Recurrent genomic instability of chromosome 1q in neural derivatives of human embryonic stem cells

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Human pluripotent stem cells offer a limitless source of cells for regenerative medicine. Neural derivatives of human embryonic stem cells (hESCs) are currently being used for cell therapy in 3 clinical trials. However, hESCs are prone to genomic instability, which could limit their clinical utility. Here, we report that neural differentiation of hESCs systematically produced a neural stem cell population that could be propagated for more than 50 passages without entering senescence; this was true for all 6 hESC lines tested. The apparent spontaneous loss of evolution toward normal senescence of somatic cells was associated with a jumping translocation of chromosome 1q. This chromosomal defect has previously been associated with hematologic malignancies and pediatric brain tumors with poor clinical outcome. Neural stem cells carrying the 1q defect implanted into the brains of rats failed to integrate and expand, whereas normal cells engrafted. Our results call for additional quality controls to be implemented to ensure genomic integrity not only of undifferentiated pluripotent stem cells, but also of hESC derivatives that form cell therapy end products, particularly neural lines.

Introduction

Whether pluripotent stem cell derivatives can eventually be used widely for therapeutic purpose after the first ongoing clinical trials (1–4) will depend upon their capacity to pass strict quality controls, among which chromosomal and genomic integrity is a key issue. Genomic instability has been demonstrated for pluripotent stem cells at the undifferentiated stage. Aneuploidies, as well as more restricted abnormalities, occur nonrandomly in cultured human embryonic stem cells (hESCs). The most frequent alterations described are whole or partial gain of chromosomes 12 and 17, aneuploidy of chromosome X, or duplication of the 20q11.21 region (5–9). hESCs exhibit indefinite self renewal and pluripotency: they have the ability to divide endlessly while maintaining their capacity to differentiate into all cell types of the organism. These are the only physiological cells of the human organism that can self renew indefinitely in culture. hESCs do not undergo senescence and can remain nontransformed over many passages. Nevertheless, genomic alteration may eventually appear, and its probability tends to accrue over time in culture. Some of these changes likely provide a proliferative or survival advantage to their bearer cells, as indicated by the progressive domination of the original cell line by these altered cells. In contrast, it is expected that derivatives of hESCs should enter senescence after a finite number of doublings, as do any somatic cells (10). However, somatic cells maintained in culture occasionally acquire mutations that allow them to escape senescence (11). Loss of evolution toward senescence observed in hESCs derivatives may therefore reflect the presence of chromosomal changes.

Within the framework of another research program using the VUB03-DM1 hESC line, we showed here that neural derivatives had escaped senescence, as they could be propagated over 34 passages (at least 100 doublings). This was specific to this cell population, as intermediate precursors of mesodermal and keratinocytic lineages systematically reached senescence before 15 passages, in keeping with known limits for somatic cells of about 50 doublings. We also examined neural derivatives of 5 other hESC lines and 1 human induced pluripotent stem (iPS) cell line, all of which showed similar spontaneous loss of a normal evolution toward senescence systematically associated with the alteration of chromosome 1 integrity.

Results

Long-term culture of neural stem cells derived from the VUB03-DM1 hESC line reveals chromosome 1q duplication. Neural derivatives of VUB03-DM1 hESC line propagated over 34 passages (at least 100 doublings) did not reach senescence, while maintaining a normal phenotype (Figure 1, A and C) and the capacity to differentiate into postmitotic neurons expressing βIII-tubulin (TUBB3; Figure 1, D and F). Whereas no chromosomal abnormality was observed in hESCs at the undifferentiated stage (Figure 2A), neural stem cells (NSCs) derived from VUB03-DM1 propagated up to passage 34 exhibited amplification of a segment of chromosome 1 in all but 1 mitosis analyzed. More specifically, a portion of chromosome 1 was translocated onto the telomeric ends of chromosomes 5p (15.4%), 8q (3.8%), and 13q (23%), or else onto the centromeric region of chromosome 13p (53.8%) (Figure 2, B and C, Table 1, and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI46268DS1). At passage 44, this latter dominant clone was apparently selected, since 100% of the cells exhibited the der(13)t(1;13) translocation, accompanied or not by additional chromosomal changes, such as loss of the long arm of chromosome X or polyploidy (Supplemental Figure 1, A and B).

Further investigation using arm-specific chromosome painting showed that the duplicated arm (i.e., 1q) was the long arm (Figure 2D). FISH with juxtacentromeric-specific probes that detected the

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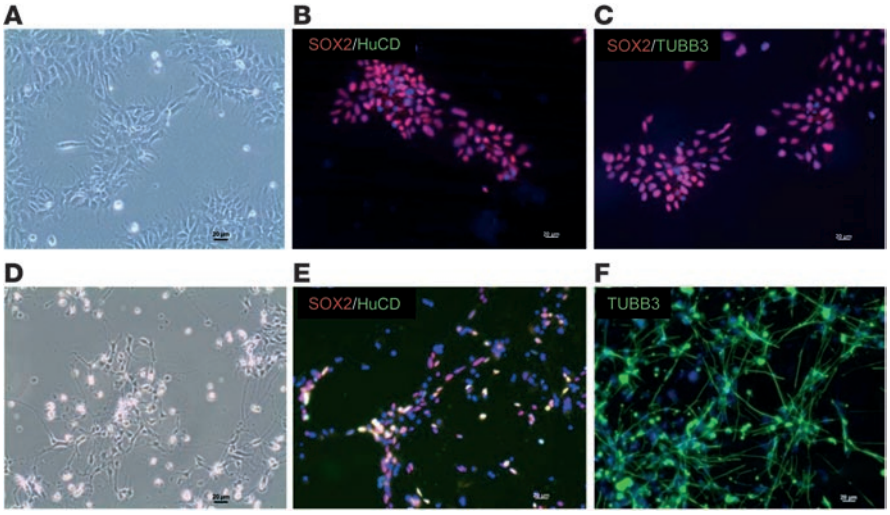

Figure 1
Characterization of the neural derivatives of the VUB03-DM1 cell line. (**A**) Morphological features of NSCs at passage 48 derived from VUB03-DM1. (**B** and **C**) VUB03-DM1 passage 48 NSCs expressed the neural marker SOX2. Note that cells did not express the neuron-specific ELAV/Hu family members HuC, HuD (HuCD) (**B**) and TUBB3 (**C**). (**D**) Morphological features of neurons derived from VUB03-DM1 passage 48 NSCs. (**E** and **F**) Neurons expressing the neuronal markers HuCD (**E**) and TUBB3 (**F**) generated after 20 days of differentiation from VUB03-DM1 passage 48 NSCs. Scale bars: 20 μm.

1q12 region revealed the presence of 3 juxtacentromeric regions of chromosome 1 in metaphases (Figure 2E) and in interphase nuclei (Supplemental Figure 1, C and D). In some interphase nuclei, 2 types of hybridization signals were observed: condensed spots (Supplemental Figure 1C) and dispersed spots (Supplemental Figure 1D), corresponding to condensed and decondensed heterochromatin, respectively. Banked NSCs were thawed at earlier passages in order to estimate roughly the moment at which the 1q duplication was first observed. Translocation of a segment of chromosome 1q onto the telomeric ends of chromosome 4q was noted in 7% of the mitoses at passage 18 (Supplemental Figure 1, E and F).

Recurrence of 1q duplication in long-term cultures of hESC-derived NSC lines. VUB03-DM1 NSCs originated from an embryo that carried a dystrophia myotonica protein kinase (*DMPK*) mutation associated with myotonic dystrophy type 1 (DM1). The absence of a relationship between this specific disorder and the observed translocation was established by analyzing other hESC lines that either did not carry a known disease-related mutation (SA001, VUB01, and H9 lines), carried another *DMPK* mutation (VUB19-DM1 line), or else carried a huntingtin (*HTT*) mutation associated with Huntington's disease (HD; VUB05-HD line). We also investigated 1 human iPS cell line reprogrammed from IMR90 fibroblasts using a classical lentiviral technique (12). At the undifferentiated stage, all of these pluripotent stem cell lines exhibited a normal diploid karyotype, as analyzed using BAC array comparative genomic hybridization (aCGH) or G-banding technologies. In contrast, in neural derivatives, BAC aCGH and multicolor FISH (mFISH) analyses systematically revealed amplification of a segment of chromosome 1 in neural derivatives of SA001 cells at passage 15, VUB05-HD cells at passages 38 and 53 (referred to herein as batch a) and 32 and 59 (batch b), VUB19-DM1 cells at passage 50, H9 cells at passage 52, VUB01 cells at passage 65, and the iPS cell line at passage 23 (Supplemental Figures 3–5, Table 1, and Supplemental Table 1). These chromosomal changes were not found at earlier passages in neural derivatives of SA001 (passage 10; Supplemental Figure 3A), VUB19-DM1 (passage 22), and VUB01 (passage 21) cells. In neural derivatives of SA001 cells, other abnormalities were observed in the absence of 1q duplication at the later passages 31 and 51, namely, polyploidy and translocation (Supplemental Figure 3, C and E). Arm-specific chromosome painting showed that the duplicated part of chromosome 1 was always the long arm (Supplemental Figure 6, A–C). FISH with the juxtacentromeric 1q12 probe revealed that the juxtacentromeric region of chromosome 1 was maintained when the fusion occurred with the p arm of acrocentric chromosomes (such as 13p, 15p, 21p, and 22p),whereas it was either lost (fusion with chromosomes 1p and 17q) or maintained (fusion with chromosomes 10q and 22q) in other cases (Supplemental Figure 6, D–K). The assessment of the organization of the juxtacentromeric heterochromatin of chromosome 1 using the 1q12 probe demonstrated heterochromatin decondensation in 2 of 6 NSC cell lines, namely, VUB01 and VUB19-DM1. The capacity of the NSC lines carrying a duplication of 1q arm at terminal neuronal differentiation was altered in 2 cases (VUB01 and batch a of VUB05-HD) but not in others that were tested, including batch b of VUB05-HD (Figure 3, A–F). In order to determine long-term consequences of the 1q duplication, we also implanted VUB01-NSCs carrying the defect or not into the brains of 10 adult female nude rats. At 7 weeks after transplantation, nonaffected grafted cells were recovered in all cases. In contrast, NSCs carrying the 1q duplication systematically failed to integrate and expand in the host brain (Figure 3, G and H).

Discussion

The present study systematically registered a recurrent gain of chromosome 1q in all NSC derivatives of pluripotent stem cell lines maintained in long-term culture. This 1q duplication was most often isolated when first observed; cells acquired at later passages demonstrated additional chromosomal abnormalities, including aneuploidy and polyploidy. In NSC lines exhibiting the 1q duplication, cell behavior may be heavily biased toward self renewal, as the potential for neuronal differentiation may be altered. NSCs may also fail to survive and/or differentiate after transplantation. The relatively small number of cell lines assayed did not permit us to determine whether these functional consequences relate directly to the 1q duplication or to its specific association with a particular chromosome. Indeed, both the size of the duplicated fragment and the recipient chromosomes differed between the 2 batches of mutant VUB05-HD NSCs that exhibited different differentiation capacities. We cannot exclude that the different ages of the control and mutant cells in the graft experiment could account for the failure of the mutant cells to engraft: control cells never had more than 15–20 passages, whereas homogeneous

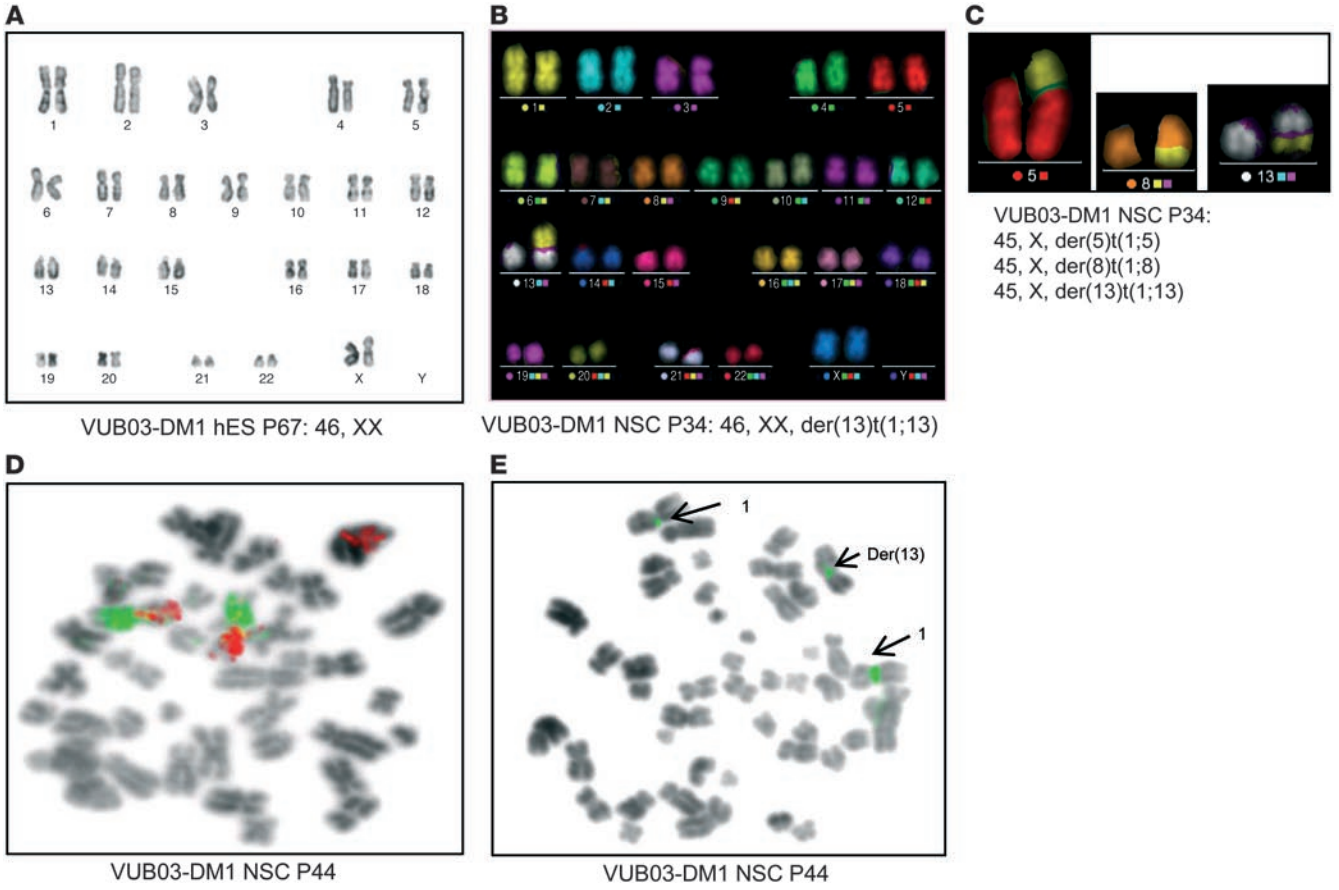

Figure 2
Control of genetic stability in the VUB03-DM1 line. (**A**) G-banding analysis of the undifferentiated VUB03-DM1 passage 67 (P67) hESCs. (**B**) mFISH analysis of neural derivatives of VUB03-DM1 passage 34 NSCs showing a segment of chromosome 1 translocated onto chromosome 13p. (**C**) Partial karyotype showing a segment of chromosome 1 translocated onto chromosomes 5p, 8q, and 13q. (**D**) Arm-specific chromosome painting revealed 2 copies of chromosome arms 1p (green) and 3 copies of chromosome arm 1q (red). (**E**) In situ hybridization of a juxtacentromeric-specific probe detecting the 1q12 region showing 3 signals on metaphases (arrows).

populations of mutant cells were always older, a technicality that precluded firm conclusion. It is worth noting, however, that 1q translocation did not systematically hamper the differentiation of NSCs in neurons in vitro (Table 1). Whether the 1q translocation observed in NSCs derived from pluripotent stem cells may be consequential for tumorigenesis is another unsolved issue in the absence of affected cell survival in our transplantation assay. Nevertheless, chromosomal rearrangements — both the duplication of a segment and its translocation onto different recipient chromosomes — have already been observed and termed *jumping translocations* (JTs; ref. 13 and Supplemental Table 2). Acquired JT aberrations are seen mostly in hematological neoplasias, in which they are associated with poor prognosis. The majority of published data concern hematological malignancies (14), which can explain why JTs have mostly been observed in hematological neoplasias. However, duplication of the 1q arm has been also observed in solid tumors, such as breast cancer, chordomas, hepatocellular carcinoma, retinoblastoma, and pediatric brain tumors (Supplemental Table 2), which suggests that this defect may affect many cell phenotypes. In the case of pediatric brain tumors, the 1q gain correlates with poor clinical outcome independent of tumor grade and histological type (15). JTs recurrently involve chromosome 1q in these cases. This common specific alteration in NSCs derived from pluripotent stem cells that escaped senescence in the present study, and in all these malignancies, suggests that causal mechanisms and functional consequences may also be similar, and this calls for caution. 1q JT randomly occurred in the present study onto recipient chromosomes 1, 4, 5, 8, 10, 13, 15, 17, 18, 21, 22, and Y, which strongly suggests that the abnormality primarily concerns the 1q region itself, rather than the recipient chromosome.

How 1q JT is related to loss of evolution toward senescence, and whether this is a direct or indirect connection, is beyond the scope of the present study. It is interesting to note, however, that chromosome 1q is the longest human chromosome arm, containing more than 1,700 genes and 40 miRNAs (NCBI Map Viewer database; http://www.ncbi.nlm.nih.gov/mapview/). Some of these genes may be deregulated after duplication and translocation, for example, as seen with the BCR-Abelson translocation in chronic myeloid leukemia. Accordingly, a study by Fournier et al. identified 1q12 chromosome translocations in B cell lymphoma and proposed that 1q12 rearrangements represent a new paradigm for long-range epigenetic deregulations in cancer (16).

Table 1
JTs in neural derivatives of human pluripotent stem cell lines

Cell line	NSC passage	JT donor	JT acceptor	Neuronal differentiation potential
VUB03-DM1	18	1q	4q	Yes
VUB03-DM1	34	1q	13p, 13q, 5p, 8q	Yes
VUB03-DM1	44	1q	22p	ND
SA001	15	1q	22p	ND
VUB05-HD[A]	38[B]	1q11.21q44	ND	ND
VUB05-HD[A]	53	1q	15p	No
VUB05-HD[A]	32[B]	1q11.21q32.21	ND	ND
VUB05-HD[A]	59	1q	10q, Y	Yes
VUB19-DM1	50	1q	1p, 5q, 18q	ND
H9	50	1q	1p, 5q, 18q	ND
VUB01	65	1q	22p	No
IMR90	24	1q	17q	ND

[A]At the undifferentiated stage, cells were carrying an amplification of the 20q11.21 region. The extra copy of the 20q11.21 region still remained present in the neural progenies. Analyses were done on 2 batches of VUB05-HD NSC lines (batch a, passages 38 and 53; batch b, passages 32 and 59). [B]Karyotype was performed using BAC-aCGH technology; consequently, the recipient chromosome is unknown. ND, not determined; JT, jumping translocation.

In contrast to prior studies using undifferentiated pluripotent stem cells themselves (17–19), there have been few investigations of chromosomal abnormalities in their differentiated derivatives, although analyses of mouse ESC derivatives have identified chromosomal changes (20) in cultured mouse neurospheres and NSC lines (Supplemental Table 3). Quite interestingly, the human 1q chromosome arm corresponds to mouse chromosomes 1 and 3, and chromosome 1 is also the most affected site in the mouse neurospheres and NSC lines analyzed (Supplemental Table 3). Long-term cell culture of human NSCs derived from fetal brain also revealed chromosomal abnormalities (ref. 21 and Supplemental Table 3). At odds with our results, however, are the results of 2 studies that reported a stable karyotype in neural derivatives of pluripotent stem cells that were extensively propagated (refs. 22, 23, and Supplemental Table 3). This discrepancy may be attributable to the capacity of some ES or iPS cell–derived neural cell lines to continue dividing over many more passages than other lineages; it would be of major interest to examine the phenotypic differences between NSCs analyzed by those teams compared with those of our group, as slight technical differences in differentiation protocols may be the cause. For instance, high levels of antioxidants may increase aneuploidy in stem cell cultures (24), and high levels of B27 were used in our study that may have exacerbated the genomic instability of the NSCs. However, antioxidants induce a variety of chromosomal abnormalities (24), whereas in our study, the chromosomal changes were clearly nonrandom. It would also be necessary to use common approaches for screening for chromosomal defects, as the other studies relied on G-banding karyotyping, whereas some abnormalities — albeit not all — required more detailed techniques to be revealed, namely, mFISH and aCGH.

Why the 1q region is especially prone to JT is a matter of speculation. Chromosome stability is regulated by constitutive heterochromatin (25). The presence of fragile sites *FRA1J* (1q12) and *FRA1F* (1q21) in the heterochromatic region may predispose the 1q arm to genomic instability. The other chromatin feature that may predispose to instability is condensation state. It has been suggested that the first step in the chromosome 1q JT may be caused by heterochromatic decondensation, leading to centromeric destabilization (26). Partial endoreduplication would then occur when heterochromatin is decondensed, facilitating the formation of a JT. This heterochromatin decondensation of chromosome 1q12, the largest heterochromatin site in the human genome, is characteristic of immunodeficiency, centromeric region instability, and facial anomalies (ICF) syndrome (27). ICF syndrome, a rare autosomal-recessive disease caused primarily by a mutated DNA methyltransferase gene (*DNMT3B*; ref. 27), is characterized by decondensation of the juxtacentromeric heterochromatin of chromosomes 1 and 16, which are then prone to breakage. This chromosomal instability is associated with demethylation of the juxtacentromeric regions of these chromosomes (27). The similarity of the centromeric instability observed in neural derivatives compared with that in patients with JT or with ICF syndrome suggests a similar etiologic mechanism.

Our present results advocate regular monitoring, not only of human pluripotent stem cells (as explicitly requested by international guidelines; ref. 28), but also of their progenies, in particular when these seem to avoid senescence. We propose checking chromosome 1q status as a regular control for genomic integrity in neural derivatives of pluripotent stem cells. Such controls will be particularly needed when neural derivatives are considered for cell therapy, as this may compromise safety, as well as for in vitro drug discovery and toxicology, because JT would likely bias results.

Methods

Human pluripotent stem cell culture. hESCs lines SA001 (obtained from Cellartis AB), H9 and H1 (obtained fromWiCell Research Institue Madison), and VUB01, VUB03-DM1, VUB19-DM1, and VUB05-HD (obtained from AZ-VUB) were maintained on a layer of mitotically inactivated murine embryonic STO fibroblasts and cultured in DEM/F12 medium. See Supplemental Methods for details. Human iPS cells, obtained from IGBMC (Department of Cell Biology and Development, Illkirch, France), were generated from fetal lung fibroblasts IMR-90 and grown as previously described (29). Cultures were fed daily and manually passaged every 5–7 days.

Human pluripotent stem cell–derived NSCs. The protocol used to obtain NSCs from hESCs or iPS cells has 2 steps. The first is the production of neuroepithelial cells (NEPs) from human pluripotent stem cells; the second is the production of NSCs from NEPs. NEPs were obtained after 8–10 days of differentiation in N2B27 medium containing neurobasal, DMEM/F12, N2 supplement, B27 supplement, and 0.55 mM β-mercaptoethanol (all from Invitrogen) complemented with human recombinant noggin (300 ng/ml; Preprotech) and SB431542 (20 μM; Tocris). The medium was changed daily until the appearance of neural rosettes containing NEPs after 8–10 days of differentiation. At this time, medium was replaced by N2B27 medium supplemented with hEGF (10 ng/ml; R&D Systems), hFGF2 (10 ng/ml; PeproTech) and human brain-derived neurotrophic factor (hBDNF, 10 ng/ml; R&D Systems). NSCs were cultured for 3–5 days until confluence and passaged (dilution factor, about 1:3 to 1:5) using 0.05% trypsin/EDTA.

Differentiation of NSCs to neurons. NSCs were counted and seeded at a density of 50,000 cells/cm² on polyornithin/laminin-precoated dishes. N2B27 medium was used in the presence of hBDNF, but without hEGF and hFGF2. Medium was changed every 2 days until the appearance of neurons, which were fully differentiated after 20 days. Phenotype was monitored based on expression of specific markers by immunochemistry.

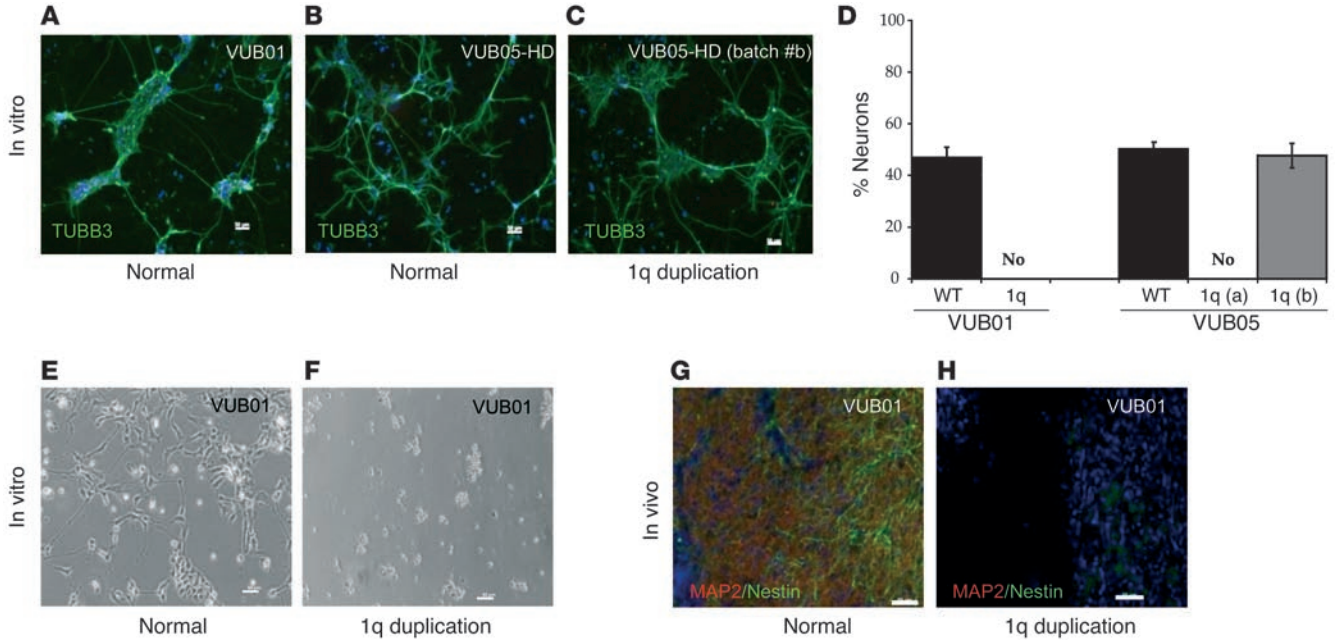

Figure 3

Differentiation potential in vitro and in vivo. (**A**–**F**) In vitro neuronal differentiation potential. 3 weeks after growth factor withdrawal, normal VUB01 passage 18 NSCs (**A**), normal VUB05-HD passage 15 NSCs (**B**), and mutant VUB05-HD (batch b) passage 61 NSCs (**C**) differentiated in vitro into TUBB3-positive neurons. (**D**) Number of neurons generated with normal VUB01 and VUB05 and mutant VUB05-HD (batch a and b) NSC lines, determined using the neuronal nuclear marker HuCD. Samples of normal and mutant cell lines were differentiated in at least 3 independent experiments. The proportion of HuCD-positive cells (± SEM) was determined in at least 1,000 cells per sample in randomly picked fields. NSC lines bearing 1q duplication failed to differentiate into neurons, except mutant VUB05-HD (batch b). (**E** and **F**) 2 weeks after growth factor withdrawal, normal VUB01 passage 18 NSCs (**E**) differentiated in vitro into neurons, whereas mutant VUB01 passage 71 NSCs (**F**) failed to give rise to neurons and died. (**G** and **H**) In vivo differentiation potential. 7 weeks after grafting, human nestin- and MAP2-positive cells were observed with rat brain transplants of normal VUB01 passage 21 NSCs (**G**), but not with mutant VUB01 passage 74 NSCs (**H**). Scale bars: 50 μm.

Cell transplantation and tissue processing. Adult female nude rats (10 weeks old, weight 190–220 g at the time of grafting; Charles River Laboratories) were used. Animals were randomly allocated to 2 groups: 5 rats grafted with VUB01 passage 21 NSCs without the 1q JT, 5 rats grafted with VUB01 passage 74 NSCs carrying the 1q JT. All surgical procedures were carried out as previously described (30) under full anesthesia using a mixture of ketamine (15 mg/kg) and xylazine (3 mg/kg; Bayer Health Care) and using a stereotaxic frame. Unilateral lesions were made by injecting 1 μl of 80 nmol/μl quinolinic acid dissolved in 0.1 M PBS into the right striatum (anteroposterior, –0.5 mm; lateral, –2.7 mm; ventral, –4.7 mm; tooth bar, +3 mm). At 1 week after the lesion, rats received transplants of cells (100,000 in 2× 1 μl HBSS supplemented with 0.05% DNaseI; Invitrogen) (anteroposterior, –0.5 mm; lateral, –2.7 mm; ventral, –5.7 mm; –4.7°). To ensure that the mutant grafted cells were still alive at the moment of transplantation, a few cells that had undergone the same treatment as the grafted cells were successfully brought back into culture. 7 weeks after transplantation, rats were terminally anesthetized with 150 mg/kg i.p. sodium pentobarbital (Ceva Santé Animale), and their brains were fixed by transcardial perfusion with 100 μl of 0.1M PBS (pH 7.4), followed by 250 ml of buffered 4% PFA. Brains were removed, postfixed overnight at 4°C in 4% PFA, and then cryoprotected in 30% sucrose solution at 4°C. Coronal brain sections (30 μm) were cut on a cryotome, collected serially, and stored at –20°C in a cryoprotectant solution until analysis. Grafted cells were first searched at the coordinates used for the injection (i.e., bregma –0.5 mm) according to cerebral structures localized on serial brain slices and the Paxinos-Watson atlas. Grafts usually appeared on cresyl violet–stained slices as a distinct pack of cells, darker than the rest of the parenchyma. If no graft was clearly identified, human-specific labeling (MAB1281, 1:500; HNA Millipore) was used to reveal potentially scattered cells in the brain sections containing the striatum.

Immunocytochemistry and immunohistochemistry. See Supplemental Methods.

Assessment of copy number variation. IntegraChip genome-wide BAC arrays of 5,245 BAC clones (526-kb median spacing) were hybridized by the manufacturer (IntegraGen). CGH data have been deposited in GEO (accession nos. GSE13565 and GSE33708; http://www.ncbi.nlm.nih.gov/geo/). See Supplemental Methods for details.

Harvest of pluripotent stem cells and their progenies for chromosome analysis. See Supplemental Methods for details of preparation of pluripotent stem cells and their progenies, G-banding, mFISH, FISH with juxtacentromeric specific probes, and arm-specific chromosome painting.

Study approval. All animal experiments were conducted with approval by the Direction des Services Vétérinaires, Ministère de l'Agriculture of France, and the European Communities Council Directive (86/609/EEC).

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