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Induced pluripotent stem cells (iPSC) created from skin fibroblasts of patients with Prader-Willi syndrome (PWS) retain the molecular signature of PWS

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ABSTRACT

Prader-Willi syndrome (PWS) is a syndromic obesity caused by loss of paternal gene expression in an imprinted interval on 15q11.2-q13. Induced pluripotent stem cells were generated from skin cells of three large deletion PWS patients and one unique microdeletion PWS patient. We found that genes within the PWS region, including *SNRPN* and *NDN*, showed persistence of DNA methylation after iPSC reprogramming and differentiation to neurons. Genes within the PWS minimum critical deletion region remain silenced in both PWS large deletion and microdeletion iPSC following reprogramming. PWS iPSC and their relevant differentiated cell types could provide *in vitro* models of PWS.

Resource tables

Table R1
PWS iPSC Line 129.

Name of stem cell line	129-CUMC/UF
Institution	Columbia University, NYSCF, University of Florida
Person who created resource	Lisa Cole Burnett (Columbia University), Dieter Egli (Columbia University, NYSCF), Carlos Sulsona (University of Florida), Daniel Driscoll (University of Florida), Rudolph Leibel (Columbia University)
Contact person and email	Rudolph Leibel, rl232@columbia.edu
Date archived/stock date	4/15/2016
Origin	Human skin fibroblasts
Type of resource	Biological reagent: induced pluripotent stem cell (iPS); derived from PWS Type 2 deletion patient fibroblasts
Sub-type	129 cell line
Key transcription factors	Oct4, Sox2, cMyc, Klf4
Authentication	Identity and purity of cell line confirmed (Fig. 1)
Link to related literature	See references at end of the manuscript
Information in public databases	
Ethics	Patient informed consent obtained/Ethics Review Board-competent authority approval obtained

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Table R2
PWS iPSC Line 139.

Name of stem cell line	139-CUMC/UF
Institution	Columbia University, NYSCF, University of Florida
Person who created resource	Lisa Cole Burnett (Columbia University), Dieter Egli (Columbia University, NYSCF), Carlos Sulsona (University of Florida), Daniel Driscoll (University of Florida), Rudolph Leibel (Columbia University)
Contact person and email	Rudolph Leibel, rl232@columbia.edu
Date archived/stock date	4/15/2016
Origin	Human skin fibroblasts
Type of resource	Biological reagent: induced pluripotent stem cell (iPS); derived from PWS Type 1 deletion patient fibroblasts
Sub-type	139 cell line
Key transcription factors	Oct4, Sox2, cMyc, Klf4
Authentication	Identity and purity of cell line confirmed (Fig. 1)
Link to related literature	See references at end of the manuscript
Information in public databases	
Ethics	Patient informed consent obtained/Ethics Review Board-competent authority approval obtained

Table R3
PWS iPSC Line 066MD.

Name of stem cell line	066MD-CUMC/INSERM
Institution	Columbia University, NYSCF, INSERM
Person who created resource	Lisa C. Burnett (Columbia University), Sanaa Eddiry (Centre de Physiopathologie de Toulouse-Purpan, INSERM), Maithe Tauber (Centre de Physiopathologie de Toulouse-Purpan, INSERM), Carlos R. Sulsona (University of Florida), Brynn Levy (Columbia University), Jean Pierre Salles (Centre de Physiopathologie de Toulouse-Purpan, INSERM), Daniel J. Driscoll (University of Florida), Dieter Egli (Columbia University, NYSCF), Rudolph L. Leibel (Columbia University)
Contact person and email	Rudolph L. Leibel, rl232@columbia.edu
Date archived/stock date	4/15/2016
Origin	Human skin fibroblasts
Type of resource	Biological reagent: induced pluripotent stem cell (iPS); derived from PWS microdeletion patient
Sub-type	066MD cell line
Key transcription factors	Oct4, Sox2, cMyc, Klf4, Lin28
Authentication	Identity and purity of cell line confirmed (Fig. 1)
Link to related literature	See references end of the manuscript
Information in public databases	
Ethics	Patient informed consent obtained/Ethics Review Board-competent authority approval obtained

Table R4
PWS iPSC Line 031M.

Name of stem cell line	031M-CUMC/INSERM
Institution	Columbia University, NYSCF, INSERM
Person who created resource	Lisa C. Burnett (Columbia University), Sanaa Eddiry (Centre de Physiopathologie de Toulouse-Purpan, INSERM), Maithe Tauber (Centre de Physiopathologie de Toulouse-Purpan, INSERM), Carlos R. Sulsona (University of Florida), Brynn Levy (Columbia University), Jean Pierre Salles (Centre de Physiopathologie de Toulouse-Purpan, INSERM), Daniel J. Driscoll (University of Florida), Dieter Egli (Columbia University, NYSCF), Rudolph L. Leibel (Columbia University)
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Table R4 (*continued*)

Name of stem cell line	031M-CUMC/INSERM
Date archived/stock date	4/15/2016
Origin	Human skin fibroblasts
Type of resource	Biological reagent: induced pluripotent stem cell (iPS); derived from PWS Type 1 deletion patient
Sub-type	031M cell line
Key transcription factors	Oct4, Sox2, cMyc, Klf4, Lin28
Authentication	Identity and purity of cell line confirmed (Fig. 1)
Link to related literature	See references end of the manuscript
Information in public databases	
Ethics	Patient informed consent obtained/Ethics Review Board-competent authority approval obtained

Introduction

Prader-Willi syndrome (PWS) is caused by a loss of paternally-expressed genes in an imprinted region of 15q11.2-q13; the syndrome affects 1 in 25,000 live births (Smith et al., 2003; Angulo et al., 2015). Most instances of PWS (70%) are due to a "large" 4–5 Mb deletion in 15q11.2-q13 (Fig. 1A). All large PWS deletions include six imprinted, protein-encoding genes (*MKRN3*, *MAGEL2*, *NECDIN*, *C15ORF2*, *SNURF*, *SNRPN*), seven non-protein coding snoRNA genes (*SNORD107*, *SNORD64*, *SNORD108*, *SNORD109A*, *SNORD116*, *SNORD115*, and *SNORD109B*), and the long non-coding RNA, *IPW* (Cassidy and Driscoll, 2009). About 25% of instances of PWS are due to uniparental maternal disomy, while <5% of cases result from unbalanced paternal translocations, imprinting defects, or microdeletions (Angulo et al., 2015; Cassidy and Driscoll, 2009). The clinical phenotypes of PWS include hyperphagic obesity, hypogonadism, low growth hormone (GH) associated with short stature, hyperghrelinemia, and relative hypoinsulinemia (Smith et al., 2003; Angulo et al., 2015; Butler et al., 2006). Although over a dozen mouse models of PWS have been generated, none develop the hallmark obesity associated with PWS. Patient-specific iPSCs could provide a novel model system with which to study the molecular etiology of the disease.

Results and discussion

Generation of iPS cell lines from PWS patients and confirmation of genotypes and pluripotency

We generated induced pluripotent stem cells (iPSCs) from three large deletion (LD) PWS patients and one microdeletion (MD) PWS patient with the smallest deletion (118 kb deletion, Chr 15q: 15:25,257,217–15:25,375,376) identified to date (Table 1) (Bieth et al., 2015). All iPSC expressed pluripotency markers at the protein and transcript level, and differentiated to teratomas consisting of all three germ layers (Supplemental Figs. 1–3). Methylation-specific multiplex ligation probe amplification assay (MS-MLPA) and comparative genome hybridization single nucleotide polymorphism (CGH SNP) arrays confirmed the genotypes of the cell lines utilized in the study (Supplemental Figs. 4 and 5). G-banded karyotyping confirms the absence of balanced translocations for all lines (Supplemental Fig. 6). The 4–5 Mb "large" deletions and the 118 kb microdeletion in the PWS patient lines are below the level of resolution for G-banded karyotyping. Microdeletion breakpoints were confirmed with the Agilent High Density SNP array to encompass only three genes, all of which are non-coding RNA genes: *SNORD109A*, *SNORD116*, and *IPW* (Supplemental Fig. 5).

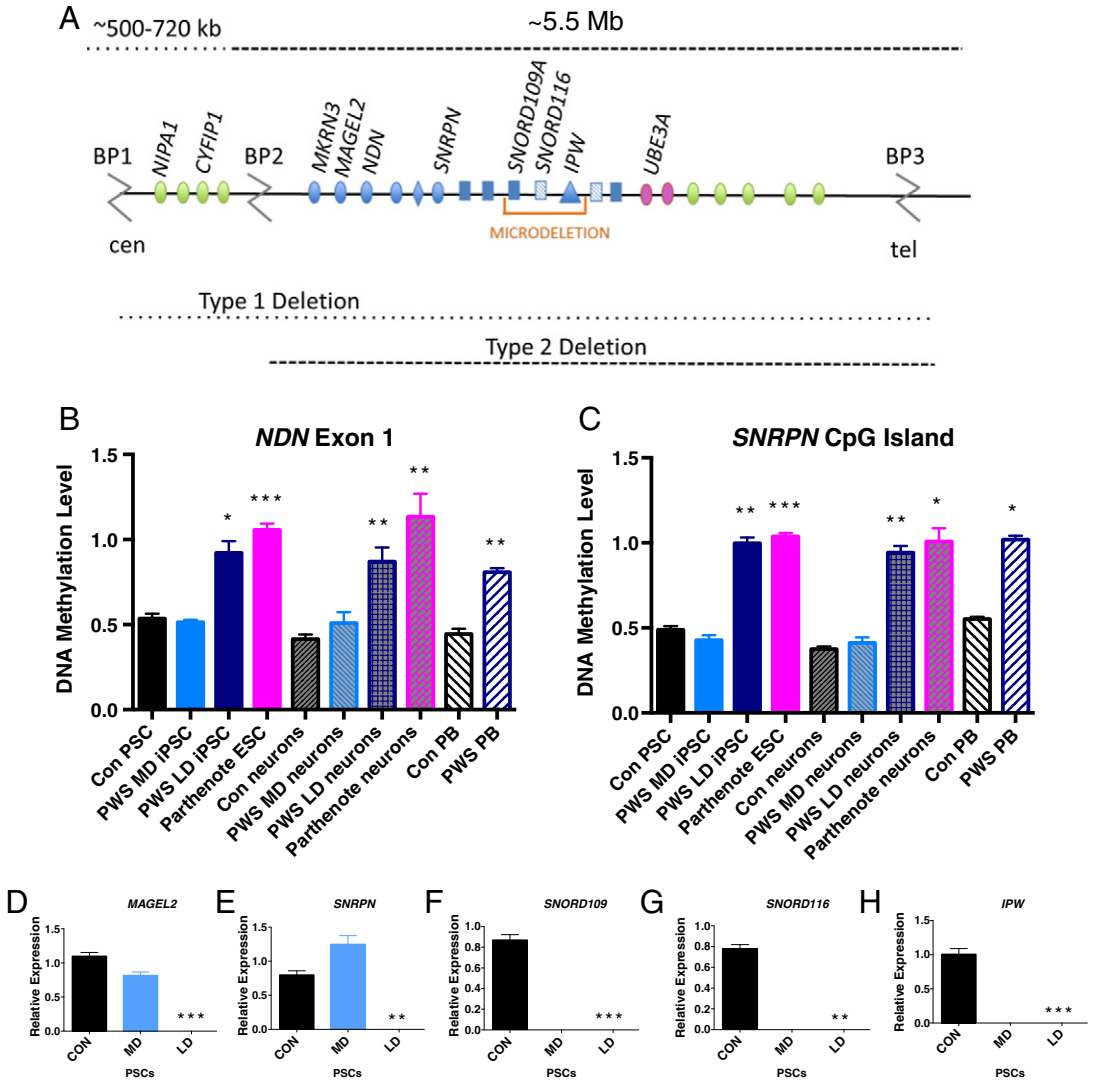

Fig. 1. PWS iPSCs retain the molecular signature of PWS. A) The PWS locus contains non-imprinted genes (green), maternally imprinted, paternally expressed genes (blue), and paternally imprinted, maternally expressed genes (pink). There are protein- coding (ovals) and non-protein coding RNA genes (squares and triangles) in the interval. Large deletions (LD) are bound by breakpoints 1 or 2 and breakpoint 3. Patients harboring microdeletions (MD) define a minimum critical deletion region highlighted in orange containing three non-coding, paternally expressed RNA genes. B, C) DNA methylation at the PWS locus persists after reprogramming to iPSC and differentiation to neurons. Genomic DNA from unaffected control (CON) peripheral white blood cells, iPSC's, and iPSC-derived neurons have methylation levels ranging from 0.38 to 0.58 indicating that one allele is methylated (methylation status is expressed as digested sample relative to non-digested sample fraction). In PWS large deletion iPSCs, iPSC-derived neurons, and peripheral white blood cells, methylation levels of genomic DNA range from 0.80 to 1.05 indicating that all gDNA screened was methylated at both the *NDN* and *SNRPN* loci. There were no differences in methylation levels of PWS large deletion, parthenogenic ESCs, and ESC-derived neurons. Parthenogenic ESC and ESC-derived neurons were used as a positive control as all genetic content is maternally derived. There were also no differences in methylation levels at *NDN* and *SNRPN* loci among unaffected control, PWS microdeletion iPSC and iPSC-derived neurons because these two genes are not included in the microdeletion interval. Furthermore, methylation differences between genotypes of reprogrammed or differentiated material were not different than those for gDNA from primary peripheral white blood cells on which no reprogramming or differentiation procedures have been performed. D-H) Genes in the PWS locus remain silenced after iPSC generation. Expression of *MAGEL2* and *SNRPN* can be detected in iPSC from unaffected control and PWS microdeletion individuals. However, *SNORD109*, *SNORD116*, and *IPW* expression are undetectable in iPSC from both PWS large deletion and PWS microdeletion patients. These transcripts are present in iPSC from unaffected control individuals. The CON PSC bars in A and B represent data averaged from the following unaffected control cell lines: 1043-D3 iPSC P11, 1058-B7 iPSC P11, S1111B iPSC P10, 1043-B1 iPSC P12, 1058-6 iPSC P12, 056LBT iPSC P10, 1016 iPSC P15, and NYSCF1 HUES P20 (*14*). The PWS MD iPSC bars in A and B represent data averaged from 066MDA iPSC P10 and 066MDC iPSC P10. The PWS LD iPSC bars in A and B represent data averaged from 031MT iPSC P10, 129C iPSC P9, and 139P iPSC P12. The parthenote ESC bars in A and B represent data averaged from pES2 P10, swaps1 P7, and swaps2 p7. The CON neurons bar represents data averaged from mature neurons between day 30 and 35 from iPSC lines 1043-D3, s1111B, 1058-6, 1043-B1, s1034-A3, 1058-B7, HUES46, and 1013ASV. The PWS MD neurons bar represents data averaged from mature neurons between day 30 and 35 from iPSC lines 066MDA, 066MDC, and 066MDE. The PWS LD neurons bar represents data averaged from mature neurons between day 30 and 35 of differentiation from iPSC lines 031MT, 129C, 129M, and 139P. The parthenote neurons bar represents data averaged from mature neurons between day 30 and 35 of differentiation from parthenogenetic ESC lines swaps1 and pES2. The qRT-PCR data in panels D-H represents averages from the following iPSC cell lines, all of which are between passage 10 and 11, CON: 1043-D3, 1058-B7, s1034-A3, 056LBT, 1058-6, s1111b, PWS MD: 066MDA, 066MDC, 066MDE, and PWS LD: 031MC, 031MT, 129C, 139P. [PSC = pluripotent stem cells, iPSC = induced pluripotent stem cells, ESC = embryonic stem cells].

Patient-derived iPSC retain the molecular signature of PWS

Maternal DNA methylation patterns persisted after reprogramming and neural differentiation (Fig. 1B–C). PWS microdeletion iPSCs and iPSC-derived neurons had mean methylation levels (expressed as digested sample peak height/non-digested sample peak height) at *NDN* and *SNRPN* that were not different from unaffected controls, including unaffected control gDNAs from peripheral blood lymphoblasts that were not subjected to manipulations such as reprogramming or neural differentiation (Noggle et al., 2011). *NDN* and *SNRPN* are not

Table 1
Anthropometric data from PWS patients and reprogramming methods.

Cell line	Genotype	Gender	BMI at biopsy or maximum lifetime BMI	Reprogramming method
031M	PWS type 1 large deletion	F	15.52*	Stemgent mRNA
129	PWS type 2 large deletion	M	49.4	Retrovirus
139	PWS type 1 large deletion	M	57.3	Retrovirus
066MD	PWS microdeletion	F	55.4	Stemgent mRNA

* Patient was still in 'failure to thrive' clinical phase at time of biopsy; maximum lifetime BMI not available.

included in the microdeletion interval (Fig. 1A). PWS large deletion iPSC and iPSC-derived neurons display methylation levels at *NDN* and *SNRPN* approximately twice those of unaffected control and PWS microdeletion iPSC and iPSC-derived neurons (Fig. 1B–C). Parthenogenetic embryonic stem cells (ESC) and ESC-derived neurons were used as positive controls as they only contain maternal genetic material (Sagi et al., 2016). DNA methylation levels at the *NDN* and *SNRPN* loci were similar in pluripotent stem cells (PSC) and PSC-derived neurons from PWS large deletion patients and parthenogenetic ESCs. Furthermore, the level of DNA methylation at the *NDN* and *SNRPN* loci did not differ between PWS large deletion peripheral blood and iPSC and iPSC-derived neurons, indicating that the manipulations of somatic cell reprogramming and neural differentiation did not cause de-methylation of genomic DNA at the PWS loci (Fig. 1B–C). Method of pluripotency induction had no impact on the genomic DNA methylation level at *NDN* or *SNRPN*.

Quantitative RT-PCR for PWS region genes was performed in iPSC (Fig. 1D–H) from unaffected controls, PWS large deletion patients, and a PWS microdeletion patient. Induced pluripotent stem cells from unaffected controls expressed all genes within the PWS interval (Fig. 1D–M). Expression levels of *MAGEL2* and *SNRPN* are similar in PWS microdeletion and control iPSC (Fig. 1D, E, I, J), as *MAGEL2* and *SNRPN* are not included in the PWS microdeletion interval. However, *SNORD109*, *SNORD116*, and *IPW* are deleted on the paternal chromosome in the PWS microdeletion patient and the expression of these genes remained silenced in PWS iPSC. All of the PWS interval genes - specifically *MAGEL2*, *SNRPN*, *SNORD109*, *SNORD116*, and *IPW* - remained silenced in PWS large deletion iPSCs (Fig. 1D–M).

In the aggregate, PWS iPSC and iPSC-derived neurons retain the molecular signature of PWS; in support of our findings, Chamberlain, et al. and Yang, et al., and Stelzer, et al. have also reprogrammed PWS fibroblasts to pluripotent iPSCs that maintain maternal DNA methylation at the PWS locus following reprogramming (Yang et al., 2010; Chamberlain et al., 2010; Stelzer et al., 2014). PWS iPSC and iPSC-derived neurons have appropriate deletion breakpoints and gene copy numbers in PWS and non-PWS regions as indicated by MLPA and CGH-SNP arrays (Supplemental Figs. 4, 5). PWS region maternal DNA methylation signatures persist following somatic cell reprogramming to iPSC and differentiation of iPSC to neurons (Fig. 1B, C). Additionally, PWS iPSC display appropriate absence of PWS region gene expression in both large deletion and microdeletion lines (Fig. 1D–M).

Materials and methods

Human subjects

All studies and consenting procedures were approved by the Institutional Review Boards of the participating institutions.

Fibroblast reprogramming to iPSC and PSC culture methods

Primary human fibroblasts from unaffected controls and PWS patients were reprogrammed to iPSC using retrovirus reprogramming, sendai virus reprogramming or mRNA reprogramming (Fusaki et al., 2009; Takahashi et al., 2007). The Stemgent mRNA reprogramming kit (00-0071) plus miRNA mixture were used. 15 K to 30 K fibroblasts were plated on gelatin-coated tissue culture 12-well dishes. Ideal density was determined by eye. One-to-two days after seeding fibroblasts, the miRNA cocktail was added to the fibroblasts (day 0 of reprogramming). The mRNA cocktail consisting of Oct4, Sox2, Klf4, C-Myc, Lin28, and nGFP was added days 1 to 3. On day 4, both the miRNA and mRNA cocktails were added. On day 5 to day 11 of mRNA reprogramming the fibroblasts were treated with only the mRNA cocktail. Transfections were performed at the same time each day. Induced pluripotent stem cell colonies emerging from the fibroblast culture were hand-picked and transferred to a 24 well dish with MEFs. Colonies were then serially expanded. Sendai virus reprogramming was performed as previously described (Fusaki et al., 2009). Human iPSC cultures were routinely retained on MEFs (Globalstem) and cultured in human ES media: KO-DMEM, 10% KO-SR, 1% NEAA, 1% glutamax, 0.1% β-ME, 1% P/S, and 10 ng/mL bFGF. Media was supplemented with Y-27632 upon passaging.

MS-MLPA assay for gene copy number, and DNA methylation of PWS region genes

Genomic DNA was isolated from PSC and PSC-derived neurons using the Roche High Pure PCR Template Preparation Kit. gDNA was then analyzed using the SALSA MLPA ME028 Prader Willi/Angelman probemix (MRC-Holland) for copy number and DNA methylation of PWS interval genes.

Neuronal differentiation

Induced pluripotent stem cells were differentiated to neurons using a modified dual smad protocol (Chambers et al., 2009). Undifferentiated iPSCs cultured on MEFs were dissociated and re-plated onto fresh MEFs at a density of 200,000 cells/well of a 6-well plate in human ES media with Y-27632 on day 0. On day 1, media was changed to EB media (HES media without bFGF) supplemented with 10 μM SB431542 and 250 nM LDN193189 (LSB). Cells were treated with EB + LSB on days 2 and 3, 70% media changes were performed each day. On day 4, 75% EB + 25% N2 (DMEM/F12 with 1× N2 supplement, 1% GlutaMAX, 1% penicillin/streptomycin, 0.2 mM ascorbic acid, and 1.6% glucose) with LSB was used. On day 5, 50% EB + 50% N2 with LSB was used. On day 6, 25% EB + 75% N2 with LSB was used. N2 supplemented with LSB was used on days 7 to 10. On day 10, cells were dissociated and plated onto poly-L-ornithine and laminin coated plates in N2 + LSB media with Y-27632. 200,000 cells per well for a 6 well plate or 80,000 cells per well for a 12 well plate were seeded. On day 11 the media was changed to N2 plus B27 without retinoic acid and 20 ng/uL recombinant BDNF (R&D systems). Neurons were harvested at D34 for RNA isolation.

qRT-PCR for PWS region genes in iPSC

RNA was isolated from PSC using the Qiagen RNeasy kit with DNAse treatment. Total RNA was converted to cDNA using the Roche Transcriptor First Strand cDNA Synthesis kit. qRT-PCR was performed using Roche LightCycler 480 SYBR Green I Master mix. Primers are listed in Supplemental Table 2.

Pluripotency analysis by immunohistochemistry

iPSC lines of at least passage 10 were fixed in 4% PFA for 10 min, washed with PBS twice for 5 min, blocked for 1 h at room temperature using 10% donkey serum, in PBS with 0.1% Triton X-100, washed with PBS three times for 5 min each, incubated in primary antibody overnight at 4 °C, washed with PBS three times for 5 min, and incubated with secondary antibody at room temperature for 2 h. Primary antibodies and dilutions used are as follows: Oct4 (1:500) (09-0023 Stemgent), Tra1-60 (1:500) (MAB4360 Millipore), Tra1-81 (1:500) (MAB4381 Millipore), and Nanog (1:300) (Cell Signaling Technologies D73G4). All Alexafluor secondary antibodies were diluted 1:1000. Hoechst 33342 (Sigma) was used to mark nuclei. Imaging was done on Olympus IX71 epifluorescence microscope with an Olympus DP30 monochrome camera. Monochrome images were then assigned the fluorophore color.

Nanostring N-Counter Pluripotency Gene Expression Assay in iPSC

iPSC were screened for endogenous expression of pluripotency markers *LIN28*, *NANOG*, *OCT4*, *SOX2*, *ZFP42*, endogenous expression of fibroblast marker *KLF4*, and exogenous expression of retroviral transgenes using the Nanostring N-Counter Gene Expression Assay. RNA was isolated from iPSC using the Qiagen RNeasy Plus kit. 100 ng total input RNA was used; the Pluri25 custom code set was used for the Nanostring N-Counter system.

Pluripotency analysis by Teratoma formation

Undifferentiated iPSCs (~250 K) were dissociated with TRP-LE and pelleted by centrifuging at 0.8 RCF for 4 min at room temperature, the pellet was re-suspended in 50 μL human ES media supplemented with Y-27632. Cells were mixed 1:1 (vol:vol) in matrigel (Corning) and were injected subcutaneously into the dorsal flank of NSG immunocompromised mice. Cells were allowed to grow for ~12 weeks; mice were monitored twice weekly for the appearance of growths and signs of distress. Mice were sacrificed and teratomas were removed and fixed overnight in 70% ethanol then paraffin embedded, sectioned, and stained with hematoxylin and eosin.

CGH SNP array

Deletion breakpoints in PWS microdeletion and PWS large deletion iPSC were confirmed by Comparative Genome Hybridization Single Nucleotide Polymorphism Array using the Affymetrix Cytoscan SOMA kit. Genomic DNA was isolated from iPSC using the Roche High Pure PCR Template Preparation Kit.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2016.08.008.

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