Research Article

Peroxiredoxin 2 (PRDX2), an antioxidant enzyme, is under-expressed in Down syndrome fetal brains


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Abstract. Suppression subtractive hybridization performed on Down syndrome (DS) versus control fetal brains revealed differential expression of peroxiredoxin 2 (PRDX2), mapped at 13q12. Peroxiredoxins are antioxidant enzymes involved in protein and lipid protection against oxidative injury and in cellular signalling pathways regulating apoptosis. The under-expression of PRDX2 observed in DS samples was confirmed by real-time PCR (0.73-fold). To test whether decreased expression is associated with enhanced sensitivity of DS neurons to reactive oxygen species, we down-regulated PRDX2 through stable transfections of SH-SY5Y neuroblastoma cells with antisense constructs of the complete PRDX2 coding sequence. In addition, we over-expressed SOD1 and compared the effects of the two genes on cell viability. Cells transfected with either construct showed similar sensitivity to oxidative stress in addition to increased apoptosis under basal conditions and after treatment with oxidative cytotoxic agents. This suggests that the decreased expression of PRDX2 may contribute to the altered redox state in DS at levels comparable to that of the increased expression of SOD1.

Key words. Down syndrome; peroxiredoxin; SOD1; oxidative stress; suppression subtractive hybridization; differential expression.

Introduction

Down syndrome (DS), the most common chromosomal aneuploidy in liveborn infants [1, 2], is caused by total or partial trisomy of chromosome 21. The complete sequence of this chromosome has been recently reported [3], but the relationship between specific DS phenotypic features and the gene dosage imbalance associated with the trisomy remains a challenging issue. This relationship could be either direct or indirect, because the extra gene copies in DS may lead to a cascade effect, involving additional genes not necessarily located on chromosome 21.

Concerning the phenotype, a broad spectrum of abnormalities related to oxidative stress, including mental retardation, early onset Alzheimer’s disease and premature ageing, has been associated with DS. In fact, several genes on chromosome 21 are involved in the cellular redox state, namely the Cu/Zn superoxide dismutase (SOD1), cystathione-β-synthase (CBS), carbonyl reductase (CBR), Down syndrome critical region gene 1 (DSCR1) and the heat-shock protein family member STCH [reviewed in ref. 4]. Deregulation of these genes may alter the levels of intracellular reactive oxygen species (ROS), produced during ordinary metabolic activities of aerobic organisms, and generate oxidative stress. Significantly, some ROS are involved in signalling...
peroxidases because they reduce H₂O₂ to water using thioredoxin domains [17, 18]. They were first named thioredoxin from bacteria to mammals, sharing highly conserved pro-mology with conventional antioxidant proteins [7, 9, 12]. Peroxiredoxins (PRDXs), initially characterized in yeast, glial cells [16].

PRDX2 is a human homologue of yeast thioredoxin peroxidase (TPX) [7–14], also named thioredoxin-dependent peroxide reductase 1 (TDPX1) or natural killer-enhancing factor-B (NEKF-B). It is located on chromosome 13q12 [15] and is expressed in human neurons but not in glial cells [16].

Peroxiredoxins (PRDXs), initially characterized in yeast, constitute a family of antioxidant enzymes with no homology with conventional antioxidant proteins [7, 9, 12]. More than a hundred homologues have been described from bacteria to mammals, sharing highly conserved protein domains [17, 18]. They were first named thioredoxin peroxidases because they reduce H₂O₂ to water using thioredoxin as an intermediate electron donor, but were later renamed, because some PRDXs do not require thioredoxin [9]. PRDXs play a key role in several cellular functions including protein and lipid protection against oxidative injury [12–14], cell proliferation, differentiation [19] and intracellular signalling pathways regulating apoptosis [20, 21]. Increased cellular levels of antioxidant enzymes confer protection against apoptosis. In this respect, cultured cells over-expressing PRDX2 are more resistant to apoptosis caused by serum deprivation, hydrogen peroxide, ceramide and etoposide [22]. PRDX1 and PRDX2 over-expression also protect thyroid cells from H₂O₂-induced apoptosis [21].

To test whether the decreased expression of PRDX2 in DS brains is related to the reported oxidative stress in DS cultured neurons, we achieved PRDX2 under-expression through stable transfections of SH-SY5Y neuroblastoma cells with antisense constructs of the PRDX2 coding sequence. We also over-expressed SOD1, which is located at 21q22.1 and whose activity is increased 1.3- to 1.6-fold in DS. We then compared the effects of the altered expression of either gene on basal cell viability and resistance against oxidative injuries produced by several cytotoxic agents. Cells transfected with either construct showed increased apoptotic cell death under both basal conditions and oxidative stress. Overall, the PRDX2 antisense-expressing clones showed similar or slightly higher sensitivity to oxidative injuries than those over-expressing SOD1.

Material and methods

Samples

Half brains (including half cerebellum) from 19 male fetuses obtained from therapeutic abortions were collected for this study. Ten of these samples were diagnosed as trisomic for chromosome 21 by amniocentesis (mean gestational age of 20.11 ± 1.18 weeks), while the remaining 9 did not show any chromosomal aberration and were used as controls (mean gestational age of 20.11 ± 1.56 weeks). Fresh tissue was directly frozen in liquid nitrogen. Initially, 6 samples (3 disomic vs 3 trisomic) were used for SSH. Differential expression was confirmed by real-time PCR quantification using 13 additional samples (6 disomic vs 7 trisomic). The use of all human material in this research was approved by the Ethics Committee of the University of Barcelona.

mRNA extraction, cDNA synthesis and SSH

Total RNA was extracted from six samples (three DS vs three control) as described elsewhere [23]. PolyA⁺ RNA was obtained using biotinylated oligo(dT) (Promega) and streptavidin-magnetic beads (Dynal), and retrotranscribed using MMLV-RT (Promega), following the manufacturer’s instructions. The cDNA second strand was obtained using the enzyme cocktail and protocol provided by the PCR-SELECT cDNA subtraction kit (Clontech). SSH was performed on DS and control mRNAs using the PCR-SELECT cDNA subtraction kit (Clontech) according to the manufacturer’s instructions with minor modifications. Differentially expressed cDNAs were cloned and sequenced after two rounds of subtractive hybridization to enrich the sample and two rounds of nested PCR amplification (20 primary and 30 secondary PCR cycles) using the Advantage Klentaq polymerase (Clontech).

Quantification of differentially expressed genes by real-time quantitative PCR

Total RNA was obtained using the ABI PRISM 6700 Automated Nucleic Acid Workstation (Applied Biosystems) and subsequent RT-PCRs were produced using the Taqman Reverse transcription reagents (Roche Molecular Systems). Quantitative PCRs were performed with the Universal Master Mix (Applied Biosystems) and analysed by the ABI PRISM 7700 sequence detection system (Applied Biosystems). The primers and Taqman probe (using FAM as reporter and TAMRA as quencher) were designed using the Primer Express software. Relative expression values of PRDX2 with respect to GAPDH and β-tubulin controls (primers and probe from Applied Biosystems) were obtained following the manufacturer’s instructions.
DNA constructs
The pBI-PRDX2 antisense-enhanced green protein (EGFP) and the pBI-SOD1 sense-EGFP constructs were obtained by cloning the corresponding cDNA coding sequences (from ATG to the stop codons) into the pBI-EGFP vector (Clontech) in the antisense and sense direction, respectively.

Cell culture and transfection
Human neuroblastoma SH-SY5Y cells (European Collection of Cell Culture) were grown under the following standard conditions: MEM-F12 (Gibco, Invitrogen) supplemented with 2 μM glutamine, 1% non-essential amino acids, 50 μg/ml gentamicin and 10% Tet-system approved fetal calf serum (Clontech). DNA constructs were electroporated into cells (40 μg for 5 × 10⁶ cells) using a Gene Pulser II (950 Ω, 300 V; Bio-Rad). Tet-on cell lines were obtained by transfection with the pTet-on vector (Clontech) and subsequent selection with G418 (0.5 mg/ml) (Gibco, Invitrogen) after 2–3 days. A second round of transfection was performed on selected pTet-on cell lines with a 1:40 mix of pTK-hygromycin empty vector and either pBI-PRDX2 antisense-EGFP or pBI-SOD1 sense-EGFP. After 2–3 days in culture, co-transfected cells were selected by adding G418 (0.5 mg/ml) and hygromycin (0.150 mg/ml) (Gibco, Invitrogen). Resistant and fluorescent clones were isolated, expanded and used in further experiments. The initially selected pTet-on clones were used as controls. The expression of the sense and antisense constructs was induced by adding tetracycline (2 μg/ml) onto the media.

SOD1 activity measurements
Total SOD activity was determined in cell homogenates by the xanthine/xanthine oxidase method using a commercial kit (Ransod, Randox).

Western immunodetection
Cells (5 × 10⁶) from each selected clone were collected, pelleted by centrifugation, rinsed once with PBS and resuspended in 75 μl of total protein extraction buffer (150 mM NaCl, 10 mM Tris HCl pH 7.4, 1 mM EDTA, 1% NP40 plus a cocktail of protease inhibitors). After 10 min on ice, samples were centrifuged for 15 min at 4°C to separate soluble protein extracts from cellular debris. Protein extracts were quantified following the Bradford method (and further confirmed by Coomassie staining of a replica gel), loaded (5 μg/lane) onto a 10% SDS-polyacrylamide gel, electrophoresed, semi-dry transferred onto a PVDF membrane, immunodetected with both a polyclonal antibody (1:5000) raised against PRDX2 (kindly provided by Dr H. Z. Chae) and a β-tubulin commercial monoclonal antibody (1:5000), and detected with ECL (Amersham Pharmacia). Relative quantification of PRDX2/β-tubulin expression in clones was performed with the NIH Image 1.62f software. For caspase-3 activity detection, the same general method and conditions were used but for loading, 30 μg of protein extract per lane onto a 7.5% SDS-polyacrylamide gel, immunodetection with an anti-fodrin antibody (Chemicon) (1:2000) and chemiluminescent detection with SuperSignal (Pierce).

Viability assays
For all experiments, growing cells were harvested after mild trypsinization and seeded at a concentration of 2 or 3 × 10⁵ cells/ml in 30-mm Petri dishes, 96-well plates or 8-chamber slides (Nunc) in growth medium. Tetracycline (2 μg/ml) was added the next day and experiments were performed 24 or 48 h (approximately at 80% of cell confluence) after induction. Basal levels of cell death in the control, PRDX2 antisense and SOD1 sense transfected cell lines grown on 96-well dishes were determined by propidium iodide staining (7 μg/ml), a fluorescent dye that binds to nucleic acids in dead cells [24]. After 1 h incubation, fluorescence was quantified using a Cytofluor 2350 fluorescence scanner (Millipore) [485 nm (20 nm band-pass) excitation and 530 nm (40 nm band-pass) emission]. Basal levels of cell death were calculated as a percentage of the maximum fluorescence obtained in wells with cells treated with 0.02% Triton X-100. The differential effects of several neurotoxic agents, namely hydrogen peroxide, thimerosal and etoposide (Sigma), on the growth and survival of gene-transfected cells and controls were analysed by the 2-(4.5-dimethylthiazo-1-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay with minor modifications [25]. MTT was added to the 96-well cultures (final concentration, 0.5 mg/ml), 2 h before the end of a 24-h exposure to the toxic agent. After incubation, absorbance was measured at 560 nm with a 620-nm reference wavelength in an iEMS (Labsystems) plate reader. Data (four to seven independent experiments) of basal cell death and response to cytotoxic agents were pooled and expressed as a percentage of controls. The results are given as the mean ± SE. Statistical analysis was performed with ANOVA procedures followed by Duncan’s multiple-range test at the significance level p < 0.05 (SPSS software).

Determination of the enzymatic activity of lactate dehydrogenase (LDH) released into the culture medium by damaged cells was performed as previously described [26] and used as a reference of cytotoxicity. Propidium iodide-stained and TUNEL processed cells (see below) cultured in chamber slides were also examined under the microscope.

Caspase-3 immunostaining and activity quantification
Transfected cells and controls were immunostained for the presence of cleaved caspase-3 after growth under standard conditions or under treatment with hydrogen peroxide or...
thimerosal. Cells on coverslips were fixed, and immunodetected with a rabbit polyclonal antibody specific for cleaved caspase-3 (Cell Signalling) (1:100) and a biotinylated goat anti-rabbit IgG (Vector) (1:200), followed by detection with the Vectastain ABC-kit (Vector).

Protein extracts of each selected clone were obtained from untreated and treated (after 3 h exposure to 100 μM hydrogen peroxide) cells (1 x 10⁶) as in Western immunodetection but for the lysis buffer (200 mM HEPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 10% sucrose, 1 mM PMSF and 1% NP-40 at pH 7.4). Caspase-3 activity was measured in a 96-well plate. Twenty-five micrograms of protein extract were loaded per well. Three values were obtained for each extract: (i) caspase-3 activity on the DEVD-AFC substrate (final concentration 10 μM); (ii) the same as (i) but adding DEVD-FMK (an inhibitor of caspase-3 activity) diluted in DMSO (final concentration 10 μM), and (iii) a control value of (ii) by adding DMSO instead of inhibitor to the lysis buffer. After incubating the 96-well plate containing all protein extracts and reagents (except the substrate) for 15 min at 37 °C, the substrate DEVD-AFC was added to each well. For each sample, caspase-3 activity was calculated as i – (ii – iii). Fluorescence (excitation λ at 400 nm, emission λ at 505 nm) was quantified at several incubation times (0, 0.5, 1, 3 and 6 h).

TUNEL assays
Tunel experiments were performed using the DeadEnd Colorimetric Apoptosis Detection System (Promega). A previous time course quantification of TUNEL-positive cells after hydrogen peroxide and thimerosal treatment at 3, 8, 15 and 24 h was performed and the best results were achieved at 15-h treatment. The percentage of TUNEL-positive cells was analysed in controls and in two transfected clones for each construct, under standard and the specified oxidative stress conditions. Positive cells were counted in five different microscope areas of 100 cells for each sample (two to four samples per clone and per experimental condition). Statistical analysis was performed with ANOVA procedures followed by Duncan’s multiple-range test at the significance level p<0.05 (SPSS software).

Results

Down-regulation of PRDX2 in DS fetal brains
To identify putatively down-regulated genes in DS, SSH was performed on brain tissue samples (half brains) from three DS and three control male fetuses, using an excess of DS cDNAs (driver population) with respect to control cDNAs. The DNA fragments obtained (ranging from 570 to 190 bp) were cloned and sequenced. Most of them corresponded to ribosomal protein genes and unidentified transcripts which were not further investigated. Differential expression of the remaining genes was assessed by densitometric analysis of Northern blot hybridizations (data not shown). Consistent data on decreased expression associated with DS was only obtained for PRDX2. Further confirmation and quantification was obtained by real-time PCR (0.73 ± 0.08 fold) on 13 additional samples, 6 disomic and 7 trisomic, using GAPDH and β-tubulin to normalize the values. Statistical significance was determined by the Mann-Whitney test (p < 0.05) (fig. 1).

Transfection of SH-SY5Y cells and selection of clones
To analyse the effect of PRDX2 under-expression in cultured neuronal cells, an antisense strategy to sequester the endogenous transcript was designed. A SOD1 sense expression construct was also produced to raise the cellular levels of SOD1 enzymatic activity. To prevent possible deleterious effects caused by constitutive expression of the PRDX2 and SOD1 constructs in stably transfected cells, we used the pTet-on inducible system (BD-Clontech).

After a round of transfection, several independent clones were selected and shown to express high levels of rtTA

Figure 1. Average differential PRDX2 expression of DS samples with respect to controls (7 trisomic vs 6 disomic) by real-time quantitative PCR. Values in arbitrary units are normalized with GAPDH as an endogenous control. The statistical error is also shown. The asterisk indicates statistical significance according to the Mann-Whitney test (p < 0.05).
Viability studies

Cell clones transfected with either construct showed higher spontaneous mortality than controls under standard conditions (see Material and methods), as revealed by propidium iodide staining (fig. 3a–e). Statistical significance of the increased mortality of clones over-expressing SOD1 (F5,79 = 5.734, p = 0.001) (fig. 3a) or under-expressing PRDX2 (F5,155 = 2.611, p = 0.026) (fig. 3b) was shown by one-way ANOVA.

The cytotoxicity of hydrogen peroxide, thimerosal and etoposide was tested at two concentrations, previously shown to cause low and moderate cell death in control cultures. The MTT test was used on transfected cells and controls to evaluate the effects on cell proliferation and viability, which was dependant on the construct and the treatment. Overall, transfected clones (with either PRDX2 antisense or SOD1 sense) were more sensitive than controls at the highest concentration of the cytotoxic agents. In the PRDX2 antisense transfectants, two-way (agent concentration and clone) ANOVA showed a significant effect of the toxic agent concentration for hydrogen peroxide (F2,807 = 1180.16, p < 0.001), thimerosal (F2,432 = 512.63, p < 0.001) and etoposide (F2,438 = 373.84, p < 0.001). However, differences between PRDX2 clones and controls were significant only for hydrogen peroxide (F5,807 = 22.77, p < 0.001) and thimerosal (F5,432 = 12.998, p < 0.001). Moreover, SOD1 transfectants were also significantly affected by the concentration of hydrogen peroxide (F5,509 = 1086.83, p < 0.001), thimerosal (F5,295 = 299.23, p < 0.001) and etoposide (F5,424 = 239.49, p < 0.001). In this case, differences between SOD1 clones and controls were significant only for hydrogen peroxide (F5,509 = 44.78, p < 0.001), although there was a significant interaction effect between both factors, clone and concentration, for thimerosal (F5,295 = 6.34, p < 0.001). MTT assay responses to hydrogen peroxide and thimerosal exposure are shown in figures 4a, b and 5a, b, respectively. For each concentration of the toxic agents, sensitivity differed significantly with respect to controls, as shown by one-way ANOVA followed by Duncan’s test (figs. 4a, b and 5a, b). Overall, cells transfected with PRDX2 antisense showed higher sensitivity than those over-expressing SOD1 to hydrogen peroxide (at all assayed concentrations, F5,901 = 37.09, p < 0.001) and thimerosal (only at the lowest concentration, F1,177 = 5.70, p = 0.017).

The LDH assay and propidium iodide staining also confirmed the MTT results with similar statistical significance (data not shown). After treatment with hydrogen peroxide or thimerosal, cell death increased in SOD1 sense and PRDX2 antisense transfected cultures with respect to controls (see results on propidium iodide staining, figs. 4c–e and 5c–e).

After treatment with etoposide, the viability of the transfected cells did not differ significantly from controls (data not shown) and, thus, they were not further studied.
Figure 3. Basal death in SH-SY5Y neuroblastoma cells stably transfected with human SOD1 sense or PRDX2 antisense constructs. (a) Percentage of propidium iodide fluorescence of SOD1-transfected clones (S1–S3) in relation to controls (C). (b) Percentage of propidium iodide fluorescence of PRDX2 antisense clones (P1–P5) in relation to controls (C). *p < 0.05 as compared with controls; see Results for statistics. (c–e) Microphotographs of cells fixed after propidium iodide staining. Faint basal staining of living cells is detectable in control cultures (c), while highly fluorescent dead cells, indicated by arrowheads, appear both in S (d) and P (e) clones. (f–h) Microphotographs of TUNEL-processed cultures of controls (f), S (g) and P (h) clones, with several positive cells for DNA fragmentation, marked by arrowheads, in both transfected clone types. Scale bar, 20 μm.
Cell apoptosis under standard and oxidative stress conditions
To test whether PRDX2 under-expression or SOD1 over-expression increased apoptosis in transfected neuroblastoma cells under standard and oxidative conditions (see Materials and methods), four approaches were undertaken: (i) TUNEL assay; (ii) immunostaining of caspase-3; (iii) immunodetection of caspase-3 activity upon addition of a substrate (fodrin) and (iv) direct quantification of caspase-3 activity.

Under standard basal conditions, nuclear fragmentation in dead cells was more evident in either SOD1 sense or PRDX2 antisense clones than in controls, as detected by the TUNEL assay (fig. 3f–h). Moreover, the number of TUNEL-positive cells increased after treatment with hydrogen peroxide (fig. 4f–h) or thimerosal (figs. 5f–h and 6). A more disruptive effect of both cytotoxic reagents on cell morphology and culture organization was evident in the PRDX2 antisense clones (figs. 4h, 5h). ANOVA showed an effect of both factors, clonal cell type (F(4,40) = 4.37, p = 0.005) and treatment (F(2,40) = 39.172, p < 0.001), in the percentage of TUNEL-positive cells when comparing controls and both types of transfected cells (fig. 6).

Direct immunostaining of the 17-kDa fragment from caspase-3 cleavage revealed that cell death induced by thimerosal and hydrogen peroxide proceeded, at least partially, through caspase-3 activation, either in control or in transfected cells (data not shown).

Figure 5. Effect of 24 h exposure to 2 or 3 μM thimerosal on the viability of SH-SY5Y cells stably transfected with human SOD1 sense or PRDX2 antisense constructs. (a) Cytotoxicity as indicated by a decrease in the percentage of MTT reduction in SOD1 transfected clones (S1–S3). (b) Cytotoxicity as indicated by a decrease in the percentage of MTT reduction in PRDX2 antisense clones (P1–P5). *p < 0.05 as compared with the corresponding treatment in controls (C); see Results for statistics. (c–e) Microphotographs of cells stained with propidium iodide after 24 h exposure to 2 μM thimerosal. Some highly fluorescent dead cells are indicated by arrowheads in control cultures (c) and S clones (d), whereas a higher number of dead cells are evident in P clones (e). (f–h) Microphotographs of TUNEL-processed representative cultures of control (f), S (g) and P (h) clones after 15 h of exposure to 2 μM thimerosal, with some positive cells indicated by arrowheads. A more disruptive effect of thimerosal is apparent on P cells. Scale bar, 20 μm.
Fodrin, produced as a 240-kDa precursor protein, generates an active 150-kDa peptide which, upon activation of caspase-3, is degraded to a 120-kDa proteolytic product.

Immunodetection of this 120-kDa fragment is a routine assay for cellular apoptosis. In our case, the cleaved 120-kDa fragment was barely detectable in controls (SH-SY5Y cells transfected only with the pTet-on vector) under standard conditions (lanes C in fig. 7a, b). In contrast, higher levels of cleaved fodrin were detected in protein preparations from all SOD1 sense (fig. 7a) and PRDX2 antisense (fig. 7b) transfected clones, pointing to an increase in apoptosis. When the immunodetection was performed after 3 h treatment with 100 μM hydrogen peroxide, fodrin cleavage was remarkably lower in both PRDX2 antisense and SOD1 sense clones than in controls: (i) untransfected SH-SY5Y cells (fig. 7c, lane W) and (ii) SH-SY5Y cells transfected with the pTet-on vector (fig. 7c, lane C).

Further assessment of the immunodetection data was obtained through direct quantification of caspase-3 activity on a fluorescent substrate. The results (given as an average of all the independent clones) confirmed that, compared to controls, caspase-3 activity in both types of transfectant clones (i) was higher under standard condi-

**Figure 6.** Percentage of TUNEL-positive cells after 15 h exposure to either 50 μM hydrogen peroxide or 2 μM thimerosal in SH-SY5Y control-transfected cells (C), and SH-SY5Y stably transfected with human SOD1 sense (S) or PRDX2 antisense (P) constructs. *p < 0.05 as compared with controls (see Results for statistics).

**Figure 7.** Substrate cleavage by caspase-3 activity. Immunodetection of fodrin in protein extracts from SOD1 sense (a) and PRDX2 antisense (b) transfected clones, under basal conditions (the symbols – and + indicate tetracycline induction). (c) Immunodetection of fodrin in protein extracts from tetracycline-induced PRDX2 antisense and SOD1 sense clones after 3 h treatment with 100 μM H2O2. (d) Fluorimetric quantification of mean caspase-3 activity in protein extracts from control cells and the stably transfected analysed clones under standard conditions (left) and after 3 h treatment with 100 μM H2O2 (right). The represented values were obtained after 60 min incubation of protein extract with the substrate. The standard deviation is also indicated. Clone P5 was not available for this set of experiments. P, PRDX2 antisense clones; S, SOD1 sense clones; C, control pTet-on cells; W, SH-SY5Y original non-tranfected cells.
tions and, (ii) increased to a much lesser extent upon addition of 100 μM hydrogen peroxide (fig. 7d).

Discussion

Gene dosage imbalance associated with aneuploidies may introduce multiple subtle differences in the expression of many genes, which will eventually converge on a complex phenotype, such as DS. In this context, understanding of DS pathogenesis requires complementary strategies to the systematic characterization of genes on chromosome 21. Among them, the search for DS differentially expressed genes, although not devoid of pitfalls, is a promising approach.

SSH performed on DS and control fetal brains revealed that PRDX2 is under-expressed in DS samples. This was further confirmed and quantified by real-time PCR analyses. Three individuals of each population were used for SSH and 13 additional samples (7 DS and 6 controls) were used to validate the data by quantitative RT-PCR. In total, 19 different samples were analysed. The number of samples is not a trivial issue, as the variability inherent to the individual genetic background hinders the detection of differentially expressed genes. Therefore, statistical significance can only be attained by increasing the number of samples analysed.

PRDX2 is exclusively expressed in large neurons, such as hippocampal pyramidal and Purkinje cells [16]. As significant neuronal loss in several brain regions, including the cerebral cortex, hippocampus and cerebellum, is associated with DS [28], PRDX2 under-expression may be a direct consequence of the reduction in this neuronal population. Concurrently, cellular PRDX2 expression levels might decrease as a direct effect of the altered expression of a gene or group of genes located on chromosome 21. Recently, and in agreement with our results, the search for DS differentially expressed genes, although not devoid of pitfalls, is a promising approach.

SCCP performed on DS and control fetal brains revealed that PRDX2 is under-expressed in DS samples. This was further confirmed and quantified by real-time PCR analyses. Three individuals of each population were used for SSH and 13 additional samples (7 DS and 6 controls) were used to validate the data by quantitative RT-PCR. In total, 19 different samples were analysed. The number of samples is not a trivial issue, as the variability inherent to the individual genetic background hinders the detection of differentially expressed genes. Therefore, statistical significance can only be attained by increasing the number of samples analysed.

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pared to controls, cell death proceeded mainly via necrosis or caspase-3-independent apoptosis (at least in hydrogen peroxide-treated cells).

Mental retardation in DS has often been associated with higher neuronal sensitivity to physiological oxidative radicals and increased neuronal apoptosis [4, 32, 33, 41]. Increased cellular activity of SOD1 as a result of gene-dosage imbalance was considered a major factor for the DS-associated neuronal oxidative stress. Now, according to our work, PRDX2 is under-expressed in DS fetal brains. In this context and at the cellular level, it may be relevant that the altered expression of either gene (i) decreased cell viability under standard growth conditions and (ii) increased sensitivity under oxidative stress. The cumulative effects of the combined PRDX2 and SOD1 alterations, and the contribution of other antioxidant enzymes, among them the PRDX family members [29, 30], on the cellular redox state of DS neurons remain to be elucidated. In any case, our data further support the relationship between a decrease in the oxidative stress defence mechanisms and the apoptosis and neurodegeneration observed in DS brains. Finally, we also postulate that under-expression of PRDX2 contributes to the DS-neuron oxidative alterations to a similar extent as SOD1 over-expression.

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