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Generation of Fibrodysplasia ossificans progressiva and control integration free iPSC lines from periodontal ligament fibroblasts

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A B S T R A C T

Fibrodysplasia ossificans progressiva (FOP) is a very rare devastating heterotopic ossification disorder, classically caused by a heterozygous single point mutation (c.617G > A) in the *ACVR1*gene, encoding the Bone morphogenetic protein (BMP) type I receptor, also termed activin receptor-like kinase (ALK)2. FOP patients develop heterotopic ossification episodically in response to inflammatory insults, thereby compromising tissue sampling and the development of in vitro surrogate models for FOP. Here we describe the generation and characterization of a control and a classical FOP induced pluripotent stem cell (iPSC) line derived from periodontal ligament fibroblast cells using Sendai virus vectors.

Resource Table:

Unique stem cell lines identifier	LUMCi009-A LUMCi010-A
Alternative names of stem cell lines	LUMC0085iCTRL (LUMCi009-A) LUMC0084iFOP (LUMCi010-A)
Institution	Leiden University Medical Center
Contact information of distributor	Prof. Peter ten Dijke, P.ten_Dijke@lumc.nl
Type of cell lines	iPSC
Origin	Human
Cell Source	Periodontal ligament fibroblast
Clonality	Clonal
Method of reprogramming	Integration free Sendai virus
Multiline rationale	Control and disease pair
Gene modification	YES
Type of modification	Spontaneous mutation
Associated disease	Fibrodysplasia ossificans progressiva
Gene/locus	c.617G4A; p. (Arg206His)
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	30/12/2015
Cell line repository/bank	N/A
Ethical approval	The study has been approved by the Vrije Universiteit Medisch Centrum (VUMC) Amsterdam Research Committee, with protocol number 2012.467

1. Resource utility

Patients with Fibrodysplasia ossificans progressiva (FOP) develop bone formation at extra skeletal sites in response to trauma. Therefore, induced pluripotent stem cells (iPSCs) generation may be useful to establish patient-derived primary cell lines in order to further understand the specific pathophysiological mechanisms induced by ALK2 R206H causing ectopic bone formation.

2. Resource details

Fibrodysplasia ossificans progressiva (FOP) is an extremely rare congenital disease (1 in 2.000.000 individuals) characterized by a heterozygous point mutation in the *ACVR1*gene, encoding the Bone morphogenetic protein type I receptor, also termed activin receptor-like kinase (ALK)2. The most common ACVR1 mutation in approximately 98% of all FOP patients c.617G > A causes the amino acid substitution

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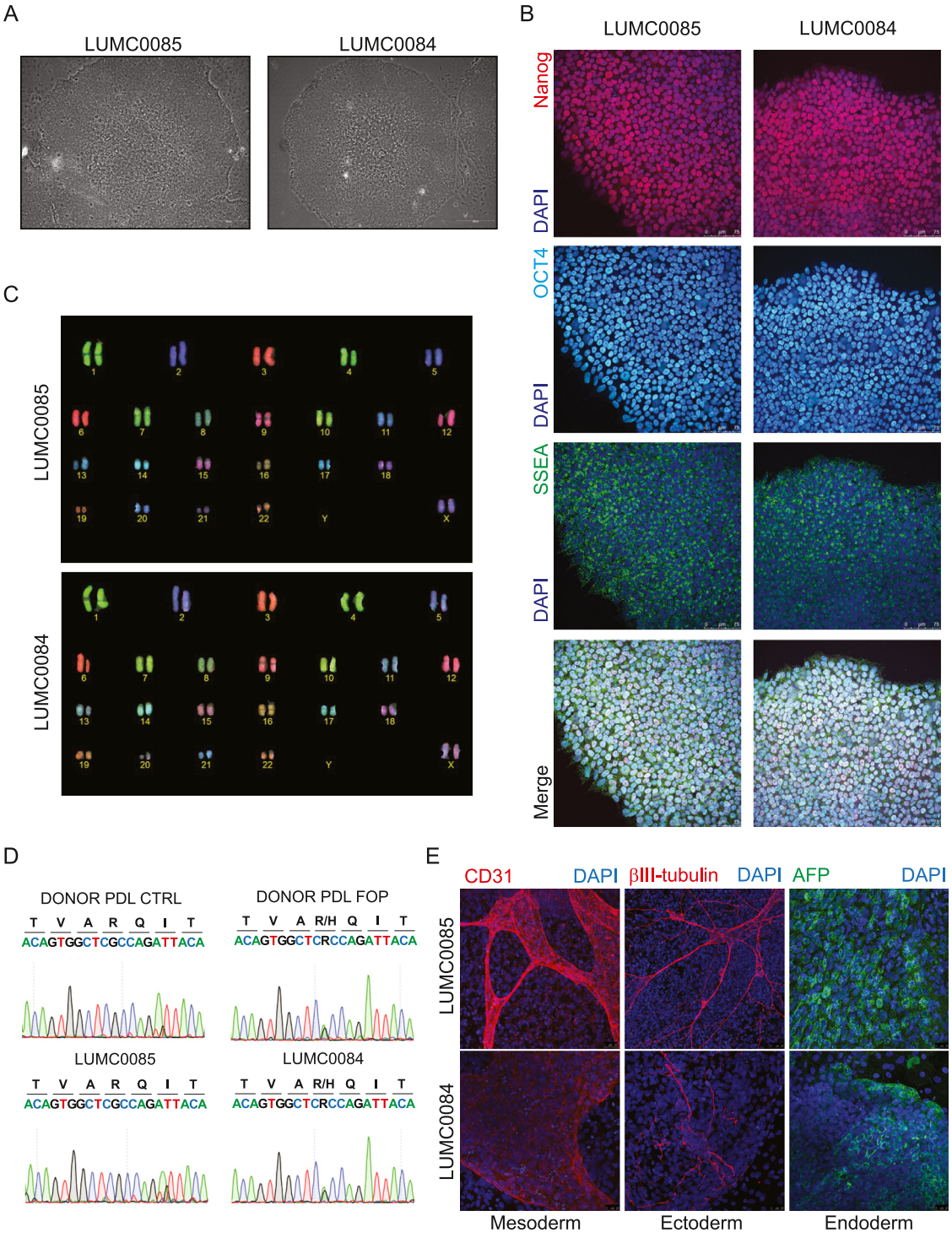

Figure 1. Characterization of the iPS cell lines LUMC0085 and LUMC0084.

R206H in the glycine serine rich intracellular domain of ALK2 (Shore et al., 2006). Here we present an iPSC line generated from periodontal ligament fibroblasts from a female patient with FOP, and a control line from a healthy donor. Periodontal ligament fibroblasts were collected from a 23 years old female with classical FOP, diagnosed with a trismus and pericoronitis of a lower wisdom tooth (Eekhoff et al., 2018), and a 30 years old control female, following the same surgical intervention to remove a wisdom tooth.

Periodontal ligament fibroblasts (PDLs), the cells that enable the anchoring of teeth into bone, were cultured from the donated biopsy samples and were frozen in liquid nitrogen at passage 3. As previously shown, periodontal ligament cells from FOP can be used to address both osteogenesis and osteoclastogenesis aspects of the disease (de Vries et al., 2018). Primary cells however have a limited life span, therefore, iPSC FOP cell models are desired. Reprogramming was performed using a Sendai virus vector containing *MYC, KLF4, SOX2*and *OCT4*, using the vector published by Nishimura et al. (2011), and clonal iPSC lines (LUMCi009-A, LUMCi010-A) were established and characterized (Fig. 1A) (Table 1). The pluripotent nature of the cells was assessed by immunofluorescent staining with specific antibodies against Nanog, Oct4 and SSEA-4 (Fig.1B), and the expression of the pluripotent gene markers *SOX2, OCT3/4, RONIN*and *REX1*by quantitative rtPCR (Supp.

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
LUMC0085iCTRL	LUMC0085	Female	30	Caucasian	c.617G	N/A
LUMC0084iFOP	LUMC0084	Female	23	Caucasian	c.617A	Fibrodysplasia ossificans progressiva

Fig. 1A).

The absence of Sendai viral particles was confirmed at passage 4 and passage 5 by immunofluorescent staining and quantitative rtPCR (Supp. Fig. 1B-C). PDL fibroblasts 36 h after transduction with Sendai particles were used as positive control. Cell line authentication using profiling of 23 STR loci demonstrated that parental and iPSC-derived lines are identical (data not shown). Multicolour FISH based molecular karyotyping was performed at cell level to detect numerical changes, interchromosomal exchanges (translocation, insertion) and large deletions, and pericentric inversions. This analysis did not reveal any large genomic aberrations and confirmed that the iPSC lines were female (46, XX) (Fig. 1C). Using Sanger sequencing we demonstrated the absence and presence of the classical FOP mutation (c.617G > A) in the *ACVR1*exon 4 in control and FOP PDL fibroblasts and iPSC lines, respectively (Fig.1D). Finally, the potential of the LUMCi009-A, LUMCi010-A iPSC lines to give rise to the three germ layers was demonstrated by their spontaneous in vitro differentiation into mesoderm (CD31), ectoderm (βIII-tubulin) and endoderm (AFP) derivatives (Fig. 1E). All cell lines generated were negative for mycoplasma. A summary of the characterization of LUMC0084 and LUMC0085 is shown in Table 2.

3. Materials and methods

3.1. Cell culture and reprogramming

Periodontal ligament fibroblast (PDL) cells were cultured as reported before (de Vries et al., 2018). At passage 6 1.10^5 cells were transduced with Sendai virus (SeVdp(KOSM)302 L) at a multiplicity of infection of 10. After 2 days 15,000 transduced cells were seeded onto a fresh layer of irradiated CD1 mouse embryonic fibroblasts (MEFs). From day 3 cells were cultured in HESC medium (DMEM/F12 (ThermoFisher Scientific) with 20% knockout serum replacement (KSR) (ThermoFisher Scientific), 10 ng/ml bFGF (Peprotech), 100 μM β-mercaptoethanol, 10 μg/ml ascorbic acid (Sigma), GlutaMax (ThermoFisher Scientific), 1% Penicillin-Streptomycin (ThermoFisher Scientific), 1% non-essential amino acids (NEAA) (ThermoFisher Scientific) and with (FOP cells) or without (control cells) 1 μM LDN-193,189 (Sigma-Aldrich) at day 8. Around week 3 visible iPSC colonies were manually transferred into a Vitronectin (StemCEll Technologies)-coated 6 well plate in TESR-E8 (StemCell Technologies) at 37 °C with 5% CO_2. iPSC clones were passaged at 1:10–1:20 ratio once a week using Gentle Cell Dissociation Reagent (StemCell Technologies).

3.2. Immunofluorescent staining

iPSCs were fixed with 2% paraformaldehyde (PFA) for 30 min at room temperature (RT), washed with 0.1 M glycine, permeabilized with 0.1% Triton X-100 and blocked in phosphate buffered saline (PBS) containing 4% normal swine serum (NSS) for one hour. Next, the cells were incubated overnight at 4 °C in blocking solution containing primary antibody. Next day, the cells were washed in PBS and incubated with secondary antibody for one hour at RT. Finally, the cells were washed and mounted in Prolong Gold containing DAPI (Invitrogen). The preparations were imaged with a Leica SP5 confocal scanning laser microscope. Antibodies are described in Table 3.

3.3. Quantitative real-time PCR analysis

Total RNA extraction was performed using NucleoSpin RNA II (Machery Nagel). 500 ng of RNA were retro-transcribed using RevertAid First Strand cDNA Synthesis Kits (Fisher Scientific), and real-time reverse transcription-PCR experiments were performed using SYBR Green (Bio-Rad) and a Bio-Rad CFX Connect device. Used oligonucleotides are shown in Table 3.

3.4. Mycoplasma detection

The absence of mycoplasma was tested using the MycoAlert mycoplasma detection kit (Lonza) according to the manufacturer's instructions.

3.5. ACVR1 mutation analysis

r100 ng of DNA were subjected to PCR to amplify the exon 4 of ACVR1/ALK2, as reported before (Shore et al., 2006). The PCR product was separated in a 1% agarose gel, purified and submitted to Sanger sequencing. Oligonucleotides used for sequencing are described in Table 3.

3.6. Human cell line authentication

The human cell lines listed below have been tested by means of the

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel A
Phenotype	Qualitative analysis	Assess staining/expression of pluripotency markers: Oct4, Nanog, SSEA4	Fig. 1 panel B
	Quantitative analysis (RT-qPCR)	Relative gene expression of *SOX2, OCT3/4, REX1*and *RONIN*.	Sup. Fig. 1, panel A
Genotype	COBRA FISH Analysis	46 XX. 5–8Mb resolution.	Fig. 1 panel C
Identity	STR analysis	Not performed.	Not shown
		23 locus STR profile. iPSCs lines match parental somatic lines	Available with the authors
Mutation analysis (IF APPLICABLE)	Sanger Sequencing	*ACVR1*exon 4 classical FOP mutation confirmed by Sanger-sequencing	Fig. 1 panel D
Microbiology and virology	Mycoplasma	Mycoplasma testing by RT-PCR. Negative.	Not shown
Differentiation potential	e.g. Embryoid body formation OR Teratoma formation OR Scorecard OR Directed differentiation	Proof of three germlayers formation	Fig. 1 panel E

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	Mouse anti-Nanog	1:150	Santa Cruz. Cat# sc-293,121, AB_2,665,475
	Mouse anti-Oct3/4	1:100	Santa Cruz. Cat# sc-5279, AB_628,051
	Mouse anti-SSEA4	1:30	Biolegend. Cat# 330,402, AB_1,089,208
Differentiation markers	Mouse anti-βIII tubulin	1:4000	Covance. Cat# MMS-435P, AB_2,313,773
	Mouse anti-CD31	1:100	Dako. Cat# M0823, AB_2,114,471
	Rabbit anti-AFP	1:25	Quartett. Cat# 2,011,200,530, AB_2,716,839
Secondary antibodies	Goat Anti-Mouse IgG2b Alexa 647	1:250	Invitrogen. Cat# A21242, AB_2,535,811
	Goat Anti-Mouse IgG3 Alexa 488	1:250	Invitrogen. Cat# A21151, AB_2,535,784
	Goat Anti-Mouse IgG1 Alexa 568	1:250	Invitrogen. Cat# A21124, AB_2,535,766
	Goat Anti-Mouse IgG Alexa 568	1:500	Invitrogen. Cat# A11031, AB_144,696
	Goat Anti-Mouse IgM-568	1:500	Invitrogen. Cat# A21206, AB_2,535,792
	Donkey Anti-Mouse IgG Alexa 488	1:200	Invitrogen. Cat# A21202, AB_141,607
	Goat Anti-Mouse IgM-568	1:500	Invitrogen. Cat# A21043, AB_2,535,712
Sendai virus antibodies	Mouse anti–TRA–1–81	1:125	Biolegend. Cat# 330,702, AB_1,089,240
	Mouse anti-SeV NP	1:1600	Non-Commercial. Provided by M. Nakanishi, National Institute of Advanced Industrial Science and Technology, AIST, Japan.
Primers			
	Target	Forward/Reverse primer (5′−3′)	
Episomal Plasmids (qPCR)	Sendai (SeV)	GCAGCTCTAACGTTGTCAAAC/ CCTGGAGCAAATTCACCATGA	
Pluripotency Markers (qPCR)	*OCT3/4*	GACAGGGGGAGGGGAGGAGCTAGG/ CTTCCCTCCAACCAGTTGCCCCAAAC	
	SOX2	GGGAAATGGGAGGGGTGCAAAAGAGG/ TTGCGTGAGTGTGGATGGGATTGGTG	
	REX1	CAGATCCTAAACAGCTCGCAGAAT/ GCGTACGCAAATTAAAGTCCAGA	
	RONIN	GAGCGGCAGTGGTGGGATACCAC/ CTAAGGCCCCAGCTTCCACTTCAG	
House-Keeping Genes (qPCR)	*GAPDH*	GCACCGTCAAGGCTGAGAAC/ TGGTGAAGACGCCAGTGGA	
Targeted FOP mutation Sanger sequencing	*ACVR1*, exon 4	CCAGTCCTTCTTCCTTCTTCC/ AGCAGATTTTCCAAGTTCCATC	

RRID Requirement for antibodies: use http://antibodyregistry.org/ to retrieve RRID for antibodies and include ID in table as shown in examples.

PowerPlex Fusion System 5C autosomal STR kit (Promega), following manufacturer's instructions.

3.7. Karyotyping

Combined binary ratio labeling (COBRA)-FISH analysis was carried out essentially following the instructions indicated in a previously published protocol (Szuhai and Tanke, 2006). Digital images were acquired with the aid of a Leica DMRA fluorescence microscope coupled to a charge-coupled device (CCD) camera.

3.8. In vitro spontaneous differentiation

Undifferentiated iPSCs were plated as clumps on Matrigel-coated coverslips in TESR-E8. At day 1 medium was replaced with DMEM/F12 (ThermoFisher) containing 20% fetal bovine serum (Gibco), 1% PenStrep (Gibco), 100μM β-mercapto ethanol and GlutaMax. Medium was changed once every three day, and after 3 weeks cells were fixed in 2% PFA for 20′ to assess lineage specific markers by immunocytochemistry.

4. Ethics

Extracted wisdom teeth are considered waste material. Informed written consent was obtained from both control and FOP patient to use the cells from their teeth for scientific use in bone research. Before the operation that resulted in the extracted wisdom teeth, the FOP patient had specifically indicated her wish that the cell material would be converted to iPSC, allowing unlimited distribution and use for the FOP research community.

STR analysis

STR analysis corresponding to somatic control and FOP periodontal ligament fibroblasts (HpdlF Cont and HpdlF FOP, respectively) and control and FOP iPSC lines (iPSC Cont and iPSC FOP, respectively) was performed and is available as supplementary material. Technical details were uploaded alongside the STR analysis results.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at 10.1016/j.scr.2019.101639.

References

Shore, E.M., Xu, M., Feldman, G.J., Fenstermacher, D.A., Cho, T.-J., Choi, I.H., Connor, J.M., Delai, P., Glaser, D.L., LeMerrer, M., Morhart, R., Rogers, J.G., Smith, R., Triffitt, J.T., Urtizberea, J.A., Zasloff, M., Brown, M.A., Kaplan, F.S., 2006. A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic Fibrodysplasia ossificans progressiva. Nat. Genet. 38, 525–527.

Eekhoff, E.M.W., Netelenbos, J.C., de Graaf, P., Hoebink, M., Bravenboer, N., Micha, D., Pals, G., de Vries, T.J., Lammertsma, A.A., Raijmakers, P.G., van Es, R.J., 2018. Flare-Up after maxillofacial surgery in a patient with Fibrodysplasia ossificans progressiva: an [^{18}F]-NaF PET/CT study and a systematic review. JBMR Plus 2, 55–58.

de Vries, T.J., Schoenmaker, T., Micha, D., Hogervorst, J., Bouskla, S., Forouzanfar, T., Pals, G., Netelenbos, C., Eekhoff, E.M.W., Bravenboer, N., 2018. Periodontal ligament fibroblasts as a cell model to study osteogenesis and osteoclastogenesis in Fibrodysplasia ossificans progressiva. Bone 109, 168–177.

Nishimura, K., Sano, M., Ohtaka, M., Furuta, B., Umemura, Y., Nakajima, Y., Ikehara, Y., Kobayashi, T., Segawa, H., Takayasu, S., Sato, H., Motomura, K., Uchida, E., Kanayasu-Toyoda, T., Asashima, M., Nakauchi, H., Yamaguchi, T., Nakanishi, M., 2011. Development of defective and persistent sendai virus vector. J. Biol. Chem. 286, 4760–4771.

Szuhai, K., Tanke, H.J., 2006. COBRA: combined binary ratio labeling of nucleic-acid probes for multi-color fluorescence in situ hybridization karyotyping. Nat. Protoc. 1, 264–275.