Supplementary Data

Discovery of a novel imprinted gene by transcriptional analysis of parthenogenetic embryonic stem cells

Hathaitip Sritanaudomchai, Hong Ma, Lisa Clepper, Sumita Gokhale, Randy Bogan, Jon Hennebold, Don Wolf & Shoukhrat Mitalipov

Supplementary Figures

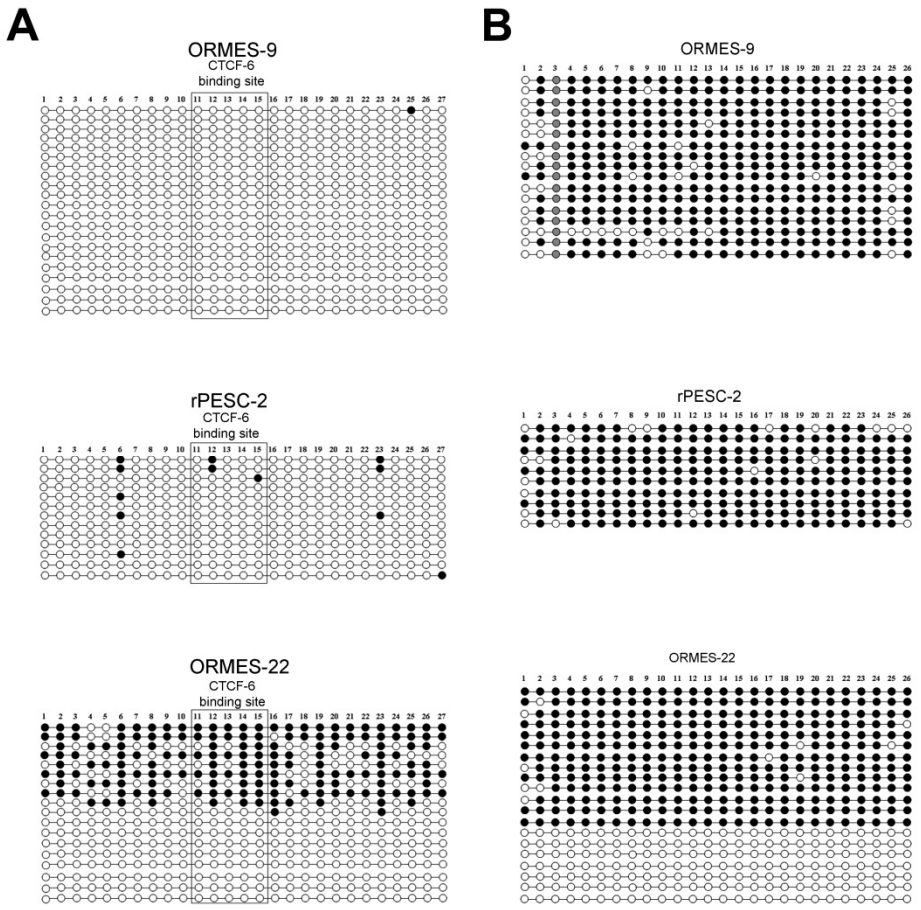

Figure S1. Analysis of known differentially methylated regions in rhesus monkey PESCs. (A) Methylation status of the *IGF2/H19* and (B) *SNURF*/*SNURPN* imprinting centers in monkey parthenogenetic (ORMES-9 and rPEC-2) and IVF-derived biparental ESCs. Genomic DNA was modified by bisulfite treatment followed by polymerase chain reaction (PCR) amplification. PCR products were cloned and a minimum of 10 randomly selected clones were sequenced for each sample. Each circle represents an individual CpG site; methylated CpG dinucleotides are depicted by black circles and the unmethylated sites by open circles. Gray circles represent ambiguous CpG sites. Boxed area corresponds to the CTCF-6 core binding site.

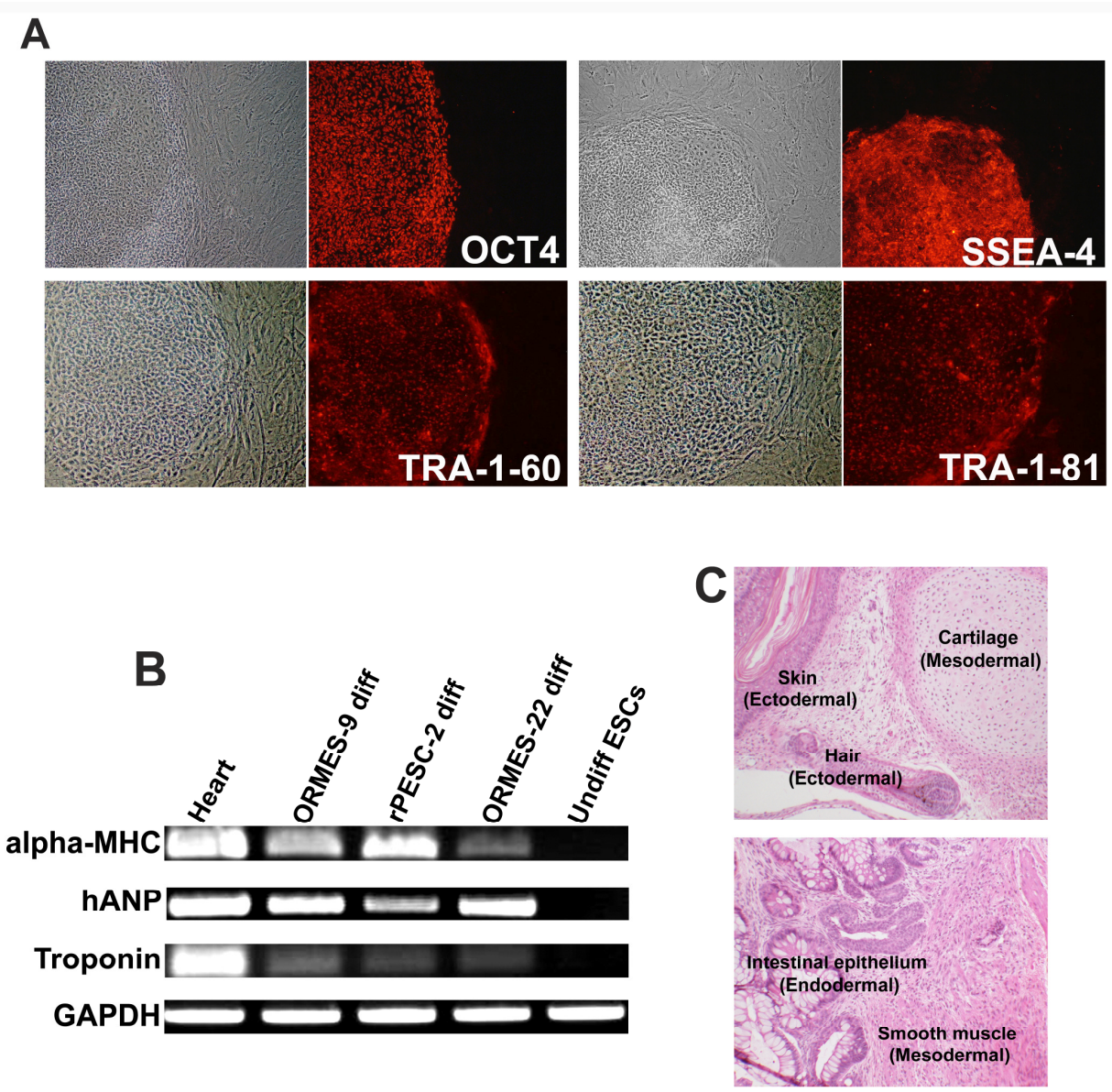

Figure S2. Pluripotency analysis of ORMES-9, a homozygous PESC line. (A) Left columns represent phase contrast images of ORMES-9 colonies and right columns demonstrate expression of primate ESC-specific markers, OCT4, SSEA-4, TRA-1-60, and TRA-1-81, detected by immuonofluoresence. (B) Expression of cardiomyocyte and muscle tissue specific markers in differentiated spontaneously contracting PESCs (ORMES-9, rPESC-2) and biparental ORMES-22 determined by RT-PCR. (C) Pluripotency analysis by teratoma assay demonstrates representative tissue originated from the three germ layers.

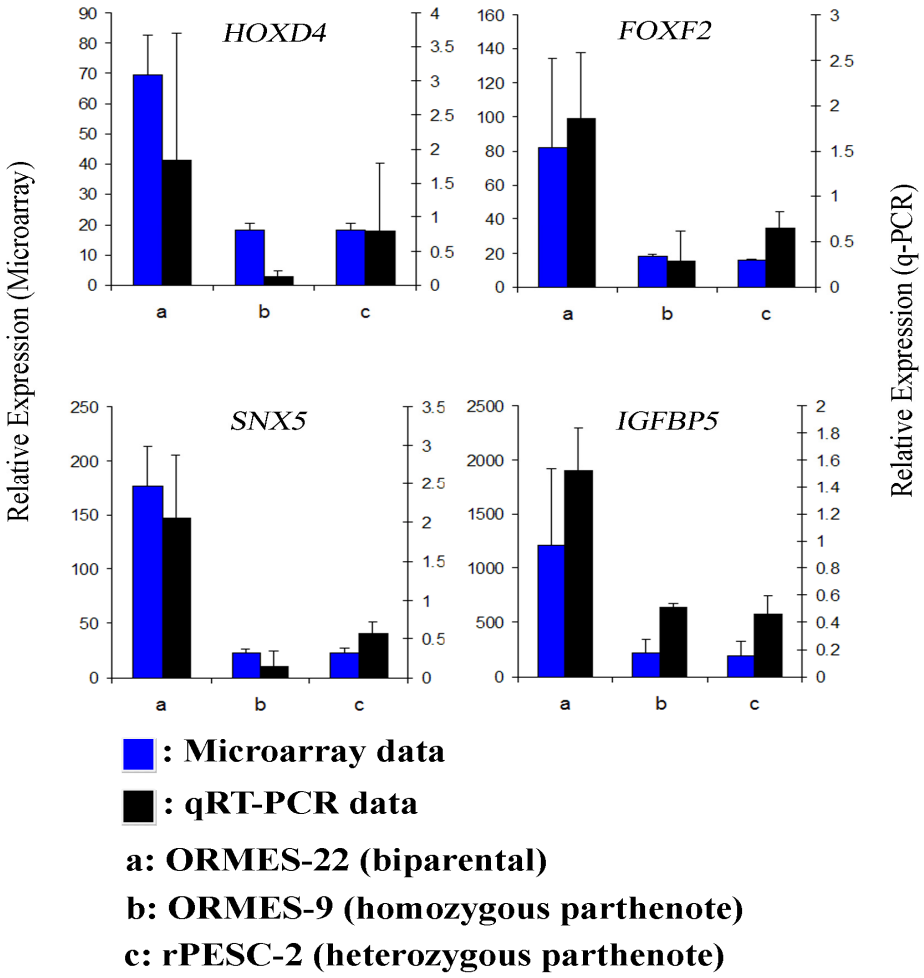

Figure S3. Validation of microarray data by qPCR. The X-axis represents analyzed cell lines (a: ORMES-22, b: ORMES-9 and c: rPESC-2). Microarray results are in blue columns and qPCR results are in black columns. The Y-axis represents RMA-normalized microarray expression values (left axis) or the target gene to *GAPDH* ratio for qPCR data (right axis). Data were analyzed by ANOVA followed by comparison between groups using the Student-Newman-Keuls test. The qPCR expression levels of *SNX5*, *FOXF2*, and *IGFBP5* in both parthenotes were significantly downregulated compared to biparental IVF-derived controls ($P < 0.05$).

Supplementary Tables

Table SI. Parentage analysis of monkey PESCs by microsatellite typing

Locus	Sperm donor for ORMES-22	Egg donor for ORMES-22	ORMES-22	Egg donor for rPESC-2	rPESC-2	Sperm donor for ORMES-9	Egg donor for ORMES-9	ORMES-9
D1S548	190/190	190/190	190/190	190/206	190/206	190/206	194/202	194/194
D2S1333	285/289	273/293	273/285	277/297	277/297	289/297	265/305	305/305
D3S1768	205/205	205/213	205/205	205/221	205/221	205/217	205/217	217/217
D4S2365	283/283	283/283	283/283	283/287	283/287	283/283	283/287	287/287
D4S413	125/141	131/145	131/141	131/131	131/131	129/145	129/137	129/129
D5S1457	132/140	132/136	136/140	132/132	132/132	132/132	132/136	132/132
D6S276	215/225	225/233	225/233	215/225	225/225	225/233	213/221	213/213
D6S291	210/216	206/216	210/216	206/208	206/206	214/216	208/210	208/208
D6S501	180/180	188/192	180/188	180/184	180/184	180/192	180/184	184/184
D6S1691	197/203	197/216	197/203	197/197	197/197	205/223	209/215	209/209
D7S513	193/199	189/217	199/217	209/239	209/239	189/209	201/213	201/201
D7S794	108/128	108/108	108/128	124/132	124/132	108/128	124/124	124/124
D8S1106	160/168	148/168	168/168	148/160	148/160	144/160	160/200	200/200
D9S921	175/195	183/195	183/195	187/195	187/195	175/179	191/195	195/195
D10S1412	160/160	157/157	157/160	157/157	157/157	157/157	157/157	157/157
D11S2002	256/260	252/252	252/256	252/252	252/252	264/264	252/260	252/252
D11S925	338/338	308/308	308/338	308/330	308/330	312/338	306/308	308/308
D12S364	268/296	282/290	268/290	290/290	290/290	282/288	278/288	288/288
D12S67	109/117	117/133	109/133	117/212	117/212	125/212	204/216	204/204
D13S765	228/228	216/236	228/236	224/232	232/232	228/228	200/200	200/200
D15S823	345/353	357/385	345/385	333/357	333/333	329/349	337/345	337/337
D16S403	152/152	152/164	152/164	162/164	162/164	158/164	160/164	164/164
D17S1300	228/284	248/252	252/284	248/260	248/260	228/248	236/280	280/280
D18S537	162/174	174/178	162/178	162/174	162/174	178/178	170/178	170/170
D18S72	306/308	308/308	308/308	308/322	308/322	308/318	312/312	312/312
D22S685	327/327	311/311	311/327	319/319	319/319	315/319	295/299	295/295
DXS2506	270/270	262/262	262/270	262/262	262/262	262/262	270/274	274/274
MFGT21	115/125	111/113	113/125	111/115	111/115	115/119	107/129	129/129
MFGT22	104/110	100/104	104/104	110/120	110/120	104/104	110/122	110/110
D6S291	210/216	206/216	210/216	206/208	206/206	214/216	208/210	208/208
G25641	273/285	269/281	ND	269/273	273/273	279/281	251/277	277/277
G51152	219/219	210/219	210/210	209/219	209/209	209/215	219/219	219/219
9P06	189/189	175/187	175/189	185/191	185/185	189/191	175/185	185/185
DRA	112/134	110/134	112/134	112/136	136/136	126/136	112/140	112/112
MICACA	194/194	200/200	194/200	194/200	194/194	200/200	200/200	200/200
246K06	277/285	283/283	283/285	275/279	275/275	275/275	277/279	279/279
162B17A	240/244	238/242	238/244	238/242	238/238	238/250	242/242	242/242
162B17B	293/315	289/309	293/309	309/309	309/309	309/311	295/303	295/295
151L13	303/309	309/309	309/309	303/309	303/303	305/307	303/311	311/311
MOGCA	121/123	123/127	123/127	121/123	121/121	123/127	121/121	121/121
268P23	148/150	150/154	150/154	148/150	148/148	150/154	148/148	148/148
222I18	167/175	167/173	173/175	167/167	167/167	167/173	165/167	167/167
D6S276	215/225	225/233	225/233	215/225	225/225	225/233	213/221	213/213
D6S1691	197/203	197/216	197/203	197/197	197/197	205/223	209/215	209/209

Heterozygous loci are shown in red. ND is not determined. ORMES-9 and ORMES-22 represent Oregon Rhesus Macaque Embryonic Stem-9 and -22, respectively and rPESC-2 represent rhesus Parthenogenetic Embryonic Stem Cell.

Table SII. Parentage analysis of monkey PESCs by single nucleotide polymorphism (SNP) assay

SNPs	Sperm donor for ORMES-22	Egg donor for ORMES-22	ORMES-22	Egg donor for rPESC-2	rPESC-2	Sperm donor for ORMES-9	Egg donor for ORMES-9	ORMES-9
BCHE:76	CG	C	CG	C	C	ND	C	C
BCHE:447	AG	G	AG	AG	G	A	A	A
CCL8:516	A	AG	A	G	G	AG	G	G
CCR1:641	AG	G	ND	G	G	G	G	G
CCR9:315	C	C	C	C	C	CT	T	T
CD40LG:738	G	A	AG	G	G	G	A	A
CD44:471	T	T	T	T	T	T	CT	C
CD69:294	C	CT	CT	CT	CT	C	CT	C
CD74:213	C	C	ND	C	C	C	C	C
CD74:344	CT	T	T	CT	CT	CT	C	C
CFTR:796	G	G	G	AG	G	G	AG	G
CX3CR1:593	AG	A	A	AG	AG	G	G	G
CXCL12:173	ND	CT	ND	C	C	CT	C	C
FAS:135	A	AG	A	A	A	A	A	A
FSHR:784	CG	C	C	CG	CG	C	C	C
HTATSF1:636	C	C	ND	C	C	C	C	C
IL1:755	T	A	A	AT	AT	AT	AT	T
IL2RA:124	C	C	C	C	C	C	C	C
INHBB:131	CT	C	CT	C	C	CT	C	C
ITGA:321	A	A	A	A	A	A	AG	A
LRP8:647	CT	C	CT	T	T	T	T	T
NDN:135	G	G	G	G	G	ND	G	G
NR3C1:458	T	T	ND	T	T	T	AT	T
PYY:151	CT	T	CT	T	T	CT	T	T
SIRT1:277	G	T	ND	GT	GT	GT	G	G
SLC5A7:79	GT	T	GT	G	G	ND	G	G
SLC6A4:132	CG	G	G	GC	C	G	G	G
STAR:522	GT	GT	GT	GT	G	GT	T	T
TLR4:735	CT	T	ND	T	T	CT	T	T
TLR5:389	C	CT	ND	TC	C	CT	C	C
TNF:82	T	T	T	T	T	T	C	C
XCL1:320	T	CT	ND	C	C	T	T	T

Heterozygous loci are shown in red. ND is not determined. ORMES-9 and ORMES-22 represent Oregon Rhesus Macaque Embryonic Stem-9 and -22, respectively and rPESC-2 represent rhesus Parthenogenetic Embryonic Stem Cell.

Table SIII. Primers, probes and PCR conditions

Primers for RT-PCR				
Genes	**Forward primer (5'-3')**	**Reverse primer (5'-3')**	**Anneal Temp (°C)**	**Product size (bp)**
Alpha-MHC	GTCATTGCTGAAACCGAGAATG	GCAAAGTACTGGATGACACGCT	61	413
hANP	GAACCAGAGGGGAGAGACAGAG	CCCTCAGCTTGCTTTTTAGGAG	61	406
Troponin	GGCAGCGGAAGAGGATGCTGAA	GAGGCACCAAGTTGGGCATGAACGA	64	150
GAPDH	GTGGTCTCCTCCGACTTCAACA	GTCTCTCTCTTCCTCTTGTGCTCT	61	217
COL3A1	TTGAATCCTAGCCCATCTGC	AAGCAGCCCCATTATTTGGT	58	695
CA3	TGTGGTCACCAAGAAACCAA	GGCCCTCCTTCAGGTAAAGA	60	695
FOXD1	GCTCGAGGAAGAAGGTAGGAA	AGGGGACAACTGAAGCCAAT	60	710
SNX5	ATGTCTCCCTTTTGCAGAGC	ATGCCCAAAGACATACACCA	60	757
LOC703703	ACTCCGGTTCCTGGTCTTTT	GCTCATAACGCTCCATCTCC	60	505
IGFBP5	TTCACAGACTCTGGCCTCCT	CTTCCGCATTCAGATGGTTT	58	503
HOXD4	TTCCAGAAGGAAGCAAAGGA	CCCCTGTTGAGAGTGATTCC	58	597
INPP5F	AAATCCCAGAAGTCCGGTTT	TCAGCCGAGAATCAATCACA	60	505
INPP5F	GAGGCTCTGTGCCTGTCTTT	TTCAGCTGCTGTTCCATGAC	58	466
INPP5F_v2	ATGTGTCGTGACGTAATTTTTATG	GCTCGCAGTGTGTGGAAATA	55	299
PTPRB	GGAGGCTCCCTTGGAATAAA	GTGCACCCTCTTGTCAAGGT	60	526
CEP68	CTGGAAGGAGGAAAGCACAG	ATGCCACATTCTGCATTCAA	60	598
ACTC1	CCTTCCAGCAGATGTGGATT	GTGGCATCTGCAGCAACTAA	60	532
SERPINE1	CCATGCAAAGCAACGACTAC	CTGCACAACAGCAACCTTGT	60	600

Gene	**Primers for qPCR (SYBR Green)**
Tel1	GGT TTT TGA GGG TGA GGG TGA GGG TGA GGG TGA GGG T
Tel2	TCC CGA CTA TCC CTA TCC CTA TCC CTA TCC CTA TCC CTA
36B4u	CAG CAA GTG GGA AGG TGT AAT CC
36B4d	CCC ATT CTA TCA TCA ACG GGT ACA A

Primers and probes for qPCR			
Genes	**Forward primer (5'-3')**	**Reverse primer (5'-3')**	**Probes**
SGCE	ACCCAAAACCTGGCGAGAT	TCCAGGTCGGTCTGGGTAAC	AGTAATGATCCCATAACATT
SNRPN	AAGCAACCAGAGCGTGAAGAA	TCCCCACGCAGCAACAC	AGCGGGTTTTGGGTCT
H19	CCTCCCCGACTCTGTTTCC	CACAACTCCAACCAGTGCAAA	CCGTCCCTTCTGAATT
IGF2	GTCGGCCCAGCCAGAGT	CGGCTACCATCATCTCCATTG	AGGAAGGAGTTTGGCC
NECDIN	TGTCTCCGAGGACTAGCCAAGT	GCCCTGGTGAGGATCAGAAA	TGGAGGCAGATGAAT
UBE3A	GAAGGAGAACAAGGAGTTGATGAAG	CCTCCACAACCAGCTGAAAAA	AGGTGTTTCCAAAGAA
PEG10	CCCTTCGAGAGCAAGTGGAA	GCGGAGCTCGATGTCATCAT	CCACCCCTGAGGATG
ATP10A	GCCCGGCCAACGTGTAC	ACCGCCGGCACGAAGT	TTGTCTTCATCGCGCTGC
CDKN1C	CATCTACGATGGAGCGTCTTGTC	GCTGGTGCGCACAAGTACTG	CCGTGGGACCTTC
DLX5	ACCAACCAGCCAGAGAAAGAAG	TTGGTTTGCCATTCACCATTC	CACCTCGGGCTCGG
GNAS	TGGCCAGCAGCAGCTACA	TCCTGCAGGCGGTTGGT	TCATCCGGGAGGACAA
MAGEL2	CAGGGCCCCTTCGAAAGA	CTGCCTTGGGAGCACAGAA	CGCATGATCTTTGCTGC
MEST	CCTGCCCTTCACTCATGGAA	GGTAGAAGATACGCAGTCCCTTGT	TCTTCAGGCAAGTTTTT
PEG3	CAAGCACCAGTCGAGGTCTAAA	CGCCGGTGGGTTGATTT	ACTATGCCTGAAGCCAA
PHLDA2	GAGCGCACCGGCAAGTAC	CAGCGGAAGTCGATCTCCTT	TGTACTTCACCATCGTCA
SLC22A18	CCCGGCCTGGTGTTCAG	GGCCTTGGTCAGCATGCT	TCTGCACACTCAATG
ZIM2	CCTCACTCAGTCCGTTCTTTCA	GGCTCCATGTCTCTGCTTCTG	TGGTGACCGGGACTG
TP73	CCAGCACGGCCAAGTCA	CTTGGCGATCTGGCAGTAGAG	CTGGACGTACTCCC
GAPDH	GGTGGTCTCCTCCGACTTCA	ACCAGGAAATGAGCTTGACAAAG	CCCACTCTTCCACCTTCGACGCTG
DIRAS3	CGGCAACTTCCGTCATGAGT	CCAGCAACTGGCAGTAGGTATTT	CCTGCCGACCATTG
XIST	CCATGCTGGGTGCTAGAGCTA	CCATTGACATTTGTATCATGCTTTAGT	AGGTGCATATTAAAGTGC

MIAME Checklist

Experimental Design

Authors:

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Type of experiment:

The transcriptomes of rhesus monkey embryonic stem cell lines derived from IVF-produced embryos (Oregon Rhesus Macaque Embryonic Stem, ORMES-22) were compared with rhesus monkey parthenogenetic embryonic stem cell lines (heterozygous rPESC-2 and homozygous ORMES-9). In addition, the transcriptome of rPESC-2 line was also compared with ORMES-9. Finally, the adult somatic skin fibroblasts were compared to each parthenote cell line as well as the biparental control ESCs.

Experimental factors:

Three biological replicates (A, B, C) of: ORMES-9, ORMES-22, rPESC-2 and adult skin fibroblasts were analyzed.

Number of hybridizations:

12 hybridizations on oligonucleotide arrays (Affymetrix Rhesus Macaque Genome Array).

Hybridization design:

ESC replicates were compared against each somatic replicate and select comparisons between ESC replicates were performed.

Type of reference used for the hybridizations:

No reference used.

Quality control steps:

Standard Affymetrix control steps. Approximately 200 ng of each sample cRNA target along with a control cRNA target is analyzed on the RNA 6000 LabChip using the 2100 Bioanalyzer (Agilent Technologies). The target quality is determined based on cRNA yield and size distribution produced from the in vitro synthesis reaction. Samples that fail quality control are discarded or relabeled.

Number of replicates:

Three biological replicates per cell line.

URL of supplemental web sites / database accession numbers:

The data have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=tjsddkusgemowbk&acc=GSE17964) and are accessible through GEO Series accession number.

Samples

Origin of sample:

Organism: Macaca mulatta

All stem and somatic cell lines were generated by us at the Oregon National Primate Research Center. Somatic fibroblasts were derived from a skin biopsy from a nine year old adult male rhesus monkey.

Sample used in microarray experiment

Sample	Microarray Name	GEO ID	Contents
A_FibroblastsA	FIB_A	GSM449698	Skin fibroblasts (Male #1) replicate A
B_FibroblastsB	FIB_B	GSM449699	Skin fibroblasts (Male #1) replicate B
C_FibroblastsC	FIB_C	GSM449700	Skin fibroblasts (Male #1) replicate C
D_ORMES22A	ORMES22_A	GSM449701	ORMES-22 (IVF derived ESCs) replicate A
E_ORMES22B	ORMES22_B	GSM449702	ORMES-22 (IVF derived ESCs) replicate B
F_ORMES22C	ORMES22_C	GSM449703	ORMES-22 (IVF derived ESCs) replicate C
G_rPESC2A	rPESC2_A	GSM449704	rPESC-2 (heterozygous PESCs) replicate A
H_rPESC2B	rPESC2_B	GSM449705	rPESC-2 (heterozygous PESCs) replicate B
I_rPESC2C	rPESC2_C	GSM449706	rPESC-2 (heterozygous PESCs) replicate C
J_ORMES9A	ORMES9_A	GSM449707	ORMES-9 (homozygous PESCs) replicate A
K_ORMES9B	ORMES9_B	GSM449708	ORMES-9 (homozygous PESCs) replicate B
L_ORMES9C	ORMES9_C	GSM449709	ORMES-9 (homozygous PESCs) replicate C

Manipulation of samples:

PESCs were grown at 37°C in 5% CO_2 atmosphere on mitotically inactivated mEF feeder cells in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) (Invitrogen) supplemented with 15% fetal bovine serum (FBS) (Hyclone), 0.1mM β-mercaptoethanol (Sigma), 1% nonessential amino acids (Invitrogen), 2mM L-glutamine (Invitrogen) and a pH of 7.2 (altered with 4N NaOH). PESC colonies were manually passaged every 4-5 days. ESCs with an undifferentiated morphology were manually removed from culture for total RNA purification.

Protocol for preparing hybridization extract:

Total RNA was extracted using Trizol® reagent (Invitrogen) followed by additional purification on RNeasy spin columns (Qiagen) following manufacturer recommendations.

Labeling protocol:

Microarray assays were performed in the Affymetrix Microarray Core of the OHSU Gene Microarray Shared Resource. Messenger RNA is amplified and labeled from 2μg of total RNA in two steps according to the standard Affymetrix one-cycle cDNA protocol (Expression Analysis Technical Manual Rev.4). In the first step, mRNA is converted to double-stranded cDNA using Superscript Reverse Transcriptase (Invitrogen) and an oligo-dT primer linked to a T7 RNA polymerase binding site sequence (Affymetrix). In the second step, amplified and labeled cRNA (the target) is produced in an in vitro transcription reaction using T7 RNA polymerase and biotin-UTP (Affymetrix). Following removal of free nucleotides, target yield is measured by UV_{260} absorbance.

External controls (spikes):

Biotinylated hybridization control oligomer and biotinylated control cRNAs for BioB, BioC, BioD and CreX (Affymetrix) at 1.5, 5, 25, and 100 pM concentrations, respectively, in hybridization buffer.

Hybridization Procedures and Parameters

Protocol and conditions:

Labeled target is fragmented at 95^{o} C in the presence of high magnesium concentration. The fragmented material is combined with biotinylated hybridization control oligomer and biotinylated control cRNAs for BioB, BioC, BioD and CreX (Affymetrix) in hybridization buffer. Ten µg of target is hybridized for 16 hours at 45°C to the GeneChip Rhesus Macaque Genome array (Affymetrix), followed by washing, staining with streptavidin-phycoerythrin (Molecular Probes), signal amplification with biotinylated anti-streptavidin antibody (Vector Labs), and a final staining step on the Fluidics Station 450 (Affymetrix). The distribution of fluorescent material on the processed array is determined using the GeneChip Scanner 3000 with the 7G upgrade (Affymetrix) and GCOS version 1.4.0 software (Affymetrix), yielding cell fluorescence intensity (.cel files). Image inspection is performed manually immediately following each scan.

Measurement Data and Specifications

Quantifications based on images:

The GeneChip expression arrays contain control probe sets for both spiked and endogenous RNA transcripts (e.g., BioB, BioC, BioD, CreX and species-specific actin and GAPDH). Following image processing and absolute analysis of the array pattern using the MAS 5.0 algorithm used within GCOS version 1.4.0 software (Affymetrix), six values are examined to assess overall assay performance: background, noise, average Signal, % Present, ratio of Signal values for probe sets representing the 5' and 3' ends of actin and GAPDH transcripts, and total Signal for probe sets for BioC, BioD and CreX. Assays demonstrating poor or marginal performance are flagged.

Type of scanning hardware and software used:

Software – GCOS version 1.4.0 (Affymetrix)

Scanning hardware – GeneChip Scanner 3000 with 7G upgrade (Affymetrix)

Type of image analysis software used:

GCOS version 1.4.0 (Affymetrix)

Description of measurements produced by the image-analysis software and measurements used in the analysis:

Probe level measurements produced by GCOS version 1.4.0 (.cel files).

Complete output of the image analysis before data selection and transformation (spot quantitation matrices):

Original Affymetrix output files (.cel files)

Data selection and transformation procedures:

The Robust Multichip Analyis (RMA) was used for signal quantification and performed using GeneSifter software (www.genesifter.com). The RMA was applied to the probe-level measurements contained in .cel files. Data analysis was performed using GeneSifter.

Final gene expression data table(s) used by authors to make their conclusions after data selection and transformation (gene expression data matrices):

All normalized microarray data sets generated from these studies can be found in Data S1-S5.

Array Design

Platform type:

Affymetrix oligonucleotide array

Surface and Coating Specifications:

Glass

Array:

Affymetrix Rhesus Macaque Genome Array

Features on the array:

See http://www.affymetrix.com/products/arrays/specific/rhesus_macaque.affx

Reporters on the array:

See http://www.affymetrix.com/products/arrays/specific/rhesus_macaque.aff