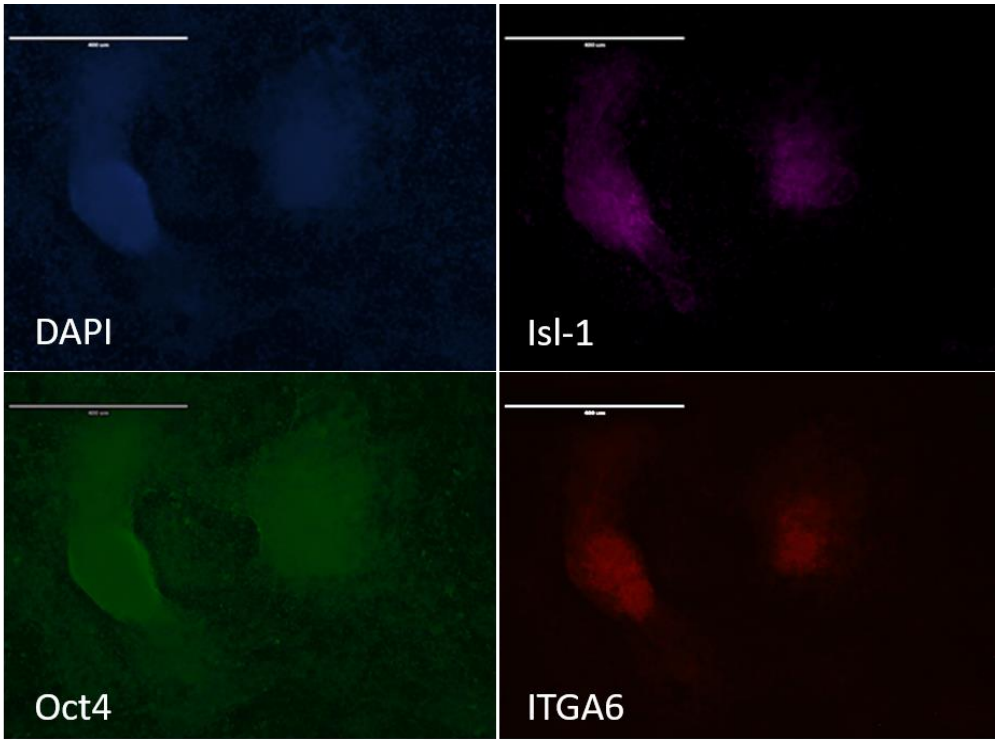

Fig. 5 Formation of a monolayer to cardiomyocytes. Representative micrographs showing the expression of ITGA6, Isl-1 and Oct4 in hESCs on day 8.

Characterization of these cell accumulations indicated that ITGA6, Isl-1 and Oct4 are expressed in them (Fig 5). This co-localization of stem cell like markers supports our hypothesis that ITGA6 is expressed in CaSCs, and may play a role in development of cardiomyocytes. These same accumulations were not found in the H9 Cas9 *ITGA6*-knockout cells. Instead a few, very small, non-contracting spheres were found. These structures contain Isl-1 and Oct4 but not ITGA6 (data below Fig 7).

Analysis of Integrin Alpha6 knockout

To verify the knockdown of ITGA6 in the CRISPR Cas9 cells, we performed flow cytometry and compared these cells to wild-type H9 cells, as controls (Fig 6). We observed that ~79% of control cells have expression CD49f, while the CRISPR human H9 cells showed only in ~16% of cells, indicating the knockdown of ITGA6. We characterized the expression of SSEA4 as control to indicate that these cells were undifferentiated hESCs. Interestingly we observed that in the control cells ~63% of cells were positive for this markers while its expression decrease in the itga6 knockdown cells to ~11%. This corroborate previous indications that interference with the expression of itga6 induce differentiation.

The H9 Cas9 ITGA6-knock out cells were differentiated using the cardiomyocyte protocol as described previously. These cells were fixed on Day 6 and Day 8. Fixing the cells on these days allowed us to view the expression of ITGA6, Isl-1 and Oct-4 before and after the major changes that occur during Day 7.

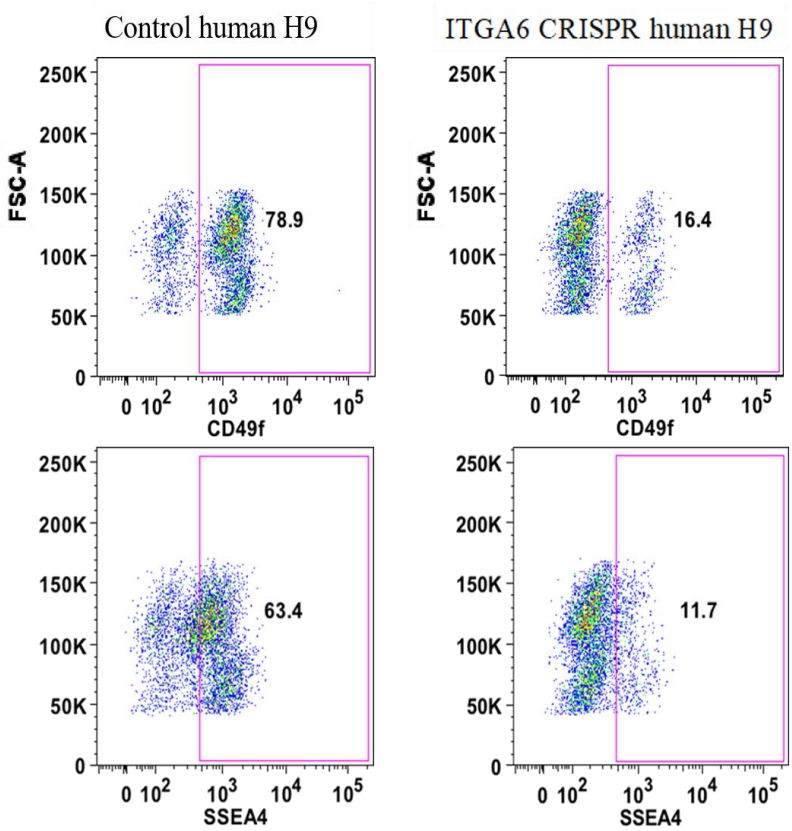

Fig. 6 Illustration of cytograms indicating the knockdown of ITGA6 to induce its knockdown. In the control H9 hESCs, ITGA6 was originally at 78.9% and after the sg10 construct knockout, it was reduced to 16.4%. SSEA4 is a marker for stemness. In the controls, there was 63.4% of expression and, after the sg10 construct knockout, there was a reduction to 11.7%.

Day 6 (144 hours) results:

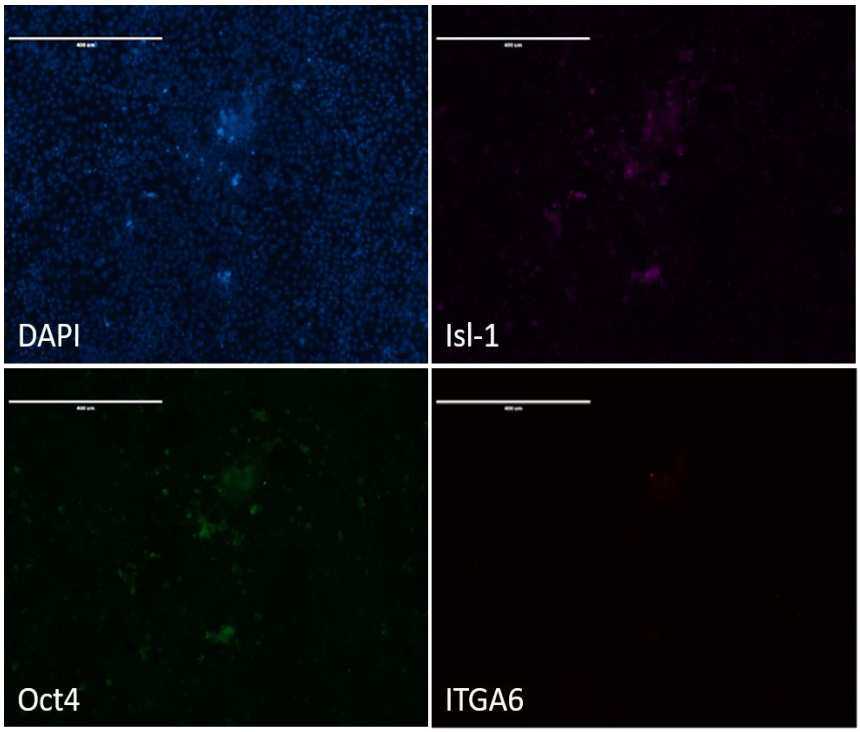

Fig. 7 CRISPR H9 cas9 sg10 knockout ICC. Representative micrographs showing the knockdown of ITGA6. These plates were fixed on day 6. No accumulations or spheres formed and beating did not occur in these cells.

As seen in figure 7, no ITGA6 was expressed in the CRISPR H9 cas9 sg10 cells. However, there seems to be only slight expression of Isl-1 and Oct4. No accumulations or spheres formed and no beating occurred in these cells. This supports that ITGA6 may play an important role in the development of cardiomyocytes.

Day 8 (192 hours) results:

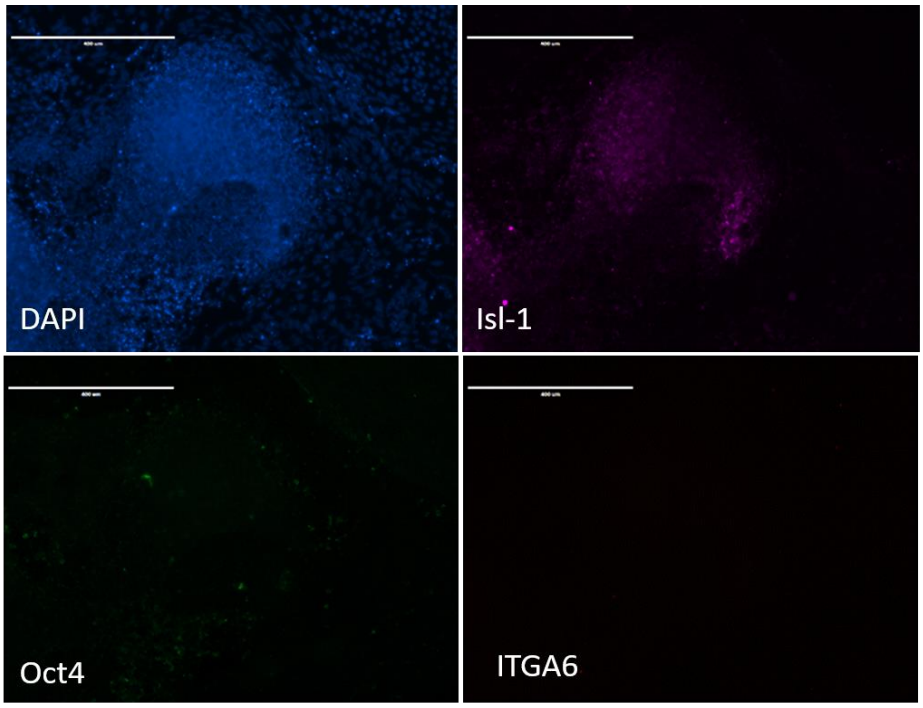

Fig. 8 CRISPR H9 cas9 sg10 knockout ICC. Representative micrographs showing the knockdown of ITGA6. These plates were fixed on day 8. No accumulations or spheres formed and no beating occurred in these cells.

As seen in figure 8, no ITGA6 was expressed in the CRISPR H9 cas9 sg10 cells. However, there seems to be only slight expression of Isl-1 and Oct4. No accumulations or spheres formed and no beating occurred in these cells. This further supports that ITGA6 may play an important role in the development of cardiomyocytes.

Discussion

Our protocol differentiated hESCs into cardiomyocytes, and somewhere during this differentiation a subpopulation of cardiac stem cells emerged temporally. The CaSCs give rise also to endothelial cells and smooth muscle cells that are involved in the heart vasculature. To confirm this we performed ICC to observe the expression levels of proteins that identify for different cell types. We focused primarily on the formation of cardiomyocytes and demonstrated the formation of both atrial and ventricular cardiomyocytes as indicated by the expression of MYL7 and MYL2, respectively. Furthermore, we observed spontaneous beating cells in our experimental control group.

ITGA6 has shown to play a role in maintenance of hESC's pluripotency, and furthermore, it has been identified in 35 populations of stem cells, including cardiac stem cells isolated from adult tissue. Therefore, we develop the hypothesis that the production of cardiomyocytes from human ESCs should rely in a cardiac stem cells that express ITGA6. We also used ICC to identify this population expressing ITGA6 and Isl-1, a transcription factor recognized as a CaSCs.

To test the role of ITGA6 in the development and function of CaSCs and downstream cell lineages, especially into cardiomyocytes, we developed a system to regulate its expression at specific times. We use a dox-inducible CRISPR Cas9 system that target specific exons.

After setting up the CRISPR Cas9 system, we used flow cytometry to determine how effective the single guide construct 10 was. In the control H9 hESCs, *ITGA6* was originally at 78.9% and after the sg10 construct knockout, it was reduced to 16.4%. SEEA4 is a marker for

stemness in cells. In the controls, there was 63.4% of expression, and after the sg10 construct knockout, it was reduced to 11.7%.

From the CRISPR Cas9 knockout, we were able to observe the effects on cardiomyocyte development without ITGA6. The morphology of the accumulation of cells containing putative CaSCs in the knockout cells were smaller than and not as frequent as previous observations. The accumulations that expressed the three markers, ITGA6, Isl-1 and Oct-4, were not as prominent or as large during growth. This is also confirmed in the ICC images, showing less Oct4 and Isl-1 expressions along with the knockout of ITGA6.

Future experiments will be performed to further analyze the Cas9 cell lines and the mechanisms of ITGA6 in the formation of cardiomyocytes. Then, experiments will be done in order to isolate the Isl-1+ subpopulation in an attempt to establish a true cardiac stem cell line. Later, a further investigation of ITGA6 in the cardiac stem cells will be done in order to discover the mechanism in the transmembrane receptor impact the development and function of these cells.

Conclusion

This experiment was performed to determine the role of ITGA6 in cardiomyocytes. ITGA6 has been shown to be expressed in many stem cell populations and play a key role in stem cell self-renewal. This experiment supports that ITGA6 may play a critical role in the formation of cardiomyocytes and, in turn, human cardiac stem cells (hCaSCs). When ITGA6 was knocked out using the CRISPR cas9 technique, we found that accumulations did not form, spheroid structures were not made and no beating tissue was obtained. Knockout of ITGA6 also yielded much lower expression of Isl-1, a marker of cardiac stem cells, and Oct4, a marker of pluripotency. Therefore, ITGA6 is likely needed in the formation of cardiac stem cells, which then yield cardiomyocytes.

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