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Supplemental Information

Efficient Designer Nuclease-Based Homologous

Recombination Enables Direct PCR Screening for

Footprintless Targeted Human Pluripotent Stem Cells

Sylvia Merkert, Stephanie Wunderlich, Christien Bednarski, Jennifer Beier, Alexandra Haase, Anne-Kathrin Dreyer, Kristin Schwanke, Johann Meyer, Gudrun Göhring, Toni Cathomen, and Ulrich Martin

Supplemental Figures



Figure S1. Optimization of transfection and characterization of hCBiPS2eGFP reporter cell clones, related to Figure 1. (A) Comparison of transfection efficiencies of hCBiPS2 and hES3 cells using our optimied protocol for the Neon® transfection system versus the optimized protocol of the AmaxaTM Nucleofection system recommended for hPSCs (mean \pm SEM of 4 independent experiments). (B) Southern Blot analysis revealed 2 clones (depicted in red) with single eGFP integration. (C) Microscopy images of the transgenic eGFP^{pos} iPSC clone hCBiPS2eGFPC7 on feeder cells. Scale bars represent 100 µm. (D) Immunostaining of clone hCBiPS2eGFPC7 cell derivatives on day 15 of differentiation showed expression of endodermal (AFP), mesodermal (sarc. alpha-Actinin, Desmin) and ectodermal (β 3-Tubulin) marker proteins (red). Nuclei are stained with DAPI (blue). Upper pictures show eGFP transgene expression. Scale bars represent 100 µm. (E) Flow cytometric analysis of derivatives of clone hCBiPS2eGFPC7 on day 28 of differentiation (continuous line) revealed consistent eGFP expression compared to undifferentiated cells (dotted line).



Figure S2. Genomic analysis of eGFP^{neg}RedStar^{pos} human iPS cell clones after eGFP-ZFN targeting and representative phenotypic characterization of one targeted iPSC clone, related to Figure 2. The analyses of two targeting experiments (E1, E2) applying different molecular ratios of ZFNs:Donor (1:1 or 1:9) are shown. (A) PCR analysis for 5' (upper row, primers p3 & p4) and 3' (middle row, primers p5 & p6) junctions as expected for correct targeted integration of the donor cassette into the eGFP locus. Bands in the lower row show the presence of additional integrations, as determined using primers p7 and p8 that bind to the donor plasmid backbone outside the homologous arms. NA, not assessed. See also figure 2A. (B) Southern blot analysis of hiPSeGFPC7 cells targeted with eGFP-ZFN and the 2A-RedStar donor plasmid. Genomic DNA was digested with BsmI and hybridized with the internal 5' probe (on top) and internal 3' probe (below). Correctly targeted clones without additional integrations are indicated in red. The 5' probe detects a 5.2 kb targeted fragment and a 4.1 kb fragment for random donor integration. The 3' probe detects a 3.5 kb targeted fragment and a 1.4 kb fragment for random donor integration. See also figure 2A. TI, targeted integration. C, control (hCBiPS2eGFPC7). (C) Clone E1 RSiPSC7 exhibits a normal karvotype (46.XY) after ZFN-mediated HR. (**D**) Assessment of pluripotency. Phase contrast images with overlay of nuclear RedStar fluorescence (upper row) and immunostaining for pluripotency markers (lower row, green) of undifferentiated E1 RSiPSC7 (passage 13 after HR and cloning). Nuclei are stained with DAPI (blue). Scale bars represent 100 µm. (E) Assessment of differentiation potential. Immunocytological detection of endodermal (AFP), mesodermal (sarc.alpha-Actinin) and ectodermal (B3-Tubulin) marker proteins (green) in differentiated iPSC derivatives of clone E1 RSiPSC7 indicates the maintenance of pluripotency. Phase contrast images with overlay of nuclear RedStar fluorescence (upper row). Nuclei are stained with DAPI (blue). Scale bars represent 100 um.



Figure S3. Genotyping and phenotypic characterization of AAVS1 targeted PSC clones, related to Figure 3. (A) PCR analysis of AAVS1-targeted CAG-eGFP or CAG-RedStar transgenic hiPSC and hESC clones for the 5' (primers p9 & p10) and 3' (primers p5/p1 & p12) junctions generated by targeted integration, for the determination of homozygous (primers p9 & p10 & p12, one band) versus heterozygous (primers p9 & p10 & p12, two bands of different size) transgene integration, and for the detection of additional donor integrations (primers p10 & p13). See the scheme in figure 3A and supplemental table S1 for details. (B) Karyotype. Human iPSC clones hCBiPS2 AAVS1eGFPC18 (upper picture) and hCBiPS2 AAVS1RedStarC8 (lower picture) exhibit a normal karyotype (46,XY). (C) Immunostaining of clone hCBiPS2 AAVS1eGFPC18 cell derivatives on day 24 of differentiation showed expression of mesodermal (sarc. alpha-Actinin, Desmin), endodermal (SOX17, AFP) and ectodermal (β 3-Tubulin) marker proteins (red). Upper pictures show eGFP transgene expression. Nuclei are stained with DAPI (blue). Scale bars represent 100 μm. (D) Immunostaining of clone hCBiPS2 AAVS1RedStarC8 cell derivatives on day 24 of differentiation showed expression of mesodermal (sarc. alpha-Actinin, Desmin), endodermal (SOX17, AFP) and ectodermal (β 3-Tubulin) marker proteins (green). The upper pictures show RedStar transgene expression. Nuclei are stained with DAPI (blue). The scale bars represent 100 µm.



Figure S4. PCR screening for ssODN donor integration in 144 iPSC clones, related to Figure 4. HCBiPS2 cells were transfected with AAVS1 specific TALENs and the ssODN donor for incorporation of a HindIII site into the AAVS1 locus. On day 3 after transfection, 10 cells per well were seeded into 96 well plates. On average, one to five cells survived per well and PCR screening for targeted integration showed positive insertion of the ssODN in 32 out of 480 analysed pools (data not shown). Limiting dilution from nine positive pools resulted in 144 single cell clones. PCR analysis for the targeted integration of the ssODN revealed 33 positive clones (marked in red) from which we randomly chose 7 clones (framed) for further evaluation (see Fig. 4). M, DNA marker (Eurogentec).

		Name	Sequence	Specificity	Product Size
		p1	CAA CGT GCT GGT TAT TGT GC	EGFP	800 bp
		p2	GGC TTC ATG ATG TCC CCA TA	sequencing	
	ח	р3	GTC CCC TTC TCC CTC TCC AG	E'iupation	1375 bp
raeti	0	р4	CCT GGC AAT TGG ACT TGC TTC	5 junction	
P ta		р5	CAT CTG ACG GTC CAG TCA TGC	2'iunation	1330 bp
eGF		р6	TGT GGA ATT GTG AGC GGA TA	Sjunction	
		р7	GCC TGA ACA CCA TAT CCA TCC	additional	2580 bp
		р8	GCA GCT GAG AAT ATT GTA GGA GAT C	integration	2300 bp
	CAG-eGFP donor	р9	CCA GCT CCC ATA GCT CAG TCT G	5'iunction	1554 bp
		p10	ATG GGG AGA GTG AAG CAG AA	5 junction	
		p11	GGG CAC AAG CTG GAG TAC A	3'iunction	1806 bp
		p12	GTG AGT TTG CCA AGC AGT CA	ojunotion	
		р9	CCA GCT CCC ATA GCT CAG TCT G		TI_1554 bp
		p10	ATG GGG AGA GTG AAG CAG AA	homo- vs.	
		р9	CCA GCT CCC ATA GCT CAG TCT G	heterozygous	WT 2032 bp
		p12	GTG AGT TTG CCA AGC AGT CA		wi_2002.bp
		p13	ATA ATA CCG CGC CAC ATA GC	additional	1932 hn
		p10	ATG GGG AGA GTG AAG CAG AA	integration	1332 66
ting	G-RedStar donor	р9	CCA GCT CCC ATA GCT CAG TCT G	5'iunction	1571 bp
Irge		p10	ATG GGG AGA GTG AAG CAG AA	ojunotori	1011.00
il te		р1	CAA CGT GCT GGT TAT TGT GC	3'iunction	2614 bp
AVS		p12	GTG AGT TTG CCA AGC AGT CA	ojunotion	
◄		p9	CCA GCT CCC ATA GCT CAG TCT G		TI_1571 bp
		p10	ATG GGG AGA GTG AAG CAG AA	homo- vs.	
		р9	CCA GCT CCC ATA GCT CAG TCT G	heterozygous	WT_2032 bp
	CA	p12	GTG AGT TTG CCA AGC AGT CA		
		p13	ATA ATA CCG CGC CAC ATA GC	additional	1962 bp
		p10	ATG GGG AGA GTG AAG CAG AA	integration	
		p14	ACA GTG GGG CCC Caa gct tG	HindIII detection	1024 bp
	ssodn	p12	GTG AGT TTG CCA AGC AGT CA		
		p15	CTT GTA GGC CTG CAT CAT CA	PCR product for	994 hn
		p16	GAA CAC CTA GGA CGC ACC AT	HindIII digestion	994 ph

 Table S1. Oligonucleotides used for PCR, related to the Experimental Procedures

Name	Class	Species	Clonality	Vendor	Dilition
anti-OCT4	lgG2b	mouse	monoclonal	Santa Cruz Biotechnologie, CA, USA	1:100
anti-NANOG	lgG1	mouse	monoclonal	Abcam, Cambridge, USA	1:500
anti-SSEA3	lgM	mouse	monoclonal	Hybridoma Bank, Iowa City, USA	1:100
anti-SSEA4	lgG3	mouse	monoclonal	Hybridoma Bank, Iowa City, USA	1:70
anti-sarc. alpha Actinin	lgG1	mouse	monoclonal	Sigma, Missouri, USA	1:800
anti-alpha-Fetoprotein	lgG1	mouse	monoclonal	R&D Systems, Minneapolis, USA	1:300
anti-beta3 Tubulin	lgG2a	mouse	monoclonal	Upstate, NY, USA	1:400
anti-Desmin	lgG1	mouse	monoclonal	Progen, Heidelberg, DE	1:20
anti-SOX17	lgG	goat	monoclonal	Millipore, Darmstadt, DE	1:200
anti-TroponinT	lgG1	mouse	monoclonal	Thermo Scientific, St. Leon-Rot, DE	1:100

Table S2. Primary Antibodies used for immunohistology, related to the ExperimentalProcedures

Supplemental Experimental Procedures

Sequences

AAVS1-specific ZFN sequence (right) (with HA-tag and NLS)

MGYPYDVPDYASRPKKKRKVGIHASPAAMAERPFQCRICMRNFSQSSNLARHIRTHT GEKPFACDICGRKFARTDYLVDHTKIHTGSQKPFQCRICMRNFSYNTHLTRHIRTHTG EKPFACDICGRKFAQGYNLAGHTKIHLRGSQLVKSELEEKKSELRHKLKYVPHEYIEL IEIARNSTQDRILEMKVMEFFMKVYGYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTK AYSGGYNLPIGQADEMQRYVKENQTRNKHINPNEWWKVYPSSVTEFKFLFVSGHFK GNYKAQLTRLNHVTNCNGAVLSVEELLIGGEMIKAGTLTLEEVRRKFNNGEINF-

AAVS1-specific ZFN sequence left (with HA-tag and NLS)

MGYPYDVPDYASRPKKKRKVGIHASPAAMAERPFQCRICMRNFSYNWHLQRHIRTH TGEKPFACDICGRKFARSDHLTTHTKIHTGSQKPFQCRICMRNFSHNYARDCHIRTHT GEKPFACDICGRKFAQNSTRIGHTKIHLRGSQLVKSELEEKKSELRHKLKYVPHEYIEL IEIARNSTQDRILEMKVMEFFMKVYGYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTK AYSGGYNLPIGQADEMERYVEENQTRNKHANPNEWWKVYPSSVTEFKFLFVSGHFK GNYKAQLTRLNHITNCNGAVLSVEELLIGGEMIKAGTLTLEEVRRKFNNGEINF-

AAVS1-specific TALEN sequence (right) (with HA-tag and NLS)

MGYPYDVPDYASRPKKKRKVGIHASAPRRRAAQPSDASPAAQVDLRTLGYSQQQQE KIKPKVRSTVAQHHEALVGHGFTHAHIVALSQHPAALGTVAVKYQDMIAALPEATH EAIVGVGKQWSGARALEALLTVAGELRGPPLQLDTGQLLKIAKRGGVTAVEAVHAW RNALTGAPLNLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPQQVVAIASHD GGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGKQALETVQRLLPVLCQAHGL TPEQVVAIASNKGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNGGGKQALETVQ RLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASN IGGKQALETVQALLPVLCQAHGLTPEQVVAIASHDGGKQALETVQALLPVLCQAHG LTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASN GGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNIGGKQALETVQ RLLPVLCQAHGLTPQQVVAIASNIGGKQALETVQRLLPVLCQAHGLTPQQVVAIASN GGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHG LTPEQVVAIASHDGGKQALETVQRLLPLCQAHGLTPQQVVAIASN GGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHG LTPEQVVAIASHDGGKQALETVQRLLPLCQAHGLTPQQVVAIASN GGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQ RLLPVLCQAHGLTPEQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASN LTGSQLVKSELEEKKSELRHKLKYVPHEYIELIEIARNSTQDRILEMKVMEFFMKVYG YRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVEENQT RNKHINPNEWWKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNHITNCNGAVLSVEEL LIGGEMIKAGTLTLEEVRRKFNNGEINF-

AAVS1-specific TALEN sequence (left) (with HA-tag and NLS)

MGYPYDVPDYASRPKKKRKVGIHASAPRRRAAQPSDASPAAQVDLRTLGYSQQQQE KIKPKVRSTVAQHHEALVGHGFTHAHIVALSQHPAALGTVAVKYQDMIAALPEATH EAIVGVGKQWSGARALEALLTVAGELRGPPLQLDTGQLLKIAKRGGVTAVEAVHAW RNALTGAPLNLTPOOVVAIASHDGGKOALETVORLLPVLCOAHGLTPOOVVAIASNG GGKQALETVQRLLPVLCQAHGLTPEQVVAIASNKGGKQALETVQRLLPVLCQAHGL TPEOVVAIASNGGGKOALETVORLLPVLCOAHGLTPEOVVAIASHDGGKOALETVO RLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASH DGGKOALETVOALLPVLCOAHGLTPEOVVAIASHDGGKOALETVOALLPVLCOAHG LTPEQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPQQVVAIASHDGGKQALETV **QRLLPVLCQAHGLTPQQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIAS** NIGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAH GLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASHDGGKQALET VQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIA SNIGGKQALETVQALLPVLCQAHGLTPQQVVAIASHDGGRPALESIVAQLSRPDPALA ALTGSQLVKSELEEKKSELRHKLKYVPHEYIELIEIARNSTQDRILEMKVMEFFMKVY GYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVEEN **QTRNKHINPNEWWKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNHITNCNGAVLSVE** ELLIGGEMIKAGTLTLEEVRRKFNNGEINF-

eGFP 2A-RedStarnuc donor DNA sequence

TACCCCGTGC	CTTCGGGGGGG	GACGGGGCAG	GGCGGGGTTC	GGCTTCTGGC	```	
GTGTGACCGG	CGGCTCTAGA	GCCTCTGCTA	ACCATGTTCA	TGCCTTCTTC		
TTTTTCCTAC	AGCTCCTGGG	CAACGTGCTG	GTTATTGTGC	TGTCTCATCA		
TTTTGGCAAA	GAATTCCTCG	AGACCATGGT	GAGCAAGGGC	GAGGAGCTGT		
TCACCGGGGT	GGTGCCCATC	CTGGTCGAGC	TGGACGGCGA	CGTAAACGGC		
CACAAGTTCA	GCGTGTCCGG	CGAGGGCGAG	GGCGATGCCA	CCTACGGCAA		
GCTGACCCTG	AAGTTCATCT	GCACCACCGG	CAAGCTGCCC	GTGCCCTGGC		∖ homology
CCACCCTCGT	GACCACCCTG	GCCTACGGCG	TGCAGTGCTT	CAGCCGCTAC	eGFP	arm left
CCCGACCACA	TGAAGCAGCA	CGACTTCTTC	AAGTCCGCCA	TGCCCGAAGG		
CTACGTCCAG	GAGCGCACCA	TCTTCTTCAA	GGACGACGGC	AACTACAAGA		
CCCGCGCCGA	GGTGAAGTTC	GAGGGCGGCA	CCCTGGTGAA	CCGCATCGAG		
CTGAAGGGCA	TCGACTTCAA	GGAGGACGGC	AACATCCTGG	GGCACAAGCT		
GGAGTACAAC	TACAACAGCC	ACAACGTCTA	TATCATGGCC	GACAAGCAGA	J	

AGAACGGCAT	CGAGGTGAAC	TTCAAGATCC	GCCAC CGTGG	TACCCCG <u>AGA</u>	ZFN-L site
TCTGGCGGCG	GAGAGGGCAG	AGGAAGTCTT	CTAACATGCG	GTGACGTGGA	2Asequence
GGAGAATCCC	GGCCCTAGG <mark>A</mark>	TGAGTAGATC	TTCTAAGAAC	GTCATCAAGG	
AATTCATGAG	ATTCAAGGTT	AAAATGGAAG	GTACTGTTAA	CGGCCACGAA	
TTCGAAATCG	AAGGTGAAGG	TGAGGGTAGA	CCATATGAAG	GTCACAACAC	
AGTCAAGTTG	AAGGTTACTA	AGGGTGGTCC	ACTGCCATTC	GCTTGGGACA	
TCTTGTCTCC	ACAATTCCAA	TACGGTTCTA	AGGTCTACGT	CAAGCACCCA	
GCTGACATTC	CAGACTACAA	GAAGTTGTCC	TTCCCAGAAG	GTTTCAAGTG	
GGAAAGGATC	ATGAACTTCG	AAGACGGTGG	CGTTGTTACT	GTTACTCAAG	
ACTCCTCCTT	GCAAGACGGT	TGTTTCATCT	ACAAGGTCAA	GCTCATTGGT	
GTCAACTTCC	CATCTGACGG	TCCAGTCATG	CAAAAGAAGA	CTATGGGTTG	RedStar nuclear
GGAAGCTTCT	ACCGAACGTT	TGTACCCAAG	AGACGGTGTC	TTGAAGGGTG	
AAATCCACAA	GGCCTTGAAG	TTGAAGGACG	GTGGTCACTA	CTTGGTCGAA	
TTCAAGTCTA	TCTACAAGGC	CAAGAAGCAA	GTCCAATTGC	CAGGCTATTA	
CTACGTTGAC	TCTAAGTTGG	ACATCATCTC	TCACAACGAA	GACTACACTA	
TCGTCGAACA	ATACGAACGT	ACTGAAGGTA	GACACCACTT	GTTCTTGTAC	
AAGTCCACCA	ACGAGAATGC	TAATACACCA	GCTGCCCGTC	TTCACAGATT	
CAAGAACAAG	GGAAAAGACA	GTACAGAAAT	GAGGCGTCGC	AGAATAGAGG	
TCAATGTGGA	GCTGAGGAAA	GCTAAGAAGG	ATGACCAGAT	GCTGAAGAGG	
AGAAATGTAA	GCTCATTTCC	TGATGATGCT	ACTTCTCCGC	TGCAGGAAAA	
CTAAGCGGGA	TCTTTTTCCC	TCTGCCAAAA	ATTATGGGGA	CATCATGAAG	
CCCCTTGAGC	ATCTGACTTC	TGGCTAATAA	AGGAAATTTA	TTTTCATTGC	
AATAGTGTAG	CCTAAGGTAG	GAGGACGGCA	GCGTGCAGCT	CGCCGACCAC	∖ ZFN-R site
TACCAGCAGA	ACACCCCCAT	CGGCGACGGC	CCCGTGCTGC	TGCCCGACAA	
CCACTACCTG	AGCACCCAGT	CCGCCCTGAG	CAAAGACCCC	AACGAGAAGC	
GCGATCACAT	GGTCCTGCTG	GAGTTCGTGA	CCGCCGCCGG	GATCACTCTC	
GGCATGGACG	AGCTGTACAA	GTAA AGATCT	TTTTCCCTCT	GCCAAAAATT	
ATGGGGACAT	CATGAAGCCC	CTTGAGCATC	TGACTTCTGG	CTAATAAAGG	
AAATTTATTT	TCATTGCAAT	AGTGTGTTGG	AATTTTTTGT	GTCTCTCACT	angle homology
CGGAAGGACA	TATGGGAGGG	CAAATCATTT	AAAACATCAG	AATGAGTATT	arm right
TGGTTTAGAG	TTTGGCAACA	TATGCCCATA	TGCTGGCTGC	CATGAACAAA	
GGTTGGCTAT	AAAGAGGTCA	TCAGTATATG	AAACAGCCCC	CTGCTGTCCA	
TTCCTTATTC	CATAGAAAAG	CCTTGACTTG	AGGTTAGATT	TTTTTTATAT	
TTTGTTTTGT	GTTATTTTT	TCTTTAACAT	CCCTAAAATT	TTCCTTACAT	J
GTTTTACTAG	CCAGATTTTT	CCTCCTCTCC	TGACTACTCC	CAGTCATAGC)
TGTCCCTCTT	CTCTTATGGA	GATCCCTCGA	CCTGCAGCCC	AAGCTTGGCG	
TAATCATGGT	CATAGCAGCC	TAA			

eGFP-2A-RedStar amino acid sequence

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVP WPTLTTLAYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVK FEGGTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIEVNFKIRHRG TP<u>RSGGGEGRGSLLTCGDVEENPGPR</u>MSRSSKNVIKEFMRFKVKMEGTVNGHEFEIE GEGEGRPYEGHNTVKLKVTKGGPLPFAWDILSPQFQYGSKVYVKHPADIPDYKKLSF PEGFKWERIMNFEDGGVVTVTQDSSLQDGCFIYKVKLIGVNFPSDGPVMQKKTMGW EASTERLYPRDGVLKGEIHKALKLKDGGHYLVEFKSIYKAKKQVQLPGYYYVDSKL DIISHNEDYTIVEQYERTEGRHHLFLYKSTNENANTPAARLHRFKNKGKDSTEMRRR RIEVNVELRKAKKDDQMLKRRNVSSFPDDATSPLQEN*