Three Huntington disease specific mutation-carrying human embryonic stem cell lines have stable number of CAG repeats upon in vitro differentiation into cardiomyocytes

Laureen Jacquet[1], Andreas Neueder[2], Gabor Földes[3], Panagiotis Karagiannis[2], Carl Hobbs[4], Nelly Jolinon[2], Maxime Mioulane[3], Takao Sakai[5], Sian E. Harding[3], Dusko Ilic[1*]

[1]Stem Cell Laboratory, Assisted Conception Unit, Division of Women's Health, Women's Health Academic Centre, King's College London, London SE1 9RT, UK; [2]Division of Genetics and Molecular Medicine, King's College London School of Medicine, Guy's Hospital, London SE1 9RT, UK; [3]National Heart and Lung Institute, Imperial College, ICTEM, 4th Floor, Hammersmith Campus, Du Cane Rd, London W12 0NN, UK; [4]Histology Laboratory, Wolfson Centre for Age-Related Diseases, School of Biomedical Sciences, King's College London SE1 1UL, UK; and [5]Department of Molecular and Clinical Pharmacology, Institute of Translational Medicine, The University of Liverpool, Sherrington Building, Ashton Street, Liverpool L69 3GE, UK

*Address correspondence to: Dusko Ilic, Assisted Conception Unit, 11th Fl. Tower Wing, Guy's Hospital, London SE1 9RT, UK. Email: dusko.ilic@kcl.ac.uk.

Running title: Modeling Huntington disease with hESC

Abstract

Huntington Disease (HD; OMIM 143100), a progressive neurodegenerative disorder, is caused by an expanded trinucleotide CAG (polyQ) motif in the *HTT* gene. Cardiovascular symptoms, often present in presymptomatic and early stage HD patients, are, in general, ascribed to dysautonomia. However, cardio-specific expression of polyQ peptides caused pathological response in murine models, suggesting the presence of a nervous system-independent heart phenotype in HD patients. A positive correlation between the CAG repeat size and severity of symptoms observed in HD patients has also been observed in HD cellular models in vitro. In order to test the suitability of human embryonic stem cell (hESC) lines carrying HD-specific mutation as in vitro models for understanding molecular mechanisms of cardiac pathology seen in HD patients, we differentiated three such HD-hESC lines into cardiomyocytes and investigated CAG stability up to 60 days after starting differentiation. To assess CAG stability in other tissues, the lines were also subjected to in vivo differentiation into teratomas for 10 weeks. Neither directed differentiation into cardiomyocytes in vitro nor in vivo differentiation into teratomas, rich in immature neuronal tissue, led to an increase in the number of CAG repeats. Although the CAG stability might be cell line-dependent, induced pluripotent stem cells generated from patients with larger numbers of CAG repeats could have an advantage as a research tool for understanding cardiac symptoms of HD patients.

Huntington Disease (HD; OMIM 143100) is an autosomal, dominantly inherited progressive neurodegenerative disorder usually with a late onset. It is caused by an expanded polymorphic polyglutamine (polyQ) trinucleotide (CAG) motif in the first exon of the *HTT* gene. *HTT* encodes huntingtin (HTT), a large 348 kD protein ubiquitously expressed, with highest levels found in the brain and testis.[1-4] HTT endogenous function is still not completely understood as it has very little homology to other known proteins.[1]

In healthy individuals, the CAG repeat number ranges from 11 to 34 while numbers greater than 36 are causative of HD. The number of repeats generally determines age of disease onset.[1,5,6] Individuals with over 55 CAG repeats tend to develop Juvenile Huntington Disease, a more severe form, with slightly different clinical manifestations that develop in their youth instead of in their third to fifth decade. HD patients bearing homozygous mutations do not automatically have a lower age of onset, but do have a more severe phenotype and disease progression.[6] The mutation shows anticipation, with both decreases and increases in repeat length occurring upon parent to offspring transmission.[1,7] Instability of the CAG repeat length has also been reported in somatic tissues, with the largest expansion being observed in the brain.[8,9] Cognitive decline, irritability and depression are often the first signs of disease, preceding clinical diagnosis and the development of motor symptoms.[10] Uncontrollable movements, difficulty in speech and swallowing lead to progressive physical deterioration, total dependency and need for full nursing care. Death is usually the result of secondary illness.

HTT is ubiquitously expressed; despite neurodegeneration being the main phenotype of HD, non-CNS HD-associated pathologies have been reported.[11-13] Orthostatic hypotension, tachycardia, impaired modulation of cardiovascular tone and attenuated heart rate responses to stress, often present in presymptomatic and early stage HD patients, have been ascribed to dysfunction of the autonomous nervous system.[14-19] However, cardiac pathology, including atrophy, has been described in HD murine models.[13,20-22] In addition, cardiomyocyte-autonomous expression of 83 polyglutamine peptide in mouse model led to reduced cardiac function and dilatation by 5 and death by 8 months, whereas 9-fold higher expression of 19 polyQ peptide in control animals had no effect on cardiac function or lifespan.[23] Taken together, the data from

animal models suggested that cardiac phenotype seen in HD patients is not exclusively a result of dysautonomia; expression of the mutant HTT may be also cardiotoxic.

Human pluripotent stem cells, bearing the endogenous *HTT* mutation, can be differentiated into multiple cell lineages and can be kept in culture ad infinitum, circumventing the limitations associated with primary cell culture or immortalized cell lines with exogenously expressed mutant *HTT*. The aim of this study was to test the stability of CAG repeats in cardyomyocytes differentiated in vitro from three HD-hESC lines, KCL027, KCL028 and KCL36, derived in our center.[24]

Material and Methods

hESC derivation, culture, expression of pluripotency markers and in vivo differentiation (teratoma)

The methods are described in detail previously.[25,26]

Cardiac differentiation

Directed cardiac differentiation was based on the work published by Laflamme et al.[27] Briefly, undifferentiated hESCs were dissociated into a single-cell suspension by a 3-5 min incubation with accutase (STEMCELL Technologies). Cells were then centrifuged, resuspended, counted and seeded in Nutristem (Stemgent) onto growth factor reduced Matrigel (BD) coated 24-well plates at a density of 2 x 10^5 cells/cm^2, with the addition of 10 μM Y-27632 (Source Bioscience) to ensure good cell attachment. This marked Day -5 of the differentiation protocol. Until Day 0, cells were fed daily with 2 ml/well of Nutristem. At Day 0, cardiac differentiation was induced by feeding the cells with 0.5 ml/well Roswell Park Memorial Institute medium (RPMI)-B27 medium (Invitrogen) supplemented with 100 ng/ml of human recombinant Activin A (Miltenyi). The following day, the medium was replaced by 1.5 ml/well RPMI-B27 supplemented with 10 ng/ml human recombinant BMP4 (Miltenyi) and left unchanged for four days. The medium was then exchanged for unsupplemented RPMI-B27 every two days for up to 60 days.

Reverse transcription (RT)-PCR and quantitative qPCR

Total RNA was extracted using the RNeasy Mini Kit (QIAGEN) following the manufacturer's instructions. A 20 min DNAse I (Qiagen) treatment step was included to eliminate potential genomic DNA contamination. cDNA was generated by reverse-transcription of total RNA (500 ng) using the Precision nanoScript Reverse Transcription kit (PrimerDesign) following the manufacturer's instruction. The qPCR reaction consisted of 5 µL of cDNA diluted 1:10 in water, 10 µL of Precision 2X qPCR Mastermix (PrimerDesign) and 300 µM of each primer for a final volume of 20 µL. qPCR cycling conditions were as follow: 1 cycle 95 °C for 10 min; 40 cycles 95 °C for 15 s, 61 °C for 30 sec followed by melt curve acquisition from 59 ºC to 95 ºC with 0.5 ºC increment. The *OCT3/4*, *NANOG, TNNT2, NKX2.5, EIF4A2* and *SDHA* primers were designed by PrimerDesign Ltd and the *HTT* primers were designed using the Primer3 website (*http://frodo.wi.mit.edu/*). Primers are listed in Supplemental Table S1. All samples were analyzed in triplicate, Ct values were determined, and the expression was calculated by the $2^{-\Delta\Delta Ct}$ method. *EIF4A2* and *SDHA* were used for internal normalisation (Supplemental Table S2).

Isolation of DNA from formalin-fixed paraffin embedded (FFPE) tissue

Seven serial sections from each paraffin block were processed for the analysis. The first and the last section were stained with hematoxylin and eosin (H&E) and the tissue types spanning both sections were determined. We purified DNA from remaining five sections and determined number of CAG repeats using human-specific primers. Wild-type allele was used as an internal control.

Isolation of DNA from FFPE tissue was adapted from Campos and Gilbert.[29] The FFPE tissue samples were digested in 500 µL alkali digestion buffer (0.1 M NaOH with 1% SDS, pH 12) for 40 min at 100°C. After a 5 min of cooling down period, 500 µL of 25:24:1 phenol:chloroform:isoamyl alcohol was added to the samples, followed by a 5 min agitation at room temperature and 5 min centrifugation at 10,000 x *g*. The upper aqueous layer was then removed and added to a new tube containing 500 µL choloroform. A second agitation and centrifugation step was performed before adding the aqueous layer to 1 volume of isopropanol and

0.1 volume of 3 M sodium acetate pH 5. The samples were then centrifuged 30 min at 10,000 x *g*. Liquid was then decanted and the DNA pellet washed twice with 1 mL 85% ethanol. After ethanol decantation, the DNA pellet was allowed to dry before resuspension in TE buffer.

CAG repeats

Preimplantation genetic haplotyping (PGH) for HD was performed as described previously.[24,25] CAG repeat size determination of the cell and tissue samples was performed by PCR as published.[30] Briefly, 5-50 ng DNA amplification was performed in 10 µl reactions containing: 0.2 mM dNTPs, 10% DMSO, AM buffer (67 mM Tris–HCl pH 8.8; 16.6 mM $(NH_4)SO_4$; 2 mM $MgCl_2$; 0.17 mg/ml BSA), 0.8 pmol FAM-labelled forward primer (GAGTCCCTCAAGTCCTTCCAGCA) and reverse primer (GCCCAAACTCACGGTCGGT) and 0.5 U AmpliTaq DNA polymerase (Applied Biosystems). Cycling conditions were: 90 sec at 94°C, 35 × (30 sec at 94 °C; 30 sec at 65 °C; 90 sec at 72 °C) and 10 min at 72 °C. After the PCR, for each sample, 1 µl of PCR product was mixed with 9 µL of HiDi Formamide (Applied Biosystems) and 0.03 µl l MegaBACE ET900-R Size standards (GE Healthcare) and denatured at 95 ºC for 5 min. Samples were then run on an ABI3730 sequencer and analysed using GeneMapper v5.2- 3730XL software (Applied Biosystem).

Results

The purpose of pre-implantation genetic diagnosis (PGD) is to identify and exclude those embryos that carry the familial mutation. Such embryos are never destined for use in treatment and are routinely discarded as soon as an affected status is diagnosed. If the patients decide to donate these embryos to research, they give consent at the time of treatment. In our center we identify HD specific mutation-carrying embryos by haplotyping using polymorphic markers flanking and intragenic to the *HTT* gene on chromosome 4p16.3. Primers designed for 15 microsatellite markers enable testing for inheritance of the at-risk HD allele. Three HD-hESC lines, KCL027, KCL028 and KCL36, were derived in our center from embryos carrying HD-specific mutation from two donor couples. One couple had eight affected embryos from which two lines, KCL027 and KCL028, were derived and the other donor couple had one affected embryo, from which KCL036 was derived.[24]

All three lines were assessed for a number of parameters that are part of standard hESC lines characterization.[26,31] The identity of the cell lines was confirmed by genotyping; amplification of polymorphic microsatellite markers was carried out, giving a unique fingerprint for each cell line (Figure 1A). The presence of pluripotency markers in all three lines was demonstrated by immunostaining using a panel of antibodies against transcription factors nanog and Oct4 and cell surface antigens TRA-1-60 and TRA-1-80. The hESC colonies were also positive for alkaline phosphatase (AP) activity using a commercially available kit (Figure 1B). As the first line test for genomic stability, we used array CGH, an oligonucleotide platform with 60,000 probes that can detect regions of imbalance down to approximately 25kb.[32] No genetic imbalance was detected during extended periods of culture (data not shown).

Next, we asked whether directed differentiation into cardiomyocytes would result in a change in number of CAG repeats. We opted for a monolayer directed differentiation protocol.[27] All three differentiated HD-hESC lines presented beating cardiomyocytes (Figure 2A). Using qPCR,

we confirmed that 30 days after the start of differentiation, in beating areas of all three hESC lines expression of pluripotency-associated genes *NANOG* and *OCT3/4* had diminished, whereas the expression of cardiac-associated genes *NKX2.5* and *TNNT2* increased. *HTT* expression remained similar between undifferentiated hESCs and hESC-derived cardiomyocytes (Figure 2B). The CAG repeat number of all three HD-hESC lines remained stable throughout differentiation (Figure 2C). We then extended culture of differentiated cardiomyocytes for an additional 30 days and showed that the number of CAG repeats in cells derived from two lines, KCL027 and KCL036, still remained unchanged (Figure 2C). To our knowledge, this is the first demonstration of differentiation of HD-mutation-carrying human pluripotent stem cells, either hESC or hiPSC, into cardiac myocytes, and assessment of the effect on the number of the CAG repeats.

Since CAG stability might differ from one tissue to another, in order to mimic more closely a physiological environment we allowed cells to differentiate spontaneously in vivo. Different techniques of generating teratomas, encapsulated tumors with tissue derivatives of all three germ layers, have been described.[33-35] We injected cells embedded in Matrigel subcutaneously into immunocompromized NOD-SCID mice and harvested them 10 weeks later. Animals homozygous for the SCID mutation have impaired T and B cell lymphocyte development, whereas the NOD background additionally results in deficient natural killer cell function. Teratomas in all lines consisted of tissue derivatives from all three germ layers (Figure 3A). All tumors contained glandular epithelium, immature neural epithelium, immature mesenchyme, cartilage and endothelium. In addition KCL027 and KCL028 teratomas contained pigmented epithelium and arachnoidal tissue. We assessed the number of CAG repeats in DNA isolated from sections of FFPE teratoma tissue samples. We found no increase in the number of CAG repeats in the mutated allele in any of the lines examined (Figure 3B). However, we detected a slight decrease in the number of CAG repeats in both the wild-type (WT) and HD allele of line KCL027. Such short truncations have been reported previously.[36,37] Taken together the data suggest that our three HD-hESC lines have a rather stable number of CAG repeats regardless of the tissue in which they were differentiated. To understand the molecular mechanisms of cardiac pathology in HD patients,

HD-hESC and -hiPSC with lower number of CAG repeats may not be the best in vitro cellular model.

Discussion

To complement HD in vivo models, HD in vitro models have been developed from mouse, rat and human cells. These are in general immortalized cell lines with a genome-integrating viral delivery system carrying a mutated *HTT* exon 1, expression of which is driven by an exogenous promoter. These cell lines recapitulated some of the hallmarks features of HD, notably an increased cell death and the formation of aggregates. Similar phenotypes have also been found in primary cell models. However, it is not until recently that stem cells have been used to model HD (Table 1).

Since the first disease-specific hESC line was derived from patients undergoing PGD for cystic fibrosis,[38] more than 20 HD-hESC lines have been derived worldwide. However, only a few of them have been thoroughly characterized or used as research tools. Two hESC lines carrying 37 and 51 CAG repeats were indistinguishable from normal control cell lines in the undifferentiated state and during differentiation into forebrain neurons. However, neural differentiation induced instability of CAG repeats and lines were gaining five to six CAG repeats upon differentiation.[39] Genes typically deregulated in HD remained unperturbed throughout differentiation. The only phenotypes observed were elevated glutamate-evoked responses in neurons differentiated from an HD-hESC line with 51 CAG repeats.[40] A marked increase in the number of repeats from 46 to 70 upon differentiation into astroglial precursors was found in one HD-hESC line derived in our laboratory.[41] On the other hand, two other groups reported that 40-48 CAG repeats were stable in five undifferentiated and differentiated HD-hESCs.[42,43] A later study involving these five HD-hESC lines reported a down-regulation of the *HTT* gene itself in HD neural cells and dysregulation of three genes, of which two, *CHCHD2* and *TRIM4*, were also dysregulated in blood cells from the HD patient.[44] In contrast, we could not detect any change in levels of *HTT* gene expression in HD cardiomyocytes (Figure 2B) indicating that this phenomenon might be either cell line-related or typical specifically for HD neurons but not for other cell types. We also found that the number of CAG repeats was stable upon in vivo and in vitro differentiation into cardiomyocytes in all three lines examined. Taking together the reports from different groups to date, it seems that some lines

are prone to CAG expansion in vitro and others are not. Molecular mechanisms behind these differences are still unknown. Although cell culture conditions might play a role, genetic background of each individual line is likely to be more important. It would be interesting to see how unstable CAG repeats in cell lines correlate with anticipation in the families that donated the embryos. However, confidentiality surrounding human embryo donation makes such comparison problematic.

Reprogramming of somatic cells into iPSC discovered by Takahashi and Yamanaka,[45] could circumvent these issues. Derivation of iPSC lines will enable comparison of disease-in-the-dish phenotypes with clinical findings in somatic cell donors and affected members of their families, leading to better understanding of the role of genetic background and molecular mechanisms in development of HD-associated symptoms. The HD iPSC consortium recently published findings showing that the positive correlation between the sizes of CAG repeats and severity of symptoms observed in HD patients could also be observed in vitro in HD-iPSC derived neurons.[46] A significant increase in lysosomal activity both during self-renewal and in iPSC-derived neurons has been noted in the iPSC lines derived from two homozygotes carrying 42/44 and 39/42 CAG repeats and one heterozygote.[47] The number of CAG repeats remained stable during the extended period of culture. HD-iPSC with 72 CAG repeats did not show phenotype when cultured in vitro or differentiated into neural precursors. However, when either subjected to oxidative stress,[48] proteasome inhibitor or engrafted into neonatal brains for 33 weeks, they exhibited signs of HD pathology.[49] Elevated caspase activity in HD-iPSCs was detected in derived neurons of HD-iSPC line with 72 repeats.[50]

The CAG-repeat expansion associated phenotypes already reported in transgenic models of HD are the result of exogenous mutation, whereas phenotypes in HD-hESC and HD-iPSC would be caused by endogenous mutation, more closely modeling in vivo disease. Our data further highlight the need to generate HD-iPSCs with a wider range of repeats and to correlate the repeat instability in different tissue types with the medical history of donors and affected members of their families.

Author contribution

D.I. conceptualized and supervised the project. L.J., A.N., G.F., P.K., C.H., N.J., and M.M. collected and analyzed the data. L.J., A.N., G.F. and S.E.H. participated in design of the experiments and writing the manuscript. L.J. and D.I. wrote the final version of the paper.

Acknowledgments

This work was supported by the UK Medical Research Council (MRC) grants G0701172 and G0801061. L.J. had MRC studentship. We thank hESC line derivation team: Emma Stephenson, Victoria Wood, Neli Kadeva, Glenda Cornwell, and Stefano Codognotto. We also thank Dr Yacoub Khalaf, Director of the Assisted Conception Unit of Guy's and St Thomas' NHS Foundation Trust and his staff for supporting the research program. We are especially indebted to patients who donated embryos. We thank Prof. Gillian Bates (King's College London), for her input and advice and Prof Caroline Ogilvie (Guy's & St. Thomas' NHS Foundation Trust) for critical reading of the manuscript.

Competing financial interests

The authors declare no competing financial interest

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Figure legends

Figure 1. HD-hESC lines characterization. Genotyping: Microsatellite markers specific for chromosomes 13, 18, 21, X and Y were amplified. The allele sizes in bp for markers on chromosomes 13, 18, and 21 are listed in the table. Pluripotency markers: alkaline phosphatase (AP) activity, TRA-1–60, TRA-1–81, OCT4, and Nanog were detected in undifferentiated cells of all three hESC lines.

Figure 2. All three HD-hESC lines have stable number of CAG repeats upon directed cardiomyocyte differentiation in vitro. (A) Beating cardiomyocytes were present in all three HD hESC lines examined. (B) Upon differentiation into cardiomyocytes, expression of plutipotency markers *nanog* and *OCT4* is nearly undetectable, whereas cardiomyocyte markers *NKX2.5* and *TNNT2* are markedly increased. There is no change in *HTT* expression. (C) Top: Analysis of CAG repeats in undifferentiated KCL027 cells at the start of differentiation [day 0] and 60-day after. Bottom: Number of CAG repeats in allele carrying HD mutation has not increased after 30-60 days differentiation into cardiomyocytes.

Figure 3. All three HD-hESC lines have stable number of CAG repeats upon spontaneous in vivo. (A) Differentiation of three germ layers in vivo: Teratomas were encapsulated and did not invade surrounding tissue. Sections are counterstained with hematoxylin and eosin and specific stains are either light blue (Alcian blue) or brown (all immunohistochemistry). Germ layer markers: Alcian blue– and periodic acid–Schiff (PAS)-stained cartilage and desmin for mesoderm, neuron-specific β-III tubulin and glial fibrillary acidic protein (GFAP) for ectoderm, GATA4 and a- fetoprotein for endoderm. (B) Number of CAG repeats in allele carrying HD mutation did not increase during 10-week teratoma formation.

Table 1. Pluripotent stem cell in vitro models of HD.

Supplemental figure legends

Supplemental Table S1. Sense and Antisense primers of target genes used for hESC-derived cardiomyocytes characterization**.**

Supplemental Table S2. Housekeeping genes designed and synthesized by PrimerDesign Ltd used for hESC-derived cardiomyocyte characterisation amplicon context. The primer sequences are commercially sensitive information but the details provided in this table are MIQE compliant.[28]

A

KCL027

Marker	Allele 1	Allele 2
D13S252	299	303
D13S325	289	293
D13S628	450	457
D13S634	394	405
D18S386	356	386
D18S390	372	372
D18S391	217	217
D18S535	486	486
D18S819	412	420
D18S976	473	483
D18S978	211	211
D21S11	245	245
D21S1409	212	220
D21S1411	312	324
D21S1435	193	193
D21S1446	311	315

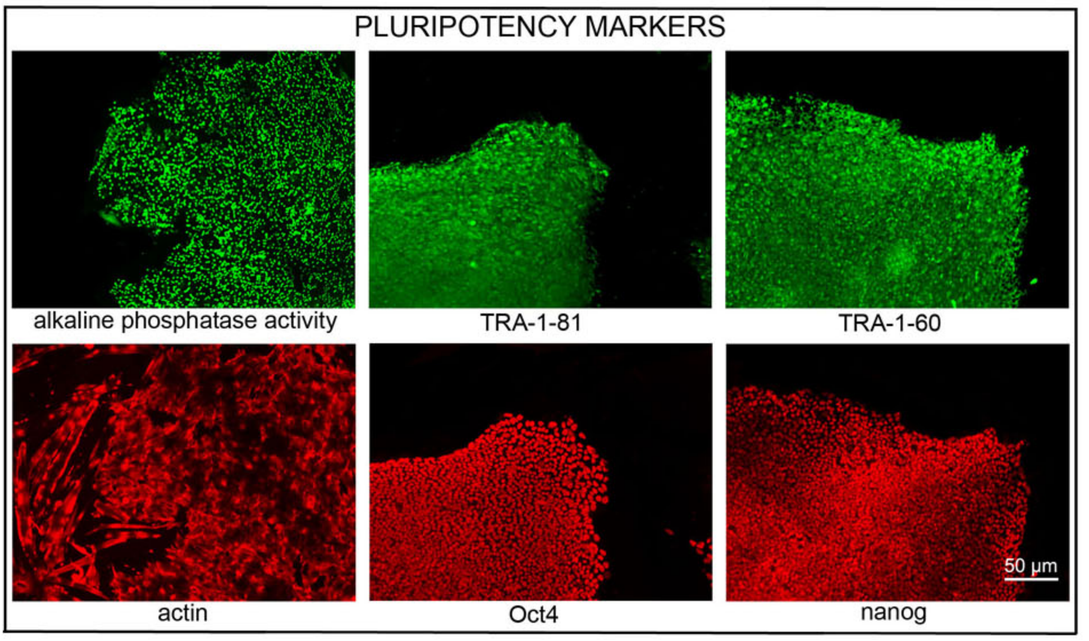
KCL028

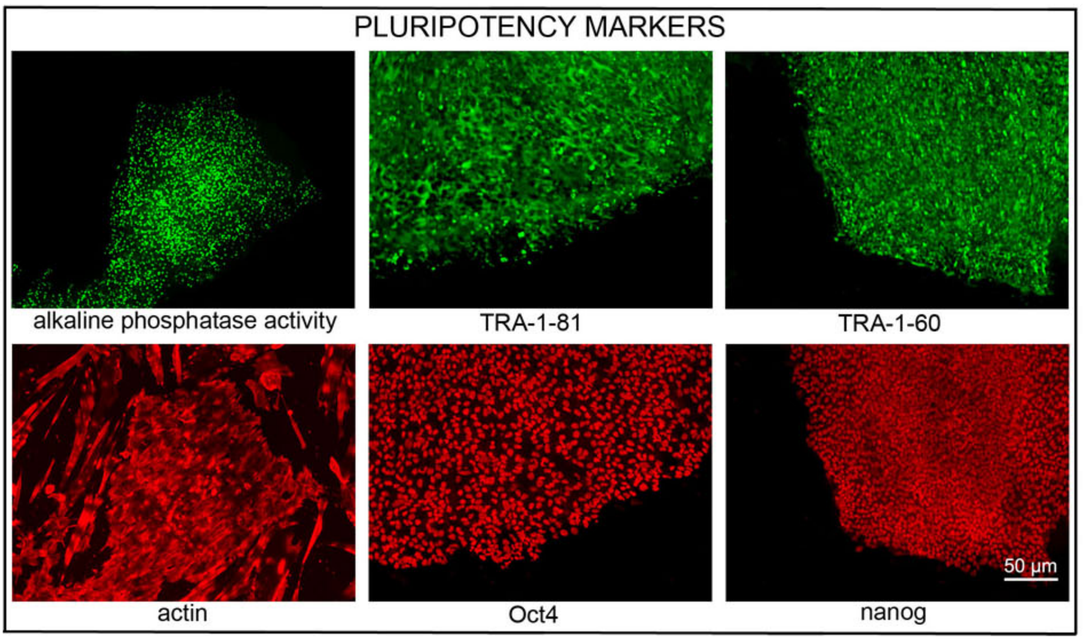
Marker	Allele 1	Allele 2
D13S252	299	303
D13S325	289	293
D13S628	450	458
D13S634	394	405
D18S386	356	386
D18S390	372	372
D18S391	217	217
D18S535	486	486
D18S819	412	420
D18S976	473	483
D18S978	211	211
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D21S1409	212	220
D21S1411	312	324
D21S1435	192	192
D21S1446	311	315

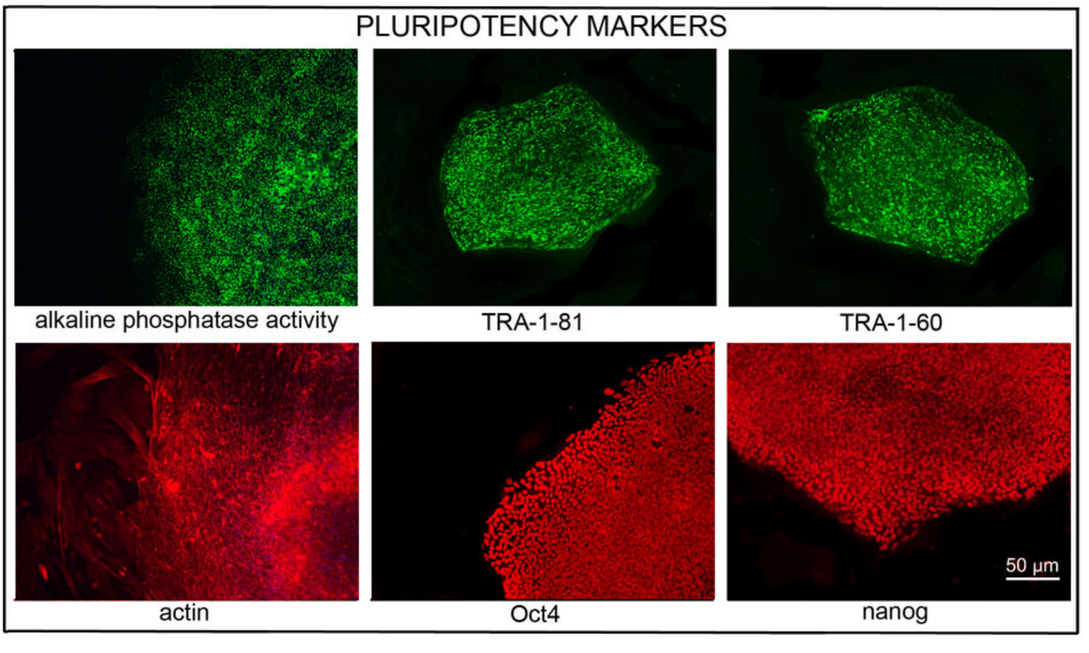
KCL036

Marker	Allele 1	Allele 2
D13S252	278	299
D13S325	293	301
D13S628	453	453
D13S634	397	401
D18S386	379	382
D18S390	368	372
D18S391	209	217
D18S535	478	482
D18S819	400	408
D18S976	476	483
D18S978	211	211
D21S11	249	259
D21S1409	216	224
D21S1411	312	316
D21S1435	188	188
D21S1446	311	325

B



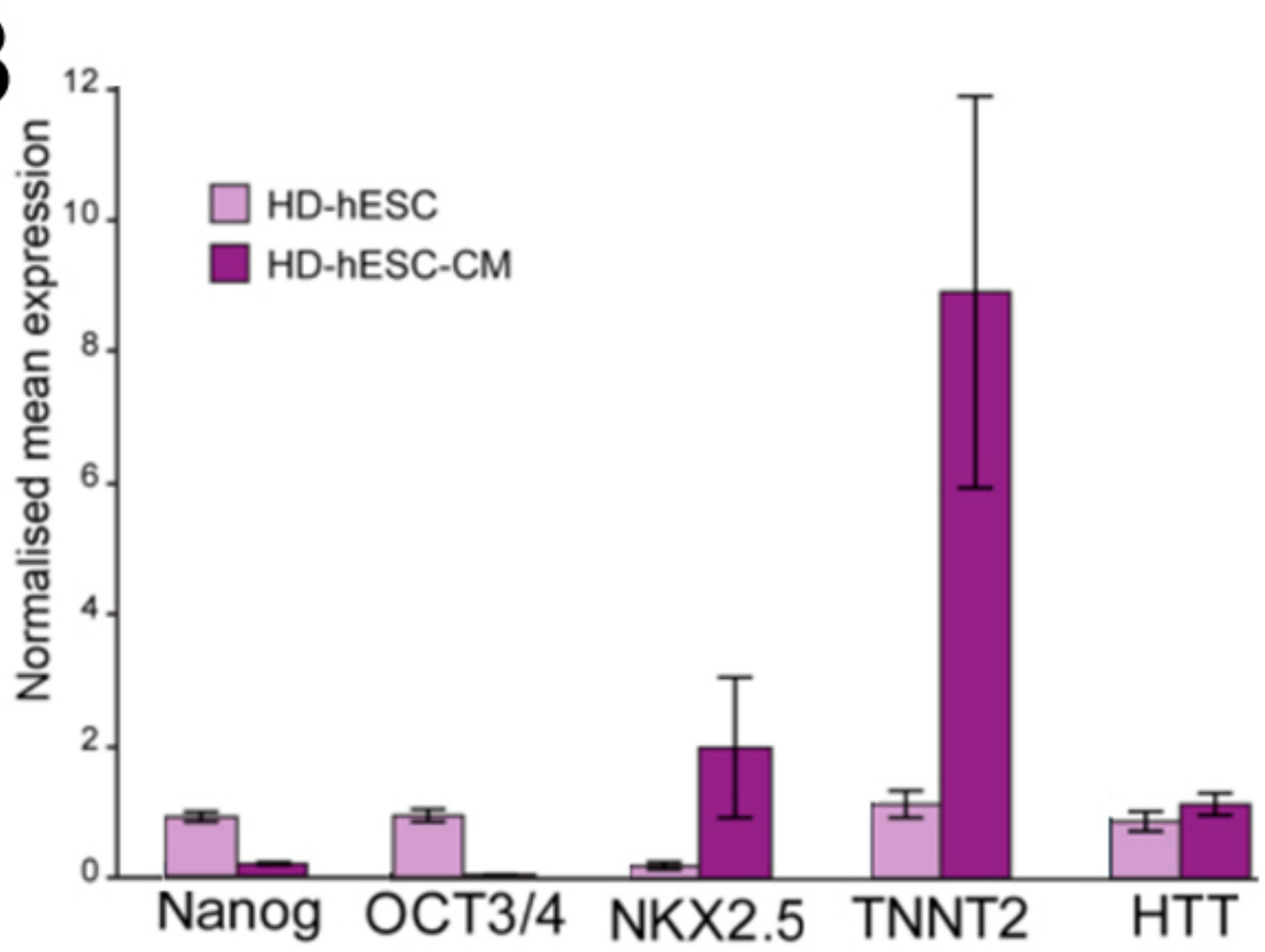




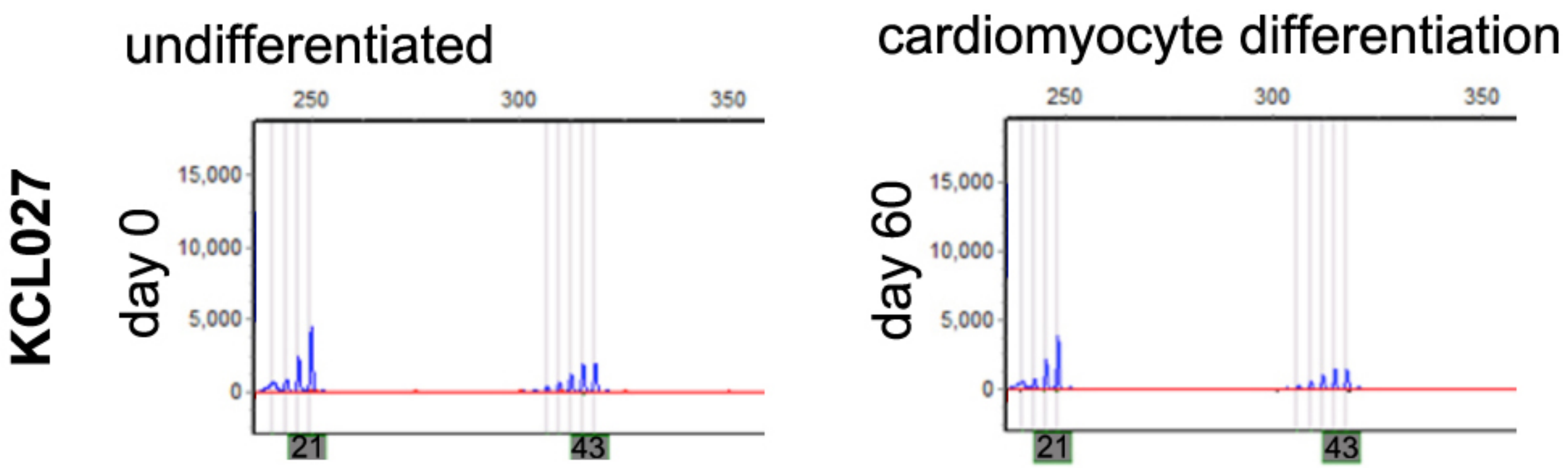
A

	Wells with beating cardiomyocytes [day 30]
KCL027	16.7%
KCL028	4.2%
KCL036	6.3%

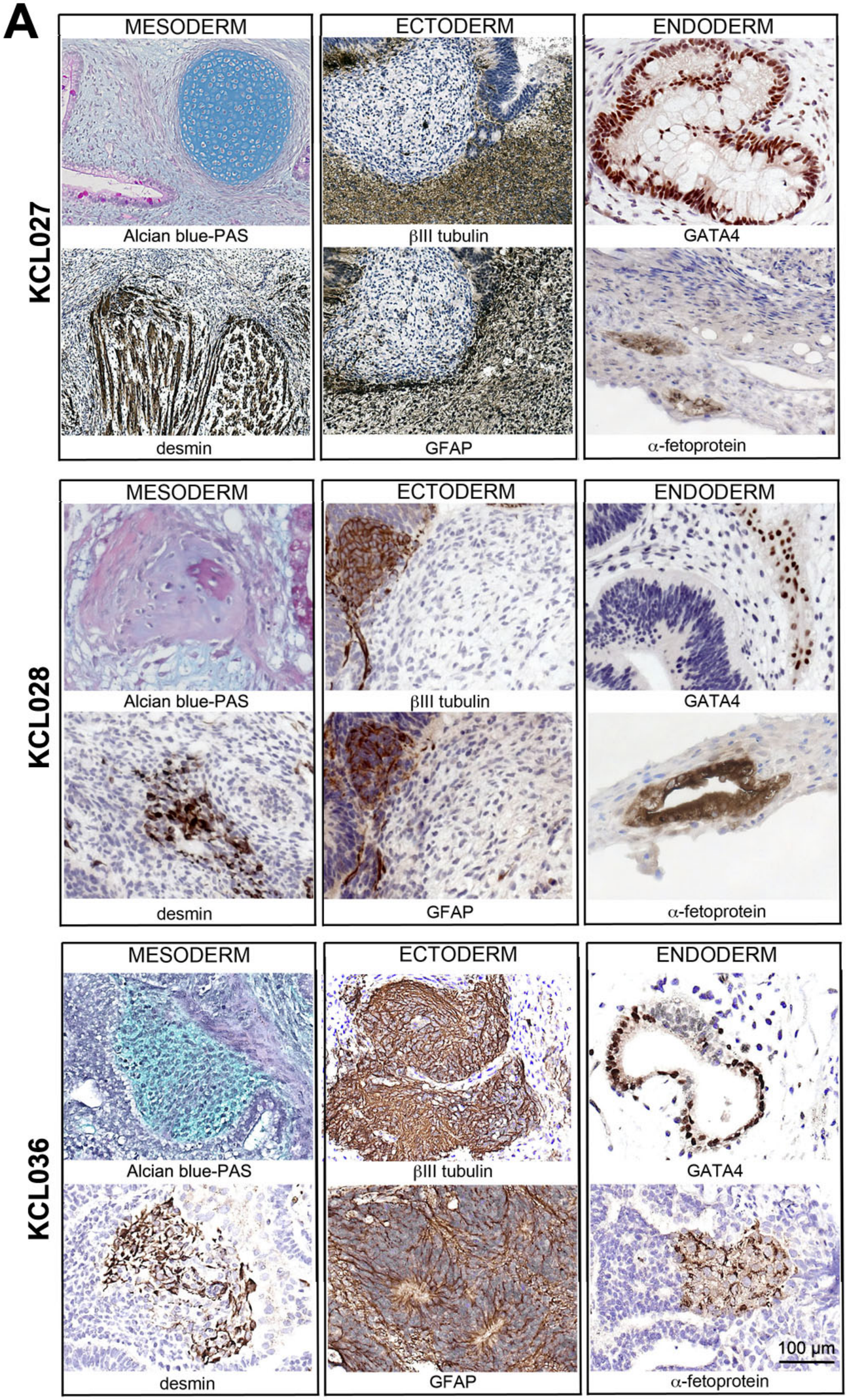
B



C



	Undifferentiated [day 0]		Cardiomyocyte Differentiation [day 30]		Cardiomyocyte Differentiation [day 60]	
	WT allele	HD allele	WT allele	HD allele	WT allele	HD allele
KCL027	21	43	21	43	21	43
KCL028	21	43	21	43	ND	ND
KCL036	14	38	14	38	14	38



B

	Embryo donors		hES cell line		teratomas	
	WT allele	HD allele	WT allele	HD allele	WT allele	HD allele
KCL027	17	43	21	43	19	37-38
KCL028	17	43	21	43	21	43
KCL036	17	42	14	38	14	37-38

Cell line	Species origin	Cell type	CAG repeat size	Phenotype	Reference
	Mouse	Neural stem cells	140/140; *HTT* KO	Impaired motility, increased ROS, dysregulated cholesterol	51
HD-iPS1	Human	iPSC	72		50, 52
*Hdh*CAG	Mouse	ESC	7, 77 and 150	Large CAG repeat sizes increase neurogenesis	53
KCL005, KCL008	Human	ESC	46	Increase in CAG repeats upon differentiation into astroglial precursors	41
KCL012, KCL013, KCL027, KCL028, KCL036	Human	ESC	42, 46	No phenotype reported	24, 25
R6/1	Mouse	ESC	127	CAG expansion, impaired DNA repair, apoptosis	54
	Mouse	ESC	20, 50, 91, 111 and *HTT* KO	Dysregulation of 73 genes	55
TrES1	Monkey	Pluripotent SC generated by the fusion of transgenic HD monkey skin fibroblast and a wild-type monkey oocyte	84	Presence of aggregates	56
rHD-ESC	Monkey	ESC			57
	Human	ESC with exogenous exon1 expression?		Insoluble HTT aggregates and neurodegeneration	58
SI-186, SI-187	Human	ESC	37 and 51	Increase in CAG repeats upon neural differentiation; elevated glutamate-evoked responses	39, 40
SI-187, SIVF017, SIVF018, SIVF020, Huez2.3, VUB05	Human	ESC	40-51	Downregulation of HTT, dysregulation of CHCHD2, TRIM4, and PKIB	42-44
HD-iPS^{hom} 4F-1, HD-iPS^{hom} 4F-2, HD-iPS^{hom} 4F-3, HD-iPS^{hom} 3F-1, HD-iPS^{hom} 3F-2, HD-iPS^{het} 3F-1	Human	iPSC	42/44, 39/42, 45	Increase in lysosomal activity	47
HD60n, HD60i.3, HD60i.4, HD109i.1, HD180n HD180i.5, HD180i6, HD180i7	Human	iPSC	60, 109, 180	Disease-associated changes in electrophysiology, metabolism, cell adhesion, and apoptosis	46
	Human	iPSC	72	Deregulated expression of oxidative stress proteins; aggregates	48, 49

Table 1. Pluripotent stem cell in vitro models of HD.