Human neural stem cells: a model system for the study of Lesch–Nyhan disease neurological aspects

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Received December 17, 2009; Revised February 5, 2010; Accepted February 14, 2010

The study of Lesch–Nyhan-diseased (LND) human brain is crucial for understanding how mutant hypoxanthine-phosphoribosyltransferase (HPRT) might lead to neuronal dysfunction. Since LND is a rare, inherited disorder caused by a deficiency of the enzyme HPRT, human neural stem cells (hNSCs) that carry this mutation are a precious source for delineating the consequences of HPRT deficiency and for developing new treatments. In our study we have examined the effect of HPRT deficiency on the differentiation of neurons in hNSCs isolated from human LND fetal brain. We have examined the expression of a number of transcription factors essential for neuronal differentiation and marker genes involved in dopamine (DA) biosynthetic pathway. LND hNSCs demonstrate aberrant expression of several transcription factors and DA markers. HPRT-deficient dopaminergic neurons also demonstrate a striking deficit in neurite outgrowth. These results represent direct experimental evidence for aberrant neurogenesis in LND hNSCs and suggest developmental roles for other housekeeping genes in neurodevelopmental disease. Moreover, exposure of the LND hNSCs to retinoic acid medium elicited the generation of dopaminergic neurons. The lack of precise understanding of the neurological dysfunction in LND has precluded development of useful therapies. These results evidence aberrant neurogenesis in LND hNSCs and suggest a role for HPRT gene in neurodevelopment. These cells combine the peculiarity of a neurodevelopmental model and a human, neural origin to provide an important tool to investigate the pathophysiology of HPRT deficiency and more broadly demonstrate the utility of human neural stem cells for studying the disease and identifying potential therapeutics.

INTRODUCTION

Lesch–Nyhan disease (LND) is an X-linked recessive disorder that occurs as frequently as one in every 380 000 live births in Canada and 1/235 000 live births in Spain (1). This disorder involves a virtually complete absence of activity of the enzyme hypoxanthine-phosphoribosyltransferase (HPRT). The HPRT gene consists of nine exons and eight introns. An opening reading frame of 654 nucleotides corresponds to the protein-encoding region (2). The HPRT gene is well known for its clinical and genetic heterogeneity. At least 2000 different mutations have been reported (3). The human HPRT amino acid sequence is similar to that of rodents, implying that it is very conservative and sensitive to any single base...
substitution (4). Molecular defects may result in partial or entire gene deletion, insertion, duplication, splicing error or formation of a stop codon, leading to an alternation in the size of the translated protein. Some molecular defects cause only a single amino acid substitution. Any changes that alter the three-dimensional shape of the protein or reduce its active sites, at least partially decrease enzyme stability, functional activity and enzyme synthesis (5).

The lack of HPRT activity results in an excessive production of uric acid and related symptoms of gout and renal dysfunction. In addition, patients with Lesch–Nyhan syndrome have relatively uniform motor phenotype that consists of severe generalized dystonia superimposed on hypotonia, sometimes with less prominent choreoathetosis or spasticity (6), mental retardation, dysarthria and very dramatic compulsive self-mutilation habits (7).

It is documented that dopaminergic neurons are damaged in affected patients (8,9). However, the mechanism by which features of Lesch–Nyhan syndrome result from impaired purine metabolism is still not well understood.

In an attempt to study the underlying causes of the complex phenotype observed in persons with Lesch–Nyhan syndrome, at least two mouse models have been created that exhibit HPRT deficiency (10,11). Mouse models contribute to our understanding of biology with respect to the similarities and differences between species and various organ systems, and provide models for testing therapeutics. Even though the HPRT-deficient mice have failed to fully recapitulate the phenotype in the human disease, they do have an abnormal phenotype at the molecular, biochemical, morphological and pharmacological levels (12). Some studies have sought to more closely model the neurobiological consequences of the enzyme defect by studying HPRT-deficient dopaminergic neurons in cultures prepared from the knockout mice (13,14). HPRT-deficient mouse dopaminergic neurons are valuable for exploring the purine synthesis pathways with regard to the interactions with the dopaminergic pathways and the resulting effects on the basal ganglia. The recent development of induced pluripotent stem (iPS) cell technology provides models of human genetic diseases. iPS cells give a new opportunity to analyse the pathways that lead to disease pathogenesis based on a particular genetic trait at the cellular level. Park and colleagues (15) generated an iPS line from a female carrier of Lesch–Nyhan syndrome (LNSc-iPS2) that will be a valuable resource for studies of homologous recombination in iPS cells, and for analysis of X chromosome reactivation during reprogramming and random inactivation with differentiation.

Dopaminergic neuronal cultures were also prepared from HPRT-deficient PC12 (16,17), HPRT-deficient neuroblastoma mutants (18,19) and HPRT2 MN9D sublines (20). Another model of LND in human cells was created by Urbach and colleagues (21) by inducing the mutation in human embryonic stem (ES) cells.

Since LND is a developmental disorder, it is not at all clear that the knockdown of the gene comes close to recapitulating the more complex developmental changes that clearly occur in this disease as defined in the literature.

A limitation of cell models is that the findings may be restricted to the in vitro environment, with little relevance for the in vivo state, especially in the brain. This limitation can be addressed by examining brain tissue from the affected Lesch–Nyhan patients.

To test the hypothesis regarding a defect in neurodevelopment, we isolated human neural stem cells (hNSCs) from LND human fetal brain specimens. These cells have two main properties: self-renewal capacity and pluripotent differentiation potential (22,23). It has been suggested that these cells could play a major role in transplantation and be able to advance our knowledge in human development. Our in vitro model provides an alternative approach to study HPRT deficiency effect since LND hNSCs are capable of differentiation into dopaminergic neurons under controlled inducing conditions in vitro and could provide new insights into LND by permitting analysis in a human system, using a large number of genetically modifiable cells but without any genetic manipulation.

To our knowledge, we isolated and studied for the first time hNSCs from brain specimens of LND human fetuses and tested the hypothesis that HPRT deficiency can adversely affect specific processes in neural development, including differentiation and proliferation. The study of the differentiation of LND hNSCs to dopaminergic neurons may offer an opportunity to delve into the developmental processes that lead to the malfunction of the dopaminergic neurons in LND.

RESULTS

In order to generate an in vitro model for LND, to our knowledge, we isolated, for the first time, hNSCs from two 14-week Lesch-Nyhan-affected fetuses according to the procedure previously described (22). This technique uses a serum-free medium containing mitogens (EGF and bFGF), which facilitate the expansion of stem cells, while differentiated cells generally become apoptotic and display poor growth capacity (22).

Characterization of the genetic mutation accounting for the deficient enzyme activity reveals that one fetus carries a r.319_402 deletion, while the other one carries a missense point mutation (c.208G > T, p.Gly70Trp). No other significant difference between the two LND hNSC lines was detected in all the analyses performed in this study.

Under these culture conditions, the morphological appearance of the Lesch–Nyhan hNSCs after 7 days of culture was typically neurospherical and showed no apparent morphological differences when compared with healthy ones. All pathological cells appeared healthy without overt signs of toxicity, such as a high frequency of non-viable cells, debris or apoptotic figures (Fig. 1A).

We analysed HPRT1 levels by real-time polymerase chain reaction (PCR) and found that LND hNSCs express HPRT1 at mRNA level.

The lack of HPRT function in LND hNSCs was confirmed by measurement of live culture incorporation of 14C-hypoxanthine into Inosine Monophosphate (IMP). The healthy hNSCs readily incorporated 14C-hypoxanthine, while LND hNSCs exhibited enzyme activity that was 0.01 ± 0.01 (Fig. 1B).
HPRT malfunction is responsible for the accumulation of uric acid; to further analyse HPRT presence in hNSCs isolated from developing Lesch–Nyhan brains, we analysed the levels of uric acid in our hNSCs (Fig. 1C). Indeed, the accumulation of uric acid was significantly higher in LND hNSCs than in healthy cells.

In addition, we compared the proliferation rate of healthy and LND cells under the selection of 6TG and HAT to confirm that the HPRT protein is not expressed in the LND hNSCs. HAT medium inhibits the de novo pathway of purine synthesis and prevents growth of cells that lack an active salvage pathway. The LND cells proliferated in the presence of 6TG and died in HAT medium, while healthy cells behaved in an opposite fashion (Fig. 1D). These results confirmed the deficiency of HPRT in LND hNSCs.

Human NSCs generated from LND fetal tissues have gross morphology that is similar to hNSCs derived from unaffected fetal tissue (Fig. 1A). On the basis of appearance, LND hNSCs grown as neurospheres do not differ from healthy neurospheres.

To determine if HPRT deficiency impaired cellular proliferation, we analysed their growth rate and the frequency of neurosphere formation. To this end, we compared the behaviour of the two LND hNSCs to five healthy hNSCs lines with similar gestational age and culture history. Proliferation rates of LND hNSCs were assessed at different passages (from passage 2 to passage 10). The growth of LND hNSCs was not significantly different from normal at all passages. As shown in Figure 2A, Lesch–Nyhan hNSCs proliferated well in serum-free conditions and showed no significant alterations.

Next, to determine the frequency of neurosphere formation, we initially plated single-cell suspensions at low cell density in growth medium containing methylcellulose to ensure that distinct colonies were derived from single cells and therefore clonal in origin. We found that LND hNSCs form neurospheres at the same frequency as those from unaffected individuals (Fig. 2B).

RT–PCR revealed the expression of neural precursor and stem cell markers such as GFAP, Nestin, β-tubulin III, NG2, SOX2, FLT4 and CD133 in healthy and LND hNSCs. As far as the expression of GFAP, NG2, SOX2 and CD133 was concerned, the data showed no particular alteration when compared with healthy hNSCs (Fig. 2C), but regarding β-tubulin III and FLT4 expression, LND hNSCs appeared to express smaller amounts than healthy ones. Western blot analysis of neural markers GFAP, β-tubulin III and Nestin, performed on undifferentiated hNSCs, evidenced Nestin and GFAP protein levels comparable to healthy cells and confirmed a reduced level of β-tubulin III in LND hNSCs (Fig. 2C).
Since one of the hallmarks of LND is neurological alteration, we induced hNSCs to differentiate as previously described (22) and performed immunofluorescence to test the morphology and number of typical neural precursors such as Nestin, GFAP, β-tubulin III and NG2 at different times (Table 1). We found interesting differences between pathological and healthy cells. In fact, LND cells revealed significant reductions for β-tubulin III positive cells already after 3 days in vitro (DIV) of differentiation and this discrepancy increased during time, whereas no significant difference was observed for GFAP expression (Fig. 3A). Oligodendrocyte precursors, still present at 5 DIV, became greater in number at 7 DIV in LND cells with respect to those in healthy ones. The data at 7 DIV were confirmed by real-time PCR (Table 2).

To determine whether the reduction in the number of LND neurons was because of an increase in cell death or to a block in differentiation, cells were stained by using the TUNEL method and by using corresponding markers for the individual cell types, at different stages of differentiation. Only a few TUNEL-positive cells were observed in the healthy differentiated hNSCs, while the number of LND TUNEL-positive cells from 3 to 7 days of differentiation (Fig. 3B) was significantly higher.

To identify the cell types of the TUNEL-positive cells, double-staining was performed using β-tubulin III, GFAP and NG2 as markers for neurons, astrocytes and oligodendrocytes (Fig. 3B and C). Interestingly, the most apoptotic processes in LND-differentiated cells were charged to astrocytes as demonstrated by double-labelling with TUNEL plus immunostaining with GFAP. The percentages of LND TUNEL-positive astrocytes at 3, 5 and 7 DIV were, respectively, 20 ± 1.5%, 18 ± 1.7% and 16.87 ± 0.9%, while the values of healthy differentiated cells were significantly lower: 0%, 1 ± 0.3%, 2.30 ± 0.6% at 3, 5 and 7 DIV, respectively. LND TUNEL-positive neurons were significantly higher than healthy ones and the values ranged from 2.98% at 3 DIV, 2.15% at 5 DIV and 4.35% at 7 DIV.

No TUNEL-and-NG2 double-positive cells were found either in LND or healthy cells at 3, 5 and 7 days upon differentiation.

We hypothesized that the differences in the extent of neuronal differentiation between Lesch–Nyhan and healthy hNSCs were probably owing to dopamine (DA) dysfunction, a distinctive aspect of LND. To investigate these findings, we performed analysis to detect, in undifferentiated hNSCs, the presence of mRNA transcripts, relevant in dopaminergic neuron development and function, such as tyrosine hydroxylase (TH), D2 receptor (D2R) and nuclear receptor-related 1 (NURR1), determined by RT–PCR. This analysis did not indicate any relevant difference (Fig. 4A). To further delve into the dopaminergic differentiative pathway of LND hNSCs, these cells were induced to differentiate towards dopaminergic neurons and immunofluorescence was performed to test the presence of key dopaminergic enzymes, such as Dopamine Transporter (DAT) and TH (Fig. 4B and C). No significant differences existed between LND and healthy hNSCs regarding the percentage of cells positive to dopaminergic markers even if the physical appearance of Lesch–Nyhan dopaminergic neurons was significantly different when compared with healthy ones. Statistical analysis to examine the overall effect of
HPRT deficiency in LND dopaminergic neurons revealed a significant reduction in soma area (Fig. 4D). Although the number of neurites per cell could not be clearly evaluated at this developmental stage, LND dopaminergic neurons’ neurite length was significantly reduced (Fig. 4D).

During human fetal development, multiple transcription factors participate in the induction and maintenance of many aspects of the neurochemical phenotype of LND neural cells (24). Although the proliferative potential of LND hNSCs appears unaltered, it is possible that the LND stem cells have distinctive gene expression patterns that have implications for later steps in development. To detect any alterations in the expression of genes related to the identification, growth and differentiation of stem cells, we performed a

| Table 1. Immunofluorescent analysis of neural markers upon differentiation of human neural stem cells (hNSCs) |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| 3 DIV                                           | 5 DIV                                           | 7 DIV                                           |                                                |                                                |                                                |                                                |
|                                                  |                                                  |                                                  |                                                |                                                |                                                |                                                |
| LND                                             | Healthy                                         | LND                                             | Healthy                                         | LND                                             | Healthy                                         |                                                |
| β-Tubulin III                                   | 2 ± 0.1%*                                       | 5.2 ± 1%*                                       | 14 ± 2.3%*                                     | 7 ± 0.2%*                                       | 20 ± 1%*                                       |                                                |
| GFAP                                            | 16 ± 3%                                         | 51 ± 4%                                         | 49 ± 6%                                         | 70 ± 2%                                         | 67 ± 5%                                         |                                                |
| NG2                                             | N.D.                                            | 1 ± 0.1%*                                       | N.D.                                            | 5.6 ± 0.4%*                                     | 2 ± 0.6%                                       |                                                |

Values are expressed as mean ± SD. LND, Lesch–Nyhan disease; N.D., not determined; DIV, days in vitro.

*P < 0.05 LND hNSCs versus healthy ones.

Figure 3. Differentiative potential of LND hNSCs (A) LND (upper) and normal (bottom) hNSCs differentiated cells stained positively for β-tubulin III (red) and GFAP (green), oligodendrocytes stained positively for NG2 (green) and for Nestin (red). DIV, days in vitro. (B) The data represent the percentages of TUNEL/GFAP or TUNEL/β-tubulin III or TUNEL/NG2 double-positive astrocytes or neurons or oligodendrocytes after double-positive cells from 10 randomly chosen fields of four independent experiments. (C) Double-staining of TUNEL-positive cells with specific cell markers. TUNEL/β-tubulin III double-positive and TUNEL/GFAP double-positive were observed in LND hNSCs upon 7 DIV. All nuclei were counterstained with DAPI (blue).
gene expression profile of stem-cell-specific markers as well as stem cell differentiation markers. The comparison between LND and healthy hNSCs revealed 10 genes between the analyzed 84 genes, whose mRNA expression was more than three-fold different in LND hNSCs (Table 3). A deeper analysis revealed that five of these genes analysed showed a statistically significant decrease in expression and play a role in neural cell differentiation (ALDH1, CD44, NCAM1, NEUROG2 and TUBB3), while PPARβ, a transcription factor expressed during neuronal in vitro maturation (25) and BMP2, key determinant of neural crest induction and development (26), were upregulated. All of the other genes misexpressed in LND hNSCs are involved in transcriptional pathways (27) (Table 3). Expression levels of ALDH1 in hNSCs were compared at the cellular level among controls and LND. Quantitative PCR confirmation (Table 3) revealed a 30-fold decrease in the expression of the ALDH1 gene.

The major biosynthetic pathway of retinoic acid (RA) from retinol is the irreversible oxidation of retinal to RA catalyzed by a cytosolic aldehyde dehydrogenase 1 (ALDH1) (28). To determine whether RA synthesis by ALDH1 has a role in the development of LND dopaminergic neurons, we aimed to compensate for ALDH1 downregulation by in vitro RA administration. The LND and healthy hNSCs were cultured in growth medium containing 5 μM RA. After 7 days, we first examined morphological changes during RA-induced neuronal differentiation (29) and found that RA restores normal neurite formation and soma area (data not shown). Characteristic patterns of TH expression were observed in LND cells cultured in RA-enriched culture medium (Fig. 4E).

In order to address the role of nitric oxide (NO) in the dopaminergic deficit in HPRT-deficient brain, the formation of NO was evaluated by measuring the stable NO and its products, nitrite and nitrate (Supplementary Material, Table S1). These results revealed that LND hNSCs’ NO production was significantly higher than that of healthy hNSCs and was prevented by the addition of N-nitro-l-arginine methyl ester (l-NAME, 100 μM). As the majority of NO produced in the brain is from the predominant NOS1 isoform, we determined its expression at mRNA level in healthy and LND hNSCs. Our results (Supplementary Material, Table S1) indicated that as far as LND hNSCs were concerned, NOS1 was not responsible for NO overproduction. To further investigate the idea that increased NO levels inhibit dopaminergic differentiation of LND hNSCs, these cells were treated with l-NAME (100 μM) upon differentiative conditions. After 7 days, we examined TH (Supplementary Fig. 1C) and β-tubulin III (data not shown). Positive cells were counted in a blind manner and no significant difference was observed between LND hNSCs with and without l-NAME addition upon differentiative condition.

### DISCUSSION

HPRT activity in patients with classic LND is typically absent or less than 1.5% of normal values. This genetic defect leads to a typical pathological pattern, which includes characteristic neurological symptoms. It is still not clear how the deficiency in HPRT activity leads to these symptoms, although it is documented that dopaminergic neurons are damaged in affected patients (8,9).

In an attempt to study the underlying causes of the complex phenotype observed in individuals with Lesch–Nyhan syndrome, HPRT-deficient mouse models have been created. These mice are useful for studying the biochemical and physiological pathways, especially involved in embryonic and fetal development, and specific functions in various organs. They provide a useful model system for the development of new therapeutic techniques.

An alternative approach is the generation of primary cultures from patients’ cells. This methodology is limited because of the specific range of tissues from which cells can be obtained.

An innovative approach to study LND pathology is to examine the phenotype and possible aberrations of human fetal LND neural stem cells. By analyzing human LND hNSCs, it is possible to study developmental abnormalities regardless of whether they are caused by direct action of HPRT1 mutations, or more likely, by a combination of direct and indirect effects of HPRT1 mutations and epigenetic factors.

As expected by prenatal diagnosis, LND hNSCs universally fail to incorporate hypoxanthine. When primary human fetal tissue is exposed to the mitogens EGF and FGF-2, aggregates of stem and progenitor cells form, which have been termed ‘neurospheres’. These neurospheres can be expanded in culture for extended periods of time (22) and as such provide an ideal alternative model to study human brain development. The neurosphere culture system allows the study of specific processes in neural development, including proliferation, migration and neuronal differentiation.

The results in this paper demonstrate that human fetal Lesch–Nyhan brain contains a population of neural stem cells, which shows the same capacity to proliferate and form neurospheres as healthy hNSCs and which has the ability to express typical neural markers (22). Cells within the neurospheres differentiate when exposed to an appropriate substrate and when deprived of growth factors to consistently produce neurons and glia. Therefore, human neurospheres offer two major advantages that make them ideal to study processes of neural development in LND: (i) tissue can be derived from human LND brain and neural stem cells can be expanded in culture for extended periods of time; (ii) neurospheres can be manipulated both genetically and epigenetically.

Here to our knowledge, we report for the first time the use of neurosphere cultures to reveal molecular and cellular differences between LND and normal hNSCs. The limitation of our study is that in vitro models are not capable of considering all
the variables of the human body. Nevertheless, this study yields relevant data useful in designing in vivo studies to explore such variables.

Human LND NSCs’ morphology was similar to that of hNSCs derived from unaffected fetal tissue. Proliferation rates and the lifespan of LND hNSCs were not significantly different from controls at any passage; the ability to grow these cells for extended periods of time allows their banking and makes them a valuable resource for LND studies in vitro.

Previous results indicate that it is the process of differentiation of dopaminergic neurons that is particularly affected adversely by a HPRT deficiency. This is consistent with the special importance of HPRT in the brain during neonatal life as indicated by the substantial increase in HPRT activity in human and rat brain during the first few weeks after birth, a period of active neuronal development (30). Thus, it is reasonable to suppose some general differences in function and expression pattern between LND and healthy hNSCs. Based on our experience in defining the pattern of neural stem cell markers, expression in healthy hNSCs, we compared the expression profile of healthy and LND hNSCs and found interesting differences. The expression of β-tubulin III and FLT4 resulted significant lowering in LND hNSCs than in healthy ones. These alterations could be implicated in the clinical picture of LND since, as previously demonstrated, the decrease in the expression of β-tubulin III could represent a neurodevelopmental abnormality (31). Furthermore, VEGF-C is required by brain neuroepithelial cells during embryonic development and has a direct neuroprotective effect on various types of neural progenitors cell expressing FLT4 (32).

We next assessed the rate of neuronogenesis by culturing hNSCs in differentiating medium and establishing the number of neurons. Interestingly, the percentage of LND cells positive to neuronal markers was significantly lower when compared with healthy ones. The reduced number of LND neurons could be owing to a block in differentiation or to enhanced cell death of differentiated neurons. The TUNEL stain results revealed a striking percentage of apoptotic events in LND astrocytes even at early stages of differentiation. As previously demonstrated by Pelled and colleagues (33), the HPRT-deficient astroglia cultures exhibited altered content of purine and pyrimidine nucleotides. In view of the quantitative

**Figure 4.** Dopaminergic differentiation and gene expression in LND hNSCs. (A) RT-PCR analysis of dopaminergic marker in LND and in normal hNSCs. There is no difference between LND and healthy hNSCs. (B and C) Dopaminergic differentiation. LND hNSCs expressed DAT (red), NF (green) (in B), and TH (red in C). (D) Morphometrical parameters after dopaminergic differentiation of LND and healthy cells. Bottom panel: Morphology of LND and healthy dopaminergic neurons stained with NF. (E) Immunostaining of LND hNSCs upon differentiation without RA (left panel) and with 5 μM RA (right panel): RA-induced dopaminergic differentiation in LND hNSCs as demonstrated by the expression of TH (red). Astrocytes were stained with anti-GFAP (green). All nuclei were counterstained with DAPI (blue).
Table 3. Fold differences in gene-expression profile in LND hNSCs

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Fold difference</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decreased gene expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34</td>
<td>2.28</td>
<td>0.003</td>
</tr>
<tr>
<td>NCAM1</td>
<td>2.14</td>
<td>0.010</td>
</tr>
<tr>
<td>NEUROG2</td>
<td>9.49</td>
<td>0.013</td>
</tr>
<tr>
<td>TUBB3</td>
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<td>0.008</td>
</tr>
<tr>
<td>ALDH1</td>
<td>39.02</td>
<td>0.001</td>
</tr>
<tr>
<td>Increased gene expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPARD</td>
<td>11.99</td>
<td>0.001</td>
</tr>
<tr>
<td>BMP2</td>
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<td>0.006</td>
</tr>
<tr>
<td>NOTCH1</td>
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</tr>
<tr>
<td>MYST1</td>
<td>4.27</td>
<td>0.005</td>
</tr>
<tr>
<td>GCNSL2</td>
<td>12.41</td>
<td>0.039</td>
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hNSCs, human neural stem cells; LND, Lesch–Nyhan disease.

dominance of glial cells in the brain, the abnormal nucleotide content in these cells, even if confined to a specific stage of development only, may be associated with the neurological deficit in LND. A hypothetical pathway involved in LND astrocytes apoptosis could be found in the accumulation of 5'-aminoimidazole-4-carboxamide riboside (AICAR), which has a toxic effect on neural cells (34). AICAR may accumulate in those individuals in which an inborn error of purine metabolism causes an increase in the rate of de novo synthesis and/or an overexpression of cytosolic 5'-nucleotidase, that appears to be the enzyme responsible for AICAR hydrolysis.

5'-Nucleotidase activity has been shown to increase in patients affected by Lesch–Nyhan syndrome. Moreover, astrocytes cell death may contribute indirectly to neuronal injury or other CNS pathologies since astrocytes provide neurotrophic support, protect against excitatory amino acid neurotoxicity, and maintain the normal homeostasis of the extracellular fluid (33).

It is documented that dopaminergic neurons are damaged in affected LND patients (8,9). The severity of symptoms in Lesch–Nyhan disease remains constant once the neurobehavioral syndrome is fully expressed (35) suggesting a developmental rather than a continuing degenerative process. The early presence of dopamine in the embryonic brain of primates (36–39) suggests a role for this neurotransmitter in the neurogenesis of the dopaminergic system and maturation of the striatum.

The establishment of an in vitro system that mimics the differentiation of undifferentiated Lesch–Nyhan and healthy neural stem cells into dopaminergic neurons gave us the opportunity to study the developmental processes that lead to the malfunction of the dopaminergic neurons in LND. When challenged to differentiate towards dopaminergic neurons, LND neurons showed a reduced neurite outgrowth and soma area. Morphological abnormalities could be a consequence of HPRT deficiency on purine metabolism. Previous studies demonstrate that guanine nucleotide depletion in neuroblastoma cell lines promotes neuronal differentiation and reduces proliferation, especially in dopaminergic neurons showing an abnormal neurite outgrowth (40–42).

Although the proliferative potential of LND hNSCs appears unaltered, it is possible that the LND hNSCs have distinctive gene-expression patterns that have implications for later steps in development. To identify pathways affected by HPRT deficiency during early cortical development, Stem Cell RT^2 Profiler PCR array was then used to compare gene expression patterns between LND and normal stem cells. The benefit of looking at gene expression in neuropehnes, as opposed to using primary fetal tissue, is that these cultures represent a relatively pure population of dividing cells at a specific time in development. We have shown that gene-expression patterns are dramatically changed in LND neuropehnes. Many of the specific signalling pathways are important for proliferation and differentiation, so the absence of significant differences in the proliferation of LND hNSCs could be explained by the fact that misexpression of these genes causes slight changes in the behaviour of the cells that we have failed to detect. The proneural gene Neurogenin 2 (NEUROG2) is a member of a family of bHLH transcription factors (43), and is important not only for neuronal differentiation (44), but also for neuronal subtype-specification in various regions of the nervous system (45).

We suggest that the dysregulation of the expression of transcription factor genes crucial for the proliferation and differentiation of hNSCs and for the maturation of neurons may be associated with the severe neurological phenotype of LND.

Along with the transcription factors described above, we have also demonstrated an altered expression of ALDH1. The downregulation ALDH1 suggests a possible involvement of aldehyde dehydrogenases (ALDH) in the pathogenesis of LND disorder. ALDH is involved in the synthesis of RA from retinal (46). RA and related retinoids are likely to be of importance for mesencephalic DA neurons since receptors and handling enzymes are expressed at high levels both in the substantia nigra neurons and in their target regions (47).

During development, expression of RA-generating enzymes precedes TH expression in DA neurons (48), indicating that retinoids may be important for the induction of the dopaminergic phenotype. In this study, we show that in vitro supplementation of RA counteracts the developmental defect in LND DA-neuron morphology differentiated from LND hNSCs. This implies that RA is crucial to induce proper DA neuronal differentiation. Most appealing, by linking local RA synthesis to LND DA neuronal development and morphology, a novel mechanism is proposed, with essential implications for LND clinical pathology.

Another important aspect of this pathology, described by Song and Friedmann (49), is that HPRT deficiency induces secondary transcriptional aberrations in other genes as observed in our study, and the expression of the corresponding secondary genetic defects could play an important role in the development of some aspects of the HPRT-deficiency phenotype, especially the neurological deficits; in fact, a relatively small number of unrelated genes are aberrantly expressed in HPRT-deficient mouse striatal tissue. These data suggest the possibility that functionally multigenic mechanisms of pathogenesis underlie this monogenic disease (49). The fact that physiological or developmental damage may occur very early in life could make it difficult to rescue the abnormalities by reintroducing a non-mutant HPRT cDNA. Nevertheless, the future development of this study will include the correction of HPRT deficiency.

Oxidative stress compromises dopamine neuron function and previous studies found that the oxidant-sensitive mitochondrial enzyme, aconitase, was partially inactivated.
by the increased production of either NO, O$_2^·$, ONOO$^-$ or H$_2$O$_2$, in HPRT-deficient brains (50,51). The present study revealed an overproduction of NO which was not correlated with NOS1 expression. Alternative sources of NO, however, should not be ignored. The inducible form of NOS, NOS2, while not normally expressed in the brain, can be induced under pathological conditions (52). It is possible that HPRT deficiency may cause an upregulation of NOS2 leading to toxicity through NO itself. LND hNSCs’ exposure to L-NAME results in reduced levels of nitrites and nitrates (reflecting reduced NO), but did not influence the differentiation of neural stem cells to neurons.

To our knowledge our results present for the first time experimental evidence that HPRT ‘housekeeping’ gene plays an important role in human fetal neurodevelopment by complex mechanisms that still need to be elucidated. Developmental neurobiology may throw light into the neurochemical and structural events that contribute to the clinical picture of LND. Such knowledge may then suggest therapeutic interventions.

**MATERIALS AND METHODS**

### Cell culture and neurosphere formation assay

Human fetal tissue was obtained from two 14-week-old legally aborted Lesch–Nyhan fetuses and from five 12–14-week-old healthy fetuses, according to the ethical guidelines of the European Network for Transplantation (NECTAR). Deficiency of HPRT was diagnosed on the basis of characterization of the genetic mutation accounting for the deficient enzyme activity: one fetus carries a c.319_402 deletion, while the other one carries a missense point mutation (c.208G > T, p.Gly70Trp).

The experimental protocol was approved by the ethics committee of the Fondazione IRCCS Istituto Neurologico ‘Carlo Besta’ and Fondazione IRCCS Policlinico-Mangiagalli-Regina Elena. hNSCs were isolated as previously described (22,53,54). hNSCs were cultured in serum-free human Medium (55). Under these culture conditions, normal values for incorporation of $[^{14}$C$]$ hypoxanthine into $[^{14}$C$]$ IMP in cells were 98–100%. M$[^{14}$C$]$ hypoxanthine was measured. HPRT activity was expressed as incorporation of $[^{14}$C$]$ hypoxanthine normalized to cellular protein and percent of healthy hNSCs. Numbers are means ± SD of five independent experiments.

### Selection media and cell proliferation

Selection media were added to healthy and LND cells to test the activity of the purine salvage pathway. Thus, the hNSCs were grown with 6TG (1 μg/ml, Sigma, St Louis, MO, USA). Alternatively, the cells were incubated with HAT (1 $\times$ 10$^{-4}$ M hypoxanthine, 4 $\times$ 10$^{-7}$ M aminopterin, 1.6 $\times$ 10$^{-5}$ M thymidine; Gibco, Invitrogen, Carlsbad, CA, USA).

Cell viability was evaluated by trypan blue exclusion test 1–3 days after adding the selection media.

### Real-time quantitative polymerase chain reaction

TaqMan real-time PCR was performed to investigate the expression of HPRT, β-III tubulin, GFAP and NG2. Three separate RNA extractions were performed on the LND hNSC lines and the pooled controls, and processed separately. Each cDNA sample (corresponding to 100 ng total RNA) was amplified in triplicate using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) in a PCR volume of 20 μl containing the TaqMan Universal PCR Master Mix (with AmpliTaq Gold DNA polymerase) and the Target Assay Mix (all from Applied Biosystems) for 35–40 cycles. The relative expression of each gene examined was normalized to GAPDH and calculated according to the formula 2$^{-\Delta\Delta$Ct} as described in the manufacturer’s instructions (Applied Biosystems).

For the detection of genes related to the identification, growth and differentiation of stem cells, real-time PCR was performed using the Stem Cells RT$^2$ Profiler$^{TM}$ PCR Array according to manufacturer’s instructions (SuperArray Bioscience Corporation, Frederick, MD, USA). Pathway-focused gene-expression analysis was performed with the PCR Array System and the PCR Array Data Analysis Web Portal. Each Stem Cells RT$^2$ Profiler$^{TM}$ PCR Array was done on separate cDNAs at least three times.

### HPRT enzymatic determination

Activity of the enzyme HPRT was determined in LND and healthy hNSCs by a high-performance liquid chromatography (HPLC)-based method (56,57). In brief, each cell line was incubated for 40 min at 37°C in 10 mM HEPES, 125 mM NaCl, 2.6 mM KCl, 5.5 mM glucose, 1 mM CaCl$_2$, 50 mM MgCl$_2$, 18 mM NaH$_2$PO$_4$ and 10 μM [$^{14}$C$]$ hypoxanthine. At the end of the incubation time, radioactive IMP was separated from hypoxanthine by HPLC, and radioactivity in the IMP peak was measured. HPRT activity was expressed as incorporation of [$^{14}$C$]$ hypoxanthine normalized to cellular protein and percent of healthy hNSCs. Numbers are means ± SD of five independent experiments.

Under these assay conditions, normal values for incorporation of [$^{14}$C$]$ hypoxanthine into [$^{14}$C$]$ IMP in cells were 98–100%.

### Western blot analysis

The LND hNSC lines and the pooled controls (5 $\times$ 10$^5$ undifferentiated cells each line) were suspended in 100 μl of cold RIPA lysis buffer (Pierce, Rockford, IL, USA) supplemented with a protease inhibitor cocktail. Protein whole extracts (40 μg) were separated by SDS–PAGE and then transferred to nitrocellulose membrane (Pierce, USA). Membranes were probed with primary antibodies for GFAP (mouse, 1:500), GAPDH (mouse, 1:500) (both from Chemicon, Temecula, CA, USA), β-III tubulin (mouse, 1:100, AXXORA, San Diego, CA, USA), Nestin (mouse, 1:100, R&D Systems, Minneapolis, MN, USA), followed by secondary antibody...
horseradish peroxidase conjugated-anti-rabbit and anti-mouse IgG (Chemicon; 1:5000). The lysate ex vivo human fetal healthy brain was used as a positive control.

TUNEL assay
To detect apoptosis in human LND and healthy differentiated NSCs, a DeadEND fluorometric TUNEL system (Promega, Madison, WI, USA) was used to carry out TUNEL method according to manufacturer’s recommendations. Approximately, 4 × 10^4 hNSCs were plated on chamber slides upon differentiating conditions.

As a negative control, cells were incubated with reaction mixture without the enzyme terminal transferase. No stained nuclei were detected.

Quantification of uric acid and NO production
Lesch–Nyhan and healthy hNSCs were grown in 4 ml media for 48 h. The medium was concentrated to 1 ml by centrifugal filter devices (Centricron, Amicon, Millipore, Bedford, MA, USA) according to manufacturer’s instructions. We assessed the level of uric acid using Cobas6000 (Roche, Mannheim, Germany) as described in the manufacturer’s instructions.

NO production was quantified by the accumulation of nitrite in the supernatants of hNSCs cultures by the standard Griess reaction according to the manufacturer’s instructions (R&D). Conversion of absorbance to NO micromolar concentrations was deduced from a standard curve. Treatment with the NOS inhibitor L-NAME (100 μM, Sigma) dissolved in culture medium of LND hNSCs and maintained for 72 h. At that time, NO production was quantified as described.

Immunofluorescence and morphological analyses and neural induction
hNSCs were plated on matrigel-coated glass-chamber slides (Nunc, Naperville, IL, USA), in media without mitogens (22). After that, cells were fixed at different times (3, 5 and 7 DIV) and analysed for the presence of neural markers such as GFAP (rabbit, 1:500), NG2 (rabbit, 1:200), β-III tubulin (mouse, 1:100) (purchased from Chemicon) by means of immunostaining as previously described (55). Three separate immunofluorescence analyses were performed on LND and healthy hNSCs; positive cells were counted in a blind manner.

Neuronal differentiation was induced by addition of cis-retinoic acid (RA, Sigma) to the differentiation media (22) on matrigel-coated glass chamber slides (Nunc), at a final concentration of 5 μM.

Neuronal differentiation was investigated upon differentiation condition after addition of 100 μM L-NAME (Sigma) after 7 days. The samples were fixed for immunocytochemical studies as described above.

For the evaluation of Dopaminergic differentiation, LND and healthy proliferative hNSCs were treated as previously described (58). Induced cultures were fixed and processed for dopaminergic markers immunostaining (mouse anti-TH, 1:500, rabbit anti-NF, 1:250; rat anti-DAT, 1:500, all from Chemicon). Phases contrast digital micrographs have been obtained by merging of single fluorescence digital images of random fields with a Nikon Eclipse TE300 microscope equipped with a Zeiss camera. Soma size and neurite length were examined using Axiovision software. At least 50 neurons were evaluated for each line.

Reverse transcriptase–polymerase chain reaction
Three separate RNA extractions were performed on two undifferentiated LND hNSC lines and the pooled undifferentiated controls and processed separately. Total RNA from Lesch–Nyhan and healthy hNSCs was isolated with TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. β-III Tubulin, GFAP, nestin, NG2, SOX2, FLT4, CD133, D2R, NURR1, TH and GAPDH primer sequences are available on request. To ensure accuracy, each reaction was repeated three times.

RT–PCR reactions were optimized for GAPDH to determine the cycle-kinetic and the cDNA concentration under non-saturating conditions. The optimal number of cycles was 28 using 25 ng of cDNA in 20 μl of the PCR reaction volume (Supplementary Material, Fig. S1). Under these cycle-kinetic and the cDNA concentration conditions, we tested the expression of GAPDH gene in LND and healthy hNSCs. GAPDH levels was comparable in the analyzed cell lines.

Statistical analysis
Data are presented as mean ± SD. Statistical analysis was performed using Wilcoxon rank sum test (P < 0.05).

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS
We thank Dr Andrea Smith for the English review of the paper. The generous collaboration of Francesca Rizzi is gratefully acknowledged.

FUNDING
This work was supported by Fondazione IRCCS Istituto Neurologico ‘Carlo Besta’ (LR8); and by The Italian Ministry of Health (RF-INN-2007-644440).

Conflict of Interest statement. We declare that we have no conflict of interest.

REFERENCES


