Generation of iPSC line from MYH7 R403L mutation carrier with severe hypertrophic cardiomyopathy and isogenic CRISPR/Cas9 corrected control

Vincent Fontaine, Laetitia Duboscq-Bidot, Charlène Jouve, Matthieu Hamlin, Angélique Curjol, Véronique Briand, Philip Janiak, Jean-Sébastien Hulot, Marie-Pierre Pruniaux-Harnist, Philippe Charron, et al.

Title: Generation of iPSC line from MYH7 R403L mutation carrier with severe hypertrophic cardiomyopathy and isogenic CRISPR/Cas9 corrected control

Authors: Vincent Fontaine[a]; Laetitia Duboscq-Bidot[b]; Charlène Jouve[c]; Mathieu Hamlin[a]; Angélique Curjol[d] ; Véronique Briand[e], Philip Janiak[e] ; Jean-Sébastien Hulot[c] ; Marie-Pierre Pruniaux-Harnist[e] ; Philippe Charron[a,b,d]; Eric Villard[a,b,*]

Affiliations:

[a] ICAN - Institute for Cardiometabolism And Nutrition, Paris F-75013 Paris, France.

[b] Sorbonne Université, INSERM, UMR_S1166, AP-HP, Hôpital Pitié-Salpêtrière, F-75013 Paris, France.

[c] Present adress : Université de Paris, INSERM, PARCC, F-75006 Paris, France.

[d] AP-HP, Referral Center for hereditary heart disease, Department of Genetics, Hôpital Pitié-Salpêtrière, F-75013 Paris, France.

[e] Sanofi R&D, Chilly-Mazarin, France.

*Corresponding author: Sorbonne University faculty of medicine, INSERM UMRS1166, 91 Blvd de l'Hôpital, 75013 Paris.

Email: eric.villard@sorbonne-universite.fr; Telephone : +33 (0)14077 9675

Abstract

MYH7 is a major gene responsible for hypertrophic cardiomyopathy (HCM). From patient's skin fibroblasts, we derived an iPSC line (CDGEN1.16) harboring the heterozygous MYH7 R403L mutation, a hot-spot codon in HCM. We subsequently corrected the mutated codon using CRISPR/Cas9 editing and obtained the isogenic control line (CDGEN1.16.40.5) preserving the genomic background of the patient. Both lines were pluripotent and could be efficiently committed to beating cardiomyocytes (CM) suitable for subsequent cell or pseudo-tissue study of HCM pathology.

Resource Table

Unique stem cell lines identifier	ICANi001-P ICANi001-P-40
Alternative names of stem cell lines	CDGEN1.16 CDGEN1.16.40.5
Institution	ICAN : Institut for Cardiometabolism And Nutrition
Contact information of distributor	Vincent Fontaine <v.fontaine@ican-institute.org>
Type of cell lines	iPSCs
Origin	Human
Cell Source	Skin Fibroblasts
Clonality	Clonal
Method of reprogramming	Episomal vectors (Okita et al, Nat Methods, 2011.)
Multiline rationale	Mutated iPSCs clone (CDGEN1.16), isogenic gene corrected control (CDGEN1.16.40.5)
Gene modification	YES

Type of modification	Disease-causing mutation present in patient's iPSC and artificial gene-mutation correction
Associated disease	Hypertrophic cardiomyopathy (HCM)
Gene/locus	MYH7, 14q11.2
Method of modification	CRISPR/CAS9
Name of transgene or resistance	pSpCas9(BB)-2A-Puro (pX459) V2.0 (Addgene #62988), puromycin
Inducible/constitutive system	N/A
Date archived/stock date	2016/08/01
Cell line repository/bank	Cardiogen iPS bank
Ethical approval	ID RCB : 2015-A01174-45

Resource utility

The mechanism by which mutations in MYH7 lead to hypertrophic cardiomyopathy (HCM) remains largely unknown, especially in human cell models. Generation of an iPSC line from an HCM patient due to the hot-spot mutation p.R403L and of its isogenic CRISPR/Cas9-corrected control will allow cellular and molecular analysis of the disease and drug screening.

Resource Details

HCM is a myocardial disease characterized by left ventricular hypertrophy not explained by abnormal loading conditions. Complications may include left ventricular diastolic and systolic dysfunction, heart failure and sudden death. Major mutated genes responsible for HCM are encoding myosin heavy chain (MYH7) and myosin binding protein C (MYBPC3), present in about half of HCM cases. Well-documented variable penetrance among mutations suggested modulation of expressivity due to modifiers such as genetic determinants. Pioneer work using iPSc-CM from patients with MYH7 mutations reported that some of the HCM features could be recapitulated using iPS-CM (Lan et al. 2013). However, this was compared to control not sharing the same genomic background as the patient, therefore introducing uncontrolled heterogeneity.

Here, we derived an iPSc line from an index patient with clinical record for severe HCM with systolic dysfunction and carrying the R403L (NM_000257.4:c.1208G>T) variant, a well-known HCM-causing mutation. Under a signed informed consent (CARDIOGEN study) a forearm skin biopsy was performed in the patient for iPSc line establishment. Skin fibroblasts were isolated and reprogrammed to iPSCs using Okita et al. protocol. After manual selection based on morphological aspects (high nuclei/cytoplasm ratio; **Figure 1C**) and phosphatase alkaline labelling at early passages, the stabilized selected clone (CDGEN1.16; passage 15) showed specific gene expression and protein immunolabelling indicating pluripotency (**Figure 1E & 1D**), high TaqMan hPSC Score confirming tri-lineage differentiation potential (**Figure 1G**) and normal karyotype reminiscent of genomic integrity (**Figure 1F**). The clone CDGEN1.16 was used for CRISPR/Cas9 directed edition in order to reverse the mutation to wild-type sequence. We used clone at passages 22 for electroporation of (i) pX459 vector (ADDGEN #62988) expressing spCas9 and a gRNA targeting a PAM 3-bp away from the mutated allele and (ii) a ssODN DNA donor for homologous recombination. ssODN harbor the wild-type C allele and an adjacent silent A>G substitution (Pro402Pro) allowing for identification of the recombinant allele in edited clones and likely preventing edited chromosome re-cutting (**Figure 1A**). Clones were selected by allele-specific ddPCR approach (Miyaoka et al. 2014) and confirmed by Sanger sequencing (**Figure 1B**). Edited isogenic iPSC clone (CDGEN1.16.40.5) was controlled for pluripotency and genomic integrity as for the original clone (**Figure 1**, CDGEN1.16.40-5 panels). Fibroblasts of the donor, iPSCs and corrected iPSCs were subjected to STR analysis that confirms filiation of the clones. No mycoplasma was detected upon repeated testing (each 5 passages).

Cardiogenicity of the clones was evaluated after differentiation in 2D cultures according to the Lian protocol (Lian et al. 2013). The 2 lines were able to differentiate into beating cellular sheets after 21 days and stained positive for aACTN2 by immunolabelling on dissociated and re-plated cells cultured for one more week (**Figure 1I**). After 3 weeks, cells were isolated, permeabilized and labelled for cardiomyocyte specific cTNT. All cell lines differentiated into cardiomyocytes with efficiency over 75% according to FACS quantitation (**Figure 1H**).

In summary, we established a pair of iPSc lines from an HCM patient, one line carrying the MYH7 p.R403L mutation and the second its isogenic corrected control, defining a model for cellular and molecular study of hypertrophic cardiomyopathy.

Materials and Methods

Cell culture, Reprogramming and Cardiomyocytes differentiation

The fibroblasts were cultured with DMEM, 10% FBS (both Life technologies) and changed every 3 days. Reprogramming of fibroblasts was carried out using episomal vector as previously described (Jeziorowska et al 2017). On Day 6 the medium was switched to TeSR-E7 medium (Stemcell) and changed every 2 days. One-month post-electroporation isolated iPS colonies were picked and expanded in daily changed mTeSR1 (Stemcell) on Matrigel pre-coated dishes at 37 °C and 5 % CO_2 incubator. Cells were passaged manually once a week. Cardiomyocytes differentiation was performed as described previously (Lian et al. 2013).

Gene editing

The gRNA was chosen using "https://zlab.bio/guide-design-resources" and the closest possible to the mutation to be edited in order to maximize HDR efficiency. HDR ssODN template was 127 bp long (Table 3) and contain the wild-type allele for mutation correction together with an adjacent silent Pro402Pro substitution for recombinant events tracking and to minimize re-cutting of the recombinant allele. 2x106 iPS cells were electroporated (Amaxa Human Stem Cell Nucleofector Starter kit, LONZA VPH-5002; prog A-013) with ssODN (IDT, 30µg) and pSpCas9(BB)-2A-Puro (pX459) V2.0 (ADDGENE #48139, 20µg) plasmid expressing Cas9 and gRNA (IDT). Cells were plated in Matrigel pre-coated 6-well plates with mTeSR1 and 10µM Y-27632 (Millipore). To select stably transduced cells, 0.5 µg/ml puromycin was applied 24-hour post-electroporation for 2 days. 3 days after electroporation, cells were isolated with StemPro accutase (Life Technologies) and dispatched in 96 well plate for sib-selection.

Recombinant clone selection - Digital PCR and Sanger Sequencing

A pair of allele-specific TaqMan probes (Table 3) measuring recombinant cells enrichment in clones was designed according to Miyaoka et al. 2014. 80 wells were screened by TaqMan assay on a Biorad ddPCR apparatus (Biorad QX200 Droplet reader). The 8 most enriched clones (1.68% to 3.34%) were dissociated and dispatched in 8 wells of 96 well culture plate for two more round of enrichment. The best clone (98.09% enrichment) was re-plated and 24 clones were sequenced by Sanger sequencing (GATC Biotech) on PCR product with primers (Table 3) outside ssODN sequence to avoid false-positive signal.

Karyotype analysis

Karyotypes on iPSC at passage 20 were performed by Cerba (Cerba laboratory). 22 metaphases were analysed.

Embryoid body formation and scorecard

Generated iPSCs were grown on matrigel, dispensed into small clumps with cell scraper, and cultured in suspension for 10 days in TeSR-E6 medium (Stemcell). At day 8, RNA was isolated from EBs (Ambion). A scorecard kit (Life Technologies) was used to evaluate the gene expression of the three germ layers. The scorecard plate was analysed on StepOneplus™ system real time PCR (Life Technologies).

Immunofluorescent staining

Cells were fixed in 4% paraformaldehyde for 10 min, incubated with Blocking Buffer (1% BSA, 0.4% Triton, x-100 in PBS) for 30 min at room temperature. Cells were incubated with primary antibodies at 4 °C overnight, washed 3 times with PBS and then incubated with secondary antibodies for 1 h at room temperature. All antibodies are listed in Table 3. Nuclei were stained with DAPI (Sigma). Microphotographs were taken using a Nikon microscope and NIS Elements software.

RNA isolation and PCR analysis

RNA was isolated with Pure link RNA mini Kit (Ambion). Reverse transcription of 1 µg of RNA was performed using Super Script IV (Invitrogen). qRT-PCR reactions were run on a LightCycler 480 Real-Time PCR System (Roche) with SYBR Select master mix (Applied Biosystems). CT-values were normalized to housekeeping gene RPL32 using ΔCT method.

Mycoplasma detection

Routine testing for mycoplasma contamination was carried out with the MycoAlert detection Kit (Lonza #LT07-318). Ratios lower than 1.2 were defined as mycoplasma negative.

Acknowledgments

We thank Mrs Saadia Jerbi for the management of patient'enrollment in the study. This work was supported by SANOFI and by the program "Investissements d'Avenir" (ANR-10-IAIHU-05) to the Institute of Cardiometabolism and Nutrition (ICAN).

References

Feng Lan, Andrew S. Lee, Ping Liang, Veronica Sanchez-Freire, Patricia K. Nguyen, Li Wang, Leng Han, Michelle Yen, Yongming Wang, Ning Sun, Oscar J. Abilez, Shijun Hu, Antje D. Ebert, Enrique G. Navarrete, Chelsey S. Simmons, Matthew Wheeler, Beth Pruitt, Richard Lewis, Yoshinori Yamaguchi1, Euan A. Ashley, Donald M. Bers, Robert C. Robbins, Michael T. Longaker, and Joseph C. Wu. Abnormal Calcium Handling Properties Underlie Familial Hypertrophic Cardiomyopathy Pathology in Patient-Specific Induced Pluripotent Stem Cells. Cell Stem Cell. 12 (2013), pp. 101–113.

Keisuke Okita , Yasuko Matsumura, Yoshiko Sato, Aki Okada, Asuka Morizane, Satoshi Okamoto, Hyenjong Hong, Masato Nakagawa, Koji Tanabe, Ken-ichi Tezuka, Toshiyuki, Shibata, Takahiro Kunisada, Masayo Takahashi, Jun Takahashi, Hiroh Saji, Shinya Yamanaka.A more efficient method to generate integration-free human iPS cells. Nat Methods. 8 (2011), pp. 409-412

Yuichiro Miyaoka, Amanda H. Chan, Luke M. Judge, Jennie Yoo, Miller Huang, Trieu D. Nguyen, Paweena P. Lizarraga, Po-Lin So, and Bruce R. Conklin. Isolation of single-base genome-edited human iPS cells without antibiotic selection. Nat Methods. 11 (2014), pp. 291–293

Xiaojun Lian, Jianhua Zhang, Samira M. Azarin, Kexian Zhu, Laurie B. Hazeltine, Xiaoping Bao, Cheston Hsiao, Timothy J. Kamp, and Sean P. Palecek. Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/β-catenin signaling under fully defined conditions. Nat Protoc. 8 (2013), pp. 162–175

Dorota Jeziorowska, Vincent Fontaine, Charlène Jouve, Eric Villard, Sébastien Dussaud, David Akbar, Valérie Letang, Pauline Cervello, Jean-Michiel Itier, Marie-Pierre Pruniaux and Jean-Sébastien Hulot. Differential Sarcomere and Electrophysiological Maturation of Human iPSC-Derived Cardiac Myocytes in Monolayer vs. Aggregation-Based Differentiation Protocols. Int. J. Mol. Sci.18 (2017), pp. 1173-1188

Additional files:

Figure 1

Table 1, 2 and 3

STR analysis

Table 1: Summary of lines

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
CDGEN1-16	CDGEN-1.16	Female	52	Caucasian	GT	HCM
CDGEN1-16.40.5	CDGEN-1.40.5	Female	52	Caucasian	GG	N/A

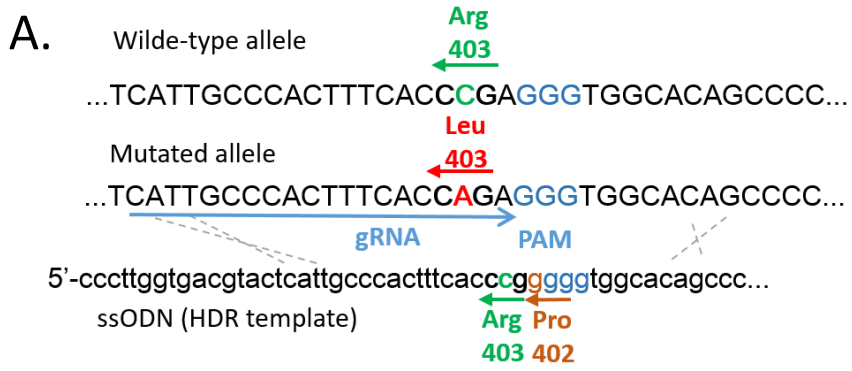
Table 2: Characterization and validation

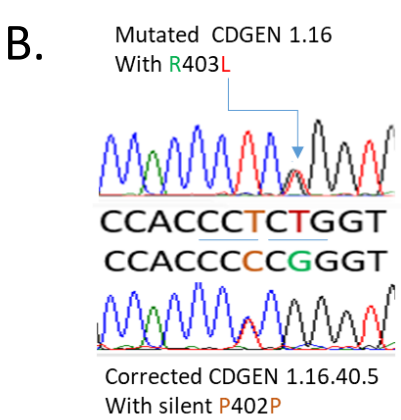
Classification	Test	Result	Data
Morphology	Photography	normal	Figure 1 panel C
Phenotype	Qualitative analysis	Positive immune staining for pluripotency markers: Oct4, Nanog, Sox2, Tra1-60, Tra1-81, SSEA4	Figure 1 panel D
	Quantitative analysis by RT-qPCR	Pluripotency markers Nanog, Sox2, Oct4, hTERT and Crypto are well expressed	Figure 1 panel E
Genotype	Karyotype (G-banding) and resolution	46XX, Resolution 450-500	Figure 1 panel F
Identity	STR analysis	STR Analysis : 18 loci tested and perfect match with the tree cell lines	submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sanger Sequencing	heterozygous type of mutation and homozygous type of wild-type reversion	Figure 1 panel B
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by luminescence. Clones were negative for Mycoplamsa testing	submitted in archive with journal
Differentiation potential	Scorecard on embryoid bodies	Genes of the tree germ layer are upregulated in spontaneous embryoid bodies (ThermoScientific #A15871)	Figure 1 panel G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Negative	not shown but available with author
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

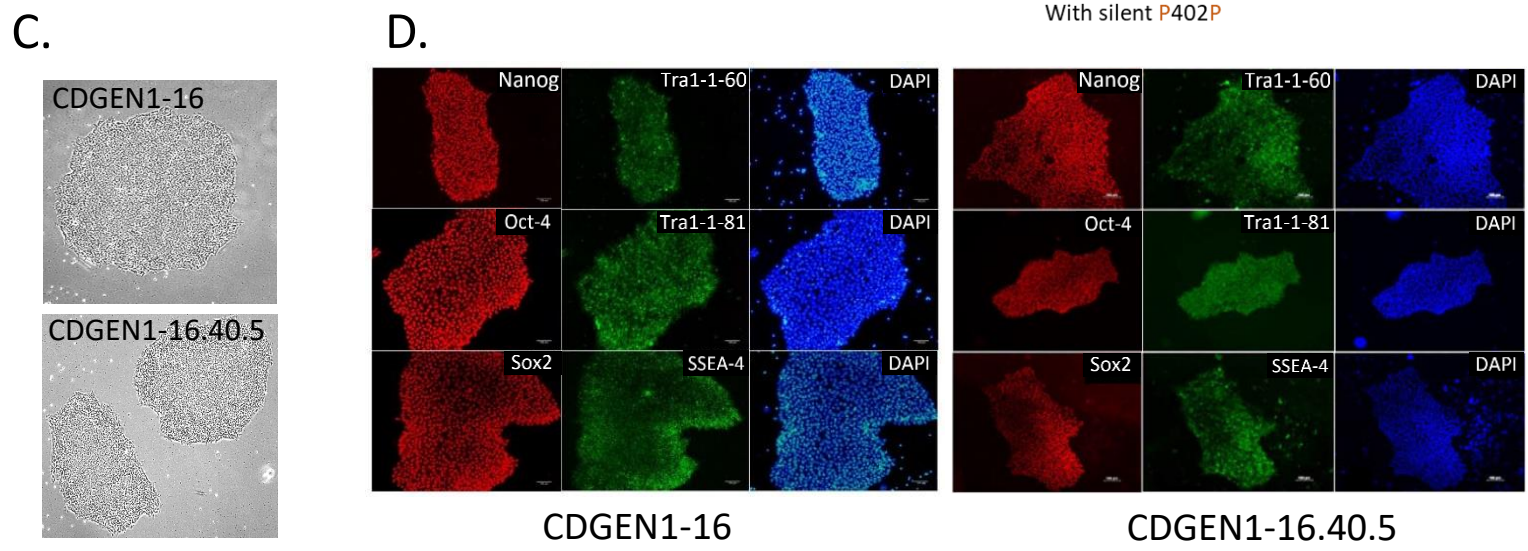
Table 3: Reagents details

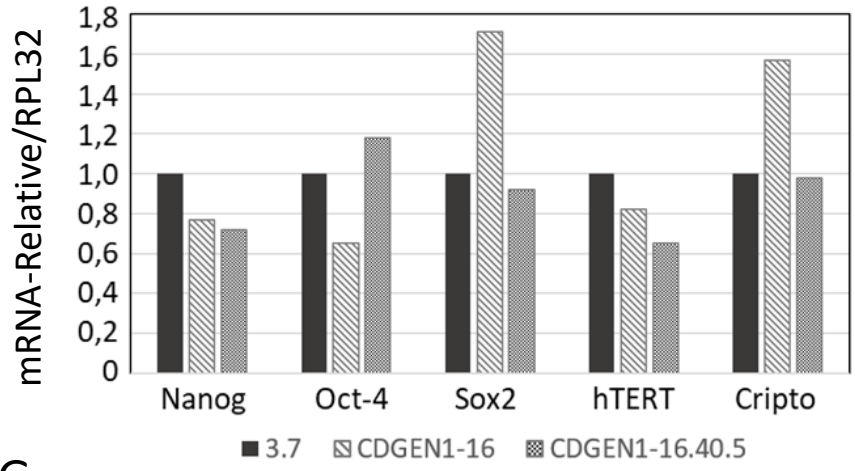
Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers	Rabbit anti-Nanog Rabbit anti-OCT4 Rabbit anti-Sox2 Mouse anti-Tra1-60 Mouse anti-Tra1-81 Mouse anti-SSEA4	1:200 1:200 1:200 1:100 1:100 1:100	Cell Signaling #4903 Clinisciences #3576-100 Millipore #AB5603 Millipore #MAB4360 Millipore #MAB4381 Santa Cruz #Sc-21704
Differentiation Markers	anti-α-actinine anti-troponineT	1:1000 1:500	Sigma-aldrich #A7811-.2ML Abcam #ab45932
Secondary antibodies	Goat anti-mouse A488 Goat anti-rabbit A546	1:1000 1:1000	Life technologies #A10680 Life technologies #A11010
Primers			
	Target	**Forward/Reverse primer (5'-3')**	
Pluripotency Markers (qPCR)	OCT-4 Sox2 Nanog hTERT	GTACTCCTCGGTCCCTTTCC/ CAAAAACCCTGGCACAAACT TTGCTGCCTCTTTAAGACTAGGA/ CTGGGGCTCAAACTTCTCTC CTCCAACATCCTGAACCTCAGC/ CGTCACACCATTGCTATTCTTCG CCTGGGTGGCACGGCTTTTGTTC/ CAGCCTTGAAGCCGCGGTTGA	
House-Keeping Genes (qPCR)	RPL32	AGTTCCTGGTCCACAACGTC/ GTGACTCTGATGGCCAGTTG	
Genotyping	MYH7	TGGTGAAGTTGATGCAGAGC GGTCCACAGCTGGCTCTAAG	
Genetic editing guide-RNA and ssODN	gRNA-403L_F gRNA-403L_R ssODN_RevertMYH7-R403	caccgCATTGCCCACTTTCACCAGA aaacTCTGGTGAAAGTGGGCAATGc TTGCTACTTGCCTTTTCCTTCCAGAGGCTGACAAGTCTGCCT ACCTCATGGGGCTGAACTCAGCCGACCTGCTCAAGGGGCT GTGCCACCC**CC**G**G**GGTGAAAGTGGGCAATGAGTACGTCACC AAGGG TGAAAGTGGGCAATGAGTACGTCACCAAGGG	

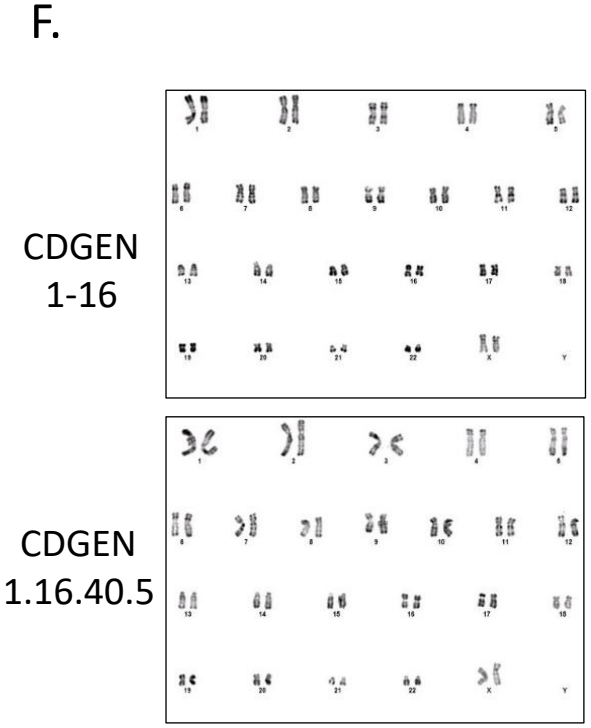
Digital PCR screening	ddPCR_R403_F	CCTTCCAGAGGCTGACAAGTCT
	ddPCR_R403_R	GAAGATGGACCCACCTGCTG
	TaqMan_403_VIC (mutant)	CACCCTCTGGTGAAA
	TaqMan_403_FAM (corrected)	CACCC**C**CGGGTGA
	TaqMan_403_FAM (wild-type)	CACCCTCGGGTGAAA
	MYH7_exon 13Fw	TTA CAG GCA TGA ACC ACA CAC C
	MYH7 exon 13rev	GTG AAC TTG AAA ACT CTC ATC CC











G.

Sample Name	Self-renewal	Ectoderm	Mesoderm	Endoderm
CDGEN1.16	-2,39	2,39	4,03	1,46
CDGEN1.16.40.5	-5,19	1,93	1,55	1,40

x<-1.5	-1.5<=x<-1.0	-1.0<=x<-0.5	-0.5<=x<=0.5	0.5<x<=1.0	1.0<x<=1.5	x>1.5
Downregulated			Comparable			Upregulated