An investigation of the role of integrin *alpha-6* in human induced pluripotent stem cell development and pluripotency

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Abstract

The general functions of integrins in attachment, gene expression, motility, polarity, shape, proliferation, and survival are well known. These critical functions provide reason for their wide expression across cell types. In particular, integrins are expressed in varying stem cell populations as well as other cell populations throughout the human body. 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 embryonic pluripotent stem cell self-renewal (Villa-Diaz 2016). Human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC) share the same morphology and gene expression but they do not share the same origin, since hESCs are obtained from the inner cell mass of blastocyst embryos, while hiPSCs are derived from somatic cells via genetic reprogramming. This similarity is what makes hiPSCs so widely applicable to regenerative medicine as they are more ethically obtained and derived. In this research, hiPSCs were utilized as a model to determine the role of *ITGA6* in hiPSC development and pluripotency.

To address this objective, we knocked out *ITGA6* in human gingival fibroblasts using 3 constructs of CRISPR-Cas9 single-guided (sg) RNAs that target specific sequences of nucleic acids in *ITGA6,* and we utilized a CRISPR-Cas9 sg control, which did not target any specific sequence of nucleic acids, and WT human gingival fibroblasts as control groups. These five groups of human gingival fibroblasts were then reprogrammed utilizing 4 crucial factors to generate iPSCs: *Oct4, Sox2*, *Klf4* and *c-Myc* (Takahashi & Yamanaka 2006). Twenty-eight days post-infection the reprogrammed fibroblasts were passaged upon colony formation, or used for RNA and protein expression analysis by qRT-PCR and immunocytochemistry (ICC), respectively. Colony formation, RNA expression, and protein translation, were compared to the wildtype fibroblast-group. Pluripotency was determined by the expression of key markers of

pluripotent stem cells such as *Sox2, NANOG, KLF-4*, and Oct4. Development of hiPSCs was tested through the proliferation of undifferentiated colonies over a two-week period.

We confirmed that vectors targeting *ITGA6* expressed significantly lower levels of ITGA6 mRNA, with Cas9 sg*ITGA6* 15 consistently having the lowest expression of *ITGA6* across the three independent replicates. As expected, formation of hiPSCs was obtained from the wild-type group. The groups from CRISPR-Cas9 sg*ITGA6* 10 and 14 groups, as well as the control developed an average of 3-5 colonies per replicate, which expressed ITGA6 and were able to passage and survive. In the Cas9/15 group only one colony developed within the thee replicates, which was consistent with its lower *ITGA6* mRNA expression. However, the one colony that did develop also expressed *ITGA6*. All together these data suggest that the knock-out of this gene did not have 100% efficiency because every colony that developed expressed *ITGA6*, which led us to the conclude that *ITGA6* is crucial for the development and maintenance of hiPSCs.

Introduction

Stem cells function in tissue homeostasis, repair, and the regeneration of tissues and organs, contributing to the maintenance of living organisms (Singh 2012). These functions have led to a deep interest in the mechanisms behind these functions. 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 pluripotent embryonic stem cells (ESC). The use of ESCs is riddled with ethical and immunological complications due to their origin. Human induced pluripotent stem cells (hiPSCs) avoid this issue because they are generated from the patient's own tissue, typically from fibroblasts, and because of their pluripotency, can then be differentiated into any cell lineage from any of the germ layers.

The characterization of hiPSCs is similar to that of ESCs because they share the expression and translation of the same genes and proteins. Human iPSCs are derived from somatic cells by overexpression of transcription factors that include *Klf4, C-myc, Sox2,* and *Oct3/4* (Takahashi & Yamanaka 2006). These critical genes, lead to the expression of *NANOG*, another pluripotent transcription factor that completes the pluripotent circuit, and the parental cells become reprogramed into iPSCs. Although these transcription factors play critical roles in the generation of iPSCs, it is possible that there may be other genes and proteins that are critical to the derivation and maintenance of iPSCs.

Integrins, in general terms, are heterodimeric transmembrane cell surface receptors that function in signaling cascades associated with attachment, differentiation, gene expression, motility, polarity, proliferation, shape, and survival of cells (Hynes 2002, Watt 2002). These proteins also associate with specific extracellular matrix components which function in interactions between neighboring cells and communication between intercellular and

extracellular environments of a cell (Hynes 2002, Watt 2002). Integrins are also utilized during attachment of stem cells to substrates *in vitro* (Vitillo 2016). Human pluripotent stem cells (hESCs and hiPSCs) require extracellular matrix proteins, such as fibronectin, laminins, and vitronectin to maintain self-renewal. Extracellular matrix proteins, such as laminin 1 and 5, are ligands of ITGα6β1 which aid in cell adhesion and these are present throughout many stem cell populations (Dogic et al. 1998). The crucial role of integrins in general cell maintenance and in particular the wide expression of ITGA6 across varying stem cell types has generated the hypothesis that this particular integrin isoform may play a critical role in development, maintenance, self-renewal, and pluripotency of hiPSCs.

Chapter I

Introduction

Integrins are heterodimeric transmembrane proteins, which initiate signal transduction cascades leading to the activation of a wide variety of proteins and transcription factors, and modifications to the cytoskeleton. Although integrins, as a whole, retain similar functions, there is a large amount of variability in the specificity of the pathways in which each integrin plays a role. Specifically, *ITGA6* is known to play roles in hESC self-renewal, attachment, survival, and other important cellular functions. This specific member of the integrin family, *ITGA6*, is expressed in more than 30 stem cell populations (Villa-Diaz personal communication), and functions critically in attachment and niche formation. Although this wide expression is well noted, the exact role of *ITGA6* in stem cells remains undetermined.

Integrins are heterodimeric cell surface receptors with alpha (α) and beta (β) subunits resulting in 24 heterodimer combinations in humans. When an α and β subunit join, the protein receptor becomes active and can sense whether a cell has bound to some extracellular site, such as the ECM or a neighboring cell. This protein complex is crucial to the maintenance of stem cells because attachment and cell-to-cell communication is crucial to the survival of cells. The gene of interest, *ITGA6* specifically functions mainly as a receptor for laminin, an ECM protein.

Induced pluripotent stem cells are indistinguishable from ESCs in morphology, proliferation, gene expression, and capability of teratoma formation but they do not share origin (Takahashi et al. 2007). In a trial experiment conducted with hESC H9-cell line and hiPSCs, the knockout of *ITGA6* by shRNA caused the differentiation of the cells, suggesting that it is crucial to cell maintenance, self-renewal, and pluripotency (Villa-Diaz et al. 2016). However, it remains

to be determined whether *ITGA6* is required for the development of pluripotent stem cells. The genetic reprogramming of somatic cells into iPSCs allow us to determine this, and therefore the present study is designed to test the following hypothesis: ITGA6 plays a critical role in hiPSC development and pluripotency.

Materials and Methods

Replicates

Each of the following reprogramming and analytic procedures were performed in triplicates.

Cell Culture

Human gingival fibroblasts (hGF) were grown in fibroblast medium in a 37˚C incubator at 5.0% CO_2 and high humidity. The fibroblast medium consisted of alpha-MEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Seradigm, Radnor, PA), 1X glutamine (Gibco), 1X non-essential amino acids (Gibco), and 1X antibiotic and antimitotic (Gibco). The hGFs were expanded in 750mL filtered flasks and media was changed every other day. The media was changed to fibroblast medium without antibiotic and antimitotic at least two days prior to reprogramming. The cells were treated with fibroblast medium with antibiotic and antimitotic (Gibco) post-infection. Four days post-infection, fibroblasts were grown in reprogramming medium, which consisted of 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 antibiotic and antimitotic (Gibco), and 10μm/mL rock inhibitor (Y27632; Stem Cell Technologies, Vancouver, Canada). Reprogramming medium was changed every other day.

Reprogramming of fibroblasts into hiPSCs

Approximately 150,000-250,000 hGFs were transfected with retrovirus containing pMXs constructs for wild type *Oct4, Sox2, Klf4,* and *c-Myc* (Takahashi & Yamanaka 2006), in fibroblast medium without antibiotic and antimitotic (Gibco), and supplemented with 10μg/mL

Polybrene (Sigma-Aldrich, Milwaukee, WI) to promote retroviral infection. Twelve hours post-infection, the medium was replaced with fibroblast medium with antibiotic and antimitotic (Gibco). Forty-eight hours post-infection, fibroblasts were detached from the plate of infection utilizing 0.25%-EDTA trypsin (Gibco), which was inactivated with fibroblast medium. Cells were re-plated on Matrigel coated plates (MCPs) of varying size (BD Matrigel hESC-qualified Matrix; BD Biosciences, San Diego, CA, http://www. bdbiosciences.com). MCPs were prepared the day prior to passage according to the manufacturer's instructions. The cells were passaged and grown in fibroblast medium with antibiotic and antimitotic (Gibco) and 10μm/mL rock inhibitor (Stem Cell Technologies) until attachment was obvious, then the media was changed to reprogramming medium to promote iPSC colony formation, maintenance, and self-renewal. Reprogramming efficiency was calculated utilizing the number of viable developed colonies divided by the total number of cells initially plated for reprogramming.

Human Induced Pluripotent Stem Cell Colony Characterization

Undifferentiated colonies were identified with tight and defined borders with the internal cells having a large nucleus and minimal cytoplasm. The cells were also defined by their expression of *Oct4* and *Sox2* by analysis of their relative mRNA and protein translation by quantitative (q)RT PCR and immunocytochemistry (ICC), respectively.

CRISPR-Cas9 Knockout

To study the effect of *ITGA6* expression during the derivation of hiPSCs, human gingival fibroblasts (hGF) that lacked the ability to express *ITGA6* 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, 0.67μL of Cas9 lentivirus

in 500µL of DMEM/F12 (Gibco) was utilized. Human gingival fibroblasts (100,000) were cultured pre- and post-infection as indicated above, in order to be infected with the lentivirus encoding the Cas9 construct. On the third day post-infection, selection of Cas9 positive cells was initiated with fibroblast medium supplemented with 0.6µg/mL blasticidin (InvivoGen, San Diego, CA), since the Cas9 construct contained a blasticidin-resistant cassette. The selection process progressed for 10 days, in which the medium with blasticidin was changed every other day. The cells were expanded until approximately 1.2 million cells were obtained, then cells were replated in 4 wells of a 6-well plate to process with the infection of 3 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. 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 500µL of DMEM/F12 (Gibco) for each respective *ITGA6* knockout sgRNA (control, 10, 14, and 15) to create the transduction medium without antibiotics and antimitotic. The fibroblasts were then infected with the transduction medium and left to incubate for 12 hours. After 12 hours, 1mL of MEF media was added. Three days after infection, the media was changed to fibroblast medium with 0.3µg/mL blasticidin (InvivoGen), and 0.5µg/mL puromycin (Gibco), to select the sgRNA positive cells utilizing a puromycin-resistant cassette. The process of selection lasted 10 days. The cells were then expanded for further experiments and then cultured with fibroblast medium supplemented with 1µg/mL doxycycline (Alfa Aesar, Haverhill, MA) to induce the recombination of the CRISPR/CAS9 system targeting *ITGA6*. After 24 hours, 1µg/mL doxycycline (Alfa Aesar) was added without media change. The fibroblasts were

maintained with 0.3μg/mL blasticidin (Alfa Aesar), and 0.25μg/mL puromycin (Gibco), expanded, and either used for further experiments or frozen and maintained in liquid nitrogen as a stock. These fibroblasts were then reprogrammed according to the reprogramming method listed above.

RNA Isolation, purification and quantification

For RNA extraction, cells were washed once with PBS, then treated with 700 μl TRIzol (Invitrogen) and stored at -80˚C until further use. RNA was then purified utilizing Direct-zol RNA Miniprep Plus (Zymo Research, Irvine, CA) with slight modifications. RNA was eluted in 25μL DNase/RNase free water rather than the 100μL recommended by the manufacturer. The purified RNA was then quantified utilizing the Nucleic Acid quantification system on a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA).

Amplification of cDNA

Reverse transcription was performed utilizing SuperScript III First Strand cDNA synthesis with random hexamers (Invitrogen, Waltham, MA). For each sample, 500ng of RNA was reverse transcribed into cDNA for use in qRT PCR. The reactions were carried out following the First-Strand cDNA synthesis instructions provided by the manufacturer; the tubes were incubated at 65˚C for 5 minutes then placed on ice for 1 minute, a cDNA synthesis mixture was added to each tube, then they were incubated at 25˚C for 10 minutes, then 50˚C for 50 minutes, then terminated at 85˚C for 5 minutes, RNase H was added and then the tubes were incubated at 37˚C for 20 minutes (Invitrogen). cDNA was kept at -20˚C until use.

Quantitative (q)RT PCR

Quantitative RT PCR was performed utilizing the cDNA prepared utilizing the method listed above which was diluted with 80μl of DNAse/RNAse-free water. The qRT-PCR was

performed utilizing 2µl of the diluted cDNA with the TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, California) and TaqMan Gene Expression Assays Probes (Applied Biosystems). The reactions were carried out as follows: 2 minutes at 50˚C, 10 minutes at 95˚C, then 40 cycles of 15 seconds at 95˚C and 1 minute at 60˚C. PCR and quantification were performed utilizing a Bio-Rad CFX Connect system (Bio-Rad Laboratories, Berkeley, CA). Relative RNA expression was analyzed with $\Delta\Delta C_q$ utilizing GAPDH as a standard. Expression levels of genes were compared to WT control samples and reported as fold changes, with a 2-fold change considered statistically significant.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature (RT). The cells were stored in PBS, sealed with parafilm, and stored at 4˚C until ICC was performed. Cells were treated with unmasking, quenching, and permeabilization solution for ten minutes each at RT. Cells were then treated with blocking solution for one hour at RT. The first protein specific antibody was incubated at 4˚C for 12 hours according to the manufacturer's recommended dilution in 1% normal serum (Table 1). After washing with PBS 3 times for 10 minutes each, the cells were incubated in secondary fluorescent-labeled antibody at RT 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 fix the antibodies and then stored at 4˚C in dark conditions. A control which contained only secondary antibody was utilized. The cells were visualized and captured utilizing a 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	1:400	Millipore
NANOG	Mouse	1:200	Millipore
Sox2	Rabbit	1:500	Millipore

Temporal Expression of *ITGA6*

An initial induction experiment was performed in order to determine when *ITGA6* is first expressed in a population of fibroblasts reprogrammed into iPSCs. Approximately 240,000 hGF cells were plated into one well of a 6-well plate with fibroblast medium without antibiotic and antimitotic. Twenty-four hours after the initial plating, hGFs were infected utilizing the reprogramming medium and procedure described above. Four days post-infection, the fibroblasts were detached from the 6-well plate then the cells were equally divided into 11 wells of a 12-well MCP and 3-60mm MCPs. The cells were passaged and grown in fibroblast medium until attachment was obvious, then the media was changed to reprogramming medium.

The 11 wells were then fixed every other day beginning with day 6 post-infection and ending on day 26 post-infection. Immunocytochemistry was performed to determine the expression of *ITGA6* and *Oct4* over the 2-week period that the experiment took place.

Results

Kinetic analysis in the expression of Integrin α6 during reprogramming of hGFs

ITGA6 expression was varied and did not appear until the first colony developed on day 26 post-infection. There was neither expression of ITGA6 nor of Oct4 in groups between day 6 to 24 days post-infection. At day 26 post-infection, several colonies formed showing co-expression of ITGA6 and Oct4 (Figure 1).

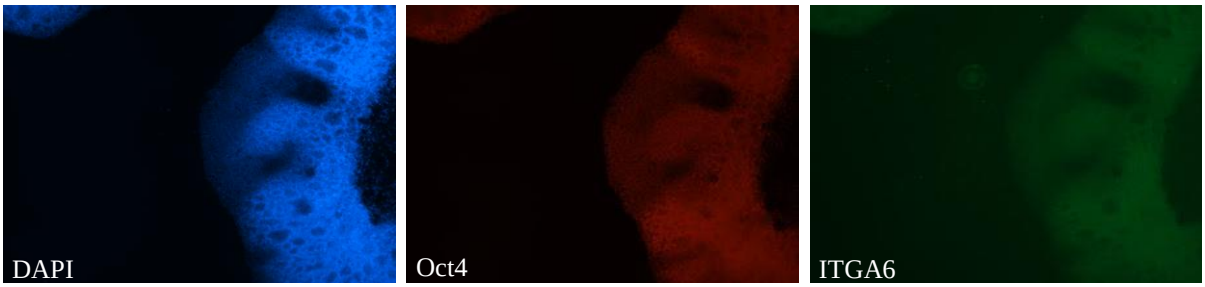

Figure 1 Representative micrographs of hiPSCs colonies co-expressing ITGA6 and Oct4 26 days post-infection

Integrin alpha-6 knockout

The reprogramming of the CRISPR-Cas9 *ITGA6* knockout sgRNA cell lines resulted in a reduction or elimination of colony development compared to the reprogrammed WT hGFs. Specific populations of cells, which were later determined to be colonies, expressed ITGA6 in CRISPR-Cas9 groups. Along with the reduction in colony development there was a reduction in *ITGA6* mRNA expression (Figure 2). The expression of *ITGA6, Oct4,* and *Sox2* was generally consistent across the three independent trials with the most consistency between trials 2 and 3. Cell populations that did not express *ITGA6* were unable to form colonies with the appropriate classical morphology and also lacked the expression of other key factors.

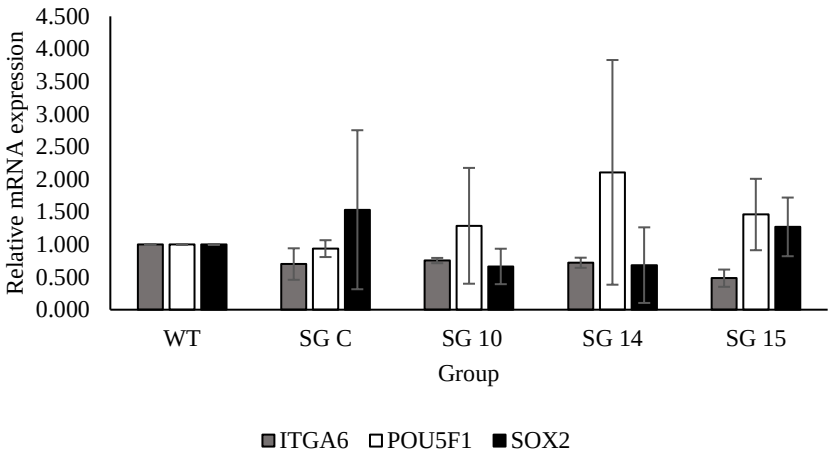

Figure 2 mRNA expression of key factors *POU5F1* (*Oct4*), *Sox2*, and *ITGA6* in reprogrammed hGFs 3 days post-infection

In the first experimental set, colonies developed in the wild-type and Cas9/10 groups (Table 2). There was a minimal reprogramming efficiency in the CRISPR-Cas9 knockout cell lines in which only one recoverable colony developed in Cas9/10. This colony tested positive for co-expression of ITGA6 and Oct4 (Figure 3). The colony was determined to be viable and fully reprogrammed according to its gene expression and morphology. The reprogramming efficiency in the wild-type group was approximately 0.05%. The colonies that developed in the other cell lines co-expressed *Sox2* and *Oct4* and each of these viable colonies also co-expressed *ITGA6* (Figure 4).

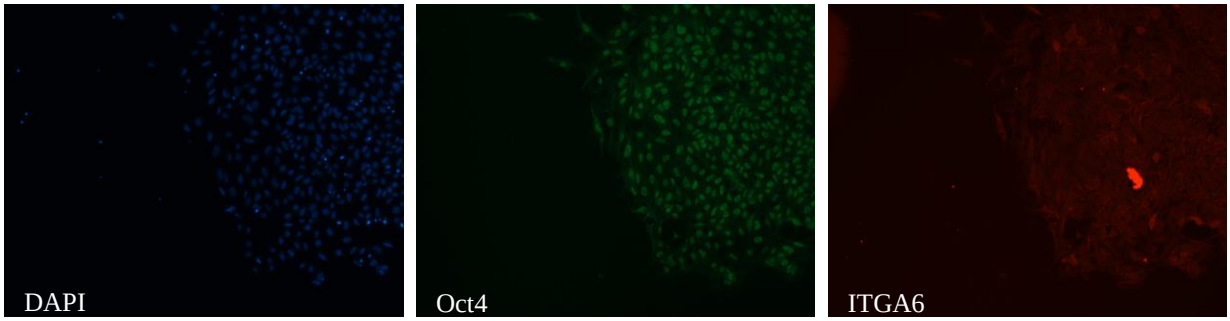

Figure 3 Representative micrographs of hiPSCs co-expressing *ITGA6* and Oct4 in reprogrammed Cas9/10 group

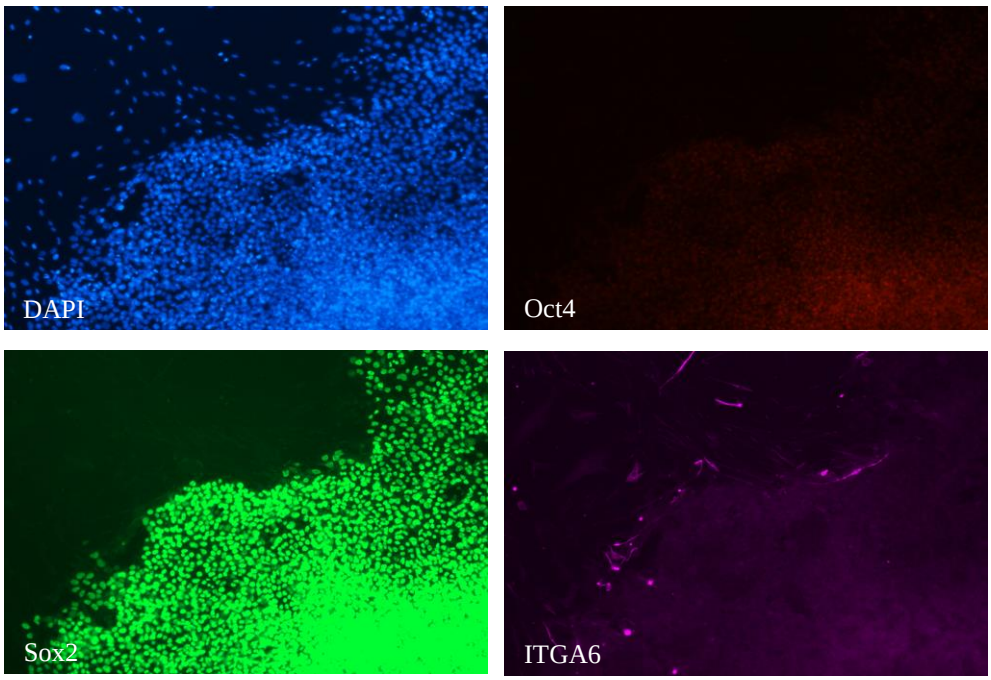

Figure 4 Representative micrographs of hiPSCs from wild type groups showing co-expression of Oct4, Sox2 and ITGA6

The second and third experimental sets were identical and performed simultaneously but they differed from the first experimental set in medium. The cells were not treated with puromycin nor blasticidin for maintenance as in the first trial. There was an increased reprogramming efficiency in trial 2 with each cell lines producing at least one viable colony (Table 2). The first Cas9/15 expandable colony developed in trial 3 and was the only colony passaged from the Cas9/15 cell line. Colonies that developed and were recovered showed co-expression of ITGA6 and Oct4 (Figure 5). The colonies that did not appear viable for passage due to maturity or improper morphology showed co-expression of *Oct4* and *Sox2* and decreased expression of ITGA6—though it was still present—compared to the reprogrammed WT. Oct4 was localized to the cytoplasm in the individual Cas9 knockout cells while those cells that resided within a colony expressed Oct4 solely in the nucleus; the cells which expressed Oct4 solely in the nucleus co-expressed ITGA6 (Figure 6).

Table 2 Average colony development per plate of reprogrammed fibroblasts

Cell line	Trial 1	Trial 2	Trial 3
WT	65	4	32
Cas9 Control	0	1	1
Cas9 10	1	1	5
Cas9 14	0	3	1
Cas9 15	0	1	1

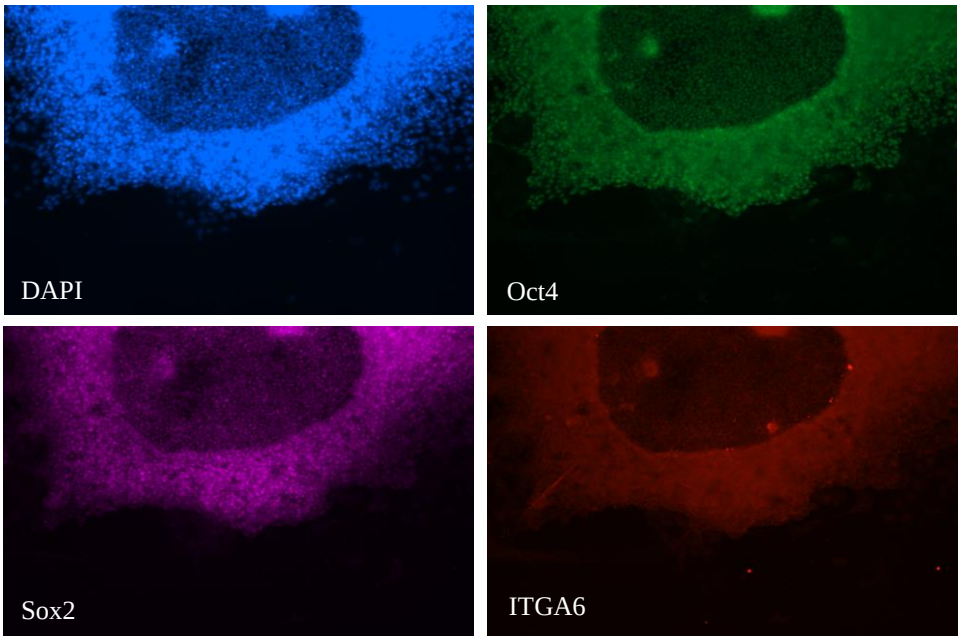

Figure 5 Representative micrographs of hiPSCS from Cas9/10 group showing co-expression of Oct4, Sox2, and ITGA6

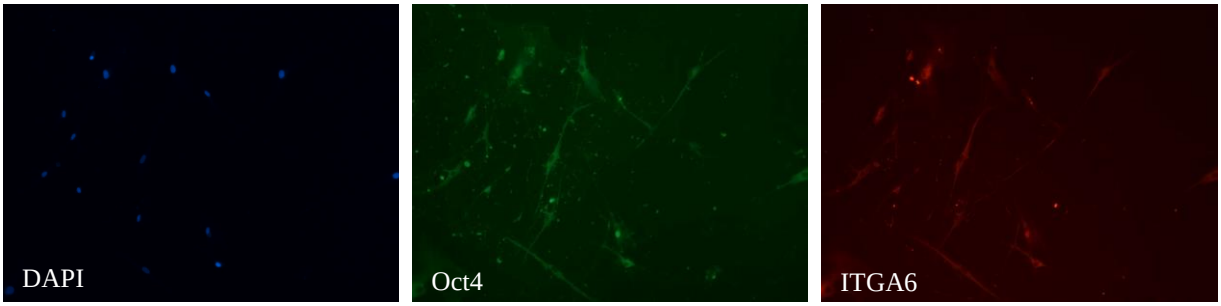

Figure 6 Representative micrographs of hiPSCs from Cas9/14 showing cytoplasmic expression of Oct4

The expression of *ITGA6* was consistently the lowest in the Cas9/15 cell line across each of the trials. Cas9/15 was determined to have the greatest knockout effectiveness and was therefore compared to the WT for further analysis. qRT PCR consistently showed the lowest expression of *ITGA6* across the three trials in the Cas9/15 cell lines (Figure 2). Cas9/15 also developed only one colony within trials 2 and 3, with only one of these colonies gaining maturity for successful passage (Table 2). This colony showed co-expression of Oct4 and ITGA6 and showed the classical morphology (Figure 7), suggesting that the CRISPR-Cas9 knockout of *ITGA6* was not 100% effective.

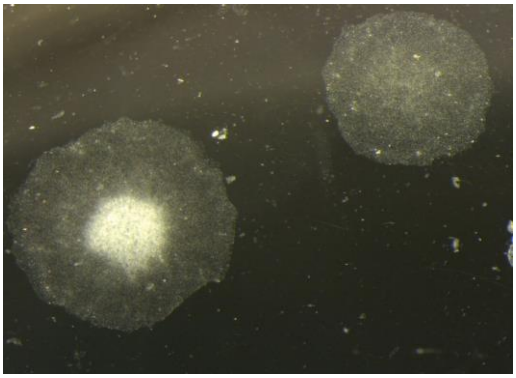

Figure 7 Classical hiPSC morphology in passaged Cas9/15 colonies

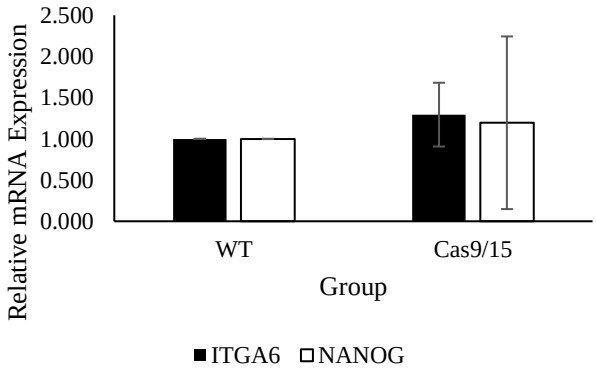

Figure 8 mRNA expression of *ITGA6* and *NANOG* in WT and Cas9/15 reprogrammed hGFs 25-30

The Cas9 control did not develop colonies in the first trial and few in the second and third trials with only one expandable colony developing in trials 2 and 3 (Table 2).

The expression of key factors tested for WT and Cas9/15 25-30 days post-infection showed an increase in both NANOG and ITGA6 when compared to the mRNA quantified 3 days post-infection (Figure 8). Solely, Cas9/15 was examined for NANOG and ITGA6 expression 30 days post infection because of its low expression of ITGA6 3 days post-infection.

Discussion

The initial experimental set showed a change in morphology and reduced reprogramming efficiency in the CRISPR-Cas9 sgRNA groups likely due to the supplementation of blasticidin and puromycin in high concentrations, which may have been toxic to the cells. This also explains the lack of colony development that may have been caused by a more efficient selection process post-infection sustaining the CRISPR-Cas9 knockouts. However, the critical observation is that the knockout was successful in the trials with and without puromycin and blasticidin as observed by decreased expression of relative *ITGA6* mRNA levels.

The Cas9 control did not develop any colonies in the first trial and only one developed in the second and third trials, despite it not targeting any specific nucleotide sequence. The amount of colony development was significantly lower than that of the WT, which suggests that the Cas9 control was not an effective control for this experiment, but the WT served this purpose instead. The WT developed viable and healthy colonies exhibiting the classical morphology, which survived when passaged and co-expressed ITGA6 in the plasma membrane and Oct4 in the nucleus.

The expression of Oct4 in the cytoplasm in Cas9 knockout cells suggests that there may be a mechanism inhibiting it from entering the nucleus after translation. This indicates that Oct4 was not performing its function as a transcription factor because it was not localized in the nucleus. This may lead to future experiments to understand the mechanism behind this mis-localized expression. The expression of Oct4 in fully-reprogrammed colonies, as expected, was localized to the nucleus suggesting that it was functioning as a transcription factor in hiPSCs which was confirmed by the expression of *NANOG*.

Future experiments will be performed to further analyze the Cas9 cell lines and the mechanisms of ITGA6 in stem cell pluripotency and self-renewal. These studies may include but are not limited to an investigation of FAK expression, as a cytoplasmic target of integrins, and the uncharacteristic cytoplasmic localization of Oct4 in *ITGA6* knockout cells.

Conclusion

This experimental procedure was performed to determine the role of ITGA6 in hiPSCs due to its wide expression in stem cell populations and key role in stem cell self-renewal. This wide expression therefore indicates that ITGA6 may play several critical roles in stem cell populations. The results of this study indicate that the consistent expression of ITGA6 in the developed hiPSC colonies as well as the lack of colonies developed without expression of ITGA6, supports the hypothesis that this integrin subunit is required for the development, self-renewal, and pluripotency of hiPSCs.

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