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Neural Stem Cells differentiated from Human induced pluripotent stem cells (iPSCs) as a novel *in vitro* model to study developmental pathways in Huntington Disease.

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AMLES…

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1. Introduction

1.1 Huntington Disease

1.1.1 Historical overview

The disease known today with the name “Huntington Disease” (HD) was described for the first time by George Huntington. He was an American physician who wrote at the age of 22 a paper entitled “On Chorea” published on the journal “Medical and Surgical Reporter” of Philadelphia on April 13, 1872 were he properly described HD as hereditary disease characterized by unwanted choreatic movements, behavioral and psychiatric disturbances and dementia.

In 1964 Blinderman, Weidner & Markham (*Kenneth et al., 1973*) discovered that HD is an autosomal dominant progressive neurodegenerative disorder where a selective neural cell loss take place with a consequent atrophy in the caudate nucleus and putamen.

In 1983 Gusella et al. mapped Huntingtin (HTT) in the chromosome 4 (it was the first disease-associated gene that was molecularly mapped to a human chromosome) *(Gusella et al., 1983*). Ten years later, scientists of the Huntington's Disease Collaborative Research Group (HDCRG) identified the gene carrying the mutation, the *IT15* gene that coding for a big protein that now is known as Huntingtin (HTT). Moreover, they determined the precise nature of the HD-associated mutation in HTT (*MacDonald et al., 1993*) and discover that the HTT gene contains a region where the triplet nucleotide CAG is repeated several times and that is longer in the individuals carrying the disease with respect to the unaffected ones.

1.1.2 The HTT gene.

The only agent causing HD is a mutation that brings to a trinucleotide CAG repeat expansion in the *HTT* gene that encodes for a 3,144-residue protein with a molecular mass of ~350 kD. Progressive motor disability, depression, personality changes, progressive decline in cognitive capabilities together with a related family history are the most common symptoms that bring clinicians to the diagnosis of the HD. However, to confirm the diagnosis of HD a genetic test to determine the number of CAG repeats in exon 1 of *HTT* (HD) gene is mandatory (*Craufurd et al., 2015*). This mutation is translated in a polyglutamine stretch in the huntingtin (HTT) protein. HD follows the autosomal dominant inheritance pattern. A parent has 50% chance of passing the mutated gene to the child. Inheriting a single mutated allele is enough to cause the disease.

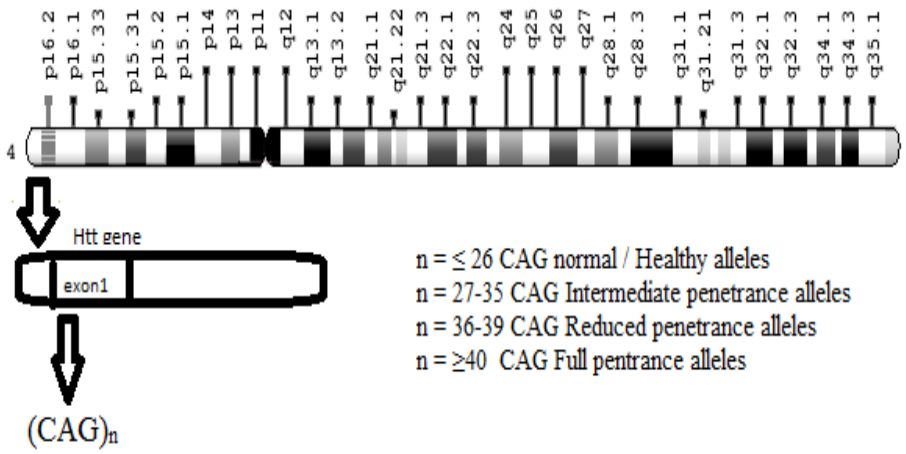


Figure 1.1 *Huntingtin gene scheme: The huntingtin gene is located on the short arm of chromosome 4; encodes a protein of 3144 amino acids, one of the largest in the human genome. HD results from a polyglutamine expansion in exon one of this gene.*

Classifying the HD alleles is still controversial but most of the researchers agree to classify the alleles as follow: normal alleles with 26 or less CAG repeats (no risk of developing the HD); reduced-penetrance HD alleles with 36-39 CAG repeats (these alleles confer to the carrier a high risk of developing the disease; asymptomatic individuals in old age with CAG repeats in this range have been identified) (*Langbehn et al., 2004*); full-penetrance HD alleles with 40 or more CAG repeats (the individuals carrying these alleles will develop the HD). Intermediate alleles with 27-35 CAG repeats (*fig.1.1*) (not at risk of developing symptoms of HD, but because of the instability in the CAG tract, may be at risk of having a child with an allele in the HD-causing range) (*Semaka and Hayden, 2014*)

Homozygous HD patients are rare, and there is still controversy over whether homozygosity for the mutation in HD is associated with a more severe phenotype. Reports from sporadic cases in which both parents are affected showed that the age-at-onset, progression and severity of the disease are similar in homozygotes and heterozygotes (*Alonso et al., 2002*; *Dürr et al., 1999*). However, this conclusion was based on clinical evaluation of eight potential homozygous and only two confirmed cases, and did not take into account differences in CAG tract sizes between siblings, or other possible genetic modifiers. A more recent detailed comparison between a large homozygous patients' series and their heterozygous counterparts in a multicenter study revealed significant clinical and neuropathological differences between the two groups (*Squitieri et al., 2003*). The fact that HD is more severe in homozygosity is supported even by works involving cell lines derived from heterozygous and homozygous HD patients (*Squitieri et al., 2010; Mormone et al., 2006*) and analyses of mouse models for HD (*Graham et al., 2006*). HD is not the only disease caused by a CAG expansion. There are at least other eight diseases, presenting a dominant pattern of inheritance

and neuronal degeneration, with a polyglutamine stretch in different proteins that share nothing with each other in homology or sequence similarity. This is a strong prove that HD, like all other polyglutamine disorders, is caused by a gain-of-function mechanism and that the expanded polyglutamine stretch is responsible for the pathogenesis (*Reiner et al., 2011*).

1.1.3 HD Neuropathology

Normal huntingtin is important for adult neuronal function and it is critical in early embryonic development, before the emergence of the nervous system (*Cattaneo et al., 2005; Nasir et al., 1995*). Cell lines from heterozygote HD patients showed expression of both normal and mutant huntingtin and the wide expression of the HTT transcript does not correlate with the pattern of neuropathology in the disease. Since 1993 many molecular events that precipitate the disease have been described. However, the role of the huntingtin (wtHTT) in healthy individuals and how the mutated huntingtin brings to the illness remain still unclear. Immunocytochemistry indicated that huntingtin is located in neurons throughout the brain, with the highest levels evident in larger neurons (*Gutekunst et al., 1995*). The pathology of the disease has been attributed to toxic gain of functions for the mutant huntingtin protein, such as protein aggregation, transcriptional dysregulation, defective energy metabolism, oxidative stress, excitotoxicity, and inflammation (*fig. 1.2*) (*Andre et al., 2016*). The mutant huntingtin (mHTT) has the propensity to misfold and aggregate, producing cellular aggregates and intranuclear inclusions (*Scherzinger et al., 1997; Gutekunst et al., 1999*). Cytoplasmic aggregations interfered with nucleocytoplasmic protein and RNA transport bringing to cellular damage (*Woerner et al., 2016*).

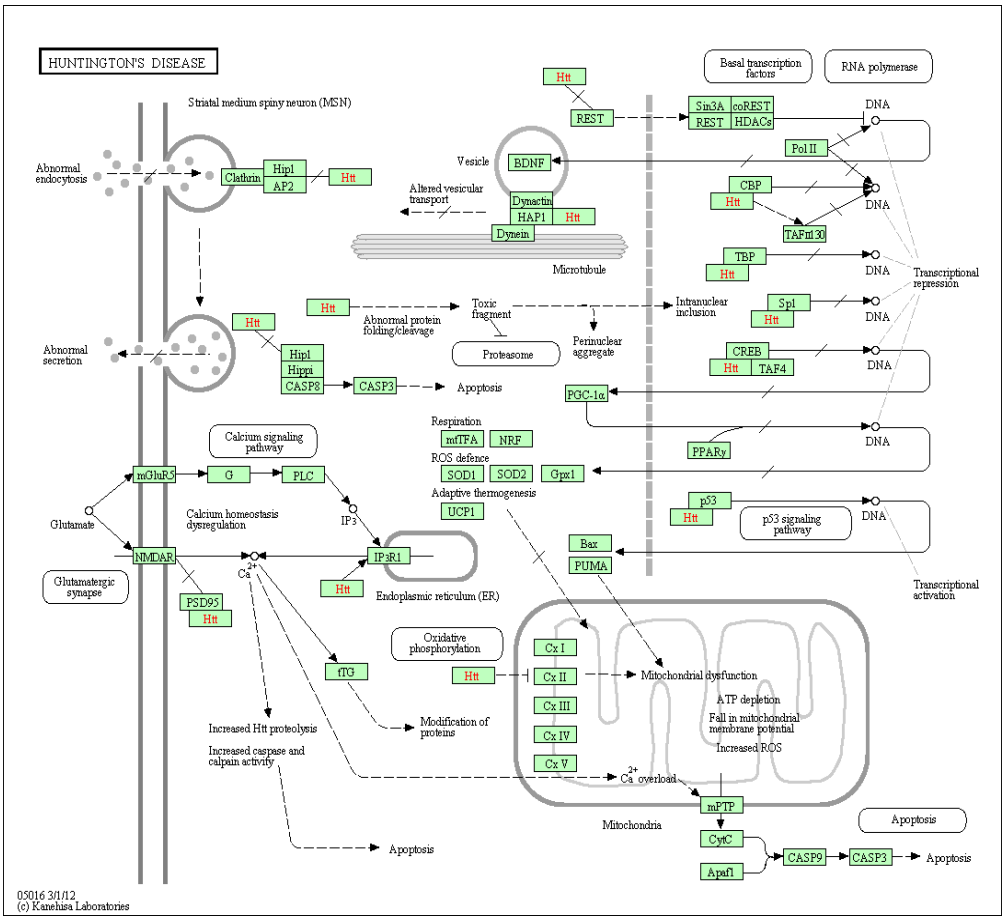


Figure 1.2 *Huntington disease (HD) pathway Mutant Htt (mHtt) has effects both in the cytoplasm and in the nucleus. In the cytoplasm, mHtt can interfere with BDNF vesicular transport on microtubules can affect Ca2+ signaling stimulating NMDAR activity, destabilizing mitochondrial Ca2+ handling and leads to mitochondrial dysfunction. The mHtt translocates to the nucleus, where it forms intranuclear inclusions. Nuclear toxicity is believed to be caused by interference with gene transcription, leading to loss of transcription. (From KEEG pathway Database).*

On the other hand, HTT aggregates, are much more common in the cerebral cortex than in the striatum. It is known that in HD there is a low cortical neuronal loss and a significant striatal neuronal loss. Also among the neuronal populations in the striatum, aggregates occurred predominantly in interneurons instead of in the more vulnerable medium spiny projection neurons (*Gutekunst et al., 1999; Kuemmerle et al., 1999).* Furthermore, inclusion bodies (IB) formation reduced the levels of diffuse mutant HTT and the risk of striatal neuronal death in a primary striatal neuronal model of HD (*Miller et al., 2010*). Even in a mouse model expressing an N-terminal human HTT fragment (exons 1 and 2) with 120Qs with frequent and widespread IB formation there were no evidence of neuronal dysfunction or neurodegeneration (*Slow et al., 2005*). All these findings suggest instead that are the soluble protein fragments, not insoluble aggregated proteins, which cause toxicity in these disorders (*Slow et al., 2006*). Recent studies have shown that oxidative stress is a key player in the pathogenesis of HD. Classical studies from postmortem brains of HD patients have demonstrated a significant increase in the level of oxidative damage (*Stack et al., 2008*).

There is a significant increase in lipid and decrease in glutathione levels in the plasma of symptomatic patients compared to that of healthy subjects. The same thing was seen when comparing levels of lipid peroxidation in the plasma of asymptomatic HD gene carriers with age and sex matched healthy subjects (*Klepac et al., 2007; Christifides et al., 2006*). The increased amounts of redox species in the plasma are probably due to the deregulation of brain energy metabolism in presymptomatic and symptomatic HD subjects.

1.1.4 Brain energy metabolism failure in HD

HD is characterized by a failure in brain energy metabolism. HD brain shows an impairment in glucose transportation, reduced glycolysis, decreased lactate concentration, altered levels of TCA cycle enzymes and diminished oxidative phosphorylation (*Naseri et al., 2015; Powers et al., 2007; Squitieri and Ciarmello, 2010; Milakovic and Johnson, 2005*). Evidences of glucose hypo-metabolism are reported in literature. There are many evidences indicating that the energetic metabolism impairment in HD patients is given by defective brain glucose uptake, especially in neurons (*Morea et al., 2017*). In membranes of post-mortem samples of caudate and cortex brain regions the quantity of GLUT1 and GLUT3 was significantly reduced in advanced HD stages patients with respect to non-HD controls. Instead at earlier stages, there was no significant difference in GLUT1 and GLUT3 membrane concentration compared with non-diseased controls (*Gamberino and Brennan, 1994*).

1.1.5 Ca(II) metabolism impairment in HD patient

HTT has also been shown to interact with a protein located in the ER membrane, the Inositol-1,4,5-triphosphate receptor (IP3R). In several cellular HD models, the Ca^{2+} regulating pathways have been found defective, in fact mutant HTT (but not the wild type protein) increases the Ca^{2+} releasing activity of the IP3 receptor rendering the neuronal cells more vulnerable to the stress induced by Ca^{2+} and could contribute to mitochondrial Ca^{2+} overload (*Tang et al., 2003; Giacomelli et al., 2011*). One of the proposals trying to explain the molecular basis of HD put at the center of the attention the mitochondrial stress caused by a dysregulation of the system for the neuronal homeostasis of Ca^{2+}.

Mitochondrial calcium abnormalities occur early in HD pathogenesis and may be a direct effect of mutant huntingtin on the organelle (*Panov et al., 2002*). Disrupted mitochondrial calcium homeostasis potentiates NMDA receptors which causes calcium influx. Calcium overload may trigger apoptosis in medium spiny striatal neurons in HD (*Bezprozvanny and Hayden 2004*). Mutant huntingtin causes the increased levels of reactive oxygen species (ROS) in neuronal and nonneuronal cells which directly contributes to cell death (*Wyttenbach et al., 2002*). Mutant HTT leads to mitochondrial dysfunction causing increased neurodegeneration of striatal neurons and motor abnormalities. (*Greenamyre et al., 2007; Sassone et al., 2015*). Mitochondrial dysfunction is considered to be one of the key defects in HD pathogenesis. Activities of complexes II, III, and IV are significantly reduced in the striatum of HD patient (Brennan et al., 1985; Stahl et al., 1974; Gu et al., 1996).

1.1.6 Autophagy in HD

Ubiquitin-proteasome system dysfunction is a consistent feature of HD pathology and HD is linked to global changes in the ubiquitin system.
(*Bennet et al., 2007*) There are many findings suggesting that autophagy plays a critical role in the degradation of N-terminal HTT and altered processing of mutant HTT by autophagy may contribute to HD pathogenesis. (*Qin et al., 2003; Ravikumar et al., 2002*) Recently a possible mechanism of the altered autophagy in HD was described. Polyglutamine domain enables wild-type ataxin 3 to interact with beclin 1, a key initiator of autophagy. This interaction allows the deubiquitinase activity of ataxin 3 to protect beclin 1 from proteasome-mediated degradation and thereby enables autophagy (*Ashkenazi et al., 2017*).

Later in life when proteasomal function becomes compromised, the autophagic pathway become of crucial importance. Interestingly, the striatal-selective pathology of HD may reflect the interaction between mHTT and the striatal-selective G-protein, Rhes protein of unclear function that has a preferential expression in the striatum. Rhes binds mHTT and acts as a SUMO (Small Ubiquitin-like MOdifier) E3 ligase to stimulate sumoylation of mHTT, a post-translation modification known to augment the mHTT solubility and therefore its toxicity. Rhes can activate autophagy by competitively displacing the inhibitory binding of Bcl-2 to Beclin-1. Thus, the sequestration of Rhes by mHTT with aging may bring to a diminished autophagic flux (*Mealer et al., 2014*).

1.1.7 Clinical features of HD

Brain abnormalities in HD are developed before evident symptoms, and involve the entire brain to a greater or lesser extent, resulting in about 25% of brain weight loss in advanced HD (*Halliday et al., 1998*). Numerous studies in recent years have used brain imaging technologies to elucidate the pathogenesis and progress of HD. Neuroimaging like MRI, CT and PET have revealed significant striatal atrophy as many as 11 years prior to clinical onset of the disease (*Aylward et al., 2004*). A selective degeneration of neurons in the caudate and putamen explain the most of the neuropathologic features of HD (*fig. 1.3*). As striatal degeneration progresses, degenerative changes occur to other brain regions connected to the striatum, in particular, the globus pallidus, the subthalamus, and the cerebral cortex and the preferential degeneration of medium spiny neurons provides the neurobiologic basis for chorea (*Mitchell et al., 1999*).

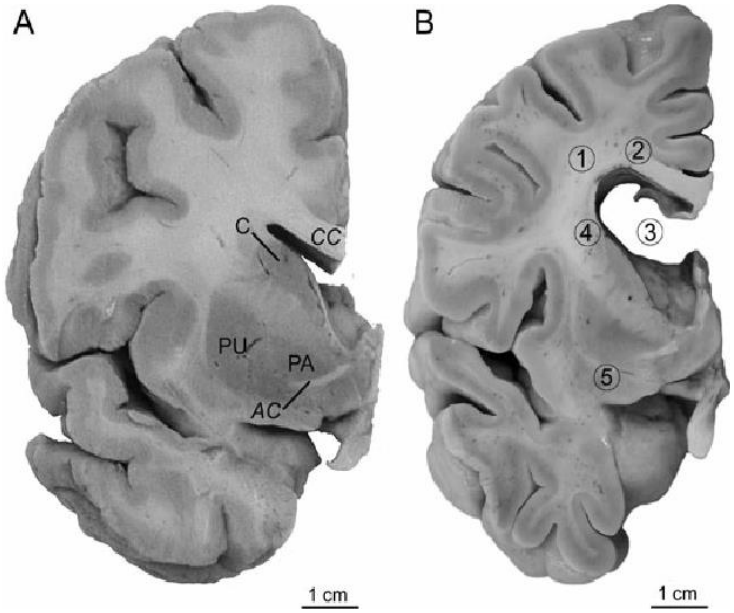


Figure 1.3 *Brain Atrophy (HD). Frontal section through the right basal forebrain of a (A)representative control individual (B)clinically diagnosed and genetically confirmed HD patient. Note the loss of deep white matter (1), the narrowed corpus callosum (CC) (2) and widened third ventricle (3). The atrophy of the caudate nucleus (C) (4) and putamen (PU) (5) AC, anterior commissure; PA, pallidum; PU, putamen. (From Rub et al., 2009).*

The classic signs of HD are progressive chorea, rigidity, dementia, psychotic and behavioral impairment. Generally, individuals that carry a mutated *HTT* allele will have a pre-symptomatic phase with a healthy life, free of clinical symptoms. Before the clinical manifestation of HD, a prodromal phase with minor changes in one or more of the following occurs: motor skills, cognition, depression and psychiatric disturbances (*Bates et al., 2002*). The mean age of onset for HD is 35 to 44 years, nearly in one fourth of the HD population, the

onset is delayed until after age 50 years, a few even after 70 years of age. The survival time after onset goes from 5 to more than 25 years (usually 15 to 18 years). The average age at death is 54 to 55 years (*Harper 2005*). In late stages of HD, motor disability becomes severe with disturbances of both involuntary and voluntary movements. Patients show serious weight loss and are unable to move, speak, swallow or take care of their own hygienic needs (*Warby et al., 2014*). A global and progressive decline in cognitive capabilities have been identified even before the onset of motor symptoms (*Rupp et al., 2010*). Individuals with HD develop significant personality changes, but the psychiatric changes tend not to progress with disease severity (*Anderson & Marder 2001; Rosenblatt, 2007*). Depression is more common in persons with HD compared to the general population with a higher suicide risk before receiving the diagnosis and when the disease advance to the point that patients are not fully independent to take care for oneself (*Baliko et al., 2004, Paulsen et al., 2005*).

1.1.8 Juvenile HD

As an accepted definition since 1968, Juvenile onset Huntington disease (JHD) include cases with onset at 20 years of age or earlier. (Bruyn 1968). The first clear description of juvenile HD (JHD), is attributed to Hoffmann in 1888. In this article, he described a family with HD and two female cousins who developed chorea in school age (*Hoffmann 1888*). Individuals with CAG repeat length >60, with few exceptions, have juvenile onset of the Huntington Disease (*fig1.4*) (*Telenius et al., 1993*). Only about 5% to 7% of individuals with HD experience the onset of symptoms before age 21 (*Nance and Myers 2001*). In the majority of JHD cases the transmitting parent is the father. This occurs in approximately 70–80% of the cases with an onset between 11 and 20 years of

age and may be over 90% in those with childhood-onset HD (*Oliver et al., 2013*). This is probably due to the instability in the CAG repeat length during spermatogenesis (*Wheeler et al., 2007*).

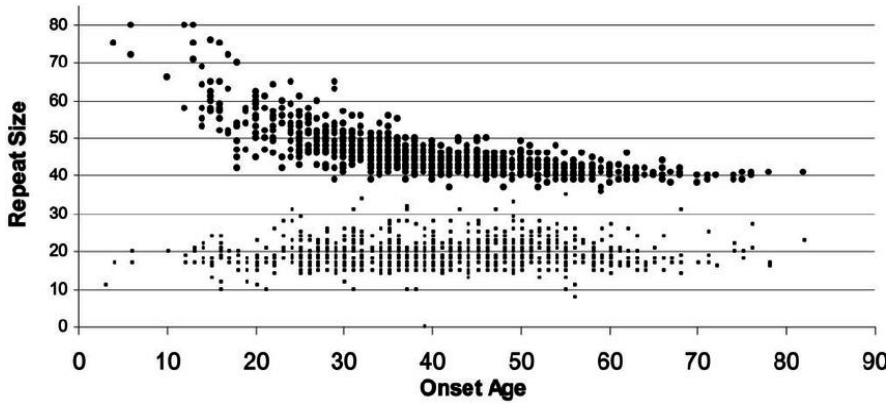


Figure 1.4. *HD repeat size and onset age. The relationship between the repeat size and the age at onset is presented. Persons with repeats of 60 or larger commonly have very young onset, before the age of 20, and among these large repeats there is a clear relationship between repeat size and onset age. (From Myers 2004).*

There is similarity between the pathology seen in HD and JHD although the juvenile onset of the disease seems to be more severe. In an early stage, JHD patients will experience bradykinesia, dystonia and parkinsonian features, while chorea, if present, is less prominent. On the contrary, classically, patients with adult HD, will demonstrate signs of chorea at the beginning of the disease, while bradykinesia and dystonia are going to be evident with the progression of the disease (*Oliver et al., 2013).*

1.1.9 Demographics of HD

HD is more common in the Caucasian population with a prevalence of 5-10 per 100,000, and a much lower prevalence in Asian and African populations. The prevalence of HD exceeds 15 per 100,000 in some populations, mostly of western European origin (*Bates et al., 2002*). About 6500 subjects are currently affected by HD in Italy and this number is prone to further increase in the next decades because of population aging, variable phenotype penetrance and improved life expectancy (*Squitieri et al., 2015*). These are huge numbers for a devastating disease but what is even more concerning is that approximately 1-4% of the general population is found to have intermediate *HTT* alleles, who are at risk of having a child with an allele in the HD-causing range (*Semaka et al., 2010*). Considering all the features of the disease more resources should be directed to the research and to the institutions dealing with HD patients

1.1.10 Treatment of HD

Although in these decades a number of disease pathways have been identified as potential therapeutic targets, there is currently no treatment known to slow the worsening of HD progression (*Banno et al., 2017*). To date therapies are limited to the clinical symptoms of the disease: chorea, impaired voluntary movement, behavioral changes and dementia (*Frank, 2014*). Recent guide lines suggest that the treatment of chorea is not necessary in most of the cases and that patients may have a better quality of life when they use no drugs to control the involuntary movements. In the cases when the chorea requires treatment the clinicians may recommend tetrabenazine (TBZ), amantadine, or riluzole (*Armstrong and Miyasaki 2012*). There is a wide variety of behavioral

and psychiatric issues that may be seen in HD. Valproate and olanzapine, given at the lowest effective doses, may be helpful for relieving both psychiatric and behavioral disorders in patients with HD (*Grove et al., 2000*). Management outside of pharmacotherapy should be considered when possible. Creatine given daily as a supplement has been shown to retard the rate of progression of HD (*Tabrizi et al., 2003*). A good diet and a regular program of physical, mental, and social stimulation is beneficial for every human to improve some of the symptoms and "halt Huntington's progression" (*Dawes et al., 2015*).

1.2 Induced pluripotent stem cells: a new source of stem cells to model rare diseases.

1.2.1 What are stem cells?

A stem cell is defined as an unspecialized cell capable of self-renewing through cell division and able to give rise to differentiated cells with specialized functions (*Pera et al., 2000*).

Stem cells can be categorized, depending upon their differentiation potential, (*Odorico et al., 2001*) as:

1. totipotent cells, that have total potential, with the ability to differentiate not only in any cell type of the organism but also into extra-embryonic tissues;
2. pluripotent cells, a specialized kind of totipotent cells that can give rise only to tissues derived from the embryonic inner cell mass
3. multipotent cells, more specialized stem cells that are committed to give rise to cells that have a particular function (i.e. hematopoietic stem cells) (See *fig.1.5* for a schematic representation)

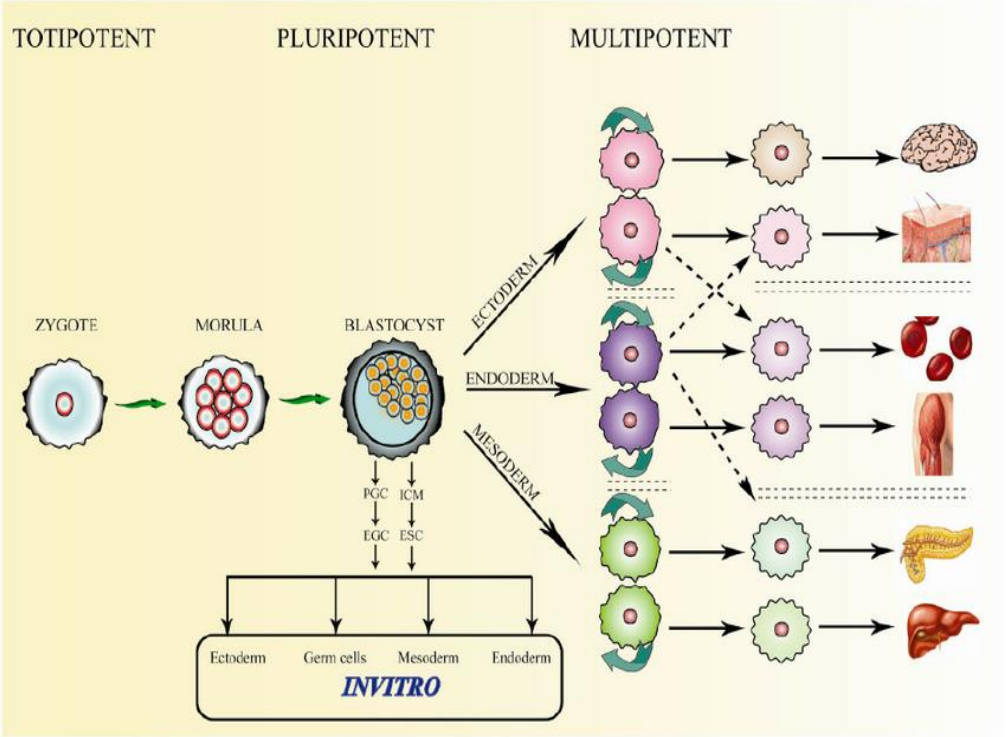


Figure 1.5 *General hierarchy for the stem cell niche a) Totipotent cells, deriving from zygote. This cells have the potential to give rise to all of embryonic and extra-embryonic cells and tissue; b) Pluripotent cells, deriving from the inner cell mass (ICM) of an embryo. can form all somatic and germline cells of a rising organism; c) Multipotent cells can only give rise to cells of their lineage. Most adult stem cells are multipotent (from Ramakrishna et al 2011).*

1.2.2 Human embryonic stem cells

Human pluripotent stem cells (hPSCs), including human embryonic stem cells and human induced pluripotent stem cells, must respect four criteria: 1) they

must origin from a pluripotent cell population 2) they must be capable of self-renewal indefinitely in the undifferentiated state 3) they must be capable of maintaining normal karyotype during growth 4) their transplantation into immune-deficient mice must lead to the formation of differentiated tumors comprising all three germ layers, resembling spontaneous human teratomas.
hESCs are able to give rise to all of the somatic and germ line cells of the fully developed organism, these cells are the "uncommitted" progenitors of the subsequent three embryonic germ layers: ectoderm, endoderm, and mesoderm (*Surani, 2001; Lovell-Badge, 2001*). In 1998, it was described the first successful human Embryonic Stem cell isolation, derived from the inner cell mass of a blastocyst during gastrulation (*Thomson et al., 1998; Donovan and Gearhart, 2001*). The use of human embryos to obtain the inner cell mass raised ethical concern and limitation (*Strong et al., 2009; Johnson, 2008*). For these reasons, effort was done to be able to obtain pluripotent stem cells without the embryos use. Chung et al. successfully derived human ESC lines from a single blastomere biopsied from patient embryos as is routinely done during preimplantation genetic test (*Chung et. al, 2008*). However, there is a variable efficiency of producing human ESC's line from individual blastomeres, this depends on properties of the isolated blastomere and the embryonic cell stage of isolation but also from the particular culture conditions to retain the totipotency of the isolated blastomere (*Desai et al., 2015*).

1.2.3 Human induced pluripotent cells

As already mentioned, hPSCs include also human induced pluripotent cells (hiPSCs). In 2006, Takahashi and Yamanaka demonstrated a

revolutionary technology able to take somatic cells back to an indifferentiated condition.

In that work 24 genes were selected as candidates for factors that induce pluripotency. They concluded that by combining just four transcription factors: octamer 3/4 (Oct3/4), SRY box– containing gene 2 (Sox2), Kruppel-like factor 4 (Klf4), and c-Myc was sufficient to generate pluripotent cells, which were called induced pluripotent stem (iPS) cells, directly from mouse embryonic or adult fibroblast cultures (*Takahashi and Yamanaka, 2006*). Only a few months later, human fibroblasts were reprogramed to iPSC's by introducing the human orthologs of the four pluripotency-associated transcription factors from the same group (*Takahashi et al., 2007*). The iPS cells so obtained shared the main characteristics of embryo-derived stem cells, were similar in morphology, growth properties, expression of pluripotency markers and were also able of self-renewal and to differentiate into cells of the three germ layers (*Takahashi et al., 2007; Takahashi & Yamanaka, 2006*). Takahashi and Yamanaka used retroviral vectors for the delivery of the reprogramming vectors. This protocol was based on insertion of foreign DNA into the reprogrammed genome with a high concern of insertional mutagenesis (*Hu, 2014*). Even the reactivation and residual expression of the randomly integrated reprogramming factors which are tumorigenic can become a serious limitation with c-Myc found to be responsible for the tumors found in iPSC chimeric mice (*Nakagawa et al., 2008*). For these reasons integrating vectors are not the best choice in the generation of iPSC's for disease modeling or even less in cell therapy where safety is the biggest concern.

In order to reduce or eliminate transgene integration almost all of the gene delivery systems have been employed in factor reprograming and many of these techniques perform direct reprogramming without integration (*Hu et al., 2014*): 1) Adenoviral reprograming with transgene dilution over time due to

cell division (*Stadtfeld et al., 2008*); 2) *PiggyBac* (PB) transposition system (*Woltjen et al., 2009*); 3) Direct delivery of reprograming proteins; (*Kim et al., 2009*) 4) Direct delivery of reprogramming RNA (synthetic mRNA, RNA virus, RNA replicon, or miRNA) (*Warren et al., 2010; Fusaki et al., 2009; Yoshioka et al., 2013; Miyoshi et al., 2011*).

1.2.4 Stem Cells for Modeling Human Disease

Animal models and immortalized cell systems are widely used as models of human diseases mechanisms and therapeutic development. Human conditions are not always reflected in animal models because of species differences and artificially manipulated cells did not represent the best choice in disease modeling. In fact, 95% of new drugs tested in artificially manipulated cells were withdrawn due to off-target effects (*Munos, 2009*).

Human tissues and many primary cell types harvested from healthy donors or patients with a relevant genetic condition could be a more reliable model. However, the difficulty to obtain fresh human disease samples is clear, especially samples able to model rare cardiovascular and neurodegenerative diseases because of limited number of suitable donors with a genetic background. What is even more limiting, most of the human samples from the heart or the brain are obtained from the end stages of the disease or postmortem. Thus, it becomes impossible to critically observe the development of pathological feature and only a few number of scientists would have the possibility to manipulate that kind of samples.

For these reasons, human pluripotent stem cells can become the principal actor in genetic disease modeling. Quantitative phenotyping on hiPSC-based models of neurodegenerative diseases provides a clear profile of these cells with severe defects in growth, migration and function compared to healthy controls. These

distinct phenotypes can offer a great help in diseases modeling and developing platforms for drug screening (*Kim, 2015; Rajamohan et al., 2013*).

2. Aim of the study

Since the discovery of the *HTT* mutation 24 years ago, more than 15,000 papers have been published on HD. However, both the role of the huntingtin (wtHTT) in healthy individuals and the molecular mechanisms by which the mutated huntingtin causes the disease remain unclear.

In order to understand the molecular processes causing the brain damage in HD patients, a vast array of genetically-modified cell and animal models has been developed. The organisms used to model various aspects of the disease includes worms (*Caenorhabditis elegans*), fruitflies (*Drosophila melangaster*), mice, rats, sheep and, more recently, pigs and monkeys (*Pouladi et al., 2013*). However, animal models have their limits in resembling human brain development and pathology because of profound morphological and physiological differences that distinguish human brain from other animal species, differences that are less marked in other organ systems (*Muzio and Consalez, 2013*). Moreover, many of the observations of the HD molecular features were based on experiments done in non-human cells transfected with cDNAs encoding mutant HTT and in primary human cell lines like fibroblasts and lymphoblasts from HD patients (*Ross and Tabrizi, 2011; Seong et al., 2005*). However, these models present limitations due to the differences between human and non-human cells and between non-neuronal and neuronal cells, thus preventing to find a good target for new drugs in HD patients.

The discovery of induced pluripotent stem cell (iPSC) technology offer the possibility to generate patient-specific iPS cells and to enable the development of *in vitro* HD models that more accurately reflect the human disease (*Golas and Sander, 2016*). iPSC's from HD patients can be propagated indefinitely and differentiated into any cell type in the human body even functional

neuronal cells (*Hargus et al., 2014*). This cell model can be used to conduct detailed studies of the disease mechanisms, cell therapy development, or the screening of new drug candidates.

Human neuronal cells carrying the mutation can become a powerful tool able to overcome some of the limitations of *in vitro* modeling of neurodegenerative disease (*Ma et al., 2012; Jeon et al., 2012*).

The main focus of this project was to obtain neuronal cell lines starting from the patient's fibroblasts using the iPSC technology. The neural cell lines obtained with this protocol will be used to study the disease development and the impairment of metabolic pathways as well as to find lead compounds able to improve the HD phenotype.

To achieve this objective, we collected fibroblast from patients carrying mutation of different length on the HTT gene. As shown in this thesis, we succeed in reprogramming the fibroblast obtained from two JHD patients, one adult HD patient and one healthy donor into iPSC. Neural stem cells obtained from the precursor differentiation were then analyzed at morphological and molecular level to underline functional and biochemical differences linked with the mutation. Moreover, we have started to study the gene expression of both iPSC and iPSC derived neural stem cells and we have discovered that proteins involved in differentiation of neural stem cell to cerebral cortex neurons and in the glucose transport are differentially expressed in the HD model with respect to the control, indicating an impairment of both the neural stem differentiation process and the energetic metabolism.

3. Results

3.1 Obtaining human dermal fibroblasts from healthy donors and HD patients.

An important part of this project concerned the recruitment of healthy donors and HD patients with different features of the disease in order to obtain skin biopsies.

Unique code	Gender	CAG repeat length		TFC	Age at biopsy
HD Patient		Allele 1	Allele 2		
HD408-02	M	18	47	9	40
HD110-08	M	14	48	11	37
HD288-03	M	18	43	7	59
HD232-02	F	18	50	10	41
HD 8YRS(JDH)	F	20	85	?	8
HD530-01	M	17	41	11	61
HD503-04	F?	17	43	12	43
HD654-01	F	17	42	11	47
HD652-01	M	17	47	6	39
HD438(JHD)	F	16	64	5	25
HD256-05	M	17	43	13	28
HD508-01	M	18	43	3	58
HD256-01	F	21	44	3	70
HD4503	M	29	43	12	34
HD229.02	M	35	43	13	48
HD 471.02.13	M	18	47	4	39
Control sub.					
MA.ON	F	Control	Control	Control	37
MO.EL	F	Control	Control	Control	59
LA.NI	M	Control	Control	Control	40
MA.HD	M	Control	Control	Control	48
HD654-02	M	Control	Control	Control	42
HD2805	M	Control	Control	Control	72

Table 3.1 *Personal features of subjects enrolled in the project.*

In table 1 the age at the time of the biopsy and the gender of both HD and control subjects are shown. The length of CAG repeats in *HTT* alleles and the Total Functional Capacity Score (TFC) are reported only for the HD patients. TFC is a standardized scale used to assess capacity to work, handle finances, perform domestic chores and self-care tasks, and live independently. The TFC scale ranges from 13 (normal) to 0 (severe disability).

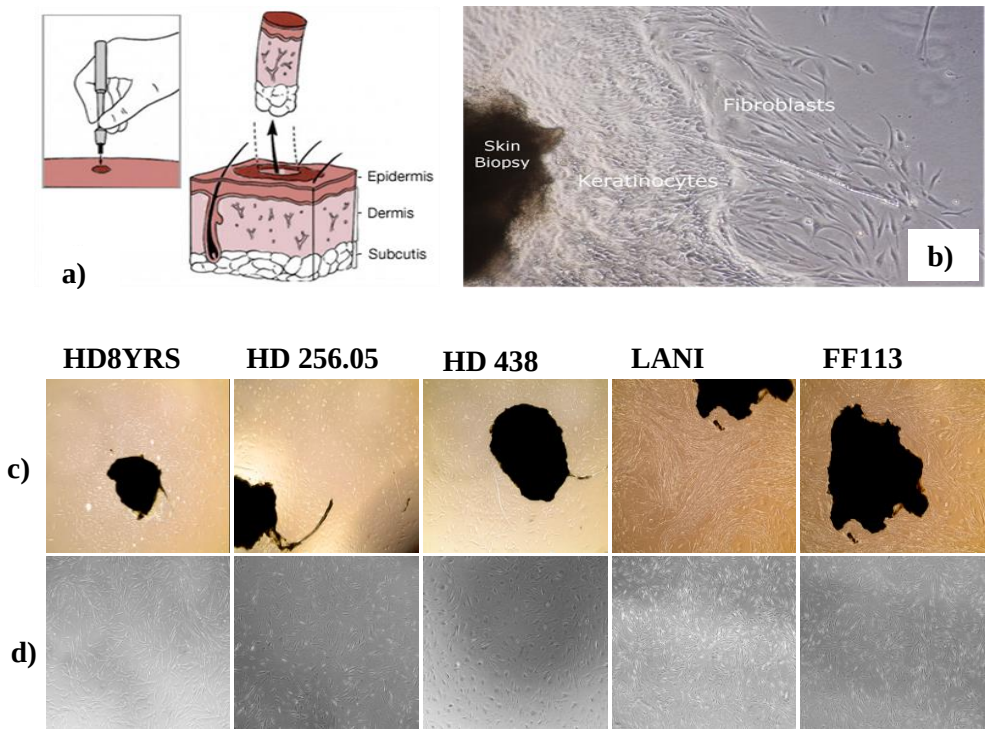


Figure 3.1 *a) cartoon of a skin biopsy b) Cells coming out from a skin biopsy after 12 days of incubation at 37°C in enriched DMEM medium;* Phase contrast images: *c) Fibroblasts growing out from the edges of each skin biopsy (only 5 biopsies shown) d) Isolated fibroblasts from the five selected biopsies.*

Two of the HD patients presented a juvenile onset of the disease and one of them an infantile onset. From the skin biopsy, human primary dermal fibroblasts were isolated and expanded for further experiments (fig. 3.1). Comparing the growth rate of dermal fibroblasts, a correlation was found with the TFC at the time of biopsy. No significant correlation was found between growth rate and CAG expansion (*fig. 3.3*).

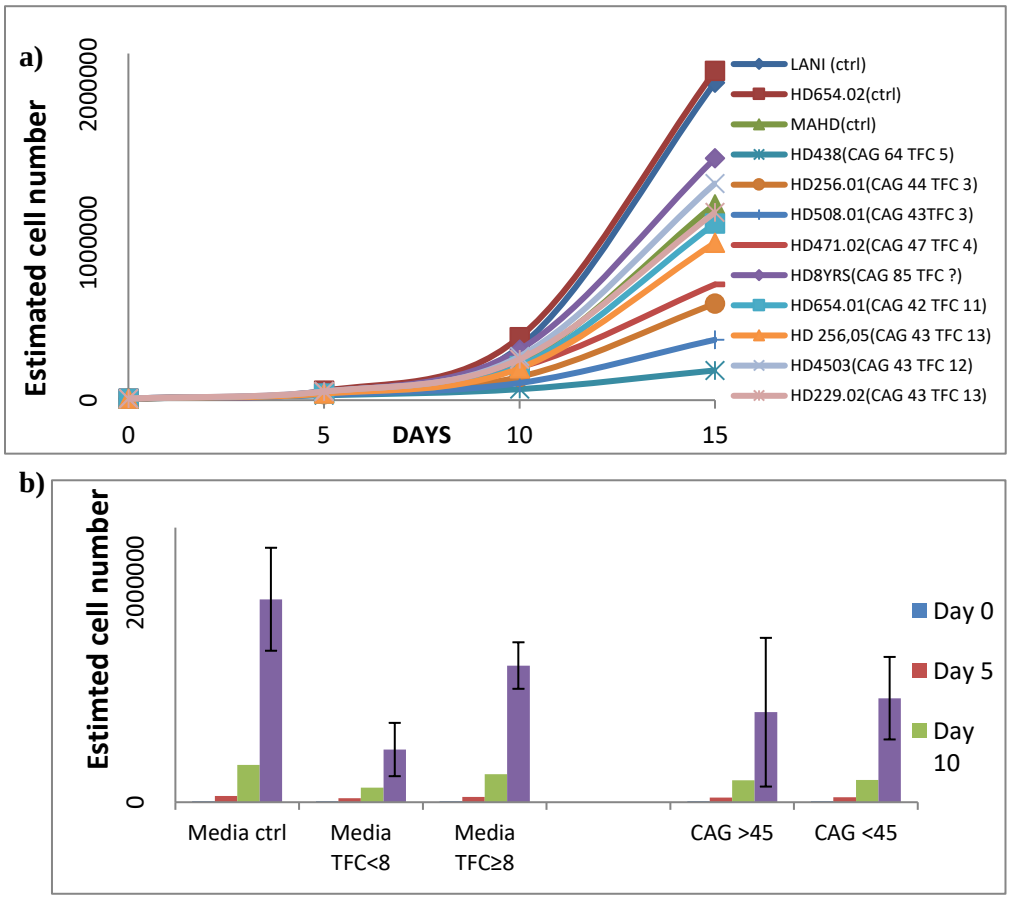


Figure 3.3 *A) Proliferation plot of primary dermal fibroblasts obtained from the skin biopsies of control and HD subjects with different TFC (total functional capacity score) and CAG repeat stretch. B) Comparison of proliferation plots of fibroblasts grouped for TFC and CAG repeat expansion.*

3.2 Induced pluripotent stem cells from wild-type and HD fibroblasts show both stemness and pluripotency features.

Primary skin fibroblasts from a healthy donor and three HD patients (two with a juvenile and one with an adult onset of the disease) were reprogrammed into induced pluripotent stem cells. The nucleofection was performed using Nucleofector4D with a protocol optimized in our lab (fig. 3.4-b). A mix of pCXLE based episomal vectors carrying the four human reprogramming factors, Oct4, Klf4, Sox2 and Myc and other stamness genes (Lin28, sh-p53) were introduced directly to the nuclei (Okita et al., 2011). From reprogrammed fibroblasts, we were able to select and expand iPSC-like colonies presenting a flat morphology and defined borders (fig. 3.4-c and d). Several clones, that resulted karyotypically normal (fig. 3.4-e), were then characterized for their stemness and pluripotency in order to be validated as *bona fide* iPSC's. Immunostaining analysis against Oct4 and Tra-1-60 demonstrated that the tested clones express both stemness markers (*fig*. 3.5-a). Real-Time PCR analysis confirmed the expression of several endogenous stemness markers in all iPSCs lines (OCT4, KLF4, LIN28, SOX2 and MYC), six divisions after the first isolation of the single clone (fig. 3.5-b). Pluripotency of the new iPSCs lines was verified in vitro through embryoid bodies (EBs) formation assay and in vivo through teratoma formation assay. When cultured in the absence of anti-differentiation factors, iPSCs spontaneously aggregate to form EBs (fig. 3.5-a).

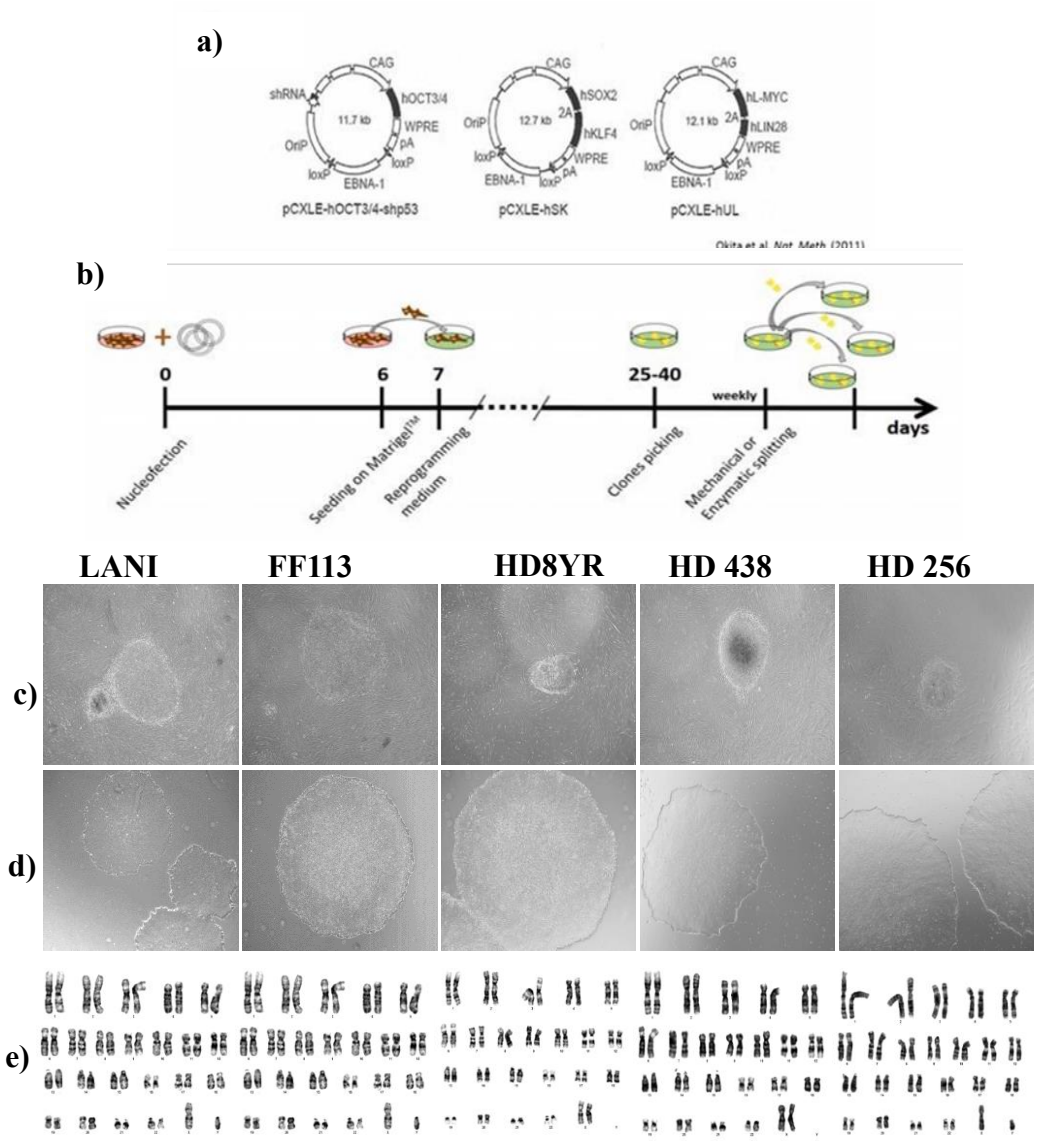


Figure 3.4 *Reprogramming experiments protocol. a) Maps of episomal vectors used for reprogramming experiments. b) Time-line of reprogramming procedures.* Phase contrast images: c*) The first iPSC's colonies of each cell line growing up in the reprogramming dish d) Isolated iPSC's clones after six passages showing a typical flat morphology. E) Karyogram of iPSC's clone showing a normal karyotype for each cell line.*

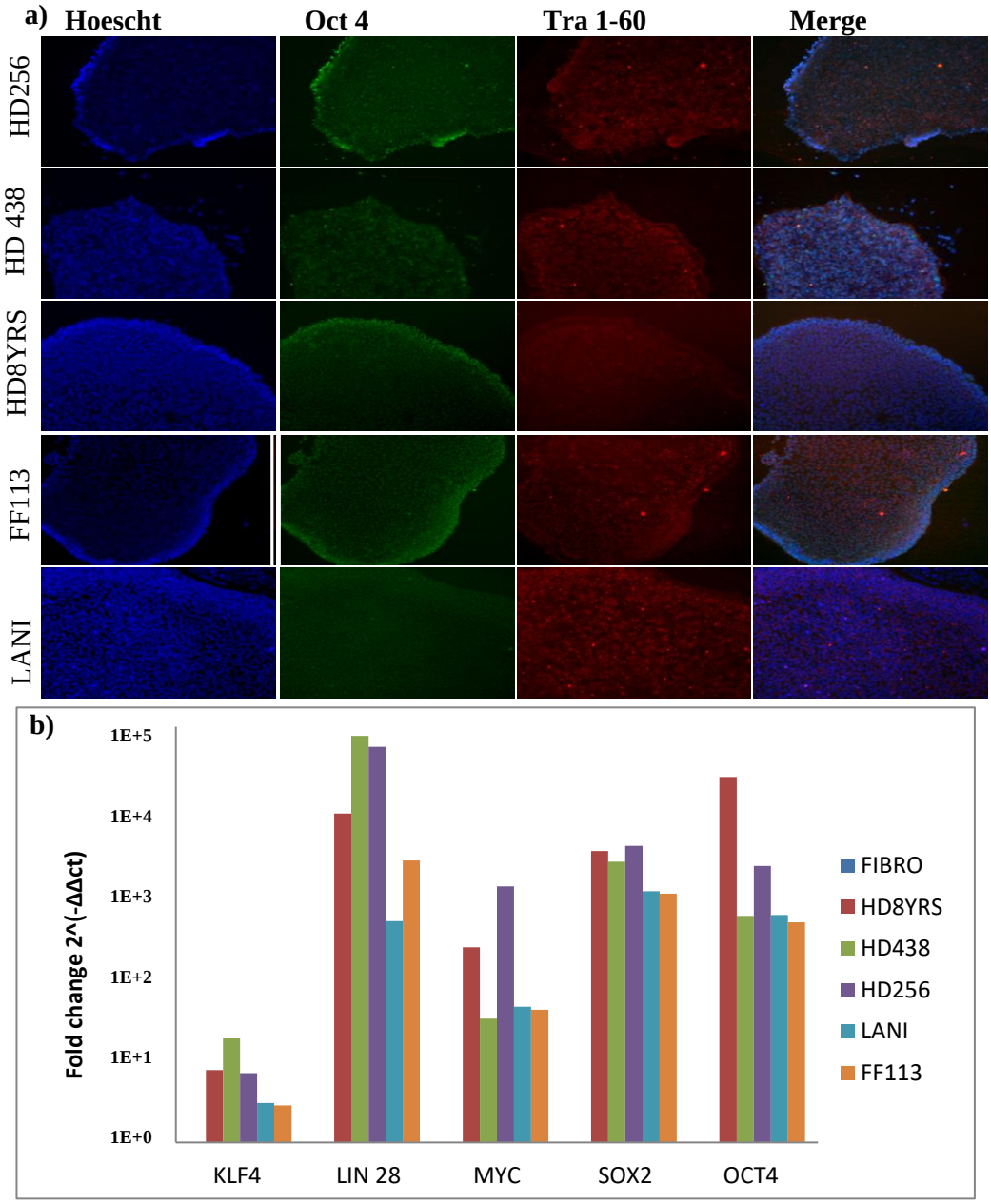


Figure 3.5 *a) Immunofluorescence analysis against Oct4 and Tra-1-60 on LANI(ctrl); FF113 (ctrl); HD 438 (CAG 64); HD 256.05(CAG 43) and HD 8YRS (CAG 85) iPSCs clones showed the expression of both stemness markers, indicating a reactivation of endogenous stemness genes after reprogramming. RED = Tra-1-60; GREEN = Oct4: BLUE = Nuclei b) Real-time PCR analysis confirmed the expression of several endogenous markers (KFL4; LIN28; MYC; SOX2; OCT4) in iPSC lines. All results have been normalized against non-nucleofected fibroblasts basal expression.*

Pluripotent stem cells within embryoid bodies undergo differentiation in the three germ lineages – endoderm, ectoderm, and mesoderm. Real Time PCR analysis showed induction of ectoderm, mesoderm and endoderm markers (fig.3.5-b). When injected subcutaneously in immunocompromised mice iPSC's are able to generate teratomas comprising tissue representatives of all three embryonic germ layers (ectoderm, mesoderm and endoderm), thus attesting to their pluripotency (fig. 3.6).

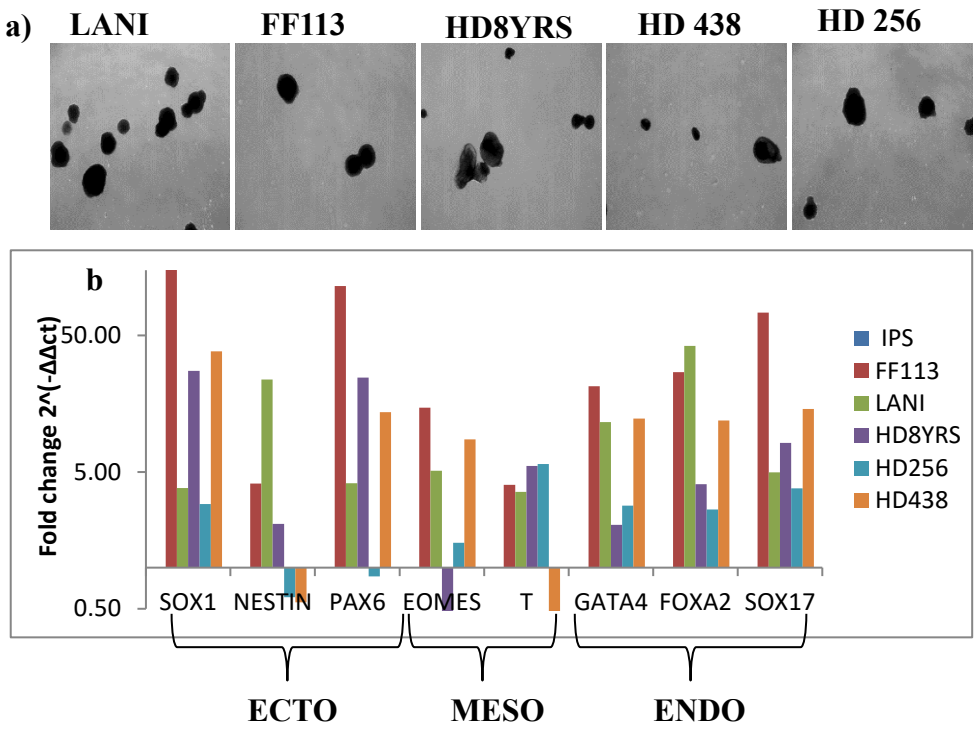


Figure 3.5 *a) Phase image of Embryoid bodies (EB's) formed 14 days after, growing iPSC from each line in floating conditions in KOSR medium b) Real-time PCR analysis carried on embryoid bodies confirmed the expression of all the three germ layers for both mutated and control cell lines, indicating pluripotency properties of cells. ECTO = ectoderm; MESO = mesoderm; ENDO = endoderm. All results have been normalized against iPSC.*

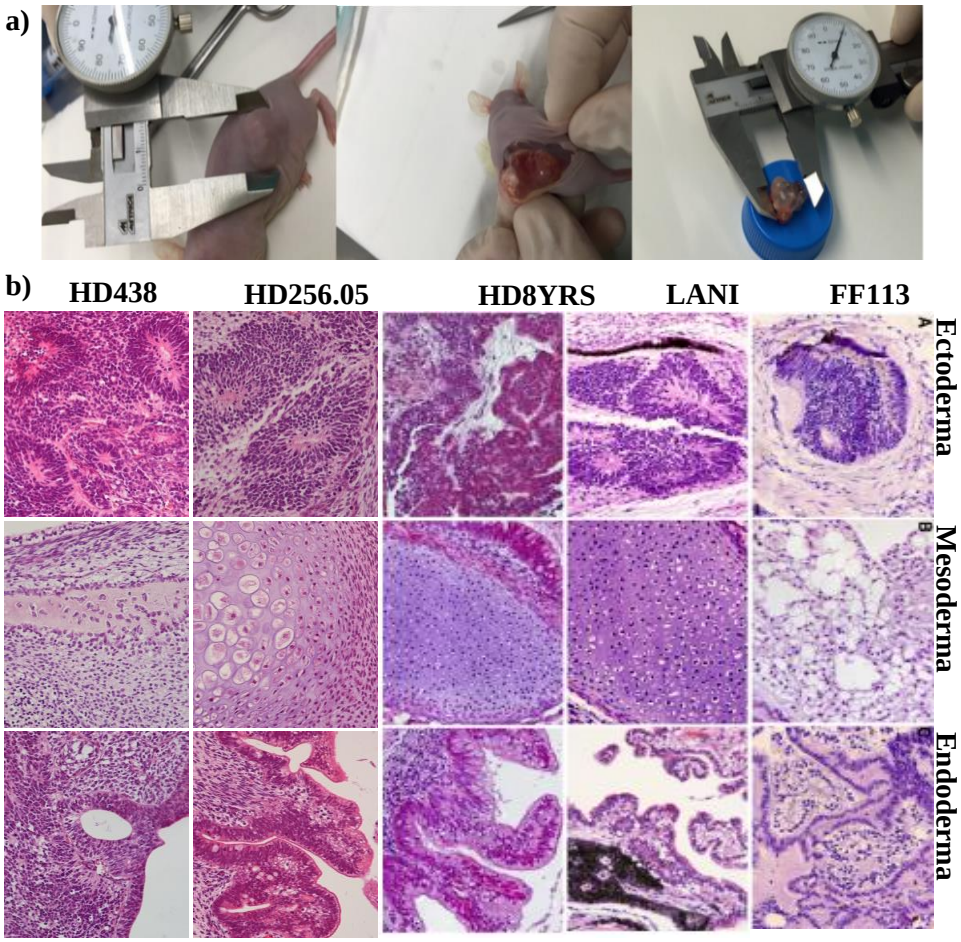


Figure 3.6 *a) Teratoma formed after subcutaneous injection of iPSC's in immunocompromised mice. b) Histological analysis of teratomas obtained through teratoma formation assays showed the presence of tissue deriving from all the embryonic layers, confirming once more pluripotency capability. of these cell lines.*

3.3 Differentiation of JHD and control iPSCs in neurospheres of Neural Precursor Cells presenting a correct morphology and self-renewal capability.

A new protocol for differentiation of iPSCs-derived embryoid bodies in Neural Precursor Cells (NPCs) was optimized in our lab. iPSC derived NPCs can be cultured as floating neurospheres, expanded and differentiated as "natural" NPCs lines.

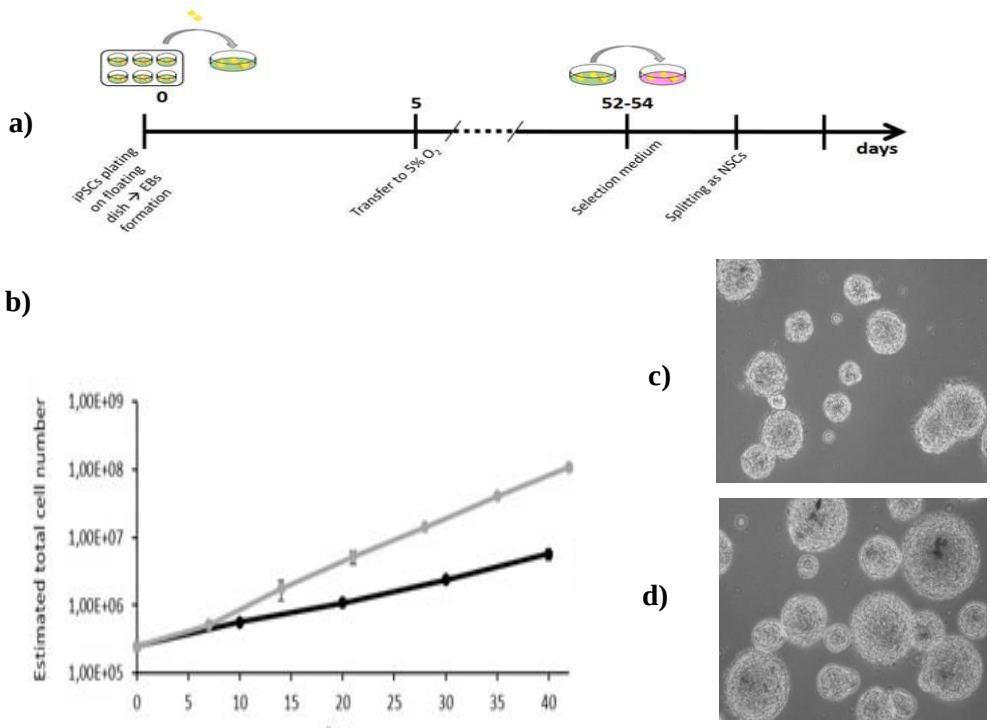
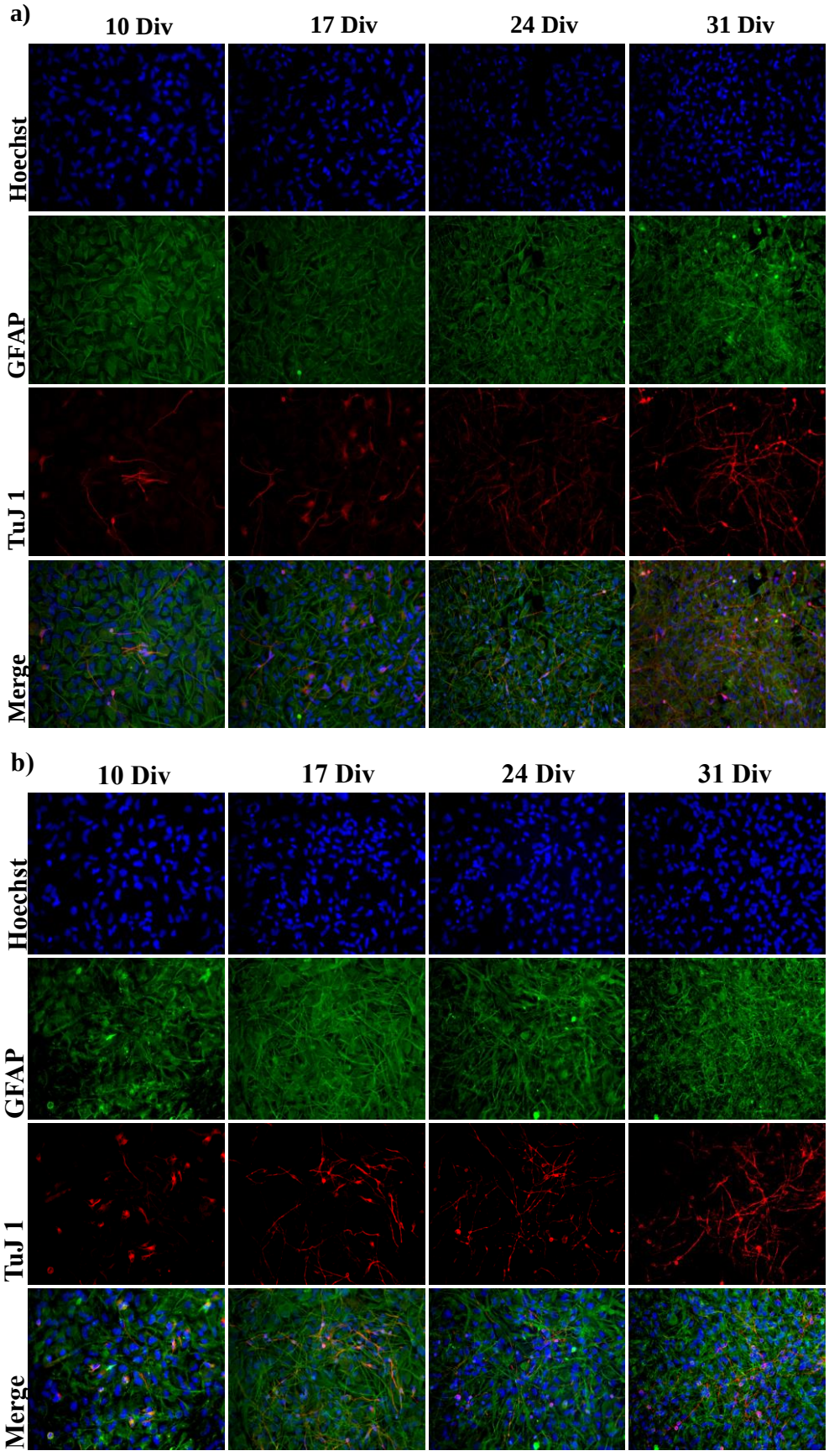


Figure 3.7 *a) Scheme of neurosphere differentiation protocols from EBs. b) Self-renewal and proliferation analysis of HD 8yrs and FF113 neurospheres. c) Neurospheres obteined from HD8yrs(JHD) and d) FF113(control) iPSCs lines both showed correct morphology and self-renewal capability.*

Briefly, this protocol includes an initial phase of embryoid bodies formation and neural-ectodermal cells enrichment through specific conditions. With this new method of differentiation there is no need to use any molecule to drive or induce iPSC's to the neuronal lineage. Conversely, the iPSC's are grown and maintained in specific conditions that mimic the early embryonic stages in vivo. This protocol is based on the spontaneous differentiation into NPCs by using culture mediums able to select only cells with neural precursor characteristics (*Vescovi et al., 1999*). Neurospheres were presented with the characteristic floating morphology and showed self-renewal properties (*fig. 3.7*).

3.4 iPSC derived neurospheres from HD and healthy individuals were able to differentiate in neurons, astroglia and oligodendrocytes.

Neurospheres, which have multipotent properties, can differentiate in all the three neural lineages (neurons, astroglia and oligodendrocytes), just by adding differentiative stimuli such as: cell adhesion matrix, bovine serum and removal of essential growth factors (FGF2 and EGF). Indeed, our neurospheres derived from both FF113 and HD8YRS iPSC lines were capable to spontaneously differentiate in different subtypes of neuronal and glial cells. We performed an immunofluorescence analysis 10, 17, 24 and 31 days after the differentiation to detect specific neuronal markers of cells originated from the spheres. We used anti-GFAP antibody to label glia cells, anti-Tuj 1 (neuron-specific class III beta tubulin) antibody to mark neurons, and Hoechst for total nuclei visualization.


a)
10 Div
17 Div
24 Div
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GFAP
TuJ 1
Merge
b)
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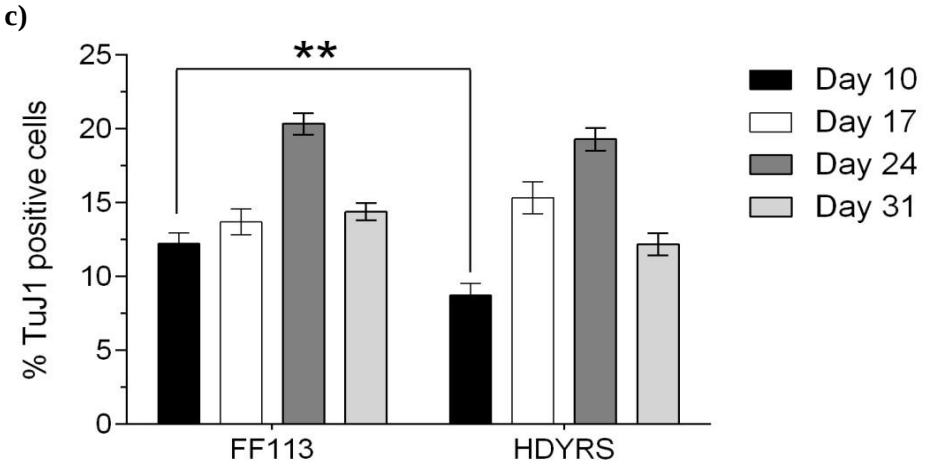

Figure 3.8 *Immunofluorescence analysis 10; 17; 24 and 31 days after spontaneous differentiated neurospheres showed the presence of both neuronal and glial cells from mutated and control cells at different time points. RED = β-Tubulin-III; GREEN = GFAP; BLUE = Nuclei. A) HD8YRS B) FF113 C) Neuronal count performed on HD8YRS and FF113 differentiation experiment showed a significant difference in percentage of TuJ1+ only in the first stages of differentiation (10 Days after spontaneous differentiation).*

We observed that the ratio between the number of neurons (TuJ1+ cells) and the total cells at 10 days after the differentiation was lower in the HD8YRS sample (8,7%) with respect to the FF113 control (12,2%). Conversely, no significant difference was detected between the two cell lines at 17, 24 or 31 days after the differentiation. The highest number of neurons (TUJ1+) was found, in both the cells lines, 24 days after differentiation (24 DIV) (~20%) (fig. 3.8). We used also anti-GABA antibody to identify gabaergic neurons, and anti-GALC (Galactocerebrsoside) to identify oligodendrocites. As shown, in (fig. 3.9) we did not observe differences in the percentage of gaba, or galc positive cells in the two compared samples (FF113 and HD8YRS).

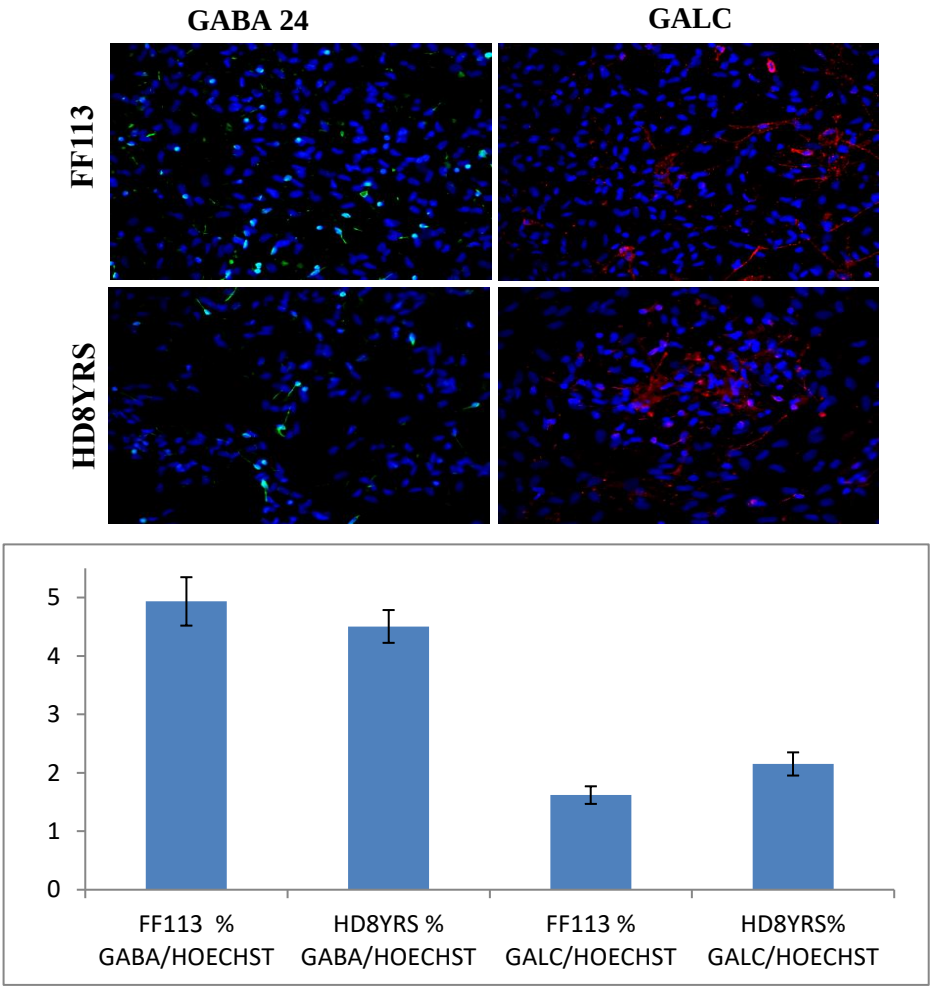

Figure 3.9 *a) Immunofluorescence analysis 24 days after spontaneous differentiated neurospheres showed the presence of Gaba-ergic and Glutaminergic neurons (data non shown for glutaminergic neurons) and oligodendrocite RED = oligodendrocytes; GREEN = gabaergic neurons; BLUE = Nuclei. b) Count performed on HD8YRS and FF113 differentiation experiment showed no significant difference in percentage of GABA or GALC 24 days after differentiation.*

3.5 iPSC HD-derived neurospheres exhibit impaired brain development processes when compared with iPSC-derived neurospheres from healthy individuals.

The list of differentially expressed genes between iPSC HD derived neurospheres, compared with iPSC-derived neurospheres from healthy individuals, were subjected to *in silico* functional enrichment analysis. In contrast, the former group exhibited a (estimated) reduced activity of the following pathways, when compared to the expression profiles of the iPSC-derived neurospheres from healthy individuals: *Synaptic Long Term Potentiation* (z-score = -0.816) and *CREB Signaling in Neurons* (z-score = -1.633). Other important pathways resulted significantly altered without the possibility to computationally estimate their activation states. These are reported in table 3.2.

In line with these pathways, we found that an array of biological functions related to the CNS were significantly considered to work at a reduced activity in HD neurospheres: *development of interneurons* (p-value = 1,16E-03, z-score = -1), *development of neurons* (p-value =5.29E-11, z-score = -1.265), *cell movement/migration of neurons* (p-value = 3,00E-05 and p-value = 5,87E-05, z-score = -1.373 and z-score = -1,694) and *proliferation of neuronal cells* (p-value = 4,27E-05, z-score = -1,392). More macroscopically, we found that the *formation of brain/forebrain* (p-value = 4,98E-04 and p-value = 4,34E-06, z-score = -1,103, z-score = -0,357) were reduced activities in HD neurospheres. (table 3.3). In fig.3.10 and 3.11 can be also be observed that respect to *iPSC-derived neurospheres from healthy individuals* in *HD-derived neurospheres* there is an impaired expression of the genes involved in the cortex differentiation and the two principal glucose transporters in the brain, glut1 (p-value 0.008; fold change 1.78) and glut3 (p-value 0.06; fold change -2.41674).

a)

Ingenuity Canonical Pathways	-log(p-value)	Ratio	z-score	Molecules
Mitotic Roles of Polo-Like Kinase	3,39E00	1,06E-01	-1,342	7
Cell Cycle: G2/M DNA Damage	2,48E00	1,02E-01	1,342	5
Wnt/β-catenin Signaling	1,62E00	4,73E-02	1,890	8
Signaling by Rho Family GTPases	1,48E00	4,05E-02	0,333	10
Synaptic Long Term Potentiation	1,42E00	0,05	-0,816	6
Glioma Signaling	1,11E00	4,55E-02	-0,447	6
Neuropathic Pain Signaling	1,06E00	4,39E-02	-1,342	5
CREB Signaling in Neurons	1,06E00	3,8E-02	-1,633	7

b)

Functions	Functions Annotation	p-Value	z-score	Molecules
organismal	organismal death	1,70E-08	3,854	119
perinatal death	perinatal death	2,39E-05	2,867	35
cognitive imp.	cognitive impairment	1,54E-04	1,982	27
Apoptosis	Apoptosis	1,04E-04	1,702	111
Congenital	malformation of brain	1,14E-04	1,664	21
cell death	cell death	1,25E-04	1,183	137
Interphase	Interphase	4,06E-04	0,872	35
Necrosis	Necrosis	5,99E-06	0,450	118
Development	of cerebral cortex	1,28E-05	0,391	15
Formation	formation of brain	4,34E-06	-0,357	34
Development	central nervous system	3,88E-07	-0,607	43
Development	development of gap junctions	8,73E-04	-0,980	15
Development	development of interneurons	1,16E-03	-1,000	4
Formation	formation of forebrain	4,98E-04	-1,103	16
Quantity	quantity of interneurons	7,09E-04	-1,187	5
Formation	intercellular junctions	9,40E-04	-1,226	17
Development	development of neurons	5,29E-11	-1,265	59
cell movement	cell movement of neurons	3,00E-05	-1,373	19
Proliferation	proliferation of neuronal cells	4,27E-05	-1,392	33
Migration	migration of neurons	5,87E-05	-1,694	18
Expression	expression of RNA	1,24E-04	-1,703	93

Transcription	Transcription	1,12E-04	-1,834	88
Transcription	transcription of RNA	8,76E-05	-1,875	81
Locomotion	Lo comotion	1,01E-03	-2,219	20
cell viability	cell viability	1,68E-06	-4,082	70
Survival	cell survival	4,23E-06	-4,263	72

Table 3.3 *In silico functional enrichment analysis. A list of differentially significantly activated biological (a) pathways b) functions) related to the CNS between iPSC derived neurospheres from HD patients, compared with iPSC-derived neurospheres from healthy individuals.*

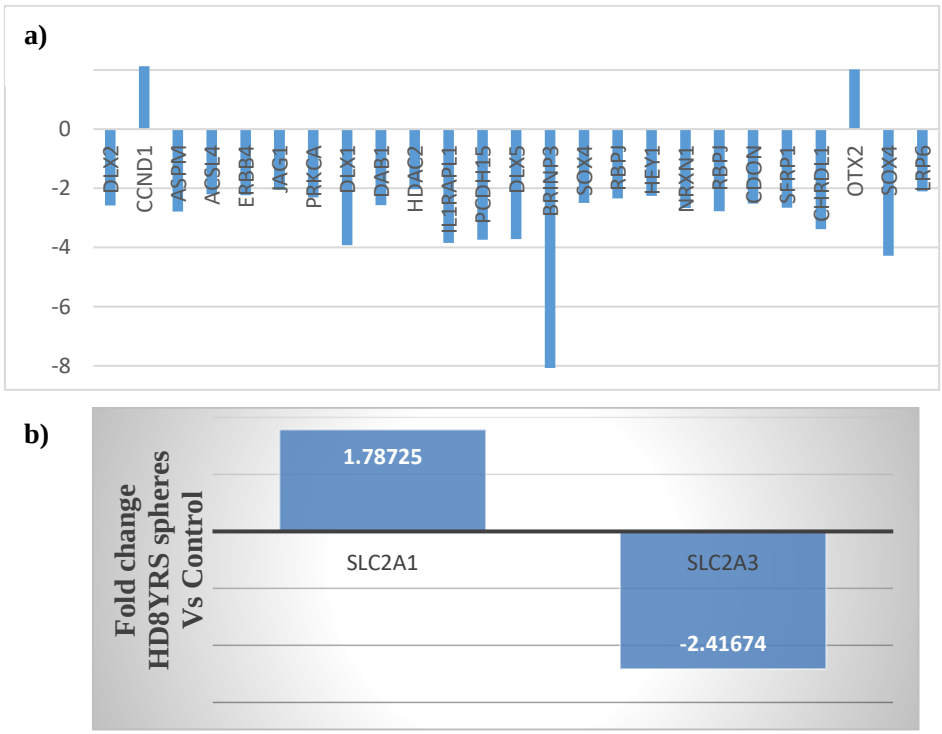

Figure 3.10 *Afymetrics human transcriptome gene chip analysis Fold change HD spheres Vs Control spheres: iPSC derived neurospheres show an impaired gene expression a) of molecules involved in the cortex differentiation. b) the two principal glucose transporters in the brain, glut1 and glut3.*

4. Discussion and conclusions

Huntington disease (HD) is a devastating, progressive autosomal-dominant neurodegenerative disease, caused by abnormal expansion of CAG repeats in the huntingtin gene, translated into a polyglutamine (polyQ) stretch in the HTT protein. The normal function of HTT, and the molecular mechanisms that contribute to the disease pathogenesis, are still unclear and currently there is no treatment known to cure or slow the disease progression. Typically, the symptoms consist of motor, cognitive, and psychiatric disorders (*Banno et al., 2017*). Before that Yamanaka and coworkers generated induced pluripotent stem cells (iPSC), directly from fibroblast cultures (*Takahashi and Yamanaka, 2006*), animal models and immortalized cell systems were the most used models of human diseases mechanisms and therapeutic development in HD. These models helped in understanding many of the impaired pathways in HD but have their limitations. Apart from man, no known animal naturally develops HD. Human conditions are not always reflected in transgenic animal models because of species differences and immortalized cells are artificially manipulated.

Induced pluripotent stem cells (iPSCs) generated from somatic cells of patients can be used to model different human diseases. The disease-specific iPSCs can be differentiated into relevant cell-types affected in HD, holding a great potential for disease modeling and drug screening (*Jang et al., 2014*).

In the present study, we have obtained dermal fibroblasts from 16 HD patients, and 6 healthy controls. The patients and the control subject were recruited in base of their CAG repeat expansion, TFC, and age at onset of the disease, with two of them presenting a juvenile onset.

We succeed in reprogramming into induced pluripotent stem cells the fibroblasts from a young adult healthy control, a young subject with 43 CAG repeats, in an early stage of the disease (TFC 13) and two JHD patients. The youngest patient (85 CAG repeats) presented an infantile onset of the disease, with appearance of the first signs of the disease at the age of 4 (the skin biopsy collected at age of 8). The other JHD patient displays 64 CAG repeats, presenting an adolescent onset. At the time of the biopsy, she was 25 years old displaying a low Total Functional Capacity Score (TFC=5), (*table 3.1*). The iPSC obtained, retained the original mutation in the *HTT* gene and presented a normal karyotype after reprogrammation. The test performed *in vitro* and *in vivo* to evaluate their stemness and pluripotency gave positive results. Indeed, the iPSCs produced in our laboratory are able to spontaneously generate embryoid bodies, expressing the three germ lineages: endoderm, ectoderm, and mesoderm and teratomas once injected subcutaneously in immunocompromised mice.

Juvenile Huntington disease (JHD) is defined by the onset of symptoms before 21 years of age. People with JHD have larger CAG repeats in the *HTT* gene compared to the individuals that will develop HD symptoms as adults. There is similarity between the pathology seen in HD and JHD. However, symptoms of juvenile and adult HD (AHD) subjects differ from each other in most of the cases. Behavioral and cognitive problems are the most common symptoms among JHD and it is well recognized that declining school performance can be a significant feature of JHD with speech and language problems that may occur early in the course of the illness (Bruyn, 1968; Ribai et al., 2007; Yoon et al., 2006). It is not rare in JHD that a child develops HD before his parent and the theory that JHD is more aggressive and presents a shorter disease duration that adult HD is widely held (Senaca et al., 2004). In a recent study gene-expanded children (CAG repeats >39) had a significantly smaller mean brain size

compared with controls, suggesting a specific deficit in brain growth, rather than a global growth abnormality. These findings suggest that abnormal HTT may have a direct effect on brain development and led to the speculation that this phenomenon is more pronounced in JHD (Lee et al., 2012; Querrell et. al, 2013). Most of the HD symptoms are related to a neuronal cell loss in the brain and it would be helpful to study molecular events taking place in first stages of neural development.

For this reason, iPS from cells from both JHD (85CAG) and healthy control, were differentiated into neurospheres of neural precursors (NPCs). NPCs were obtained using a new protocol, that consist in culturing iPSC-derived embryoid bodies and then selecting neural precursor with a selective NPCs medium that allowed the survival of cells with acquired neural properties. These "induced" NPCs demonstrated to have the same properties of "natural" NPCs: they grew as floating spheres, known as neurospheres capable of self-renewal and proliferate *in vitro*; their growth is dependent upon the presence of specific growth factor, such as bFGF and EGF (data not shown) and they can differentiate in all of three neural lineages (neurons, astroglia and oligodendrocytes). This new protocol may represent a valid model to study brain development in vitro. The iPSC derived neurospheres grow as 3D structures mimicking the natural conditions (*Muniany et al., 2016*). Spontaneous differentiation of the spheres from AHD and JHD compared with control ones, can give a lot of information about the metabolic and development impairments during neurogenesis (*Kang et al., 2007*). Neuronal and glial cells generated by this new approach can offer a better understanding of the disease features (*Szlachcic et al., 2017*). They can also become a valuable tool to test the efficiency of potential new drugs (*Zhang et al., 2016*). Gene expression profiling was performed on iPSC HD-derived neurospheres using GeneChip Human Transcriptome Array 2.0. iPSC HD-derived

neurospheres exhibit an impaired brain development processes when compared with iPSC-derived neurospheres from healthy individuals. Several genes involved in the cortex differentiation resulted hypo-expressed in JHD spheres (*fig. 3.10*). Studies on transgenic rodents and postmortem HD brains revealed that cortical atrophy and loss of CPNs is a neuropathological hallmark of HD. HD is characterized by dysregulated information flow through the cortico-striato-cortical pathway. This problem, moreover, emerges early in the course of HD, even before some neurological signs are present, suggesting a key role in the development and subsequent progression of the HD behavioral phenotype. Further research on alterations in cortical processing in HD, including the contribution of thalamic inputs and cortical interneurons, is emerging as fertile ground for further insight into the neuronal mechanisms underlying HD and for the development of effective therapeutic strategies. (Estrada-Sánchez and Rebec, 2013).

The experiments also showed that GLUT3 (encoded by the SCL2A3 gene) is down- regulated in the HD adult neurosphere with respect to the control neurosphere (fold=-2.41, $p<0.05$) whereas GLUT1 (encoded by SLC2A1 gene) is up-regulated in the HD neurospheres with respect to control neurospheres (fold=1.78, $p<0.05$) (*figure 3.10*). GLUTs are transporter molecules that allow glucose to move from blood to brain (Olson and Pessin 1996). All mammalian cells contain one or more members of this family. The human genome encodes 14 different GLUT proteins that facilitate the passage of sugars (e.g., glucose, fructose) across cell and intracellular membranes. The most highly expressed GLUTs in the brain are GLUT1 and GLUT3. Glucose is then taken up into astrocytes GLUT1, and into neurons by GLUT3. GLUT3 is highly and specifically expressed in neurons. It has both higher glucose affinity and transport capacity than GLUT1, and its expression increases concomitantly with the maturation of synaptic connections (Simpson et al.

2008). In recent papers, an early glucose hypo-metabolism was already shown in HD patients (Squitieri and Ciarmello 2010). Several evidences connect the impairment of the energetic metabolism to a defective transportation of glucose in the brain (Adanyeguh et al. 2015). Even if our results have been obtained on neurospheres i.e. neuronal stem cells, and not in differentiated cells, they demonstrate that glucose transport is impaired in the HD patients cells. Finding a way to restore the brain glucose uptake or providing efficient alternative sources to glucose can offer future therapeutic strategies to contrast the disease (Morea et al., 2017). This indicates that this analysis can give a great help in trying to understand metabolic impairments during development.
Since one of the properties that NPCs is the ability to differentiate in all neural lineages, we performed a spontaneous differentiation of neurospheres from the JHD patient and the control donor. Differentiation assay confirmed that they possessed this potentiality, being capable subtypes of neuronal and glial cells.
Immunofluorescence analysis after 10, 17, 24 and 31 days from the differentiation demonstrated that FF113 and HD8YRS spheres were able to spontaneously differentiate in different cells positive for different markers of glial or neuronal cell sub-types. We confirmed that neurospheres derived from the HD iPSC's differentiate in glutaminergic (data not shown) and gabaergic neurons and in oligodentrocytes. These three types of cells are known to be susceptible to degeneration in HD.
This study aimed to produce a valid model of the Huntington disease. We are confident that this novel *in vitro* model will permit us to have a closer view to neuronal development networks by morphologically and physiological studies on the cell types obtained from the neuropheres of NPC.
We are also interested in studying the basis and the mechanisms that bring to impaired metabolism pathways in HD such as glucose transportation and

calcium homeostasis and to offer this results to the scientific community in order be able to reverse the disorders caused from this impaired functions.

5. Materials and methods

5.1 Skin biopsies collection

Dermal skin biopsies were collected using a monouse biopsy punch. Skin fragments were then handly cutted in smaller fragment and plated on a 35mm tissue-culture dish with fetal bovine serum (FBS – Sigma Aldrich) at 37°C 5%CO2 over-night. The day after, dermal fragments were transferred in Dulbecco's Modified Eagle Medium (DMEM) High Glucose (Sigma Aldrich) supplemented with 20% FBS (Sigma Aldrich),
2mM L-Glutamine (Sigma-Aldrich), 100U/ml Penicillin Streptomicin (Sigma Aldrich) and 1X Non-essential Amino Acids (Sigma Aldrich) and cultured until fibroblasts, spreading out from skin fragments, reached confluency. Then cells were treated as a normal primary fibroblast culture (see section 5.2).

5.2 Primary skin fibroblasts culture.

All primary skin fibroblasts were cultured in DMEM High Glucose supplemented with 20% FBS, 2mM L-Glutamine, 100U/ml Penicillin-Streptomycin and 1X Non-essential Amino Acids.

5.3 Episomal vectors

For cell reprogramming was used a mix of three pCXLE-based episomal vectors previously described in Okita et al. (*Okita et al., 2011*).
Episomal vectors has been bought as glycerol-stock from Addgene (plasmids #27077, #27078 and #27080), were amplified and plasmids were extracted with Endo-Free Maxi Kit for low-copy number plasmids (Qiagen) according to manufactor instructions, then were verified through restriction analysis.

5.4 iPSCs production and culture

For reprogramming experiments, 300,000/reaction JS and control fibroblasts were nucleofected using the optimized pulse/buffer condition with 0.5 and 3μg of the previously described mix of episomal vectors (*Okita et al., 2011*) with the standard nucleofection protocol given by the producer. Briefly, after nucleofection, cells were plated in fibroblasts medium without antibiotics overnight and transferred in normal culture conditions the day after. Six days after nucleofection, 140,000 fibroblasts were seeded on a 100mm dish previously coated with Matrigel (Corning). The next day, medium was changed with Nutristem-XF (Biological Science). After 25-40 days from nucleofection, first iPSC colonies were manually picked, fragmented and individually passaged in a 24-well plate (passage I, pI) coated with Matrigel. From each reprogramming experiment, at least 3-5 iPSC clones, selected by morphology (flat and uniform colonies with defined borders), were expanded from pI. From pII, established iPSC lines were maintained in 6well plate and

passaged weekly with 1 mg/ml dispase (Gibco) in DMEM F-12 (Sigma-Aldrich).

5.5 Embryoid bodies formation assay.

iPSCs from an entire 6-well dish were detached using 1mg/ml dispase and then plated in a petri dish in floating condition. Nutristem-XF medium was then gradually switched to KOSR medium (DMEM F-12, 20% Knock-out serum replacement (Gibco), 0.1mM β-mercaptoethanol, 1X NEAA, 50U/ml Penicillin-Spreptomicin, 2mM L-glutamine) in 3 days and cells mantained in culture for 14 days.

5.6 Teratoma formation assay.

iPSCs from an entire 6-well dish were detached using 1mg/ml dispase, resuspended in 100ml of Matrigel and injected subcutaneously in the hind leg of immunocompromised mice. Teratoma growth was monitored up to 80 days after injection; then animals were sacrified and tumors hystologically analyzed.

5.7 Neurospheres production, culture and growth curves.

Embryoid bodies were produced as described in section 4.6. Unlike the classic embryoid bodies formation assay, cells were transferred at 37°C, 5% CO2, 5% O2 at day 5 after their production and maintained in these conditions for 52-54 days. At the end of this period, as previously described in Vescovi et al. cells were cultured as “natural” neuroprecursors cell lines (NPCs): cultures

were harvested, mechanically dissociated, and replated under the same conditions at a density of 104 cells/cm2 every 7–10 days in the same growth medium (*Vescovi et al., 1999*).

For growth curves, 250,000 cells were plated in growth medium in a 25cm2 flask, harvested and counted every 7-10 days. Then 250,000 cells were replated and counted in the same way. At least 5 time-points in triplicate for each cell line were collected.

5.8 Neurospheres differentiation assay.

To study NPCs differentiation capacity, their multipotency was determined by in vitro differentiation tests, in which the simultaneous presence of neurons, astrocytes and oligodendrocytes was detected by immunocytochemical labeling (see section 5.10). Neurospheres were mechanically dissociated and cells were seeded on a cultrex layer (Cultrex® Basement Membrane Extract, Trevigen) and incubated at 5% O2, 5% CO2 and 37°C for 3 days' growth medium used during their routine culturing. Then culture medium was replaced with DMEM/F12 supplemented with 2% FBS, without growth factors. Cells differentiation was monitored at 10, 17, 24 and 31 days after FBS addition.

5.9 Immunostaining and immunofluorescence imaging

5.9.1 Fibroblasts:

Cells were fixed in ice-cold methanol at

4°C for 5 minutes. Aspecific sites on fixed cells were blocked with 10% Bovine Serum Albumine (BSA – Sigma Aldrich), 1% Normal Goat Serum

(NGS – Sigma Aldrich) in 1X Phosphate Buffered Saline with Ca2+ and Mg2+ (PBS++ - Sigma Aldrich) and then cells were incubated at 4°C overnight with primary antibodies diluted in 5% BSA in 1X PBS++.

5.9.2iPSCs:

Cells were fixed in 4% parafolmaldehyde for 20
minutes at room temperature. Fixed cells were then permeabilized and blocked with 10% NGS, 1% BSA, 0.1% Triton X-100 for 45 minutes at room temperature and then incubated overnight at 4°C with primary antibodies diluted in 5% BSA.

5.9.3 Neural cells:

Cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature. Fixed cells were then permeabilized with 0.1% Triton X-100 for 10 minutes at room temperature and blocked with 10% NGS for 1 hour at room temperature. Cell were after incubated overnight at 4°C with primary antibodies diluted in blocking solution and then incubated with the proper fluorescent secondary antibodies for 1h at room temperature.

Nuclei were labeled in every immunostaining with Hoechst 33342 (Life technologies).

After staining, coverslips were mounted with Vectashield Antifade Munting Medium (VectorLabs)

All immunofluorescence images were acquired using a Nikon C2 microscope with 10X (iPSCs), 20X (Neural cells), 40 and 60X (Fibroblasts) objectives and NIS Elements 1.49 program. For every group of experiments were used the same camera settings.

For a complete list of antibodies see section 5.12.

5.10 Antibodies

Primary antibodies: anti-Oct4 (1:100 – Life technologies), anti-Tra-1-60 (1:100 – Life Technologies), anti-TuJ1 (1:400 – BioLegend), anti-GFAP (1:200 – GAKO), anti-GalC (1:200 – Merck Millipore), antiRabbit AlexaFluor 488 (Invitrogen), anti-Mouse AlexaFluor 555 (Invitrogen), anti-Mouse HRP-linked antibody (1:10000 – Amersham), anti-Rabbit HRP-linked antibody (1:10000 – Amersham).

5.11 RNA extraction and quality evaluation

Total RNAs were isolated using TRIzol reagent (Life Technologies) according to manufacturer's instructions. RNAs quality was assessed by determining UV 260/280 absorbance ratios at Nanodrop 1000 (Thermo Scientific) and examining RNA size distribution on RNA 6000 Nano LabChips (Agilent Technologies) processed on the Agilent 2100 Bioanalyzer using the total RNA electrophoresis program. Only RNAs with a RNA Integrity Number (RIN) ≥ 8 were used for subsequent analysis.

5.12 Reverse-transcription and Real-Time PCR

Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions after digestion with DNAse I (Life Technologies).
Real-Time PCR was performed in a 7900HT Fast Real-Time PCR system (Applied Biosystem). For each gene of interest, qRT-PCR was performed as

follows: each RNA sample was tested in duplicate and β-Actin was used to normalize transcript abundance and calculations were performed with the delta Ct method. Statistical analyses were performed on three indipendent experiments.

Sybr green reactions were performed using Power SYBR Green PCR Master Mix (Applied Biosystem) with this PCR program: denaturation 95°C for 10 minutes; amplification 95°C for 10 seconds, 60°C for 10 seconds, 72°C for 30 seconds all repeated for 50 cycles; final elongation 72°C for 7 minutes; final dissociation step 95°C for 15 seconds, 60°C for 15 seconds, 95°C for 15 seconds.

TaqMan reactions were carried out using TaqMan Universal PCR Master Mix (Applied Biosystem) and PCR were performed according to manufacturer's instructions.

Gene	**Primer**
hOct4 Totale	Fwd: CCC CAG GGC CCC ATT TTG GTA CC Rev: ACC TCA GTT TGA ATG CAT GGG AGA GC
hLin28 Totale	Fwd: AGC CAT ATG GTA GCC TCA TGT CCG C Rev: TCA ATT CTG TGC CTC CGG GAG CAG GGT AGG
hKfl4 Totale	Fwd: ACC CAT CCT TCC TGC CCG ATC AGA Rev: TTG GTA ATG GAG CGG CGG GAC TTG
h-Myc Totale	Fwd: GCG AAC CCA AGA CCC AGG CCT GCT CC Rev: CAG GGG GTC TGC TCG CAC CGT GAT G
hSox2 Totale	Fwd: TTC ACA TGT CCC AGC ACT ACC AGA Rev: TCA CAT GTG TGA GAG GGG CAG TGT GC

β-ACTIN	Fwd: GGC ATC CTC ACC CTG AAG TA Rev: GGG GTG TTG AAG GTC TCA AA
18S	Fwd: GGC CCT GTA ATT GGA ATG AGT C Rev: CCA AGA TCC AAC TAC GAG CTT

Table 2 – TaqMan primers.

Gene	Probe
SOX17	Hs00751752_s1
PAX6	Hs00240871_m1
FOXA2	Hs00232764_m1
NESTIN	Hs04187831_g1
T	Hs00610080_m1
EOMES	Hs00172872_m1
SOX1	Hs01057642_s1
GATA4	Hs00171403_m1
GFAP	Hs00157674_m1
SLC	Hs00188193_m1
FABP	Hs00361426_m1
DLX2	Hs00269993_m1
EN1	Hs00154977_m1
FOXG1	Hs01850784_s1

HOXB4	Hs00256884_m1
IRX3	Hs00735523_m1
OTX2	Hs00222238_m1
SOX2	Hs01053049_s1
AXIN2	Hs00610344_m1
βTubIII	Hs00801390_s1
CycD2	Hs00153380_m1
CycD1	Hs00765553_m1
Myc	Hs00153408_m1
βActin	Hs 99999903_m1

5.15 Whole genome expression profiling and in silico functional enrichment analysis of iPSC HD-derived neurospheres compared with iPSC-derived neurospheres from healthy individuals.

Gene expression profiling was performed using GeneChip Human Transcriptome Array 2.0 following the manufacturer's instructions (Affymetrix, Santa Clara, CA). Briefly, on 100 ng of total RNA, a random priming method was used to generate cDNA from all RNA transcripts present in a sample. The cDNA was fragmented and labeled with biotin using terminal deoxynucleotidyl transferase (TdT) before hybridization in a GeneChip Hybridization Oven 645 (Affymetrix). Following hybridization and post-hybridization washes, the arrays were scanned using the Affymetrix GeneChip Scanner 3000 7G to generate the raw data (CEL file). The quality control steps of the experiment were performed using Expression Console v1.3 (Affymetrix).

Expression data were analyzed with Partek Genomics Suite 6.6 and R-3.1.2. In particular, quality assessment and signal normalization were performed with Partek. Filtering and statistical analyses were carried out with R. Raw data were log-transformed and quantiles normalized. Probes not mapping to any Entrez gene were removed. In cases where several probe sets mapped to the same gene, the one exhibiting the highest variance was chosen for further analysis. Batch effects were removed by Partek's batch effect removal algorithm. Sample distribution among groups was assessed using principal component analysis (PCA). Correction for multiple test was achieved by the Benjamini-Hochberg procedure. Statistical differences in gene expression were assessed by the ANOVA test. The significance threshold was set to 0.05.

5.16 Functional enrichment analysis.

Function, and pathway analyses were conducted using Ingenuity Pathway Analysis (IPA; QIAGEN, Redwood City, CA; www.qiagen.com/ingenuity). The entire procedure was based on the prior calculation of the activation z-scores, which infer the activation states of predicted transcriptional regulators, functions, and pathways. Inference of activating or inhibiting molecules or biological functions is based on confirmation by the literature of the results of our experiments. An enrichment score (Fisher's exact test, P-value) was calculated to measure the overlap between observed and predicted regulated gene sets. We considered P-values < 0.05, positive z-scores (activation) and negative z-scores (inhibition), as significant.

5.15 Statistical analysis.

Results are shown as the mean and standard error of the mean from at least three independent repeated experiments Statistical analyses were carried out by analysis of variance. Statistical significance was evaluated by a 2-tailed unpaired Student *t*-test. A p-value <0.05 was considered statistically significant.

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