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PRINCIPAL INVESTIGATOR: Roy Geoffrey Sargent

CONTRACTING ORGANIZATION: University of California, San Francisco
San Francisco, CA 94103

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Roy Geoffrey Sargent, PhD

E-Mail: Geoffrey.Sargent@ucsf.edu

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UNIVERSITY OF CALIFORNIA, SAN FRANCISCO
1855 FOLSOM ST STE 425
SAN FRANCISCO CA 94103-4249

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14. ABSTRACT

This research addresses metabolic pathologies resulting from hypoparathyroidism, parathyroid disease, and injury. The major goals of the project are to establish genetically modified primary parathyroid cell lines, develop protocols to differentiate pluripotent stem cell lines into parathyroid tissues using a genetically marked pluripotent stem cell line, and demonstrate efficient transplantation of in vitro differentiated parathyroid tissues in a hypoparathyroid mouse model. This report discusses the successful creation of an induced pluripotent stem cell line with a red fluorescent protein marker inserted into the GCM2 gene. GCM2 is a unique marker expressed in parathyroid tissues and serves as an easily detected reporter for cells that have differentiated into parathyroid tissues. Several growth conditions and substrates for growth of parathyroid primary cells have been tested that have supported short-term growth, but apparent long term survival, of parathyroid primary cell lines in culture. Three different induced pluripotent stem cell lines have been used to test in vitro differentiation protocols to generate parathyroid tissue. These experiments have met with mixed success as demonstrated by the transient expression of parathyroid hormone and GCM2, both markers of parathyroid tissues.

15. SUBJECT TERMS

Induced pluripotent stem cells, ips cells, parathyroid, Crispr/cas9, TALENS, pluripotent stem cells, hypoparathyroidism, 2 human homolog (Gcm2/GCMB), parathyroid hormone (PTH) and calcium sensing receptor (CaSR)

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1. Introduction

This research addresses the metabolic pathologies resulting from hypoparathyroidism. Parathyroid endocrine function can be compromised or lost through injury, therapeutic intervention, e.g., surgery or radiation or as a result of disease (genetic or autoimmune), and can lead to parathyroid gland (PTG) dysfunction and a disruption of calcium homeostasis and metabolism. Hypoparathyroidism is particularly devastating and is becoming more and more prevalent because of the increase in thyroidectomies and thyroid radiotherapy. Due to the multi-organ pathological metabolic manifestations of hypoparathyroidism and the limitations of the present pharmacological therapies, it would be advantageous for patients if there were a cellular therapy that could mimic the native PTG responses to serum calcium fluctuations and compensate for lost PTG function. Induced pluripotent stem cells (iPSCs) have created a new opportunity to develop cellular therapies to repair tissues damaged by injury, therapeutic intervention or disease. This proposal will take a patient's somatic cells, reprogram them into iPSCs, and then differentiate the resultant iPSCs into parathyroid progenitors as an autologous/personalized cellular therapy that bypasses immune-rejection. This is an innovative strategy for restoring parathyroid function, since it bypasses the need for pharmacological supplementation and does not depend on rescuing remnant parathyroid glands for transplantation. The directed differentiation of iPSCs into parathyroid progenitors and mature PTG cells relies on exposure to conditions that mimic, to a degree, embryonic parathyroid organogenesis. The iPSCs are being modified with CRISPR or TALEN technology for sequence specific insertion of a GFP reporter into the parathyroid-specific glial cell missing 2 human homolog (Gcm2/GCMB), parathyroid hormone (PTH) and calcium sensing receptor (CaSR) regulatory sequences to monitor the differentiation of the iPSCs into parathyroid progenitors and mature PTG cells. The studies test the hypothesis that iPSCs can be directed to differentiate along an endodermal lineage pathway and progress to cells that have characteristics consistent with parathyroid cell function, e.g., secrete PTH in low Ca+2, GMCB, express CaSR, express vitamin D receptor (VDR), recapitulate PTG function/morphology in an ex vivo bioactive matrix and a GATA3 heterozygote (+/-) mouse model of hypoparathyroidism. Functional efficacy and reversal of hypoparathyroidism in the GATA3 (+/-) mouse is critical to helping define the parameters for the generation and selection of the parathyroid progenitors for transplantation. This research described in this project lays the groundwork for developing a patient-specific cell-based therapy for hypoparathyroidism and provides the preliminary information to support a more in-depth proposal that could lead to future clinical trials.

2. Keywords

Induced pluripotent stem cells, ips cells, parathyroid, Crispr/cas9, TALENS, pluripotent stem cells, hypoparathyroidism, 2 human homolog (Gcm2/GCMB), parathyroid hormone (PTH) and calcium sensing receptor (CaSR)

3. Accomplishments

What were the major goals of the project?

Specific Aim 1: **AIM 1** will involve **(a)** the establishment and characterization of primary cultures of parathyroid gland epithelial cells, and **(b)** the generation of a genomic GCMB/GCM2 promoter-GFP reporter iPSC line that will be directed to differentiate along an endodermal lineage specific pathway and that will be assessed of differentiation stage-specific gene expression features.	**Timeline**	**Status**
Major Task 1: The establishment and characterization of primary cultures of parathyroid gland epithelial cells.	Months	
Subtask 1: Obtain tissues from patient specimens following surgery.	1-18	ongoing
Subtask 2: Optimize culture conditions for long-term culture of primary cells and characterize cells for parathyroid features.	1-6	ongoing
Subtask 3: Establish GFP-labeled primary PTG cells with high efficiency gene delivery systems	3-9	ongoing
Local IRB/IACUC Approval IRB and GESCR approvals are in place. IACUC approval pending.	1-18	• IRB and GESCR approvals transferred to Dr Sargent. • IACUC approval under Dr Sargent's name in progress
Major Task 2: Generation of a genomic GCMB/GCM2 promoter-GFP reporter iPSC line that will be directed to differentiate along an endodermal lineage specific pathway and that will be assessed of differentiation stage-specific gene expression features.		
Subtask 1: Generate CRISPR/Cas targeting construct for inserting GFP into GCMB promoter region of a Sendai virus reprogrammed iPSC cell line. Isolate a GFP containing clone by PCR screening or secondary drug selection.	2-3	completed
Subtask 2: Develop differentiation protocol for the progression of iPSCs along a lineage pathway that will lead to parathyroid progenitors	3-12	ongoing

and their more mature descendants.		
Subtask 3: Monitor and characterize the differentiation of the cells as function of the expression of various stage-specific transcription factors and through expression of GCMB-GFP	3-12	ongoing
Specific Aim 2: **AIM 2** will be directed at developing a 3-D bioactive matrix that will promote functional PTG "organogenesis" *ex vivo* and evaluating a mouse model of hypoparathyroidism for increased parathyroid function by measuring changes in serum calcium and phosphate levels upon transplantation with iPSC-derived parathyroid progenitor cells.		
Major Task 3: Develop a 3D bioactive matrix that will promote functional *ex vivo* PTG organogenesis		
Subtask 1: Generate bioactive matricies to test for primary parathyroid cell growth and maintenance of function.	6-12	ongoing
Subtask 2: Establish parathyroid spheroid culture.	5	ongoing
Subtask 3: Generate a *GATA3*$^{(+/-)}$*Rag1*$^{(-/-)}$ mouse model for organoid and iPSC-derived parathyroid progenitor transplantation. It is expected that 10 mice will be needed to make this animal with 2 crosses.	3-9	ongoing
Subtask 4: Transplant and assess functional efficacy of the transplanted PTG gland tissue/cells. There will be 6 animals/ experiment and it is anticipated that a there will be 2 conditions (primary cells and iPSC-derived), with 10 experiments overall. Thus it is expected that there will be 120 animals required for the planned assessments	9-18	to be done

What was accomplished under these goals?

Specific Aim 1; Major Task 1: ***The establishment and characterization of primary cultures of parathyroid gland epithelial cells.***

Subtask 1: Obtain tissues from patient specimens following surgery. We received several parathyroid samples from Dr Jon George, a surgeon in the Department of Otolaryngology Head and Neck Surgery at the UCSF Mission Bay campus, but this supply has been limited. We have enlisted a second surgeon in the Department of Otolaryngology Head and Neck Surgery, Dr Marika Russell, who is based at the San Francisco General Hospital to acquire more human parathyroid samples. We are contacting other surgeons at different UCSF hospitals to increase our access to parathyroid samples for subtasks 2 and 3.

Subtask 2: Optimize culture conditions for long-term culture of primary cells and characterize cells for parathyroid features. Our initial experiments to optimize culture conditions focused on testing combinations of growth substrates and media and monitoring cell growth. This work is being performed by Dr Sargent and Cristina Barilla, a graduate student in the laboratory.

On receipt of surgical samples, the parathyroid glands are dissected from surrounding tissue, minced, and treated with collagenase and trypsin to dissociate into a single-cell dispersion. The cell suspensions were pooled and plated equally among 8 wells of a 24-well plate with the substrates and medium indicated in tables 1 and 2 below.

Table 1 Experiment 1

Substrate	**Medium**	
	MLHC	KSFM
Plastic	No growth, cell death	No growth, cell death
Fibronectin	Low survival	Nice colonies but they don't grow
Matrigel	Cell death	Cell death
Fibronectin+matrigel	Differentiation to fibroblast-like cells→change to KSFM medium look better	Nice colonies

Table 2 Experiment 2

Substrate	**Medium**	
	KSFM	KSFM+5uM Y27632
Laminin	Good survival and attachment	Good survival and attachment No difference from -Y27632
Fibronectin	Nice colonies but no cell growth	Nice colonies but no cell growth. Appearance of fibroblast-like cells
Laminin+matrigel	Less efficient than laminin alone	Less efficient than laminin alone
Fibronectin+matrigel	Cell death	Cell death

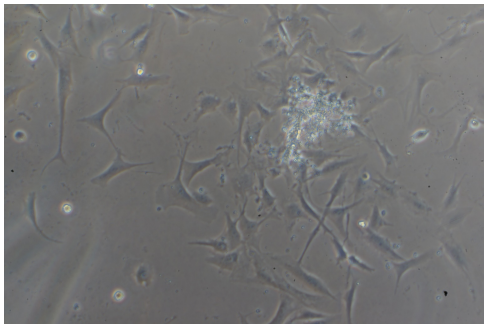


Figure 1. Laminin+KSFM+ Y27632

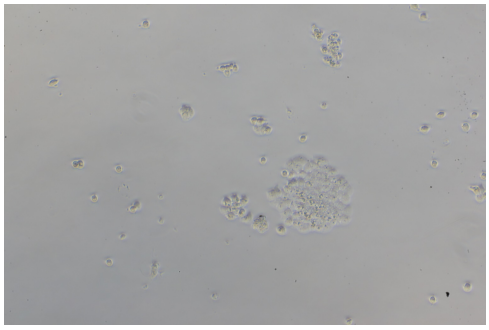


Figure 2. FN+ KSFM+ Y27632

Cell suspensions from parathyroid glands were plated on substrates used in our laboratory for growing epithelial and stem cell lines in combination with medium developed for culture of primary epithelial cell lines and primary keratinocyte cell lines. The Rho-associated kinase inhibitor Y-27632, which has been used to prevent cell death in culture of human pluripotent stem cells and has been reported to reversibly immortalize primary epithelial cells, was also tested for its potential to improve cell survival and promote cell growth.

The best conditions for plating parathyroid primary cell cultures appears to be a combination of fibronectin substrate with KSFM medium as determined by retention of the original cell morphology over several weeks, followed by cell death. Laminin with KSFM also appears to support cell attachment, however the cellular morphology changes over several days of culture to resemble fibroblast cells, suggesting the laminin substrate may not prevent differentiation or outgrowth of contaminating fibroblast cells from the parathyroid samples. For all the conditions tested, over a period of approximately 16 weeks, cell growth was poor.

We are continuing to test combinations of growth medium and substrates to improve plating efficiency of parathyroid primary cells and to stimulate cell division for long-term growth of parathyroid cells.

Subtask 3: Establish GFP-labeled primary PTG cells with high efficiency gene delivery systems
Construction of the GFP-reporter vector for establishing GFP-labeled primary PTG cells is underway. The GFP targeting vector construction is similar to that of the RFP vector used in experiments discussed below.

Specific Aim 1; Major Task 2: ***Generation of a genomic GCMB/GCM2 promoter-GFP reporter iPSC line that will be directed to differentiate along an endodermal lineage specific pathway and that will be assessed of differentiation stage-specific gene expression features.***

Subtask 1: Generate CRISPR/Cas targeting construct for inserting GFP into GCMB promoter region of a Sendai virus reprogrammed iPSC cell line. Isolate a GFP containing clone by PCR screening or secondary drug selection. This task has been accomplished. We chose to substitute the red fluorescent protein (RFP) instead of the green fluorescent protein (GFP) to distinguish between ips cells and

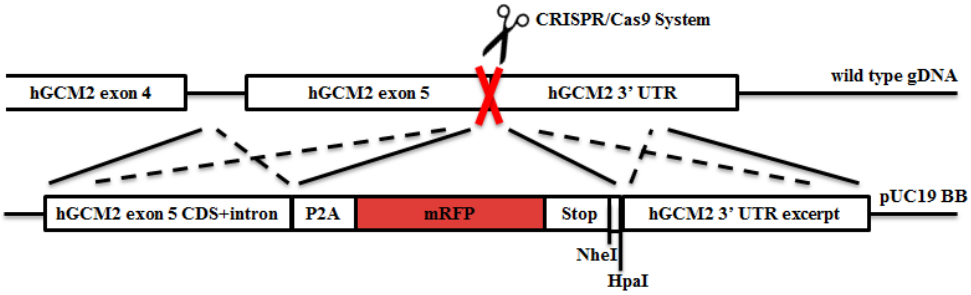
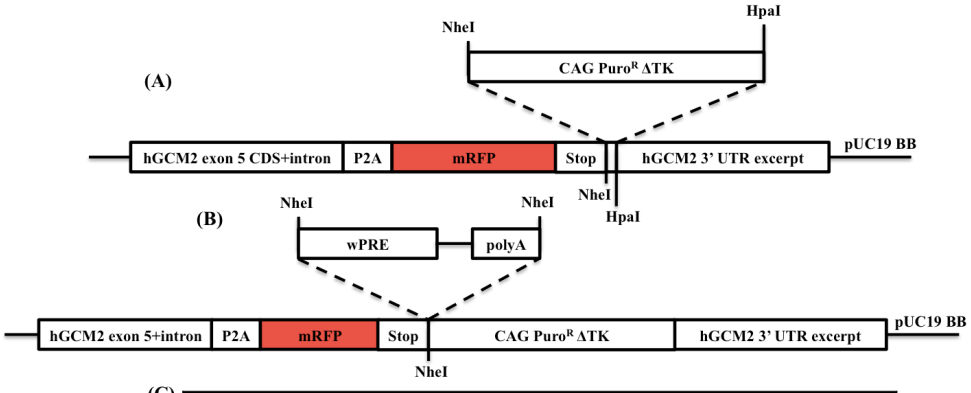


Fig. 3: GCM2 Donor DNA and gDNA Homologous Replacement Enhanced by CRISPR/Cas9 A scheme of homologous recombination between the donor DNA and wild type hGCM2 gDNA. The 5' homology arm is a 967 bp long sequence derived from amplifying the last exon of the wild type GCM2 gene and part of the intron immediately upstream of the coding sequence. The GCM2 stop codon was not included as part of the PCR amplicon. The 3' homology arm is a 925 bp long sequence derived from amplifying part of the 3' GCM2 untranslated region. The CRISPR/Cas9 system enhances homologous recombination through inducing DSB (double strand DNA break) around the stop codon.

primary cells labeled with one of the other marker. The donor DNA targeting the last exon, exon 5, of the hGCM2 gene was created using In-Fusion cloning technology and sub-cloning techniques with restriction enzyme digestions and subsequent ligations. The initial construction was carried out to insert the P2A and mRFP sequences derived from amplifying CFTRex27-mRFP-pUC19 into the GCM2 donor DNA. This template sequence contained a 2A peptide sequence immediately upstream of the mRFP sequence, and was amplified as a part of the mRFP fragment. The 5' homology arm was constructed through amplifying the genomic DNA of immortalized human bronchial epithelial cells, specifically 16HBE14o- immediately before the hGCM2 exon 5 stop codon. The 3' homology arm was constructed through similar amplification of the 3' UTR (untranslated region) in the same cell line (Fig. 3). The forward PCR primer targeting the 3' UTR (NheIHpaI - hGCM2ex5 3'UTR Fw) and the reverse PCR primer targeting the mRFP sequence (mRFP-hGCM2ex5 NheIHpaI Rv) introduced NheI and HpaI restriction endonuclease sites immediately downstream of the mRFP stop codon for the eventual insertion of a drug-selectable marker. The final construction inserted the PCR fragments into a pUC19 backbone. This intermediate construct was named hGCM2-P2A-mRFP-NheI/HpaI-pUC19. Upon completion of this construction, the CAGPuroΔTK drug cassette sequence was sub-cloned from CF2B-CAGPuroTK (a plasmid with the cassette insertion) into the newly constructed plasmid by digesting both vectors with NheI and HpaI. After gel purification of the drug cassette from the CF2B-CAGPuroTK construct, these two fragments were ligated. This new intermediate construct was named hGCM2-P2A-mRFP-PTK-pUC19 (Fig. 4). Since the insertion of the drug cassette disrupts the availability of the hGCM2 3' UTR region containing a poly-A sequence, a wPRE-polyA sequence derived from pCXLE-EGFP (Addgene #27082) was sub-cloned into the vector plasmid immediately upstream of the drug cassette, by appending an NheI cut site to both the 5' and 3' ends of the wPRE-polyA sequence via PCR.



(C)

NheI-wPREpolyA Insert Construction Primer Table		
Primer Length	**Primer Name**	**Primer Sequence**
34 bp	NheI wPREpolyA Fw	5'-TGAAGCTAGCTTCAAGCTTCGGGGACTAGTCATA-3'
30 bp	NheI wPREpolyA Rv	5'-ACAAGCTAGCTTGTTCATGGCAGCCAGCAT-3'

Fig. 4: Sub-cloning CAGPuroR ΔTK and wPRE-polyA hGCM2 Donor DNA (A) Both CF2B-CAGPuroTK and hGCM2-P2A-mRFP-NheI/HpaI-pUC19 were digested with NheI and HpaI. The vector plasmid and drug cassette were gel-purified and ligated together(B) The hGCM2-P2A-mRFP-CAGPuroTK-pUC19 backbone was digested with NheI, and the NheI-wPRE-polyA PCR fragment derived from pCXLE-EGFP amplification was ligated into the backbone. (C) Primers P7 and P8 targeted pCXLE-eGFP pDNA to generate the wPRE-polyA sequence insertion.

CRISPR/Cas9 System Design and Construction The CRISPR/Cas9 system tested in this experiment were derived from the *Staphylococcus aureus* (Sa) and *Streptococcus pyogenes* (Sp) bacterial species, both of which are known for their induction of DSBs in mammalian cells. Four sgRNA (single guide RNA) sequences per bacterial species were tested for induction of DSBs and consequent non-homologous end joining (NHEJ) around the hGCM2 exon5 stop codon in CFBE41o- immortalized human bronchial epithelial cells. Both Sa and Sp sgRNAs were designed using the web-based software "CCTop" (http://www.crispr.cos.uni-heidelberg.de/) and "Optimized CRISPR Design – MIT" (http://www.crispr.mit.edu/). Construction of Sa and Sp sgRNA were carried out using published protocols. As an overview, double-strand DNA fragments with overhangs that complement the ligation site of the sgRNA backbone vector were created from sense and anti-sense oligos, denatured and then slowly annealed, consisting of the desired GCM2 targeting sequence. The annealed fragments were then ligated into the plasmid BPK2660 (Addgene #70709).

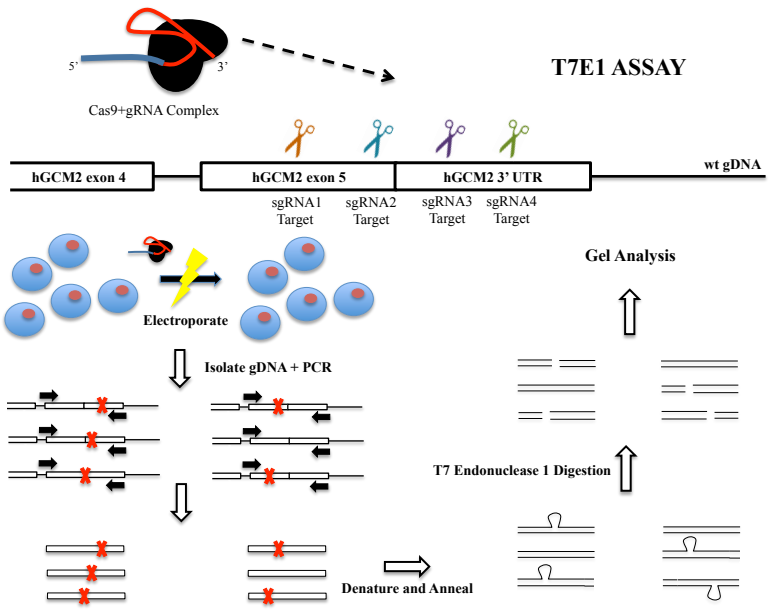


Fig. 5: T7E1 Assay The basic template to determine approximate %NHEJ efficiency for a given CRISPR gRNA sequence. Five days after CRISPR/Cas9 transfection, the gDNA was isolated and amplified using the T7E1 Primers. The PCR products were denatured by incubating at 95°C for 10 minutes and then annealed to create heteroduplexes by ramping down at -5°C/min. T7 Endonuclease 1 then cleaves the annealed PCR products at mismatched sites, and the digested products can be analyzed on an agarose gel.

Optimization of a Targeting Site via CRISPR/Cas9 System CFBE41o- cells were nucleofected using the SF Cell line solution (Lonza Group) according to their 4D-Nucleofector Protocol with the optimized pulse code, DS120. Transfection conditions are shown in Table 3. The gDNA was harvested 5 days after transfection, and analyzed by performing a T7E1 Assay (Fig. 6).

Sp CRISPR/Cas9 system successfully targets and introduces DSBs in the GCM2 targeting site. The induction of DSB in a target sequence dramatically improves site-specific homologous recombination. Therefore, an optimized CRISPR/Cas9 system was sought to target and introduce DSBs at or near the stop codon of the GCM2 gene. For testing Sp gRNAs, transfection conditions (Table 3), as optimized in previous experiments, compared the four gRNA sequences with an empty px330 vector negative control, which demonstrated low-level yet detectable %NHEJ incidences that produced T7E1-digested fragments about 400 bp in length (Fig. 6). While Gb11 sgRNA demonstrated the greatest %NHEJ incidences at ~19.3%, the band intensity analysis of the gel may have included the negative control's non-specific digestion band patterns to yield a %NHEJ value higher than the actual percentage of DSB on-target occurrences,

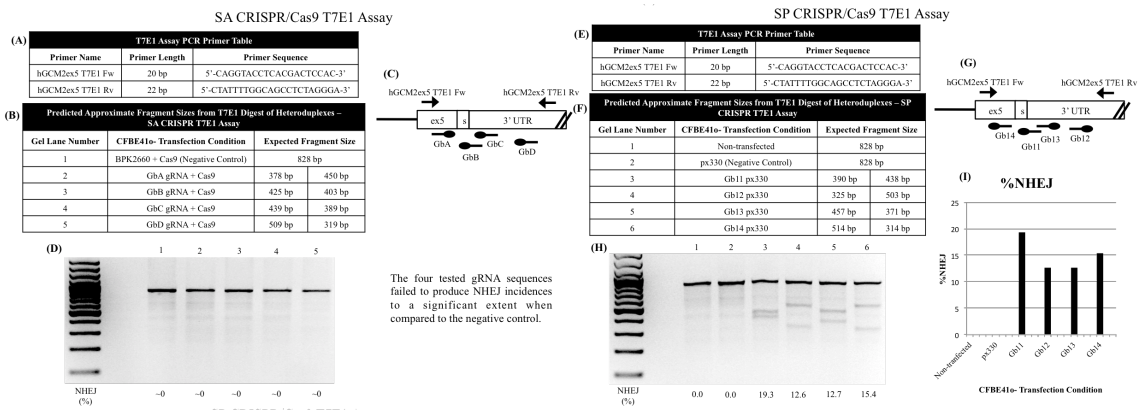


Fig. 6: CRISPR/Cas9 T7E1 Assay Results (a,e) The PCR primers to amplify the modified gDNA are shown. (b,f) The predicted band sizes that result from T7E1 enzymatic activity based on gRNA targeting sequences are shown, assuming on-target DSB induction per gRNA sequence for Sa and Sp systems, respectively. (c,g) Relative targeting sites of both Sa and Sp gRNA sequences are shown, respectively. (d,h) T7E1-digested fragments run on 2% TBE gels. (i) The results suggested that the tested Sp sgRNA were significantly more effective at inducing NHEJ incidences than the tested Sa sgRNA targeting the last exon of the GCM2 gene. Gb11 Sp sgRNA in px330 was selected to enhance homologous recombination in CF3iPS2 upon co-transfection with the hGCM2 donor DNA constructed earlier.

suggesting that on-target cutting efficiency was roughly the same for all Sp sgRNAs tested in this experiment. Ultimately, Gb11 sgRNA was chosen as the targeting gRNA sequence.

Additionally, four corresponding Sa bacterial CRISPR/Cas9 systems were also tested for their on-target cutting efficiency (Fig. 6). Interestingly, none of the four Sa sgRNA sequences successfully enhanced %NHEJ according to a T7 Endonuclease 1 Assay. Other possible candidates for Sa gRNA sequences targeting GCM2 gene must be tested in order to determine if Sa bacterial CRISPR systems preferentially are unable to target the specific GCM2 gene.

Table 3: CFBE41o- Transfection Conditions for T7E1 Assay

T7E1 Assay – CFBE41o- Transfection Conditions					
Cell Number	**Cas9**	**gRNA**	**gRNA+PAM Sequence**	**Top Oligo Sequence**	**Bottom Oligo Sequence**
$2x10^5$ per transfection	MSP1830 (800ng)	BPK2660 (200ng)	*	*	*
		GbA SAsgRNA (200ng)	5'-TCCGCAGTCAGTTACTCAGA cagagt-3'	5'-CACCgTCCGCAGTCAGTTACTCAGA-3'	5'-AAACTCTGAGTAACTGACTGCGGAc-3'
		GbB SAsgRNA (200ng)	5'-TGTCCCCTGGATTGTCTTTC aaaaat-3'	5'-CACCgTGTCCCCTGGATTGTCTTTC-3'	5'-AAACGAAAGACAATCCAGGGGACAC-3'
		GbC SAsgRNA (200ng)	5'-GCACACTGCTATTATGTCCC ctggat-3'	5'-CACCgGCACACTGCTATTATGTCCC-3'	5'-AAACGGGACATAATAGCAGTGTGCc-3'
		GbD SAsgRNA (200ng)	5'-ACACAAGGTGAATCTCCCAA ctcaat-3'	5'-CACCgACACAAGGTGAATCTCCCAA-3'	5'-AAACTTGGGAGATTCACCTTGTGTC-3'
$3x10^5$ per transfection	px330 (1.5µg)		*	*	*
	Gb11 SPsgRNA in px330 (1.5µg)		5'-CACACTGCTATTATGTCCCCtgg-3'	5'-CACCgCACACTGCTATTATGTCCCC-3'	5'-AAAcGGGGACATAATAGCAGTGTGc-3'
	Gb12 SPsgRNA in px330 (1.5µg)		5'-GCAATGATCTCTTATTGAGTtgg-3'	5'-CACCgGCAATGATCTCTTATTGAGT-3'	5'-AAAcACTCAATAAGAGATCATTGCc-3'
	Gb13 SPsgRNA in px330 (1.5µg)		5'-CATAATAGCAGTGTGCATGCagg-3'	5'-CACCgCATAATAGCAGTGTGCATGC-3'	5'-AAAcGCATGCACACTGCTATTATGc-3'
	Gb14 SPsgRNA in px330 (1.5µg)		5'-CTCTGCTTCATCTGTCCTAGagg-3'	5'-CACCgCTCTGCTTCATCTGTCCTAG-3'	5'-AAAcCTAGGACAGATGAAGCAGAGc-3'
*Undigested BPK2660 + MSP1830 is the SA negative control. Undigested px330 is the SP negative control. px330 is a chimeric sequence that contains both the gRNA and Cas9 sequence in a single plasmid construct.					

Percentage of NHEJ incidences (%NHEJ) was determined by the following formula:

$$f_{cut} = \frac{CleavageBand_1 + CleavageBand_2}{CleavageBand_1 + CleavageBand_2 + UncleavedBand} \quad \text{Eq.}$$

$$\%NHEJ = 100 * \left(1 - \sqrt{1 - f_{cut}}\right) \quad \text{Eq. 2}$$

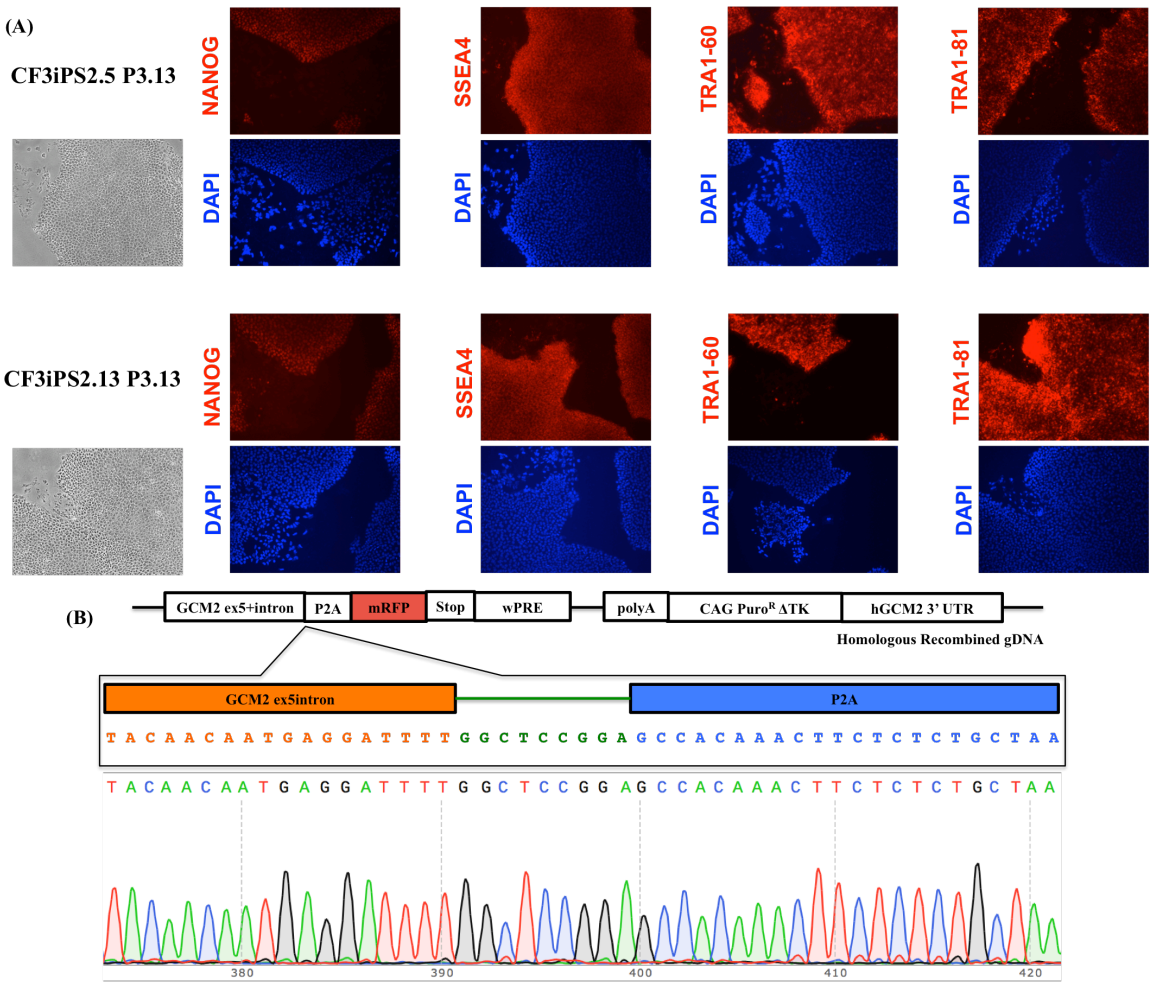
Co-transfection of GCM2 Donor DNA and CRISPR/Cas9 system yields the insertion of a reporter system in iPSCs. The CF3iPS2 cell line was co-transfected with the constructed GCM2 donor DNA and Gb11 sgRNA in the px330 backbone. CF3iPS2 was initially characterized through immunostaining and cytogenetic analysis, followed by additional immunocytochemical analysis of positive clones post-modification. A total of 2 x 10^6 cells were nucleofected in each experiment with a 1:1 ratio of donor DNA to gRNA/Cas9 DNA using P3 Primary Cell Solution (Lonza Group) in a 100 μL large cuvette. Significant cell death was observed immediately following nucleofection. The cells were plated on a 60 mm Matrigel-coated dish with 10 μM Y27632-containing mTeSR1 feeder-free media for one day post-transfection, after which Y27632 concentration was decreased to 5 μM. Puromycin (0.5 μg/mL) was added to the mTeSR1 feeding media starting from day 2 for two weeks.

On day 9 through 11 post-transfection, individual puromycin resistant ($Puro^R$) clones were picked. A total of 42 clones out of the approximately 100 colonies observed were individually isolated and propagated to continue growth.

The 42 puromycin resistant CF3iPS2 clones were analyzed by PCR (Fig 8) to identify GCM2 homologous recombinants. Primer pairs AP1.3/AP2 and AP3/AP4 only amplify gDNA sequences with the correctly integrated reporter system into the genomic GCM2 site. Additional PCR analysis was performed using AP3 and AP4.2. The AP4.2 primer is complementary to DNA sequence in the 3'UTR that was part of the donor DNA 3' homology arm. A positive PCR product for the AP3/AP4.2 PCR but negative PCR product for AP1.3/AP2 and AP3/AP4 primers would suggest random integration(s) of the donor DNA to a different site(s) of the genomic DNA. For example, clones 15 and 29, among others, demonstrate PCR product patterns that suggest random integration of the donor DNA. Finally, primer pair P7F and P6r amplifies the $Puro^R$ gene to confirm presence of the drug selectable marker. Positive signals for all 4 analysis PCRs conducted indicated successful insertion of P2A and mRFP sequences immediately downstream of the GCM2 gene. Co-transfection of the Gb11 in px330 and the GCM2 Donor DNA yielded a targeted modification efficiency of 21.4% (9 out of the 42 clones). Overall, the insertion of mRFP and the puromycin resistance drug cassette demonstrates that site-specific modification of gDNA is a viable procedure to edit pluripotent stem cells using a CRISPR/Cas9 system to enhance the frequency of HDR (homology-directed repair) events.

However, certain clones, such as clone 2 and clone 28, exhibit AP3/AP4 band patterns that are smaller than expected, suggesting a mutation in the 3' region of the PCR amplicon. This mutation is possibly due to continued CRISPR/Cas9 cutting activity after the integration of the reporter system, such that Gb11 in px330 targets the modified 3' arm downstream of the integrated drug cassette and cuts the gDNA again, introducing a noticeable deletion in the 3'UTR region. Although the deletion is in the untranslated region and therefore has no effect on the GCM2 coding region, it is uncertain how large of an impact the deletion has on mRNA transcript stability. Further research will be necessary in order to determine the nature of the deletion events.

Two puromycin resistant clones identified by PCR as homologous recombinants, clone 5 (CF3ips2.5) and clone 13 (CF3ips2.13), were selected for further analysis to confirm that the modified GCM2 cell lines still expressed markers consistent with pluripotence. Both clonal cell



(C)

Immunocytochemical Analysis Antibodies	
PRIMARY ANTIBODY	**VENDOR**
Anti-NANOG antibody, mouse monoclonal IgG1	BD Biosciences 560482
Anti-SSEA-4 antibody, mouse monoclonal IgG3	Abcam Inc. ab16287
Anti-TRA-1-60, mouse monoclonal IgM	BD Biosciences 560071
Anti-TRA-1-81, mouse monoclonal IgM	Millipore MAB4381
SECONDARY ANTIBODY	**VENDOR**
Alexa Fluor 488 goat anti-mouse IgG1	Invitrogen A21121
Alexa Fluor 546 goat anti-mouse IgM	Invitrogen A21045

Fig. 8: CF3iPS2 Characterization: (A) Immunocytochemical analysis for CF3-iPS2 P3.9 pluripotency markers: NANOG, SSEA4, Tra1-60, and Tra1-81. A phase contrast image demonstrated typical pluripotent cell culture morphology in feeder free culture. (B) CF3iPS2 c13 was sequenced and shows the correct junction sequence between GCM2 exon 5 and the 2A Peptide. (C) Primary and secondary antibodies for immunostaining are shown.

lines express NANOG, SSEA4, Tra1-60, and Tra1-81 as detected by immunofluorescence (Fig 8A). The DNA sequence from Clone 13 demonstrated the GCM2ex5-P2A junction sequence was intact (Fig. 8B). Further analysis is underway to demonstrate these clones can differentiate into the 3 germ layers, that they have a normal diploid karyotype, and that the RFP reporter is co-expressed with GCM2.

An issue encountered during this experiment was the generation of Sa CRISPR gRNA targeting sequences that successfully induced DSBs. Currently, there exists no readily available system for determining predicted on-target cutting efficiencies for Sa gRNA sequences. Rather, present methods base gRNA efficacy predictions solely on off-targeting DNA cutting potential. As a result, all of four Sa gRNA sequences tested did not demonstrate the induction of on-target NHEJ, as determined in the T7E1 assay when compared with the negative control. Unlike the Sa CRISPR/Cas9 system, the Sp CRISPR/Cas9 system has been used since its development as the first working CRISPR/Cas9 system in mammalian cells, and its prediction algorithms of its targeting site were developed and optimized by many research groups. All of the Sp gRNA targeting sequences (Gb11-Gb14) tested using the T7E1 Assay introduced at least 12.6% of %NHEJ when compared with a non-transfected and non-targeting Cas9 transfected negative controls.

Subtask 2: Develop differentiation protocol for the progression of iPSCs along a lineage pathway that will lead to parathyroid progenitors and their more mature descendants.

See Subtask 3

Subtask 3: Monitor and characterize the differentiation of the cells as function of the expression of various stage-specific transcription factors and through expression of GCMB-GFP.

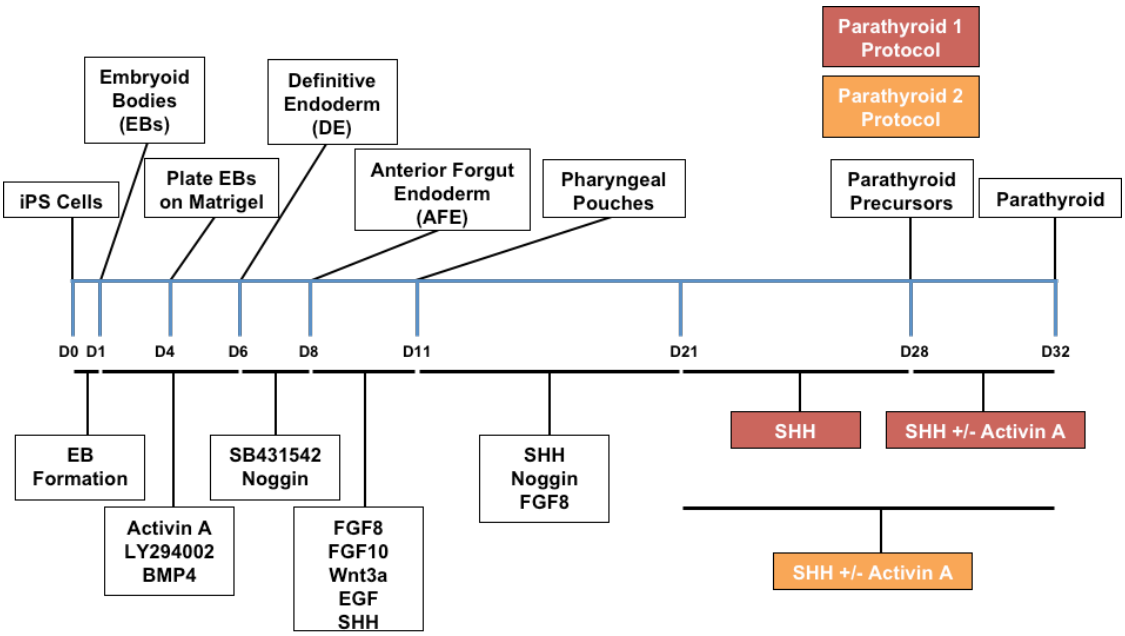


Figure 9: Differentiation Timeline

Subtasks 2 and 3 are closely related and summarized here.

We have tested both differentiation protocols outlined in Figure 9 and measured gene expression levels of HoxA3, PBX1, Parathyroid hormone (PTH) and GCM2. HoxA3 and PBX1 are transcription factors expressed during development of the pharyngeal arches in definitive endoderm with HoxA3 expression detected in endodermal tissues of the 3rd and 4th pharyngeal

arches about E28 and PBX1 expressed in parathyroid and thyroid progenitor cells at about E32. PTH and GCM2 are genes expressed in parathyroid tissues.

We compared the quality of differentiation of three different human iPS cell lines using embryoid bodies, as indicated in the differentiation timeline (Fig 9) without controlled aggregation (EB-aggregation) or by controlling the number of iPS cells used to form EB using Aggrewell plates. Regardless of the method used for EB formation, addition of cytokines to the medium to initiate differentiation into parathyroid tissues resulted in cell death. This appears to be influenced by the iPS cell line used as well as the method used for EB formation (Table 4; Experiments 1-4). Direct plating of iPS cells onto tissue culture plates, however, showed the best long-term cell survival and was used for preliminary differentiation experiments into parathyroid lineages.

Table 4. Summary of Differentiation Experiments

Experiment	iPS Cell Line	EB or Direct	Comments
1	FHTF	EB – aggregation	Cell death by 28 days post cytokine addition
	CF3iPS2eGFP b5.1	EB – aggregation	Cell death by 28 days post cytokine addition
	CF3iPS2eGFP b5.2	EB – aggregation	Cell death by 28 days post cytokine addition
2	FHTF	EB + aggregation	Cell death by 5 days post cytokine addition
3	FHTF	EB + aggregation	Cell death by 5 days post cytokine addition
4	CF2iPS3Ic8e11	EB + aggregation	Cell death by 24 days post cytokine addition
	CF2iPS3	EB + aggregation	Cell death by 24 days post cytokine addition
5	CF3iPS2eGFP b5.1	Direct plating	Cell survival 35 days post cytokine addition
	CF3iPS2eGFP b5.2	Direct plating	Cells survival 35 days post cytokine addition

EB-aggregation protocol utilizes dispase treatment of growing iPS cell cultures followed by replating cell clumps into media in low-attachment tissue culture plates. EB+aggregation protocol utilizes accutase treatment of growing iPS cell cultures and aggregation into cell clumps of known cell density by centrifugation into Aggrewell plates before replating into media in low–attachment tissue culture plates. Direct plating indicates replating iPS cells into tissue culture plates for differentiation bypassing EB formation.

Gene expression analysis by quantitative PCR of HOXA3, PBX1, PTH, and GCM2 are shown in figure 10. Expression of HOXA3 and PBX1 are found in the pharyngeal pouch and surface ectoderm whereas GCM2 and PTH are characteristically expressed in parathyroid tissues. Both HOXA3 and PBX1 are transiently expressed at D11 in cells differentiated by a protocol leading to airway epithelium, whereas expression of these two transcription factors is weak or nonexistent in cells differentiated towards pharyngeal pouches and ultimately parathyroid tissues. Expression of PTH and GCM2 was observed at D8-D11, however expression of these two genes, characteristic of parathyroid tissues, diminished after D11 suggesting the CF3iPS2eGFPb5.1 cell line cell line or the current differentiation protocol is not generating parathyroid tissue. The b5.1 cell line does not have a GFP reporter integrated in the GCM2 gene and we will use the GCM2-GFP modified cell line in future experiments.

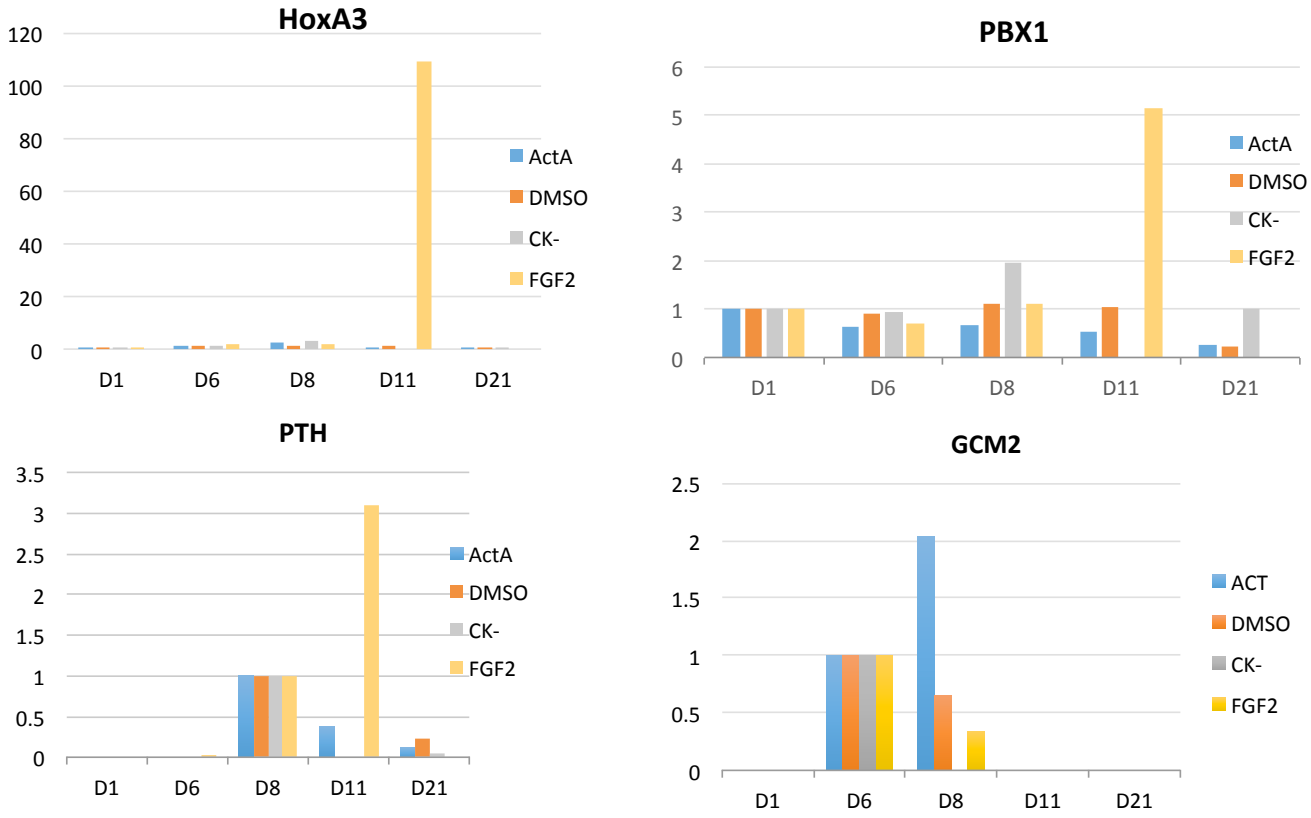


Figure 10: Gene expression analysis of cell lines differentiated to parathyroid lineages. Cells were sampled on the indicated days during differentiation for 4 different differentiation conditions and HOXA3, PBX1, PTH, and GCM2 expression measured by quantitative PCR. Blue histograms were cells differentiated by Protocol 1 (Fig 10), Orange histograms were medium with DMSO, Grey histograms were medium alone, and Yellow histograms were cells subjected to an airway epithelium differentiation protocol. Gene expression is normalized to D1 expression for HOXA3 and PBX1 and normalized to D8 for PTH and D6 GCM2.

Quantitative PCR was also performed on RNA samples from previous parathyroid differentiation experiments to quantify expression levels of PTH, HOX3A, and PBX1 expression. Expression of HOXA3 in protocol 1 only occurs in the presence activin A treatment whereas in the protocol 2 experiment. HOXA3 expression did not depend on activin A treatment. There is expression of GCM2 in cells differentiated by either protocol and at approximately the correct time suggesting protocols 1 (Fig 11) and 2 (Fig 12) are proficient for differentiation cells into parathyroid tissue. Transient expression of parathyroid hormone was detected in a control protocol for differentiation of cells in to airway epithelium suggesting that it might be possible to modify the airway epithelium differentiation protocol to generate parathyroid tissues.

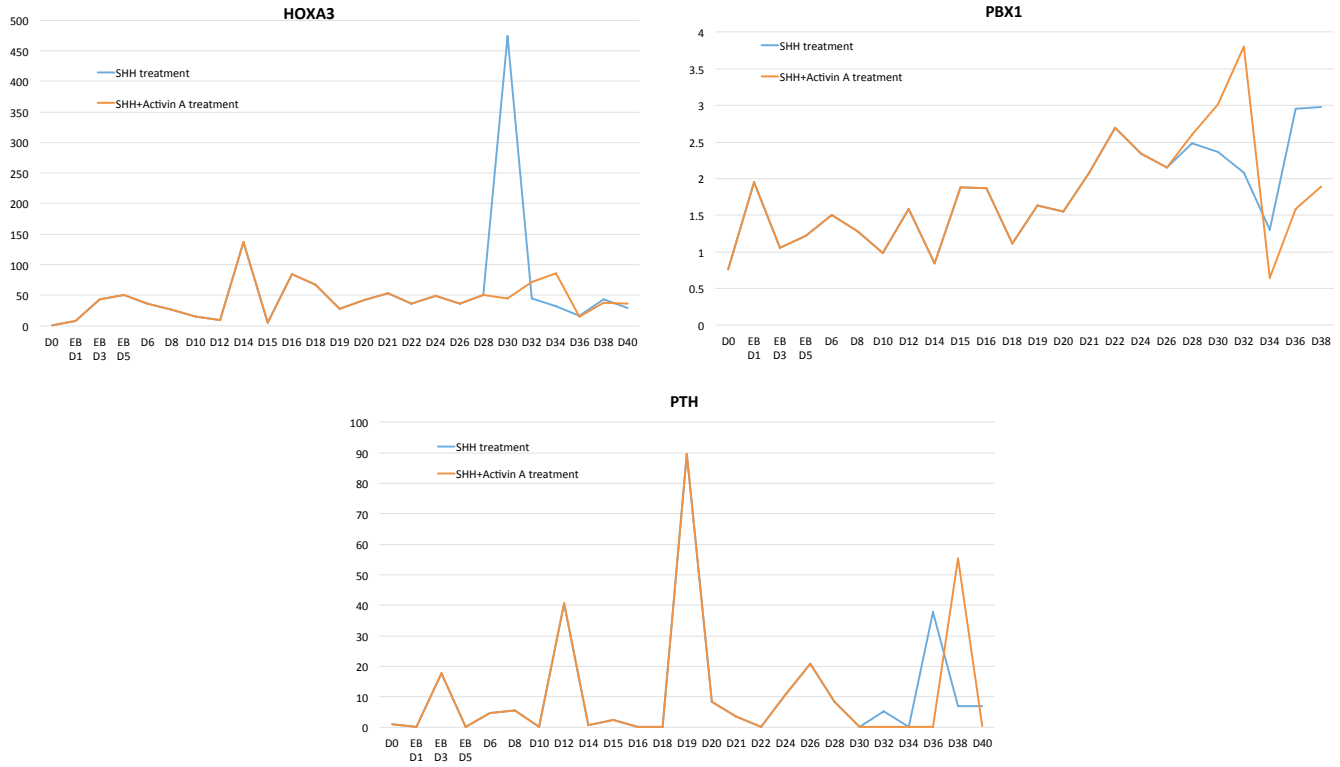


Figure 11. Differentiation of CFNP7 iPS cells by protocol 1

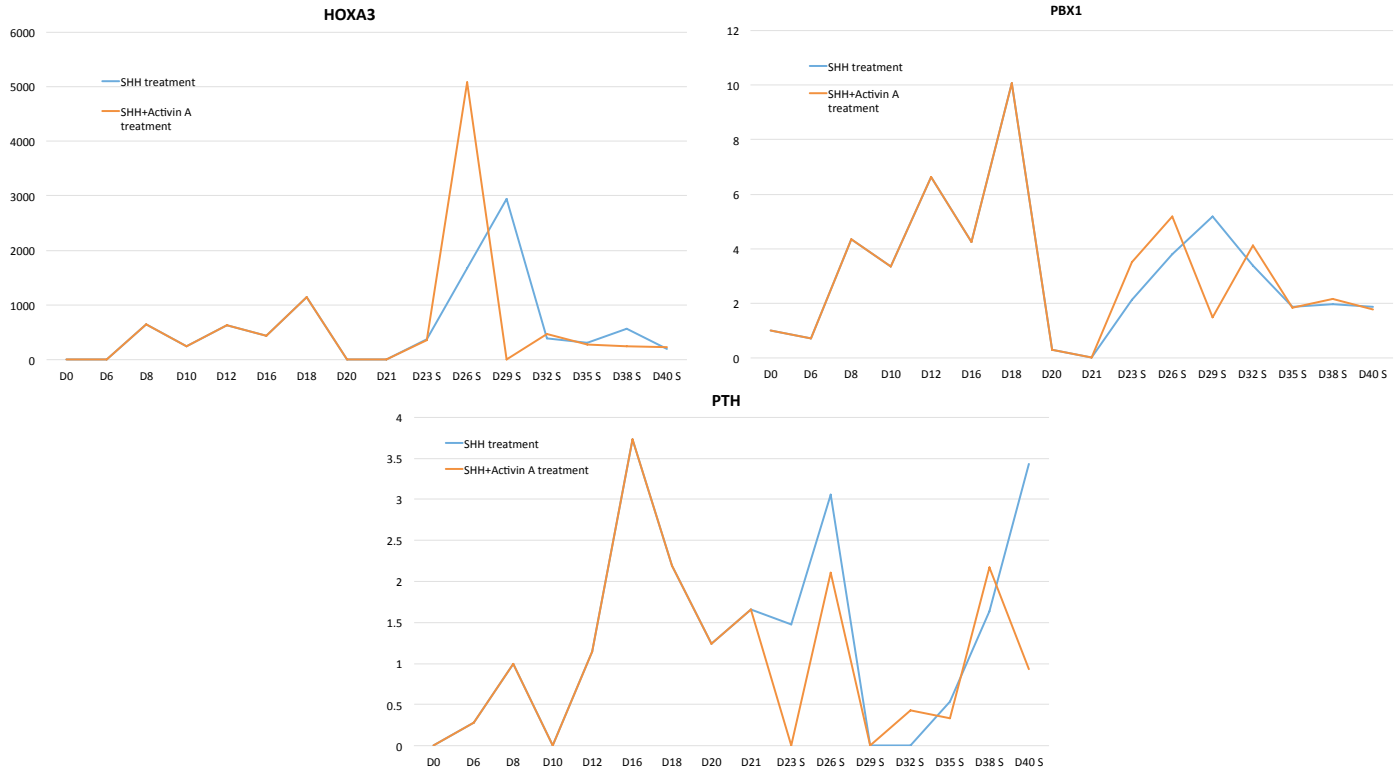


Figure 12. Differentiation of CFNP7 iPS cells by protocol 2

Specific Aim 2; Major Task 3: ***Develop a 3D bioactive matrix that will promote functional ex vivo PTG organogenesis***

Subtask 1: Generate bioactive matricies to test for primary parathyroid cell growth and maintenance of function.

The research for subtask 1 is incorporated into subtasks associated with Specific Aim 1. We have tested several bioactive matrices to establish long-term culture of parathyroid primary cells and will be assessing the influence of these matrices on differentiation of iPS cells into definitive endoderm and parathyroid cells.

Subtask 2: Establish parathyroid spheroid culture.

Nothing to report. These experiments depend on a regular source of parathyroid tissues and development of differentiation protocols. We have a new surgical source of parathyroid tissues that will allow us to test formation of spheroid cultures and test the influence of bioactive matrices on the formation and survival of parathyroid spheroids.

Subtask 3: Generate a GATA3(+/-)Rag1(-/-) mouse model for organoid and iPSC-derived parathyroid progenitor transplantation. It is expected that 10 mice will be needed to make this animal with 2 crosses.

See subtask 4

Subtask 4: Transplant and assess functional efficacy of the transplanted PTG gland tissue/cells. There will be 6 animals/ experiment and it is anticipated that a there will be 2 conditions (primary cells and iPSC-derived), with 10 experiments overall. Thus it is expected that there will be 120 animals required for the planned assessments

We plan to acquire Tbx1 hypomorph mice for the transplantation studies from Deepak Srivastava. We are resubmitting the IACUC application and anticipate the colony will be ready Winter of 2017 to begin transplantation experiments.

What opportunities for training and professional development has the project provided?

Drs Gruenert, Sargent and Suzuki were responsible for mentoring students (Barilla, Chosa, and Yao) for research activities associated with the grant.

Dr Suzuki, Keisuke Chosa, and Cristina Barilla attended the International Society for Stem Cell Research meeting in San Francisco, CA June 22-25, 2016.

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

Since Dr Gruenert's death we have been involved with transferring IRB, GESCR, and BUA protocols to Dr Sargent. That is almost complete. In order to perform the mouse experiments Spring 2017, we are beginning the IACUC approval process and will establish a colony of TBX1 mice.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. Changes/Problems

Changes in approach and reasons for change

None to report.

Actual or anticipated problems or delays and actions or plans to resolve them

Dr Dieter Gruenert, the original Principle Investigator on the grant, died unexpectedly on April 10, 2016. The grant has been transferred to Dr Sargent for the remaining grant term. However, this unfortunate circumstance caused a delay in laboratory operations and has required completion/transfer of required laboratory certifications to Dr Sargent. At this time the Biological Use Application has been transferred to Dr Sargent (appendix) and the IRB/GESCR applications are under review. Dr Gruenert was preparing the IACUC application for the proposed mouse studies at the time of his death. Dr Sargent is completing the application for IACUC review.

An extension for the grant term from February to July 2017 was requested for the transfer of the grant to Dr Sargent. This additional time will allow for reorganizing our efforts and for completion of the IACUC and mouse studies.

During the transition period, we did not have access to human parathyroid surgical samples to complete studies on establishing primary human parathyroid cell cultures and identifying bioactive matrices to support parathyroid cell growth. Dr Marika Russell, a surgeon in the Department of Otolaryngology Head and Neck Surgery at UCSF, has agreed to help us acquire more human parathyroid samples to complete these specific aims. We are contacting other surgical staff in the Department of Otolaryngology to enlist help in acquiring parathyroid tissue.

Changes that had a significant impact on expenditures

Dr Sargent's salary is less than that of Dr Gruenert's, which allowed for rebudgeting of expenditures to request an extension of the grant term to July 2016. The transition request, with supporting documentation, was submitted to the U.S. Army Medical Research Acquisition Activity on June 03, 2016.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Enlisted an additional source for acquisition of human parathyroid samples.

Significant changes in use or care of vertebrate animals.

Delay in proposed mouse experiments from delayed completion of IACUC due to Dr Gruenert's death.

Significant changes in use of biohazards and/or select agents

Nothing to report

6. Products

Nothing to report

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name	Dieter C Gruenert (deceased)
Project Role	Ex-PI
Research Identifier	NA
Nearest Person Month Worked	4
Contribution to Project	Dr Gruenert was the Principle Investigator and died April 10, 2016
Funding Support	National Institutes of Health-NIDDK 1P01DK08876001A1 and National Institutes of Health R01DK104681

Name	Roy Geoffrey Sargent
Project Role	PI
Research Identifier	NA
Nearest Person Month Worked	6
Contribution to Project	Dr Sargent is the current PI and assumed responsibility for the grant in June, 2016.
Funding Support	1P01DK08876001A1, National Institutes of Health-NIDDK, Development of iPS Cells for Treatment of Hemoglobinopathies, Kan (PI), No Cost Extension.

Name	Shingo Suzuki
Project Role	Post Doctoral Fellow
Research Identifier	NA
Nearest Person Month Worked	3
Contribution to Project	GCMB/GCM2 reporter-gene knockin and mentoring Keisuke Chosa, Cristina Barilla, and Michael Yao
Funding Support	Postdoctoral fellowship from Japan

Name	Cristina Barilla
Project Role	Graduate Student
Research Identifier	NA
Nearest Person Month Worked	3

Contribution to Project	Defining growth conditions for human primary parathyroid tissues and differentation of parathyroid tissues from pluripotent stem cells.
Funding Support	Graduate student fellowship from University of Padua

Name	Keisuke Chosa
Project Role	Graduate Student
Research Identifier	NA
Nearest Person Month Worked	3
Contribution to Project	
Funding Support	Graduate student fellowship

Name	Michael Yao
Project Role	Volunteer/Intern
Research Identifier	NA
Nearest Person Month Worked	2
Contribution to Project	GCMB/GCM2 reporter-gene vector construction and design of CRISPR/Cas9 oligonucleotides
Funding Support	Laboratory volunteer

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Please note that funding changes were accounted for in the transition documents for transfer of the grant to Dr Sargent.

No cost extension
National Institutes of Health-NIDDK 1P01DK08876001A1
Development of iPS Cells for Treatment of Hemoglobinopathies
Core B Co-Director 40 % effort Kan (PI) 09/30/2011 to 07/31/2016

Consortium Contract Terminated due to Dr Grunert's Death
National Institutes of Health R01DK104681
Activating cystic fibrosis transmembrance conductance regulator: the therapeutic potenital of RNA directed gene activation
Scientist 15% Effort Gruenert(Consortium-PI) 04/01/2015 to 03/31/2020

What other organizations were involved as partners?

Nothing to report

8. Special Reporting Requirements

Nothing to report

9. Appendices
BUA Authorization Letter to Dr Sargent

Institutional Biosafety Committee
Office of Research
University of California, San Francisco

DATE: September 20, 2016

TO: Roy Sargent PhD
Otolaryngology
Box 1330

FROM: Michael McGrath, M.D., Ph.D.
Chair, Institutional Biosafety Committee
Box 0942

RE: **APPROVAL:**
BUA #: BU085708-01B
Expiration Date: February 01, 2018
NIH Level: Section III-D-1
Generation of Mammalian iPS Cells for Use in Evaluating Disease Models and Therapies and Transformation and Immortalization of Mammalian Cells

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The Institutional Biosafety Committee (IBC), through the Biosafety Officer, approved the modification application referenced above on **March 24, 2016**.

***The IBC approved the following minimum containment conditions*:**

- Human tissues and cell lines must be used following Universal Precautions and BSL 2 containment criteria.
- Sendai virus and MMLV must be used following BSL 2 containment criteria.
- Cos-7 cells must be used following BSL 2 containment criteria.
- Mouse cell lines, non-pathogenic E. coli, and plasmids may be used following BSL 1 containment criteria.

NOTE: It is the PI's responsibility to ensure that all personnel listed on the BUA have read the approved BUA. The PI must hold a laboratory meeting to review and discuss the BUA and answer all questions. All lab personnel must sign the Safety Training Verification Form confirming that they fully understand potential hazards and applicable safety measures. This form must be uploaded to the "Documents" page of your BUA in RIO and will be audited by your EH&S Specialist.

Required Documentation:

- Print the approved BUA and file it in your laboratory's UCSF Biosafety Logbook
- The approved BUA is on the "Documents" page of your BUA in RIO. Print the most recent summary document with "FINAL" in the file name.
- Print your BUA's ancillary documents, which have been uploaded on the "Documents" page.

cc: Biosafety Officer
EH&S Specialist