ROZPRAWA DOKTORSKA

Nowe modele komórkowe iPSC choroby Huntingtona do zastosowań w eksperymentalnej terapii komórkowej oraz w badaniach funkcji neurorozwojowych normalnej i zmutowanej huntingtyny

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Spis treści

Streszczenie

Choroba Huntingtona (HD) jest monogenową chorobą neurodegeneracyjną dziedziczoną w sposób dominujący. Najczęściej rozwija się u osób dorosłych, jednakże istnieją jej formy młodzieńcza oraz dziecięca. U chorych występują objawy motoryczne, poznawcze i psychiatryczne a głównym klinicznym symptomem neuropatologicznym jest śmierć neuronów kolczystych prążkowia. Przyczyną choroby jest zwiększenie liczby powtórzeń CAG obecnych w egzonie 1 genu *HTT*, co skutkuje powstaniem toksycznego białka huntingtyny o wydłużonej domenie poliglutaminowej (poliQ). Choroba rozwija się w obecności powyżej 36-40 powtórzeń. Huntingtyna jest wielofunkcyjnym białkiem biorącym udział m.in. w procesach regulacji transkrypcji, transportu i podziałów mitotycznych. Z tego względu jego mutacja wywołuje szerokie spektrum molekularnych zaburzeń w funkcjonowaniu komórek. Liczne modele komórkowe i zwierzęce HD znacznie przyczyniły się do zrozumienia tej choroby, jednak jak dotąd nie opracowano jej skutecznej terapii.

Potencjalną strategią terapeutyczną dla HD jest autologiczna terapia komórkowa, w której pochodzące od pacjenta i poddane terapii *ex vivo* komórki byłyby dostarczane do uszkodzonych regionów mózgu tej samej osoby. Tam tworzyłyby funkcjonalne neurony, regenerując uszkodzone obszary oraz pełniąc funkcje ochronne. Optymalną, trwałą metodą terapeutyczną dla tych autologicznych komórek wydaje się być eliminacja ekspresji zmutowanej huntingtyny. Z kolei technologia indukowanych pluripotencjalnych komórek macierzystych (iPSC) umożliwia pozyskanie od dorosłych pacjentów komórek, które można przekształcić do dowolnego typu komórek organizmu.

Jednym z celów zawartych w tej pracy badań było stworzenie modelu komórkowego, który mógłby być użyty w eksperymentalnej terapii komórkowej HD u myszy. W tym celu wyprowadziłem i scharakteryzowałem komórki iPSC z mysiego modelu HD, YAC128. Następnie stworzyłem linie tych komórek ze stabilną ekspresją reagentów typu shRNA umieszczonych w transpozonie piggyBac, które w sposób trwały miały wyciszać ekspresję zmutowanej, patogennej huntingtyny. Jeden z reagentów (shHTT2) obniża ekspresję zmutowanej huntingtyny o około 85% w komórkach iPSC i o około 60% w zróżnicowanych z nich neuronalnych komórkach macierzystych (NSC). Uzyskane komórki NSC mogą być użyte w modelu terapii komórkowej *in vivo* u myszy lub w modelach terapii *in vitro*.

Reagenty shRNA wprowadziłem również do ludzkich komórek iPSC od pacjentów HD (HD71, HD109) i od osób zdrowych, uzyskując przy użyciu reagenta shHTT2 nieallelospecyficzne wyciszenie normalnej i zmutowanej huntingtyny. Są to pierwsze opisane ludzkie komórki iPSC z trwałym wyciszeniem normalnej huntingtyny, co pozwala wykorzystać je do badań nad funkcjami rozwojowymi tego białka u ludzi.

HD jest tradycyjnie uznawana za neurodegeneracyjną chorobę dorosłego mózgu. Jednak badania z ostatnich lat wskazują, że istotnym elementem patogenezy tej choroby są defekty nabyte w trakcie neurorozwoju. Dowody na istotność fazy neurorozwojowej pochodzą z badań klinicznych oraz badań w modelach zwierzęcych i w modelach tworzonych na bazie komórek pluripotencjalnych. W ramach poszukiwania markera użytecznego dla oceny skuteczności terapii już na etapie pluripotencji, zidentyfikowałem w komórkach iPSC zaburzenia w ekspresji białek ścieżek sygnałowych Wnt i ERK, białek odpowiedzi na stres oksydacyjny, i przede wszystkim białka p53. Najważniejszą obserwacją jest obniżenie ekspresji białka p53 w komórkach YAC128-iPSC oraz HD109 i odwrócenie tego fenotypu w komórkach YAC128-iPSC z wyciszoną ekspresją zmutowanej huntingtyny. Białko p53 potencjalnie może więc być markerem skuteczności terapii HD w komórkach pluripotencjalnych. Pytania nad biologiczną istotnością zaobserwowanych zmian pozostają otwarte i będą one przedmiotem dalszych badań bazujących na wynikach uzyskanych w wyprowadzonych modelach iPSC.

Abstract

Huntington disease (HD) is a monogenic, dominantly inherited neurodegenerative disorder. HD onset usually occurs in adulthood; however, HD can appear in youth or children in its rare juvenile form. The clinical picture of HD include motor, cognitive and psychiatric symptoms. Death of striatal medium spiny neurons is the main neuropathological manifestation of the disease. The cause of HD is a CAG repeat expansion within exon 1 of HTT gene, which results in expression of a toxic huntingtin protein with an extended polyglutamine (polyQ) domain. The disease develops when the number of repeats exceeds 36-40. Huntingtin is the multifunctional protein involved in processess such as transcription regulation, transport and mitotic division. Therefore, the huntingtin mutation results in a wide spectrum of molecular defects in cell functioning. Although multiple cellular and animal HD models have considerably increased understanding of the disease, a successful therapy has not been developed yet.

An autologous cell therapy is a potential therapeutic strategy for HD. In such a therapy, cells derived from a patient would be treated *ex vivo* and transplanted into affected brain regions of the same person. Grafted cells would develop into functional neurons executing regenerative and protective roles. Silencing of the mutant, pathogenic huntingtin expression seems to be an optimal therapeutic strategy for the treatment of autologous cells. Additionally, an induced pluripotent stem cell (iPSC) technology permits establishment of patient-derived cells with a potency to be turned into any cell type of a body.

One of research aims included in this thesis was to create a cellular model for an experimental HD cell therapy in mice. Therefore, I have established and characterized induced pluripotent stem cells (iPSCs) from a YAC128 mouse model of HD. Subsequently, I have stably introduced piggyBac transposon-driven shRNA reagents into the YAC128 iPSC, with the aim of permanent silencing of mutant huntingtin expression. One of the reagents, shHTT2, reduces mutant huntingtin expression by about 85% in iPSC lines and by about 60% in neural stem cells (NSCs) derived from the iPSC lines. The established NSC can be used for an *in vivo* cell therapy of YAC128 mice or for *in vitro* models of a cell therapy.

I have also introduced the shRNA reagents into human iPSCs derived from both HD patients (HD71, HD 109) and healthy people. The potent shHTT2 reagent efficiently silences normal and mutant huntingtin expression in these cells. These are the first human iPSCs with stable knock down of normal huntingtin, which makes them a valuable tool for research on developmental functions of huntingtin in humans.

Huntington disease is traditionally considered as a neurodegenerative disease of an adult brain. However, research from recent years suggests that defects acquired during neurodevelopment are a considerable part of HD pathogenesis. The evidence for the neurodevelopmental phase impact comes from clinical research, as well as from studies in animal models and in pluripotent cell-derived models. During a search for an early biomarker of therapeutic efficiacy in pluripotent cells, I identified deregulated expression of various proteins in HD iPSCs. The observed changes included effectors of Wnt and MAPK/ERK signaling pathways, oxidative stress response proteins and, most importantly, p53 protein. The p53 protein was downregulated both in YAC128-iPSCs and human HD109 cells, and the mutant huntingtin silencing reversed the phenotype in YAC128-iPSCs. Thus, p53 potentially might be a biomarker for HD therapy in pluripotent cells. The questions on biological relevance of the observed phenotypes remain opened and will be a subject of further research based on results obtained in the derived iPSC models.

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1 Wprowadzenie

1.1 Choroba Huntingtona

Choroba Huntingtona (HD) jest dziedziczoną w sposób dominujący, monogenową chorobą neurodegeneracyjną występującą z częstością ok. 10-14 na 100 000 osób w populacjach Zachodu. Początek klinicznych objawów HD to średnio 45. rok życia a mediana czasu przeżycia pacjentów po ich pojawieniu się wynosi ok. 18 lat (Bates i in., 2015). U chorych progresywnie rozwijają się prowadzące do niepełnosprawności zaburzenia motoryczne takie jak pląsawica, bradykinezja, dystonia i zesztywnienie. Pojawiają się także objawy kognitywne (m. in. zaburzenia uczenia i pamięci krótkotrwałej, funkcji wykonawczych, mowy) i psychiatryczne (m.in. zmiany nastroju i osobowości, depresja, psychozy, skłonności samobójcze, zmiany cyklu dobowego) (Walker, 2007). Najbardziej charakterystycznym elementem neuropatologii mózgu w HD jest atrofia prążkowia wynikająca z selektywnego obumierania GABA-ergicznych średnich neuronów kolczystych (ang. medium spiny neurons, MSN). W dalszych etapach rozwoju choroby widoczna staje się również degeneracja innych regionów mózgu, takich jak kora mózgu, gałka blada czy istota czarna (Waldvogel i in., 2015). Jednak HD to nie tylko choroba mózgu. W ostatnich latach coraz więcej uwagi zwraca się na patologię poza centralnym układem nerwowym, obejmującą przykładowo. utratę wagi, zaburzenia metaboliczne czy dysfunkcje układów mięśniowego lub immunologicznego (Carroll i in., 2015). Co ciekawe głównymi fizjologicznymi przyczynami zgonów pacjentów HD są zachłystowe zapalenie płuc, niewydolność serca, zaburzenia połykania czy niewydolność oddechowa (Abildtrup i Shattock, 2013; Heemskerk i Roos, 2012). Mimo licznych prób terapeutycznych HD jak dotąd pozostaje chorobą nieuleczalną.

HD jest najczęstszą z chorób poliglutaminowych (poliQ), do których należą również ataksje-rdzeniowo móżdżkowe (SCA) typu 1, 2, 3, 6, 7 i 17, rdzeniowo-opuszkowy zanik mięśni (SBMA) oraz zanik jądra zębatego, czerwiennego, gałki bladej i jądra Luysa (DRPLA). W każdej z tych chorób neurodegeneracyjnych ekspansja powtórzeń trójnukleotydu CAG w sekwencji kodującej specyficznego dla danej choroby genu prowadzi do ekspresji białka z nadmiernie długim łańcuchem glutamin, który zmienia strukturę białka powodując utratę części jego funkcji oraz/lub nabycie nowych, toksycznych właściwości, co razem prowadzi do rozwoju choroby. Różnice w patogenezie pomiędzy chorobami poliQ wynikają z odrębnych właściwości każdego z białek oraz profilu jego ekspresji w organizmie. Z kolei część wspólnych objawów jest niezależna od kontekstu białkowego i do ich wystąpienia wystarczy obecność długiego polipeptydu poliglutaminowego. Charakterystycznym, wspólnym elementem patogenezy chorób poliQ jest powstawanie rozpuszczalnych i nierozpuszczalnych agregatów białkowych, zwanych inkluzjami, w skład których wchodzi zmutowane białko lub jego fragmenty pochodzące z proteolizy, jak również molekuły z nimi oddziałujące (Hoffner i Djian, 2014; Woerner i in., 2016). Na skutek związanej z powtórzeniami translacji niezależnej od kodonu ATG (tzw. RAN translacja) z loci zmutowanego genu poliQ powstają również toksyczne białka polialaninowe, poliserynowe, polileucynowe czy policysteinowe (Bañez-Coronel i in., 2015; Cleary i Ranum, 2017). Czynnikiem patogennym w chorobach poliQ mogą być również cząsteczki RNA z wydłużonym ciągiem powtórzeń CAG tworzącym toksyczną strukturę typu spinki do włosów, która sekwestruje białka, przykładowo czynniki splicingowe, i tym samym zmniejsza ich funkcjonalnie aktywną pulę w komórce (Martí, 2016). Mutacja ekspansji powtórzeń CAG ma charakter dynamiczny – im więcej powtórzeń tym większa szansa dalszego zwiększenia liczby

powtórzeń, zarówno podczas gametogenezy (preferencyjnie spermatogenezy), jak i w komórkach somatycznych. Wydłużenie traktu poliQ w kolejnych pokoleniach rodzin dotkniętych chorobami poliQ może prowadzić do antycypacji choroby czyli coraz wcześniejszego jej wystąpienia oraz jej intensywniejszego przebiegu (McMurray, 2010). W przypadku HD istotny wpływ na wystąpienie i przebieg choroby mają również czynniki genetyczne (geny modyfikujące), epigenetyczne i środowiskowe (Bates i in., 2015; Genetic Modifiers of Huntington's Disease (GeM-HD) Consortium, 2015; Moss i in., 2017). Tak więc pomimo zdefiniowanych mutacji wywołujących choroby poliQ ich etiologia jest skomplikowana.

W HD wydłużeniu ulegają powtórzenia CAG w egzonie 1 położonego na chromosomie 4 genu *HTT*, którego produktem jest białko huntingtyna (The Huntington's Disease Collaborative Research Group, 1993). HD rozwija się, gdy liczba powtórzeń przekroczy ok. 36-40 (Bates i in., 2015). Objawiająca się przed 21. rokiem życia forma młodzieńcza tej choroby występuje przy ponad 60 powtórzeniach (Quigley, 2017; Squitieri i in., 2006). Huntingtyna jest niezwykle interesującym, wielofunkcyjnym białkiem, które mimo intensywnych badań nadal w dużej mierze pozostaje nie w pełni poznane (Saudou i Humbert, 2016). Jest to duże białko o masie 230 kDa składające się z 3142 aminokwasów, które powszechnie występuje w organizmie, lokalizuje w różnych kompartmentach komórki i bierze udział w wielu procesach komórkowych, m.in. regulacji transkrypcji (Zuccato i in., 2001, 2003), transporcie komórkowym (Gauthier i in., 2004; Trushina i in., 2004) i synaptycznym (Smith i in., 2005), orientacji mitotycznego wrzeciona podziałowego (Godin i in., 2010) i adhezji komórek (Lo Sardo i in., 2012), oraz chroni przed apoptozą (Rigamonti i in., 2000; Zhang i in., 2006). Mnogość procesów komórkowych, w które uwikłana jest huntingtyna czyni patogenezę HD niezwykle złożonym procesem.

Dzięki zdefiniowanej w przypadku każdej z chorób poliQ mutacji przyczynowej możliwe jest tworzenie precyzyjnych genetycznie modeli komórkowych oraz zwierzęcych tych chorób. Dodatkową zaletą specyficznej mutacji dynamicznej jaką jest ekspansja powtórzeń CAG, jest możliwość modulacji siły fenotypu poprzez manipulację długością ciągu CAG. Pod względem genetycznym modele możemy podzielić na modele transgeniczne, w których wprowadzony transgen integruje z genomem biorcy w sposób losowy oraz modele typu knock-in, w których transgen projektuje się tak aby integrował w pożądanym locus, najczęściej genu wywołującego daną chorobę. Poprzez dobór odpowiedniego promotora i ewentualne wykorzystanie sekwencji regulujących można regulować siłę, miejsce (typ komórek) oraz czas (tzw. modele warunkowe) ekspresji transgenu.

Modele zwierzęce chorób poliQ oraz podejścia terapeutyczne z ich użyciem zostały szerzej opisane w stworzonych w naszym zespole dwóch pracach przeglądowych (Figiel i in., 2012; Switonski i in., 2012) oraz internetowej bazie danych (Szlachcic i in., 2015). Badania z wykorzystaniem licznych modeli zwierzęcych chorób poliQ znacznie przyczyniły się do zrozumienia tych chorób oraz ogólnych mechanizmów neurodegeneracji. Dzięki nim możliwe było również zaproponowanie szeregu podejść terapeutycznych testowanych później w badaniach klinicznych.

1.2 Modele komórkowe choroby Huntingtona

Do badań nad HD w modelach komórkowych tradycyjnie wykorzystywano unieśmiertelnione linie takie jak HEK293T (ludzkie embrionalne komórki nerki), nowotworowe linie guza chromochłonnego PC12 lub neuroblastomy SK-N-SH, w których wywoływano krótkotrwałą, stabilną, bądź indukowalną ekspresję huntingtyny, najczęściej jej N-końcowych fragmentów, z wydłużonym ciągiem

poliglutaminowym. Wyprowadzano również mysie i szczurze pierwotne prekursory neuronalne, które można było przekształcić w unieśmiertelnione linie, bądź neurony (Trettel i in., 2000). Pozyskiwanie komórek bezpośrednio od pacjentów długo ograniczało się do fibroblastów bądź komórek krwi. Modele neuronalne HD wykazują złożoną patologię, na którą składa się akumulacja zmutowanej huntingtyny i wynikające z niej zaburzenia w metabolizmie komórki. Tradycyjne modele niezmiernie przyczyniły się do zrozumienia funkcji normalnej i zmutowanej huntingtyny, jednakże żaden z nich nie jest w stanie w pełni odtworzyć procesu patogenezy HD zachodzącej w naturalnie dotkniętych chorobą komórkach pacjentów (Kaye i Finkbeiner, 2013).

1.2.1 Modele pluripotencjalne

Nową jakość w badaniach nad patogenezą HD w warunkach *in vitro* przyniosła technologia wyprowadzania i hodowli komórek pluripotencjalnych. Pluripotencja to zdolność komórek do różnicowania w dowolną wyspecjalizowaną komórkę organizmu pochodzącą z jednego z trzech listków zarodkowych tworzących organizm. Hodowane *in vitro* komórki pluripotencjalne, takie jak embrionalne komórki macierzyste (komórki ES, ang. embryonic stem cells), są odpowiednikiem embrionalnych komórek węzła zarodkowego (ICM, ang. inner cell mass) i wywodzącego się z niego epiblastu (Smith, 2017). W odpowiednich warunkach kultury *in vitro* komórki pluripotencjalne dzięki zdolności samoodnawiania oraz aktywnym mechanizmom blokującym różnicowanie mogą być teoretycznie hodowane w nieskończoność z zachowaniem normalnego kariotypu (De Los Angeles i in., 2015). W warunkach *in vitro* komórki pluripotencjalne można zróżnicować w pożądany dla specyfiki badań typ komórek, np. w przypadku HD w średnie neurony kolczyste. Możliwości wydają się ograniczone jedynie dostępnością odpowiednich protokołów różnicowania a niezwykle szybki postęp technologiczny nieustannie te granice przesuwa. Intensywnie rozwijają się technologie organoidów czyli miniorganów tworzonych z komórek pluripotencjalnych różnicowanych w hodowli 3D. Organoidy umożliwiają lepsze odwzorowanie rozwoju i różnorodności komórkowej organów *in vivo* w warunkach hodowli 3D *in vitro* (Kelava i Lancaster, 2016). Przykładem są np. organoidy siatkówki (Nakano i in., 2012), minimózgi (ang. cerebral organoids) (Lancaster i in., 2013) czy minijelita (ang. miniguts) (Sato i Clevers, 2013).

1.2.2 Technologia iPSC

Jedynym źródłem tradycyjnych ludzkich komórek ES są przedimplantacyjne zarodki, a ich pozyskanie wiąże się z ze zniszczeniem zarodka, co utrudnia ich tworzenie ze względów na kontrowersje etyczne. Przełomowe odkrycie w 2006 roku technologii indukowanych pluripotencjalnych komórek macierzystych (iPSC, ang. induced pluripotent stem cells) przez zespół prof. Yamanaki (Takahashi i Yamanaka, 2006) umożliwiło pozyskiwanie podobnych do komórek ES komórek pluripotencjalnych bezpośrednio z somatycznych komórek pobranych od pacjentów w dowolnym wieku. Proces przeprogramowania dorosłych, wyspecjalizowanych komórek do stanu pluripotencji jest tradycyjnie wywoływany przez wymuszenie w tych komórkach ekspresji białkowych czynników transkrypcyjnych charakterystycznych dla komórek ES, takich jak OCT3/4, KLF4 czy SOX2. Obecność tych białek wywołuje kaskadę zdarzeń prowadzącą do resetu epigenetycznego genomu komórki i uruchomienia endogennych szlaków utrzymania, w odpowiednich warunkach hodowlanych, stanu pluripotencji (Smith i in., 2016). Opracowano wiele metod uzyskiwania iPSC przy użyciu różnych systemów

wprowadzania do komórek czynników przeprogramowujących oraz różnych zestawów tych czynników, wspomaganych lub zastępowanych związkami niskocząsteczkowymi (Li i Belmonte, 2016). W trakcie dekady gwałtownego rozwoju technologia iPSC znacznie zwiększyła znaczenie komórek pluripotencjalnych w badaniach podstawowych, takich jak modelowanie chorób czy badania nad rozwojem, i stosowanych, wpływając na postępy medycyny spersonalizowanej poprzez stworzenie nowych możliwości testowania leków czy terapii komórkowej. Zostało to docenione w 2012 roku przyznaniem nagrody Nobla w dziedzinie Medycyny lub Fizjologii dla Johna Gurdona. i Shinyi Yamanaki. Modele pluripotencjalne wprowadziły również nową jakość w badaniach nad HD, o czym będę pisał w dalszych rozdziałach.

1.3 Podejścia terapeutyczne w leczeniu HD

Większość stosowanych dotąd podejść terapeutycznych ukierunkowana była na łagodzenie widocznych objawów klinicznych choroby lub któregoś z wielu molekularnych efektów obecności zmutowanego białka, co nie przyniosło spodziewanych rezultatów. W przypadku leczenia objawowego stosuje się przykładowo leki uspokajające, psychotropowe, prokognitywne oraz neuroprotekcyjne, które nie zapobiegają progresji choroby. Próby zapobiegania efektom toksyczności białka obejmują przykładowo podejścia zmniejszające ekscytotoksyczność, chroniące mitochondria, stymulujące pracę systemów degradacji białek, zmniejszające produkcję toksycznych fragmentów huntingtyny i hamujące ich agregację, przeciwapoptotyczne oraz regulujące ekspresję genów (szczególnie stymulujące ekspresję białka BDNF) (Bates i in., 2015; Wyant i in., 2017). Rozpatruje się również terapie skierowane na tkanki i narządy poza ośrodkowym układem nerwowym (Carroll i in., 2015).

1.3.1 Podejścia celujące w ekspresję huntingtyny

Wielowątkowość patogenezy HD wywołanej obecnością zmutowanego allelu *HTT* sugeruje, że celowanie w jeden lub nawet kilka aspektów tego procesu może być niewystarczające do stworzenia skutecznej terapii. Dlatego racjonalne wydaje się dotarcie do źródła HD, czyli zablokowanie ekspresji zmutowanej huntingtyny. Badania z wykorzystaniem mysiego modelu o warunkowej, chemicznie wyłączalnej, ekspresji zmutowanej huntingtyny wykazały, że usunięcie toksycznego białka u myszy objawowej znacząco poprawia jej fenotyp (Yamamoto i in., 2000). Różne podejścia do leczenia przyczynowego metodami, które można by zastosować u pacjentów, z pierwszymi próbami opisanymi już w 1997 roku (Haque i Isacson, 1997), przyniosły obiecujące rezultaty w badaniach *in vivo* w mysich i szczurzych modelach, co szerzej opisano w pracy przeglądowej (Keiser i in., 2016). W zebranych w niej oraz w nowszych badaniach (Datson i in., 2017; Didiot i in., 2016; Miniarikova i in., 2016, 2017) do obniżenia ekspresji zmutowanego białka wykorzystywano oligonukleotydy antysensowe (ASO) lub reagenty interferencji RNA w formie siRNA lub w wektorach wirusowych jako shRNA, w sztucznym bądź bazującym na endogennym miRNA (tzw. shmiR (Fiszer i Krzyzosiak, 2014)) kadłubie. Badania te potwierdzają, że redukcja ekspresji zmutowanego białka jest w stanie zredukować fenotypy motoryczne i neuropatologiczne oraz wydłużyć długość życia, przynajmniej u gryzoni. U myszy skuteczne było również podejście z wykorzystaniem nukleazy typu palca cynkowego specyficznie blokującej transkrypcję zmutowanego allelu HTT poprzez wiązanie

ciągu CAG (Agustín-Pavón i in., 2016; Garriga-Canut i in., 2012).

W części spośród powyższych podejść terapeutycznych wykorzystano reagenty alleloselektywne mające wyciszać ekspresję wyłącznie zmutowanego allelu huntingtyny, poprzez specyficzne nakierowanie na wydłużony ciąg powtórzeń lub różnicujące polimorfizmy jednonukleotydowe (SNP). Istotnym zagadnieniem dla terapii wyciszającej jest bowiem pytanie do jakiego poziomu i jak długo ekspresja normalnego allelu *HTT* może zostać obniżona, jako efekt uboczny wyciszenia ekspresji allelu zmutowanego, bez wywołania negatywnych skutków w organizmie (Fiszer i Krzyzosiak, 2014). Badania u gryzoni wskazują, że czasowe obniżenie (Drouet i in., 2009; Kordasiewicz i in., 2012; McBride i in., 2008) bądź całkowite wyłączenie (Wang i in., 2016) ekspresji huntingtyny w mózgu dorosłych myszy nie wywołuje ewidentnych negatywnych skutków. Sugeruje to, że mimo znanych funkcji huntingtyny w dorosłych neuronach, nie jest ona w nich niezbędna (Liu i Zeitlin, 2017). Również przedkliniczne badania efektów częściowego obniżenia ekspresji normalnej huntingtyny w mózgu dorosłych rezusów nie wykazały oczywistych negatywnych efektów motorycznych i neurodegeneracyjnych, jednak badania te ograniczone były do kilku tygodni lub miesięcy po dostarczeniu reagentów (Grondin i in., 2012; McBride i in., 2011). Istotnym zagadnieniem są również potencjalnie toksyczne efekty niespecyficzne, niezależne od sekwencji nukleotydowej, reagentów interferencji RNA (Olejniczak i in., 2016). W badaniach *in vivo* u myszy HD wykazano, że transfer reagenta ze sztucznego kadłuba shRNA do naturalnego kadłuba miRNA (shmiR) znacznie redukuje efekty neurotoksyczne i neurozapalne wywołane przez reagent dostarczony w formie wektorów wirusowych AAV (Boudreau i in., 2009; McBride i in., 2008). Innymi problemami związanymi z wyciszaniem huntingtyny *in vivo* jest sposób dostarczania i dawki reagentów oraz trwałość uzyskanego efektu terapeutycznego (Keiser i in., 2016). Pozytywne wyniki u krótko żyjącej myszy o małym mózgu nie muszą się przekładać na rezultaty uzyskane u większych zwierząt, np. rezusów, oraz pacjentów. Mimo że wykazano możliwość obniżenia ekspresji huntingtyny w mózgu zdrowego rezusa reagentami ASO (Kordasiewicz i in., 2012), siRNA (Grondin i in., 2015, 2012) lub shmiR (McBride i in., 2011), nie opisano jak dotąd efektów terapeutycznych u rezusów HD. Efektem tych badań są pierwsze próby kliniczne z zastosowaniem reagentów ASO (obecnie w fazie badań I/II, Ionis Pharmaceuticals), w których celem jest ekspresja zmutowanej huntingtyny.

Oprócz potencjalnie nietrwałych podejść wykorzystujących blokowanie transkrypcji lub translacji w terapii HD rozważane są również metody polegające na bezpośredniej ingerencji w sekwencję DNA zmutowanego allelu HTT. Przykładem jest jego usunięcie na drodze rekombinacji homologicznej (An i in., 2012). Jednak ze względu na niską wydajność metoda ta nie może być wykorzystana w warunkach *in vivo*, a jedynie w połączeniu z terapią komórkową. Rozwijane są również podejścia wykorzystujące systemy edycji genomu z użyciem technologii ZFP, TALEN czy CRISPR. Technologii CRISPR/Cas9 z powodzeniem użyto ostatnio do usunięcia zmutowanej huntingtyny w ludzkich iPSC (Shin i in., 2016; Xu i in., 2017) oraz *in vivo* w prążkowiu mysiego modelu (Merienne i in., 2017; Monteys i in., 2017; Yang i in., 2017). Możliwość całkowitego usunięcia zmutowanego allelu jest obiecująca ale technologie edycji genomów wymagają szczególnej ostrożności w kontekście możliwości wystąpienia niepożądanych mutacji w niespecyficznych loci (Fellmann i in., 2017).

1.3.2 Terapia komórkowa

Ponieważ śmierć neuronów jest podstawową cechą HD, obiecującym podejściem terapeutycznym może być regeneracyjna terapia komórkowa. Zachęcające wyniki uzyskano w mysich i szczurzych genetycznych modelach HD, w których stosowano różne typy zdrowych komórek, m.in.

neuronalne komórki macierzyste (El-Akabawy i in., 2012; Yang i Yu, 2009), neurony prążkowia uzyskane poprzez różnicowanie komórek ES (Delli Carri i in., 2013; Shin i in., 2012) oraz komórki nieneuronalne – mezenchymalne komórki macierzyste (Dey i in., 2010; Fink i in., 2013; Krystkowiak i in., 2007; Lin i in., 2011; Rossignol i in., 2015, 2014), tłuszczowe komórki macierzyste (Im i in., 2010; Lee i in., 2009), jak również przeszczep szpiku kostnego (Kwan i in., 2012). Prowadzono również ewaluację kliniczną, często wieloletnią, transplantacji z wykorzystaniem ludzkich płodowych tkanek neuronalnych (Bachoud-Lévi i Perrier, 2014; Gallina i in., 2014), lub ksenograftów z użyciem tkanek świni (Fink i in., 2000), jednakże wyniki uzyskane w tych próbach były niejednoznaczne (Bachoud-Lévi i Perrier, 2014; Fink i in., 2015). Długotrwałe badania wykazały, że przeszczepy, które nie uległy odrzutowi, mogą przetrwać przez wiele lat w mózgu pacjentów, mimo że ulegają stopniowej degeneracji (Cicchetti i in., 2011), co może częściowo tłumaczyć nietrwałość pozytywnych efektów terapii obserwowanych u niektórych pacjentów. Powyższe badania nie tylko zasugerowały potrzebę znalezienia lepszego źródła bezpiecznych komórek terapeutycznych ale również konieczność zwiększenia wiedzy podstawowej na temat mechanizmów molekularnych wpływających na skuteczność terapii komórkowej mózgu (Dunnett i Rosser, 2014; Freeman i in., 2011; Sackett i in., 2016).

Aby zapewnić przetrwanie komórek w mózgu i zapobiec odrzuceniu przeszczepu, stosuje się immunosupresję, z którą jednak związane są liczne potencjalne komplikacje mogące wpływać na jakość i długość życia pacjentów (Sackett i in., 2016). Alternatywnym rozwiązaniem są neuronalne przeszczepy autologiczne, które są obecnie możliwe dzięki technologii iPSC. W większości badań wykazano, że przeszczepy z autologicznych iPSC u myszy i naczelnych nie wywołują istotnej odpowiedzi immunologicznej i raczej nie ulegają odrzuceniu (Almeida i in., 2014; Araki i in., 2013; Emborg i in., 2013; Guha i in., 2013; Morizane i in., 2013). Wstrzyknięcie komórek pluripotencjalnych oczywiście skutkowałoby tworzeniem potworniaków, dlatego niezbędne jest ich zróżnicowanie do pożądanego typu komórek lub ich prekursorów, co w przypadku neuronalnych komórek macierzystych dodatkowo obniża ryzyko odpowiedzi immunologicznej ze strony mózgu (Gao i in., 2016; Sackett i in., 2016). W przypadku choroby Huntingtona mogą to być neuronalne komórki macierzyste, prekursory neuronalne bądź glejowe (Benraiss i in., 2016; Mattis i in., 2015).

Autologiczne komórki przeszczepione do mózgu pacjenta wcześniej czy później rozwiną fenotyp chorobowy związany z obecnością w nich zmutowanej huntingtyny. Dlatego poprzedzające transplantację „wyleczenie" ich w warunkach *ex vivo* poprzez trwałe wyciszenie ekspresji zmutowanego allelu wydaje się być niezbędne. Jednak taka kombinowana terapia z dużym prawdopodobieństwem nadal będzie niewystarczająca. W HD jednym z mechanizmów patogenezy jest komórkowo nieautonomiczna propagacja choroby. Komórki przeszczepu trafią do chorego środowiska biorcy, w wyniku czego będą pod wpływem toksycznego mikrośrodowiska (Cicchetti i in., 2011). Dodatkową komplikacją jest powszechność zjawiska transferu różnych typów RNA, w tym mRNA i miRNA, oraz białek pomiędzy komórkami, również w neuronach i gleju, będącego narzędziem komunikacji między komórkami (Frühbeis i in., 2013; Hu i in., 2012; Vlassov i in., 2012; Wang i in., 2010). W warunkach hodowli *in vitro* wykazano, że mRNA zmutowanej huntingtyny może być transportowane pomiędzy komórkami w egzosomach (Zhang i in., 2016). Najnowsze doniesienia wskazują, że także białko zmutowanej huntingtyny jest transportowane pomiędzy komórkami, również z mózgu pacjenta do przeszczepu, w sposób przypominający choroby prionowe (Cicchetti i in., 2014; Jeon i in., 2016; Pecho-Vrieseling i in., 2014; Tan i in., 2015). Mechanizm propagacji prionopodobnej jest pomocny w wyjaśnieniu sekwencyjnego rozprzestrzeniania się choroby z prążkowia najpierw do regionów mózgu sąsiadujących a w dalszej kolejności bardziej oddalonych (Pecho-Vrieseling, prezentacja na

konferencji FENS 2016). Ponadto zaobserwowano zjawisko fuzji komórek transplantu z dorosłymi komórkami biorcy (Brilli i in., 2013). Wszystkie wymienione powyżej czynniki – mikrośrodowisko, transport zmutowanego mRNA i białka oraz fuzja komórek – mogą wywoływać w komórkach przeszczepu fenotyp HD. Z drugiej strony, wykorzystując te naturalne zdolności egzosomów do transportu miRNA pomiędzy komórkami, wykazano że w warunkach *in vitro* możliwe jest zmniejszenie ilości zmutowanej huntingtyny w komórkach hodowanych w kokulturze z komórkami o stabilnej ekspresji reagentów typu shRNA celowanych w huntingtynę (Olson, Kambal i in., 2012). Terapia kombinowana może również wykorzystywać dodatkowe czynniki, takie jak nadekspresja neurotrofin, które pełniłyby funkcje ochronne względem komórek transplantu, i neuronów w mózgu (Deng i in., 2016; Maucksch i in., 2013; Olson, Pollock i in., 2012). Perspektywa zastosowania autologicznej terapii komórkowej w HD daje pacjentom nadzieję. Jednakże zaprojektowanie skutecznej terapii, w obliczu złożoności interakcji komórek przeszczepu ze środowiskiem mózgu, wymaga lepszego poznania mechanizmów w nie zaangażowanych.

2 Cel pracy

Celem badań prowadzonych w ramach pracy doktorskiej było stworzenie i scharakteryzowanie linii iPSC z mysiego modelu choroby Huntingtona oraz stabilne wyciszenie w tych komórkach ekspresji zmutowanego białka przy użyciu narzędzi, które można by wykorzystać w komórkach pochodzących od pacjentów. Uzyskane modele iPSC będą w przyszłości wykorzystane w terapii *in vivo* u myszy oraz w badaniach nad mechanizmami terapii komórkowej w modelach terapii *in vitro*. Takie badania mogą przyczynić się do opracowania bardziej skutecznej terapii komórkowej choroby Huntingtona i innych chorób neurodegeneracyjnych.

Kolejnym celem pracy doktorskiej, realizowanym w ramach charakterystyki uzyskanych linii iPSC, było zbadanie czy już w stadium pluripotencji, odpowiadającemu wczesnym stadiom rozwoju organizmu, występują zaburzenia molekularne wywołane obecnością zmutowanej huntingtyny. Zidentyfikowane zmiany mogłyby wytyczyć nowe kierunki badań nad wczesną patogenezą HD oraz stać się wczesnymi markerami oceny efektywności terapii komórkowej lub leków testowanych w liniach iPSC od pacjentów.

3 Streszczenie prac i ich interpretacja na tle piśmiennictwa przedmiotu

3.1 Wstęp

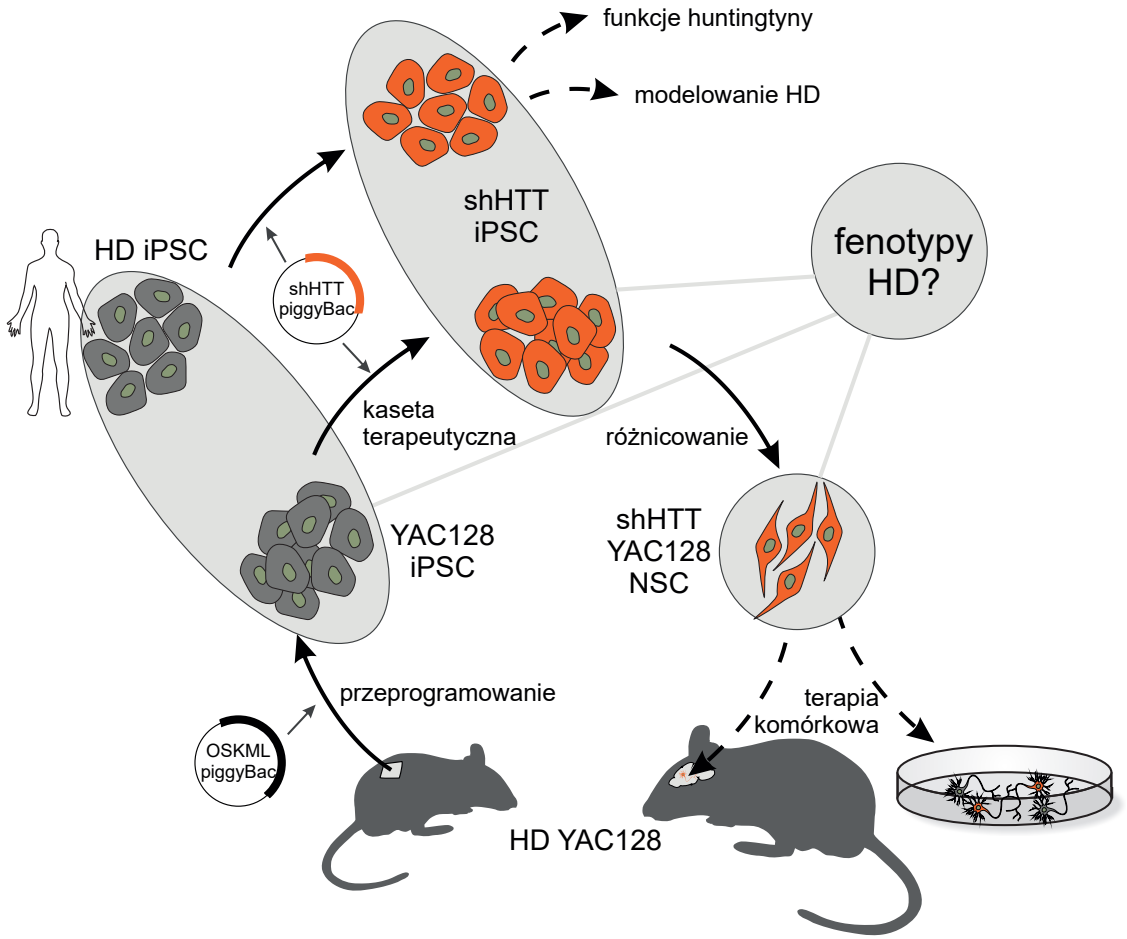
Choroby neurodegeneracyjne, będące najczęściej chorobami wieku dorosłego, w związku z wydłużającą się średnią długością życia stają się coraz większym problemem i obciążeniem dla nowoczesnych społeczeństw. Niestety skomplikowana etiologia tych chorób jak dotąd uniemożliwiła opracowanie skutecznych terapii. Niniejsza praca doktorska jest odpowiedzią na potrzeby stworzenia nowych modeli komórkowych do badania patogenezy i poszukiwania terapii dla tych chorób. HD i inne choroby poliglutaminowe są szczególnie ciekawym przykładem chorób neurodegeneracyjnych, ponieważ ich etiologia, mutacja w specyficznym genie, jest ściśle zdefiniowana a jednocześnie spektrum zaburzeń w nich obserwowanych w wielu aspektach przypomina choroby o bardziej skomplikowanym podłożu, takie jak choroba Parkinsona czy Alzheimera. Dlatego HD może być traktowana jako model chorób neurodegeneracyjnych, którego właściwości mogą być częściowo ekstrapolowane na inne choroby tego typu.

Potencjalnym podejściem terapeutycznym w HD, które z jednej strony mogłoby powstrzymać dalszy rozwój choroby a z drugiej pozwoliłoby również zregenerować już uszkodzone rejony mózgu, jest terapia komórkowa. Technologia iPSC, dzięki możliwości wyprowadzenia pluripotencjalnych iPSC bezpośrednio od pacjentów HD, daje nadzieję na stworzenie spersonalizowanej autologicznej terapii, która znacznie zmniejszyłaby ryzyko odrzutu przeszczepu. Jednakże komórki iPSC pochodzące od pacjentów produkują zmutowaną huntingtynę, co może wywołać zaburzenie ich dojrzewania i integracji w mózgu biorcy oraz przyspieszyć ich późniejszą degenerację. Zasadne wydaje się więc zastosowanie w iPSC terapii genowej, która trwale wyciszyłaby w nich ekspresję zmutowanego białka i jednocześnie chroniła je przed toksycznym środowiskiem mózgu biorcy oraz wydzielała czynniki terapeutyczne.

Wysoko rozwinięty poziom technologii mysich komórek pluripotencjalnych, duża liczba dostępnych mysich modeli HD, jak również ekonomiczne i praktyczne aspekty badań z wykorzystaniem myszy, czynią ten gatunek odpowiednim modelem dla wstępnych badań nad terapią komórkową. Mysim modelem, który uznaliśmy za najlepszy do naszych badań, mających łączyć terapię genową (reagentami uniwersalnymi również dla ludzkich komórek) z terapią komórkową, jest transgeniczna mysz YAC128 (Slow i in., 2003). W modelu tym znajduje się ok. 4 kopii genomowego regionu ludzkiego genu *HTT* o długości ok. 308 tys. pz (sam gen *HTT* ma 170 tys. pz), z których zachodzi ekspresja huntingtyny z ciągiem 128 glutamin. Poziom ekspresji zmutowanego białka w prążkowiu dorosłej myszy jest na poziomie około 0.63x ekspresji mysiej huntingtyny pochodzącej z w dwóch endogennych alleli *Htt* (Pouladi i in., 2012). Istotną kwestią w wyborze mysiego modelu do terapii jest również możliwość oceny zmian w fenotypach: motorycznym, behawioralnym, neurologicznym i innych. Typowy fenotyp motoryczny pojawia się w modelu YAC128 u osobników młodszych niż pół roku. Dodatkowo u tych myszy, podobnie jak u pacjentów, można zaobserwować dwufazowy fenotyp aktywności lokomotorycznej (u młodych myszy hiper- a u starszych hipoaktywność) oraz śmierć neuronów prążkowia. Te właściwości spowodowały, że myszy YAC128 były najczęściej wykorzystywane w badaniach nad terapią HD wśród modeli z ekspresją pełnej długości zmutowanej huntingtyny (Switonski i in., 2012; Szlachcic i in., 2015).

W kolejnych rozdziałach przedstawię syntezę artykułów wchodzących w skład niniejszej roz-

prawy doktorskiej. W pierwszej kolejności omówię pracę, w której opisano wyprowadzenie i charakterystykę komórek iPSC z modelu YAC128 (Szlachcic i in., 2015), a następnie artykuł opisujący trwałe wyciszenie ekspresji huntingtyny z wykorzystaniem reagentów typu shRNA w mysich i ludzkich komórkach iPSC (Szlachcic i in., 2017). Na końcu przedstawię artykuł przeglądowy zwracający uwagę na neurorozwojowe aspekty patogenezy HD oraz możliwości ich badania z wykorzystaniem technologii iPSC (Wiatr i in., 2017). W tekście będę odwoływał się zarówno do rycin umieszczonych bezpośrednio w niniejszej rozprawie (Ryc.) lub rycin w omawianych artykułach (Fig.). Schemat stworzonego w niniejszej pracy modelu do eksperymentalnej terapii komórkowej HD w systemie mysim przedstawia Ryc. 1.



Ryc. 1: Ogólny schemat badań. Fibroblasty skóry z mysiego modelu HD YAC128 przeprogramowałem do komórek iPSC z wykorzystaniem technologii transpozonu piggyBac. Następnie do komórek YAC128-iPSC i komórek HD iPSC od pacjentów i osób zdrowych wprowadziłem z użyciem transpozonu piggyBac reagenty typu shRNA, w celu wyciszenia ekspresji patogennego białka huntingtyny (linie shHTT). Linie shHTT YAC128-iPSC po zróżnicowaniu do neuronalnych komórek macierzystych (NSC) będą w przyszłych badań użyte w modelach terapii *in vivo* i *in vitro*. Ludzkie komórki z reagentami shRNA mogą być użyte do badań nad funkcjami huntingtyny i w modelowaniu HD. Celem projektu była również identyfikacja fenotypów w komórkach iPSC od myszy i pacjentów oraz ocena efektów wyciszenia huntingtyny na zidentyfikowane fenotypy. Linie przerywane oznaczają planowane przyszłe badania wykorzystujące narzędzia stworzone w ramach niniejszej rozprawy.

3.2 Huntington disease iPSCs show early molecular changes in intracellular signaling, the expression of oxidative stress proteins and the p53 pathway

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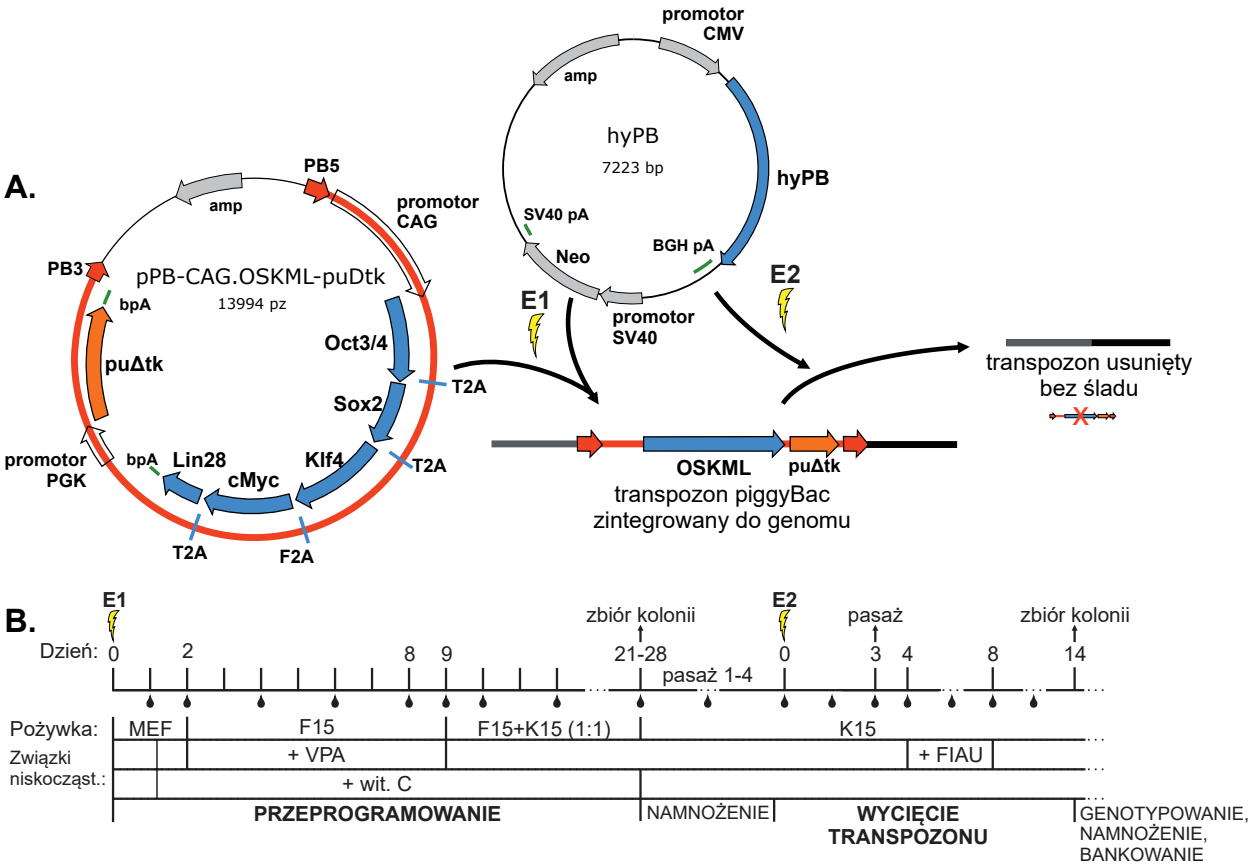
Pierwszym etapem badań w projekcie stworzenia modelu do eksperymentalnej terapii komórkowej HD było wyprowadzenie i charakterystyka iPSC z mysiego modelu HD, YAC128. Następnie, wykorzystując wyprowadzony model YAC128-iPSC oraz komórki iPSC pochodzące od pacjentów HD, chcieliśmy odpowiedzieć na pytanie czy w stadium pluripotencji występują zaburzenia ekspresji genów związane z HD, które mogłyby stanowić biomarkery w podejściach terapeutycznych z wykorzystaniem tych komórek.

Bezpieczeństwo pacjentów jest jednym z najważniejszych czynników, które należy wziąć pod uwagę przy projektowaniu podejść wykorzystujących technologię iPSC do medycyny regeneracyjnej, i ściśle wiąże się ono z metodą ich uzyskania. Przez długi czas najczęściej stosowaną metodą uzyskania komórek iPSC było dostarczenie DNA kodującego czynniki przeprogramowujące za pomocą wektorów retro- lub lentiwirusowych, co skutkuje wbudowaniem egzogennego DNA do losowych loci w genomie komórki. Trwała obecność tych egzogennych czynników, w tym onkogenów takich jak cMyc, stwarza możliwość ich ekspresji w komórce po procesie przeprogramowania, zwiększając ryzyko nowotworzenia. Ponadto losowa natura takiej integracji stwarza szansę nabycia potencjalnie niebezpiecznych mutacji. Komórki iPSC uzyskane metodą trwale modyfikującą genom nie zostałyby dopuszczone do terapii u pacjentów (Shi i in., 2016). Biorąc pod uwagę powyższe okoliczności linie iPSC z fibroblastów pobranych od dorosłych myszy wyprowadziłem metodą wykorzystującą transpozon piggyBac jako nośnik cDNA czynników transkrypcyjnych Oct4, Sox2, Klf4, Myc i Lin28 (Ryc. 2A) (Yusa i in., 2009, 2011). Transpozon ten integruje do genomu biorcy w wyniku aktywności transpozazy piggyBac. Po zakończonym procesie przeprogramowania transpozon może zostać usunięty w całości i bez pozostawienia śladu z pomocą ponownej ekspozycji komórek na transpozazę (Ding i in., 2005).

W celu poprawienia wydajności przeprogramowania użyłem zmodyfikowanej transpozazy o zwiększonej aktywności (hyPB) (Yusa i in., 2011) oraz dodatkowo zbadałem wpływ szeregu związków niskocząsteczkowych na proces przeprogramowania. Ostatecznie usprawniłem proces poprzez zastosowanie dwóch związków wspomagających epigenetyczny reset genomu – witaminy C, która wzmacnia aktywność demetylazy histonów (Esteban i in., 2010; Hore i in., 2016), i kwasu walproinowego (VPA), inhibitora deacetylaz histonów (Huangfu i in., 2008). Wydajność zoptymalizowanego protokołu w przeprogramowaniu komórek testowych, którymi były mysie embrionalne fibroblasty (MEF), wynosiła około 160 uzyskanych kolonii na milion elektroporowanych komórek. Dla porównania wydajność w transgenicznych komórkach MEF z wbudowaną kasetą przeprogramowującą o ekspresji kontrolowanej przez doksycyklinę była ponad 10-krotnie wyższa. Docelowy protokół dostosowany do fibroblastów pobranych od dorosłej myszy (Ryc. 2B, Fig. S1) umożliwił uzyskanie linii iPSC z częstością około 7 kolonii na milion komórek. Większa wydajność przeprogramowania embrionalnych fibroblastów w porównaniu z dorosłymi fibroblastami była efektem różnic w biologii tych komórek, które mają znaczny wpływ na przebieg tego procesu (Brouwer i in.,

2016). Dodatkową przyczyną różnic w wydajności była niższa efektywność elektroporacji dorosłych fibroblastów w porównaniu do komórek MEF. Niemniej jednak wydajność przeprogramowania była wystarczająca do uzyskania odpowiedniej dla dalszych badań liczby linii iPSC.



Ryc. 2: Strategia przeprogramowania mysich dorosłych fibroblastów do komórek iPSC. *(A)* W użytym systemie piggyBac w plazmidzie pPB-CAG.OSKML-puδtk umieszczony jest transpozon (czerwona linia), w obrębie którego znajdują się dwie kasety ekspresyjne: pięciu czynników przeprogramowujących (niebieskie), które tworzą pojedynczą jednostkę transkrypcyjną, oraz genu fuzyjnego puδtk, umożliwiającego selekcję pozytywną i negatywną. W trakcie translacji powstający peptyd jest cięty na indywidualne białka dzięki obecności sekwencji samotnących peptydów T2A i F2A. Dodatkowy plazmid, hyPB, koduje transpozazę. Transpozon na skutek koelektroporacji (E1) plazmidów pPB i hyPB był wbudowywany do genomu fibroblastów, wywołując proces przeprogramowania. Po uzyskaniu linii iPSC był bez pozostawienia śladu wycinany z genomu, na skutek ponownej elektroporacji (E2) wektorem hyPB i selekcji FIAU. *(B)* Chronologia procesu tworzenia linii komórek iPSC.

System piggyBac funkcjonował z najwyższą wydajnością w porównaniu z innymi testowanymi w naszym zespole metodami przeprogramowania, które nie pozostawiają czynników przeprogramowujących w genomie. Były to metody czasowej ekspresji czynników z plazmidów (Okita i in., 2008), stałej ekspresji czynników z ich usunięciem przy użyciu rekombinazy Cre (Kaji i in., 2009), system minicircle (Jia i in., 2010) oraz system episomalny (Yu i in., 2009). Dla ludzkich komórek metodą powszechnie stosowaną stał się system episomalny, jednak w przypadku mysich komórek nie funkcjonował. Prawdopodobną przyczyną jest wykorzystanie w tym systemie wektorów opartych na wirusie Epsteina-Barra, które nie replikują w mysich komórkach (Hu, 2014). W trakcie trwania projektu zespół prof. Hongkui Denga uzyskał mysie komórki iPSC przy użyciu wyłącznie związków niskocząsteczkowych (Hou i in., 2013; Zhao i in., 2015). Z pewnością metoda ta stanie się w przyszłości powszechnym sposobem przeprogramowywania, szczególnie w przypadku komórek do zastosowań klinicznych. Jak dotąd jednak nie otrzymano ludzkich iPSC przy użyciu wyłącznie

związków niskocząsteczkowych.

Po uzyskaniu linii iPSC transpozon z kasetą przeprogramowując był usuwany z genomu poprzez ponowną elektroporację iPSC plazmidem kodujacym transpozazę hyPB i negatywną selekcję z zastosowaniem analogu nukleozydów fialurydyny (FIAU), który jest toksyczny dla komórek, w których nie nastąpiło wycięcie transpozonu. Taka negatywna selekcja jest możliwa dzięki obecności w systemie piggyBac białka fuzyjnego puroδtk, które składa się z białka oporności na puromycynę oraz funkcjonalnego fragmentu kinazy tymidynowej wirusa opryszczki typu 1, dla której FIAU jest substratem (Chen i Bradley, 2000). Fosforylowany przez kinazę tymidynową FIAU jest wbudowywany do DNA blokując jego replikację (Borrelli i in., 1988). Podsumowując po procesie usuwania transpozonu uzyskałem około 50 klonalnych linii iPSC pochodzących od fibroblastów pobranych z dorosłych myszy YAC128 (n=3) i typu dzikiego (n=3). Usunięcie kasety potwierdziłem metodą genotypowania PCR (Fig. S1). Spośród uzyskanych linii do dalszych badań losowo wybrałem 11 linii (YAC128 n=5 i WT n=6), w tym co najmniej jedną z każdej myszy.

W trakcie przeprowadzania komórek somatycznych do stanu pluripotencji oprócz prawidłowo przeprogramowanych pluripotencjalnych komórek można uzyskać nie w pełni przeprogramowane komórki o niskiej jakości. Mimo że morfologia i zdolność do samoodnawiania takich komórek są podobne do komórek dobrej jakości, ich potencjał do różnicowania może być upośledzony. Dlatego niezbędne jest przeprowadzenie charakterystyki jakościowej linii uzyskanych iPSC. Jest to możliwe, ponieważ stan pluripotencji charakteryzuje się specyficznymi właściwościami molekularnymi i funkcjonalnymi (De Los Angeles i in., 2015; Martí i in., 2013). Wyprowadzone przez mnie linie iPSC posiadają podstawowe molekularne cechy pluripotencji takie jak ekspresja alkalicznej fosfatazy (Fig. 1C) i antygenu powierzchniowego SSEA1 (Fig. 1D) oraz przede wszystkim poziomy ekspresje czynników transkrypcyjnych niezbędnych dla utrzymania tego stanu, co analizowałem analizowałem metodami RT-PCR (Fig. 1A), real-time qPCR (Fig. S2), western blot (Fig. S2) oraz immunofluorescencji (Fig. 1D). Ponadto część linii iPS była pozyskana z krzyżówki myszy YAC128 i myszy Oct-eGFP (Lengner i in., 2007), w której ekspresja białka eGFP zachodzi z intronu endogennego białka Oct3/4 tylko w przypadku ekspresji białka Oct3/4, co umożliwiło przyżyciowe monitorowanie ekspresji tego białka (Fig. 1B). Jednakże ocena molekularna, nawet w przypadku zastosowania nowych wysokoprzepustowych analiz transkryptomu (Müller i in., 2011; Panopoulos i in., 2017), proteomu (Baud i in., 2017) czy stanu epigenetycznego komórki (Lenz i in., 2015), nadal nie jest wystarczająca. Na chwilę obecną jedynie przy pomocy testów funkcjonalnych, określających zdolność komórek pluripotencjalnych do różnicowania w komórki wszystkich listków zarodkowych, można tę zdolność w sposób jednoznaczny ocenić (De Los Angeles i in., 2015). Z tego względu przeprowadziłem testy funkcjonalne pluripotencji wyprowadzonych linii iPSC w warunkach różnicowania *in vitro* z wykorzystaniem ciał embrionalnych (ang. embryoid bodies, EB) (Fig. 1E) oraz *in vivo* z użyciem testu formowania potworniaków (ang. teratoma formation assay) (Fig. S3), które potwierdziły pluripotencję uzyskanych komórek iPSC. Jako metodę analityczną w obu przypadkach wykorzystywałem metody mikroskopowe, natomiast możliwe jest obecnie zastosowanie metod analizy ekspresji transkryptów genów biomarkerowych poszczególnych listków zarodkowych (D'Antonio i in., 2017; Tsankov i in., 2015). Podsumowując, wykonane testy molekularne i funkcjonalne potwierdziły obecność w wyprowadzonych liniach charakterystycznych cech pluripotencji, w tym najważniejszą dla ich późniejszych zastosowań w eksperymentalnej terapii HD zdolność do różnicowania w komórki neuroektodermy.

Model komórkowy choroby musi umożliwiać obserwację charakterystycznych dla niej fenotypów. Wydawałoby się, że w przypadku HD, tradycyjnie uważanej za chorobę dojrzałego mózgu,

efekty obecności zmutowanej huntingtyny można będzie obserwować dopiero po zróżnicowaniu iPSC do dojrzałych neuronów. Wskazywałaby na to również niska ekspresja zmutowanej huntingtyny w niezróżnicowanych iPSC w porównaniu do komórek poddanych krótkiemu różnicowaniu w kierunku linii neuronalnych (Fig. 2). Wyższą ekspresję huntingtyny w komórkach zróżnicowanych w stosunku do stanu pluripotencji wykazano również we wcześniejszych badaniach nad mysimi (Feyeux i in., 2012) oraz ludzkimi komórkami ES (Metzler i in., 1999). Jednakże obserwacje z innych modeli pluripotencjalnych, jak również dane z badań nad funkcjami huntingtyny na wczesnych etapach rozwoju oraz z badań dotyczących neurorozwojowych aspektów HD (streszczone przeze mnie w dyskusji nad ostatnim artykułem) sugerują, że subtelne efekty obecności zmutowanej huntingtyny mogą być obserwowane już w stanie pluripotencji. Ponieważ w HD zaburzeniom ulegają ścieżki sygnałowe zaangażowane w procesy rozwojowe, postanowiłem sprawdzić czy ich deregulację można zaobserwować w pluripotencjalnych komórkach YAC128-iPSC.

Porównanie ekspresji białek metodą western blot pomiędzy liniami YAC128-iPSC (n=5) i WT-iPSC n=6) wykazało obniżoną indukcję ścieżki sygnałowej kinaz aktywowanych sygnałami zewnątrzkomórkowymi (ang. extracellular signal-regulated kinase, ERK) w odpowiedzi na aktywujący tę ścieżkę zasadowy czynnik wzrostu fibroblastów (ang. basic fibroblast growth factor, bFGF). Indukcja ścieżki była mierzona poziomem fosforylowanych białek ERK1/2 względem całkowitej ilości tych białek (Fig. 3). Zaobserwowałem również obniżoną aktywność kanonicznej ścieżki sygnałowej Wnt, mierzoną zwiększoną ilością fosforylowanego białka beta-kateniny (β-catenin) oraz ekspresją transkryptu (qPCR) kinazy Gsk3β (Fig. 4), dla której beta-katenina jest substratem. Dodatkowo pomiary ekspresji białek odpowiedzi na stres oksydacyjny wykazały podwyższoną ekspresję białka dysmutazy ponadtlenkowej SOD1 (Fig. 5). Największą zmianę, blisko dwukrotne obniżenie ekspresji, zaobserwowałem w białku p53, które fizycznie oddziałuje z białkami każdej z analizowanych ścieżek sygnałowych i z huntingtyną (Fig. 7, 8). Aby przekonać się czy zmiany obserwowane w liniach iPS z mysiego modelu HD YAC128 mają odzwierciedlenie w ludzkich komórkach iPS od pacjentów HD, postanowiłem wykonać podobne analizy w pozyskanych systemem episomalnym liniach dostępnych w repozytorium amerykańskiego Narodowego Instytutu Chorób Neurologicznych i Udarów (NINDS). Wykorzystałem linie HD z traktem 71 powtórzeń CAG (HD71, dwie linie od jednego pacjenta) oraz z traktem 109 powtórzeń CAG (HD109, dwie linie od jednego pacjenta) i porównałem je do linii kontrolnych od osób zdrowych (WT, dwie linie, każda od innej osoby) (Fig. 6, 8). W analizie western blot zaobserwowałem podobne jak w przypadku mysich komórek zmiany w ścieżkach ERK, Wnt oraz białkach SOD1 i p53, szczególnie w liniach HD109 pochodzących od pacjenta z dziecięcą formą HD. Podobne rezultaty uzyskałem więc w dwóch niezależnych modelach iPSC HD. Należy jednak mieć świadomość, że każda z badanych ścieżek w sposób wielopłaszczyznowy wpływa na procesy samoodnawiania, utrzymania i wyjścia ze stanu pluripotencji (Huang i in., 2015; Lin i Lin, 2017; Ma i in., 2016; Sineva i Pospelov, 2014). Dlatego odkrycie potencjalnego biologicznego znaczenia zidentyfikowanych fenotypów HD wymaga kompleksowych badań z użyciem nowych, precyzyjnych narzędzi i modeli badawczych, co omówię w dalszych częściach pracy.

Podsumowując, w tym artykule wyprowadziłem i scharakteryzowałem komórkowy model YAC128-iPSC oraz wskazałem potencjalne biomarkery terapeutyczne, które w pierwszej kolejności należało zweryfikować z wykorzystaniem narzędzi opracowanych w następnej omawianej publikacji (Szlachcic i in., 2017)

3.3 The generation of mouse and human Huntington disease iPS cells suitable for *in vitro* studies on huntingtin function

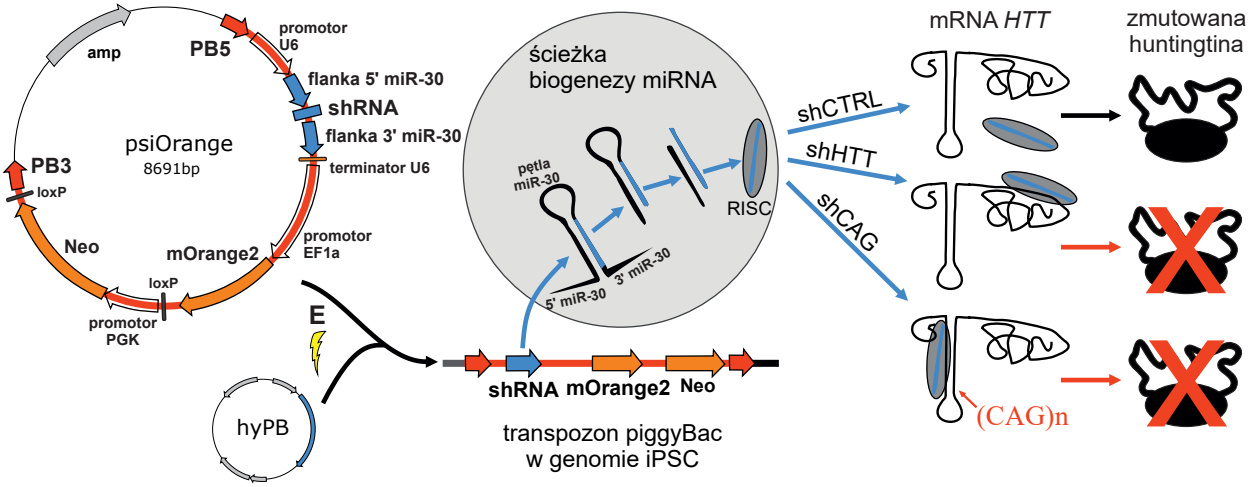
Szlachcic WJ, Wiatr K, Trzeciak M, Figlerowicz M, Figiel M.
Fronters in Molecular Neuroscience. 2017. 10:253
DOI: 10.3389/fnmol.2017.00253

Niniejsza publikacja opisuje finalny etap tworzenia modelu do terapii komórkowej HD, czyli wyprowadzenie linii YAC128-iPSC ze stabilnym wyciszeniem ekspresji huntingtyny. Celem badań przedstawionych w tej pracy było również potwierdzenie bezpośredniej zależności fenotypów HD zaobserwowanych w poprzedniej pracy od ekspresji huntingtyny. Udowodniona zależność byłaby silną przesłanką dla użyteczności tych fenotypów jako biomarkerów terapeutycznych. Równocześnie celem pracy było prześledzenie losów zidentyfikowanych fenotypów oraz efektów działania reagentów po zróżnicowaniu komórek iPSC do stadium neuronalnych komórek macierzystych (NSC), w którym to stadium miałyby być dostarczane do mysiego mózgu.

Linie YAC128-iPSC zostały stworzone z zamiarem badania i modelowania autologicznej terapii komórkowej. Model ten ma odzwierciedlać podejście terapeutyczne, w którym pluripotencjalne komórki iPSC wyprowadzone od pacjenta zostaną trwale "wyleczone"*ex vivo* i wstrzyknięte jako prekursory neuronalne do mózgu. Jako metodę terapeutyczną wybraliśmy cząsteczki typu shRNA (Silva i in., 2005) umieszczone w kontekście endogennej cząsteczki mikroRNA miR-30 (Paddison i in., 2004) i trwale wbudowane do genomu przy użyciu transpozonu piggyBac (Ryc. 3, Fig. 1). Reagenty shRNA zaprojektowaliśmy tak aby były specyficzne dla ludzkiego genu *HTT* (reagenty z serii shHTT) lub tak aby celowały bezpośrednio w region powtórzeń CAG (reagent shCAG). W konstrukcie umieściliśmy również gen kodujący białko oporności na neomycynę (neoR), który umożliwił selekcję komórek ze zintegrowanym transpozonem, oraz gen białka fluorescencyjnego mOrange2, upraszczającego śledzenie i sortowanie zmodyfikowanych komórek.

Analizy ekspresji huntingtyny metodą western blot w zmodyfikowanych liniach YAC128-iPSC wykazały efektywność reagentów z serii shHTT (Fig. 2, Fig. S3), a szczególnie reagenta shHTT2, który obniżał ilość huntingtyny o około 85% w porównaniu do linii z ekspresją reagenta kontrolnego (shCTRL) celującego w gen GFP. Po zróżnicowaniu komórek YAC128-iPSC do komórek NSC, hodowanych jako neurosfery (Fig. S2), poziom wyciszenia wynosił około 60% (Fig. 2). Taka efektywność reagenta shHTT2 predysponuje linie YAC128-iPSC z jego ekspresją do wykorzystania w modelu autologicznej terapii eksperymentalnej. Z podobną wydajnością jak w mysich liniach iPSC reagent ten obniżał ekspresję normalnej i zmutowanej huntingtyny w ludzkich liniach HD71 iPSC oraz WT iPSC (Fig. 3). Co ciekawe w przypadku ludzkiej linii HD109 jego efektywność była niższa i wynosiła około 50-60%. Wytłumaczeniem tych różnic w efektywności wyciszenia mogą być zidentyfikowane przeze mnie różnice w poziomie ekspresji huntingtyny normalnej i zmutowanej pomiędzy liniami WT, HD71 i HD109. W tych liniach zaobserwowałem, że im dłuższy trakt CAG w zmutowanym allelu, tym całkowita ekspresja huntingtyny jest niższa. W wyniku wyciszenia reagentem shHTT2 ekspresja całkowitej huntingtyny zostaje zrównana do podobnego poziomu we wszystkich ludzkich liniach HD i WT. Ponieważ reagent shHTT2 jest specyficzny dla ludzkiej huntingtyny i w związku z tym w ludzkich komórkach obniża ekspresję obu alleli, uzyskane ludzkie linie iPSC z ekspresją tego reagenta mogą być niezwykle przydatne do badań nad funkcjami HTT. W opisywanej przeze mnie

w kolejnym rozdziale rozprawy pracy przeglądowej (Wiatr i in., 2017) przywołujemy niezwykle istotne obserwacje dotyczące funkcji huntingtyny uzyskane dzięki wykorzystaniu mysich modeli pluripotencjalnych pozbawionych ekspresji huntingtiny. Do tej pory nie opisano ludzkich komórek pluripotencjalnych z wyciszeniem huntingtyny umożliwiających prowadzenie podobnych badań w systemie ludzkim.



Ryc. 3: Strategia trwałego wyciszenia huntingtyny reagentami typu shRNA. Transpozon piggyBac (czerwony fragment plazmidu pomiędzy PB5 i PB3) z kasetą terapeutyczną shRNA (niebieski) jest wbudowywany do genomu komórek iPSC na drodze koelektroporacji wektora psiOrange, nośnika transpozonu, oraz wektora hyPB kodującego transpozazę. Reagenty shRNA ulegają ekspresji i dojrzewaniu z użyciem endogennej ścieżki biogenezy miRNA. Nić efektorowa zostaje inkorporowana do kompleksu RISC, efektora interferencji RNA, i przyłącza się do docelowej sekwencji mRNA HTT skutkując zablokowaniem translacji.

Reagent shHTT2 celuje w fragment sekwencji ludzkiego genu *HTT*, który różni się w mysim genie *Htt* jednym nukleotydem w pozycji 9 reagenta. W efekcie tej nieznacznej różnicy w mysich komórkach YAC128-iPSC reagent shHTT2 oprócz ludzkiej zmutowanej huntingtyny wyciszał również o około 50% huntingtynę mysią. Ponieważ w komórkach YAC128 ekspresja mysiego genu *Htt* zachodzi z dwóch alleli, reagent shHTT2 teoretycznie redukuje ekspresję normalnej huntingtyny do poziomu jednego allelu obecnego u heterozygotycznych myszy HD knock-in oraz u heterozygotycznych pacjentów HD. Dotychczasowe badania sugerują, że jest to poziom wystarczający do prawidłowej embriogenezy i różnicowania, nie skutkujący nieprawidłowym fenotypem u myszy, jednakże nie można wykluczyć subtelniejszych zmian wynikających z tego obniżenia. W porównaniu do reagenta shHTT2, dwa inne testowane reagenty, shHTT1 i shHTT3 , wykazywały niższą efektywność w liniach YAC128-iPSC (Fig. S3). Reagenty te nie wykazują podobieństwa do mysiego genu *Htt*, a więc nie skutkowały wyciszeniem normalnej mysiej huntingtyny.

W badaniu funkcji huntingtyny i testowaniu komórkowych terapii HD niezwykle pomocne mogą okazać się reagenty alleloselektywne, wyciszające jedynie zmutowaną huntingtynę. Ponieważ wariant ludzkiego gen *HTT* u myszy YAC128 nie posiada żadnych charakterystycznych dla zmutowanego allelu polimorfizmów SNP, które umożliwiałyby zaprojektowanie reagenta uniwersalnego dla części pacjentów, postanowiliśmy przetestować reagent celujący w wydłużony trakt powtórzeń CAG. Wybraliśmy w tym celu reagent A2 opisany wcześniej przez zespół prof. Krzyżosiaka (Fiszer i in., 2013), który wykazywał wysoką efektywność i alleloselektywność w ludzkich fibroblastach HD w formie zarówno samodupleksujacych oligonukleotydów siRNA, jak i reagentów shRNA w wektorze lentiwirusowym. Na bazie tego reagenta zaprojektowałem reagent shCAG dopasowany do naszego

systemu shRNA bazującego na wektorze piggyBac i endogennym miR-30. Niestety reagent nie wykazał efektu wyciszenia ani w liniach YAC128-iPSC (Fig. 2) ani w ludzkich liniach iPSC (Fig. 3). Co ciekawe po zróżnicowaniu komórek YAC128-iPSC do neuronalnych komórek NSC reagent shCAG obniżał ekspresję zmutowanej huntingtyny o około 40% (przy ok. 60% efektywności reagenta shHTT2) (Fig. 2). Zaobserwowane różnice w efektywności poszczególnych shRNA pomiędzy różnymi typami komórek sugerują konieczność testowania ich w docelowym typie komórek.

W poprzedniej pracy zidentyfikowałem w komórkach HD iPSC białka o zmienionym poziomie ekspresji lub fosforylacji z zamiarem ich wykorzystania jako markerów efektu terapeutycznego shRNA. Efekty te zostały zbadane i opisane w niniejszej pracy a w Tabeli 1 zebrałem wyniki wszystkich opisanych w załączonych pracach badawczych analiz western blot w mysich i ludzkich liniach iPSC. Analiza wpływu wyciszającego efektu reagenta shHTT2 na ekspresję białka efektorowego kanonicznej ścieżki Wnt, beta-kateniny, wykazała w komórkach YAC128-iPSC podwyższenie całkowitego białka, natomiast wpływ na fosforylację był różny pomiędzy poszczególnymi grupami linii izogenicznych i1 i i2 (Fig. 4a, b). Podobne różnice pomiędzy liniami izogenicznymi z grup i1 i i2 można było zaobserwować teście aktywacji ścieżki ERK (Fig. 4c, d). Zarówno w przypadku ścieżki Wnt, jak i ścieżki ERK obniżenie ekspresji zmutowanej huntingtyny nie odwracało kierunków zmian pomiędzy liniami YAC128-iPSC i WT, sugerując bardziej złożoną interakcję tych ścieżek ze zmutowaną HTT. W ludzkich liniach HD109, HD71 i WT (Fig. 5, Fig.) nie zaobserwowałem jednoznacznego efektu wyciszenia całkowitej huntingtyny na ekspresję i fosforylację badanych białek ścieżek Wnt i ERK.

Tabela 1. Podsumowanie opisanych w Szlachcic i in. 2015 oraz 2017 analiz western blot linii iPSC

		Linie	YAC128-iPS		HD109		HD71		WT	
		shHTT2 / Białko	-	+	-	+	-	+	-	+
Ścieżka		HTT								
	ERK	ERK1/2								
		p-ERK1/2		izo	n/s		n/s			
	Wnt	B-catenin				n/s		n/s		
		p-B-catenin		izo	n/s	*				
	p53	p53								
		p-p53	n/a	n/s	n/p	n/a				
	Stres oksyd.	PRDX1	n/s	n/p		n/a		n/a		n/a
		SOD1		n/p		n/a	n/s	n/a		n/a

Legenda: czerwony/zielony/biały – obniżenie/podwyższenie/brak zmian w stosunku do kontroli (shHTT2–: do WT; shHTT2+: do shCTRL); n/s – trend nieistotny statystycznie, n/a – nieanalizowane; n/p – nieopublikowane; izo – różny efekt w zależności od linii izogenicznych; * podobne zmiany również w reagencie shCAG

Jedynie dla białka p53 (Fig. 6) wyciszenie ekspresji zmutowanej huntingtyny przez reagent shHTT2 skutkowało jednoznacznym odwróceniem fenotypu – podwyższeniem ekspresji – w modelu mysim i to zarówno w iPSC, jak i komórkach NSC. Podobną reakcję na reagent shHTT2 wykazywała fosforylowana forma białka p53 (Fig. S6). Ponadto analiza linii iPSC ze wszystkimi

reagentami shHTT oraz shCTRL wykazała wysoką ujemną korelację poziomu białka p53 z poziomem zmutowanej huntingtyny (współczynnik korelacji Pearsona r = 0.6952). W ludzkich iPSC, nie zaobserwowałem wpływu wyciszenia huntingtyny przez reagent shHTT2 na ekspresję białka p53 zarówno w przypadku wyciszenia wyłącznie normalnej huntingtyny w komórkach WT, jak i obu alleli w liniach HD71 i HD109 (Fig. 7). Przyczyny mogą być różne, począwszy od różnicy w stopniu wyciszenia ekspresji zmutowanego białka między ludzkimi i mysimi komórkami, a skończywszy na niewystarczającej liczbie linii.

Podsumowując, uzyskałem linie YAC128-iPSC ze stabilną ekspresją reagenta efektywnie wyciszającego zmutowaną ludzką huntingtynę, do wykorzystania w modelach terapii HD. O ich przydatności do terapii komórkowej świadczy również zdolność przeżycia komórek NSC z reagentem shHTT2 w mysim mózgu przez okres 8 tygodni (Fig. S1). W komórkach YAC128-iPSC potwierdziłem także zależność ekspresji białka p53 od ekspresji huntingtyny. Dodatkowo wprowadziłem efektywny reagent shHTT2 do ludzkich linii iPSC, uzyskując pierwszy opisany knock-down całkowitej huntingtyny zarówno w komórkach od osób zdrowych, jak i od pacjentów. Komórki te mogą zostać wykorzystane do badań nad funkcjami huntingtyny, przy czym model ten można jeszcze ulepszyć, przykładowo poprzez wyprowadzenie linii klonalnych oraz cofnięcie do stanu pluripotencji naiwnej. Dyskusję nad zasadnością, możliwością i sposobem prowadzenia podobnych badań przeprowadziliśmy w ostatniej załączonej do tej rozprawy publikacji (Wiatr i in., 2017), którą omówię w kolejnym rozdziale.

3.4 Huntington Disease as a Neurodevelopmental Disorder and Early Signs of the Disease in Stem Cells

Wiatr K, Szlachcic WJ, Trzeciak M, Figlerowicz M, Figiel M.
Mol Neurobiol. 2017 May 11.
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Praca przeglądowa.

Opisane przeze mnie w poprzednich pracach poszukiwania wczesnych fenotypów HD już na etapie pluripotencji mogą dziwić w kontekście tradycyjnego spojrzenia na HD jako choroby wieku dorosłego. Jednakże liczne badania ostatnich lat wskazują, że HD ma również komponent neurorozwojowy, który w istotny sposób może determinować procesy neurodegeneracyjne (Humbert, 2010; Nopoulos, 2016). W pierwszej części niniejszej pracy przeglądowej podsumowujemy obecną wiedzę na temat roli normalnej i zmutowanej huntingtyny w procesach rozwojowych oraz zwracamy uwagę na wynikające z tego implikacje dla potencjalnej terapii HD. W dalszej części pracy opisujemy istniejące modele pluripotencjalne HD w kontekście zaburzeń w ścieżkach molekularnych związanych z procesami rozwojowymi, które zostały zaobserwowane w tych modelach oraz w uzyskanych z ich różnicowania komórkach neuorektodermalnych.

Do dogłębnych analiz neurozwojowej natury HD skłaniają obserwacje z mysich modeli oraz, co istotniejsze, dane kliniczne. Długotrwałe programy kliniczne wskazują bowiem na obecność subtelnej fazy prodromalnej HD, której neurobiologiczne komponenty, w tym zmiany morfologii mózgu, można zaobserwować na wiele lat przed objawową fazą kliniczną HD (Paulsen i in., 2014; Quaid i in., 2017). Wydawałoby się, że faza prodromalna mogłaby być jedynie emanacją początków neurodegeneracji. Jednakże jednym z elementów fazy przedobjawowej HD jest mniejsza objętość śródczaszkowa u mężczyzn nosicieli HD (Nopoulos i in., 2011). Objętość śródczaszkowa uznawana jest za miarę maksymalnego przyrostu mózgu w trakcie rozwoju, a więc jego mniejsza objętość sugeruje nieprawidłowy rozwój mózgu. Podobne wnioski można wysnuć z obserwowanego u dzieci z mutacją HD mniejszego obwodu głowy (Lee i in., 2012). Wydaje się jednak, że w rzadkich przypadkach form młodzieńczej i dziecięcej HD, występujących przy dużych ekspansjach powtórzeń CAG (nawet powyżej 100), zaburzenia neurorozwojowe są dużo silniejsze, skutkując nieco odmienną i bardziej dynamiczną formą choroby (Quigley, 2017). Pytanie w jakim stopniu zaburzenia neurorozwojowe wpływają na rozwój i przebieg choroby pozostaje otwarte.

Huntingtyna jest niezbędna w rozwoju, przynajmniej u myszy (Fig. 1). Bezsprzeczny dowód na to trzy niezależne grupy badawcze uzyskały już w 1995 roku (Duyao i in., 1995; Nasir i in., 1995; Zeitlin i in., 1995), dwa lata po identyfikacji mutacji w tym białku jako przyczyny HD. Wykazano wtedy, że całkowity (homozygotyczny) knock-out *Htt* jest u myszy letalny i skutkuje śmiercią embrionów w początkowej fazie tworzenia układu nerwowego. Ekspresja normalnej huntingtyny na poziomie ok. 10-15% naturalnego poziomu lub jej brak na dalszych etapach rozwoju embrionalnego oraz postnatalnego skutkuje wadami rozwojowymi, fenotypem neurologicznym i przedwczesną śmiercią myszy (Auerbach i in., 2001; Dragatsis i in., 2000). Przywrócenie normalnej ekspresji *Htt* we wczesnym okresie postnatalnym nie jest w stanie całkowicie naprawić skutków wad rozwojowych (Arteaga-Bracho i in., 2016). Równocześnie brak huntingtyny u dorosłych myszy nie wywołuje negatywnych skutków (Wang i in., 2016).

Jakie są funkcje huntingtyny w trakcie rozwoju embrionalnego i rozwoju mózgu? Przywołane w pracy przeglądowej badania wskazują na istotne znaczenie tego białka w regulacji powstawania

listków zarodkowych, symetrii podziałów komórkowych, cyklu komórkowego i dojrzewania neuronów prążkowia. Mutacja huntingtyny zaburza prawidłowy przebieg tych procesów. Najnowsze badania wykazały również negatywnie wpływ mutacji na migrację post-mitotycznych neuronów kory mózgowej a tym samym jej architekturę (Barnat i in., 2017). W kontekście patogenezy HD wykazano, że ekspresja zmutowanej huntingtyny u myszy wyłącznie w trakcie rozwoju (do 21. dnia po urodzeniu) jest wystarczająca do wywołania objawów charakterystycznych dla mysiej formy HD, aczkolwiek słabszego niż w przypadku ciągłej ekspresji (Molero i in., 2016). Zestawiając te wyniki z pozytywnymi efektami terapeutycznymi usunięcia ekspresji zmutowanej huntingtyny u dorosłych myszy (Kordasiewicz i in., 2012; Yamamoto i in., 2000), można się zastanawiać jak silny efekt terapeutyczny można uzyskać już po wystąpieniu defektów neurorozwojowych. Zarówno bardzo niska ekspresja normalnej jak i obecność zmutowanej huntingtyny zaburza prawidłowy rozwój połączeń komunikacyjnych kora-prążkowie u myszy. Nieprawidłowe sygnały z kory do prążkowia mogą wpływać na prawidłowe dojrzewanie neuronów prążkowia, czyniąc je w fazie neurodegeneracyjnej choroby bardziej podatnymi na wywołaną stresem apoptozę.

Te spostrzeżenia implikują szereg pytań kluczowych dla terapii HD. Jakie znaczenie dla rozwoju choroby oraz skuteczności terapii ma komponent neurorozwojowy? Czy i w jaki sposób można naprawić defekty neurorozwojowe? Jak wcześnie należy rozpocząć terapię HD i młodzieńczego/dziecięcego HD? W jaki sposób w regeneracyjnej terapii komórkowej uzyskać dojrzałe i funkcjonalne neurony, odporne na toksyczne środowisko zewnętrzne?

Do odpowiedzi na te pytania mogą przyczynić się modele terapii komórkowej wykorzystującej technologię komórek pluripotencjalnych. Dotychczas utworzone modele, opisane w Tabeli 1 publikacji, znacznie przyczyniły się do zrozumienia mechanizmów patogenezy HD, w tym związanej z rozwojem. Modele te umożliwiają śledzenie kolejnych etapów rozwoju fenotypów HD w trakcie różnicowania, co opisujemy w drugiej części publikacji. Co istotne, fenotypy związane z obecnością zmutowanej huntingtyny mogą być obserwowane już w stadium pluripotencji, chociaż ich intensywność wzrasta na dalszych etapach rozwoju (Fig. 1, Fig. 3). Nie jest znany wpływ wczesnych zaburzeń obecnych w stadium pluripotencji na dalsze etapy rozwojowe. Do takich badań niezbędne w mojej opinii jest stworzenie nowej generacji modeli komórkowych.

Czynnikiem limitującym badania HD z użyciem istniejących modeli pluripotencjalnych od pacjentów jest duża zmienność genetyczna i epigenetyczna pomiędzy liniami pochodzącymi od różnych osób. Wynikające z tego różnice molekularne i funkcjonalne są istotnym, ogólnym problemem w badaniach z użyciem ludzkich komórek pluripotencjalnych (Ortmann i Vallier, 2017). W przypadku modelowania chorób takich jak HD różnice pomiędzy liniami wynikające ze zmienności genetycznej i epigenetycznej mogą utrudniać identyfikację fenotypów chorobowych. W HD istotnym modyfikatorem fenotypu jest również liczba powtórzeń CAG. Właściwie wszystkie dotychczasowe badania nad HD, w tym moje, bazują na liniach iPSC od co najwyżej kilku pacjentów, często z istotnie różną liczbą powtórzeń CAG. Jedno z rozwiązań tego problemu – pozyskanie dużej liczby linii od różnych pacjentów – nie jest proste, szczególnie w przypadku rzadszych przypadków młodzieńczej HD, i trudne do zrealizowania nawet dla dużych konsorcjów naukowych (Consortium, 2012; HD iPSC Consortium, 2017). Drugą możliwość stanowi tworzenie linii izogenicznych, genetycznie różniących się od linii HD tylko brakiem zmutowanego allelu *HTT* lub stabilnym obniżeniem jego ekspresji, tak jak w przypadku wyprowadzonych przeze mnie linii z częściowym wyciszeniem *HTT*. Możliwe jest również wprowadzenie mutacji do komórek od osoby zdrowej, dzięki czemu można by stworzyć serie izogenicznych linii allelicznych o różnej długości powtórzeń CAG, co pozwoliłoby w sposób nieobciążony zmiennością genetyczną i epigenetyczną zbadać wpływ liczby powtórzeń na

patogenezę. W badaniach nad HD dotychczas uzyskano linie izogeniczne jedynie poprzez redukcję liczby powtórzeń w zmutowanym allelu HTT komórek od pacjentów. Dokonano tego w grupach Lisy Ellerby, na drodze rekombinacji homologicznej (An i in., 2012; Ring i in., 2015), oraz Mahmouda Pouladiego, na drodze edycji genomu z wykorzystaniem technologii CRISPR/Cas9 (Xu i in., 2017). Niestety jak dotąd nie powstały linie ludzkich komórek pluripotencjalnych z całkowitym knockoutem *HTT* ani linie o warunkowej ekspresji genu *HTT*, które byłyby bardzo przydatne w badaniach nad funkcjami tego białka..

Istotnym ograniczeniem w inżynierii genetycznej ludzkich komórek ESC i iPSC jest stadium pluripotencji, w którym tradycyjnie występują one w hodowlach *in vitro*. Stadium to jest określane jako pluripotencja aktywowana (ang. primed) i w trakcie rozwoju embrionalnego myszy jest ono charakterystyczne dla komórek występujących w epiblaście zarodka po jego implantacji. W odróżnieniu od komórek ludzkich, mysie komórki pluripotencjalne hoduje się w stadium tzw. pluripotencji naiwnej (ang. naïve), który odpowiada komórkom izolowanym z epiblastu mysiego zarodka przed jego implantacją (Ying i Smith, 2017). Ze względu na nietrwałość stadium pluripotencji naiwnej u naczelnych przez długi czas ludzkie naiwne komórki pluripotencjalne były nieuchwytne. Niedawno kilku grupom, przede wszystkim zespołom prof. Rudolfa Jaenischa (Theunissen i in., 2016, 2014) i prof. Austina Smitha (Guo i in., 2017; Takashima i in., 2014), udało się w ludzkich komórkach aktywowanych wywołać i utrzymać w warunkach hodowli *in vitro* stan przypominający pluripotencję naiwną. Jedną z ważnych cech odróżniających te dwa stadia pluripotencji jest łatwość selekcji klonalnej komórek, do czego konieczna jest dysocjacja do pojedynczych komórek. W stanie pluripotencji aktywowanej tak zdysocjowane komórki wkraczają na ścieżkę apoptozy (Chen i in., 2010; Ohgushi i in., 2010). W stanie pluripotencji naiwnej brak kontaktu z innymi komórkami nie stanowi problemu, co znacznie ułatwia wyprowadzanie klonalnych linii zmodyfikowanych genetycznie i od lat jest wykorzystywane przy tworzeniu myszy transgenicznych z mysich naiwnych komórek pluripotencjalnych. Z tego powodu zastosowanie metod inżynierii genetycznej, szczególnie wieloetapowych, w tradycyjnych kulturach ludzkich komórek pluripotencjalnych jest trudniejsze niż w przypadku komórek mysich. Możliwa jest ochrona przed apoptozą komórek aktywowanych poprzez zastosowanie inhibicji kinaz związanych z białkiem Rho (ang. ROCK) (Watanabe i in., 2007), jednak nie do końca poznany jest wpływ długotrwałego stosowania takiej inhibicji na metabolizm komórek (Gharechahi i in., 2014; Vernardis i in., 2017).

Pośród wielu innych różnic pomiędzy tymi dwoma stadiami pluripotencji (Grzybek i in., 2017), szczególnie istotne dla badań nad HD są różnice w stanie epigenetycznym genomu. Chromatyna komórek w stanie pluripotencji naiwnej ulega otwarciu na skutek demetylacji histonu H3 (konkretnie metylacji H3K27me3) i globalnej demetylacji DNA (Grzybek i in., 2017). Genom komórek w stanie aktywowanym pozostaje w stanie bardziej upakowanym, a DNA nie podlega intensywnej demetylacji. W tym stanie znaczniki epigenetyczne uzyskane w przeszłości, np. w trakcie życia pacjentów lub w trakcie procesu przeprogramowania, mogą zostać nie w pełni usunięte i wpływać na właściwości funkcjonalne komórek. Z tego powodu pomiędzy liniami ludzkich komórek pluripotencjalnych występuje duża zmienność epigenetyczna, mogąca kontrybuować do różnic w zdolności do różnicowania obserwowanej pomiędzy poszczególnymi liniami (Grzybek i in., 2017; Ortmann i Vallier, 2017). Stan pluripotencji aktywowanej wprowadza więc dodatkową zmienność utrudniającą badania nad aspektami neurorozwojowymi HD w modelach komórkowych. Jak dotąd nie uzyskano ludzkich komórek HD w stanie pluripotencji naiwnej, które niwelowałyby tą zmienność.

4 Podsumowanie i perspektywy

Wśród opisanych w niniejszej rozprawie badań, najważniejszymi rezultatami są:

- wyprowadzone mysie linie YAC128-iPSC oraz WT-iPSC,
- wyprowadzone linie YAC128-iPSC z ekspresją efektywnych reagentów shRNA wyciszających huntingtynę oraz linie kontrolne,
- wyprowadzone ludzkie komórki HD109, HD71 i WT z reagentami shRNA wyciszającymi huntingtynę oraz linie kontrolne,
- odkryte zaburzenia w ekspresji białek w mysich i ludzkich komórkach iPSC, w szczególności białka p53, które mogą być wczesnymi zaburzeniami fazy rozwojowej HD.

Wyniki te będą podstawą do dalszych badań w kierunkach modelu terapii komórkowej HD oraz badań nad rolą huntingtyny w rozwoju i wczesną patogenezą HD.

4.1 Terapia HD

Linie YAC128-iPSC z reagentem shHTT2 zostały stworzone z myślą o terapii *in vivo* u myszy YAC128 i wstępne badania wykazały, że po wstrzyknięciu do mysiego prążkowia zróżnicowanych z tych linii komórek NSC są one w stanie przetrwać w mózgu przez co najmniej 2 miesiące. Użyteczna w tej materii okazała się metoda CLARITY/PACT (Tomer i in., 2014; Yang i in., 2014). CLARITY/PACT to metoda przygotowania tkanki w której staje się ona przezroczysta a tło związane z autofluorescencją zostaje zredukowane. W mózgu poddanym CLARITY/PACT fluorescencja białka reporterowego mOrange2 jest utrzymana (Szlachcic i in., 2017; Fig. S1). Metoda ta umożliwia immunofluorescencyjne barwienie całych organów, a więc ułatwi precyzyjną analizę 3D regionów mózgu, do których wstrzyknięto komórki. Taka charakterystyka transplantów pozwoli wyjaśnić m.in. do jakiego rodzaju komórek się przekształcają oraz czy funkcjonalnie zintegrowały z mózgiem biorcy. Analizy te pozwolą zoptymalizować technikę transplantacji, co uważam za niezbędny krok przed badaniem efektów terapeutycznych u myszy.

Oprócz użycia w modelu terapii *in vivo*, uzyskane modele – w tym ludzkie komórki iPSC – można wykorzystać w modelach *in vitro* terapii, które pozwolą dokładniej przyjrzeć się mechanizmom terapii komórkowej, takim jak interakcje pomiędzy komórkami transplantu i biorcy, i wskazać możliwe sposoby jej ulepszenia. Przykładem takich modeli mogłaby być kokultura komórek HD z komórkami HD z wyciszoną ekspresją huntingtyny zarówno w formie adherentnej jaki i w strukturach 3D takich jak organoidy. Modele *in vitro* umożliwiłyby wykonanie pomiarów niedostępnych w warunkach *in vivo*. Przykładem byłoby zbadanie czy reagenty shRNA są transportowane pomiędzy komórkami, co mogłoby skutkować wyciszeniem huntingtyny w komórkach niezmodyfikowanych.

Kolejnym ważnym aspektem badań nad terapią komórkowa jest wybór konkretnych linii do dalszych badań. Selekcja powinna zostać dokonana na podstawie nie tylko wysokiego poziomu wyciszenia ekspresji huntingtyny ale również na podstawie locus/loci integracji genomwoej transpozonu z kasetą ekspresyjną shRNA. Taka analiza może zostać wykonana metodą odwróconego PCR. Do terapii komórkowej powinny być wybrane linie z transpozonem zintegrowanym w regionach pozagenowych. Moim zdaniem niezbędna w selekcji odpowiednich linii jest również analiza integralności ich genomu, co najmniej na poziomie prawidłowego kariotypu.

4.2 HD jako choroba neurorozwojowa

Uzyskane przeze mnie pierwsze ludzkie komórki iPSC z częściowym wyciszeniem huntingtyny pozyskane od osób zdrowych i pacjentów z młodzieńczą formą HD, będą przydatne do badań nad funkcjami rozwojowymi u ludzi. Uzyskany w nich poziom wyciszenia huntingtyny koresponduje z poziomem, który u myszy wywołuje silne wady rozwojowe. Model ten można usprawnić przez transfer do stanu pluripotencji naiwnej, który ułatwiłby wyprowadzenie linii klonalnych, co umożliwiłoby wybranie linii z wysokim poziomem wyciszenia huntingtyny. Co istotne w stanie naiwnym znaczniki epigenetyczne zostałyby usunięte, zmniejszając wynikającą z różnic epigenetycznych zmienność pomiędzy komórkami. Bardzo użyteczne do tego typu badań i komplementarne do uzyskanego modelu byłyby również nowe linie izogeniczne, które można by stworzyć z użyciem technologii CRISPR i które umożliwiałyby warunkowy knock-out ekspresji normalnej i/lub zmutowanej huntingtyny o różnej długości powtórzeń.

Badania nad neurorozwojowymi aspektami HD z użyciem naiwnych komórek iPSC można by wykonać w sposób systematyczny na wielu etapach odpowiadających kolejnym fazom rozwoju embrionalnego, począwszy od pluripotencji naiwnej a skończywszy na dojrzałych organoidach. Możliwe jest też wykorzystanie dwuwymiarowych adherentnych metod typu mikrowzorów (ang. micropatterning), które ułatwiają kontrolę nad organizacją przestrzenną komórek w modelowaniu embriogenezy *in vitro* (Deglincerti i in., 2016). Idealne byłoby użycie wysokoprzepustowych analiz transkryptomicznych, proteomicznych i epigenetycznych, o ile to możliwe wykorzystujących indywidualną analizę tysięcy komórek. Dane uzyskane z tych badań pozwolą na zaprojektowanie precyzyjnych testów funkcjonalnych badających realny wpływ zmutowanej i normalnej HTT na rozwój organizmu.

Moim zdaniem dopiero przeprowadzenie tego typu szczegółowych badań pozwoli ocenić znaczenie biologiczne zaobserwowanych przeze mnie zmian. Pozwolą one na określenie czy ich obecność na etapie pluripotencji ma znaczenie dla późniejszych procesów rozwojowych (efekt kuli śniegowej) czy dopiero zmiany na tych dalszych etapach nabierają funkcjonalnych konsekwencji. Badania nad rozwojowymi aspektami HD mogą mieć istotne znaczenie dla projektowania terapii tej choroby.

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OŚWIADCZENIE

Dotyczy rozprawy doktorskiej mgr inż. Wojciecha J Szlachcica:

„Nowe modele komórkowe iPSC choroby Huntingtona do zastosowań w eksperymentalnej terapii komórkowej oraz w badaniach funkcji neurorozwojowych normalnej i zmutowanej huntingtyny"

Rozprawa doktorska Wojciecha J Szlachcica jest związana z badaniami dotyczącymi tworzenia nowych modeli komórkowych iPSC choroby Huntingtona do zastosowań w terapii komórkowej a także do badania funkcji huntingtyny. Będąc promotorem Wojciecha J Szlachcica oraz autorem korespondencyjnym wszystkich prac składanych przez doktoranta w ramach rozprawy doktorskiej mogę zapewnić, że jego wkład w każdą z nich był znaczący i dotyczył wszystkich kwestii związanych z powstaniem publikacji – od współtworzenia koncepcji pracy, przez planowanie i wykonywanie eksperymentów, aż po interpretacje danych i współtworzenie manuskryptów. Mój udział w tych publikacjach związany był z zainicjowaniem w IBCH PAN badań nad komórkami iPSC jako komórkowymi modelami chorób poliQ, opracowaniem koncepcji platformy eksperymentalnej terapii w której komórki iPSC są generowane z modelu lub z pacjentów a następnie „wyleczone" poprzez modyfikacje reagentami shRNA celującymi w HTT, stworzeniem koncepcji i planu publikacji, a także projektowaniem i koordynowaniem przeprowadzonych eksperymentów, analizą wyników, pisaniem manuskryptów i wykonywania prace eksperymentalnych. Wszystkie z prac były elementami oraz były całkowicie finansowane z moich autorskich grantów w których byłem kierownikiem projektów.

W poniższych oświadczeniach przedstawiam swój udział w publikacjach składanych przez doktoranta w ramach rozprawy doktorskiej.

Huntington disease iPSCs show early molecular changes in intracellular signaling, the expression of oxidative stress proteins and the p53 pathway
Szlachcic WJ, Switonski PM, Krzyzosiak WJ, Figlerowicz M, Figiel M.
Dis Model Mech. 2015. 8(9):1047-57. doi: 10.1242/dmm.019406.

Mój udział w niniejszej publikacji polegał na stworzeniu koncepcji i planu pracy, projektowaniu eksperymentów, wykonywaniu eksperymentów i koordynowaniu pracy zespołu. Wspólnie z Wojciechem J Szlachcicem analizowaliśmy uzyskane dane, które doprowadziły nas do interpretacji że zidentyfikowane deregulowane molekuły są wczesnymi zmianami HD odkrytymi już w komórkach typu macierzystego i że są powiązane interakcjami. Na potrzeby publikacji wykonałem eksperymenty in vivo polegające na wstrzyknięciach komórek celem wykonania testu formowania potworniaków. Następnie wykonałem manualną dysekcję uformowanych potworniaków i barwienia części preparatów histologicznych. Mój udział polegał również na napisaniu większości manuskryptu, późniejszym redagowaniu i przygotowaniu wersji publikacyjnych.

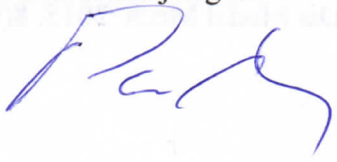
Huntington Disease as a Neurodevelopmental Disorder and Early Signs of the Disease in Stem Cells
Wiatr K, Szlachcic WJ, Trzeciak M, Figlerowicz M, Figiel M.
Mol Neurobiol. 2017 May 11. doi: 10.1007/s12035-017-0477-7. Praca przeglądowa.

W pracy przeglądowej mój udział polegał na zaplanowaniu koncepcji publikacji, zaproponowaniu koncepcji analiz bioinformatycznych wykonanych na potrzeby pracy oraz koordynowaniu prac podczas pisania manuskryptów. Ryciny do pracy wykonane zostały zgodnie z moimi pomysłami. Różne części pracy zostały napisane przez: Kalinę Wiatr, Wojciecha J Szlachcica oraz przeze mnie. Dodatkowo mój udział w pisaniu manuskryptu polegał na gruntownym redagowaniu wszystkich rozdziałów i nierzadko przepisywaniu/poprawianiu znacznych części niektórych rozdziałów.

The generation of mouse and human Huntington disease iPS cells suitable for in vitro studies on huntingtin function.
Szlachcic WJ, Wiatr K, Trzeciak M, Figlerowicz M, Figiel M.
Front Mol Neurosc. 2017. 10:253. doi: 10.3389/fnmol.2017.00253

W powyższej pracy mój udział polegał na zaplanowaniu kształtu publikacji oraz planowaniu, wykonywaniu i koordynowaniu eksperymentów. Zaproponowałem koncepcję leczenia ex-vivo drogą stałej ekspresji regentów shRNA i byłem współautorem konstruktów do takiej terapii. Wykonałem prace eksperymentalne związane z wyprowadzaniem ludzkich linii komórek HD iPSC z ekspresją regentów. Zaplanowałem i przeprowadziłem eksperymenty in vivo związane z iniekcjami komórek do mózgu myszy. Na potrzeby badań in vivo wykonałem manualną dysekcję mózgów zawierających grafty. Następnie wykonałem analizę konfokalną preparatów histologicznych z tych mózgów i elektroniczną obróbkę zdjęć. Mój udział polegał również na napisaniu większości manuskryptu, późniejszym redagowaniu i przygotowaniu wersji publikacyjnych.

dr hab. Maciej Figiel



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Poznań, 11 września 2017

Prof. dr hab. Marek Figlerowicz
Instytutu Chemii Bioorganicznej PAN
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OŚWIADCZENIE O WSPÓŁAUTORSTWIE PUBLIKACJI

Dotyczy:

1. Szlachcic WJ, Switonski PM, Krzyzosiak WJ, Figlerowicz M, Figiel M.
Huntington disease iPSCs show early molecular changes in intracellular signaling, the expression of oxidative stress proteins and the p53 pathway
Dis Model Mech. 2015 Sep;8(9):1047-57.

2. Wiatr K, Szlachcic WJ, Trzeciak M, Figlerowicz M, Figiel M.
Huntington Disease as a Neurodevelopmental Disorder and Early Signs of the Disease in Stem Cells
Mol Neurobiol. 2017 May 11. doi: 10.1007/s12035-017-0477-7.

3. Szlachcic WJ, Wiatr K, Trzeciak M, Figlerowicz M, Figiel M.
The generation of mouse and human Huntington disease iPS cells suitable for in vitro studies on huntingtin function
Front Neurosc. 2017. doi: 10.3389/fnmol.2017.00253.

W powyższych pracach moja rola polegała na krytycznej analizie prezentowanych badań i ich wyników oraz na korekcie manuskryptów.

Marek Figlerowicz

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Poznań, 12 września 2017

OŚWIADCZENIE

o mojej roli w powstaniu publikacji wchodzącej w skład rozprawy doktorskiej
mgr. inż. Wojciech J. Szlachcica

Dotyczy:

Szlachcic WJ, Świtoński PM, Krzyżosiak WJ, Figlerowicz M, Figiel M. Huntington disease iPSCs show early molecular changes in intracellular signaling, the expression of oxidative stress proteins and the p53 pathway. Dis Model Mech. 2015 Sep;8(9):1047-57.

Moja rola polegała na współprojektowaniu kierunku prowadzonych doświadczeń, na kontroli nad przebiegiem prac badawczych w formie seminariów oraz na sprawdzaniu manuskryptu na różnych etapach jego powstawania. Ponadto dla tej części badań, która była prowadzona w kierowanym przeze mnie Zakładzie, zapewniłem możliwość użytkowania przestrzeni laboratoryjnej oraz sprzętu badawczego.

Włodzimierz J. Krzyżosiak

Poznań, 21 marca 2016

OŚWIADCZENIE

W niniejszym oświadczeniu pragnę wskazać mój udział w publikacjach, w których jestem współautorem wraz z Wojciechem J. Szlachcicem.

1. *Figiel M, Szlachcic WJ, Switonski PM, Gabka A, Krzyzosiak WJ. Mouse models of polyglutamine diseases: review and data table. Part I. Mol Neurobiol. 2012 Oct;46(2):393-429.*

W niniejszej pracy przeglądowej brałem udział w stworzeniu koncepcji publikacji, w tym w stworzeniu elektronicznej tabeli katalogującej genotypy prezentowane przez mysie modele chorób poliglutaminowych. Mój udział polegał na zebraniu danych literaturowych dotyczących mysich modeli choroby Huntingtona i napisaniu tej części manuskryptu.

2. *Switonski PM, Szlachcic WJ, Gabka A, Krzyzosiak WJ, Figiel M. Mouse models of polyglutamine diseases in therapeutic approaches: review and data table. Part II. Mol Neurobiol. 2012 Oct;46(2):430-66.*

Byłem współautorem koncepcji drugiej części pracy przeglądowej opisującej mysie modele chorób poliglutaminowych. Zebrałem dane literaturowe potrzebne do napisania pracy i stworzyłem strukturę publikacji. Ponadto przygotowałem ryciny znajdujące się w publikacji.

3. *Switonski PM, Szlachcic WJ, Krzyzosiak WJ, Figiel M. A new humanized ataxin-3 knock-in mouse model combines the genetic features, pathogenesis of neurons and glia and late disease onset of SCA3/MJD. Neurobiol Dis. 2014 Oct 7. pii: S0969-9961(14)00293-9. doi: 10.1016/j.nbd.2014.09.020.*

W niniejszej publikacji byłem współtwórcą strategii transgenezy skutkującej uzyskaniem mysiego modelu knock-in ataksji rdzeniowo-móżdżkowej typu 3. Wykonałem większość eksperymentów i analiz, za wyjątkiem eksperymentów immunohistochemicznych oraz testu

statycznego pręta służącego do oceny zaburzeń motorycznych u myszy. Stworzyłem strategię genotypowania i przeprowadziłem analizę genotypów wszystkich zwierząt. Przeprowadziłem także analizy western blot i real-time PCR obrazujące wzór ekspresji szeregu genów ulegających deregulacji u pacjentów SCA3 i innych modelach chorób poliglutaminowych. Przeprowadziłem również część eksperymentów behawioralnych opisujących fenotyp neurologiczny u myszy Ki91. Przygotowałem również większość rycin do publikacji, a także brałem udział w pisaniu manuskryptu.

4. *Szlachcic WJ, Switonski PM, Krzyzosiak WJ, Figlerowicz M, Figiel M. Huntington disease iPSCs show early molecular changes in intracellular signaling, the expression of oxidative stress proteins and the p53 pathway. Dis Model Mech. 2015 Sep;8(9):1047-57.*

W niniejszej publikacji moja rola polegała na analizie fenotypu choroby Huntingtona metodą western blot w ludzkich komórkach iPS wyprowadzonych od pacjentów. Brałem również udział w pisaniu manuskryptu.

5. *Szlachcic WJ, Switonski PM, Kurkowiak M, Wiatr K, Figiel M. Mouse polyQ database: a new online resource for research using mouse models of neurodegenerative diseases. Mol Brain. 2015 Oct 29;8(1):69..*

W niniejszej pracy byłem współtwórcą koncepcji bazy danych oraz wprowadzaniu do niej części rekordów.

Poznań, 25. sierpnia 2017

OŚWIADCZENIE WSPÓŁAUTORSTWA

W niniejszym oświadczeniu pragnę wskazać mój udział w publikacjach, w których jestem współautorką wraz z Wojciechem J. Szlachcicem.

1. *Wiatr K, Szlachcic WJ, Trzeciak M, Figlerowicz M, Figiel M. Huntington Disease as a Neurodevelopmental Disorder and Early Signs of the Disease in Stem Cells. Mol Neurobiol. 2017 May 11. doi: 10.1007/s12035-017-0477-7.*

Moja rola w przygotowaniu tej publikacji polegała na projektowaniu, pisaniu i korekcie manuskryptu, przygotowywaniu rycin i tabel oraz przeprowadzeniu analizy bioinformatycznej.

2. *Szlachcic WJ, Wiatr K, Trzeciak M, Figlerowicz M, Figiel M. The generation of mouse and human Huntington disease iPS cells suitable for in vitro studies on huntingtin function. Front Mol Neurosci. 2017 Aug 08. doi: 10.3389/fnmol.2017.00253.*

Moim udziałem w powyższych badaniach była analiza aktywacji ścieżki ERK w komórkach YAC128-iPSC, hodowla neuronalnych komórek macierzystych oraz analizy ekspresji białek metodą western blot w tych komórkach.

Z poważaniem

K. Wiatr

Kalina Wiatr

Oklahoma City, 06. września 2017

OŚWIADCZENIE WSPÓŁAUTORSTWA

W niniejszym oświadczeniu pragnę wskazać mój udział w publikacjach, w których jestem współautorką wraz z Wojciechem Szlachcicem.

1. *Wiatr K, Szlachcic WJ, Trzeciak M, Figlerowicz M, Figiel M. Huntington Disease as a Neurodevelopmental Disorder and Early Signs of the Disease in Stem Cells. Mol Neurobiol. 2017 May 11. doi: 10.1007/s12035-017-0477-7.*

Moja rola w przygotowaniu tej publikacji polegała na poszukiwaniu referencji, pisaniu fragmentów manuskryptu oraz przygotowaniu rycin.

2. *Szlachcic WJ, Wiatr K, Trzeciak M, Figlerowicz M, Figiel M. The generation of mouse and human Huntington disease iPS cells suitable for in vitro studies on huntingtin function. Front Mol. Neurosc. 2017 Aug 08. doi: 10.3389/fnmol.2017.00253.*

Moim udziałem w powyższych badaniach była hodowla neuronalnych komórek macierzystych oraz analizy ekspresji białek metodami immunofluorescencji i western blot w tych komórkach.

Marta Trzeciak

Marta Trzeciak

© 2015. Published by The Company of Biologists Ltd | Disease Models & Mechanisms (2015) 8, 1047-1057 doi:10.1242/dmm.019406

RESEARCH ARTICLE

Huntington disease iPSCs show early molecular changes in intracellular signaling, the expression of oxidative stress proteins and the p53 pathway

Wojciech J. Szlachcic, Pawel M. Switonski, Wlodzimierz J. Krzyzosiak, Marek Figlerowicz and Maciej Figiel*

ABSTRACT

Huntington disease (HD) is a brain disorder characterized by the late onset of motor and cognitive symptoms, even though the neurons in the brain begin to suffer dysfunction and degeneration long before symptoms appear. There is currently no cure. Several molecular and developmental effects of HD have been identified using neural stem cells (NSCs) and differentiated cells, such as neurons and astrocytes. Still, little is known regarding the molecular pathogenesis of HD in pluripotent cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Therefore, we examined putative signaling pathways and processes involved in HD pathogenesis in pluripotent cells. We tested naïve mouse HD YAC128 iPSCs and two types of human HD iPSC that were generated from HD and juvenile-HD patients. Surprisingly, we found that a number of changes affecting cellular processes in HD were also present in undifferentiated pluripotent HD iPSCs, including the dysregulation of the MAPK and Wnt signaling pathways and the dysregulation of the expression of genes related to oxidative stress, such as *Sod1*. Interestingly, a common protein interactor of the huntingtin protein and the proteins in the above pathways is p53, and the expression of p53 was dysregulated in HD YAC128 iPSCs and human HD iPSCs. In summary, our findings demonstrate that multiple molecular pathways that are characteristically dysregulated in HD are already altered in undifferentiated pluripotent cells and that the pathogenesis of HD might begin during the early stages of life.

KEY WORDS: Huntington disease, MAPK pathway, SOD1, Wnt pathway, iPS, iPSC, p53 pathway

INTRODUCTION

Huntington disease (HD) is a hereditary dominant neurodegenerative disorder evoked by the excessive expansion of the CAG repeats in the *HTT* gene (The Huntington's Disease Collaborative Research Group, 1993). The mutant *HTT* allele produces mutant huntingtin protein containing a long polyglutamine region, which evokes pathological changes in cellular physiology resulting in neuronal death and the degeneration of neuronal networks within the brain. Pathologic changes in neuronal brain cells, for instance in the cerebral cortex and striatum, elicit the development of chorea and cognitive impairments and lead to earlier patient death (Papoutsi et al., 2014; Roos, 2010).

Although the symptoms of HD appear relatively late, during the third and fourth decade of life, the impact of the presence of mutant and normal huntingtin can be identified substantially earlier. First, HD can be detected in affected individuals decades before the classical neurological diagnosis is reached (Paulsen et al., 2008). In both affected humans and HD mouse models, neuronal degeneration in the cortex and striatum and dysregulation of neurodevelopmental pathways in these brain structures occurs long before the onset of classical HD symptoms and phenotypic changes (Cummings et al., 2006; Hodges et al., 2006; Kuhn et al., 2007; Milnerwood et al., 2006; Schippling et al., 2009). In addition, the presence of huntingtin is required during the early stages of embryogenesis and during differentiation (Conforti et al., 2013; Duyao et al., 1995; Woda et al., 2005; Zeitlin et al., 1995; Nasir et al., 1995). Mutant huntingtin can induce the precocious specification of a neuroectodermal fate (Nguyen et al., 2013). In HD mouse models, stem-cell-mediated neurogenesis in the striatum and the deployments of the core pluripotency factors SOX2 and NANOG are impaired (Molero et al., 2009). Similarly, the development of the cerebral cortex is impaired in HD mice (Molina-Calavita et al., 2014). In summary, several studies have suggested that the pathogenic process of HD might start early in life, and it has been postulated for some time that the pathological processes might develop successively, starting at birth (Penney et al., 1997). The questions that remain are how early the molecular and functional changes related to HD occur, and which of them occurs first.

Recently, an HD phenotype was demonstrated in HD neural stem cells (NSCs) and HD neuronal cells that were differentiated from patient induced pluripotent stem cells (iPSCs) (An et al., 2012; Mattis et al., 2015; The HD iPSC Consortium, 2012). In addition, treating iPSCs with a proteasome inhibitor induced the production of HD inclusions (Jeon et al., 2012). Moreover, iPSCs generated from R6/2 mice showed defects in the expression of proteins involved in lysosomal formation, cholesterol synthesis and pluripotency (Castiglioni et al., 2012). Therefore, we investigated the HD pathogenic processes and markers using both HD YAC128 iPSCs that we generated and human HD iPSC lines. Both cell types showed a number of dysregulated cellular processes, indicating that these events might be among the earliest markers of HD. The dysregulated processes included ERK signaling, β-catenin phosphorylation, SOD1 accumulation and the expression of p53, which is a common interactor of ERKs and GSK3β. In summary, we demonstrate that a host of key HD pathogenic processes are active under the embryonic stem cell (ESC)-like cellular conditions that are characteristic of iPSCs. Our data indicate that the pathogenesis and alteration of signal transduction in HD begins in the early stages of life.

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TRANSLATIONAL IMPACT

Clinical issue

Huntington disease (HD) is one of several genetic neurodegenerative diseases that are caused by the expansion of trinucleotide CAG repeats in certain causative genes (the huntingtin gene in the case of HD). The classical motor and cognitive symptoms of HD are associated with the expression of mutant huntingtin mRNA and protein, which are particularly toxic for neurons, and occur late, during the third and fourth decades of life. Interestingly, some alterations, such as neuronal degeneration and dysregulation of neurodevelopmental pathways, are detectable in both human patients and HD cell and mouse models long before classical HD symptoms manifest. This suggests that the disease process begins substantially earlier than indicated by the onset of classical HD symptoms. The questions that remain are how early in the disease process the molecular and functional changes related to HD occur, and which of them occurs first. Recent data indicate that transcription factors and other proteins involved in intracellular cascades might be responsible for the early disease alterations seen in HD.

Results

In this study, to identify early markers of HD, the authors used human HD-induced pluripotent stem cells (iPSCs) from HD- and juvenile-HD-affected individuals, and generated a model of undifferentiated naïve cells (HD YAC128 iPSCs). These cells expressed mutant huntingtin and resembled embryonic stem cells (ESCs). None of the tested cells contained reprogramming factors that could affect the occurrence of non-specific signs of HD. The authors discovered that some key molecular pathways, including the ERK1/2 and Wnt pathways, certain oxidative-stress-related genes and p53 are dysregulated in HD iPSCs. These results suggest that dysregulation of such signaling pathways is a very early event in the pathogenesis of HD and that these alterations occur in cells at the stage of pluripotency.

Implications and future directions

Detecting early molecular signs of HD already in pluripotent cells can change and advance our understanding of the onset of HD, which is typically regarded as an age-related disease. The current data indicate that the HD presymptomatic stage is characterized by early molecular processes that might lead to the symptomatic stage of the disease. This study also identified candidate molecules that can be further investigated as potential targets for early protective therapeutic approaches and for validating anti-HD drugs.

RESULTS

Generation of iPSCs using skin fibroblasts isolated from the YAC128 HD model

We generated HD iPSC lines by reprogramming adult skin fibroblasts isolated from YAC128 (Slow et al., 2003), YAC128/Oct-eGFP and wild-type (WT) mice. The fibroblasts were reprogrammed using a piggyBac transposon-based system (Yusa et al., 2009, 2011) that drove the expression of five reprogramming factors (OCT3/4, SOX2, KLF4, cMYC and LIN28), which we adapted for use with adult fibroblasts (supplementary material Fig. S1A). We established more than 50 lines of primary iPSCs containing the genome-integrated reprogramming cassette. For the seamless excision of the reprogramming cassette, the selected clones were electroporated with the piggyBac transposase construct and negatively selected through incubation with the thymidine-kinase substrate fialuridine (FIAU). The surviving colonies were individually harvested and confirmed to be free of the transposon through PCR genotyping (supplementary material Fig. S1B). Five HD and six WT iPSC cell lines were selected for further analysis. The morphology of the selected cell lines was indistinguishable from that of the control mouse ESCs. RT-PCR analysis of the selected cell lines revealed expression of the pluripotency markers *Sox2*, *Klf4*, *cMyc*, *Esrrb*, *Utf1*, *Nanog*, *Oct3/4*, *Cripto*, *Dnmt3l*, *Rex1*, *Ecat1*, *Eras*, *Zfp296*, *Dax1* and *Dppa* (Fig. 1A). The expression levels of the analyzed markers were comparable to those observed in mouse ESCs. Both HD and WT iPSCs exhibited similar expression levels of OCT3/4 mRNA and protein (supplementary material Fig. S2A,B), and the iPSC lines generated from YAC128/Oct-eGFP cells showed green fluorescence corresponding to Oct-eGFP expression (Fig. 1B). The HD and WT iPSCs also showed similar expression levels of other pluripotency genes (*Dax1*, *Dnmt3l* and *Rex1*; supplementary material Fig. S2A) and of alkaline phosphatase activity, which is another hallmark of pluripotency (Fig. 1C). The HD and WT iPSC colonies both exhibited intense nuclear immunostaining for OCT3/4 and NANOG, and cell-surface immunostaining for SSEA1 (Fig. 1D). To analyze their differentiation potentials, the HD and WT iPSCs were tested using *in vitro* and *in vivo* differentiation assays. Following the formation of embryoid bodies, the cells readily differentiated into neuronal lineage cells, showing long neuronal processes that were positive for TUJ1 expression (Fig. 1E). Cells of lineages originating from the other germ layers were also present, e.g. beating cardiomyocytes (data not shown). Upon injection into SCID and FVB/J mice, our iPSCs formed teratomas in which we detected tissues originating from all three germ layers (supplementary material Fig. S3).

Neuronal differentiation of YAC128 iPSCs preferentially increased the level of mutant huntingtin protein

The YAC128 and YAC128/Oct-eGFP iPSC lines contained a human *HTT* transgene (supplementary material Fig. S4A) and expressed both mutant human huntingtin mRNA (supplementary material Fig. S4B) and protein (supplementary material Fig. S4C). In immunoblots, the mutant huntingtin protein was represented by bands of noticeably lower intensity compared with those of mouse huntingtin. Similarly, bands of mutant huntingtin of lower intensity than that of the bands of normal huntingtin were observed in samples of human 4281 HD fibroblasts. We investigated the levels of the expression of the mutant and normal huntingtin proteins upon iPSC differentiation. We found that, in cells that differentiated toward neurons, the level of mutant huntingtin was increased several fold, whereas the degree of increase in mouse WT huntingtin was not significant (Fig. 2; time of differentiation×allele interaction, P=0.0192; main effect of time, P=0.0002; two-way ANOVA; Bonferroni post-test, *P<0.01). Our results indicated that mutant huntingtin had accumulated in neuronal cells at an early stage of differentiation.

YAC128 iPSCs showed a decrease in MAPK-ERK pathway signaling

The ERK pathway has been implicated in HD pathogenesis (Bowles and Jones, 2014), and upregulation of its activity might be beneficial in mouse HD models (Maher et al., 2011). Therefore, we investigated the profile of ERK activation in YAC128 iPSCs expressing human mutant huntingtin and investigated whether HD cells in an ESC-like state exhibited dysregulation of the ERK pathway. We stimulated both YAC128 and WT iPSCs using bFGF for 5, 10 or 15 min and compared their levels of ERK pathway activation (Fig. 3A). We observed different profiles of induction of this pathway for WT and HD YAC128 iPSCs (time×genotype interaction, P=0.0013; main effect of time, P<0.0001; two-way ANOVA). In WT iPSCs, we observed a maximum of approximately

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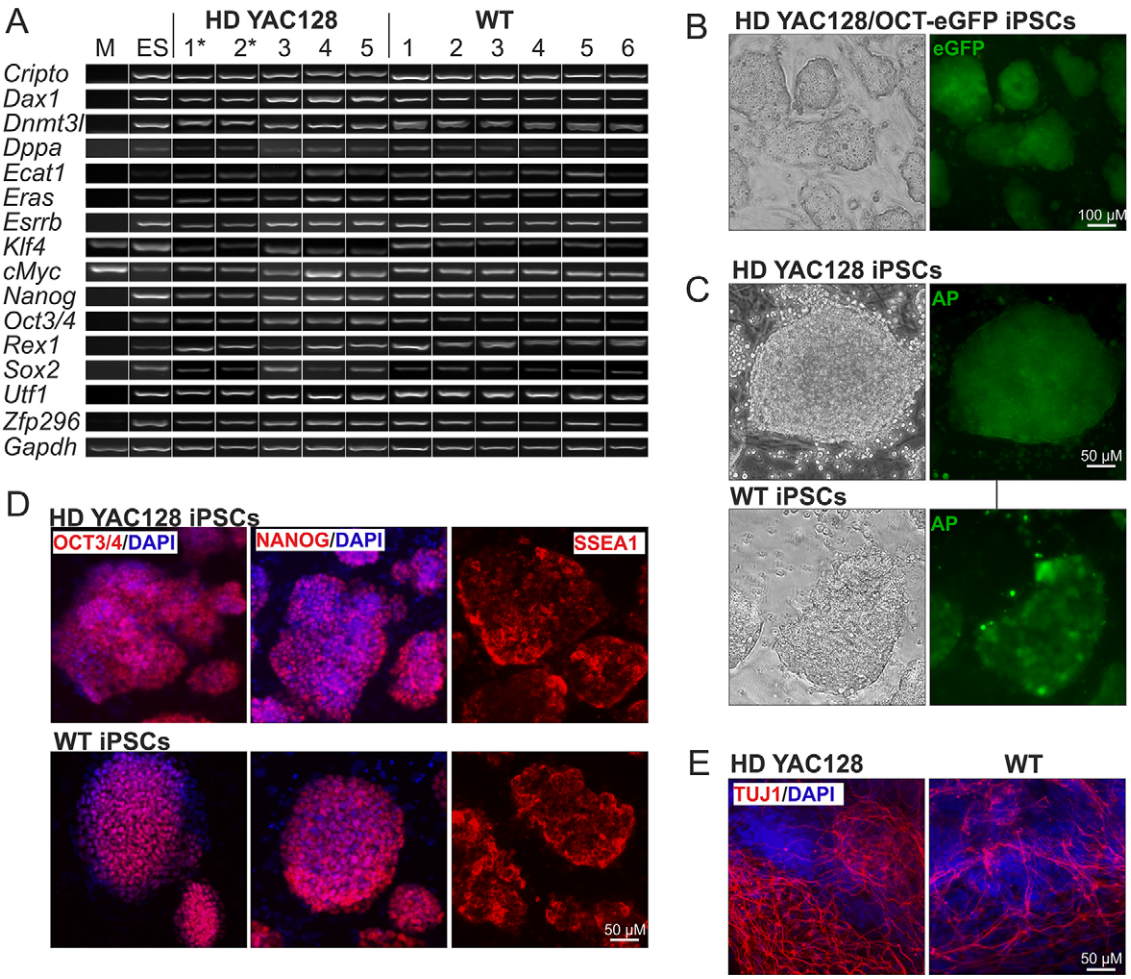

Fig. 1. Generation of iPSCs using cells isolated from the YAC128 HD model. (A) Detection of pluripotency markers in HD YAC128 iPSCs (numbered clones), wild-type (WT) mouse iPSCs (numbered clones), a mouse embryonic stem cell line (ES) and mouse embryonic fibroblasts (M) using RT-PCR analysis. * indicates individual YAC128/Oct-eGFP cell lines. (B) The YAC128/Oct-eGFP iPSC lines showed eGFP fluorescence, indicating the expression of endogenous Oct3/4 protein. (C) Alkaline phosphatase (AP) was present in HD YAC128 and WT iPSCs. (D) The HD YAC128 and WT iPSCs demonstrated the expression of Oct3/4, Nanog and SSEA1 proteins, as determined using immunostaining. (E) Immunostaining for neuronal marker Tuj1 after 14 days of *in vitro* differentiation of HD YAC128 and WT iPSCs.

a 29-fold increase in the ERK1/2 phosphorylation at 5 min compared with basal levels, whereas the level of activation observed at 15 min after stimulation had decreased to 11-fold above basal levels. In YAC128 iPSCs, we observed a similar peak of ERK1/2 activation at 5 min after stimulation. However, the activation of ERK1/2 recorded at 10 min after stimulation had decreased 2.3-fold, whereas the level of ERK1/2 activation in WT iPSCs remained unchanged ($P<0.01$, Bonferroni post-test; Fig. 3B). In addition, we found that the level of basal activation of ERK1/2 in YAC128 iPSCs was 1.56-fold higher than that in WT iPSCs, although this difference was not significant ($P=0.08$). Moreover, the analyses of the levels of the total ERK1 and 2 proteins revealed that the total ERK1 protein levels were significantly lower in the YAC128-iPSC cells (Fig. 3C,D).

HD YAC128 iPSCs showed increased phosphorylation of β-catenin

The Wnt pathway is another transduction cascade that has been implicated in the pathogenesis of HD (Godin et al., 2010); therefore, we questioned whether YAC128 iPSCs also showed dysregulation of the Wnt pathway. We monitored the phosphorylation of β-catenin at serines 33/37, 41/45 and 675 using phospho-specific antibodies. Western blotting analysis revealed an increase in the level of β-catenin phosphorylation at serines 33/37 in YAC128 iPSCs versus WT iPSCs (Fig. 4A,B), whereas the levels of serines 41/45 and 675 phosphorylation were similar in the YAC128 and WT iPSCs (supplementary material Fig. S5). However, there was no difference in levels of total β-catenin protein in these cells. Interestingly, a trend toward an increased expression of *Gsk3β* mRNA in the YAC128 iPSCs was observed (Fig. 4C); however, this change was not significant.

The YAC128 iPSCs demonstrated increased SOD1 expression and the presence of other SOD1-immunoreactive compounds

HD is associated with the increased production of free radicals and the resultant oxidative stress, which leads to neuronal dysfunction (Hands et al., 2011; Valencia et al., 2013). Antioxidant-related proteins defend cells from oxidative stress and free radicals. To determine whether the expression of such proteins was induced in early undifferentiated HD YAC128 iPSCs, we investigated the expression levels of *Sod1*, *Gpx1* and *Prdx1*. The *Sod1* mRNA expression level was increased in the HD YAC128 iPSCs, and the differences found in the expression levels of *Gpx1*

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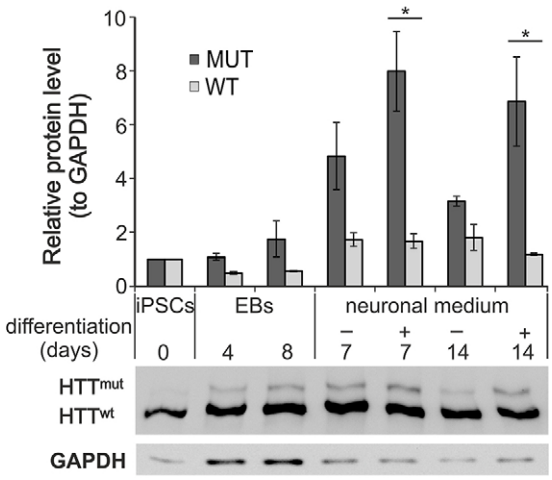

Fig. 2. The expression of mutant huntingtin increased upon the differentiation of the YAC128 iPSCs. Protein lysates for western blotting analysis were prepared on days 4 and 8 of the formation of non-adherent embryoid bodies (EB4 and EB8 lanes, respectively), on days 7 and 14 of adherent differentiation (after 4 days of EB formation) in either FBS-containing medium (–) or N2B27 neuronal medium (+). The relative level of expression of mutant huntingtin increased several fold during adherent differentiation, whereas the increase in mouse wild-type (WT) huntingtin was not significant. The level of increase in mutant huntingtin was significant under neuronal-cell culture conditions. **P*<0.01 in Bonferroni post-test.

and *Prdx1* genes were less statistically significant (Fig. 5A). Subsequently, we analyzed SOD1 protein expression levels and found that expression of its 16-kDa monomeric form was also increased in the YAC128 iPSCs (Fig. 5B,C). A similar increase in the level of SOD1 expression was observed in the human HD fibroblast cell line (GM04281, Fig. 5D). Interestingly, other SOD1-immunoreactive bands were also selectively present in the protein lysates of the YAC128 iPSCs. We detected SOD1-immunoreactive compounds with apparent molecular weights of approximately 29 and 36 kDa (Fig. 5B). These compounds might represent SOD1 oligomers, and they were virtually undetectable in the WT iPSCs.

Human HD iPSCs established from HD and juvenile HD patients showed dysregulation of HD markers

We next tested whether the phenotypes observed in mouse iPSCs could also be detected in human HD patient-derived iPSCs. Several HD iPSC lines were acquired from the NINDS repository. Two lines contained 71 CAG repeats in *HTT* and originated from a 20-year-old HD patient, and another two lines contained 109 CAG repeats and originated from a 9-year-old juvenile HD patient. All HD lines contained both normal and mutant huntingtin, whereas the control lines contained only normal huntingtin (Fig. 6A). The total protein was isolated from human iPSCs, and the expression levels of phosphorylated and total ERK1/2, phosphorylated and total β-catenin, and SOD1 were examined by immunoblotting (Fig. 6A). In our studies (two lines of each genotype; three technical replicates), we observed a twofold decrease in the phosphorylation of ERK1/2 in the juvenile HD 109 CAG line and a milder 1.4-fold decrease in the HD 71 CAG line when compared with control lines (Fig. 6B). Notably, human pluripotent stem cells are dependent on an active ERK1/2 pathway and Essential 8 medium contains bFGF. Therefore, relatively high levels of ERK1/2 phosphorylation were observed in western blots without performing additional bFGF stimulation, which was inevitable for ERK assay in mouse iPSCs. Additionally, the SOD1 expression was dysregulated in human HD iPSCs, showing an increase of 1.88- and 1.6-fold in juvenile HD 109 CAG lines and HD 71 CAG lines, respectively (Fig. 6D). This increase in SOD1 expression reached statistical significance (1-way ANOVA, $P=0.0182$; $P<0.05$, Bonferroni post-test). The levels of PRDX1 protein (Fig. 6D) and β-catenin (Fig. 6C) remained unchanged. In conclusion, the protein expression patterns observed in human HD and juvenile-HD iPSCs were similar to the changes detected in YAC128 iPSCs.

The p53 pathway was dysregulated in human HD iPSCs and YAC128 iPSCs

In our search for molecular pathways that were dysregulated in the YAC128 iPSCs, we detected altered activation profiles of the ERK and Wnt pathways and the accumulation of mutant huntingtin and SOD1. p53 is a pleiotropic molecule that binds huntingtin (Steffan et al., 2000), interacts with ERK and Wnt pathways and plays a role in

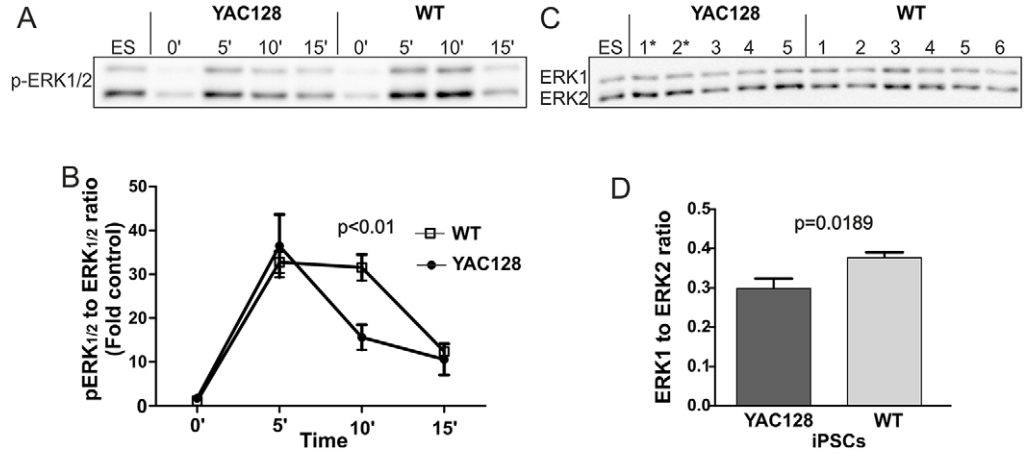

Fig. 3. The MAPK pathway is dysregulated in the YAC128 iPSCs. (A) Representative time-course of ERK1/2 phosphorylation stimulated by bFGF in the YAC128 and wild-type (WT) iPSC lines. The ES lane represents a standard protein lysate that was used to normalize the band intensity among experiments. (B) Diagram demonstrating the profile of ERK1/2 activation (levels of pERK1/2). The data for the diagram were collected by investigating the level of pERK1/2 in several different clonal lines of HD YAC128 ($n=5$) and WT iPSCs ($n=6$). ERK1/2 activation was dramatically decreased in the HD YAC128 iPSCs 10 min after stimulation using bFGF, whereas ERK1/2 activation in the WT iPSCs remained high. Two-way ANOVA; time×genotype interaction, $P=0.0013$; Bonferroni post-test. (C) Western blot analysis of the levels of total ERK1/2 proteins. (D) The analysis revealed a decreased level of total ERK1 in the YAC128 lines (*t*-test, $P=0.0189$). * indicates the YAC128/Oct-eGFP lines.

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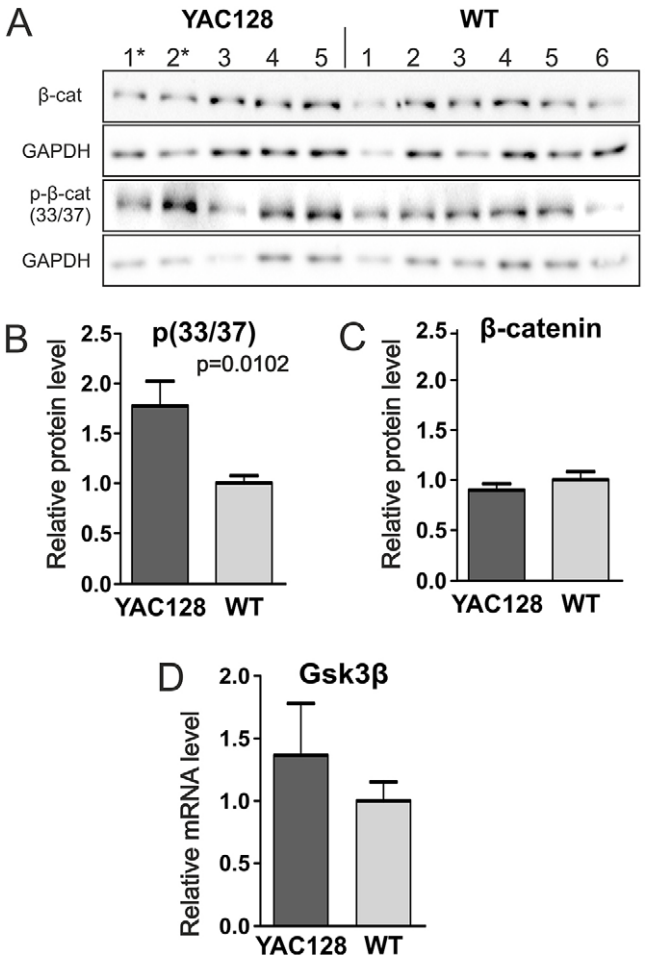

Fig. 4. The level of β-catenin phosphorylation was increased in YAC128 iPSCs. (A) The levels of total β-catenin, p-β-catenin (S33/37) and GAPDH were investigated in clonal lines of HD YAC128 (*n*=5) and wild-type (WT) iPSCs (*n*=6) using western blotting. (B) Quantification of the level of p-β-catenin (S33/37) showed increased phosphorylation in the HD YAC128 iPSC lines without changes in (C) the level of total β-catenin protein. (D) qPCR analysis revealed a trend toward the increased expression of *Gsk3β*, but the extent of the increase was not significant. * indicates the YAC128/Oct-eGFP lines.

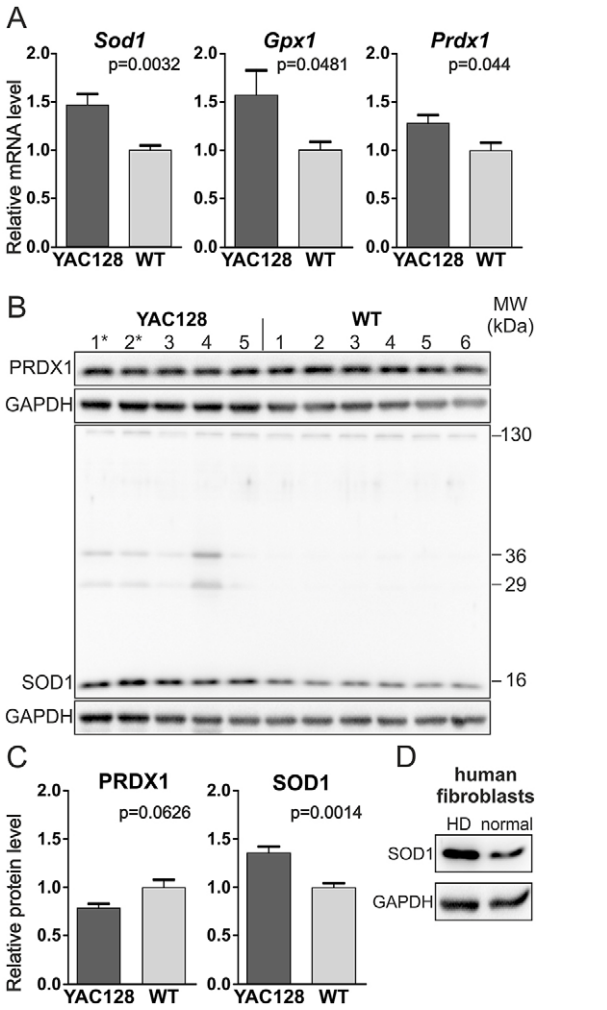

Fig. 5. SOD1 was overexpressed in the YAC128 iPSCs. (A) Real-time qPCR analysis of the expression of oxidative-stress-related genes revealed the increased expression of *Sod1* and less significant differences in the levels of *Gpx1* and *Prdx1* mRNAs. (B,C) Western blotting analyses of several clonal lines revealed the increased expression of SOD1 protein but not PRDX1 protein in HD YAC128 iPSCs. (B) Notably, additional SOD1-immunoreactive bands of approximately 29 and 36 kDa were observed in HD YAC128 iPSCs but not in WT iPSCs. * indicates the YAC128/Oct-eGFP lines. (D) Increased expression of SOD1 protein was also observed in the human HD fibroblast line GM04281 (Coriell) using western blotting.

oxidative stress. Data mining using the STRING 9.1 database (Franceschini et al., 2013) revealed that all molecules with dysregulated processes in HD YAC128 iPSCs interact with p53 (Fig. 7). In our studies, investigation of the level of p53 expression revealed the induction of the expression of *Trp53* mRNA (Fig. 8A); however, the level of the protein was strongly reduced in the HD YAC128 iPSCs (Fig. 8B,C). Interestingly, in human iPSCs, we observed a strong (tenfold) decrease in p53 protein in the juvenile HD 109 CAG line (Fig. 8D). However, this effect was absent in the HD 71 CAG line (P=0.0285, 1-way ANOVA; P<0.05, Bonferroni post-test).

Expression of other genes in HD YAC128 iPSC

The H2A histone family, member Y (*H2afy*) gene, which encodes the macroH2a1 histone variant, has been found to be a clinically relevant HD biomarker that is overexpressed in the blood (mRNA) and brains (protein) of HD patients, as well as in the brains of two HD mouse models (Hu et al., 2011). Consistent with these results, we detected the upregulated expression of *H2afy* mRNA in YAC128 iPSCs (supplementary material Fig. S6A); however, this upregulation was not accompanied by an increased level of macroH2A1 protein (supplementary material Fig. S6B).

We found no differences in the levels of mRNA expression (supplementary material Fig. S7) for genes involved in cholesterol biosynthesis (*Dhcr7a*) and the TGF-β (*Lefty1/2*) pathway, which are dysregulated in HD R6/2 iPSCs (Castiglioni et al., 2012) and in HD knock-in ESCs (Jacobsen et al., 2011), respectively.

DISCUSSION

We investigated HD molecular markers using both HD YAC128 and human HD iPSCs. YAC128 and other HD mouse models faithfully recapitulate the pattern of molecular changes observed in human postmortem caudate tissue from individuals with HD using high-throughput mRNA expression profiling (Hodges et al., 2006; Kuhn et al., 2007). The HD phenotype in the YAC128 mouse model progresses slowly and corresponds relatively well to the human condition. However, there are reports that suggest that the YAC128 phenotype is similar to juvenile HD (Brooks et al., 2012). Interestingly, its 128 CAG repeats originate from a juvenile HD patient (Slow et al.,

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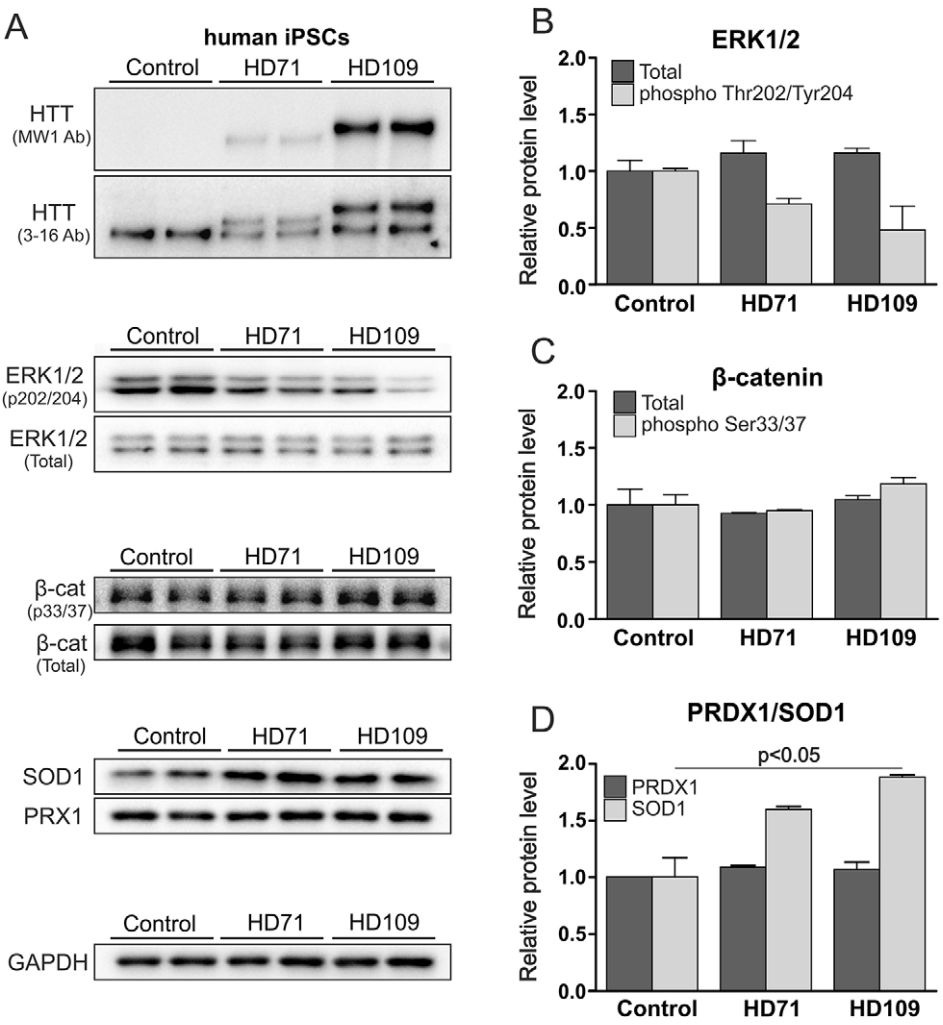

Fig. 6. Human HD iPSCs show dysregulation of HD markers. (A) HD 109 CAG and HD 71 CAG cell lines contained both normal and mutant huntingtin. Western blots demonstrate decreased ERK1/2 phosphorylation, increased SOD1 expression, and unchanged PRDX1 and β-catenin protein levels. Ctrl, control. (B) A twofold decrease in the phosphorylation of ERK1/2 in the juvenile HD 109 CAG line and a milder 1.4-fold decrease in the HD 71 CAG line were detected after quantification. (C) The levels of total and 33/37 phosphorylated β-catenin remained unchanged. (D) SOD1 expression was dysregulated in human HD iPSCs, showing an increase of 1.88- and 1.6-fold in the juvenile HD 109 CAG cells and HD 71 CAG lines, respectively (1-way ANOVA P=0.0182; P<0.05, Bonferroni post-test).

2003). Overall, the YAC128 model possesses relatively high construct and face validity, making it a good candidate for the generation of iPSCs. An important advantage of using mouse iPSCs is their defined genetic background, originating from somatic cells of donor mice. In this respect, individual human iPSC lines bring greater genetic variability, which might affect the discovery of HD-related phenotypes. In addition, mouse iPSCs exist in a more primitive 'naïve' pluripotent state compared with the more primed state of human iPSCs (Rossant, 2015). Taken together, both HD iPSC models are suitable and complementary for determining early HD phenotypes.

YAC128 and WT iPSCs with excised reprogramming cassettes expressed the mRNA and protein of key endogenous pluripotency markers, including OCT3/4 and NANOG. In addition, the iPSCs were able to differentiate into cells of lineages that represented all germ layers. Most importantly, the YAC128 iPSCs possessed a neuronal differentiation potential and expressed mutant huntingtin. The accumulation of mutant huntingtin is the major event in HD (Chan et al., 2010) and, upon the neuronal differentiation of the YAC128 iPSCs, the expression of mutant huntingtin increased at a substantially greater rate than did the expression of mouse huntingtin. This result might indicate the decreased turnover of the mutant protein, leading to its accumulation already during the early stage of neuronal differentiation. It is likely that the level of mutant huntingtin might also be affected by differences in the proliferative characteristics of iPSCs and iPSC-derived cells that were differentiating into neurons.

Mouse HD YAC128 iPSCs and human HD iPSCs exhibited divergent basal ERK phosphorylation levels because of different pluripotency cell stages. In mouse HD YAC128 iPSCs, the unstimulated level of ERK phosphorylation was almost undetectable, whereas the level observed in human iPSCs was the result of high ERK and bFGF signaling. Regardless of these differences, both HD YAC128 iPSCs and human HD iPSCs showed decreased levels of ERK phosphorylation, exhibiting altered profiles of ERK induction minutes after stimulation (HD YAC128) and decreased levels of ERK phosphorylation under conditions of constant activation of the pathway (human HD iPSC). Symptoms of early and late HD have been demonstrated in the YAC128 HD mouse model, including early susceptibility and later resistance to excitotoxicity induced by quinolinic acid as well as early hyperactivity and later decreases in activity (Slow et al., 2003). Mutant huntingtin is known to affect the activation of the MAPK signaling pathway (Bodai and Marsh, 2012), and the dysregulation pattern is biphasic, depending on the stage of HD. Young YAC128 mice show decreased phosphorylation of ERK, whereas 1-year-old YAC128 mice show an increased level of p-ERK (Gladding et al., 2014). Differences in ERK phosphorylation are also observed in the R6/2 HD mouse model, in which an increased level of phosphorylation is apparent during the later stages of the disease (Saavedra et al., 2011). Therefore, the decreased level of p-ERK in iPSCs demonstrates that the initial molecular changes associated with HD are present early on, when the cells are in the pluripotent stage.

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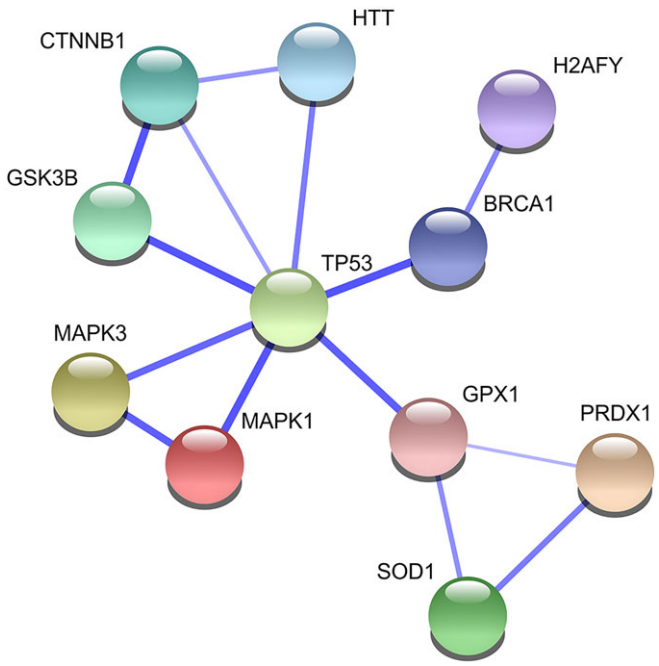

Fig. 7. Molecules participating in the pathways that were dysregulated in the HD YAC128 iPSCs interact with p53 protein. The graph was generated using data obtained from the STRING 9.1 database.

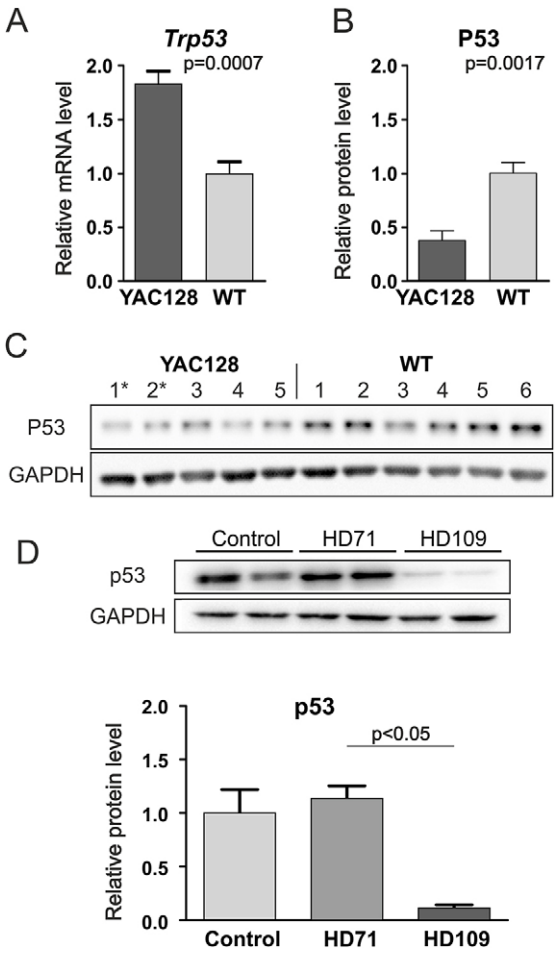

Fig. 8. The levels of p53 (*Trp53*) mRNA and protein were altered in HD iPSCs. (A) Real-time qPCR analysis revealed the increased expression of *p53* mRNA in HD YAC128 iPSCs. (B,C) Western blotting and densitometric analysis revealed that p53 protein levels were decreased in the HD YAC128 iPSCs. * indicates the YAC128/Oct-eGFP lines. (D) p53 protein levels were decreased in human juvenile HD iPSC lines but not in human HD 71 iPSC lines (P=0.0285, 1-way ANOVA; P<0.05, Bonferroni post-test).

The decrease in the level of ERK1/2 phosphorylation in the HD iPSCs could be explained by several mechanisms. Because huntingtin is responsible for the vesicular trafficking of receptors (Caviston and Holzbaur, 2009; Liot et al., 2013), a plausible explanation might be that the trafficking of fibroblast growth factor receptors (FGFRs) was altered. Other conditions that might affect ERK1/2 signaling are the recently reported enhancement of the expression of protein tyrosine phosphatase (Saavedra et al., 2011) or the activation of proteases, such as calpain (Gladding et al., 2014). Interestingly, conditions that activated ERK signaling improved the HD phenotype of model mice (Maher et al., 2011). Collectively, the data indicated that enhancing ERK1/2 signaling in non-symptomatic HD might be a valid therapeutic strategy.

Several authors have suggested that there is crosstalk between the ERK1/2 and Wnt pathways (Bikkavilli and Malbon, 2009; Ji et al., 2009). In addition, dysregulation of the Wnt pathway and the accumulation of phosphorylated forms of β-catenin both interfere with polyglutamine toxicity in cellular and *in vivo* models of HD (Carmichael et al., 2002; Dupont et al., 2012; Godin et al., 2010). Therefore, we investigated whether canonical Wnt signaling was dysregulated in HD YAC128 iPSCs and human HD iPSCs. We found that unstimulated YAC128 iPSCs maintained a higher level of β-catenin phosphorylation at positions 33/37 than WT iPSCs. Human juvenile HD 109 CAG iPSCs and HD 71 CAG iPSCs did not show changes in β-catenin phosphorylation. The differences between mouse and human iPSCs might be due to differences in the pluripotent state or in the polyglutamine (polyQ) content of huntingtin proteins.

Residue 33/37 of β-catenin is phosphorylated by GSK3β, and we detected a non-significant increase in the level of *Gsk3β* mRNA expression in HD YAC128 iPSCs. In addition, young presymptomatic HD animals accumulate GSK3β and huntingtin in lipid rafts, and inhibiting GSK3β expression increased the survival rate of HD neurons (Carmichael et al., 2002; Valencia et al., 2010). Moreover, it was demonstrated that β-catenin directly binds to FOXO to enhance FOXO transcriptional activity in mammalian cells (Essers et al., 2005). Integration of canonical β-catenin signaling, sirtuin and FOXO signaling protects against mutant huntingtin toxicity (Parker et al., 2012). In turn, FOXO neuroprotective activity is repressed by induction of Ryk expression in HD neurons (Tourette et al., 2014). Together, a decreased level of canonical Wnt activation might represent a promising marker of early HD symptoms, and restoration of dysregulated β-catenin and FOXO transcriptional activity might serve as a possible therapeutic strategy in HD. In addition, increased β-catenin phosphorylation in HD pluripotent cells might influence several future stages of neuronal differentiation. For instance, inhibition of Wnt–β-catenin signaling with Dkk1 during the mid and late stages of cortical neurogenesis suppresses neuronal production (Munji et al., 2011), whereas, in adult mice, it triggers degeneration of striatal synapses and impairs motor coordination (Galli et al., 2014).

A study of symptomatic R6/1 animals demonstrated that they had increased levels of inhibitory phosphorylation of GSK3β, suggesting the decreased activity of this molecule in the HD R6/1 model (Lim et al., 2014). Therefore, it cannot be excluded that signaling dependent on GSK3β can change, depending on the stage of HD.

In addition, the 41/45 serines of β-catenin are generally phosphorylated before serines 33/37, and we detected the

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phosphorylation of β-catenin at serines 41/45 and 675. The phosphorylation levels of serines 41/45 and 675 were relatively high and were similar in the WT and HD YAC128 iPSCs, suggesting similar activities of CK1α and PKA in our iPSCs. Normal somatic brain cells generally demonstrate low levels of phosphorylated β-catenin (Godin et al., 2010).

Oxidative stress is a molecular hallmark of HD (Ribeiro et al., 2012), and HD brain tissue shows greatly increased expression of multiple genes involved in oxidative-stress-related pathways (Sorolla et al., 2008). We also detected changes in YAC128 iPSCs regarding the expression of *Sod1*, *Prdx1* and *Gpx1*, which are genes involved in protection against oxidative stress. SOD1 expression was also increased in human HD fibroblasts and HD iPSCs. The additional SOD1-immunoreactive bands observed in mouse iPSC might represent complexes or aggregated forms of SOD1, alone or with other proteins. Interestingly, human iPSCs derived from an HD patient with 72 CAG repeats showed decreased SOD1 expression (Chae et al., 2012).

The above-mentioned dysregulation of processes involving *Mapk1* (ERK2), *Mapk3* (ERK1), *Gsk3β*, *Ctnnb1* (β-catenin), *Gpx1*, *Prdx1* and *Sod1*, and the presence of mutant HTT, seem to build a network of molecular interactions. The STRING database predicted p53 as a molecule that interacts with all of the above-identified pathways and molecules. p53 protein levels were lower in HD YAC128 iPSCs than in WT iPSCs, but the level of *Trp53* mRNA was higher in HD YAC128 iPSCs, which might be the result of increased degradation and/or the disturbed production of p53 protein in HD YAC128 iPSCs. The decreased p53 protein expression was also observed in human juvenile HD 109 CAG cells but was unchanged in human HD 71 CAG cells. Interestingly, the observed decrease in p53 protein was not detected in NSCs or differentiated HD cells, where p53 expression is typically increased and is associated with apoptotic processes (Bae et al., 2005; Ehrnhoefer et al., 2014; The HD iPSC Consortium, 2012). Summarizing, the observed dysregulation of p53 expression in HD iPSCs supports the involvement of the p53 pathway in the early stages of HD pathogenesis. In addition, further research including functional assays is needed to elucidate developmental effects of early HD changes.

The identification of very early molecular signs of HD could enhance our understanding of neurodegenerative diseases that are typically regarded as age-related processes. In particular, our findings will facilitate more precise identification of the onset of HD and will have implications for therapeutic approaches, including cellular therapy using autologous iPSCs. Moreover, our study identified early markers that can be investigated in iPSCs to validate the efficacy of drugs in cell culture as well as *in vivo*.

MATERIALS AND METHODS

HD mice and derivation of fibroblasts

YAC128 HD mice and WT littermates were obtained from The Jackson Laboratory (Bar Harbor, ME, USA; JAX Mice #004938, FVBN/J background). Additionally, for the assessment of fibroblast reprogramming, we generated a crossbreed of YAC128 and Oct-eGFP mice (Lengner et al., 2007) (JAX Mice #008214, B6;129S4/SvJae background). The cultured mouse adult fibroblasts were derived from back-skin biopsies of 8- to 10-week-old mice. Briefly, the skin fibroblasts were cultured using the explant technique in HEPES-buffered DMEM (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% FBS originating from New Zealand (Sigma-Aldrich), 2 mM L-Gln (Sigma-Aldrich), 1× antibiotic-antimycotic mixture (Sigma-Aldrich) and 10 µg/ml L-ascorbic acid (Sigma-Aldrich). Human HD fibroblasts, line GM04281, were obtained from Coriell (Coriell Cell Repository, Camden, NJ, USA). The protocols for the maintenance and handling of the animals were approved and monitored by the Local Ethical Commission for Animal Experiments in Poznan.

Generation and culturing of the mouse iPSC lines

The HD YAC128 and WT iPSCs were generated using a five-factor (Oct3/4, Sox2, Klf4, cMyc and Lin28) piggyBac transposon-based system (Yusa et al., 2009, 2011) optimized for mouse adult fibroblasts. We also established iPSCs from YAC128/Oct-eGFP crossbred mice to monitor the expression of endogenous OCT3/4 during reprogramming, culturing and differentiation.

Briefly, 2×10^6 detached fibroblasts at passages 2-4 were electroporated (BTX, ECM 830) with 6 µg of pPB-CAG.OSKML-puΔtk and 4 µg of the hyPBase plasmids in DMEM-HEPES cell suspension. Subsequently, cells were seeded onto 60-mm dishes (1×10^6 fibroblasts/dish) coated with mitomycin-C-inactivated MEF feeder cells in fibroblast medium. The medium was changed the next day (day 1). The medium used later, during reprogramming, consisted of Knockout DMEM (Life Technologies, Carlsbad, CA, USA), 2 mM L-Gln, 1× antibiotic-antimycotic mixture, 1× MEM non-essential amino acids (Sigma-Aldrich), 1000 U/ml LIF (ORF Genetics, Kopavogur, Iceland), 0.1 mM β-mercaptoethanol (Sigma-Aldrich) and 10 µg/ml L-ascorbic acid (Sigma-Aldrich) containing either 15% FBS (F15 medium) or 15% KnockOut Serum Replacement (Life Technologies; K15 medium). From day 2 to 8, the cells were grown on F15 medium supplemented with 2 mM valproic acid (VPA, Sigma-Aldrich), whereas, from day 9, F15:K15 (1:1 mix) medium lacking VPA was used. The medium was changed every 2 days. The medium used during reprogramming was supplemented with L-ascorbic acid, which was removed after the initial selection of clones. Individual iPSC clones were harvested between days 15 and 28 and were expanded in K15 medium without L-ascorbic acid, with daily medium renewal. The cells were dissociated using the TrypLE Select reagent (Life Technologies). To excise the reprogramming cassette, the iPSC clones (1×10^6 cells; passages 2-4) were electroporated with 4 µg of hyPBase and were seeded on a feeder layer. At day 3, the iPSCs were passaged and, from day 4, they were grown for 5 days under selection conditions using 0.2 µM FIAU (Moravek Biochemicals, Brea, CA, USA). After another 5 days of growth without the selection reagent, the emerging colonies were selected, expanded and genotyped to confirm the excision of the transposon. The ESCs used in the study were Gibco (C57BL/6) mouse ESCs (Life Technologies), and the TrypLE Select reagent (Life Technologies) was used to dissociate the pluripotent stem cells for passaging.

Human HD iPSCs

Human episomal HD and control iPSC lines were obtained from the NINDS Human Genetics Resource Center DNA and Cell Line Repository (https://catalog.coriell.org/1/ninds). For the analysis, we used two clonal HD lines with 71 CAG repeats (ND42228, ND42230; derived from a 20-year-old patient), two juvenile HD clonal lines with 109 CAG repeats (ND42223, ND42224; derived from a 9-year-old patient) and two control lines with 17/18 (ND41658) and 21 (ND42245) CAG repeats. Human iPSCs were cultured in chemically defined conditions in Essential 8 medium (Life Technologies) and grown on recombinant human vitronectin-coated surfaces (VTN-N, Life Technologies). Cells were passaged using gentle dissociation with 0.5 mM EDTA in PBS. Total protein was isolated after three to five passages after thawing of the NINDS samples.

Genotyping, RT-PCR and real-time qPCR

For genotyping, DNA was isolated using a Spin Column Genomic DNA Kit (Bio Basic Inc., Markham, Canada). Genotyping to determine the excision of the transposon was performed using multiplex PCR using a set of primers specific for the *Tcrd* gene (the internal wild-type control) and either pPB-OTS or pPB-KM2 primers specific for the OCT/T2A/SOX or KLF/T2A/cMYC linker in the reprogramming cassette, respectively. A list of the primers used in this study is provided in supplementary material Table S1. Genotyping to determine the presence of the YAC128 transgene was performed using a pair of primers specific for the human intronic *HTT* sequence and a pair of primers specific for the internal wild-type control

(*Tcrd*). All genotyping analyses were performed using Touchdown PCR with the following cycling conditions: 3 min at 94°C; 12× [35 s at 94°C, 45 s at 64°C (–0.5°C/cycle) and 45 s at 72°C]; 25× (35 s at 94°C, 30 s at 58°C and 45 s at 72°C); and, finally, 2 min at 72°C. The reaction products were separated on 1.3% agarose gels in TBE buffer and were stained using ethidium bromide.

The total RNA was isolated using TriReagent (MRC, Cincinnati, OH, USA) and reverse transcription was performed using SuperScript® III reverse transcriptase (Life Technologies). For the real-time qPCR, SYBR® Select Master Mix (Life Technologies) was used, and the reactions were performed using a LightCycler 480 II (Roche) instrument. The data was analyzed using LightCycler 480 software (release 1.5.1.62). For each primer pair a standard curve was prepared using the Second Derivative Maximum method. The efficiencies of standard curves and Ct values for each sample are given in supplementary material Table S2. The relative expression was calculated with the E-method with Gapdh as a reference (Ref) gene. Target/Ref ratios for each sample were normalized to mean ratio of WT samples and these values were used for statistical analyses.

Both the human *HTT* and mouse *Htt* mRNAs were amplified by RT-PCR using primers that result in products spanning the CAG-repeat-containing exon 1 and the exon 2 junction. Because the primers were designed for human *HTT* and there was one non-complementary nucleotide for the mouse *Htt* in the reverse primer, it cannot be excluded that the amplification of the human and mouse huntingtin mRNA products proceeded with slightly different efficiencies. Thus, the direct comparison of the levels of the *HTT* and *Htt* mRNAs was not possible.

Immunostaining

For immunostaining, the cells were cultured in 12-well dishes on gelatin- and feeder-cell-coated coverslips. The cells were washed using PBS, fixed by incubation with 4% PFA for 15 min at room temperature (RT), washed and permeabilized (except when immunostaining SSEA1, which is a surface marker) using 0.3% Triton X-100 in PBS for 10 min at RT. Blocking was performed in 5% normal goat serum (Jackson ImmunoResearch) in PBS for 1 h at RT, and the primary-antibody incubation was conducted overnight at 4°C in the same buffer. The primary antibodies used were as follows: anti-SSEA1 (1:500, Millipore, MAB4301); anti-OCT3/4 (1:500, Santa Cruz Biotechnology, sc-5279); anti-NANOG (1:700, Abcam, ab80892); and anti-TUJ1 (1:500, Millipore, MAB1637). After washing using PBS, the cells were incubated for 1 h at RT with a secondary antibody, ether Cy3-conjugated goat anti-mouse or anti-donkey antibodies (Jackson ImmunoResearch) at a 1:500 dilution in PBS. A 5-min incubation in DAPI (1:10,000) dissolved in water was used for counterstaining. Additionally, the primary antibodies were omitted in the negative controls. The coverslips containing the cells were mounted on slides using anti-fade glycerol/propyl gallate mounting medium. The specimens were analyzed using an Olympus IX70 fluorescence microscope and images were captured using an Olympus DP71 camera. The alkaline-phosphatase Live Stain (Life Technologies) assay was used to determine the level of alkaline phosphatase activity, according to the manufacturer's protocol.

In vitro differentiation

The iPSCs or mouse ESCs were dissociated and then transferred to differentiation medium (K15 without LIF) in non-adherent six-well plates (Corning) at a density of 5.5×10^5/well. Embryoid bodies were allowed to form until day 4, with daily changes of the medium, and then they were transferred into gelatin-coated wells containing EB medium supplemented with FBS or N2B27 instead of KSR and were cultured until day 14, with a medium change every 2 days.

Teratoma formation

A total of 1×10^6 cells suspended in 100 µl of 1:1 PBS/Geltrex (Life Technologies) solution were subcutaneously injected into both dorsal flanks of SCID mice. Teratomas generally formed in 4-8 weeks and were collected when they reached approximately 1 cm in diameter and then were transferred to 4% PFA and fixed for 1 week at 4°C. The tumors were then transferred to 10%, 20% and 30% sucrose, each for 3 days, frozen on a specimen holder and cut into 10-µm sections using a cryostat (Leica). The sections were stained using H&E.

ERK activation assay

The day before the assay was performed, the medium on mouse iPSCs was exchanged for serum-free medium without LIF, and the cells were starved for 24 h. Then, without changing the medium, 20 ng/ml bFGF was added, and the cells were incubated for 5, 10 or 15 min. After each incubation period, the medium was quickly discarded and the cells were immediately lysed using a protein-lysis buffer (see next subsection for buffer formulation). The ERK phosphorylation was directly tested in human iPSCs because the Essential 8 medium contains bFGF, which is required for growth of human iPSCs. The protein lysates were subsequently used for western blotting using ERK1/2 phospho-specific antibodies.

Western blotting

For protein isolation, the cells were washed using PBS, lysed in a protein-lysis buffer containing 60 mM Tris base, 2% SDS, 10% sucrose, 2 mM PMSF (Sigma-Aldrich) and 1× Halt Phosphatase Inhibitor Cocktail (Thermo Scientific), and homogenized. An aliquot of 20-30 µg of total protein per lane was dissolved in loading buffer containing 2-mercaptoethanol and was boiled for 5 min. The proteins were separated using SDS-PAGE (5%/10% stacking/resolving gels) and Laemmli buffer. Huntingtin was separated in 4% stacking/5% resolving gels using commercial XT Tricine running buffer (Bio-Rad), as previously described (Fiszer et al., 2013). The proteins were transferred to nitrocellulose membranes and the blots were blocked using 5% nonfat milk and 0.1% Tween 20 in TBS and were subsequently incubated overnight at 4°C with primary antibody diluted in TBS-Tween containing 5% milk or BSA. The antibodies used were purchased from Cell Signaling Technology (Leiden, The Netherlands) unless otherwise stated and were as follows: rabbit anti-β-catenin (1:1000, cat. 8480); rabbit anti-phospho-β-catenin (Ser675) (1:1000, 4176), (Thr41/Ser45) (1:1000, 9565) and (Ser33/37) (1:1000, 2009); rabbit anti-p44/42 MAPK (ERK1/2) (1:2000, 4695), rabbit anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (1:1000, 4370), rabbit anti-MacroH2A1 (1:1000, 8551); mouse anti-Oct3/4 (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA; sc-5279); mouse anti-p53 (1:1000; 2524); mouse anti-huntingtin (1:2000, Millipore, Billerica, MA, USA; MAB2166), rabbit anti-huntingtin N-terminal, amino acids 3-16 (1:1000, Sigma-Aldrich, H7540), mouse anti-polyQ [1:1000, MW1, Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA; Ko et al., 2001]; and mouse anti-GAPDH (1:10,000, Millipore, MAB374). The blots were then incubated for 2 h at RT with HRP-conjugated secondary antibodies raised against rabbit or mouse antibodies (1:2000-1:20,000 dilution, Jackson ImmunoResearch, Suffolk, UK), and the labeled bands were detected using the ECL-based SuperSignal West Pico (Thermo Scientific) substrate. All analyses were performed as three independent technical replicates.

Statistics

The two-group comparisons of the gene expression data were conducted using the unpaired Student's *t*-test. The data regarding huntingtin expression during differentiation and ERK1/2 activation were subjected to a two-way ANOVA, followed by Bonferroni post-tests. *P*-values of less than 0.05 were considered significant. Error bars on all graphs represent s.e.m.

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Competing interests

The authors declare no competing or financial interests.

Author contributions
W.J.S., M. Figiel and P.M.S. conceived, designed and performed the experiments, and analyzed the data. M. Figiel planned and executed the live animal experiments (teratoma injections). W.J.K. and M. Figlerowicz critically revised the article. W.J.S. and M. Figiel wrote the paper. M. Figiel was responsible for concept and obtaining funding.

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Supplementary Figures

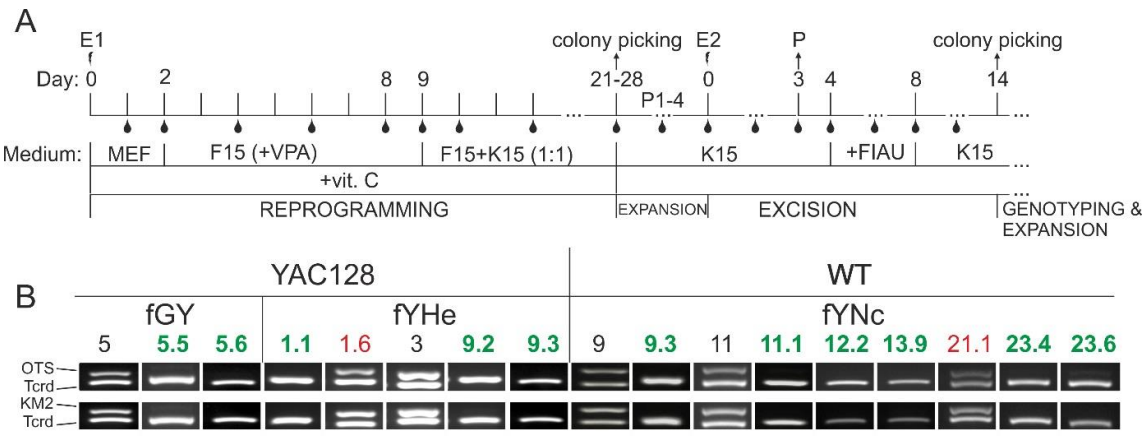

Fig. S1

Schematic illustration of the method used for iPSC generation using the piggyBAC system. (A) Reprograming of fibroblasts into iPSCs, harvesting iPSC colonies and the excision of reprogramming cassette. The black drops, P and E indicate medium changes, passage and electroporation, respectively. (B) The excision of the cassette was validated using multiplex PCR genotyping which lack of reprogramming cassette. Oct-to-Sox (OTS) was a PCR product for which primers were designed to bind to the linker between Oct3/4 and Sox2. Similarly, KM2 was a PCR product for which the primers bound to the linker between Klf4 and cMyc. The majority of the clones exhibited the excision of the cassette after selection using FIAU and are labeled with green numbers, whereas only a few of the clones still contained the cassette (red numbers). Pre-excision clones (positive control) are labeled with black numbers. Only the clones in which excision occurred (green labeled) were used in the studies described herein.

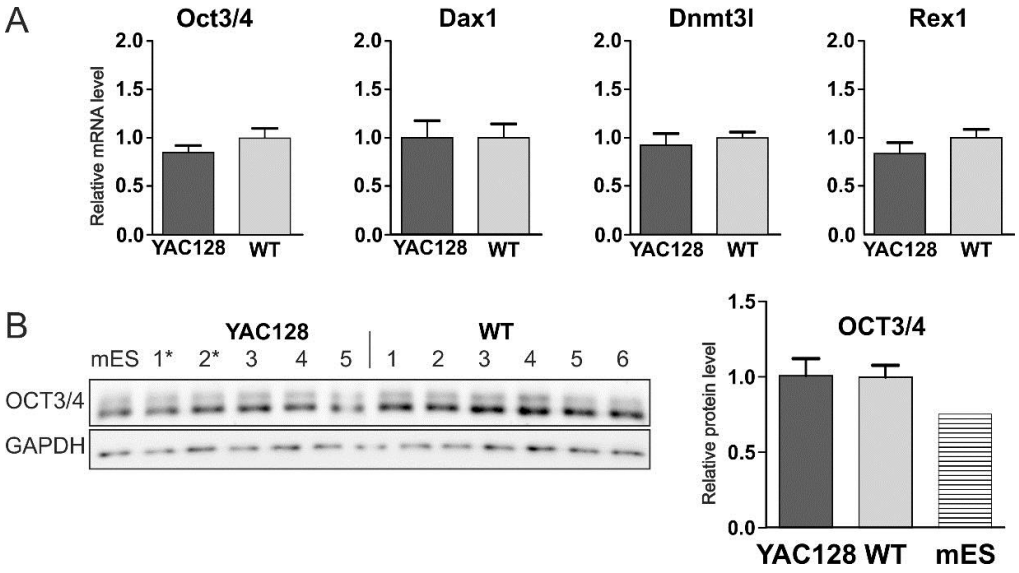

Fig. S2

Analysis of the expression of the pluripotency genes Oct3/4, Dax1, Dnmt3l and Rex1 using (A) qPCR and (B) western blotting demonstrated no differences between the YAC128 and WT lines. *YAC128/Oct-eGFP lines.

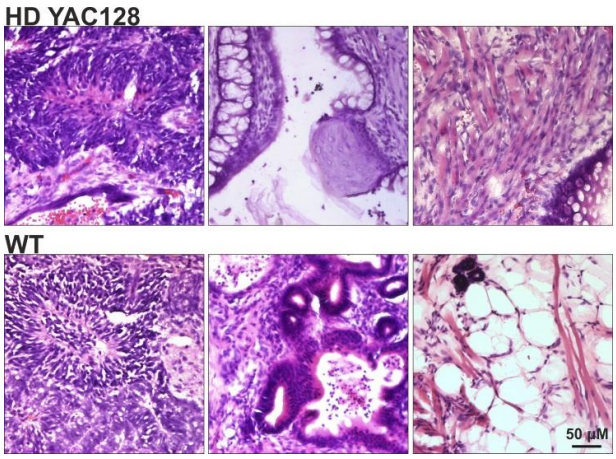

Fig. S3

Teratomas generated from HD-YAC128 and WT iPSCs contained cell types originating from the three germ layers: neuroepithelial cells (ectoderm); gut and respiratory epithelial cells, glands (endoderm); and muscle fibers, adipose tissue and cartilage (mesoderm).

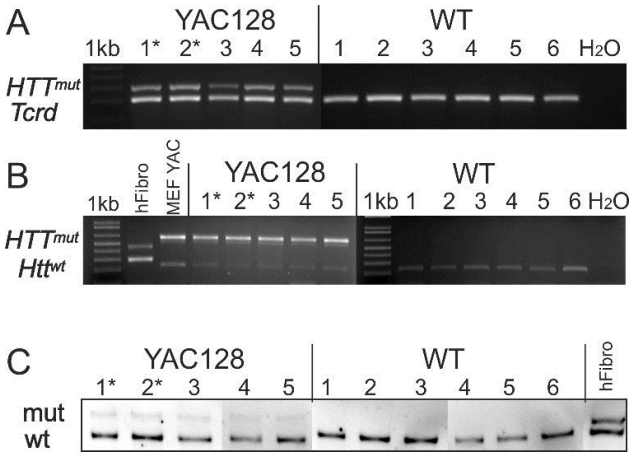

Fig. S4

Mutant huntingtin was expressed in YAC128 iPSCs (A) Genotyping for the presence of the mutant *HTT* gene in YAC128 and WT iPSCs. The multiplex touchdown-PCR reaction utilized endogenous *Tcrd* gene as the internal control (B) RT-PCR analysis of *HTT* mRNA expression. (C) Western blotting analysis of huntingtin expression in the HD YAC128 and WT iPSCs. (*) YAC128/Oct-eGFP lines; hFibro, human HD fibroblasts (GM04281, Coriell) containing 17 and 68 CAGs; MEF YAC, MEFs obtained from YAC128 mice.

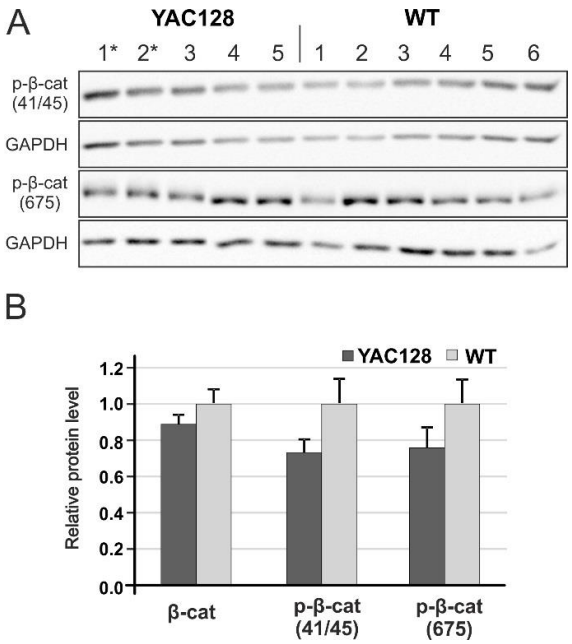

Fig. S5

The levels of p41/45-β-catenin and p675-β-catenin were similar in YAC128 iPSCs and WT iPSCs. (A) The levels of p41/45-β-catenin, p675-β-catenin and GAPDH were investigated in clonal lines of HD YAC128 (n=5) and WT iPSCs (n=6) using western blotting. (B) The quantification diagram for the levels of expression of p41/45-β-catenin and p675-β-catenin demonstrates similar degrees of activation in the HD YAC128 and WT iPSC lines. *YAC128/Oct-eGFP lines.

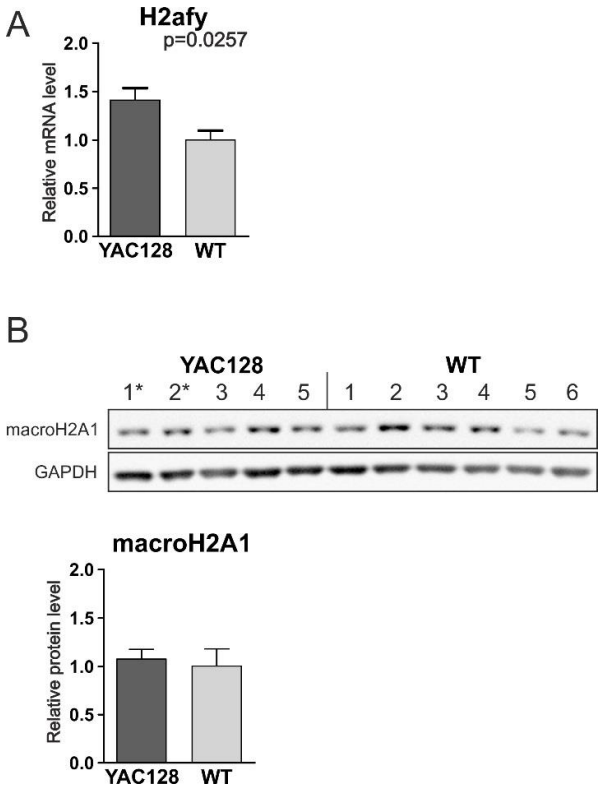

Fig. S6

The expression of the *H2afy* gene was increased (A) in the YAC128 iPSCs. The level of the resulting histone macroH2A1 (B) protein was similar in YAC128 and WT iPSCs. *YAC128/Oct-eGFP lines.

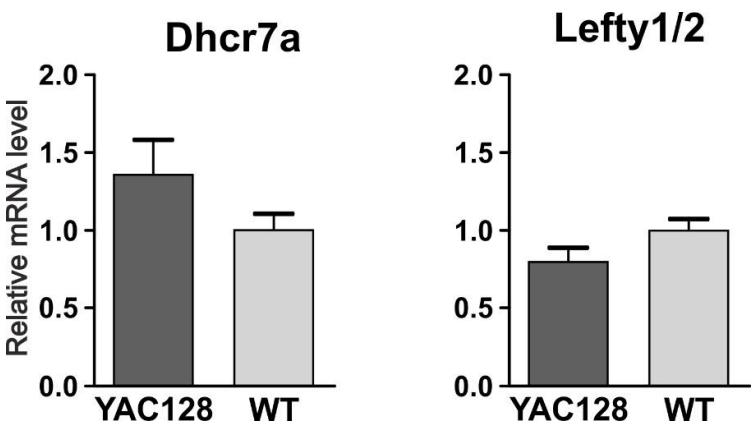

Fig. S7

The levels of mRNA expression of genes that play roles in lipid metabolism (*Dhcr7*), and signaling pathways (*Lefty1/2*) were similar in YAC128 and WT iPSCs, as revealed using real-time qPCR analysis.

SUPPLEMENTARY TABLE 1. List of primers used

Primer name		Sequence 5' -> 3'	Comment/Source
Genotyping			
pPB-OTS	F	TTCCCAACGAGAAGAGTATGAGGCTACA	transposon removal
	R	GCTCCGTCTCCATCATGTTATACATTGG	
pPB-KM2	F	AGGCGAGAAACCTTACCACTGTGACTG	transposon removal
	R	AGTCGAGGTCATAGTTCCTGTTGGTGAA	
NI26_27	F	CCTCTTATATATGGATGCTAATCTCATTC	YAC128 transgene
	R	AATACACAACACATGAGAGCATATAGAAC	
Tcrd	F	CAAATGTTGCTTGTCTGGTG	internal control
	R	GTCAGTCGAGTGCACAGTTT	
RT-PCR			
mCripto (Tdgf1)	F	ATGGACGCAACTGTGAACATGATGTTCGCA	(1)
	R	CTTTGAGGTCCTGGTCCATCACGTGACCAT	
mDax1 (Nr0b1)	F	TGCTGCGGTCCAGGCCATCAAGAG	(1)
	R	GGGCACTGTTCAGTTCAGCGGATC	
mDnmt3l	F	CCCTCTTCCTGTATGATGATGATGG	(2)
	R	CCTCTGCAGCAGTCCACTCCGTGAG	
mDppa3	F	GAGGACGCTTTGGATGATACAGACG	(2)
	R	CAACAAAGTGCGGACCCTTCTCTTG	
mEcat1 (Khdc3)	F	TGTGGGGCCCTGAAAGGCGAGCTGAGAT	(1)
	R	ATGGGCCGCCATACGACGACGCTCAACT	
mERas	F	ACTGCCCCTCATCAGACTGCTACT	(1)
	R	CACTGCCTTGTACTCGGGTAGCTG	
mEsrrb	F	AACCTGCCGATTTCCCCACCTGCTA	(2)
	R	GGCTCATCTGGTCCCCAAGTGTCAG	
mGapdh	F	AATGGTGAAGGTCGGTGTG	
	R	AAGATGGTGATGGGCTTCC	
emKlf4 endo	F	GGCGAGAAACCTTACCACTGT	(3)
	R	TACTGAACTCTCTCTCCTGGCA	
emcMyc endo	F	TCAAGCAGACGAGCACAAGC	(3)
	R	TACAGTCCCAAAGCCCCAGC	
emNanog endo	F	GTGCATATACTCTCTCCTTCCC	(3)
	R	AGCTACCCTCAAACTCCTGGT	
emOct3/4 endo (Pou5f1)	F	CCAACGAGAAGAGTATGAGGC	(3)
	R	GTGCTTTTAATCCCTCCTCAG	
mRex1 (Zfp42)	F	ACGAGTGGCAGTTTCTTCTTGGGA	(1)
	R	TATGACTCACTTCCAGGGGGCACT	
emSox2 endo	F	TCTGTGGTCAAGTCCGAGGC	(3)
	R	TTCTCCAGTTCGCAGTCCAG	
mUtf1	F	GGATGTCCCGGTGACTACGTCTG	(1)
	R	GGCGGATCTGGTTATCGAAGGGT	
mZfp296	F	CCATTAGGGGCCATCATCGCTTTC	(1)
	R	CACTGCTCACTGGAGGGGGCTTGC	
HD-dl	R	CACGGTCTTTCTTGGTAGCTG	human huntingtin mRNA (mismatch with mouse sequence) ; (4)
	F	CCCTGGAAAAGCTGATGAAG	

qPCR			
qmDax1 (Nr0b1)	R	ATCTGCTGGGTTCTCCACTG	(5)
	F	CTATCTGAAAGGGACCGTGC	
qmDnmt3	R	GCTTGCTCCTGCTTCTGACT	(5)
	F	GGTGTGGAGCAACATTCCAG	
qmOct4 (Pou5f1)	R	TCTTCTGCTTCAGCAGCTTG	(5)
	F	GTTGGAGAAGGTGGAACCAA	
qmRex1 (Zfp42)	R	TATGACTCACTTCCAGGGGG	(5)
	F	AGAAGAAAGCAGGATCGCCT	
Dhcr7	R	TGAGGTCACAGACGACCAAT	(5)
	F	ACAGGCCAGTCTGATGGAAG	
Gapdh	R	TTGATGGCAACAATCTCCAC	(5)
	F	CGTCCCGTAGACAAAATGGT	
Gpx1	R	CAATGTAAAATTGGGCTCGAA	(5)
	F	GTTTCCCGTGCAATCAGTTC	
GSK3β	R	GTGGTTACCTTGCTGCCATC	(5)
	F	GACCGAGAACCACCTCCTTT	
H2afy	R	TGCTCACTTCTCCCTGCTTC	
	F	GCCAAAAAGGCCAAGTCTCC	
Lefty1/2	R	TGCAGTAGACTGCTCAGGACC	(5)
	F	CATGAAGTCCCTGTGGCTTT	
Prdx1	R	TTGATGGTATCACTGCCAGG	(5)
	F	CCGCTCTGTGGATGAGATTA	
Sod1	R	TACTGATGGACGTGGAACCC	(5)
	F	GAACCATCCACTTCGAGCA	
Trp53	R	TCCGACTGTGACTCCTCCAT	(5)
	F	CTAGCATTCAGGCCCTCATC	

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SUPPLEMENTARY TABLE 2.

Click here to Download Table S2

frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
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The Generation of Mouse and Human Huntington Disease iPS Cells Suitable for *In vitro* Studies on Huntingtin Function

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Huntington disease (HD) is an incurable neurodegenerative disorder caused by expansion of CAG repeats in huntingtin (HTT) gene, resulting in expanded polyglutamine tract in HTT protein. Although, HD has its common onset in adulthood, subtle symptoms in patients may occur decades before diagnosis, and molecular and cellular changes begin much earlier, even in cells that are not yet lineage committed such as stem cells. Studies in induced pluripotent stem cell (iPSC) HD models have demonstrated that multiple molecular processes are altered by the mutant HTT protein and suggested its silencing as a promising therapeutic strategy. Therefore, we aimed to generate HD iPS cells with stable silencing of HTT and further to investigate the effects of HTT knock-down on deregulations of signaling pathways e.g., p53 downregulation, present in cells already in pluripotent state. We designed a gene silencing strategy based on RNAi cassette in piggyBAC vector for constant shRNA expression. Using such system we delivered and tested several shRNA targeting huntingtin in mouse HD YAC128 iPSC and human HD109, HD71, and Control iPSC. The most effective shRNA (shHTT2) reagent stably silenced HTT in all HD iPS cells and remained active upon differentiation to neural stem cells (NSC). When investigating the effects of HTT silencing on signaling pathways, we found that in mouse HD iPSC lines expressing shRNA the level of mutant HTT inversely correlated with p53 levels, resulting in p53 level normalization upon silencing of mutant HTT. We also found that p53 deregulation continues into the NSC developmental stage and it was reversed upon HTT silencing. In addition, we observed subtle effects of silencing on proteins of Wnt/β-catenin and ERK1/2 signaling pathways. In summary, we successfully created the first mouse and human shRNA-expressing HD iPS cells with stable and continuous HTT silencing. Moreover, we demonstrated reversal of HD p53 phenotype in mouse HD iPSC, therefore, the stable knockdown of HTT is well-suited for investigation on HD cellular pathways, and is potentially useful as a stand-alone therapy or component of cell therapy. In addition, the total HTT knock-down in our human cells has further implications for mutant allele selective approach in iPSC.

Keywords: Huntington disease, iPS cells, NS cells, YAC128, shRNA, huntingtin, p53, juvenile HD

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1. INTRODUCTION

Huntington disease (HD) is an incurable autosomal dominant neurodegenerative disorder caused by CAG repeat expansion in exon 1 of the huntingtin (HTT) gene (The-Huntington's-Disease-Collaborative-Research-Group, 1993). A prominent feature of HD is neuronal loss, with medium spiny neurons predominantly affected (Bates et al., 2015). Disease pathogenesis is primarily caused by the presence of mutant HTT that contains a polyQ stretch of over 40 glutamines, encoded by the CAG repeats; however, RNA toxicity might also be involved (Marti, 2016; Urbanek et al., 2016) The polyglutamine tract in the protein interferes with the physiological activity of the HTT protein, causing both loss of function and acquisition of new toxic functions (Bates et al., 2015). HTT is a multifunctional protein that is both essential in development and important for adult brain homeostasis (Wiatr et al., 2017). Mutant HTT alters multiple physiological pathways, including transcriptional regulation, signal transduction, apoptosis, intracellular vesicle trafficking, cytoskeleton assembly, and centrosome formation, making the disease pathology highly complex (Bates et al., 2015).

Despite such profound and widespread effects of mutant HTT on cellular function, disease onset usually occurs at age 30–50, and its average duration is 15–20 years (Bates et al., 2015). In rare cases of longer CAG tracts (70 or more CAG repeats), HD can develop early in life, with onset before age 20 or in childhood; such cases are called juvenile HD (Squitieri et al., 2006; Quigley, 2017). Interestingly, the process of neurodegeneration in distinct brain regions can be observed many years before the onset of motor symptoms (Tabrizi et al., 2013), even in typical HD. Although, traditionally considered a late-onset neurodegenerative disorder, a growing amount of compelling evidence has suggested that HD may be considered a neurodevelopmental disease (Wiatr et al., 2017). HTT is essential in development; lack of HTT expression results in embryonic lethality in mice at E6.5 (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). Embryos of HdhQ111 mice, a model with mild HD features, exhibit an altered cell cycle and impaired differentiation of striatal neural progenitor cells, resulting in abnormal striatal development at E13.5–E18.5 (Molero et al., 2009). Moreover, cortical and striatal synaptic development is similarly disturbed in HD and conditional HTT knockout models (McKinstry et al., 2014). Another study has shown that the expression of mutant HTT only during mouse development is sufficient to induce HD-like phenotypes (Molero et al., 2016).

Recently, this new idea about a developmental role for mutant HTT has been strongly supported by a growing amount of research using new cellular models, including patient-derived induced pluripotent and neuronal stem cells (iPSCs and NSCs, respectively) (Mattis and Svendsen, 2015; Zhang et al., 2015; Wiatr et al., 2017). We previously demonstrated that similar molecular changes can be observed in the iPSC stage in both YAC128 mouse- and juvenile HD patient-derived cells (Szlachcic et al., 2015). The common alterations included decreased MAPK (mitogen-activated protein kinase) signaling activity and increased expression of the antioxidative protein SOD1 (superoxide dismutase 1). Finally, expression of p53 protein, which interacts with HTT and is involved in the above pathways, was decreased in both YAC128 mouse- and juvenile HD iPSCs. In addition, results from HD patient tissues and animal models demonstrate involvement of multiple signaling pathways, including the MAPK and p53 pathways, in HD pathogenesis (Bowles and Jones, 2014; Wiatr et al., 2017).

Gene silencing is one of the therapeutic strategies (Kordasiewicz et al., 2012; Miniarikova et al., 2016; Rué et al., 2016) which can potentially be used for neurodegenerative disease treatment such as cell therapy to correct patient cells or to determine how the level of mutant protein (e.g., HTT) interferes with the deregulated disease pathways. Therefore, our aim was to establish stable silencing of HTT in mouse and human HD iPS cells and subsequently to investigate the effects of HTT knock-down on deregulations of signaling pathways characteristic for HD. We designed a gene silencing strategy based on RNAi cassette in piggyBAC vector for constant shRNA expression. The HD lines with stable expression of anti HTT shRNA possess the same genetic background as the parental lines (i.e., they are isogenic) therefore another aim of isogenic line generation in the present work was the improved quality of comparison of HD phenotypes between genetically similar lines with and without stable HTT knockdown. For this we have selected the most effective HTT silencing reagents and investigated MAPK, Wnt, and p53 deregulations, which are important molecules affected in HD.

2. MATERIALS AND METHODS

This study was carried out in accordance with the recommendations of Local Ethical Commission for Animal Experiments in Poznan. The protocol was approved by the Local Ethical Commission for Animal Experiments in Poznan.

2.1. Mouse iPS Cells Culture

The HD YAC128 and WT iPSC lines were described previously (Szlachcic et al., 2015). These lines were reprogrammed using the piggyBac transposon system (Yusa et al., 2009, 2011) and were shown to be free of the reprogramming cassette after its seamless excision. Cells were cultured on gelatin-coated mitomycin C-inactivated mouse embryonic fibroblast (MEF) feeders in a medium consisting of Knockout Dulbeccos modified Eagle medium (DMEM), 15% KnockOut Serum Replacement (both Thermo Fisher Scientific, Waltham, MA), 2 mM L-Gln, 1x antibiotic antimycotic mixture, 1x MEM non-essential amino acids, 0.1 mM β-mercaptoethanol (all SigmaAldrich, St. Louis, MO), and 1,000 U/mL leukemia inhibitory factor (LIF, ORF Genetics, Kopavogur, Iceland). iPSCs were passaged with TrypLE Select (Thermo Fisher Scientific).

NSCs medium consisted of a 7:3 mixture of DMEM with Hams F12 Nutrient mix, 2% B27 supplement, 1x CTS GlutaMAX-I supplement, 1x penicillin-streptomycin (all Thermo Fisher Scientific), 5 μg/mL heparin (Sigma-Aldrich), 20 ng/mL basic fibroblast growth factor (bFGF), and 20 ng/mL

Abbreviations: HD, Huntington disease; iPSCs, induced pluripotent stem cells; NSC, neural stem cells; HTT, huntingtin.

epidermal growth factor (EGF; both ORF Genetics). Floating NSCs were derived from iPSCs by gentle dissociation of colonies with collagenase type IV (Thermo Fisher Scientific). The enzyme was aspirated while colonies were still attached to a plate followed by detachment with a cell scraper in DMEM/F12 plus 0.075% bovine serum albumin (BSA) Fraction V (both Thermo Fisher Scientific), and collection with a 5-mL pipette. Cell clumps were then centrifuged for 3 min at 1,300 rpm. Clumps were gently resuspended in NSC culture medium with the bFGF and EGF concentrations increased to 100 ng/mL, and the cells were seeded onto wells that had been precoated with polyHema (Santa Cruz Biotechnology, Dallas, TX, USA) to prevent adhesion. One near-confluent well of iPSCs was used for the induction of NSCs in two wells of a 6-well plate. The medium was changed every other day by allowing the spheres to settle to the bottom of a tube, after which the old medium was aspirated, and the spheres were gently resuspended in fresh medium and returned to the plates. NSCs were passaged every 4–6 days using a chopping method (Svendsen et al., 1998; Ebert et al., 2013). After 2–3 passages, bFGF and EGF concentrations were reduced to 20 ng/mL.

2.2. Human iPS Cells Culture

Human episomal HD and control iPSCs lines were previously acquired (Szlachcic et al., 2015) from public repository (NINDS Human Genetics Resource Center DNA and Cell Line Repository; https://catalog.coriell.org/1/ninds). For establishing the lines containing the stable expression of the reagents, we used HD lines with 71 CAG repeats (HD71; ND42228; derived from a 20-year-old patient), juvenile HD line with 109 CAG repeats (HD109; ND42224; derived from a 9-year-old patient), and a control line with 21 CAG repeats (ND42245). Human iPSCs were cultured in Essential 8 medium (Life Technologies) on human vitronectin-coated surfaces (VTN-N, Life Technologies) and were passaged using 0.5 mM EDTA in PBS.

2.3. Construct and Isogenic Line Derivation

Constructs (**Figure 1A**) composed of a U6 promoter, a miR-30 5′ flank (151 bp), an shRNA sequence, a miR-30 3′ flank (128 bp), a U6 terminator (TTTTTT), an EF1alpha promoter, an mOrange2 reporter gene, and an SV40 pA site were were synthesized by Genscript (Piscataway, NJ) and cloned into a pPB-HKS-neoL vector obtained, by removing the EGFP reporter gene, from a pPB-UbC.eGFP-neo plasmid (Yusa et al., 2009). The shRNA sequences (**Figure 1B**, Table S1) targeting human huntingtin (shHTT) and EGFP (control reagent, shCTRL) were designed using the RNAi Codex database (Olson et al., 2006) with a mir-30 loop between the passenger and guide strands. The allele-specific shCAG reagent targeting the CAG tract in mutant HTT was adapted from ref. (Fiszer et al., 2013), along with the miR-25 loop. To generate cell lines stably expressing the shRNA construct, 0.56 x 10^6 cells from two iPS lines derived from YAC128 animals were electroporated with 10 μg of the piggyBac transposase-encoding plasmid (hyPBase) (Yusa et al., 2011) and 2 μg of each shRNA plasmid in HEPES-buffered DMEM. Cells were seeded in K15 medium and selected on G418 (300 μg/mL) (Thermo Fisher Scientific) for 8 days. For derivation of clonal lines, after another 7 days without selection, colonies expressing the mOrange2 reporter gene were picked and expanded. In the case of human iPSCs, the cells were gently detached in clumps containing several cells. For each electroporation, 1/3 well of a confluent 6-well plate was used. The same plasmid concentration, electroporation, and selection protocols were used as for mouse cells. After the antibiotic selection all cells were mOrange2 positive and were passaged after reaching confluence. Material for protein expression analysis was collected after at least three passages.

2.4. PCR Genotyping

For genotyping, DNA was isolated using a Spin Column Genomic DNA Kit (Bio Basic Inc., Markham, Canada), and GoTaq G2 polymerase (Promega GmbH, Mannheim, Germany) was used for PCR. Genotyping to confirm insertion of the shHTT and shGFP constructs was performed using multiplex PCR with a set of primers specific for the YAC128 transgene [intron 26–27 of human HTT; forward (F): 5′-CCTCTTATA TATGGATGCTAATCTCATTC-3′ and reverse (R): 5′-AAT ACACAACACATGAGAGCATATAGAAC-3′] as the internal control, and primers specific for the construct. The forward, universal primer (U6: 5′-CGGCAGCACATATACTAGTCGA-3′) was designed to be in the U6 promotor-miR30 boundary, while the reverse primers were specific for each construct (shHTT: 5′-GCCTCTATATATTCTGGGCGCT-3′, shCTRL: 5′-GAAGTTCACCTTGATGCCGG-3′). The genotyping analyses were performed using Touchdown PCR with the following cycling conditions: 3 min at 94°C; 12 x (35 s at 94°C, [45 s at 64°C - 0.5°C/cycle], and 45 s at 72°C); 25 x (35 s at 94°C, 30 s at 58°C, and 45 s at 72°C); and finally, 2 min at 72°C. Genotyping for the CAG-composed shCAG construct was conducted using two pairs of primers in separate reactions: pair 1 with the universal U6 forward primer and the shCAG-specific reverse primer (A2_R: 5′-TGTGACAGGAAGCAGCTGC-3′); and pair 2 with the shCAG-specific forward primer (A2_F: 5′-CTGCTGCTGCTTTGCCTACT-3′) and the universal EF1a-promoter specific primer (EF1a: 5′-GGGGCGAGTCCTTTTGTATGA-3′). Standard PCR cycling was used for these reactions. Reaction products were separated on 1.3% agarose gels in TBE buffer and were visualized using ethidium bromide.

2.5. ERK Activation Assay

The ERK assay in iPSCs was performed as described previously (Szlachcic et al., 2015). Briefly, the day before the start of experiments, the medium was exchanged for serum-free medium without LIF, and the cells were starved for 24 h. Then, without changing the medium, 20 ng/mL bFGF was added, and the cells were incubated for 5, 10, or 30 min. After each incubation period, the medium was quickly discarded, and the cells were immediately lysed using a protein-lysis buffer. As NSC culture media containing bFGF and NSCs depend on the MAPK signaling pathway, the basal levels of pERK1/2 were measured in cell lysates taken directly from cultures.

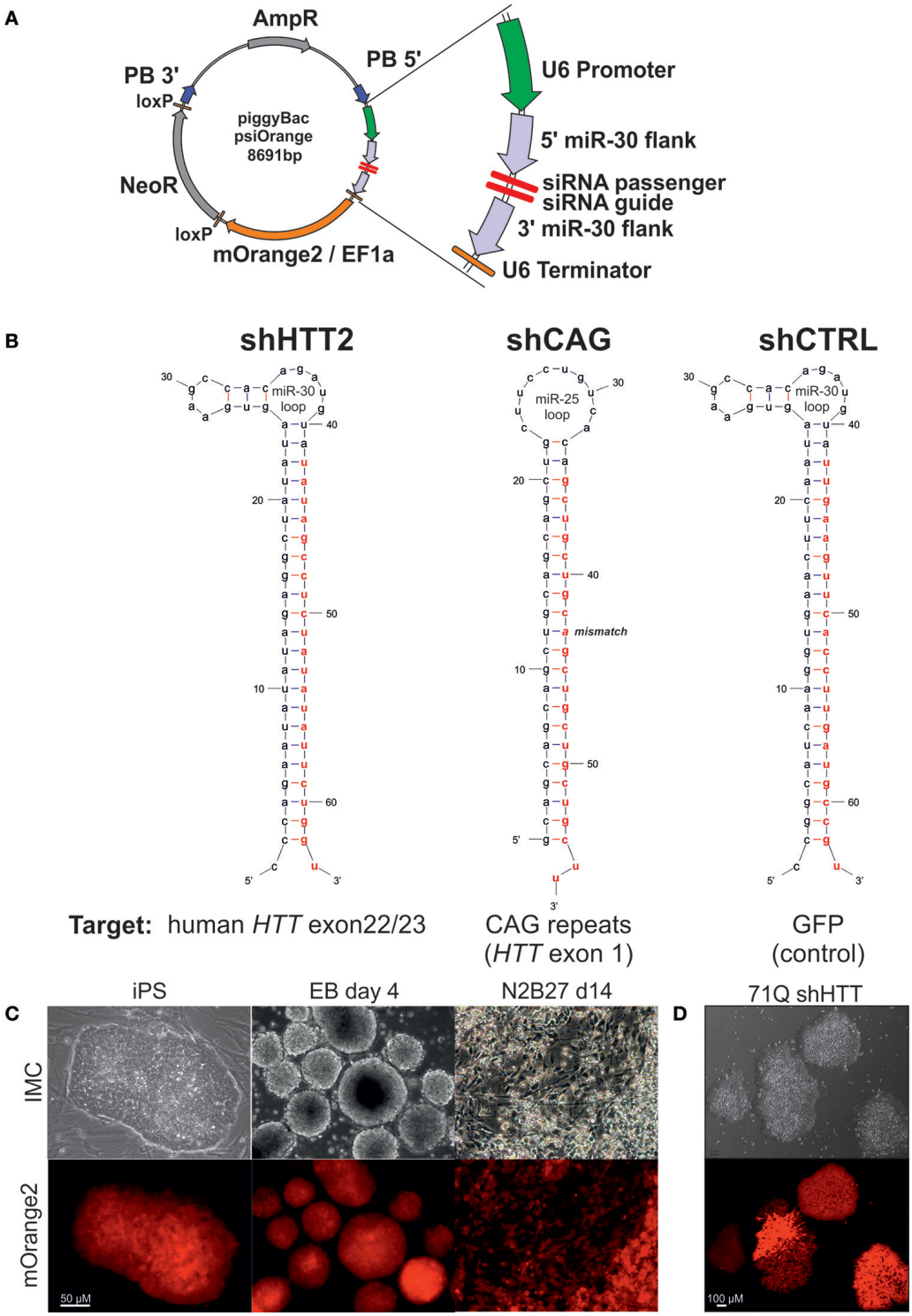

FIGURE 1 | The silencing cassette design and derivation of the iPSC lines. **(A)** Schematic of the psiOrange vector. The silencing cassette is inserted into the iPSC genome as a piggyBac (PB) transposon bordered by 5′ and 3′ PB arms. An shRNA is expressed under regulation of a U6 promoter and is flanked by pri-miR-30 5′ and 3′ sequences, which are 151 and 128 bp long, respectively. Additionally, the mOrange2 fluorescent reporter and NeoR resistance genes are included within the transposon. **(B)** Schematic of shRNA sequences. Effector guide strands are marked in red. **(C)** mOrange reporter expression is sustained in the mouse iPSC state and upon differentiation as embryonic bodies (EB) or neuroectoderm (N2B27 conditions). **(D)** mOrange reporter is expressed in human iPSCs with shRNA cassettes.

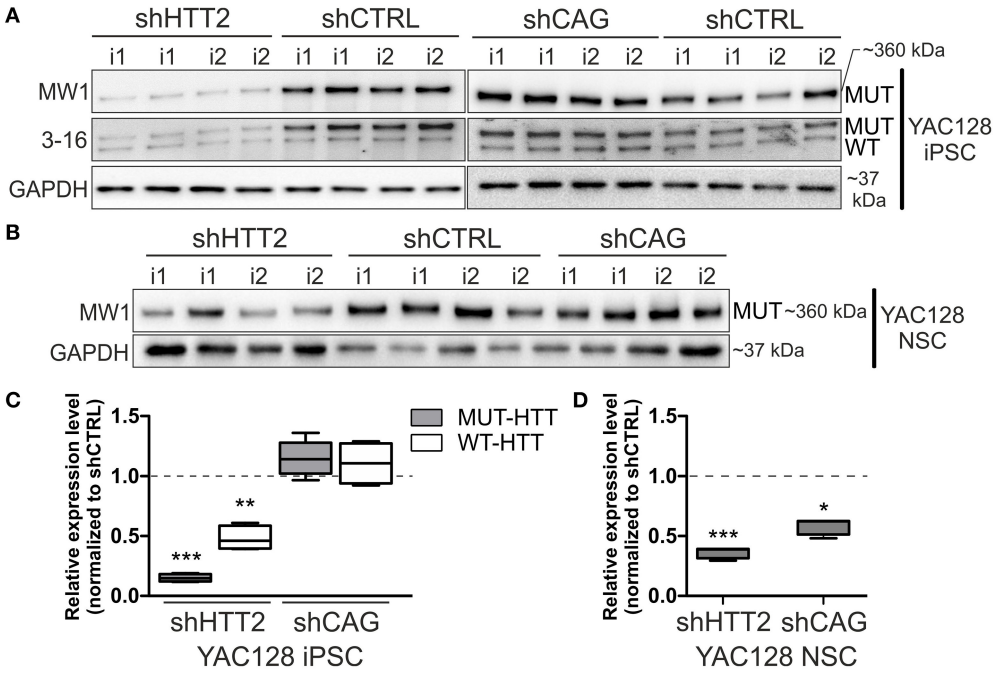

FIGURE 2 | Isogenic YAC128 iPSC and NSC lines with efficient shRNA-mediated silencing of mutant HTT. **(A,B)** Western blot analysis reveals efficient mutant HTT silencing in iPSC lines with shHTT2 but not shCAG reagent, compared to shCTRL lines. **(C,D)** Mutant HTT is continuously silenced by the shHTT2 reagent after iPSCs differentiating into the NSC state, as assessed by western blots. However, the shCAG reagent changes its mode of action, decreasing mutant HTT expression in NSCs. *$p<0.05$, **$p < 0.01$, ***$p<0.001$; $N = 4$ lines for each reagent for both iPSC and NSC analysis (the same lines were used); i1, i2 isogenic lines derived from separate parental lines 1 and 2. In (**A**, iPSCs) blots were cropped; full-length blots are presented in Figure S8.

2.6. Western Blotting

For protein isolation, the cells were washed using PBS, lysed in a protein-lysis buffer containing 60 mM Tris base, 2% SDS, 10% sucrose, 2 mM PMSF, and 1x Halt Phosphatase Inhibitor Cocktail (Thermo Scientific), and then homogenized. An aliquot of 20–30 μg of total protein per lane was dissolved in loading buffer containing 2-mercaptoethanol and was then boiled for 5 min. The proteins were separated using SDS-PAGE (5/10% stacking/resolving gels) and Laemmli buffer. For comparison of NS WT vs HD cell lines, we used 10% TGX Stain-free FastCast Acrylamide gels (Bio-Rad, Hercules, CA, USA). HTT was separated in 4% stacking/5% resolving gels using commercial XT Tricine running buffer (Bio-Rad). The proteins were semi-dry-transferred (Transblot Turbo, Bio-Rad) to nitrocellulose or PVDF (huntingtin) membranes and the blots were blocked using 5% nonfat milk in TBS-Tween. Blots were subsequently incubated overnight at 4°C with primary antibody diluted in TBS-Tween containing 5% milk or BSA. The antibodies used were purchased from Cell Signaling (Danvers, MA) unless otherwise stated and were as follows: rabbit anti-β-catenin (1:1,000, cat. 8480); rabbit anti-phospho-β-catenin (Ser33/37) (1:1,000, cat. 2009); rabbit anti-p44/42 MAPK (ERK1/2) (1:2,000, cat. 4695); rabbit anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (1:1,000, cat. 4370); mouse anti-p53 (1:1,000, cat. 2524); rabbit anti-p53 (DO-1, 1:600, Santa Cruz, sc-126); mouse anti-phospho-p53 (S15) (1:1,000, cat. 9284); mouse anti-OCT3/4 (1:1,000, Santa Cruz, sc-5279); mouse anti-nestin [Rat-401 (Hockfield and McKay, 1985), 1:100; DSHB, Iowa City, IA]; rabbit anti-PAX6 (1:1,000; Millipore, Billerica, MA; AB2237); rabbit anti-SOX1 (1:1,000, cat. 4194); mouse anti-TUBB3 [6G7 (Halfter et al., 2002), 1:100, DSHB]; anti-huntingtin antibodies: mouse MW1 (Ko et al., 2001) (1:1,000, DSHB), 4–19 (Macdonald et al., 2014) (1:1,000; CH00146, CHDI Foundation, Corriel Cell Repositories), 3–16 (1:1,000; Sigma-Aldrich; H7540), and MAB2166 (1:2,000, Millipore); and mouse anti-GAPDH (1:10,000, Millipore, MAB374). The blots were then incubated for 2 h at RT with HRP-conjugated secondary antibodies raised against rabbit or mouse antibodies (1:2,000–1:20,000 dilution, Jackson ImmunoResearch, West Grove, PA), and the labeled bands were detected using the ECL-based WesternBright Quantum (Advansta Inc., Menlo Park, CA) or homemade ECL reagent. Data was collected using ChemiDoc XRS+ System with Image Lab v5.2 Software (Bio-Rad). To avoid overexposure of any band, image acquisition times were set based on image histograms. Images were not processed before quantitation. All analyses were performed as three independent technical replicates. Data within a gel were normalized to GAPDH or total protein (WT vs, HD NSC analyses), and data between gels were normalized to the average of WT or isogenic shGFP samples.

2.7. Immunostaining

For immunostaining, the cells were cultured in 24-well dishes on gelatin- and feeder cell-coated coverslips. The cells were washed

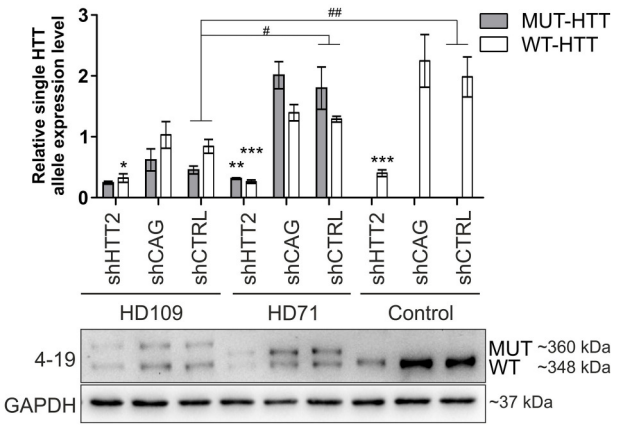

FIGURE 3 | Huntingtin can be effectively and stably silenced with shRNAs in human HD and control lines Western blot analysis of mutant (MUT) and wild-type (WT) huntingtin protein expression in iPSC lines from patients with 109 CAGs (HD109) and 71 CAGs (HD71) and healthy controls (Control, 21 CAGs) with shRNA reagents reveal efficient silencing with the shHTT2 reagent. Despite differences in initial alleles expression levels between cell lines the reagent lowered both alleles to similar levels. *Statistically significant difference vs. isogenic shCTRL line; #Statistically significant difference vs. Control shCTRL line; * and #p <0.05; ** and ##p<0.01; ***p<0.001. Note that the results for Control lines were normalized to reflect the level of one HTT allele (densitometry values of detected HTT level in control lines was divided by 2). For each patient, one cell line per each reagent was used (9 modified cell lines in total).

using PBS, fixed by incubation with 4% paraformaldehyde for 15 min at RT, washed, and permeabilized using 0.3% Triton in PBS for 10 min at RT. Blocking was performed in 3% BSA, 0.1% Tween-20 in PBS for 30 min at RT, and the primary-antibody incubation was conducted overnight at 4°C in an antibody dilution solution composed of 5% normal serum of secondary antibody species (Jackson Immunoresearch) and 0.1% Tween-20 in PBS. The primary antibodies used were as follows: anti-OCT3/4 (1:500, Santa Cruz, sc-5279), rabbit anti-nestin (1:400, Abcam, ab27952), mouse anti-nestin (1:50, DSHB, Rat-401), rabbit anti-PAX6 (1:50, Millipore, AB2237), rabbit anti-SOX1 (1:100, cat. 4194), and mouse anti-TUBB3 (Tuj1) (1:400, Millipore, MAB1637). After washing with PBS, the cells were incubated for 1 h at RT with a proper Cy3- or AlexaFluor488-conjugated secondary antibody (1:500, Jackson Immunoresearch) in the antibody dilution solution. A 5-min incubation in DAPI (1:10,000) dissolved in water was used for counterstaining. Additionally, the primary antibodies were omitted in the secondary antibody controls. The coverslips containing the cells were mounted on slides using anti-fade glycerol/propyl gallate mounting medium. The specimens were analyzed using a DMIL LED inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany) and Leica Application Suite Software. Confocal microscopy was performed using a Leica TCS SP5 microscope.

2.8. Statistics

Two-group comparisons of the gene expression data were conducted using the unpaired Students *t*-test. The data for ERK1/2 activation in iPSCs were subjected to a two-way ANOVA, followed by Bonferroni *post-hoc* tests. Pearsons simple correlation was used to determine relationships between mutant HTT and other analyzed protein expression levels. *P*-values of less than 0.05 were considered significant. Whiskers in box plots represent 5–95 percentile, while error bars on bar graphs are presented as SEM.

Full description of methods is provided in the Supplementary Materials online.

3. RESULTS

3.1. Generation of Mouse YAC128-HD-iPSCs and Human HD109, HD71, and Control iPS Isogenic Cell Lines with Stable Expression of shRNA Targeting Mutant HTT

Cell lines with continuous expression of RNAi constructs that effectively silence target genes can be used as tools in cell therapy and for the generation of shRNA isogenic lines to specifically assess the effect of mutant HTT on early HD phenotypes. We have assembled a silencing construct and stably integrated it into the iPSC genome; this construct is based on the piggyBac transposase system (Yusa et al., 2011) and contains anti-HTT or control shRNA in the mir-30 backbone (Paddison et al., 2004), and the gene encoding mOrange2 fluorescent protein (Shaner et al., 2008) as a reporter (**Figure 1A**). To establish mouse isogenic iPSC, we used our previously generated HD iPSC lines (Szlachcic et al., 2015) derived from YAC128 mice (Slow et al., 2003) and several shRNA silencing constructs. Using the constructs, we first evaluated the efficiency of 3 anti-HTT shRNAs (shHTT1-3) in iPSCs (see next section, Table S1). Then, we used the most effective reagent (shHTT2), as well as a reagent specifically targeting the CAG repeats (shCAG) (Fiszer et al., 2013) or targeting EGFP as a control (shCTRL), and we generated 12 isogenic iPSC lines expressing these shRNAs from two HD iPSC lines (two clones per line and reagent; **Figure 1B**, Table S1). The lines were genotyped for the presence of a proper shRNA construct (Figure S1). We also established human HD iPSCs expressing the sHTT2, shCAG, or shCTRL from HD109 (109 CAG repeats), HD71 (71 CAG repeats), and Control (21 CAG repeats) iPSC lines.

Floating neurospheres (non-adherent neural stem cells, NSCs) with and without the reagents were generated by iPSC differentiation and expressed characteristic cellular markers (Figures S2A–E). To investigate survival of cells containing reagents in the mouse brain, we injected the cells into the mouse striatum, and using the PACT method (Yang et al., 2014) we found that they survived for the 8-week test period (Figure S2F). The mOrange2 reporter exhibited a strong red fluorescent signal in pluripotent shRNA iPSCs, embryoid bodies and throughout adherent differentiation (**Figures 1C,D**). Summarizing, we have generated both mouse and human HD lines containing construct with several shRNA reagents targeting various parts of mRNA for human HTT able to differentiate to NSC and able to survive in mouse brain upon delivery by injection.

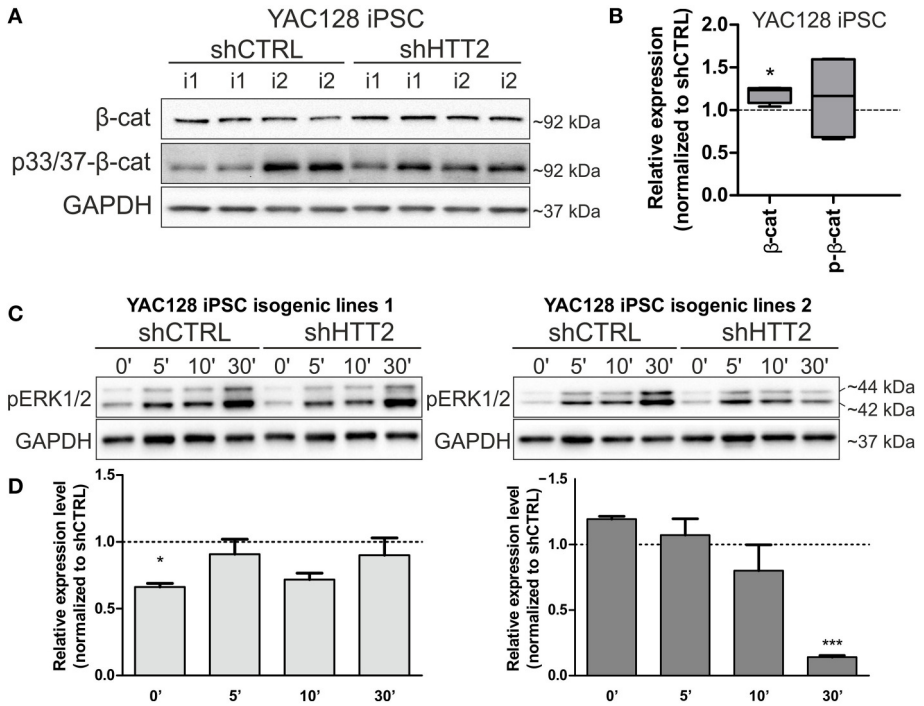

FIGURE 4 | Effects of mutant HTT knockdown on MAPK and Wnt pathways in mouse iPSCs. **(A,B)** Western blot analysis of shHTT2 effects on the Wnt pathway revealed a significant increase in total β-catenin levels but not phospho-β-catenin (S33/37) levels. $N = 4$ lines for each reagent; **(C,D)** Isogenic lines ($N = 2$ for each reagent) originating from separate parental lines (1 and 2) show different responses to bFGF-induced activation of ERK1/2 phosphorylation (Thr202/Tyr204) after 30 min of stimulation. $^{*}p<0.05$, $^{***}p<0.001$. In Panel **(A)** blots were cropped; full-length blots are presented in Figure S8.

3.2. Continuously Expressed shRNA Reagents Can Efficiently Silence Mutant HTT in Mouse iPSCs and NSCs

We first evaluated efficiency of three anti-huntingtin shRNA reagents (shHTT1, shHTT2, and shHTT3) in mouse HD iPSCs without clonal selection. shHTT1 and shHTT3 reagents lowered levels of mutant huntingtin by 54 ± 8% ($p = 0.0042$) and 35 ± 7% ($p = 0.004$), respectively (Figure S3), as assessed by western blotting. The most efficient reagent, shHTT2, which lowered HTT expression up to 85% was used in further studies and derivation of clonal mouse HD iPSC lines. The lines containing shHTT2, shCAG, or shCTRL reagents were tested for the expression of mutant and normal HTT. Western blotting with polyQ-specific antibody revealed that HTT was effectively silenced in iPSC lines containing the shHTT2 reagent (−85 ± 3%, $p = 0.0043$; shHTT2 vs. shCTRL; **Figures 2A,C**). HTT was not silenced, and in some cases was upregulated, in lines containing stable expression of the shCAG reagent; however, the upregulation was non-significant (19.5 ± 13%, $p = 0.2$; shCAG vs. shCTRL). We also analyzed the effects of shRNA reagents on expression of wild-type mouse HTT. Its expression was reduced in shHTT2-iPSC lines (−53 ± 13%; $p = 0.032$) but was unchanged in shCAG-iPSC lines.

Next, we assessed whether the effect of HTT silencing with shRNA reagents was preserved after differentiation from iPSCs into a neural lineage. Therefore, we differentiated iPSCs containing shHTT2 to the state of non-adherent NSCs in bFGF and EGF conditions (Figure S1). Similar to iPSCs, mutant HTT was also effectively silenced in shHTT-NSC lines but with a slightly lower efficiency (−62 ± 19%, $p = 0.0005$; shHTT vs. shCTRL; **Figures 2B,D**). Surprisingly, the shCAG reagent, which was previously ineffective in iPSCs, became effective in the NSC state and decreased mutant HTT protein levels by 40 ± 10% ($p = 0.01$; shCAG vs. shCTRL). Summarizing, we have selected a shHTT2 reagent which is suitable for continuous expression iPSC and evokes stable silencing of mutant HTT with high efficiency in mouse cells.

3.3. Stable Expression of shRNA Reagents Silenced Total HTT in Human HD Cells

Human HD109, HD71, and Control iPSC lines expressing the HTT targeting shHTT2 reagent revealed effective silencing of both mutant (HD109: −51 ± 22%, $p = 0.059$; HD71: −83 ± 21%, $p<0.01$) and normal HTT (HD109: −62 ± 18% $p<0.05$; HD71: −79 ± 4% $p<0.001$; Control: −80 ± 17% $p<0.001$; **Figure 3**). In addition we have also tested the total level of HTT mRNA and found its effective silencing (Figure S4). Similarly to mouse HD iPSCs, the shCAG reagent was ineffective in human iPSC lines. We have also noticed major differences in expression levels of mutant and normal HTT which seemed to be dependent on CAG length in human iPSC lines. The expression level of normal HTT and total HTT was most significantly decreased in HD109

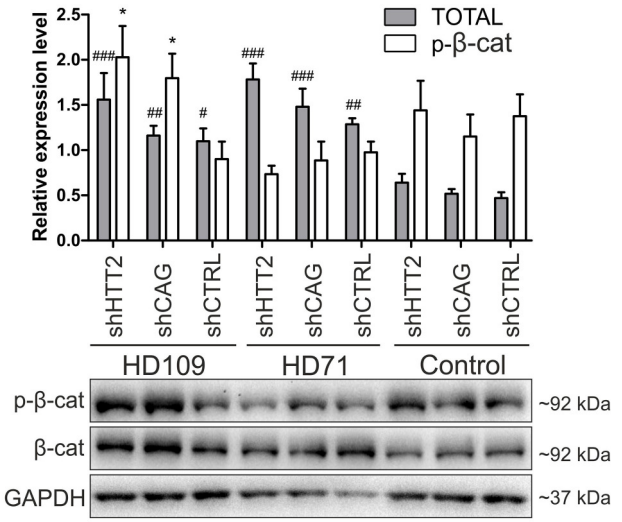

FIGURE 5 | Effects of mutant HTT knockdown on Wnt pathway in human iPSCs. Western blot analysis of shHTT2 effects on the Wnt pathway revealed a significant increase in total β-catenin levels in HD109 and HD71 lines as compared to healthy Control lines, but no effects of shRNA reagents. Phospho-β-catenin (S33/37) did not differ between lines with different CAG numbers, however its level was affected by both effective shHTT2 and ineffective shCAG reagents in HD109 iPSCs only. *Statistically significant difference vs. isogenic shCTRL line; #Statistically significant difference vs. Ctrl shCTRL line; * and #$p < 0.05$; ##$p<0.01$; ###$p<0.001$. For each patient, one cell line per each reagent was used (9 modified cell lines in total).

(normal allele: $-63 \pm 17.5\%$ $p<0.05$ as normalized to a single allele; total HTT level: $-72 \pm 16\%$, $p<0.01$; HD109 vs. Control) while the level of normal HTT have revealed the trend toward decreased HTT level in HD71 (normal allele: $-39 \pm 17\%$ $p = 0.0599$ as normalized to a single allele; total HTT level: $-25 \pm 17\%$ $p =$ ns; HD71 vs. Control). Moreover, HD109 had lower protein expression level of both HTT alleles as compared to HD71 lines (mutant allele: $-78 \pm 21\%$, $p< 0.01$; normal allele: $-40 \pm 10\%$ $p<0.05$; total HTT: $-62 \pm 15\%$ $p<0.05$). Regardless of the basal level of the HTT, the level of both mutant and normal allele dropped to comparable levels after silencing with shHTT2 in all lines. Therefore, some border level of HTT protein remains after silencing by a given shRNA and is independent of the initial level of the HTT. The low level of the normal HTT in HD109 iPSC is distinct to the much higher normal mouse HTT level in mHD-YAC128 iPSC (Figure S5). In summary, the silencing of HTT in human cells was efficient with shHTT2 reagent however the experiments additionally revealed a general decrease in expression of both mutant and normal HTT in HD109 iPSC vs. Control iPSC.

3.4. The Effects of HTT Silencing on Wnt and ERK Signaling in HD shRNA-iPS Cell Lines

We next asked whether downregulation of mutant HTT protein in shHTT2 iPSC lines would affect the pathways that were identified for HD and which were also affected in HD iPSC (Bowles and Jones, 2014; Szlachcic et al., 2015; Wiatr et al., 2017). Therefore, we examined β-catenin, phospho-β-catenin and phospho-ERK 1/2 by western blotting in HD YAC128 iPSC (**Figure 4**) and human HD109 and HD 71 iPSCs (**Figure 5**, Figure S5). In our previous study, we observed that more β-catenin protein is tagged for decay by Wnt-mediated phosphorylation at serines 33 and 37 in HD iPSCs (Szlachcic et al., 2015). The downregulation of mutant HTT did not affect this phenotype; however, the total β-catenin expression was increased ($19 \pm 6\%$, $p = 0.0126$) in mouse shHTT2-iPSC lines (**Figures 4A,B**). MAPK signaling is suppressed in HD YAC128 iPSCs, as indicated by the weaker ERK1/2 phosphorylation observed upon bFGF stimulation (Szlachcic et al., 2015). In the present study we observed an inconsistent response to HTT silencing (**Figures 4C,D**) showing no rescue (in isogenic lines derived from lines 1) or further decrease of ERK1/2 phosphorylation (in isogenic lines derived from line 2) after 30 min of bFGF stimulation ($-87 \pm 14\%$; shHTT2 vs. shCTRL Bonferroni *post-hoc* test $p<0.001$).

In the case of Wnt in human cells (**Figure 5**) we observed a general increase in phospho-β-catenin in HD109 cells with HTT knockdown ($+125 \pm 44\%$; $p<0.05$; shHTT2 vs shCTRL), while it was not changed in HD71 upon HTT knockdown. In HD109 and HD71 shCtrl lines total β-catenin level was increased as compared to Control shCtrl cells ($+134 \pm 40\%$, $p< 0.05$ and $174 \pm 21\%$, $p<0.01$, respectively). Silencing of HTT with shHTT2 in HD71 further increased the level of total β-catenin ($39 \pm 15\%$; $p<0.05$; shHTT2 vs. shCTRL) but no significant increase was present in HD109 ($42 \pm 30\%$, not significant, shHTT2 vs. shCTRL). ERK1/2 phosphorylation was not affected in human HD109 and HD71 cells with shHTT vs shControl (Figure S6). In general we observed moderate effects of the HTT silencing on Wnt and Erk1/2 pathways in both mouse and human HD iPSC.

3.5. HTT Silencing Is Able to Reverse p53 Deregulation in Mouse Isogenic iPSCs and NSCs

Along with our previous data showing decreased levels of p53 expression in YAC128 iPSCs and human juvenile HD iPSCs (Szlachcic et al., 2015), we found a similar decrease in p53 expression in NSCs originating from YAC128 iPSCs ($-52 \pm 7\%$, $p = 0.0006$) (**Figures 6A,B**). Subsequently, we investigated the YAC128 iPSCs and NSCs with shHTT and shCAG reagents vs isogenic shCTRL lines. In **Figures 6C,D**, we show that shHTT2 is able to rescue the decrease in p53 protein expression and to drive p53 expression well above the levels seen with the shCTRL reagent in both iPSCs and NSCs ($56 \pm 11\%$, $p = 0.0003$ and $44 \pm 19\%$, $p = 0.053$, respectively). Interestingly, the shCAG reagent did not rescue p53 expression in iPSCs or NSCs and led to further decreases in the p53 levels (**Figures 6C,D**). The pattern of p53 deregulation was followed by similar deregulation of phospho-p53 (S15) (Figure S7). The correlation study on isogenic lines expressing all anti-HTT reagents (shHTT1-3) or shCTRL reagents revealed that the levels of mutant HTT and p53 were

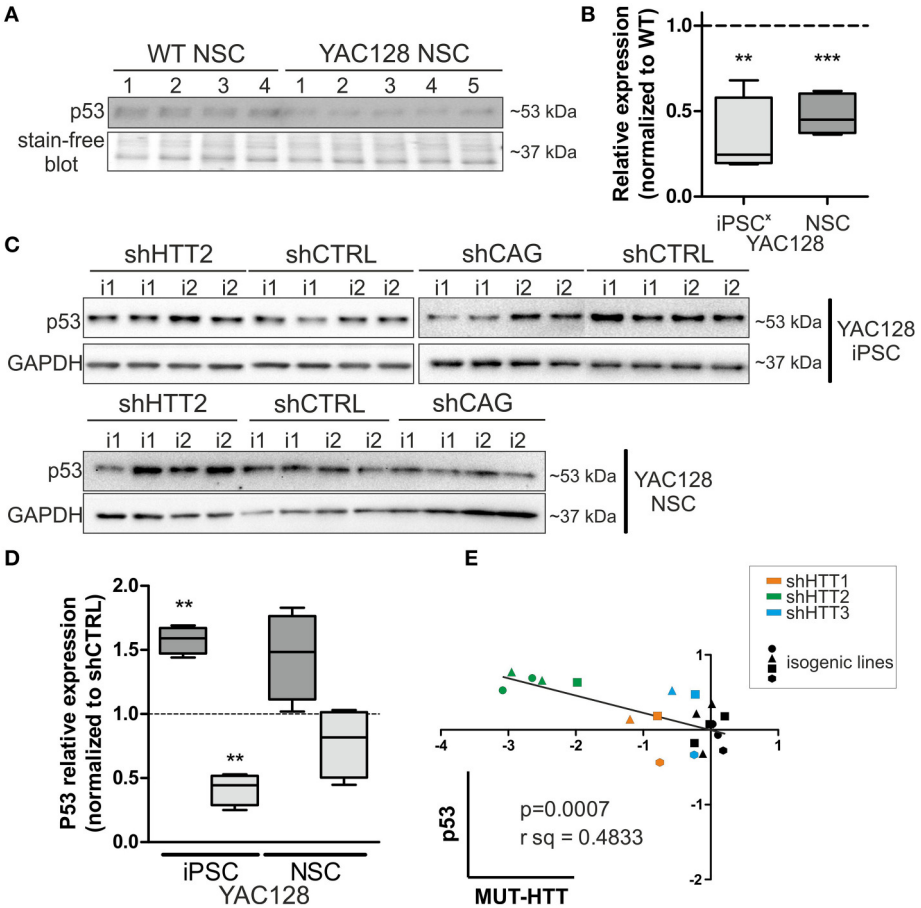

FIGURE 6 | p53 protein levels in iPSC and NSC states are dependent on mutant HTT levels. **(A,B)** The decreased p53 level is maintained in HD YAC128 NSCs after differentiation of iPSCs, as assessed by western blotting ($N = 4$ for WT and $N = 5$ for YAC128). The data was normalized to total protein visualized on blots using Bio-rads stain-free technology. Data from YAC128 iPS cells (X) was adapted from our previous work (Szlachcic et al., 2015) ($N = 6$ for WT, $N = 5$ for YAC128). shHTT iPSC **(C)** and NSC **(D)** lines show reversal of the p53 phenotype, whereas shCAG reagents further decrease the p53 expression level. $N = 4$ for each reagent in both iPSC and NSC. **(E)** Pearson correlation reveals an inverse correlation of mutant HTT and p53 expression levels ($N = 11$ for HTT reagents and $N = 9$ for control shCTRL reagent). In NSCs, the correlation is not significant $^{**}p < 0.01$, $^{***}p < 0.001$. In Panel **(A)** and (**C**, iPSCs) blots were cropped; full-length blots are presented in Figure S8.

inversely correlated in iPSCs (Pearson $r = -0.6952$; $p = 0.0007$; **Figure 6E**).

Although, p53 level was strongly decreased in human 109Q lines as compared to healthy control lines with shRNAs expression ($-85 \pm 15\%$, $p<0.001$ between shCTRL lines), we found no effects of HTT silencing with shHTT2 reagent on the p53 expression level (**Figure 7**). In summary, we found the that the low level of p53 in mouse YAC128 iPSC and derived NSC was reversible after shHTT2, while the decreased level of p53 in human cells did not react to silencing.

4. DISCUSSION

An experimental system in which the expression of a causative gene can be constantly depleted or eliminated should be considered for studying the pathogenesis of genetic neurodegenerative disorders and for therapeutic approaches. Therefore our aim was to generate HD iPSC lines with stable depletion of mutant HTT and to identify whether phenotypes characteristic for HD can be affected by HTT knockdown in HD iPSC. In addition, such approach allows for generation of cell lines with or without HTT silencing on the same, homogenous genetic background, i.e., isogenic lines. Use of isogenic lines reduces variability between compared cell lines that could mask discovery of relevant phenotypes.

One of the suitable strategies for genetic correction of HD cells is the constant expression of shRNA and gene silencing. We used a piggyBac transposase system (Yusa et al., 2011) and anti-HTT shRNA in the mir-30 backbone (Paddison et al., 2004) which provides additional possibility for future excision of the reagent if desired. Using the strategy (**Figure 8**), we derived isogenic mouse YAC128 HD and human HD iPSCs with continuous expression of shRNA targeting HTT. In the case of mouse iPSC, we tested 3 reagents targeting human HTT shHTT1-3 and a reagent targeting CAG repeats in mutant HTT (shCAG). The most potent anti-HTT reagent, shHTT2,

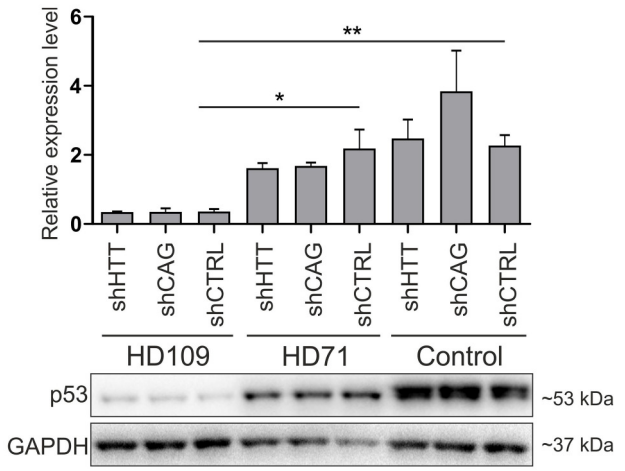

FIGURE 7 | Non-allele selective silencing of HTT does not affect p53 expression level in human juvenile HD and control iPSCs. Western blot analysis of p53 expression in human HD109, HD71 and Control iPSCs with shHTT2, shCAG and shCTRL reagents. Although, in HD109 shCTRL line expression of p53 was significantly lower than in shCTRL HD71 ($p = 0.0104$) or Control line ($p = 0.0045$), shHTT reagent did not affect the phenotype in any of lines. *$p<0.05$; **$p< 0.01$. For each patient, one cell line per each reagent was used (9 modified cell lines in total).

was continuously effective in both mouse iPSC and NSC lines with approximately 85 and 62% silencing of the mutant human protein, respectively; the silencing was similar between isogenic lines with different reagents. However, the shHTT2 reagent also reduced by approximately 53% expression of wild-type mouse huntingtin, which is expressed in YAC128 mice from 2 alleles. The shHTT2 reagent was also further used to generate shRNA expressing human HD109, HD71 and Control iPSCs. In human lines the reagent was silencing both mutant and normal allele with similarly high efficiency (over 80% silencing in HD71 and Control iPSCs). We also observed lower levels of both normal and mutant HTT in HD109 cells; however after knockdown with shHTT2 reagent the expression level of each allele reached similar levels in all lines, irrespectively of their expression levels in isogenic lines without silencing. The factor interfering with the HTT protein and transcript level may be a transcript retention in the nuclear foci demonstrated for HD cells which may result in prevention of the effective transport and translation of both mutant and normal transcripts (Urbanek et al., 2016). This may result in knock down of normal and mutant HTT in stem cells and insufficiency of HTT during development in juvenile patients. Low level of normal huntingtin may also indicate that its silencing via non-allele specific strategies might be detrimental in the case of juvenile HD.

The action of the shCAG reagent whose design was based on published allele-selective reagent revealed a dependency on cell type. In mouse iPSCs, the reagent increased mutant HTT expression, in mouse NSCs it induced a 40% silencing effect, while in human iPS cells it remained ineffective. In previous studies, the shCAG reagent was very effective at silencing HTT in fibroblasts (Fiszer et al., 2013); the observed differences in shCAG activity might result from differences in processing of the reagent in different cell types (Meijer et al., 2014; Tan et al., 2014). The described phenomena may depend on differences in the construct properties and the reagent delivery, e.g., our piggyBac construct vs. a lentiviral construct (Fiszer et al., 2013). Therefore, the shCAG reagent might be effective and should be examined in terminally differentiated neurons. Moreover, our results indicate the necessity of tailoring the therapeutic shRNA and delivery systems for disease-specific cell types, e.g., terminally differentiated neurons or astrocytes in the case of HD. In addition, the shCAG reagent demonstrates a new feature of our system namely as a reagent testing pipeline where the effects of reagents can be more precisely tested in consecutive cellular stages.

We have previously reported that YAC128 iPSCs exhibit phenotypes of early HD and share these phenotypes with human iPSCs from juvenile HD patients (Szlachcic et al., 2015). Among the changes, we identified decreased MAPK1 activation and p53 levels and increased β-catenin-p(33/37) levels. Using the established system for continuous silencing we have assessed whether the HD phenotypes demonstrated a dependency on the level of mutant HTT. In the case of the MAPK pathway and iPSCs, we observed minor changes in ERK1/2 activation depending on the iPSC line. In the case of mouse iPSC and Wnt pathway, the total β-catenin level had a slight dependency on the HTT level. Also in HD71 we have seen a greater total HTT level which may indicate recovery of Wnt signaling. In the case of HD109 we have demonstrated even higher phosphorylation of β-catenin, which may indicate an adverse effect on Wnt. We conclude that more human iPSC more HD iPS cell lines with mutant allele selective silencing originating from several patients are needed to investigate a fine relation of HTT expression and the pathways in iPSC. The differences in the phenotypes between HD109 and HD71 iPSC may be also attributed to number of CAG repeats in HTT but we can also not exclude the effect of the genetic background.

A large difference in p53 expression was found in the isogenic mouse iPSC and NSC. First, we demonstrated that p53 downregulation continued through to the NSC stage and the difference was similar to one previously seen by us for iPSC (Szlachcic et al., 2015). Moreover, shRNA isogenic cell lines at the iPSC and NSC stages demonstrated a clear dependency of p53 level on the mutant HTT level, although the effect was less prominent in NSCs. Interestingly, we observed recovery of decreased p53 expression with the shHTT2 reagent and a lack of recovery and a further decline in p53 expression with shCAG in both cell types. In the case of human HD109 iPS cells expressing the shHTT2 we did not observe the recovery of p53 level. Previously, p53 was shown to be involved in HD pathogenesis, with total levels in the brain increasing with HD severity and particularly being upregulated in late HD stages, in grade 3 and 4 patients (Bae et al., 2005). Although, p53 protein expression was upregulated in YAC128 mice, this upregulation was not observed in primary neuronal culture from E16.5 YAC128 mice unless cells were treated with the p53 activator camptothecin (Ehrnhoefer et al., 2013). Moreover, p53 phosphorylation and activation of the ATM DNA-damage-response pathway are downregulated

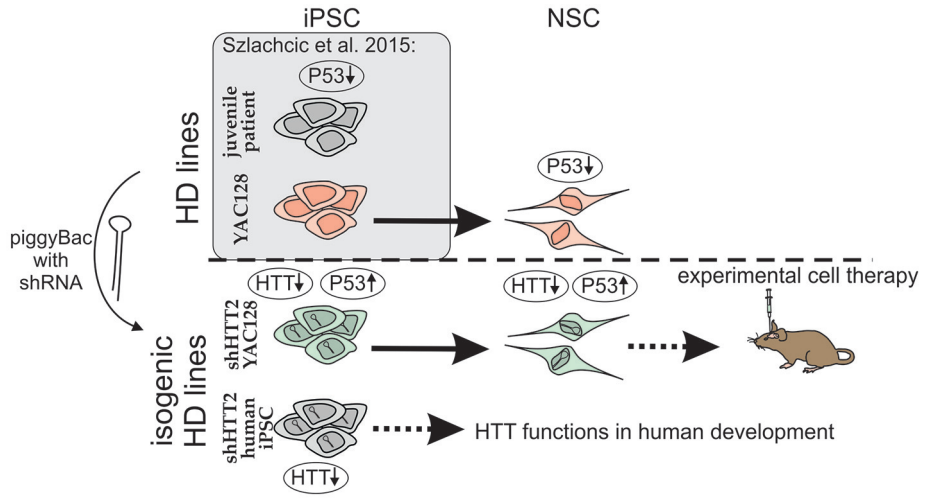

FIGURE 8 | Schematic illustration of the isogenic experimental system. Isogenic iPSC lines with HTT silencing by shHTT2 were derived from previously described HD-YAC128 iPSCs and human HD109, HD71, and Control iPSCs. Knockdown of HTT in both YAC128 iPSCs and NSCs resulted in rescue of p53 downregulation phenotype. iPSCs with HTT knockdown can be used for experimental cell therapy (mouse YAC128 iPSCs) or research on huntingtin developmental functions (human iPSCs). Gene expression changes are indicated by upward (upregulation) or downward arrows (downregulation).

in HD iPSCs (Tidball et al., 2015). Our results, together with the published data, may indicate that the gradient of p53 expression changes from downregulation in early HD stages (stem and NSCs) to upregulation in the adult brain during neurodegeneration. In general, it is well established that fine regulation of p53 expression in early developmental stages is essential to maintain the necessary balance between stem cell self-renewal and differentiation (Yang et al., 2014). In addition, high p53 expression levels may lead to terminal differentiation and growth arrest (Mendrysa et al., 2011). Decreased p53 levels in neurodegenerative diseases may lead to excessive NSC activation and insufficient differentiation potential, similar to what is observed in fragile X syndrome model (Li et al., 2016). NSCs from HD mouse models also show enhanced late-stage self-renewal, delayed cell cycle exit and impaired differentiation into striatal medium spiny neurons subtype (Molero et al., 2009; Molina-Calavita et al., 2014). Therefore, p53 may be a valid early therapeutic target in neurodegenerative diseases. Similar to p53 the Wnt is one of the major signaling pathways during development, it remains active in adult brain and is implicated in brain diseases (Noelanders and Vleminckx, 2017). In addition, the clear dependency of p53 on HTT expression in mouse cells and its downregulation in HD109 iPSCs implicates p53 in potential therapy in juvenile HD.

Sustained silencing of HTT via shRNA in iPSCs and NSCs, together with the observed p53 phenotype reversal, supports the idea of combining shRNA and autologous cell therapy. In therapeutic applications for HD, simple excision of the mutant allele via homologous recombination (An et al., 2012) or genome editing using, e.g., a CRISPR-Cas9 system, may be insufficient and could be accompanied by stable shRNA expression. An important reason for combining both approaches or even for selecting shRNA for cell therapy is evident from several demonstrations of neurodegeneration (including HTT aggregate formation) of healthy cells grafted into a brain undergoing neurodegeneration (Cicchetti et al., 2009, 2011, 2014; Jeon et al., 2016). One of the underlying mechanisms is the shuttling of HTT mRNA or HTT protein aggregates between cells, thus allowing them to spreading through the graft in a prion-like manner (Brundin et al., 2010; Herrera and Outeiro, 2012; Costanzo et al., 2013; Pecho-Vrieseling et al., 2014; Jeon et al., 2016; Zhang et al., 2016). Moreover, it is known that cells, including neurons and glia, can mediate exosomal and non-exosomal transfer of both proteins and RNAs (Vlassov et al., 2012; Frühbeis et al., 2013), including miRNAs (Wang et al., 2010; Hu et al., 2012) and synthetic mature shRNAs (Olson et al., 2012). Therefore, shRNA from grafted cells, if transferred via exosomes, may confer a therapeutic effect on host cells. Taken together, sustained expression of an HTT-silencing agent that also reverses the molecular phenotype has the capacity to protect grafts from non-cell-autonomous degeneration caused by surrounding HD-affected host cells.

To our knowledge, our human HD and Control iPSC lines with integrated shHTT2 are the first human iPSCs with stable huntingtin knock-down, and they can be helpful for *in vitro* research on huntingtin functions in human development.

AUTHOR CONTRIBUTIONS

WS and MacF conceived, designed, performed the experiments, and analyzed the data. KW performed ERK assay and consecutive western blotting analyses. KW and MT cultured and analyzed the phenotype of NSC. MacF planned and executed the live animal experiments (mouse brain injections). MarF critically revised the article. WS and MacF wrote the paper. MacF was responsible for concept and obtaining funding.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnmol.2017.00253/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers

New mouse and human Huntington disease iPS cells with stable expression of anti-huntingtin shRNAs suitable for research on HD pathogenesis and HTT function

SUPPLEMENTARY DATA

Supplementary Methods

Cell culture

Adherent NS (aNSC) culture was initiated by dissociation of spheres with a single round of chopping followed by incubation with TrypLE. Spheres were dissociated into single cells and plated into gelatin-coated vessels at a density of 30,000 cells/cm^2. The aNSC medium consisted of DMEM/F12 (1:1) with 5 mM HEPES, additional glucose (6 mg/mL in total), 1x Glutamax, 1x N2 supplement, 50 μg/mL apo-transferrin (Sigma-Aldrich), 50 μg/mL BSA, 5 μg/mL heparin, 20 ng/mL bFGF and 20 ng/mL EGF. The N2 supplement consisted of 5 μg/mL insulin, 30 nM sodium selenite, 51 nM triiodo-L-thyronine (T3), 6 ng/mL progesterone, and 16 μg/mL putrescine (all Sigma-Aldrich). For the initial passages, cells were passaged 1:2 to 1:3 every 4-6 days; for later passages (>5), confluence was reached between 2-4 days when cells were passaged 1:3 to 1:4. The aNSCs were passaged with TrypLE, and culture medium was gently replaced every other day. For neuronal differentiation, cells were transferred into poly-DL-ornithine- and poly-D-lysine-coated wells in NSC medium without bFGF, EGF or heparin. For astrocyte differentiation, dishes were coated with gelatin and NSC medium without growth factors and was supplemented with 1% FBS. Neurons and astrocytes were immunostained after 14 days.

Striatal transplantation

NSC spheres were prepared for transplantation by priming cultures for 3 days without EGF53. Spheres were transferred to an animal facility in NSC medium on ice, where they were dissociated into single cells using chemical dissociation (NeuroCult Chemical Dissociation Kit; StemCell Technologies, Vancouver, Canada) and passed through a mesh filter (BD cell strainer, BD Biosciences, San Jose, CA). Then, 1x10^5 cells suspended in 2 μl of NS medium (w/o EGF) were stereotactically injected (Angle Two^{TM} Stereotaxic Instrument, Leica) using a Hamilton syringe with a 35G needle into the striatum at coordinates of AP +0.5, ML+1.8, DV-3.5. Mice were sacrificed at 8 weeks post-transplantation. The mOrange2 signal from transplanted cells was visualized after passive clearing of the brain using the PACT method (Yang et al., 2014).

qPCR analysis of HTT expression

Total mRNA was isolated from human HD iPSCs using RNAzol RT (Molecular Research Center, Cincinnati, OH) according to manufacturer's protocol. Reverse transcription was performed using Maxima H Minus Reverse Transcriptase (Thermo Fisher) (200U per reaction) on 2 μg of RNA in 20 μl of total reaction according to the manufacturer's protocol. For priming a mixture of random hexamers (25 pmol) and oligo(dT)18 (25 pmol) was used. Additionaly RiboLock RNase inhibitor was added

to the reaction mix (20U). Before adding the enzyme and the inhibitor, templates were denatured in 65°C for 5 minutes; after mixing all reaction reagents reaction was incubated for 10 min at 25°C followed by 15 min at 50°C. Resulting cDNA was further 10 times diluted with nuclease-free water and stored in -20°C. qPCR was performed using 5x HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) on 1 μl of cDNA in 10 μl of total reaction volume. The reaction mix included 250 nM primers. Primers used are: exon1-Forward 5'-GAGCCGCTGCACCGAC-3', exon2-Reverse 5'-CTGACAGACTGTGCCACTATGTTT-3 (both adapted from (Sathasivam et al., 2013), exon52-Forward 5'-TCTCACGCCATTGCTAAGGA-3', exon53-Reverse 5'-TGACCATCCAAGCTTCCACA-3, PGK1-Forward 5'-CTGTGGCTTCTGGCATACCT-3', PGK1-Reverse 5'-CGAGTGACAGCCTCAGCATA-3'. Thermocycling parameters were as follows: 15 min of initial denaturation at 95°C and 45 two-step cycles with 20 s denaturation at 95°C, 20 s annealing at 60°C and 20 s elongation at 72°C. The reactions were run on CFX96 instrument (Bio-Rad). Specificity of reaction for each primer pair was confirmed by Melting curve analysis and agarose gel electrophoresis. RT- and no template controls were included in analysis. Data was obtained and analyzed using CFX Manager 3.1 (BioRad). Cq values were determined in software using regression model and were exported to Excel for further analyses. Relative expression level was calculated using dCt method with PGK1 used as a reference gene.

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Table S1. Sequences of used shRNA guide strands

Name	Sequence	Target	Source
shHTT1	5'-UUUCGUAAACAAGUCAGCAGCC-3'	human HTT exon 67	HP_124353; RNAi Codex database
shHTT2	5'-UAUAGCCUCUAUAUAUUCUGGU-3'	human HTT exon 22/23	HP_7232; RNAi Codex database
shHTT3	5'-AAUACAAAGCCAAUAAACACCU-3'	human HTT exon 33/34	HP_220452; RNAi Codex database
shCAG	5'-GCUGCUGC**A**GCUGCUGCUGCUU-3'	CAG repeats in HTT **(one mismatch)**	A2_R reagent; (Fiszer et al., 2013)
shCTRL	5'-UUGAAGUUCACCUUGAUGCCGU-3'	GFP	this research

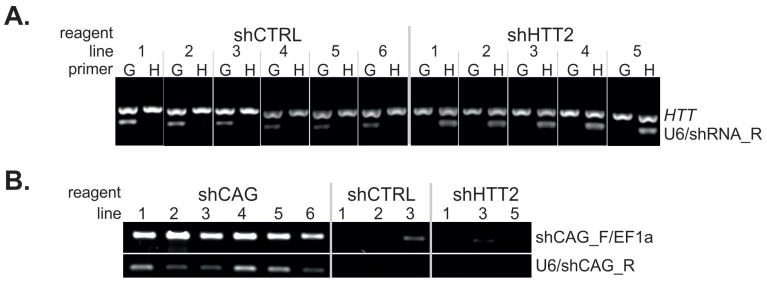

Figure S1: Genotyping analysis results on mouse iPSC lines to determine proper integration of shRNA transposons. In (A), multiplex PCR was performed with upper bands being the amplification products of human HTT, used as an internal reaction control, while lower bands are shHTT (H) or shCTRL (G) construct-specific products. In (B), we used two pairs of primers specific for 3' (lower panel) or 5' (upper panel) parts of shCAG.

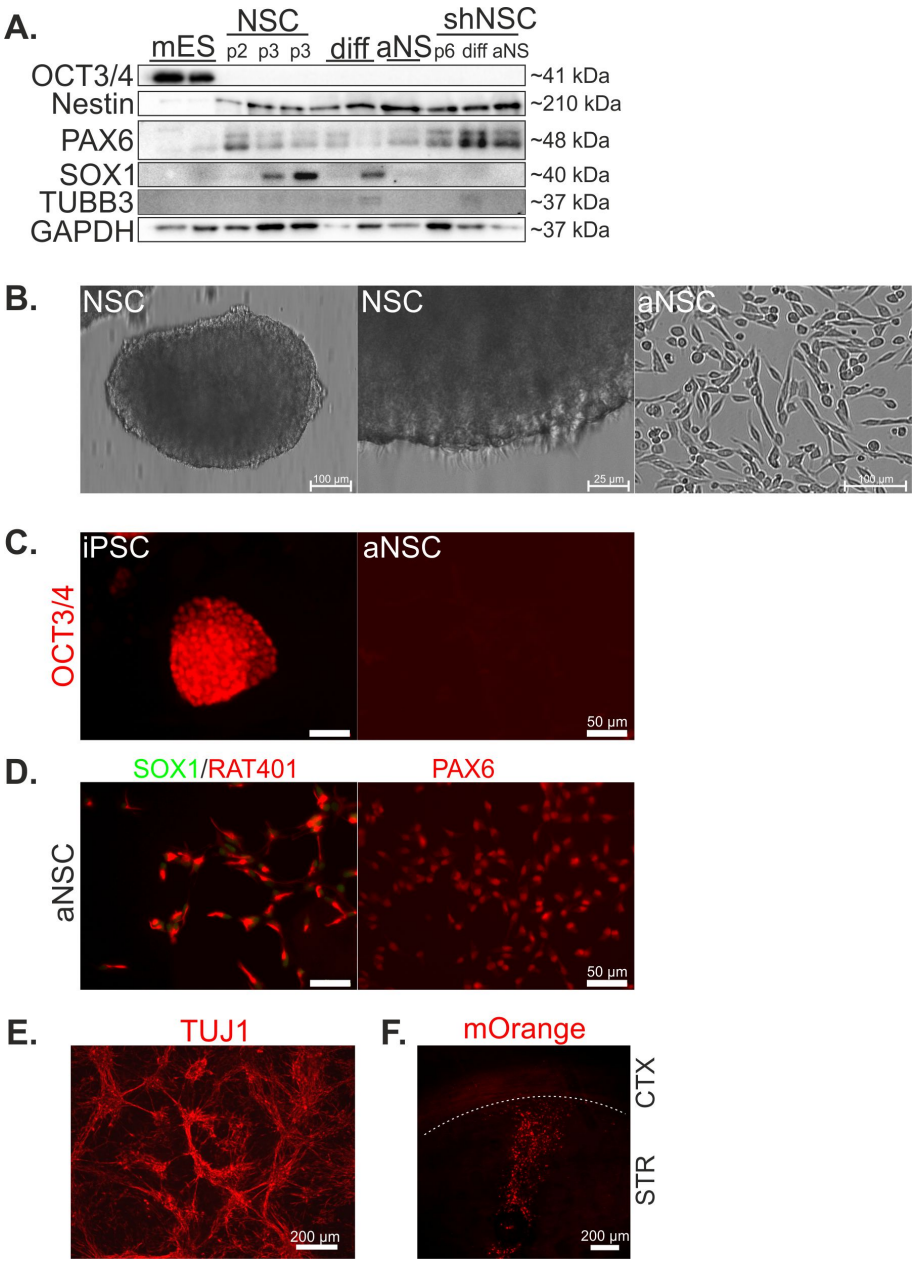

Figure S2: Characteristics of NSCs. (A) Western blot analysis of NSCs. Upon differentiation of iPSCs into floating NSC spheres, OCT3/4 expression is lost; this is maintained upon further passages, transition to adherent culture (aNSC) and further differentiation (diff). The expression of the general NSC markers, nestin and PAX6, is acquired and maintained upon passaging. In contrast, SOX1 expression peaks transiently in lower passages and is lost upon continuous culture. The neural marker TUBB3 is not present until after short-term differentiation of NSCs. (B) Morphology of NSCs. NSC spheres develop characteristic cilia (middle panel). After transfer of floating NSCs into adherent aNSCs, the cells acquire bipolar morphology. (C) Immunostaining of iPSCs and aNSCs for OCT3/4 confirms loss of its expression in the latter. (D) Nestin (RAT401 antibody) and PAX6 are expressed in the aNSC state, while SOX1 expression is not detected. (E) NSCs can be differentiated towards TUJ1-positive neurons. (F) Maximum-intensity projection of mOrange2 fluorescence in a whole graft within a CLARITY/PACT-cleared mouse brain, acquired via confocal microscopy. mOrange2-positive cells survive for at least 8 weeks after NSCs are transplanted into the mouse striatum.

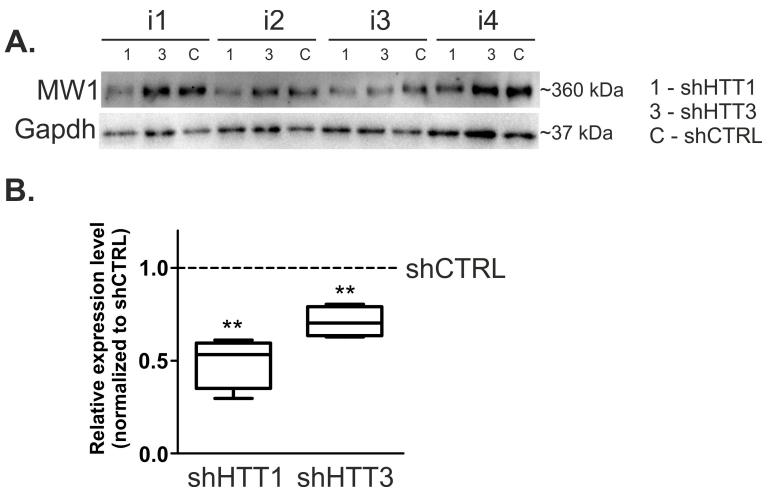

Figure S3: Efficiency of shHTT1 and shHTT3 reagents in mouse HD iPSCs. (A, B) Western blot analysis of mutant huntingtin expression level using polyQ-specific antibody MW1 as compared to shCTRL lines. * $p<0.05$, *** $p<0.001$; i1-i4 – isogenic lines derived from separate parental lines 1 to 4.

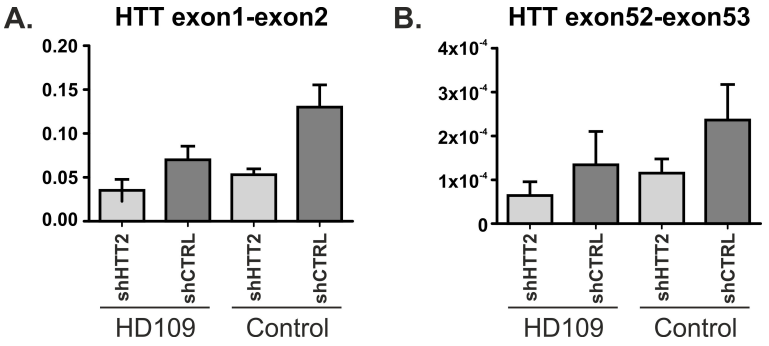

Figure S4: qPCR analysis of total HTT expression in HD109 and Control lines with shHTT2 vs shCTRL lines. Amplification of 5' region (exon1/exon2 junction) and of more terminal region (exon52/53 junction) HTT transcripts reveal similar decrease upon shHTT2 reagent-mediated silencing. Note lower levels of total HTT mRNAs in HD109 vs Control in shCTRL lines.

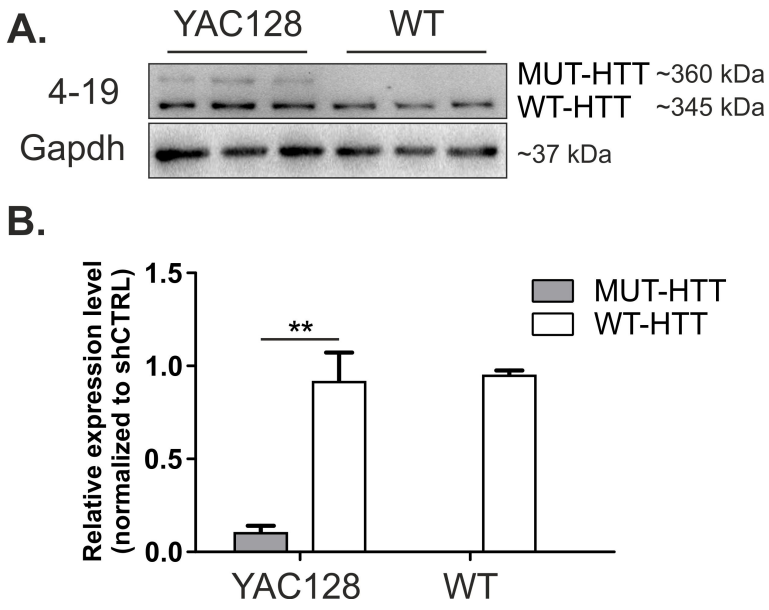

Figure S5: Human mutant (MUT) and mouse wild-type (WT) HTT protein expression in YAC128 and WT iPSC analyzed with western blot using antibody specific to N-terminal part of HTT (aminoacids 4-19). The presence of mutant HTT does not influence level of wild-type HTT and mutant HTT expression is approximately 9 times lower than mouse HTT in YAC128 iPSC.

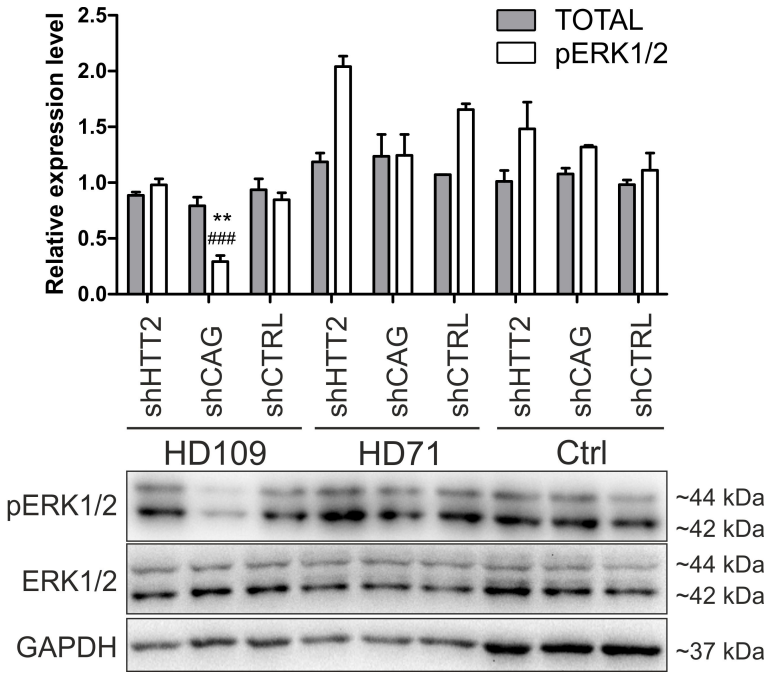

Figure S6: Effects of mutant HTT knockdown on Wnt pathway in human iPSCs. We observed no effects on total or phospho-ERK1/2 (Thr202/Tyr204) human shRNA iPSC lines. * - statistically significant difference vs isogenic shCTRL line; # - statistically significant difference vs Ctrl shCTRL line; ** and ## $p<0.01$; *** and ### $p<0.001$. For each patient, one cell line per each reagent was used

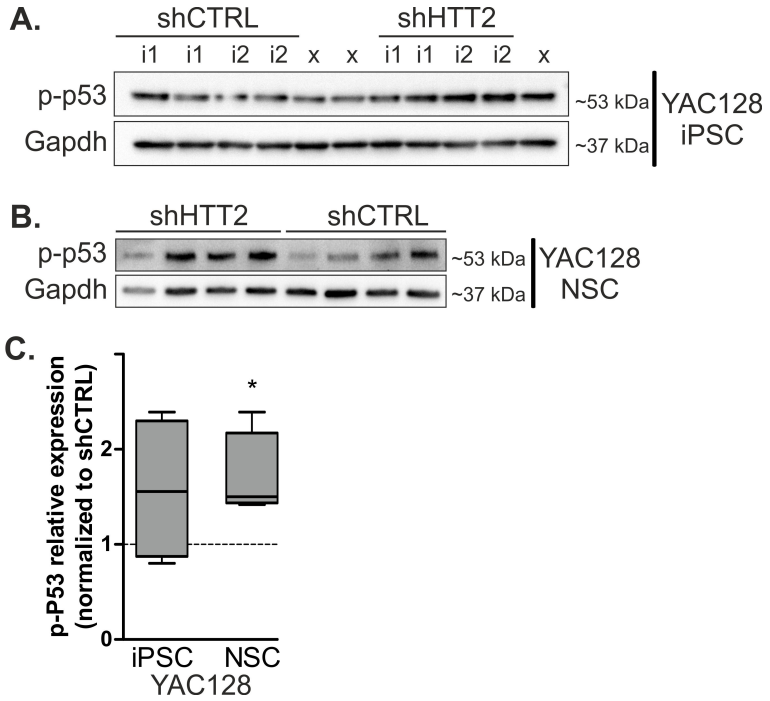

Figure S7: p53 phosphorylated at serine 15 is affected in shHTT2 iPSC (A, C) and NSC (B, C) lines, similarly to total p53. *p<0.05

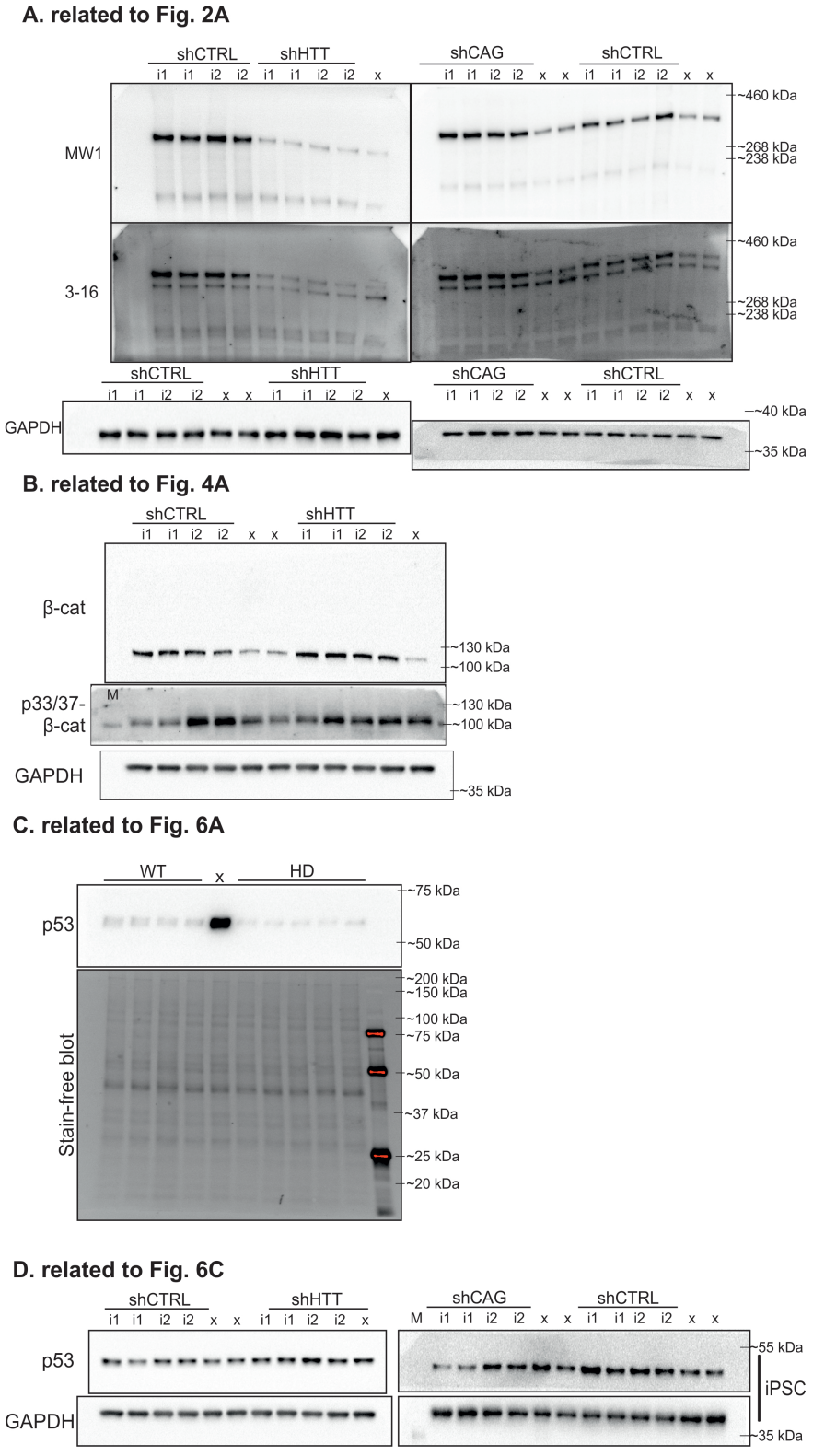

Figure S8: Full-length blots for blots with cropped lanes in the main text figures, i.e. Fig. 2B (A), Fig. 4A (B), Fig. 6A (C) and C (D). Note, that whole-gel blots were cut into pieces to simultaneously stain for various proteins of different molecular weights. Lanes marked with X- were removed from main text figures; these lanes are positive controls and calibrator samples. M – protein ladder lane.

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Huntington Disease as a Neurodevelopmental Disorder and Early Signs of the Disease in Stem Cells

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Abstract Huntington disease (HD) is a dominantly inherited disorder caused by a CAG expansion mutation in the huntingtin (HTT) gene, which results in the HTT protein that contains an expanded polyglutamine tract. The adult form of HD exhibits a late onset of the fully symptomatic phase. However, there is also a long pre-symptomatic phase, which has been increasingly investigated and recognized as important for the disease development. Moreover, the juvenile form of HD, evoked by a higher number of CAG repeats, resembles a neurodevelopmental disorder and has recently been the focus of additional interest. Multiple lines of data, such as the developmental necessity of HTT, its role in the cell cycle and neurogenesis, and findings from pluripotent stem cells, suggest the existence of a neurodevelopmental component in HD pathogenesis. Therefore, we discuss the early molecular pathogenesis of HD in pluripotent and neural stem cells, with respect to the neurodevelopmental aspects of HD.

Keywords Stem cells · Polyglutamine diseases · ESC · iPS · iPSC · NSC · Neurodegenerative disease · Neurodevelopmental disease · polyQ disease · Huntington disease

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Introduction

The onset of serious motor and cognitive symptoms is late in Huntington disease (HD). However, extensive longitudinal research in the PREDICT-HD [1] and other clinical programs [2] indicates that premanifest HD patients develop subtle HD-mediated changes decades before classical diagnosis. These changes include cognitive, functional, and psychiatric symptoms; altered brain morphology and connectivity; and even subtle motor deficits [3–8]. Moreover, neuronal degeneration and the deregulation of neurodevelopmental genes occur long before the onset of classical HD symptoms and phenotypic changes in both mouse models and patients [9–14]. These findings raise the question of whether premanifest symptoms in HD are a consequence of neurodevelopmental abnormalities. Neurodevelopmental deficits typically occur in childhood. For instance, children at risk of HD exhibit smaller head size, indicating a deficit in brain growth [15]. In addition, lower body weight index (BMI) is also present in prodromal HD children and body weight deficit was identified for juvenile HD suggesting a developmental deficit probably due to mitochondrial dysfunction [16]. The HD juvenile form, also known as "Westphal variant," is characterized by a high number of CAG repeats, onset under 20 years of age, and disease manifestation different than that of adult-onset disease [17, 18].

Multiple roles have been established for normal and mutant huntingtin (HTT) in pre- and postnatal development via in vivo and in vitro developmental research on animal models. Evidence from recent stem cell studies supports the idea that mutant HTT-dependent changes may be detected early, even at the naïve pluripotent cell stage (see discussion and references in the following sections). Therefore, it is important to elucidate the pathogenesis of HD along the differentiation axis (pluripotent stem cells (PSC) → neural stem cells (NSC) → mature neurons, Fig. 1) to identify the early processes relevant to developmental defects and disease onset. Understanding

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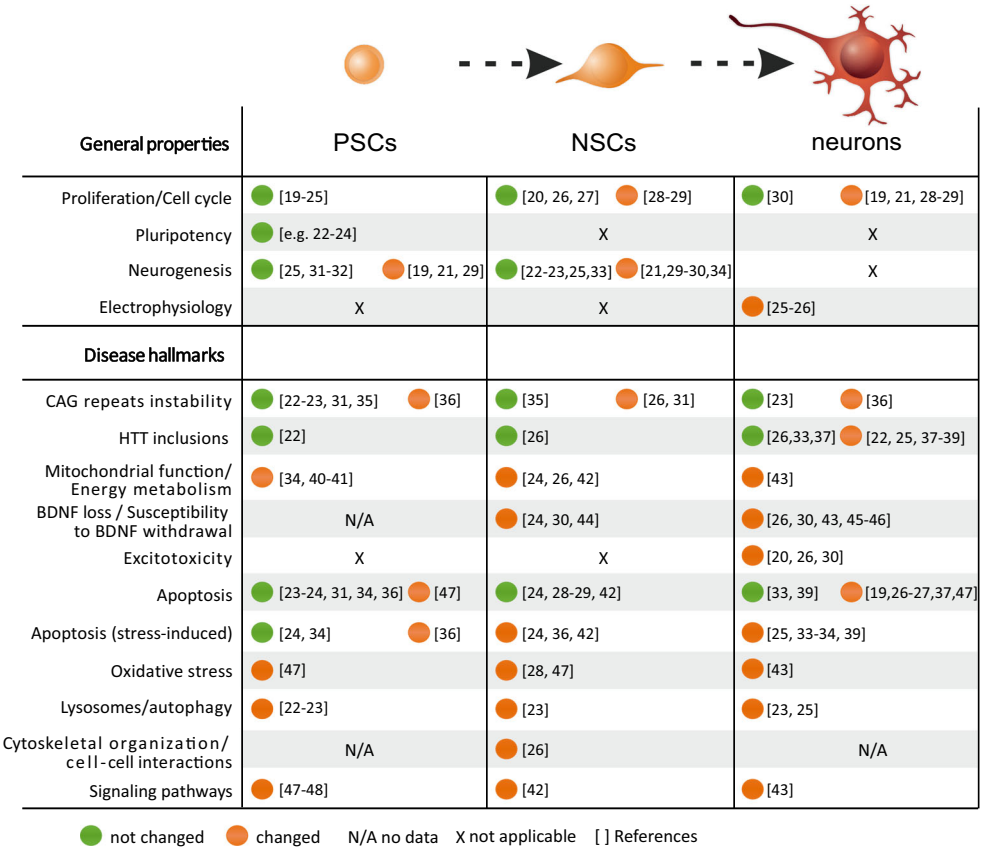


Fig. 1 HD pathogenic changes occur along the differentiation axis: pluripotent stem cells → NSC → mature neurons. The figure summarizes HD pathogenesis in the indicated cell types. Many HD-associated changes are present in the early stages of development, beginning with pluripotent stem cells and continuing throughout differentiation to NSC and mature neurons. The data form high-throughput transcriptomics and proteomics were not included in the table. Changes detected (*orange*), absence of change (*green*), discrepant results (*gray*), data non-available (*N/A*), and non-applicable (*X*) (Color figure online)

the pathogenesis in these cell types is particularly important for the development of effective cell therapies and determination of the therapeutic impact.

The aim of this work is to review the current knowledge regarding the most important facts about the role of normal and mutant HTT in organism development. Subsequently, we will discuss early molecular pathogenesis of HD identified in PSC and NSC, which may underlie the developmental deficits in HD. Additionally, we have performed a meta-analysis to determine whether previously identified deregulated genes/proteins in HD PSC, NSC, and neurons are related to developmental Gene Ontology (GO) terms.

HTT Is Necessary for Development

HTT is crucial for the organism development (Fig. 2), and the absence of HTT is lethal in mice around developmental day E7.5–8.5 [49–51]. This lethality may be caused by early embryonic patterning deficits, including shortened primitive streak and lack of headfolds, which denote a failure in the development of the head [52]. The expression of wild-type HTT at approximately 10–15% of normal levels may rescue this embryonic lethality; however, the HTT insufficiency causes abnormal brain development and mild movement abnormalities [53]. On the other hand, similar low levels of mutant Q50 or Q100 HTT lead to perinatal death [53, 54]. The discrepancy in the effects of low levels of wild-type vs mutant HTT may result from both the loss of function of mutant HTT during development and/or the protective role of normal HTT, interfering with the gain of function of mutant HTT. The decreased level of wild-type HTT (10–15% of normal levels) in mouse embryos, followed by reconstitution of HTT expression to normal level on postnatal day 21, resulted in progressive striatal and cortical neuronal degeneration and motor incoordination later in life [55]. In addition, when mice are exposed to normal levels of mutant HTT 97Q until postnatal day 21, they develop a HD-like phenotype including neuropathology and motor deficits. The phenotype is not as severe as in the mice with lifelong expression of HTT 97Q [56]. Summarizing, low level of HTT or expression of mutant HTT, limited to the time of embryonic development and short postnatal time when striatal neurogenesis occurs, is sufficient for generation of the neurological phenotype in mice. In addition, the conditional reduction of HTT in the mouse forebrain, which is initiated at later embryonic stages and reached 84% reduction of expression by postnatal day 60, leads to progressive neurodegeneration and premature death [57]. In addition, the phenomenon called "huntingtin holiday" also suggests that the disease symptoms in HD may be reversed but the reversal is not complete [58]. HTT knockout (KO) in Wnt1-

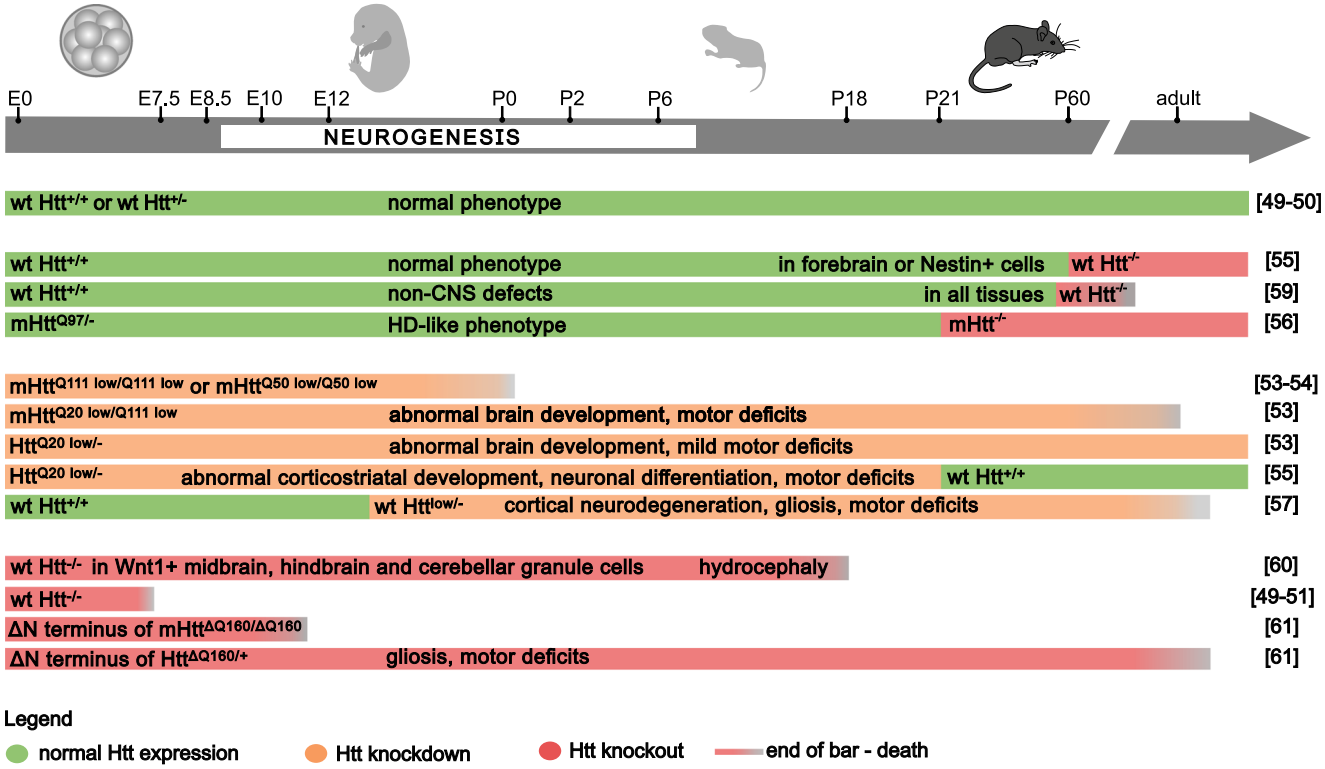


Fig. 2 Phenotypic effects of the HTT gene manipulation during mouse development. The diagram illustrates the knockout and knockdown of normal and mutant HTT, at several points in time of mouse embryonic and postnatal development, indicating CNS and non-CNS consequences. The mouse age and the duration of HTT knockout (*red bars*) or knockdown (*orange bars*) in mouse life are indicated by the *relevant length* of *bars*. *Green* indicates non-manipulated, wild-type HTT expression. Premature death or embryonic death is indicated by a *shorter bar*, ending with a *gray-colored gradient*. The phenotypic effects of the mutant and WT HTT gene manipulation are described directly on the *bars*. The mutant and WT HTT gene manipulation is indicated as genotype for each experiment at the beginning of each segment of the *bar* (Color figure online)

expressing cells of the midbrain, hindbrain, and cerebellar granule cells results in hydrocephaly and death at postnatal P6–18 [60]. In contrast, mice remained unaffected when HTT was knocked down in the forebrain or Nestin-positive cells at 2, 4, or 8 months of age [55]. HTT knockdown to the level hardly identified by immunoblotting in all mouse tissues at 2 months of age leads to death as a result of non-CNS defects, such as acute pancreatitis [59]. However, no neuronal deficits were identified in these mice. In addition, the deletion of polyglutamine (polyQ) or proline-rich regions within the N-terminus of HTT did not affect normal mouse development, whereas the N-terminus alone is insufficient to rescue lethality in embryonic and young mice [61–63].

Developmental Functions of HTT

The neural rosettes are radial arrangements of cells in the culture, indicating that embryonic stem cells (ESC) differentiate and form NSC. Therefore, neural rosettes in culture are a developmental marker resembling the radial arrangements of NSC forming neural tube during development [64]. Mouse ESC-derived NSC with low expression of HTT are able to form rosettes; however, NSC which are deprived of HTT (HTT-null cells) are unable to form neural rosettes in vitro [65]. The phenotype, which is referred to as rosetteless, is reflected in the impaired acquisition of proper polarity during neurulation in HTT-null zebrafish embryogenesis [65]. It is a consequence of defective cell adhesion function of HTT, which depends on the N-terminal portion of the HTT protein, and is mediated by ADAM10/N-cadherin [65]. The cell adhesion function of N-terminus of HTT is a recent evolutionary step which probably enabled more complex development of the CNS [65].

HTT is essential for the formation and orientation of a proper mitotic spindle [66]. Its depletion during embryonic cortical neurogenesis by in utero electroporation, using HTT siRNA, causes incorrect spindle orientation, which results in a decreased pool of proliferating progenitors and increased differentiation due to an imbalance in symmetric vs asymmetric divisions [66, 67]. Similarly, the expression of mutant HTT in the absence of normal HTT in cells derived from $Hdh^{Q111/Q111}$ mice causes mitotic spindle misorientation along with defects in the proliferation of neuroprogenitors [68].

Conditional reduction of HTT (less than 10% of the normal level), occurring selectively in cortical excitatory Emx1-expressing neurons, produces low HTT expression already at E 9.5, prior to early postnatal synaptic development. Notably,

the depletion also includes cortical layer 5, which projects to the striatum. Such experimental setup demonstrated altered cortical and corticostriatal connectivity and the increase in excitatory synapse formation in the striatum, which suggests a non-cell-autonomous effect on maturation of striatal medium spiny neurons (MSNs) [69]. Similar changes have been identified in the corticostriatal development of HD knock-in zQ175 mice, which suggests HTT loss of function in the development of corticostriatal synaptic connectivity [69]. Aberrant cortical inputs may affect the proper maturation of striatal MSNs, since generation of striatal neural progenitors (NPC) is also compromised in HdhQ111 knock-in mice [70] and patients [71]. Abnormal specification and maturation of MSNs impair the acquisition of the proper mature striatal cytoarchitecture. Improperly matured MSNs may be vulnerable to stress-mediated cell death in the symptomatic stages of the disease. The overall evidence indicates a neurodevelopmental stage in HD and its significant role in the disease development.

Considerations for HD Modeling in PSC and NSC

The earliest molecular phenotypes of HD pathology were identified in pluripotent stem cellular models. PSC recapitulate the cellular stages occurring during early stages of organism development. ESC are isolated from inner cell mass of the blastocysts, whereas the induced pluripotent stem cells (iPSC) are produced by cellular reprogramming of somatic cells, with the use of genetic mechanism described by Takahashi and Yamanaka [72]. Table 1 summarizes and provides an overview of the existing animal and HD patient stem cell models. This overview indicates that the existing HD stem cells possess highly variable characteristics, such as technology (derivation method, cell source, and epigenetic status), disease (number of CAG repeats), and experimental design (e.g., the number of lines directly compared in order to determine the phenotypes, and the lines compared had uniform or differing genetic backgrounds, i.e., isogenic or non-isogenic/mutation silenced/corrected). For example, the majority of HD patient iPSC were produced using stable genomic integration of reprogramming factors, which remain in iPSC genome after the reprogramming process and may affect the observed HD changes. In contrast, the newest techniques use the reprogramming systems which involve significant control over the expression of exogenous reprogramming factors and prevent integration of these factors with the genome of somatic cells during the reprogramming process. Such systems, e.g., based on Sendai virus, messenger RNA (mRNA), or episomal vectors, are denoted as integration free [73]. In addition, for identification of the phenotypes, iPSC from HD patients were compared to iPSC from other, genetically unrelated, non-affected patients. This type of comparison is characterized by a limited resolution due to genetic modifiers, which may affect phenotypes. For example, the CAG tract length in HTT is not entirely correlative with the disease onset and duration, pointing to the existence of additional genetic modifiers [91, 92]. HD phenotypes can also be modified by the epigenome states of iPSC, which may convey transcriptional characteristics of source somatic cells. Importantly, the epigenome states in iPSC are related to different types of pluripotency, namely primed and naïve pluripotency [93]. The currently available human iPSCs commonly occur as primed pluripotent cells, and such cell state first appears in later blastocyst stages [94]. Naïve pluripotent state is characteristic of cells of the inner cell mass of the early blastocyst, and such stage is typical of mouse ESC and iPSC. Naïve pluripotent cells self-renew rapidly and almost indefinitely in vitro, and their self-renewal is dependent on the leukemia inhibitory factor (LIF) [93]. An epigenetic state of both naïve and primed iPSC may be recognized by the methylation status of their genome. Naïve PSC have transcriptionally active hypomethylated genome, while the primed PSC already exhibit some methylation patterns characteristic of more advanced stage of the cell linage [93]. The available protocols for generation of human iPSC can be used to reprogram somatic cells until primed stage but are not suitable for direct reprogramming to developmentally earlier, naïve cell stage characterized by fewer epigenetic marks. Therefore, the genome of primed human iPSC exhibits remaining epigenetic marks of source cells and additionally acquires new epigenetic marks by reprogramming or continuous culture [95, 96]. Such epigenetic marks can affect the differentiation potential and development of HD phenotypes [80].

One potential solution to overcome variability in determining HD phenotypes is to increase the statistical power by increasing the number of patients and iPSC lines. However, the best solution is to employ genetic technologies, such as genome editing or constant short hairpin RNA (shRNA) expression, to generate corrected, patient-specific isogenic cells suitable for use as control cell lines. A complementary solution is to use the iPSC from healthy patients, to generate the isogenic iPSC with incorporated mutant genes. Therefore, the current knowledge of HD-mediated changes will have to be verified using more technologically advanced stem cell systems.

NSC are multipotent, self-renewing cells that differentiate into neural, glial, and oligodendrocyte lineages and represent the next stage of differentiation in the process of nervous system development. In the adult mammalian brain, NSC contribute to the brain plasticity and are located in the dentate gyrus of the hippocampus and the telencephalic subventricular zone [98]. During postembryonic and embryonic neurogenesis, NSC undergo symmetric and asymmetric divisions to generate NPC, which are unipotent or oligopotent; thus, they have a limited ability to self-renew and are committed to neural fate. Both NSC and NPC require growth factors and extracellular signals to regulate proliferation and differentiation. NSC may be maintained in vitro, in multiple, distinct

Table 1 Pluripotent models of HD

Species and cell type	Name (mutQ number)	Model/cells of origin	Derivation method (factors)	Isogenic	References
Mouse ESC	Hdh CAG150 (150)	Hdh CAG150 knock-in	Embryo	Syngeneic	[19]
	$Hdh^{Q20/7}$ (20) $Hdh^{Q50/7}$ (50) $Hdh^{Q91/7}$ (91) $Hdh^{Q111/7}$ (111)	129Sv mES	Knock-in	Yes	[40] [21, 27, 29, 32]
	$Htt^{F140Q/7Q}$	mES	Knock-in	Yes	[28]
	HD ESC (127)	R6/1-HTTex1	Embryo	Syngeneic	[36]
	$Hdh^{Q20/7}$ (20) $Hdh^{Q140/7}$ (140)	CAG140 knock-in	N/d	Syngeneic	[27, 41]
Monkey ESC	TrES1 (72) [tetraploid]	rHD-HTTex1-GFP fibroblasts/WT oocyte	Hybrid embryo	No	[73]
	rHD-ES—7x (28–48, one line 131)	rHD-HTTex1-GFP	Embryo	No	[39, 74]
Human ESC	hESC-184 hESC-196	IVF/PGD[b]	Embryo	No	[75, 76]
	VUB05_HD (44)	IVF/PGD[b]	Embryo	No	[35, 76, 77, 78]
	SI-186 (37) SI-187(51)	IVF/PGD[b]	Embryo	No	[31]
	STR-155-HD (44) a.k.a. Huez2.3	IVF/PGD[b]	Embryo	No	[79, 76, 78]
	SIVF017-HD (40) SIVF018-HD (46) SIVF020-HD (48) SIVF046-HD (45) a.k.a. GENEA017,18,20,46	IVF/PGD[b]	Embryo	No	[80, 34, 76]
	Q23 (23) Q73 (73) Q145 (145)	H9 hESC	piggyBAC transposon w/ HTTex1 cDNA	Yes	[37]
	GENEA089 (41) GENEA090 (46) GENEA091 (42)	IVF/PGD[b]	Embryo	No	[34]
	KCL027 (43) KCL028 (43) KCL036 (38)	IVF/PGD[b]	Embryo	No	[81]
Mouse iPSC	HD-iPS (two mice) (144)	R6/2-HTTex1 transgenic fibroblasts	Retroviral (OSKM)	Syngeneic	[22]
	YAC-HD-iPS (128)	YAC128 transgenic fibroblasts	piggyBac (OSKML), excised from genome	Syngeneic	[48]
Monkey iPSC	RiPS-3 (72) +3 other	rHD-HTTex1-GFP fibroblasts	Retroviral (OSK)	No	[82]
	HD-14 (27/65)	rHD-HTTex1-GFP dental pulp stromal cells	Retroviral (OSK)	No	[39]
Human iPSC	HD-iPS-4 (GM23225) and HD2 (72)	Fibroblasts: GM04281	Retroviral (OSKM)	No	[83] [24, 38, 42, 44, 47, 84]
	Corrected-HD-iPS4 clones C127 (21) and C116 (20)	HD-iPS4 (72) line	Homologous recombination	Yes	[42]
	$HD\text{-}iPS^{HOM}$ 4F/3F (42/44)[c] $HD\text{-}iPS^{HOM}$ 4F (39/43)[c] $HD\text{-}iPS^{HET}$ 3F (45)	Fibroblasts: HD509 HD832 HD1657	Lentiviral (OSKM/OSK)	No	[23]
	F-HD-iPSC (50) D-HD-iPSC (109)	Fibroblasts	Retroviral (OSKM)	No	[85]
	HD60i (60) HD109i.1 (109) HD180i (180)	Fibroblasts: GM03621 ND39258 GM09197	Lentiviral (OSKMNL) Retroviral (OSKM)	No	[26]
	HD1—5x (n/d) HD2—5x (86Q)	Fibroblasts: GM04693 GM05539	Lentiviral (OSKM)	No	[43]
	HD70 (70) HD180 (180)	Fibroblasts: GM21756 GM09197	Episomal (OSKML+sh-p53)	No	[86, 87]

Table 1 (continued)

Species and cell type	Name (mutQ number)	Model/cells of origin	Derivation method (factors)	Isogenic	References
	HD60n (60) HD109n (109) HD180n (180)	Fibroblasts: GM09197 ND39258 GM03621	Episomal (OSKML+sh-p53)	No	[30]
	ND4228, ND4229, ND4230 (71) ND4222, ND4223, ND4224 (109)	Fibroblasts: GM04281	Episomal (OSKML+sh-p53)	No	NINDS[a] [48]
	ND41657 (57)	Fibroblasts: ND33392	Episomal (OSKML +sh-p53)	No	NINDS[a] [88, 89]
	HD-iPSC-A1, −A7 (43) HD-iPSC-B4, −B16 (43)	Fibroblasts	Retroviral (OSKMN)	No	[33]
	Q47 (47) Q70 (70)	Fibroblasts	Lentiviral (OSKM)	No	[46]
	iPSHD11 (40) iPSHD22 (47) iPSHD34 (42)	Fibroblasts	Lentiviral (OSKM)	No	[25]

[a] These cell lines have been deposited at the National Institute of Neurological Disorders and Stroke (NINDS) repository, https://stemcells.nindsgenetics.org, and were obtained from there by authors of the cited articles

[b] IVF/PGD—embryos donated after preimplantation diagnostics of in vitro fertilization procedure

[c] These lines were derived from a homozygous patient

cellular stages of nearly homogenous cells, which may reflect the ongoing transitional developmental progress of their in vivo counterparts [99, 100]. Notably, these distinct expandable states are composed of previously established, selected cell populations, and end-point analyses of these populations may not capture events relevant to step-wise in vivo development. Therefore, for modeling a disease, NSC and NPC should be individually derived from PSC for each experiment, as this method might be more accurate than using the high-passage NSC.

Pluripotency and Self-Renewal

HTT is not required for the maintenance of a pluripotent state in mouse cells [101]. Additionally, HD mutation does not influence iPSC generation or other features of the pluripotent state, including the expression of pluripotency network genes and the general ability to differentiate into cells that originate from all germ layers [22, 23, 26, 48].

The self-renewal and cell cycle of NPC are altered in the developing and adult HD mice, as well as postmortem HD patient brains [66, 70]. However, no differences have been identified in the proliferation and cell cycle of human HD ESC/iPSC or derived NSC [21, 23, 24]. In contrast, lower proliferation rates have been identified in heterozygous (ESC-derived) and homozygous (brain-derived) NSC from HD 140CAG KI mice vs WT NSC [28]. At another laboratory, heterozygous NSC from the same mice did not exhibit differences in the proliferation or cell cycle, despite similar culture conditions [27]. Similarly, there were no differences between isogenic HD Q20, Q50, and Q111 NSC [27]. These studies used established, high-passage monolayer NSC cultures, which represent a homogenous population of NSC. In contrast, Nguyen et al. used a step-wise neuralization protocol, with NSC analyzed at each step, and they identified an increased proliferation of HD Q111 ESC-derived NSC [29]. Therefore, the discrepant effect of full-length mutant HTT on cell growth characteristics using mouse cell lines may be the result of several variations between cell lines, such as differential culture protocols, exact source of stem cells, and developmental timing of cell isolation.

Differentiation Potential

HTT is required for NSC rosette formation [65]; however, its loss does not affect the derivation and identity of postrosette, radial glia-like, NS populations [27, 102]. Interestingly, pre-rosette [26, 30], rosette [24, 42] and NS states [27] may be derived and maintained in HD cells. Reports by Nguyen and colleagues, which are more closely focused on cellular identity transitions, have indicated that both the loss and mutation of HTT in mouse ESC impair the specification and maturation of progenitors within all germ layers [21, 29]. Mutant HTT has been shown to promote neuroectodermal fate (increased numbers of Sox1+ and Nestin+ cells) and advanced neuronal maturation, as well as increased acquisition of oligodendrocyte fate, at later stages of NSC maturation.

The iPSC derived from transgenic R6/2 mice did not present overt neural differentiation deficits [22]. In contrast, differentiation of ESC from mouse HD 150Q knock-in resulted in more neural precursors (Sox3+) and neurons (β-tubulin+), than differentiation of WT NSC [19]. Similar results were

obtained from adult HD 150Q NSC isolated from SVZ [19]. Furthermore, an increase has been observed in population of Nestin+ NPC after 42 days of differentiation of human juvenile HD iPSC (60, 109, and 180Q) towards striatal-like fates [30]. Interestingly, this retained Nestin+ population, and not mature neuronal cells, appeared to account for the previously shown population of striatal-differentiated juvenile HD cells susceptible to excitotoxic death induced by BDNF withdrawal [26]. BDNF is deficient in HD [103] and it is an important regulator of adult neurogenesis [104]. Therefore, the results indicate that the deficit in BDNF affects immature neuronal progenitors, rather than mature cells, possibly reducing a pool of endogenous cells, which may be able to regenerate the affected brain regions. Two additional groups have reported similar findings, with decreased neural differentiation using a paradigm for efficient differentiation of late-passage, adherent mouse NSC into matured GABAergic neurons [105]. In one report, homozygous, mouse HD Q140 knock-in NSC resulted in fewer Nestin+ NPC, fewer βIII-tubulin+ neurons, and more Gfap+ glial cells than WT cells [28]. Similarly, fewer neuronal cells (Map2+ and Tau+) were identified in HTT KO cells and heterozygous Q20–140 knock-in cells [27]. However, increases in glial populations (Gfap2+ and S100β+) were only present in KO cells, which suggests that these putative loss-of-function mechanisms may be rescued by the mouse WT HTT allele in heterozygous HD cells. GABAergic neurons, which are mostly affected in HD, have not been directly assessed in the previously described studies; however, a decreased acquisition of GABAergic cell fate was evident in KO and Q111 mouse ESC [21]. In addition, a decreased neurite length was demonstrated in GABAergic neurons that originated from juvenile human HD iPSC (86Q) [43]. In contrast, the differentiated human ESC displayed an increased number of GABAergic neurons, increased number of neural nodes, but no change in neurite length [34]. Additional studies using human ESC and iPSC with lower adult HD CAG numbers indicated no deficit of maturation into GABAergic projection neurons [20, 23, 25, 33], which suggests greater developmental deficits in GABAergic maturation in the case of juvenile-onset HD than in case of adult-onset HD.

Evidence from investigation of differentiation of HD pluripotent models, together with the brain region-specific effects of HTT identified in vivo in mice and patients, indicates that the true effects of mutant HTT may sometimes be masked in artificial cell culture systems. Therefore, researchers should consider using conditions closer to an in vivo situation, e.g., cerebral organoids, to delineate the developmental deficits in HD more precisely, using adult and juvenile human HD cells.

CAG Number and Genomic Stability

In HD the CAG length is dynamic and may undergo changes during gametogenesis and in somatic cells [106, 107]. The length of the parentally transmitted mutation correlates with the disease onset and severity. However, progression of the disease can be modified by somatic expansions. For instance, vast somatic expansions were identified in premanifest and late-phase, postmortem brains [108]. Large expansions in HD stem cells may change the interpretation of research data, since the length of the mutation might influence the severity of the cellular phenotype. The expansion rate in mice is cell-type dependent and occurs in postmitotic neurons as a consequence of a defective DNA repair of stress-induced DNA breaks [107, 109]. ESC and NSC are characterized by an increased DNA damage response and repair systems, which are essential for their developmental roles [110]. Therefore, the absence of changes [23, 27, 35] or mild [26, 31] changes in the CAG length in human and mouse pluripotent and neural HD stem cells is not surprising. Following differentiation, HD cells do not undergo overt repeat instability for up to 10 weeks. This was assayed in vitro in neuronal [23] and non-neuronal lineages (cardiomyocytes) [90], as well as in vivo in teratomas [35, 90]. The main exception to the previously described research includes data obtained from R6/1 mouse-derived ESC, in which repeat instability was identified in both pluripotent and neural differentiated states [36]. On average, there was a threefold instability increase when cells were challenged by peroxide-induced oxidative stress. Importantly, peroxide induced the upregulation of specific DNA repair system genes compared with WT cells. These findings suggest that increased susceptibility and reaction to stress-induced DNA are features of HD PSC, compared with non-affected lines.

The overall rate of genomic mutations is lower in PSC than in somatic cells; however, the reprogramming process and prolonged in vitro self-renewal conditions increase the chances of acquiring mutations [111]. Human HD iPSC have an increased rate of genomic instability during reprogramming, when p53 silencing is used in the process [96]. Reprogramming evokes increased replication stress [112] as a result of genome reorganization and extensive proliferation, which may explain the increased rate of CAG expansion identified in the reprogrammed HD iPSC [95]. However, in a long-term cell culture, the genomic integrity of iPSC remained unaffected by the mutant *HTT* gene [23, 96].

These experiments indicate that CAG instability in HTT gene is limited in PSC and NSC in vitro. Moreover, mutant HTT does not increase genomic mutation rate during continuous culture of PSC in comparison to control cells. Therefore, HD cells should be assessed for mutations as frequently as control PSC. In addition, the substantial expansions characteristic of in vivo conditions may not be identified prior to months of culture of differentiated neurons. The application of stress to cells may enhance CAG and genomic instability.

Developmental Hallmarks of Disease in Stem Cell Modeling HD

HTT Expression and Aggregation

HTT is an ubiquitously expressed protein [113]; however, its level increases along with brain development [114] or following the in vitro differentiation of PSC into neural lineages [48, 85]. Moreover, multiple non-canonical HTT isoforms that result from alternative splicing have been described in normal human cells and HD ESC [115]. One alternatively spliced transcript, which excludes HTT posttranslational cleavage-regulating exon 10, has been downregulated during neuronal differentiation, which suggests a role in development. Of the 3 isoforms previously identified in HD patients and mice [116–118], none has been identified in ESC [115].

Mutant HTT forms various oligomeric and polymeric aggregates that differ in terms of protein composition, solubility, cellular localization, and toxicity [119, 120]. The presence of antibody-detectable inclusions in cells of the CNS and peripheral tissues is a hallmark of disease. However, they are absent in immature PSC and NSC and only begin to emerge in mouse [22] and rhesus [39] transgenic HTT-exon 1 (144Q and 65-72Q, respectively) cells after 1.5–3 weeks of neural differentiation. The formation of inclusions is preceded by the formation of soluble oligomers in rosette-stage NSC, which may be detected via immunoblotting. In human transgenic ESCs with mutant HTT, an exon 1 fragment with juvenile CAG repeat range inclusions and soluble oligomers were identified approximately 2 months after differentiation [37]. However, a longer time span is required for the formation of detectable inclusions in juvenile and adult patient-derived cells because inclusions were detected in neurons after 6 months of culture [25] or transplantation [38], but not up to 2–3 months of differentiation [26, 33]. Aggregation may be enhanced with the use of a proteasome inhibitor, e.g., MG132. In one study, the use of MG132 enabled researchers to identify inclusions already in human iPSC with 72Q [38]. Interestingly, mutant HTT RNA also aggregates into toxic foci, which may be identified in human iPSC and derived NPC with only 57 CAG repeats [97, 98]. Observations in human cells reveal the late onset of aggregation identified in HD patients and lack of aggregates in early developmental stages of cellular HD models.

Differences in Gene Expression

Gene expression alterations may be the most common and earliest difference detectable in HD cells at the PSC or NSC stage. We have reviewed the research regarding alterations in gene expression and performed a simple meta-analysis to retrieve level 5 GO terms (most detailed GO level) related to developmental biology and signaling pathways related to developmental processes using lists of names of deregulated genes provided by 8 research works. The "Methods" section contains detailed meta-analysis paradigm, and Table 2 contains a list of research works that were included in the analysis.

Methods

Eight works listed in Table 2, all investigating human cells, were selected for the meta-analysis. The names of the human deregulated genes or proteins represented as names of genes were retrieved from 8 original works and were sorted into 3 separate lists. Names of genes and names of genes corresponding to deregulated proteins were subsequently listed as HUGO Gene Nomenclature Comity symbols (HGNC symbol). We established a list containing names of genes deregulated in NSC and iPSC, a list containing names of genes deregulated in NSC, both ESC and iPSC derived, and a third list containing names of genes deregulated in neurons. We did not distinguish between the deregulated genes/proteins identified in transcriptomic and proteomic experiments, since the aim of the meta-analysis was to identify level 5 GO terms and overrepresented signaling pathways related to developmental biology to generate a global overview reflecting consequences of deregulations of genes and proteins for the cellular phenotype. The cell types, culture conditions, and high-throughput methods used by the authors to identify the deregulations, together with the cutoff values selected in the 8 studies are indicated in Table 2 and Suppl. Table 1. The genes overlapping between lists, and the genes reported in more than one of the 8 studies included in the meta-analysis, were identified using MS Excel formulas (Fig. 3a, b, Suppl. Table 2).

For the retrieval of level 5 GO terms, each list was separately subjected to overrepresentation analysis using the respective tool included in an online version of ConsensusPathDB (CPDB) [121]. "Biological process," "molecular function," and "cellular component" GO terms (level 5, p value cutoff = 0.01) have been selected for the analysis. The retrieved GO terms for each gene list were further manually sorted into 22 arbitrary categories (Suppl. Table 4). The GO annotations related to development were presented in the form of a heatmap (Fig. 3c) [122, 123] using the q values (adjusted p values) calculated with the use of overrepresentation analysis in CPDB. The green heatmap color denoted the lack of GO term, and the increasing dose of warm colors denoted the presence and increasing statistical significance of GO terms (decreasing q value). For retrieval of biological pathways, each list of genes was again separately subjected to overrepresentation analysis using the respective tool of CPDB, and statistically significant pathways were retrieved using the p value cutoff = 0.01, and minimum overlap with the input list equaled 10 genes (Suppl. Table 5).

Table 2 Studies included in the meta-analysis

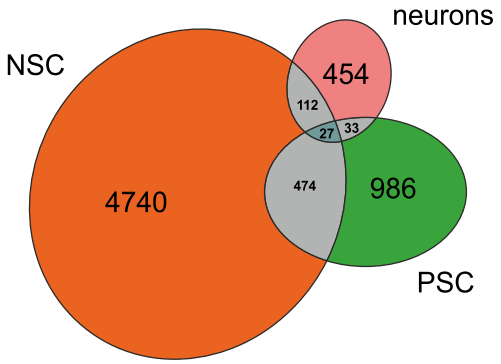
Cell type	Markers of cell identity	Method	Cutoff	Reference
iPSC and iPSC-derived NSC	Nestin (NSC)	RNA-seq	FDR <0.05	[42]
ESC and ESC-derived NSC	Nestin, PAX6, Ki67 (NSC)	Gene microarray	$P < 0.001$	[76]
iPSC	Markers for pluripotency	Gene microarray	FDR <0.05	[24]
iPSC-derived NSC and neurons	Nestin, PAX6 (NSC) TUBB3,MAP2A/B, DARPP-32, BCL11B (neurons differentiated towards striatal-like)	Gene microarray	absolute value of fold change >2	[26]
iPSC-derived neurons	TUJ1, MAP2, GABA, GAD65, DARPP-32, Calbindin (neurons differentiated towards GABAergic striatal like)	Gene microarray	Adjusted $P < 0.05$ and fold change >1	[33]
iPSC-derived neurons	TUBB3, DARPP-32, GAT1 (neurons differentiated towards GABAergic MS-like)	Gene microarray	$P < 0.05$	[25]
ESC and ESC-derived neurons	MAP2, GABA, GAD65 (neurons differentiated towards GABAergic-like)	IPG-IEF and LC-MS/MS	$P < 0.05$	[34]
iPSC and iPSC-derived neurons	MAP2 (neurons)	Western blot, 2D electrophoresis and LC-MS/MS	$P < 0.05$	[47]

GO Terms Identified for Deregulated Genes

The list of names of differentially expressed genes/proteins, used for meta-analysis, contained 986, 4740, and 454 names of genes for PSC (ESC and iPSC), NSC, and neurons, respectively. Among the listed genes, several deregulated genes overlapped between the analyzed lists; however, only 27 genes were present in all 3 lists (Fig. 3a, b, Suppl. Table 2). Supplementary Tables 2, 3, 4, and 5 present the detailed results of our meta-analysis, including the genes/proteins overlapping between the lists, all GO terms, and genes/proteins reported in more than one study and biological pathways that were represented by more than ten genes. As also described in the "Methods" section, we have established a total number of 22 arbitrary categories. We have selected ten arbitrary categories which represented biological processes related to the developmental aspect of HD and categories with already well-established HD phenotypes. Table 3 indicates the selected categories, with the number of related GO terms and the top 5 GO terms in a particular category. The top 5 GO terms were selected by the highest number of genes/proteins from the input list. In addition, the number of GO terms in a particular category was normalized to the total number of GO terms identified for a list of genes of a particular cell type and also presented as the percentage of total number of GO terms (Table 3). The same or similar top 5 GO terms have been shared for 2 or 3 lists of genes in several of the arbitrary categories. The categories sharing GO terms between all gene lists included "differentiation," "neurodevelopment," "cytoskeletal organization, cell-cell interactions," and "apoptosis". The categories sharing GO terms between two gene lists included "proliferation" (NSC and neurons), "development," "control of gene expression," and "metabolism" (PSC and NSC). These findings suggest that similar biological processes, resulting from deregulation of genes/proteins, occur in PSCs and may be sustained during differentiation, until reaching the stage of mature neurons. Noteworthy, similar top 5 GO terms occurred in the analysis, regardless of the protocols for ESC/iPSC derivation, NSC differentiation, neuronal identity, and the inclusion of genes from transcriptomic and proteomic studies. This analysis also confirms that the investigation of HD-associated changes, along the differentiation axis of ESC/iPSC-NSC-neurons, is justified. Finally, the categories most strongly represented by GO terms in PSC and NSC namely, differentiation, neurodevelopment, and development highlight the role of developmental processes, resulting from gene deregulations in HD.

Nevertheless, there are also differences between the analyzed lists of genes. As expected, there were fewer GO terms related to development for neurons than for PSC or NSC. A plausible explanation is the fact that the genes and processes that play a role in the developmental process may have biological functions in mature neurons. There were twice as many GO terms in the neurodevelopment category for NSC than for other lists of genes, which reflects the developmental stages of the analyzed cells. Another evident difference was identified in the metabolism category. The GO terms related to metabolism of nucleic acids dominate among the deregulated genes listed for PSC and NSC, which can likely be attributed to high proliferation rates, whereas in neurons, the category contains mainly GO terms related to metabolism of proteins

a



b

27 genes overlapping between PSC, NSC and neurons		
ACACA, ANGPTL4, AXL, CFL1, COL3A1, COL5A1, COL5A2, COMT, CYTL1, DDR2, DLK1, DPYSL5, EFEMP2, GPX1, GSTO1, ITGA11, MAN1A1, NPNT, PRDX1, PRDX6, PSMD5, S100A11, S100A4, SDC2, TENC1, THBS1, VIM		
Top 5 GO terms for the list of 27 genes overlapping between PSC, NSC and neurons		
GO term	**q-value**	**Genes**
positive regulation of signal transduction	0.016482	NPNT, GSTO1, PSMD5, THBS1, GPX1, S100A4, COL3A1, AXL
positive regulation of cell communication	0.016482	NPNT, GSTO1, PSMD5, THBS1, GPX1, S100A4, COL3A1, AXL
regulation of intracellular signal transduction	0.018076	NPNT, GSTO1, PRDX1, THBS1, GPX1, S100A4, COL3A1, AXL
calcium ion binding	0.000213	EFEMP2, THBS1, NPNT, DLK1, S100A11, S100A4, MAN1A1
negative regulation of cell death	0.040684	GPX1, ANGPTL4, CFL1, AXL, THBS1

c

◀ **Fig. 3** Meta-analysis reveals common genes/proteins and developmental GO terms for PSC, NSC, and neurons.**a** Names of genes that overlap between the lists of deregulated genes for PSC, NSC, and neurons. Venn diagram indicates number of genes/proteins included in the meta-analysis that overlap between only two or all three of the analyzed lists of genes. Detailed list of overlapping genes is available in Supplementary Fig. 2 (Venn diagram generated with eulerAPE v3). **b** *Top*: list of 27 genes/proteins that overlap between all three lists of genes for PSC, NSC, and neurons. *Bottom*: top 5 GO terms (level 5) containing the highest number of genes/proteins from the input list with 27 genes. The *q* values and list of members are provided. **c** Heatmap demonstrates GO terms (level 5) related to development, which were detected by the meta-analysis in the lists of genes for PSC, NSC, and neurons. Absence of developmental GO term (*green*), the *increasing dose* of *warm colors* (*orange* to *red*) denotes the increasing statistical significance of GO term (decreasing *q* value for a particular term). GO terms in the heatmap are ordered according to germ layers and general events (Heatmap generated with R software v3.3.0) (Color figure online)

and neurotransmitters. Furthermore, there is no GO term related to lipid metabolism in neurons, whereas it is present in the top 5 GO terms in the "cell response" category for PSC and NSC (Suppl. Table 4), which may be associated with a higher demand for cholesterol in the early stages of embryonic development [22]. Interestingly, NSC and neurons are enriched in GO terms related to cell stress compared to fewer GO terms in PSC. This finding corresponds with the demonstration revealing that after differentiation, HD cells are more vulnerable to stress and death evoked by withdrawal of BDNF and other growth factors [24]. There have been several, additional interesting categories and GO terms representing biological processes, implying their role in HD. For instance, in the meta-analysis, there have been such GO terms as "response to wounding" or "axon regeneration" (Suppl. Tables 3 and 4), which emphasizes the occurrence of processes related to neural degeneration in HD or GO terms related to immune response underlining its important role in HD [124]. In our analysis, we did not detect GO terms directly related to epigenetics. However, these changes were detected in mouse and human ESC, NPC, and neurons that linked mutant HTT and chromatin status during development [32, 34].

Signaling Pathways

Several signaling pathways involved in embryonic development are also affected in HD [125]. For example, the MEK/ERK signaling pathway, which is a part of the MAPK signaling pathway, plays a neuroprotective role in HD. In addition, the TGF-beta signaling pathway is upregulated in HD human cells and a rat model [125]. The meta-analysis performed for the purposes of this review also identified overrepresented, developmental signaling pathways among the deregulated genes and proteins identified in iPSC and NSC, collected from the eight research works and listed in Table 2. In iPSC, the developmental signaling pathways [126], represented by at least ten deregulated members (proteins/genes) as a cutoff (cutoff = 10, $p < 0.01$), included the TGF-beta, Wnt, PI3K-Akt, Hippo, MAPK, EGFR1, and Rap1 signaling pathways semaphorin interactions, and other signaling pathways. In NSC, the pathways included TGF-beta, beta catenin, PI3K-Akt, EGFR1, PDGFR-beta, Rap1, Hippo, BDNF, semaphorin interactions, and other pathways (cutoff = 10, $p < 0.01$). In terminally differentiated neurons, the signaling pathways with at least ten deregulated proteins included the MAPK family, PI3K-Akt, EGFR1, PDGF, NGF, and other (cutoff = 10, $p<0.01$) (Suppl. Table 5). In addition to high-throughput screens, the HD signaling pathways have also been investigated in a more detailed way, and early HD phenotypes have been identified in stem cells. For instance, the deregulation of the MAPK, Wnt, and p53 pathways was identified in mouse and human iPSC [48]. A study by Ring et al. indicates that NSC derived from HD human iPSC (72Q) were rescued from apoptosis when TGF-beta was added to growth factor-deprived culture medium (without bFGF and LIF) [42]. However, the apoptotic phenotype was masked in a complete culture medium, potentially because the TGF-beta signaling, which is putatively protective, was upregulated in the HD NSC. Therefore, it must be noted that the signaling pathway inhibitors and activators used during stem cell culture and differentiation may modify or mask the HD phenotypes related to their activity in vivo.

The p53 tumor suppressor protein is a regulator of cell fate specification, as the p53-null mutation in mice leads to multiple developmental defects, including brain exencephaly [127, 128]. Embryonic lethality may be induced by disrupting p53 transactivation functions via the KO of the p53 regulators, the E3 ubiquitin-protein ligases: MDM2 or MDM4 [129], or the transcription activation domains, vital for interactions [130]. In HD, p53 influences the disease phenotype via multiple pathways, which have previously been reviewed in a comprehensive way [125]. In addition, the absence of p53 in $Hdh^{140Q/140Q}$ mouse, which presents a number of repeats in the juvenile range, results in increased formation of aggregates in the brain [131]. Research on adult HD cells or HD patient brains indicates that the p53 expression or activity was upregulated at later stages of disease and mediated apoptosis [125].

In human undifferentiated and neuronally differentiated HD iPSC (86Q), p53 was extensively translocated into mitochondria, which was accompanied by p53-dependent activation of pathways that led to neuronal maturation defects and apoptosis [43]. Both defects were repaired by p53 silencing or mitochondria fission inhibition [43]. Moreover, Chae et al. identified increased levels of p53 phosphorylation in human HD iPSC (71 CAG) [47]. In contrast, we have shown that p53 is downregulated in undifferentiated mouse iPSC from the YAC128 model (128 CAG) and also in human iPSC sampled from a juvenile HD patient (onset at 3 years; 109 CAG) [43]. The p53 remained unchanged in iPSCs independently derived from the same patient as in the work of Chae et al. containing

Table 3 Meta-analysis summary: number of GO terms and names of top 5 GO terms grouped into 10 arbitrary categories describing relevant biological and developmental processes and HD phenotypes. Each arbitrary category contains the indicated number of GO terms, which is also expressed as a percentage of total number of GO terms for particular cell type. Numbers in brackets indicate number of genes from the input list, which were assigned by CPDB for each GO term

Category	PSC—292 total GO terms, 986 genes	NSC—445 GO terms, 4740 genes	Neurons—191 GO terms, 454 genes
Proliferation, cell cycle, and growth	13 GO terms; 4.45 %	21 GO terms; 4.80 %	10 GO terms; 5.23 %
Top 5 terms	Positive regulation of cell proliferation (62), negative regulation of cell proliferation (50), regulation of mitotic cell cycle (32), regulation of epithelial cell proliferation (31), positive regulation of cell cycle (21)	Positive regulation of cell proliferation (276), negative regulation of cell proliferation (224), regulation of cell cycle process (146), negative regulation of cell cycle (139), mitotic cell cycle phase transition (130)	Positive regulation of cell proliferation (34), negative regulation of cell proliferation (27), regulation of cell cycle process (20), negative regulation of cell cycle (19), positive regulation of cell cycle (16)
Differentiation	24 GO terms; 8.22 %	24 GO terms; 5.39 %	9 GO terms; 4.71 %
Top 5 terms	Cell morphogenesis involved in differentiation (89), positive regulation of cell differentiation (67), negative regulation of cell differentiation (53), epithelial cell differentiation (45), stem cell differentiation (42)	Cell morphogenesis involved in differentiation (378), positive regulation of cell differentiation (297), negative regulation of cell differentiation (221), epithelial cell differentiation (204), stem cell differentiation (151)	Cell morphogenesis involved in differentiation (58), negative regulation of cell differentiation (30), positive regulation of cell differentiation (29), epithelial cell differentiation (27), stem cell differentiation (19)
Development	59 GO terms; 20.21 %	93 GO terms; 20.90 %	12 GO terms; 6.28 %
Top 5 terms	Regulation of cell development (61), chordate embryonic development (58), regulation of cell morphogenesis (43), striated muscle tissue development (38), regulation of vasculature development (30)	Regulation of cell development (303), chordate embryonic development (243), regulation of cell morphogenesis (193), striated muscle tissue development (138), camera-type eye development (117)	Regulation of cell development (35), chordate embryonic development (25), striated muscle tissue development (16), endochondral bone morphogenesis (12), mesenchymal cell development (12)
Neurodevelopment	13 GO terms; 4.45 %	30 GO terms; 6.74 %	12 GO terms; 6.28 %
Top 5 terms	Neurogenesis (121), neuron differentiation (109), axon development (58), Regulation of nervous system development (54), axon guidance (44)	Neurogenesis (578), neuron differentiation (504), regulation of nervous system development (282), axon development (264), axon guidance (184)	Neurogenesis (74), neuron differentiation (60), axon development (36), regulation of nervous system development (30), axon guidance (27)
Control of gene expression	18 GO terms; 6.16 %	16 GO terms; 3.60 %	4 GO terms; 2.09 %
Top 5 terms	Regulation of gene expression (206), transcription, DNA-templated (179), sequence-specific DNA binding (63), regulatory region DNA binding (52), transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding (21)	Regulation of gene expression (1057), transcription, DNA-templated (901), sequence-specific DNA binding (281), regulatory region DNA binding (240), transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding (91)	Structure-specific DNA binding (16), DNA conformation change (12), protein-DNA complex assembly (10), establishment of protein localization to chromosome (2)
Signaling pathways	18 GO terms; 6.16 %	29 GO terms; 6.52 %	8 GO terms; 4.19 %
Top 5 terms	Cell surface receptor signaling pathway (166), intracellular signal transduction (151), positive regulation of signal transduction (94), regulation of intracellular signal transduction (90), negative regulation of signal transduction (71)	Cell surface receptor signaling pathway (839), intracellular signal transduction (805), regulation of intracellular signal transduction (454), positive regulation of signal transduction (430), negative regulation of signal transduction (325)	Cell surface receptor signaling pathway (90), intracellular signal transduction (82), regulation of intracellular signal transduction (50), positive regulation of signal transduction (42), negative regulation of signal transduction (37)
Cytoskeletal organization, cell-cell interactions	13 GO terms; 4.45 %	22 GO terms; 4.94 %	14 GO terms; 7.33 %
Top 5 terms	Positive regulation of cell communication (106), negative regulation of cell communication (75), actin cytoskeleton (33), positive	Positive regulation of cell communication (506), microtubule cytoskeleton (274), negative regulation of cell communication	Positive regulation of cell communication (52), negative regulation of cell communication (38), actin cytoskeleton (29), positive

Table 3 (continued)

Category	PSC—292 total GO terms, 986 genes	NSC—445 GO terms, 4740 genes	Neurons—191 GO terms, 454 genes
	regulation of cell adhesion (25), regulation of cell-cell adhesion (24)	(355), actin cytoskeleton (182), actin filament organization (117)	regulation of cell adhesion (20), actin filament organization (18)
Oxidative and other cell stress	3 GO terms; 1.03 %	11 GO terms; 2.47 %	10 GO terms; 5.24 %
Top 5 terms	Cellular response to reactive oxygen species (12), stress fiber (8), response to X-ray (5)	Stress-activated MAPK cascade (82), response to UV (43), cellular response to reactive oxygen species (40), cellular response to alcohol (37), stress fiber assembly (32)	Cellular response to reactive oxygen species (11), stress fiber (7), regulation of oxidative stress-induced cell death (6), cellular response to ionizing radiation (6), regulation of response to oxidative stress (6)
Apoptosis	6 GO terms; 2.05 %	8 GO terms; 1.80 %	10 GO terms; 5.24 %
Top 5 terms	Apoptotic process (112), regulation of programmed cell death (97), negative regulation of cell death (67), positive regulation of cell death (40), neuron apoptotic process (24)	Apoptotic process (570), regulation of programmed cell death (447), negative regulation of cell death (289), apoptotic signaling pathway (198), positive regulation of cell death (197)	Apoptotic process (73), regulation of programmed cell death (66), negative regulation of cell death(48), apoptotic signaling pathway (27), positive regulation of cell death (26)
Metabolism	42 GO terms; 14.38 %	61 GO terms; 13.71 %	33 GO terms; 17.28 %
Top 5 terms	Regulation of cellular biosynthetic process (203), regulation of nucleobase-containing compound metabolic process (198), regulation of macromolecule biosynthetic process (187), positive regulation of cellular metabolic process (183), RNA biosynthetic process (182)	Regulation of cellular biosynthetic process (1055), regulation of nucleobase-containing compound metabolic process (999), regulation of macromolecule biosynthetic process (996), RNA biosynthetic process (926), regulation of RNA metabolic process (907)	Positive regulation of cellular metabolic process (87), positive regulation of macromolecule metabolic process (76), regulation of protein metabolic process (75), regulation of cellular protein metabolic process (68), negative regulation of cellular metabolic process (65)

71 CAG repeats (disease onset at 14 years) [48]. Moreover, p53 has been identified as a top 5 gene regulator of the pathways altered after HTT mutation correction in hiPSC-derived NSC [42]. It is plausible that the p53 deregulation profile in juvenile HD stem cells is dependent on the number of CAG repeats. It is also possible that the expression level and activity of p53 may be different in juvenile HD and in adult HD. However, to verify these hypotheses, data from more HD iPSC lines, preferably isogenic and ranged from low to high CAG repeat numbers, are needed. The mitochondrial and apoptotic effects that may result from altered p53 activity will be discussed in subsequent sections.

Mitochondrial Dysfunction

One of the most extensively investigated features of HD pathology includes complex mitochondrial impairments involving multiple aspects of organelle biology [132–134]. However, their genesis and impact on disease pathogenesis are insufficiently understood. The downstream consequences of these changes may include susceptibility to excitotoxicity, reactive oxygen species (ROS)-induced DNA damage, and apoptosis.

The mitochondrial content, life cycle, activity, and dynamics are important regulators of embryonic and brain development, and their dysfunction may lead to neurodevelopmental disorders [135]. Remarkably, Ismailoglou et al. reported that mouse ESC with HTT KO had increased glucose uptake from the medium. The cells were incapable of sufficient mitochondrial ATP synthesis and therefore turned to glycolytic respiration and lowered their oxygen consumption [41]. The mitochondria in these cells were aberrantly structured; however, no polarization defects were identified. Similarly, the ESC containing mutant HTT(140Q) from knock-in HD model exhibited increased glucose consumption; however, in contrast to KO cells, the oxidative phosphorylation respiration increased, with no evidence of alterations in the mitochondrial structure. On the other hand, Jacobsen et al. reported no ATP/ADP ratio changes in KO mouse ESC and a decreased ATP/ADP ratio in Q111 knock-in cells [40]. The discrepancy between these two studies likely resulted from the different culture conditions and different reference cells. Ismailoglou et al. cultured cells in feeder-free and defined serum-free ground-state (2i+LIF) conditions [136], whereas Jacobsen et al. maintained the cells in undefined serum replacement conditions on feeders, with LIF only. Nevertheless, these studies, combined with proteomic research on human ESC and iPSC [34, 47], have identified a substantial number of alterations in the metabolome and expression of genes related to mitochondrial function, energy

metabolism, and metabolite synthesis. The metabolic impairments induced by the mutation or loss of the *HTT* gene include dysregulation of the lipid and cholesterol synthesis pathways, which have previously been shown to be affected in patients [137], PSC [22, 34, 41], and differentiated neurons [26].

Research regarding human iPSC-derived NSC has indicated that calcium signaling and the ATP/ADP ratio are decreased in these cells [26], and genetic correction of mutant HTT alleles enhanced the maximum respiration rate towards control cell levels [24]. Guo et al. focused on mitochondrial fission deficits in multiple cellular and mouse models of HD [43]. In their models, excessive accumulation of the fission-driving protein Drp1 causes the translocation of p53 to mitochondria, which leads to accumulation of ROS, mitochondrial fragmentation, and, consequently, apoptosis. In HD iPSC, they identified increased levels of Drp1 and p53 in mitochondria, whereas iPSC-derived GABAergic neurons, including MSNs, had defective, fragmented mitochondria neurites with a decreased membrane potential, decreased ATP/ADP ratios, increased ROS, and enhanced apoptosis. Both the selective Drp1 inhibitor P110-TAT and p53 silencing rescued these phenotypes and normalized the neurite lengths. The treatment also turned out to be beneficial in R6/2 mice, which validated research in iPSC HD cellular models.

The altered ROS accumulation and oxidative stress response represent other mitochondria-related impairments featured in HD [138]. As described, an increased ROS content has been identified in human MSNs [43]. If not neutralized, excess ROS may induce DNA damage and alter ROS signaling pathways, which may regulate proliferation and differentiation processes [139]. The phenotype was also shown in mouse knock-in ESC- and brain-derived NSC with 140Q [28]. Interestingly, the loss of HTT in KO-NSC did not cause ROS accumulation. In human iPSC, the expression levels of multiple antioxidant proteins were altered, including the downregulation of SOD1 and GST and the upregulation of proteins in the PRX family [47]. These changes were sustained throughout differentiation into mature neurons. We have also shown [48], in both adult and juvenile human iPSC and mouse YAC128 iPSC, that SOD1 is altered; however, in our case, it was upregulated. This discrepancy again points to the potential effects of the culture conditions, as we used feeder-free defined Essential 8 conditions for culture of human iPSC. Nevertheless, these changes may indicate that an altered redox homeostasis and increased susceptibility to oxidative stress, including the ultimate solution, apoptosis (which will be described later), are present in iPSC.

Another crucial mitochondria-related pathomechanism of HD is excitotoxicity, in which *N*-methyl-D-aspartate receptors (NMDARs) are overactivated in response to glutamate. NMDAR overstimulation leads to dysregulation of the cellular Ca^{2+} homeostasis, which is typically maintained by mitochondria and endoplasmic reticulum, and ultimately leads to cell death [140, 141]. The propensity for neuronal excitotoxicity in mature brains has been evaluated using ESC/iPSC-derived neurons. The calcium homeostasis was disrupted in MSN-like cells derived from human HD iPSC [25]. The abnormal increase in the cellular calcium entry, which was regulated by calcium store-operated channels, was effectively blocked by EVP4593, thereby leading to decreased cell death rates. Exposure to both physiological and pathological glutamate concentrations exacerbated the Ca^{2+} imbalance and led to increased apoptosis in striatal-like HD cell lines, from adult and juvenile patient iPSC [26]. It has subsequently been demonstrated that the cells that died in this in vitro culture were not mature neurons; instead, they were Nestin+ striatal-like NPC, and the excitotoxicity may be mediated by the loss of neuroprotective BDNF via the TrkB pathway [30]. BDNF is a key player in HD pathogenesis [141], and its downregulation has been identified following differentiation to NSC [24, 44].

It is still to be determined how mitochondrial deficits correspond to defects in the differentiation and maturation of HD cells. Moreover, the early presence of a mitochondrial phenotype in pluripotent models may provide additional insights into the mitochondrial impairments in HD, which are of particular interest as a result of several contradictory observations concerning metabolism and mitochondria in HD [132].

Autophagy and UPS

Autophagy is a part of the stress response system responsible for the degradation of dysfunctional or toxic protein aggregates and organelles, including mitochondria, and pathogens. In this process, proteins and organelles to be cleared are delivered to lysosomes, the effectors of degradation. Autophagy is important for stem cell self-renewal and development, and it is essential for the maintenance of stress-sensitive, postmitotic neurons [142, 143]. In HD, mutant HTT impairs the autophagosome trafficking and thus the fusion of autophagosomes with lysosomes [144]. The mRNA expression of *Tfeb*, a master regulator of autophagy and lysosomes, along with their targets, *Tpp1* and *Ctsf*, has been shown to be increased in iPSC and neurons derived from R6/2 mice [22]. These increases resulted in a greater number of lysosomes in iPSC. An increased number of lysosomes have also been identified in an adult patient's iPSC and the derived neurons [23]. Additional studies have confirmed an increased number of autophagosomes, lysosomes, and mitophagy (mitochondrial autophagy) in iPSC-derived MSN-like neurons [25]. In these cells, lysosome content was decreased following treatment with the Ca^{2+} influx-repairing agent EVP4593. Interestingly,

astrocytes derived from the juvenile HD patient iPSC had an increased content of cytoplasmic vacuoles, some of which were autophagosomes [94].

Another mechanism for clearance of unwanted proteins is the unfolded protein response (UPR)/ubiquitin-proteasome system (UPS) pathway. In HD, the accumulation and aggregation of mutant HTT result from a failure of the UPS to efficiently deal with polyQ-expanded HTT [144, 145], which may be influenced by deficits in the preceding UPR pathway [146]. Consistent with this evidence, human ESC and derived neurons demonstrate altered levels of the ubiquitination pathway proteins [34], whereas correction of the HD mutation in human iPSC results in the upregulation of UPR-related gene expression [24, 146].

Apoptosis

Programmed cell death may be mediated by apoptosis, which is activated by a caspase cascade. Caspase activity is elevated in cellular and animal models of HD and postmortem brain tissues obtained from HD patients [147]. In vivo experiments have also shown that modulation of the HTT level results in apoptotic responses in the developing brain. For instance, the depletion of HTT in neuroepithelial cells by the shRNA construct may lead to disturbed cell migration from ventricular zone to caudoputamen and reduced proliferation or increased apoptosis in the cortex of developing 12.5-day-old embryo. Both the neuronal survival and proliferation may be partially rescued by over 40% caspase-9 knockdown [148]. Notably, mutant Hdh CAG knock-in NSC exhibit overactivity of caspase-3/7 [147, 149]. Similarly, the activity of caspase-3/7 plays a key role in apoptosis in HD, and it is increased in a CAG repeat length-dependent manner in mouse ESC-derived NSC during differentiation [27]. Even the cells without endogenous HTT exhibit high levels of caspase activity [150, 151]. However, mutation of the *HTT* gene in human ESC or iPSC did not induce programmed cell death, as indicated by the activated caspase-3 staining [23]. Furthermore, there was no difference in the caspase-3/7 activity between genetically corrected (21 CAG repeats) and uncorrected human isogenic HD iPSC (72 CAG repeats) [24]. Both corrected and uncorrected HD iPSC were differentiated into NSC, followed by stress induction through withdrawal of growth-supporting factor from the culture medium. The uncorrected HD NSC responded to such a withdrawal with increased apoptosis as assessed by increased TUNEL staining and increased caspase-3/7 activity, while the corrected NSC were “cured” of these effects. The results suggest that polyglutamine expansion makes iPSC and NSC more sensitive to cell death on their differentiation route towards neurons; however, they become sensitive to polyQ-induced apoptosis at a relatively early stage of neuronal development.

Apoptosis was also increased following stress conditions in cells obtained from HD patients or animal models [147, 152, 153]. In the pluripotent HD models, after 3 weeks of differentiation of juvenile human NSC, there has been a gradual decrease in the number of active neurons, with eventual cell death by the end of the third week [26]. The HD cultures developed a severe phenotype and exhibited high levels of mutant HTT expression. Furthermore, after differentiation using a modified, more protective protocol, neurons exhibited increased caspase-3/7 activity and mortality following BDNF withdrawal. This effect was reversed by the addition of BDNF, at 4 times of its normal concentration, to the media of the HD cell cultures [26]. The selective inhibition of ATM-mediated signaling may also confer the protection of HD iPSC-derived striatal neurons from BDNF withdrawal [45]. Multiple reports have identified stressors that induce increased levels of apoptosis in PSC-derived HD neurons, e.g., H_2O_2 (oxidative stress) [33, 39], 3-methyladenine (autophagy inhibitor), MG-132 (proteasome inhibitor) [105] and staurosporine (broad-spectrum kinase inhibitor) [34]. In HD, neuronal dysfunction and death are more widespread, notably in the cases of longer CAG repeats, which suggests that the toxicity mediated by stress factors may not be exclusively limited to striatal neurons. It should be noted that severe MSN death characteristic for HD is not as relevant in mouse models. For example, in knock-in mice, the phenotype can hardly be observed until late life unless mice are homozygous and have CAG repeat numbers over >140. Therefore, human and primate HD PSC are more suitable for research on apoptosis.

Cell Adhesion

Cell adhesion and cytoskeletal molecules, including N-cadherin and actins, are indispensable for normal brain development because of their vital roles in cell orientation, migration, communication (including apoptotic signaling), and the formation of brain structures [154, 155]. As previously discussed, in the absence of HTT, the rosetteless phenotype may occur in NSC as a result of increased activity of ADAM10 metalloproteinase, which mediates increased cleavage of N-cadherin [65]. Moreover, N-cadherin deficits have been identified in the brains of HD Q111 knock-in mice [156].

The N-cadherin pathway is directly affected by mutant HTT, and genetic correction of the mutant allele has resulted in upregulated expression of several protocadherins in human iPSC and iPSC-derived NSC [24, 42]. Moreover, human iPSC-derived NSC lines with 60Q and 180Q bound less phalloidin peptide, which suggests changes in the actin cytoskeleton, and displayed a decrease in the adhesive capabilities via cell cluster formation assays [26]. The existence of actin deficits in HD NSC is further supported by the motility

reduction identified in mouse ESC-derived NSC, with a 110Q knock-in mutation or the loss of HTT [28], as well as the expression changes in the motility and cytoskeleton pathways, identified following neuronal differentiation of 140Q knock-in mouse ESC, human ESC, and iPSC [19, 26, 34, 47]. Taken together, these findings suggest that cytoskeletal and adhesion molecules may influence the functional differentiation and survival deficits that occur in stem cell models of HD and during in vivo development of HD models.

Conclusion

In conclusion, this review summarizes current evidence for the existence of a developmental, pathogenic phase in the progression of HD, which suggests that the traditional view that HD is solely an adult, neurodegenerative disease should be revisited. The most convincing evidence includes the effects of induced expression and the selective deficiency of normal or mutant HTT in developmental stages. Potential mechanisms are presented, based on data from in vivo models. Pluripotent, neuronal stem cells and mature neurons derived from these cells also exhibit an array of phenotypes characteristic of HD patients and models. Finally, the GO terms retrieved for the deregulated genes in HD cell models and deregulated signaling pathways (most importantly, TGF-β, Wnt, and MAPK signaling) are directly related to development and neurogenesis, affected in vitro and in vivo. Taken together, ESC/iPSC appear to be the best cellular models, which are available at present to investigate the impact of neurodevelopmental defects in HD. The data also indicate the need for optimization of human iPSC models, including reprogramming and differentiation protocols, the use of isogenic human stem cells, cerebral organoids, and defined media, for the purposes of consistent observation of HD phenotypes. The selective differentiation of ESC/iPSC into neuronal cell types provides an additional opportunity to model late HD phenotypes because the neurons may be maintained for extended periods of time in culture. In this model, later phenotypes may be enhanced by the application of stressors or aging factors that induce age-related events, such as progerin [157, 158]. The developmental characteristics of HD have crucial implications for therapy. It remains unclear which therapeutic strategy is most appropriate and when the treatment should be initiated. For cell therapy, a transplant may directly provide protective and trophic agents, or it may be designed to mature in vivo, developing functional neurons within the network of host cells.

Neurodegenerative disorders are traditionally recognized as diseases of late onset; however, this perception may be shifting. A suitable example demonstrating the need for this perception change can be the evidence showing that healthy 3-year-olds in a genetic risk group of developing Alzheimer disease achieved lower scores on working memory and attention and also had smaller hippocampi than the non-risk group representatives [159].

CNS, central nervous system; DG, dentate gyrus; ESC, embryonic pluripotent stem cell; GO, Gene Ontology; HD, Huntington disease; HTT, huntingtin; iPSC, induced pluripotent stem cells; KO, knockout; MSN, medium spiny neuron; NPC, neural progenitor; NSC, neural stem cells; polyQ, polyglutamine; PSC, pluripotent stem cells; SVZ, subventricular zone; WT, wild type

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Compliance with Ethical Standards

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